# Harderoporphyria: A Variant Hereditary Coproporphyria

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A B S T R A C T   Three siblings with intense jaundice and hemolytic anemia at birth were found to excrete a high level of coproporphyrin in their urine and feces; the pattern of fecal porphyrin excretion was atypical for hereditary coproporphyria because the major porphyrin was harderoporphyrin (>60%; normal value is <20%). The lymphocyte coproporphyrinogen III oxidase activity of each patient was 10% of control values, which suggests a homozygous state. Both parents showed only mild abnormalities in porphyrin excretion and lymphocyte coproporphyrinogen III oxidase activity decreased to 50% of normal values, as is expected in heterozygous cases of hereditary coproporphyria. Kinetic parameters of coproporphyrinogen III oxidase from these patients were clearly modified, with a Michaelis constant 15–20-fold higher than normal values when using coproporphyrinogen or harderoporphyrinogen as substrates. Maximal velocity was half the normal value, and we also observed a marked sensitivity to thermal denaturation. The possibility that a mutation affecting the enzyme on the active center which is specifically involved in the second decarboxylation (from harderoporphyrinogen to protoporphyrinogen) was eliminated by experiments on rat liver that showed that coproporphyrinogen and harderoporphyrinogen were metabolized at the same active center. The pattern of porphyrin excretion and the coproporphyrinogen oxidase from the three patients exhibited abnormalities that were different from the abnormalities found in another recently described homozygous case of hereditary coproporphyria. We suggest naming this variant of coproporphyrinogen oxidase defect "harderoporphyria."

## INTRODUCTION

Coproporphyrinogen III oxidase (EC 1.3.3.3.) is the enzyme of the heme pathway that catalyzes the sequential decarboxylation of coproporphyrinogen to protoporphyrinogen. The reaction is shown in Fig. 1: The propionyl groups in position 2 and 4 of coproporphyrinogen are decarboxylated and oxidized to yield the two vinyl groups of protoporphyrinogen. Several lines of evidence imply that the tricarboxylic intermediate is harderoporphyrinogen; the propionyl group on position 2 of coproporphyrinogen is decarboxylated first (1).

Hereditary coproporphyria (HC)[1] is a genetic disorder of heme and porphyrin biosynthesis and is inherited as an autosomal dominant disorder clinically resembling two other forms of inherited hepatic porphyria, intermittent acute porphyria and porphyria variegata (2). This disorder is characterized biochemically by the excretion of large amounts of coproporphyrin III, mainly in feces. Data from several investigations (3–5) support the idea that coproporphyrinogen III oxidase deficiency (50%) is the primary gene defect in HC. Although it is usually expressed in the heterozygous state, a case of homozygous HC was recently described (6, 7). The patient in this case was found to excrete very large amounts of coproporphyrin in the urine and feces; lymphocyte coproporphyrinogen III oxidase activity was only 2% of the control level.

This paper describes a previously unreported variant of porphyria that is characterized by the accumulation of harderoporphyrin in feces of homozygous patients.

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[1] *Abbreviations used in this paper:* ALA, δ-aminolevulinic acid; HC, hereditary coproporphyria; HPLC, high pressure liquid chromatography.

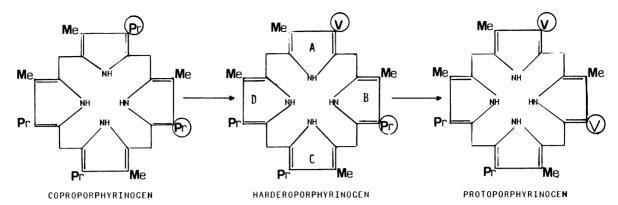COPROPORPHYRINOGEN     HARDEROPORPHYRINOGEN     PROTOPORPHYRINOGEN

FIGURE 1 Biosynthesis of protoporphyrinogen from coproporphyrinogen III. This stepwise reaction is catalyzed by coproporphyrinogen oxidase. Harderoporphyrinogen is the natural intermediate between copro- and protoporphyrinogen. Isoharderoporphyrinogen (vinyl group on pyrrole B instead of pyrrole A) has not been isolated from tissues synthesizing protoporphyrin IX; however, isoharderoporphyrinogen is a known substrate of coproporphyrinogen oxidase (17). Me, methyl ($-CH_3$); Pr, propionyl ($-CH_2-CH_2-COOH$); V, vinyl ($-CH=CH_2$).

