*UROPORPHYRINOGEN III COSYNTHETASE IN HUMAN CONGENITAL ERYTHROPOIETIC PORPHYRIA**

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Abstract.—Activity of the enzyme uroporphyrinogen III cosynthetase in hemolysates from five patients with congenital erythropoietic porphyria was much lower than the activity in control samples. The low cosynthetase activity in patients was not due to the presence of a free inhibitor or some competing enzymatic activity, because hemolysates from porphyric subjects did not interfere either with the cosynthetase activity of hemolysates from normal subjects or with cosynthetase prepared from hematopoietic mouse spleen. This partial deficiency of cosynthetase in congenital erythropoietic porphyria corresponds to that shown previously in the clinically similar erythropoietic porphyria of cattle and explains the overproduction of uroporphyrin I in the human disease.

Erythropoietic porphyria is a rare congenital disorder of man and cattle, characterized by photosensitivity, erythrodontia, hemolytic anemia, and porphyrinuria.[1] Many of the clinical manifestations of the disease can be explained by the production in marrow, deposition in tissues, and excretion in the urine and feces, of large amounts of uroporphyrin I and coproporphyrin I, which are products of the spontaneous oxidation of uroporphyrinogen I and its decarboxylated derivative, coproporphyrinogen I. In cattle, the condition is inherited as an autosomal recessive trait,[2, 3] and the statistical data in humans are consistent with a similar mode of genetic transmission.[1]

Uroporphyrinogen I and coproporphyrinogen I are not intermediates in the biosynthesis of protoporphyrin IX. *In vitro*, the enzyme uroporphyrinogen I synthetase catalyzes the formation of uroporphyrinogen I from the monopyrrole compound porphobilinogen.[4, 5] In the presence of a second enzyme, uroporphyrinogen III cosynthetase, the product formed from porphobilinogen by the synthetase is uroporphyrinogen III,[6, 7] which is converted to heme and chlorophyll by biologic systems.[8] The mode of action of the cosynthetase is unknown; it does not catalyze interconversion of the uroporphyrinogen isomers I and III,[6, 9] nor does it affect the rate or stoichiometry of the reaction catalyzed by synthetase.[6, 7] Either an absolute decrease in the activity of cosynthetase or a relative increase in the activity of the synthetase could account for the increased formation of uroporphyrinogen I in congenital erythropoietic porphyria.[6]

Cosynthetase activity in crude tissue extracts can be assayed by measuring its effect on the amount of uroporphyrinogen III formed by partially purified uroporphyrinogen synthetase.[7] When assayed by this method, the activity of cosynthetase in hemolysates from four mature porphyric cattle was found to be much lower than in hemolysates from four mature normal animals.[10] Similar results have now been obtained in hemolysates from five human patients with congenital erythropoietic porphyria. This reduction in cosynthetase activity is

not due to the presence of a free inhibitor of cosynthetase or to the activity of an enzyme catabolizing uroporphyrinogen III.

Materials and Methods.—The patients with congenital erythropoietic porphyria and their primary controls are listed in Table 1. Four of the five porphyric patients have been described in the literature previously.[12-15] Heparinized blood was obtained from each patient with congenital erythropoietic porphyria and the corresponding primary controls on the same day. The blood was frozen immediately in solid CO_2 and stored at a temperature of $-20°$ or less until assayed. In each case an initial cosynthetase assay was carried out on the porphyric hemolysate paired with one of the control hemolysates within 48 hr of the time the blood was drawn. A second cosynthetase determination was done on the porphyric hemolysate paired with the other control hemolysate as soon as possible thereafter. Assays repeated over a period of several weeks gave no indication that human blood cosynthetase is unstable at deep-freeze temperatures over this time span.

