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Heme synthetase deficiency in human protoporphyria. Demonstration of the defect in liver and cultured skin fibroblasts.

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Research Article

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Heme Synthetase Deficiency in Human Protoporphyria

DEMONSTRATION OF THE DEFECT IN LIVER AND CULTURED SKIN FIBROBLASTS

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ABSTRACT The final step in heme biosynthesis is chelation of porphyrin with Fe^{++} catalyzed by the mitochondrial enzyme heme synthetase. We have employed a sensitive radiochemical assay for this enzyme, using ^{59}Fe and deuteroporphyrin or protoporphyrin as substrates. In this method iron is maintained in the ferrous state, oxygen is excluded from the incubation system, and labeled heme product is extracted into ethyl acetate.

This assay has been used to measure the activity of heme synthetase in homogenates of liver, obtained by needle biopsy, and in sonicates of human skin fibroblasts, cultured in vitro. In addition, activity of the first enzyme of the heme synthetic pathway, δ-aminolevulinic acid synthetase, has been measured in fibroblast lysates.

Lysates of fibroblasts from eight patients with protoporphyria had activities of δ-aminolevulinic acid synthetase which did not differ significantly from those of eight normal fibroblast lines, whereas activity of heme synthetase, with either deuteroporphyrin or protoporphyrin as substrate, was markedly decreased in sonicates of skin fibroblasts from these patients, the mean being 8% of control with deuteroporphyrin and 14% with protoporphyrin as substrate.

In homogenates of liver from five patients with protoporphyria, activity of heme synthetase was also significantly less than that found in six patients without porphyria, the mean being 13% of control with protoporphyrin as substrate.

These results provide evidence that decreased activity of heme synthetase is the basic defect in the heme synthetic pathway in protoporphyria. This deficiency is probably responsible for protoporphyrin accumulation and hence the biochemical and clinical features observed in protoporphyria.

Portions of this work have appeared in abstract form (1, 2) and were presented at the National Meeting of the American Federation for Clinical Research, 3–4 May 1975, Atlantic City, N. J.

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INTRODUCTION

Of the recognized inherited disorders of porphyrin metabolism, the last to be described (3, 4) has been called "erythropoietic protoporphyria."[1] The biochemical hallmark of this disorder is an increase in protoporphyrin concentration in red cells, stool, or both (6). It is inherited as an autosomal dominant with variable penetrance and clinically is characterized by photosensitivity, which runs a benign course. However, several patients have developed cirrhosis and hepatic failure (7–10). The livers of these and some other, nonfatal cases have shown protoporphyrin pigment deposition in the biliary system, including the canaliculi, and in Kupffer cells and hepatocytes (11, 12).

[1] The alternative name "erythrohepatic protoporphyria" (5) has also been suggested. In this paper we shall simply use the term "protoporphyria."

The nature and locus of the metabolic defect of heme biosynthesis have been unresolved questions in protoporphyria. A profound deficiency of erythropoietic heme synthesis does not usually occur, although patients may be anemic (7, 9, 10, 12). Furthermore, hepatic heme biosynthesis appears to be normal although this is difficult to assess and has not been studied in detail.

Normally, the rate of heme biosynthesis is thought to be controlled primarily at the level of δ-aminolevulinic acid (ALA)[2] synthetase (succinyl-CoA-glycine succinyl transferase, EC 2.3.1.37), the first enzyme of the pathway, which is subject both to end-product (heme) repression and inhibition (6). An increase in hepatic ALA synthetase activity occurs in the dominantly inherited hepatic porphyrias, acute intermittent porphyria (6, 13), variegate porphyria in relapse (13), and hereditary coproporphyria in relapse (14). In the case of acute intermittent porphyria, this increase is believed to be secondary to a genetically determined deficiency of uroporphyrinogen I synthetase which may lead to partial heme deficiency and subsequent derepression and/or disinhibition of ALA synthetase until adequate amounts of end product are formed (15-18). Other factors outside the heme pathway affect the level of activity of ALA synthetase as well (6, 17-19). A reasonable postulate is that the characteristic biochemical features of each of these diseases result from accumulation of different heme precursors behind an inherited partial enzyme deficiency unique to that disease, coupled with a secondary increase in ALA synthetase activity.

In protoporphyria, one might predict a partial deficiency of heme synthetase activity (protoheme ferrolyase, EC 4.99.1.1) to explain the observed findings. In an early in vitro study using liver and bone marrow from two brothers with protoporphyria, Porter failed to demonstrate a deficiency of incorporation of ^{59}Fe into heme (20). The significance of this is uncertain, however, since optimal conditions for assay (21, 22) were not employed. More recently two groups of workers independently have found evidence for decreased erythroid heme synthetase activity in three patients with protoporphyria (23, 24).