The molecular basis of this disease is shown to be a mutation leading to the presence (at least in lymphocytes) of a coproporphyrinogen III oxidase with modified kinetic properties; this indicates a structural abnormality of the enzyme which is distinct from a case previously described.

## METHODS

Case reports. The patients are three siblings born in 1973, 1975, and 1980 from healthy, nonconsanguineous French parents. Porphyria was first discovered in the second child, S.M., at the Department of Pediatrics of Lille Hospital when he developed an intense jaundice shortly after his birth. The perinatal history revealed normal pregnancy and delivery. Physical findings in addition to jaundice included hepatosplenomegaly. The total serum bilirubin level was 16.7 mg/dl with an unconjugated bilirubin value of 12.1 mg/dl. The blood group of the baby was the same as that of the mother (O Rh negative). Hematologic data are shown on Table I; the erythrocyte morphology was normal. Exchange transfusion was immediately performed and followed by phototherapy. Soon afterwards, the baby showed a rash with vesicles and blisters and a diagnosis of pemphigus was considered. A second rash appeared a few days later when the

TABLE I

Representative Hematologic Values of the Patients and Their Parents

| Patients | Date | RBC | Hb | Retics | Ht | Platelets |
|---|---|---|---|---|---|---|
| | | $\times 10^6/\mu l$ | g/100 ml | % | | $\times 10^3/\mu l$ |
| S.M. | 25 August 1975* | 4.0 | 12.4 | 10.0 | 38 | 83 |
| | 25 November 1975 | 2.7 | 6 | 15 | 25 | — |
| | 10 July 1977 | 3.7 | 9.1 | 3.5 | 31 | 396 |
| | 1 February 1978 | 4.3 | 8.5 | 2.6 | 31 | 350 |
| D.M. | 11 October 1973* | 4.3 | 10.8 | 12 | 40 | 82 |
| | 2 November 1973 | 3.0 | 8.0 | 25 | 24 | — |
| | 10 July 1977 | 4.0 | 9.2 | 5 | 30 | 230 |
| A.M. | 13 October 1980* | 5.1 | 12.0 | 10.0 | 43 | 85 |
| | 22 October 1980 | 4.00 | 8.8 | 16.0 | 30 | — |
| | 28 September 1981 | 3.85 | 9.7 | 5.5 | 32 | 600 |
| Parents | | | | | | |
| Mother | 2 March 1983 | 4.50 | 13.4 | 1.0 | 41.0 | 486 |
| Father | 2 March 1983 | 4.88 | 14.7 | 1.2 | 46.5 | 311 |

Abbreviations used in this table: RBC, erythrocytes; Retics, reticulocytes; Ht, hematocrit; Hb, hemoglobin.
* Date of birth.

primary blisters were not yet completely healed. At this time, a red discoloration of the urine was observed, and the diagnosis of inherited porphyria was confirmed by the high levels of uro- and coproporphyrin found in the urine (Table II). No further biological investigation was done in 1975 on this child.

The elder brother, D.M., had presented similar symptoms on the day of his birth. However, like his younger brother, he was sent home with the diagnosis of hemolytic anemia; the cause remains unknown at this time. During the next two years, his hepatosplenomegaly disappeared and a compensating hemolytic process persisted. His growth and development remained normal; neither abnormal cutaneous features nor red discolorations of the urine or teeth were noticed. However, in 1975, when urine porphyrins were studied, a very high level of coproporphyrin was found (Table II).

In 1980, the birth of a girl, A.M., with clinical features similar to those of her two brothers prompted the physicians to send us samples of urine, feces, and blood from the three children for biochemical investigations. After the neonatal period, hepatomegaly disappeared in all patients but slight splenomegaly was still found together with pallor and a persistent hemolytic process (Table I). Growth and development remained normal. Neither abnormal cutaneous features nor red coloration of teeth or urine were noticed. Abdominal and neurological symptoms that are typical of hepatic porphyrias never appeared in either the children or the parents. All hematologic values of the parents were normal.

*Procedures.* Chemicals were obtained from the following sources: [4-$^{14}$C]δ-aminolevulinic acid (ALA), Amersham; [2,3-$^3$H]ALA, Commissariat a l'Energie Atomique, France; coproporphyrin and protoporphyrin, Sigma Chemical Co., St. Louis, MO. Standard hardero- and isoharderoporphyrin were gifts from Dr. K. M. Smith (University of California, Davis); Ficoll, Pharmacia Fine Chemicals, Piscataway, NJ; Na-metrizoate, Nyegaard, Oslo, Norway; and Aquasol, New England Nuclear Boston, MA. All other chemicals used were of reagent grade and were obtained from the usual commercial sources.