For the assay of uroporphyrinogen III cosynthetase, thawed hemolysates were diluted with an appropriate volume of 0.05 *M* buffer. Portions of this dilution were incubated for 30 min at 31° with 50 μmoles of buffer, 0.12 μmole of tritiated porphobilinogen, and enough uroporphyrinogen I synthetase to catalyze the formation of 5 mμmoles of uroporphyrinogen (I + III), in a volume of 0.5 ml. The uroporphyrinogen formed was oxidized to the porphyrin with iodine.[7] The uroporphyrin was isolated on talc, eluted with NH_4OH, esterified, and extracted into chloroform by the method of Bogorad, scaled down fivefold.[16] The concentration of uroporphyrin methyl esters in the chloroform extracts was determined fluorometrically.[7] From each extract an aliquot containing 0.25 μg of esters was evaporated to dryness under a jet of nitrogen. The residue was redissolved in a few drops of chloroform and chromatographed in a double-development system.[17] Segments containing uroporphyrin I and III methyl esters, located by fluorescence under long-wave ultraviolet light or from appropriate guide strips, were placed in 5 ml of toluene containing 20 mg of 2,5-diphenyloxazole and 0.5 mg of *p*-bis[2-(5 phenyloxazolyl)]-benzene, and the radioactivity was counted in a Beckman CPM-100 liquid scintillation system. One unit of cosynthetase activity is defined as the amount needed to give 50% III (2.5 mμmoles of uroporphyrinogen III) under these conditions.

The isomer composition of the product was determined in other experiments by chemically decarboxylating the enzymatically formed uroporphyrinogen and separating isomers of the resulting coproporphyrin. For these experiments the reaction mixture was the same as that described previously, except that the amount of mouse-spleen synthetase was doubled, so that 10 mμmoles of uroporphyrinogen were formed in 30 min at 31°. At the end of the incubation the uroporphyrin was adsorbed on talc and eluted as before, after which the eluate was placed in a combustion tube and evaporated to dryness under a jet of nitrogen. The residue was redissolved in 0.5 ml of 1.0 *M* HCl and decarboxylated by the method of Edmondson and Schwartz.[18] The solution was diluted with 5 ml of water, the pH was adjusted to about 4 with 0.1 ml of saturated sodium acetate solution, and the coproporphyrin isomers were extracted into 2–4 ml of ethyl acetate.[19] The extract was washed twice with water. The coproporphyrins were then transferred to a minimal volume of 0.7 *M* NH_4OH. Carrier coproporphyrins I and III were added. Aliquots of each sample were spotted on Cellulose MN 300 thin-layer sheets (Brinkman Instruments)[20] and chromatographed in 2,6-lutidine–0.7 *M* NH_4OH (10/7, v/v),[21] ascending. Coproporphyrins I and III were located under long-wave ultraviolet light and eluted with 0.5 ml of 0.7 *M* NH_4OH. To this eluate were added 15 ml of dioxane containing 90 mg of 2,5-diphenyloxazole and 1.5 gm of naphthalene. The samples were stored in the dark overnight to allow disappearance of the chemiluminescence, and the radioactivity was counted.

Tritium-labeled porphobilinogen (8.8 mCi/mmole) was prepared enzymatically[5] from δ-aminolevulinic acid-3,5-^{3}H (New England Nuclear, 250 mCi/mmole) which had been diluted 25-fold with unlabeled material (Nutritional Biochemicals). The radioactive porphobilinogen contained no detectable porphyrin, as determined by the absence of a Soret band. It was used without further dilution of the label for experiments in which the

isomers of coproporphyrin were analyzed, or was diluted fourfold with unlabeled porphobilinogen for the experiments in which isomers of uroporphyrin methyl esters were analyzed. Uroporphyrinogen I synthetase for the cosynthetase assay and uroporphyrinogen III cosynthetase for control experiments were prepared from hematopoietic mouse spleen.[5] Uroporphyrinogen I synthetase activity was measured in terms of the rate of disappearance of porphobilinogen,[5] and uroporphyrinogen formation was calculated from the stoichiometry.[7] Uroporphyrin I and III esters for use as chromatographic markers were obtained as previously described.[5] Methyl esters of coproporphyrins I and III were purchased from Calbiochem and hydrolyzed in 6 *M* HCl overnight. The buffers used were dilutions of 1.0 *M* potassium phosphate, pH 7.65, measured at 22°.