In this study, we have measured heme synthetase activity in homogenates of liver and sonicates of cultured skin fibroblasts from controls and patients with protoporphyria. In addition ALA synthetase activity has been measured in lysates of the fibroblasts, both to serve as a mitochondrial "marker" and to provide further information regarding this enzyme in protoporphyria.

[2] *Abbreviation used in this paper:* ALA, δ-aminolevulinic acid.

METHODS

Preparation of tissues

Fibroblasts. 4-mm punch biopsies of skin, from areas not chronically exposed to sunlight, were placed into tissue culture medium to establish fibroblast lines. Culture medium was Eagle's minimum essential medium with Earle's salts (Grand Island Biological Co., Grand Island, N. Y.) and 10% fetal calf serum (Flow Laboratories, Inc., Rockville, Md.) plus kanamycin (Grand Island Biological Co.), 100 μg/ml.

Fibroblasts were harvested at confluence from monolayer culture into Ca^{++}-Mg^{++}-free phosphate-buffered saline, pH 7.4, with 0.25% trypsin. They were centrifuged (1,000 *g*, 10 min, 4°C) and evenly resuspended in 5–7 ml 0.25 M sucrose-0.05 M Tris Cl (pH 7.5). Cell counts and estimates of viability (trypan blue exclusion) were performed on a suitable dilution of this suspension. Viability was 70–90%.

A portion of the cell suspension ($15–20 \times 10^6$ cells) was centrifuged again and the cell button resuspended in 1–2 ml 0.154 M NaCl-0.04 M Tris Cl (pH 7.4) which was stored at −70°C until assay of ALA synthetase, 1–10 wk later.

The rest of the cells were prepared immediately for assay of heme synthetase by adding 0.05 ml. of Tween-20 per ml of suspension and sonifying three times for 20 s at a setting of 100 W-S (Heat Systems-Ultrasonics, Inc., Plainview, N. Y., model W 185D sonifier) alternating with 20-s periods without sonication.

Liver. Rats were fasted 16–24 h and killed by decapitation. The liver was removed, rinsed with iced saline, homogenized (Potter-Elvehjem) in 9 vol 0.25 M sucrose-0.05 M Tris Cl (pH 7.5), and sonified in the same way as the fibroblast suspensions.

Human liver was obtained by aspiration needle biopsy after informed consent had been obtained. Biopsies from patients with protoporphyria and from control patients 1–5 (Table II) were obtained as part of their evaluation and to aid in management. Liver was obtained from control 6 shortly after death and was stored and assayed simultaneously with the specimens from protoporphyria patients 9 and 10 to serve as a direct control for these patient specimens.

The biopsies were immediately placed into iced saline and divided. One part was placed into fixative for histologic study; the other was blotted, weighed, and frozen or homogenized fresh in 3.5 ml 0.25 M sucrose-0.05 M Tris Cl (pH 7.5) with a ground-glass semi-microhomogenizer (MicroMetric Corp., Cleveland, Ohio). Final sonication was done as for the fibroblasts. All specimens not obtained at Yale-New Haven Hospital (Table II, patients 8–11 and control 6) and some obtained at Yale (Table II, controls 1–3 and 5) were kept at −70°C until assays were performed, 2–10 wk later. Storage of rat liver in this way did not affect activity of heme synthetase as routinely assayed herein. There was no systematic difference in activities in human livers between those assayed immediately compared with those assayed after frozen storage.

Preparation of reagents

$^{59}Fe^{++}$. Ferrous sulfate in ~0.05 M H_2SO_4, 2 mCi ^{59}Fe/2 ml (New England Nuclear, Boston, Mass.) was added to 6.0 ml of a 5-g/100 ml solution of ascorbic acid rendered free of O_2 by boiling, cooling under an ultrapure N_2 stream, and storing tightly capped. Ascorbic acid and O_2 exclusion

were used to insure maintenance of the iron ion in the ferrous state. This stock $^{59}Fe^{++}$ solution was flushed with N_2 periodically. Working iron solutions were prepared from suitable volumes of stock $^{59}Fe^{++}$, carrier $FeSO_4$ solutions (10 or 200 mM), and water, the latter solutions also O_2-free, such that the final working solution contained 1–2 μCi $^{59}Fe^{++}$ and 100 nmol (for fibroblasts) or 500 nmol (for liver) Fe^{++} per 0.05 ml.

Porphyrins. The dimethylesters of protoporphyrin (Sigma Chemical Co., St. Louis, Mo.), mesoporphyrin, and deuteroporphyrin (ICN K&K Laboratories, Inc., Plainview, N. Y.) were hydrolyzed by refluxing for 4–5 h 15 mg of the ester in 20 ml of a solution prepared by dissolving 1 g KOH in 96 ml methanol, 2 ml water, and 1 ml Tween-20. Undissolved ester was removed by filtration and the final concentration of free porphyrin determined by spectrophotometry on a 1/100 dilution of the filtrate in HCl (25). The solutions were stored at −25°C, protected from light.