Lymphocytes were isolated from heparinized blood by centrifugation using the Ficoll-metrizoate mixture (8). For elimination of possible erythrocyte contamination, lymphocytes were treated with 0.15 M NH$_4$Cl for 15 min at 37°C. After being washed with 0.15 M NaCl, the cells were stored as a pellet at −20°C until required for enzyme assay (usually 24 h). They were then thawed (in 0.15 M NaCl) and frozen twice. After centrifugation, the supernatant was retained for assay (more than 95% of the total enzyme activity was usually found). Protein concentration was estimated by the method of Lowry et al. (9) using bovine serum albumin (BSA) as standard. Rat liver homogenate was prepared as described previously (10).

*Porphyrin data.* Urinary, fecal, and erythrocyte porphyrins were determined spectrophotometrically after extraction by the usual methods (11). Determinations of ALA and porphobilinogen in the urine were done according to the method of Mauzerall and Granick (12). The concentrations of porphyrins and porphyrin methyl esters were measured by using spectrophotometry (13); 180 was used as millimolar extinction coefficient for the harderoporphyrin trimethyl ester (14). The absorption spectrum of harderoporphyrin trimethyl ester was obtained on a Beckman spectrophotometer (model 35, Beckman Instruments, Inc., Fullerton, CA). Total fecal porphyrin was calculated as the sum of the coproporphyin and protoporphyrin fractions. To perform high pressure liquid chromatography (HPLC) analysis, acid ex-

tracts obtained by solvent extraction were adjusted to pH 3–4 and extracted in ethylacetate-acetic acid (3:1, vol/vol). The mixture was evaporated under reduced pressure at 45°C and porphyrins were treated overnight with 50 ml of methanol-sulfuric acid (95:5, vol/vol). Porphyrin esters were extracted into chloroform as previously described (15) and an aliquot was then injected into a Perkin-Elmer high pressure liquid chromatograph (model 604, Perkin-Elmer Corp., Norwalk, CT). The column used was a 30 × 0.4 cm (10 μm) Porasil (Waters Instruments, Inc., Rochester, MN). The chromatogram was recorded at 404 nm using an LC 55 Perkin-Elmer detector. Peak areas were determined with a computing integrator (icap 5, LTT, Paris, France). Analysis was carried out successively in two different solvent systems at a flow rate of 1.5 ml/mn. The first one (ethylacetate/cyclohexane, 55:45, vol/vol) separated porphyrin esters with 2 to 8 carboxylic groups. The second system of lower polarity (ethylacetate/cyclohexane, 1:3, vol/vol) allowed a better separation of porphyrin esters with 2, 3, and 4 carboxylic groups. The isomeric type of coproporphyrin was determined after hydrolysis of corresponding porphyrin esters (16) isolated by HPLC.

Mass spectra were done by Dr. Beaucourt from Commissariat a l'Energie Atomique (Saclay) using an electronic impact technique with a mass spectrometer (CH 7 A, Varian Associates, Palo Alto, CA)

*Preparation of radiolabeled substrates.* Radioactive ALA from the commercial source was diluted with cold ALA to certain specific activities. [$^{14}$C]Coproporphyrin III was obtained from 50 μCi of [4-$^{14}$C]ALA (2.5 mCi/mmol) using a human erythrocyte hemolysate as described previously (4, 10). Tritiated coproporphyrin III and harderoporphyrin were synthesized similarly using 1 mCi of [2,3$^3$H]ALA (1.5 mCi/μmol), except that the incubation was carried out under aerobic conditions.

Radioactive harderoporphyrin was isolated by thin-layer chromatography as described for coproporphyrin (10). The specific activities of all radioactive porphyrins synthesized matched those expected from stoichiometric conversion of ALA. Porphyrin esters were hydrolyzed with 200 μl of 6 N HCl at room temperature in the dark for 48 and 16 h for coproporphyrin and harderoporphyrin, respectively. Then, the hydrochloric solution was dried in vacuo over KOH. Porphyrins were conserved in 0.05 N KOH at −20°C for [$^{14}$C]coproporphyrin and at 4°C for [$^3$H]porphyrins.