Results.—Cosynthetase activity of porphyric and control hemolysates: The results of the first cosynthetase assay performed in this study are shown in Figure 1. With both the control subject and the porphyric patient, there was a linear relationship between the percentage of isomer III in the product and the amount of hemolysate added, in the range from 15 to 75 per cent III. The volume of hemolysate required for 50 per cent III under these conditions, defined as one unit of cosynthetase,[7] could be determined by inspection of the lines, and the number of units per ml of blood could be calculated. An experiment like that shown in Figure 1 was carried out for each of the porphyric and primary control subjects, with the results shown in Table 1. In every case the cosynthetase activity per unit volume was much less in the porphyric hemolysate than in the nonporphyric one. Because cosynthetase activity is not detectable under these conditions in normal human plasma, the results in Table 1 are also shown corrected for variations in hemoglobin concentration. Regardless of which way the results are expressed, there is no overlap between the porphyric and control values. The highest cosynthetase activity in any porphyric subject was observed with patient

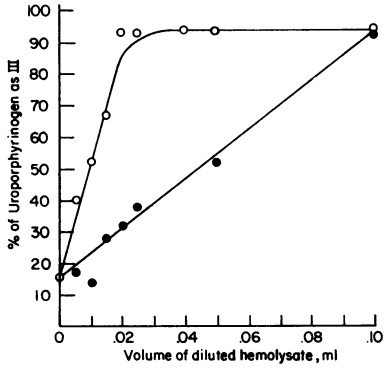

FIG. 1.—Cosynthetase activity of diluted (1/4) hemolysates. *Solid circles:* porphyric patient 1; *open circles:* control subject I*a*.

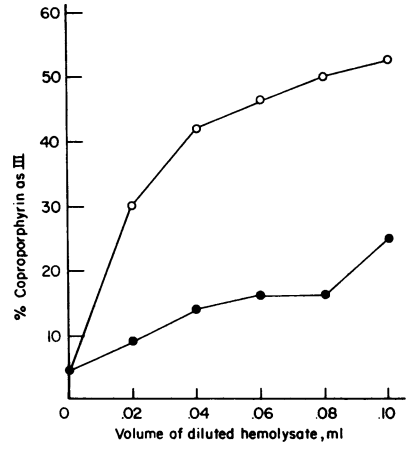

FIG. 2.—Cosynthetase activity of diluted (1/4) hemolysates. The uroporphyrinogen formed in the assay was chemically decarboxylated, and the resulting coproporphyrin isomers were separated chromatographically. *Solid circles:* porphyric patient III; *open circles:* control subject III*a*.

V; in this case the activity of cosynthetase per gram of hemoglobin was 41 per cent of the activity of the lower of the two controls. All other values in porphyrics were less than 30 per cent of the corresponding primary controls.

Patients I and V were each restudied about two months after the experiments shown in Table 1, again with freshly drawn blood samples. On these occasions their respective cosynthetase activities were 720 and 860 units per gram of hemoglobin, while the values on new control subjects (1 and 10 in Table 2) were 6480 and 1900 units per gram of hemoglobin.

Cosynthetase activity of hemolysates from other nonporphyric individuals: In addition to the ten controls described in Tables 1 and 2, blood samples from 15 other nonporphyric individuals have been tested for cosynthetase activity. The results obtained on these subjects and pertinent clinical information are described in Table 2. The lowest activity found in this group was 1670 units per gram of hemoglobin (subject 7), or twice the highest activity observed in a porphyric subject. These data provided confirmatory evidence that the cosynthetase activities measured in the blood of the porphyric individuals described in Table 1 are below the normal range.

Control experiments: If the amount of mouse-spleen synthetase used in the assay was doubled so as to form 10 mμmoles of product in 30 minutes, the amount of porphyric or normal hemolysate required for 50 per cent III was also doubled. This is the expected behavior of a synthetase–cosynthetase system.[7] Also, the cosynthetase activity of the hemolysates was completely destroyed in five minutes at 60°, which is consistent with the known thermal lability of cosynthetase from human red blood cells.[22]

TABLE 1. *Cosynthetase activity in hemolysates from porphyric patients and controls.*

Subject	Race,* sex, age	Diagnosis	Hemoglobin (gm/100 ml of blood)	Reticulocytes (% circulating red blood cells)	Cosynthetase Activity (units/ml)	Cosynthetase Activity (units/gm hemoglobin)
Patient I[11]	WM 2	Porphyria	11.8	0.8	90	760
Control I*a*	BM 4	Aseptic meningitis	9.8	1.4	445	4540
Control I*b*	WM 2	Pneumonia	10.7	2.0	800	7480
Patient II[12]	WF 23	Porphyria	14.0	2.2	62	440
Control II*a*	WF 32	Well	13.5	0.6	800	5930
Control II*b*	WF 26	Thalassemia minor	10.0	2.0	400	4000
Patient III[13]	RF 16	Porphyria	10.4	3.3	80	770
Control III*a*	WF 17	Well	13.8	0.6	400	2890
Control III*b*	WF 26	Well	15.9	1.2	425	2680
Patient IV[14]	WF 22	Porphyria†	10.0	12.0	40	400
Control IV*a*	WF 35	Well‡	13.8	2.4	480	3480
Control IV*b*	WF 22	Well	14.2	1.0	279	1970
Patient V[15]	WF 22	Porphyria	12.0	1.7	102	850
Control V*a*	WF 24	Well	14.4	1.1	300	2080
Control V*b*	WF 21	Well	12.5	1.4	634	5070