Hematin. 5 mg hemin (ICN Pharmaceuticals Inc., Cleveland, Ohio) was dissolved in 20 ml 0.1 M Na_2CO_3, and 0.5 ml of this freshly prepared solution was added at the end of incubation as heme carrier and as a means of correcting results for heme recovery, which was always in the range of 75–90%.

Other reagents. Ethyl acetate was washed with water and crystalline $FeSO_4 \cdot 6H_2O$ before use.

Enzyme assays

ALA synthetase. ALA synthetase activity was estimated by the method of Ebert and Pearson (26), with [5-^{14}C]α-ketoglutarate as substrate. For each assay two to four separate determinations were done, with different amounts of protein per flask, incubated for 20 min. In all instances, the amount of ALA formed increased linearly with protein content.

Heme synthetase. The conditions employed for assay of heme synthetase were based on the results of previous studies (21, 22, 27–29) and on preliminary experiments with rat liver and normal human fibroblast sonicates as the source of enzyme. For assay of activity in fibroblast sonicates, 1.0 ml of a sonicate of $5–10 \times 10^6$ cells/ml was added to a 25-ml Erlenmeyer flask containing 0.1 ml Tween-20, 840 μmol Tris Cl (pH 7.5), and 100 nmol porphyrin in a final volume of 3.95 ml. The flasks, kept on ice, were capped tightly with rubber diaphragms and flushed with N_2 for 3 min. They were then shaken 5 min at 37°C on a Dubnoff shaker after which 0.05 ml $^{59}Fe^{++}$ solution was added through the diaphragm to start the reaction, which was allowed to run for 2 h.

Sonicates of liver were incubated similarly except that the flasks contained 750 μmol Tris Cl and 500 nmol porphyrin, and the duration of incubation was 45 min.

In all experiments, a tissue blank was prepared by boiling a portion of the sonicate for 10 min. Active enzyme preparations were run in duplicate or triplicate concurrently with a single tissue blank. In many experiments a reagent blank (no tissue added) was included as well. Heme synthesis by tissue blanks was equivalent to that of reagent blanks, which for normal fibroblasts was 2–10% that of active enzyme preparations. All results were corrected for nonenzymatic formation of product.

At the end of incubation, hematin was added and the flask contents extracted with 5 and then 2.5 ml ethyl acetate: glacial acetic acid, (4/1 vol: vol). The combined organic layers were washed twice with 5 ml water and once with 2.5 ml 1.5 N HCl, which removed remaining porphyrin. The washed ethyl acetate was counted in a Packard scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.; ^{59}Fe counting efficiency 20–30%). After counting, the ethyl acetate was removed by heating to 50–60°C under an N_2 stream. The heme residue was dissolved in 0.7 ml pyridine, 3.0 ml water, and 0.30 ml 1 N NaOH were added; and heme concentration of the resulting solution was measured by the pyridine chromogen method of Porra and Jones (22).

Heme sp act was then determined, and total heme synthesized was calculated as follows: heme synthesized/flask (picomoles) = (heme sp act × heme carrier added × Fe added [picomoles])/^{59}Fe added (counts per minute). When high levels of heme synthetase activity were present, it was possible to estimate activity by determining the rate of porphyrin disappearance. For this purpose, porphyrin concentration in the HCl wash of ethyl acetate was determined spectrophotometrically (25).

Chromatography of heme

Column chromatography was performed by the method of Richmond et al. (30).

Other methods

Porphyrins were quantitated spectrophotometrically or fluorimetrically after extraction by the methods of Schwartz et al. (31). Proteins were determined by the method of Lowry et al. (32), with crystalline bovine serum albumin as standard. Student's *t* test (33) was used to compare data from controls and patients with protoporphyria.

Clinical and biochemical characterization of patients studied

Salient features of the patients with protoporphyria who were studied are summarized in Table I. The diagnosis of protoporphyria was made on the basis of typical history and physical findings of photosensitivity, a positive family history, and demonstration of increased erythrocyte protoporphyrin. Liver biopsy was done in six of the patients and hepatic heme synthetase activity was estimated in five (Table I). As summarized in Table II, the specimens

TABLE I

Biochemical Characteristics of the Protoporphyria Patients Studied

Patient	Age	Sex	Protoporphyrin levels: Packed RBC	Protoporphyrin levels: Stool	
	yr		*μg/100 ml*	*μg/day*	*μg/g dry wt*
1. L. R.	26	F	1,426	37,500	1,428
2. C. A.	27	F	955	—	—
3. J. A.	19	M	500	—	—
4. B. R.	16	M	1,258	2,100	112
5. K. R.	12	F	272	1,000	95
6. R. McG., Jr.	10	M	575	—	—
7. P. McG.	5	F	1,428	—	—
8. R. McG., Sr.	41	M	107	380	18
9. W. H.	56	M	2,850	111, 600	2,206
10. R. F.	9	M	697	—	813
11. S. F.	15	M	7,749	—	827
12. J. F.	31	M	1,725	—	198
Normal range			0–30	<1,000	Trace-120