*Measurement of coproporphyrinogen oxidase activity.* The standard method using [$^{14}$C]coproporphyrinogen (20 mCi/mmol) as substrate was described in detail elsewhere (10). Briefly, [$^{14}$C] coproporphyrin was reduced with sodium amalgam and incubated for 1 h with the enzymatic preparation (~0.2 to 0.3 mg protein) in a reaction mixture of 0.55 ml containing Tris-HCl, 110 mmol/l; ascorbate, 4.5 mmol/l; albumin, 2.3 mg/ml; and coproporphyrinogen, 1.3–1.6 μmol/l. The products formed (protoporphyrin and harderoporphyrin) were isolated by methylation, extraction, and thin-layer chromatography, and then were quantitated by scintillation counting (10). In all experiments, a blank without enzymes was included.

*Kinetic studies.* For Michaelis constant ($K_m$) and maximum velocity ($V_{max}$) determinations, tritiated coproporphyrin and harderoporphyrin were diluted to a specific activity of 100 mCi/mmol; the corresponding cold porphyrin was then reduced to correspond to porphyrinogen with sodium amalgam as already described (4, 10).

*Thermal denaturation.* Thermal denaturation was studied by preincubating the enzyme at 50°C in the reaction mixture in the absence of substrate for 15 min. The tube was

then cooled in ice and incubated at 37°C for 1 h after addition of radioactive coproporphyrinogen. The activity of the enzyme without thermal denaturation was measured simultaneously.

## RESULTS

*Overproduction of porphyrins.* Stool porphyrin content of the cases 1, 2, and 3 was strongly elevated (Table II). Analysis by HPLC showed a very peculiar pattern (Fig. 2), which was identical for the three children, with the prominence of a porphyrin with a retention time that was intermediate between those of coproporphyrin and protoporphyrin. This porphyrin was further identified as "Harderoporphyrin." A small amount of harderoporphyrin was also noted in the feces of the parents. Fecal coproporphyrin from the children was 80% type III. Study of their urine revealed a large amount of coproporphyrin and a trace of harderoporphyrin (Table II and Fig. 2). The father had elevated coproporphyrin, uroporphyrin, ALA, and porphobilinogen excretion in his urine, while the mother demonstrated only increased urinary ALA and porphobilinogen (Table II). Erythrocyte protoporphyrin from the three children was slightly elevated, but no harderoporphyrin could be found by HPLC (data not shown).

Harderoporphyrin ester isolated from feces was identified as follows: (*a*) HPLC analysis showed that its retention time was identical to that of standard harderoporphyrin methyl ester (Fig. 2). (*b*) Its absorption spectrum in chloroform was identical to the spectrum of standard harderoporphyrin methyl ester (peaks at 403, 503, 536, 572, and 624 nm). (*c*) When harderoporphyrin ester from feces was hydrolyzed, reduced

with sodium amalgam to harderoporphyrinogen, and incubated with a rat liver homogenate, complete conversion to protoporphyrin IX occurred (data not shown).

*Coproporphyrinogen oxidase activities.* Coproporphyrinogen oxidase activities in lymphocytes from patients 1, 2, and 3 were decreased to 10% of the mean control value, whereas both parents had an activity in the range of coproporphyric patients (Table III). Harderoporphyrin that had formed was also quantitated and the ratio of harderoporphyrin/harderoporphyrin plus protoporphyrin was calculated (Table III). This ratio was equally increased in all three patients. The proportion of harderoporphyrin synthesized by lymphocytes of both parents was in the normal range.

*Kinetic characteristics of coproporphyrinogen oxidase from the patients.* Subsequent studies were performed using lysates of pooled lymphocytes from the three children. This was necessary to obtain enough material for further experiments.

The kinetic characteristics of the abnormal enzyme were determined using [$^3$H]coproporphyrinogen and [$^3$H]harderoporphyrinogen as substrates. When coproporphyrinogen was the substrate, the amounts of protoporphyrin and harderoporphyrin formed were estimated: With control cells, the proportion of harderoporphyrin from harderoporphyrin and protoporphyrin formed ranged between 30 and 45%, and the lowest ratios of harderoporphyrin/harderoporphyrin plus protoporphyrin were found at low substrate concentrations (data not shown). In contrast, with lymphocytes from the three patients, the proportion of harderoporphyrin was high (60–70%) and was indepen-