* Race: B, black; W, white; R, American Indian.
† Pregnant, 12th week.
‡ Pregnant, 20th week.

It is known that human red blood cells contain uroporphyrinogen synthetase activity[22] and that porphyric erythrocytes fluoresce because of the presence of uroporphyrin I.[1] Neither the synthetase nor the uroporphyrin present in the hemolysates interferes with the cosynthetase determinations in the present study because of the small volumes of hemolysate used for the assay. The activity of the endogenous synthetase in the largest amount of hemolysate assayed for cosynthetase (0.1 ml of a 1:4 dilution of hemolyzed whole blood) was less than 5 per cent of the activity of the synthetase added as the mouse-spleen preparation, except in patient IV, who had a reticulocyte count of 12 per cent; in this case the synthetase activity of this volume of hemolysate was 10 per cent of the activity of the mouse-spleen synthetase. The amount of fluorescence recovered when porphyric hemolysates were added to reaction mixtures from which mouse-

TABLE 2. *Cosynthetase activity in hemolysates from nonporphyric individuals.*

Subject	Race, sex, age	Diagnosis	Hemoglobin (gm/100 ml)	Reticulocytes (% red blood cells)	Cosynthetase Activity (units/ml)	Cosynthetase Activity (units/gm hemoglobin)
1	BM 4	Psychosocial dwarfism	8.8	3.0	570	6,480
2	WM 1	Diarrhea and dehydration	8.6	1.8	460	5,350
3	RF 5	Strabismus	11.5	1.5	750	6,520
4	WM 1	Recurrent vomiting	12.5	0.6	559	4,470
5	WF 1	Cleft lip repair	11.7	0.7	343	2,930
6	WM 3	Aspirin ingestion	12.0	1.0	218	1,820
7	WF 23	Well	14.4	0.7	240	1,670
8	WF 29	Well	14.0	2.0	308	2,200
9	BF 41	Systemic lupus erythematosis	7.4	2.8	500	6,760
10	WF 19	Well	13.6	0.7	258	1,900
11	RM 67	Pyridoxine-responsive anemia	7.0	12.0	800	11,400
12	WM 30	Well	15.0	1.8	460	3,070
13	WM 26	Well	15.6	1.0	370	2,370
14	WM 29	Well	15.2	0.6	667	4,380
15	WM 36	Well	14.9	1.6	750	5,040

spleen cosynthetase had been omitted or to complete reaction mixtures which had not been incubated was up to 20 per cent greater than in similar blanks containing normal hemolysates. However, the maximum amount of fluorescence recovered in these blanks was only 10 per cent of the amount recovered in complete reaction mixtures incubated in the usual way.

To evaluate the possibility that cosynthetase activity appeared to be low in porphyric blood because of the presence of an inhibitor of this enzyme, porphyric hemolysates were tested as inhibitors of the cosynthetase activity of normal hemolysates and of the cosynthetase activity of hematopoietic mouse spleen. No inhibition was observed; the cosynthetase extracted from porphyric hemolysates seemed in most experiments to function additively with cosynthetase from other sources. These experiments also indicate that the lower amount of uroporphyrinogen III in the product from reaction mixtures containing porphyric hemolysates was not the result of an increased activity of enzymes which convert

uroporphyrinogen III to other products. Direct experiments showed that under the conditions used in the cosynthetase assay, the amounts of hemolysate tested did not catalyze significant decarboxylation of the newly formed uroporphyrinogen III to coproporphyrinogen III.[23] It should be noted that the presence of an active decarboxylase in porphyric hemolysates would have decreased the amount of fluorescence recovered after esterification, because coproporphyrin is not eluted from talc by the method used in these studies. However, no decrease in the recovery of fluorescence was observed when the amounts of hemolysate were increased, nor was there any discrepancy between the amount of fluorescence recovered in porphyric compared to control samples. The partially purified mouse-spleen synthetase does not contain significant uroporphyrinogen decarboxylase activity under these conditions.