TABLE II

Clinical, Biochemical, and Histological Features of Subjects on Whose Liver Biopsies Heme Synthetase Activity Was Estimated

Patient	Age	Sex	Diagnosis	Liver function tests				Liver histology
				SGOT	AlkP'ase	Alb	Glob	
	yr			*kU/liter*	*IU/liter*	*g/dl*		
Controls								
1. S. Z.	55	F	Mild, chronic hepatitis	55	38	4.0	3.0	Mild, minimally active chronic hepatitis
2. T. T.	79	F	Villous adenoma of rectum	35	53	3.4	3.0	Subcapsular fibrosis without other abnormality
3. A. G.	31	M	Psoriasis. Took methotrexate 7 yr before	24	25	4.1	2.6	Normal except for mild fatty infiltration
4. C. M.	51	F	Sarcoidosis	44	14	3.9	3.9	Normal architecture. Several small parenchymal noncaseating granulomas
5. J. W.	22	M	Fever of unknown origin	56	39	4.2	3.1	Focal hepatitis with acidophilic bodies
6. C. F.	21	M	Normal. Kidney donor	—*	—*	—*	—*	Normal surgical specimen
Protoporphyria								
7. R. McG., Sr.	41	M	Protoporphyria	21	33	3.8	3.5	Normal; no protoporphyrin
8. W. H.	56	M	Protoporphyria	21	61	5.2	2.3	Mild portal inflammation; no protoporphyrin
9. R. F.	9	M	Protoporphyria	18‡	—	—	—	Normal; no protoporphyrin
10. S. F.	15	M	Protoporphyria	130‡	253§	3.9	3.9	Cirrhosis with massive protoporphyrin deposition
11. J. F.	31	M	Protoporphyria	79	76	4.3	3.2	Mild portal inflammation; focal protoporphyrin deposits.
Normal range, except as noted				5–40	10–85	3.5–5.0	1.5–3.0	

SGOT, serum glutamic oxaloacetic transaminase; AlkP'ase, alkaline phosphatase; Alb, albumin; Glob, globulin.

* Patient died of accidental head injuries within 1 h of hospital admission; no tests of liver function were obtained. He had no history of hepatobiliary disease or exposure to possible hepatotoxins.

‡ IU, normal range 5–13.

§ Normal range 60–200.

obtained showed a range of histologic features, from normal without protoporphyrin deposits to cirrhosis and considerable pigment deposition. The serum glutamic oxaloacetic transaminase was abnormal in all patients except R. McG., Sr., and W. H.; Bromsulphalein retention (45 min) was 3.5% in R. McG., Sr., and 9% in W. H. (normal, < 5%). None of the patients had a history of other liver disease. Fibroblasts were cultured from skin biopsies of patients 1–8 of Table I.

Control skin fibroblasts were cultured from people without personal or family history of any disorder of porphyrin metabolism, ranging in age from 4 to 50 yr. Control liver biopsies were obtained from nonporphyric patients summarized in Table II. They had normal or only mildly abnormal liver function tests and hepatic histology; none had pigment deposition in the liver.

RESULTS

Validity of radiochemical assay of heme synthetase. Disappearance of deutero-, meso-, or protoporphyrin provided a suitable means for estimating levels of heme synthetase found in homogenates of 50–200 mg normal rat liver. However, this approach was not sufficiently sensitive to assay the levels of activity in sonicates of cultured human fibroblasts or human liver obtained by needle biopsy. Therefore, a method was used which measured $^{59}Fe^{++}$ incorporation into heme.

Incubations carried out in the dark gave the same results as those in light. Removal of O_2 from the incubation flasks was important, however, in that the enzyme activity in air was only 10–20% that in N_2, perhaps owing to oxidation of the iron ion to inactive ferric state and in that ^{59}Fe counts in heme decreased after 30 min incubation in air, suggesting that heme catabolism was occurring. Indeed heme oxygenase, which catalyzes the formation of biliverdin from heme, has an absolute O_2 requirement (34).