TABLE II
*Porphyrin Concentration in Urines, Feces, and Erythrocytes of Patients and Family*

| Subject | Age | Urine | | | | Feces | | | | Erythrocytes | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ALA | PBG | URO | COPRO | Total | COPRO‡ | HARDERO‡ | PROTO‡ | COPRO | PROTO |
| | yr | μmol/liter | μmol/liter | nmol/liter | nmol/liter | nmol* | % | % | % | nmol/liter | nmol/liter |
| Father | 28 | 55 | 19 | 156 | 1,093 | 54 | 47 | 10 | 43 | Traces | 320 |
| Mother | 28 | 50 | 14 | 24 | 247 | 48 | 32 | 8 | 60 | Traces | 402 |
| Patient 1 (S.M.) | 6 | 57 | 20 | 150 | 2,144 | 342 | 26 | 66 | 8 | Traces | 1,535 |
| Patient 2 (D.M.) | 8 | 50 | 11 | 35 | 1,980 | 656 | 29 | 66 | 4 | Traces | 1,740 |
| Patient 3 (A.M.) | 1 | 40 | 11 | 320 | 2,560 | 272 | 21 | 65 | 14 | 42 | 2,398 |
| Coproporphyric patients (asymptomatic carriers) (*n* = 10) | | 27.2±0.51 | 8.1±5.2 | 20±10 | 295±24 | 735±543 | 85 | 2.5 | 12.5 | <150 | <1,240 |
| Normal controls | | <38 | <9 | <40 | <382 | <170 | 31±16 | 12±5 | 57±19 | <150 | <1,240 |

*Abbreviations used in the table:* ALA, δ-aminolevulinic acid; PBG, porphobilinogen; COPRO, coproporphyrin; HARDERO, harderoporphyrin; PROTO, protoporphyrin.
* Results are expressed per gram dry weight.
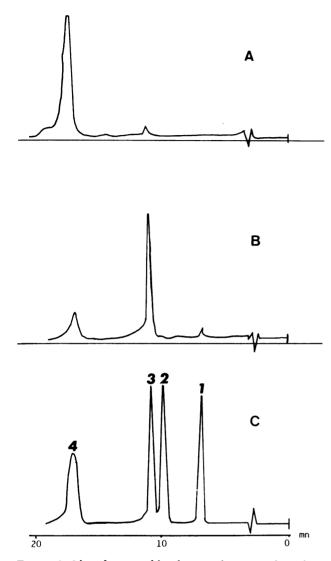‡ The percentage of each porphyrin in feces was determined by mean±SD (*n* = 10).

FIGURE 2 Identification of harderoporphyrin. High performance liquid chromatography of porphyrin methyl esters from urine (A) and feces (B) of the patients was performed as described in Methods (solvent system, ethyl acetate:cyclohexane, 1:3, vol/vol). A mixture of standard porphyrin esters was also separated (C) under the same conditions. 1, protoporphyrin; 2, isoharderoporphyrin; 3, harderoporphyrin; 4, coproporphyrin.

dent of coproporphyrinogen concentration. Therefore, the sum of protoporphyrin and harderoporphyrin was used for determination of kinetic parameters.

Fig. 3 shows double-reciprocal plots of the rate of products formed as a function of the concentrations of [³H]coproporphyrinogen (Fig. 3 A) and [³H]-harderoporphyrinogen (Fig. 3 B). With the patients' coproporphyrinogen oxidase, similar abnormalities were found with both substrates (Table IV): the $K_m$ values
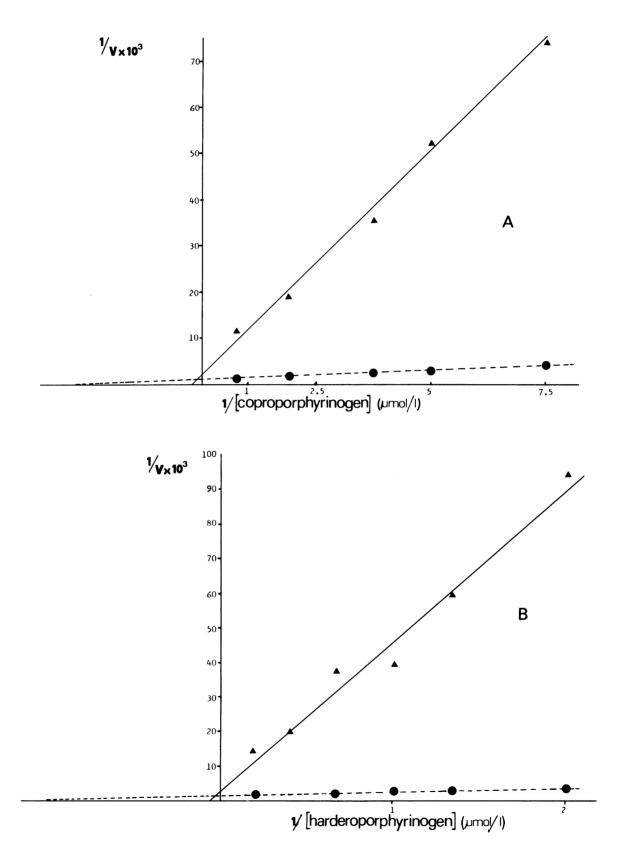
were greatly increased (>10 times the normal values) and $V_{max}$ values were decreased by ~50%.