Cosynthetase activity of hemolysates assayed by separation of coproporphyrin isomers: The chromatographic method for the separation of uroporphyrin methyl esters has certain limitations. The R_f values for the I and III isomers are very different, but when mixtures of the two are chromatographed, each of the spots is contaminated with some of the other, apparently held in an intermolecular complex.[9] This difficulty can be minimized by spotting the same amount of ester from each sample,[17] and the method can be used as a basis for comparing one preparation of cosynthetase to another.[16] Nevertheless, to confirm the results obtained with this method, the isomer composition of the product formed when mouse-spleen synthetase was incubated with porphobilinogen in the presence of porphyric and normal hemolysates has also been examined by a different analytical procedure. In these experiments the uroporphyrinogen which had been formed enzymatically was decarboxylated chemically, and the resulting coproporphyrin isomers were separated chromatographically. Figure 2 shows the data from an experiment of this kind, carried out on the hemolysates from patient III and control III*a*. The results observed were similar to those seen when the uroporphyrin methyl esters were separated; once again, the percentage of III was much higher when normal blood was added to the reaction mixture than when porphyric blood was added. Experiments like that shown in Figure 2 have been performed on the other four porphyric patients as well, along with one primary control for each patient, with similar results. The conclusion that cosynthetase activity is lower in porphyric blood is thus not dependent on the method used to separate the porphyrin isomers.

Discussion.—These data show that the amount of enzymatically active uroporphyrinogen III cosynthetase in hemolysates from patients with congenital erythropoietic porphyria is from one tenth to one third of that in hemolysates from nonporphyric individuals. These results are like those reported previously for porphyric cattle[10] and support the view that the human and animal disorders are biochemically similar.[1] A complete absence of the catalytic activity of cosynthetase in porphyric tissues, analogous to the enzymatic defect in some other metabolic diseases, would not be expected, because the reaction in which cosynthetase is involved is essential to life. The partial deficiency of cosynthetase is a reasonable explanation for the production of uro- and coproporphyrin I in this disease. Formation of large amounts of porphyrins of the I series seems to be

responsible for the photosensitivity, erythrodontia, and porphyrinuria, and perhaps for the hemolytic anemia.[1]

It has been proposed that the enzymatic defect in congenital erythropoietic porphyria is limited to bone marrow,[24] because the overproduction of type I porphyrins has only been observed at this site. However, it is possible that cosynthetase activity is lower by two thirds in all porphyric tissues, but that only in erythropoietic tissue is the rate of porphyrin synthesis great enough to exceed the reduced capacity of the cosynthetase which remains. The enzymatic lesion would thus be present in all tissues, while the metabolic error occurred only in erythropoietic tissue. Clarification of this point awaits measurement of cosynthetase levels in nonhematopoietic organs from normal and porphyric individuals.

It seems probable that the demonstrated low cosynthetase activity in patients with erythropoietic porphyria is the primary genetic defect in this disease. Alternatively, it has been proposed by Watson *et al.*[25] that the basic enzymatic lesion is an increased activity of uroporphyrinogen I synthetase, which generates excessive amounts of an intermediate normally converted to uroporphyrinogen III in a reaction catalyzed by cosynthetase. The accumulation of this intermediate would exceed the capacity of cosynthetase to metabolize it, and it would spontaneously form uroporphyrinogen I. This would explain the reported overproduction of uroporphyrin III as well as uroporphyrin I in this disease.[25, 26] This hypothesis could be reconciled with the observations reported here that the activity of cosynthetase in porphyric tissues is low, because it has been shown that *in vitro* uroporphyrinogen III cosynthetase is inactivated during the reaction catalyzed by uroporphyrinogen I synthetase.[7] If this inactivation also occurs *in vivo*, it is possible that elevated synthetase levels deplete the tissue stores of cosynthetase, permitting uroporphyrinogen I formation to proceed. To distinguish between a primarily deficient cosynthetase and a secondarily depleted one will require measurement of the levels of synthetase found in normal and porphyric tissues and an investigation of the possibility that synthetase inactivates cosynthetase *in vivo*.

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