Tween was added to the stock porphyrin solutions to mitigate polymer formation (29) and was used in tissue sonication to provide a more homogeneous suspension. When added to the incubations it was found approximately to double rates of formation of product. Furthermore, Tween has been found to inhibit nonenzymatic

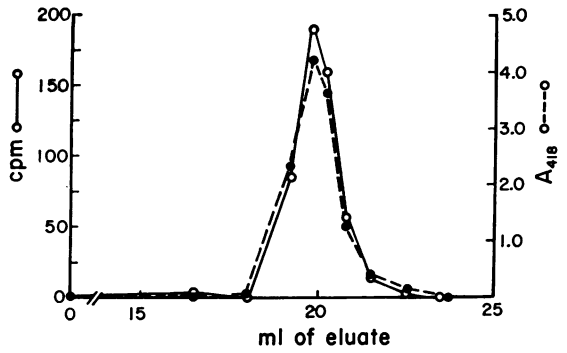

FIGURE 1 Column chromatography of heme. A homogenate of 330 mg wet wt rat liver was incubated 60 min with ^{59}Fe and protoporphyrin as outlined in Methods. After addition of carrier, heme product was extracted into ethyl acetate: glacial acetic acid which was washed successively (see Methods). After the organic solvent had been evaporated, the heme residue was redissolved in 0.05 ml pyridine plus 0.10 $CHCl_3$ and chromatographed on a 1×7.5-cm column by the method of Richmond et al. (30). Portions of eluate were counted, and heme concentration was then measured spectrophotometrically (25). The sp act of the heme applied to the column was 42 cpm/A_{418} unit. No counts or heme appeared in the eluate beyond 23 ml; total elution volume was 75 ml.

protoheme formation in aqueous media (35). Determinations were run in triplicate, and the coefficient of variation averaged 15%.

Several lines of evidence supported the validity of purifying heme product by ethyl acetate extraction as outlined in Methods. First, two 5-ml water washes were sufficient to remove virtually all water-soluble ^{59}Fe from the ethyl acetate. Second, as shown in Fig. 1, when heme residue from washed ethyl acetate was chromatographed, all radioactivity was recovered with the heme, and heme sp act of the eluates was virtually constant and equivalent to that of the starting material. Third, as shown in Fig. 2, the amount of heme synthesized was similar to the amount of porphyrin utilized when enzymic activity was high.

The equimolar substrate concentrations used in the assays of heme synthetase activity were in the range used previously by other investigators (21, 22, 28, 29, 36). No excess substrate inhibition with respect to iron or protoporphyrin was observed by Bottomley (21) at concentrations above the maximal concentrations (125 μM) used here. With 40-100 mg rat liver, an iron concentration ninefold greater produced an activity only 1.4 times that obtained with the 125 μM concentration. This latter concentration was always used in the human liver incubations because it yielded nearly maximal activity with rat liver and because it was felt that higher concentrations would have produced too much dilution of the $^{59}Fe^{++}$ tracer in incubations in which total product formation was relatively low.

As shown in Fig. 2, under the conditions of the assay, the amount of product formed increased as a function of the amount of liver used and as a function of the time of incubation. The lack of strict linearity has been observed previously (22); the reason for this is unknown. In Fig. 3 are shown similar curves for sonicates of fibroblasts from control subjects, in which linear results were obtained for at least 2 h of incubation (Fig. 3A) and for 0-5.5 mg protein (representing 0-13 $\times 10^6$ cells/flask, Fig. 3B).

Enzyme activities in fibroblasts. Results of enzyme assays on fibroblast sonicates are summarized in Figs. 4 and 5. Fig. 4 shows that mean activity of ALA synthetase was nearly identical in cells from patients and normals; the mean value (±SD) was 35.6±13.9 for controls and 38.7±18.5 pmol ALA/mg protein·h for patients.

In contrast, activity of heme synthetase, whether assayed with deuteroporphyrin (Fig. 5A) or protoporphyrin (Fig. 5B) as substrate, was much lower in sonicates of fibroblasts from patients with protoporphy-

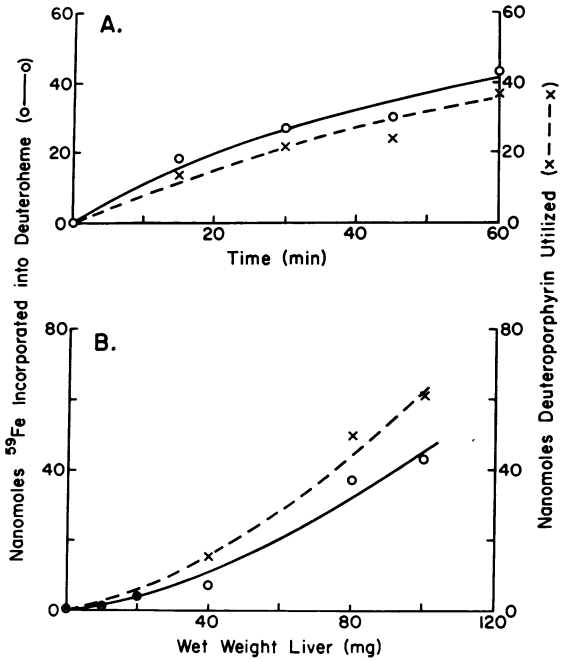

FIGURE 2 Heme synthetase activity in rat liver homogenates. Comparison of ^{59}Fe incorporation into deuteroheme and deuteroporphyrin disappearance. In A, enzyme activity of homogenates of 50 mg wet wt of liver from a male Wistar rat is shown as a function of time of incubation. In B, incubations were carried out for 45 min, and activity is expressed as a function of the wet wt liver from another male rat. Incubation and assay conditions were as described in Methods.