With lymphocytes from the parents, studies of the formation of harderoporphyrinogen plus protoporphyrinogen as a function of coproporphyrinogen concentration (Fig. 4) gave results compatible with a biphasic double-reciprocal plot. Intercepts of the lines with the abscissa allowed us to calculate two $K_m$ values: the lower one was similar to the $K_m$ value of normal subjects while the higher one was almost identical to the value found with children's enzyme.

To provide additional information about the properties of the coproporphyrinogen oxidase from patients, the thermal denaturation was studied at 50°C. Results (Table V) indicate the greater thermosensitivity of the patients' enzyme. The enzyme in typical coproporphyric patients did not differ from controls.

The thermal inactivation pattern of the parents' enzyme (Table V) also indicated a greater thermosensitivity than normal controls. However, it has to be kept in mind that the parents still have ~50% of the normal enzyme, which presumably explains why their coproporphyrinogen oxidase is not as thermosensitive as the children's enzyme.

*Evidence for a single site of decarboxylation for coproporphyrinogen oxidase.* It appeared that the accumulation of harderoporphyrin in vivo and in vitro (as was observed in the coproporphyrinogen oxidase assay

TABLE IV

Kinetic Parameters for Normal and Patient's Lymphocyte Coproporphyrinogen Oxidase

| | Substrate | Controls mean±SD | Patients |
|---|---|---|---|
| $K_m$* ($\mu mol/liter$) | Coproporphyrinogen | 0.34±0.06 ($n = 4$) | 4.8 |
| | Harderoporphyrinogen | 0.74±0.40 ($n = 3$) | 15 |
| $V_{max}$‡ | Coproporphyrinogen | 1,010±240 ($n = 3$) | 498 |
| | Harderoporphyrinogen | 1,360±229 ($n = 3$) | 740 |

* $V_{max}$ and $K_m$ were obtained from analyses of Fig. 3.
‡ $V_{max}$ are expressed in picomoles of product(s) formed per milligram protein per hour.

with their lymphocytes) needed to be discussed in relation to the properties of the normal enzyme. The apparent selective impairment of the second decarboxylation in the patients, which is reported here, does not appear to corroborate with the hypothesis of Elder et al. (17), which suggests that the two coproporphyrinogen oxidase-catalyzed decarboxylations take place at the same active site. Therefore, we decided to reinvestigate this question with kinetic experiments using the rat liver enzyme: The products formed from various concentrations of either [³H]coproporphyrinogen or [³H]harderoporphyrinogen were quantitated in the presence or absence of the cold alternate substrate. In agreement with other reports (18), it was found that cold harderoporphyrinogen competitively inhibited the decarboxylation of labeled coproporphyrinogen. Moreover, when [³H]harderoporphyrinogen was used as substrate, the formation of radioactive protoporphyrinogen was also competitively inhibited by cold coproporphyrinogen. $K_m$ and inhibition constant ($K_i$) values and $V_{max}$ were calculated (Table VI) for both substrates (when considered as inhibitors and assuming a competitive type of inhibition); $K_m$ and $K_i$ remained similar. In addition, coproporphyrin III competitively inhibited the decarboxylation of both substrates coproporphyrinogen and harderoporphyrinogen with apparently the same $K_i$ (Fig. 5).

Fig. 6 illustrates experiments that use tritiated coproporphyrinogen as a substrate in which the ratio of [³H]harderoporphyrinogen to [³H]harderoporphyrinogen plus [³H]protoporphyrin was calculated. For a large range of concentrations of radioactive copropor-

phyrinogen, the ratio was constant and remained unmodified by addition of cold harderoporphyrinogen to the incubation. These data supported the idea that a fraction of harderoporphyrinogen synthesized from coproporphyrinogen does not leave the active center of coproporphyrinogen oxidase before being decarboxylated to yield protoporphyrinogen (17). Thus, this fraction is not susceptible to isotopic dilution by adding cold harderoporphyrinogen. Under conditions of low substrate concentrations and with the absence of cold harderoporphyrinogen, the fraction of radioactive harderoporphyrinogen released from coproporphyrinogen oxidase can bind again to the enzyme and be further metabolized into protoporphyrinogen.