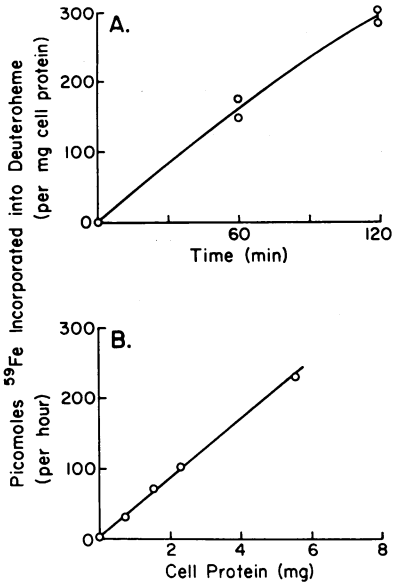

FIGURE 3 Heme synthetase activity in sonicates of skin fibroblasts from control subjects. In A, enzyme activity is shown as a function of the time of incubation. As shown, duplicates were run at each time point. In B, activity is presented as a function of amount of cell protein. Incubation, with deuteroporphyrin as substrate, and assay conditions were as described in Methods.

ria than from controls. There is no overlap between the range of normal and patient values with either substrate. The mean values (±SD), with deuteroporphyrin as substrate, are 141±81.6 for controls and 10.0±5.3 for patients; with protoporphyrin as substrate, corresponding values are 24.9±12.8 and 3.6±1.5 pmol heme/mg protein·h, respectively.

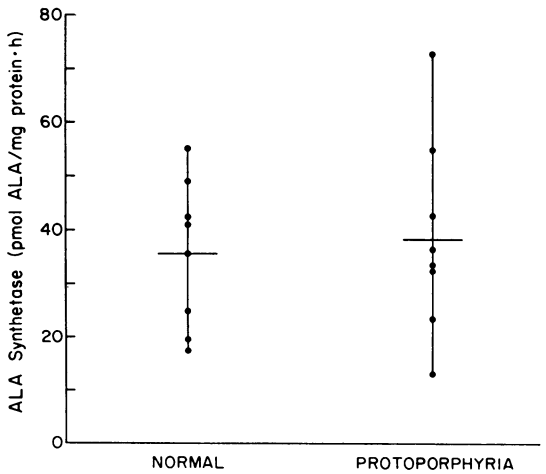

FIGURE 4 ALA synthetase activities in lysates of skin fibroblasts from normal controls and patients with protoporphyria. Each point represents activity of cells from a single patient. The horizontal lines are the mean values, which are not significantly different from each other.

The ratio of heme synthetase activity with protoporphyrin as substrate/activity with deuteroporphyrin as substrate in normals ranged from 0.09 to 0.35. In cells from patients with protoporphyria this ratio was similar (0.15–0.39) in six cases, but in patients 2 and 8 (Table I), the ratio was 1.61 and 0.90, respectively. In both instances the ratio was high because activity with deuteroporphyrin as substrate was very low (1.2 for patient 2 and 6.2 pmol heme/mg protein·h for patient 8). No apparent cause for these low activities could be recognized. There was no correlation between the levels of activity of ALA synthetase and heme synthetase in control or patient cells.

Enzyme activities in liver biopsies. Results of assays of heme synthetase in human liver biopsies are shown in Fig. 6. Activities in the five livers from patients with protoporphyria were all low. The lowest value was obtained for patient S. F., who had cirrhosis and heavy pigment deposition on liver biopsy.

Activity of heme synthetase in five of the six control liver biopsies ranged between 273-580 pmol heme/mg wet wt·h. In one control (J. W., Table II) the activity was 67.3, only slightly above the range of values for protoporphyric subjects. This patient was moderately ill and had had a fever of unknown origin for several weeks before biopsy. He denied drug ingestion which might have accounted for diminished heme synthetase activity, e.g., chloramphenicol (37), and no explanation for the finding is apparent. The mean activity (±SD) for all controls was 352±171 while for patients with protoporphyria the value was 44.2±20.5 pmol heme/mg wet wt·h, a highly significant difference. With protoporphyrin as substrate the mean heme synthetase activity in livers of patients with protoporphyria was 13% of control and in fibroblasts was 14%, a closely similar value.

DISCUSSION

These studies demonstrate a striking decrease in activity of heme synthetase in sonicates of cultured skin fibroblasts and liver from patients with protoporphyria. In interpreting the significance of this finding, a number of questions may be considered.