## DISCUSSION

The three children reported here have a porphyria with a very early clinical onset of hemolysis. The pattern of porphyrin excretion was clearly different from any other previously described case since the major fecal porphyrin was harderoporphyrin while a large amount of coproporphyrin was found in the urine. Assuming a dry weight of feces of 20 g/d with a urinary volume of 0.8 liter, one can compute from Table II that roughly two-thirds of the total porphyrin excretion per 24 h was harderoporphyrin. For this reason, we propose to name this previously uncharacterized porphyria "Harderoporphyria."

Coproporphyrin is excreted predominantly in urine, whereas harderoporphyrin appears mostly in feces. This difference presumably relates to the lower polar-

FIGURE 3 Double-reciprocal plot of the effect of coproporphyrinogen III (A) and harderoporphyrinogen (B) concentration on lymphocyte coproporphyrinogen oxidase activity. Substrate concentrations varied from 0.13 $\mu$mol/liter to 1.3 $\mu$mol/liter for coproporphyrinogen (A) and from 0.5 $\mu$mol/liter to 5 $\mu$mol/liter for harderoporphyrinogen (B). Under the experimental conditions, <10% of the substrate was consumed during the incubation (30 min). V is calculated as the rate of harderoporphyrinogen plus protoporphyrinogen (A) or protoporphyrinogen (B) formed in picomoles per milligram protein per hour. ●, normal human lymphocytes (0.1 mg protein); ▲, porphyric patients' lymphocytes (0.3 mg protein).
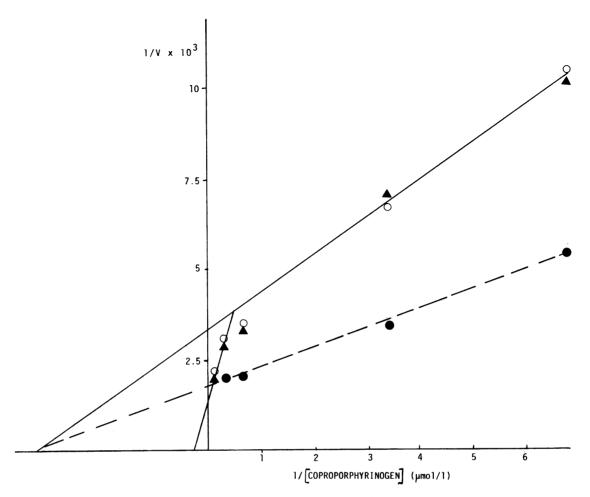
FIGURE 4  Double-reciprocal plot of the effect of coproporphyrinogen III concentration on lymphocyte coproporphyrinogen oxidase activity from parents. Coproporphyrinogen varied from 0.15 $\mu$mol/liter to 7.5 $\mu$mol/liter. $V$ is calculated as the rate of harderoporphyrinogen plus protoporphyrinogen formed in picomoles per milligram protein per hour. ●, normal human lymphocytes (0.2 mg protein); ○, mother lymphocytes (0.3 mg protein); ▲, father lymphocytes (0.3 mg protein).

ity of harderoporphyrin as compared with copropor-phyrin and hence, to its lower water solubility and limited presence in urinary excretion.

The clinical symptomatology was dominated by the very early onset of hemolytic anemia which improved during the first year and is mild at the present time.

TABLE V
Thermal Inactivation of Lymphocyte Coproporphyrinogen Oxidase

| | Controls ($n$ = 7) mean±SD | Coproporphyric patients ($n$ = 6) mean±SD | Harderoporphyric patients | | |
|---|---|---|---|---|---|
| | | | Cases 1, 2, and 3 | Mother | Father |
| Percentage of initial activity | 56±8.7 | 56±6 | 4° | 30 | 24 |

Activities of coproporphyrinogen oxidase were measured before and after thermal denaturation (15 min at 50°C).
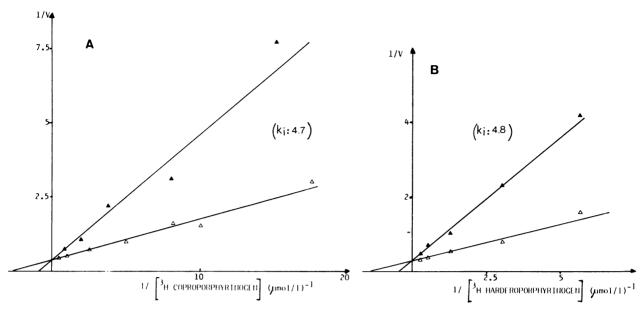° Mean of two determinations.