First, the question may be raised as to whether the low activities could be due to enzyme inhibition by excess substrate, either iron or porphyrin. In preliminary studies, Fe^{++} at 10 times the concentration used in our assay did not diminish activity, suggesting that mammalian hepatic heme synthetase, like erythroid heme synthetase (21), is not inhibited by excess Fe^{++}. In any event, iron as estimated by histochemical stain was not increased in the livers of the patients with protoporphy-

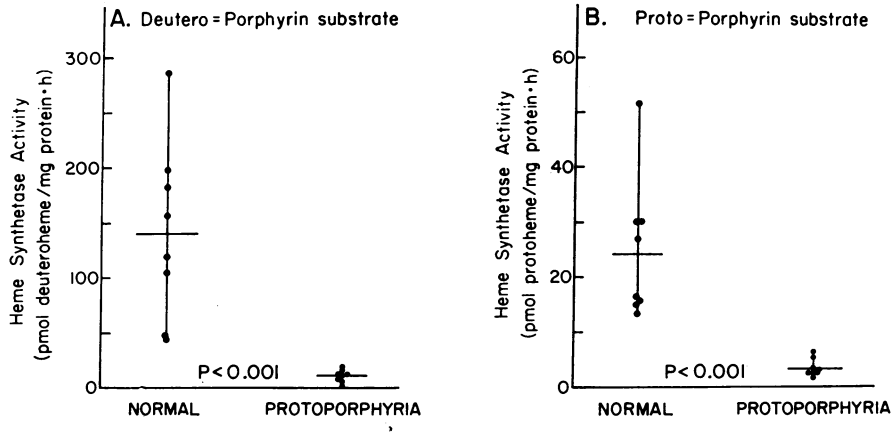

FIGURE 5 Heme synthetase activities in sonicates of skin fibroblasts from normal controls and patients with protoporphyria. Each point represents activity of cells from a single patient. The horizontal lines are mean values. (A) Activities obtained with deuteroporphyrin as porphyrin substrate; (B) activities with protoporphyrin as porphyrin substrate. Note that enzyme activity is three to six times higher with deuteroporphyrin than with protoporphyrin.

ria or in the controls, indicating that the concentration of iron in the assay mixture was the same for both groups of patients.

On the other hand, concentrations of protoporphyrin (420 μM or higher) have been found to inhibit erythroid heme synthetase (21). The concentration of exogenous protoporphyrin used in our assays both for fibroblast and liver sonicates was well below such inhibitory concentrations. The possibility that inhibitory concentrations of protoporphyrin could be reached from that endogenously present in the tissue sonicates is ruled out by the following considerations: The amount of endogenous porphyrin which would have to have been present in tissues from protoporphyria subjects is very large if final protoporphyrin concentration in the assays are to approach inhibitory levels. For example, in patient livers the protoporphyrin concentration would have to have been about 3% (by wet wt), and in fibroblasts, even higher. However, under the growth conditions employed, no or only trace protoporphyrin accumulation, could be detected by fluorescence microscopy or by extraction and fluorimetric analysis (of fibroblasts from either patients or controls). While quantitative measurement of endogenous protoporphyrin could not be carried out on the liver biopsies because of limited amounts of tissue, no or only trace amounts of protoporphyrin were detected by fluorescence and polarization microscopy in four of the five patients with protoporphyria (Table II), whereas this porphyrin would have been readily detectable by these means if the hepatic concentration had been only a fraction of the amount added exogenously. Thus, as for iron, the concentration of protoporphyrin used in the assays was virtually equivalent for both groups.

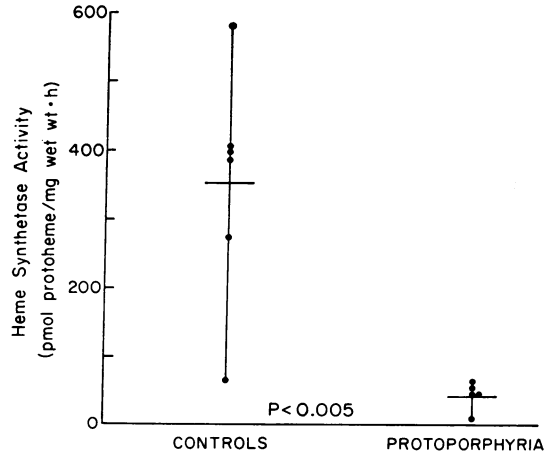

FIGURE 6 Heme synthetase activities in homogenates of liver biopsy specimens from control subjects and patients with protoporphyria. Each point represents activity of liver homogenate from a single patient. The horizontal lines are mean values, which differ significantly from each other, as shown.

Another question raised by our findings relates to the nature of the genetic defect which underlies protoporphyria. The degree of decrease in heme synthetase activity observed in this study (14% of control) is similar to that recently observed by Bottomley et al. (24) in erythroid cells from protoporphyric subjects. An autosomal, structural gene mutation might be expected to lead to enzyme activity about 50% of normal. Explanations previously suggested for a more profound defect in protoporphyrin metabolism have re-

lated to excess substrate inhibition (24) or abnormal lability of mRNA for heme synthetase (38); however, neither of these seems likely to explain our findings. Furthermore, the low activities observed in patient fibroblasts, which had been cultured in vitro for several generations, rule out the possibility of inhibitor(s) which might have originated from another tissue of the patient.

Since heme synthesis in normal cells appears to be controlled primarily by the level of ALA synthetase activity, which is subject to end-product inhibition and repression (6), the question arises why ALA synthetase activity, as measured here, was not increased in the fibroblasts in which heme synthetase activity was markedly decreased. In considering this finding it should be borne in mind that the assay used for ALA synthetase measures the overall conversion of α-ketoglutarate to ALA in the presence of pyridoxal phosphate and glycine in high concentration. Thus, it is theoretically possible that the generation of succinyl-CoA from α-ketoglutarate was rate limiting, not the activity of ALA synthetase itself. Previously, however, whole liver homogenate, similarly incubated, has been found to generate succinyl-CoA at apparently optimal rates, even for very high levels of ALA synthetase (39). In frozen-thawed preparations of viral-transformed cells cultured in vitro, induction of ALA synthetase has been demonstrated by using our radioassay (26), and this induced activity remains intact after several weeks of frozen storage.[3] Furthermore, activities observed in the present studies, in preparations of fibroblasts stored frozen, were, if anything, higher than those we previously observed in preparations from fibroblasts assayed immediately after harvesting (19). Thus, cell freezing and storage do not decrease activity of ALA synthetase as determined by this assay, and apparent induction can be demonstrated in frozen-thawed preparations of cell lines grown in vitro. These considerations suggest that our assay would have detected an increase in ALA synthetase had it been present.

From the lack of porphyrin accumulation by the intact cells of patients or controls, it may be concluded that activity of the steps in the heme pathway between uroporphyrinogen and heme were not rate limiting under the growth conditions used. Rather, it seems likely that generation of ALA was rate limiting in both types of intact cells, as is the case for normal liver (6), a suggestion supported by the observation that skin fibroblasts grown in the presence of exogenous ALA accumulate protoporphyrin.[4] The lack of apparent induction of ALA synthetase in fibroblasts from porphyric subjects, despite a partial "block" in heme synthesis distal to ALA may simply be due to the fact that the demand for heme synthesis by these cells is insufficient to derepress ALA synthetase. Several other possible explanations have been discussed previously (19).

Protoporphyrin production and excretion is increased in another heritable disorder of porphyrin metabolism, variegate porphyria (6), as well as in protoporphyria. The metabolic basis for the apparent increase in protoporphyrin (and other porphyrin) production in variegate porphyria is imperfectly understood; a reasonable postulate would be that there is an inborn deficiency of heme synthetase activity. Were such a decrease to be found, it would complicate further interpretation of the observations reported here. However, in the single study thus far published (40), heme synthetase activity was normal in muscle tissue from variegate subjects. This finding, if confirmed, indicates that in this disease protoporphyrin accumulates for reasons other than that demonstrated here for protoporphyria, perhaps due to deficient activity of protoporphyrinogen oxidase with increased biliary secretion of protoporphyrinogen which is oxidized to protoporphyrin in the gut or during fecal analysis.

The tissue sources responsible for protoporphyrin overproduction in protoporphyria remain uncertain. Experiments of similar design in two patients (5, 23) led to divergent interpretations concerning the relative importance of liver and erythroid tissue as possible sources. While our studies did not address this problem directly, the deficiency of hepatic heme synthetase activity in our patients is compatible with the liver as a source under certain circumstances. In none of the patient livers was heme synthetase activity less than the rate at which normal mammalian liver appears to synthesize ALA or require heme (41). However, the one patient with hepatic pigment deposition (patient S. F.), had heme synthetase activity of only 10.1 pmol heme/mg wet wt·h. If this figure is close to the maximal rate at which hepatic heme can be synthesized by this patient, only a modest induction (three-times normal) of liver ALA synthetase would be required to exceed the capacity of heme synthetase to metabolize endogenously produced protoporphyrin. Since hepatic ALA synthetase can be induced to very high levels and since the rate of total hepatic aminoketone synthesis has been found increased above three-times normal in a few protoporphyria patients previously (42), it seems possible that the liver overproduces protoporphyrin, at least episodically, in some patients with this disorder.

In this regard it should also be stressed that an increase in ALA synthetase activity, leading to an increased pool of protoporphyrin, might need to be only a temporary or episodic phenomenon, until a new steady

[3] Ebert, P. S. Unpublished observations.
[4] Bloomer, J. R. Unpublished observations.

state is reached, wherein further ALA and protoporphyrin production rates are balanced by their rates of utilization for heme synthesis and their rates of excretion or other disposition. If the rates of alternate disposal are only slightly elevated or normal, little or no increase in ALA synthetase activity at the new steady state would be required.

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