FIGURE 5 Double-reciprocal plot of the inhibitory effect of coproporphyrin III (8 $\mu$mol/liter) on rat liver coproporphyrinogen oxidase. (A) With [$^3$H]coproporphyrinogen as substrate. (B) With [$^3$H]harderoporphyrinogen as substrate. The apparent $K_i$ of coproporphyrin has been calculated from these plots. $\triangle$, no inhibitor; $\blacktriangle$, with inhibitor.

In patient 1, although no relapse of photosensitivity was noted, phototherapy for hyperbilirubinemia induced a bullous eruption. Cutaneous manifestations are commonly seen in different types of porphyria, except acute intermittent porphyria (2), and they are thought to be related to porphyrin-mediated phototoxicity at the skin level (19). Such a mechanism may explain the photosensitivity induced by phototherapy. In contrast, the relationship between the anemia and the biological abnormalities reported here was unclear. Hemolytic anemia sometimes occurs in congenital erythropoietic porphyria (2) and it has also been reported in a few cases of erythrohepatic porphyria (20). In those cases, anemia is presumably related to the high porphyrin content of erythrocytes. In the present cases, only a moderate increase of erythrocyte protoporphyrin level was noted; however, measurement was done when the anemia was mild and a high erythrocyte porphyrin level at birth remains a possibility. However, no harderoporphyrin was found in children's or their parents' erythrocytes.

Enzymatic studies of coproporphyrinogen oxidase in lymphocytes from these patients clearly revealed modified kinetic parameters and a marked sensitivity to thermal denaturation. The very low activity measured with our standard assay (Table III) was mainly attributed to a highly increased $K_m$ of the enzyme for coproporphyrinogen; the standard concentration of the substrate ($\simeq$1.4 $\mu$mol) that was used was obviously

much too low to obtain the $V_{max}$ of the abnormal enzyme. These findings strongly suggest that no detectable normal enzyme was present in lymphocytes from these patients. Studies of the $K_m$ and the thermosensitivity of coproporphyrinogen oxidase in lymphocytes from the parents suggest that their intermediate coproporphyrinogen oxidase activity results from a mixture of normal and abnormal enzymes. The most probable interpretation is that the three children studied were homozygous for a gene coding for a structurally modified coproporphyrinogen oxidase, while their parents are both heterozygotes for the same defect.

Although the mutant enzyme was only assayed in lymphocyte lysates, it is likely that the defect is not restricted to those cells, but is also present in other tissues, as demonstrated in other types of porphyria (2).
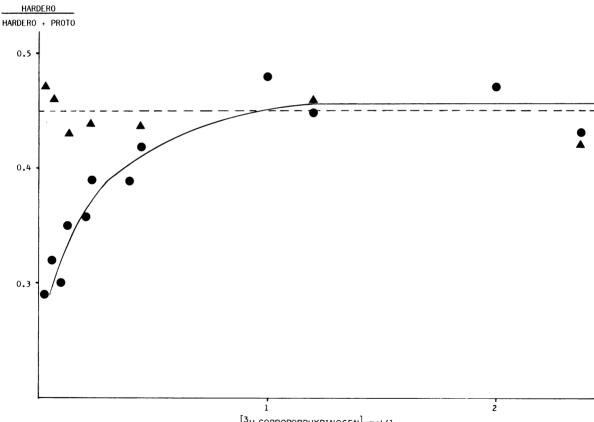
The large overproduction of porphyrins is in agreement with the commonly held idea that an enzymatic defect along the heme pathway leads to derepression of the first and rate-limiting enzyme, ALA-synthetase, in the liver (21) and possibly in some other organs (22). The derepression of the first enzyme is followed by an increased synthesis of intermediate substrates of the metabolic pathway, which are lost by the cell as their intracellular concentration increases (23). The unique pattern of fecal porphyrin excretion reported here (the predominance of harderoporphyrin) can be explained by the nature of the enzyme abnormality in relation

to the properties of coproporphyrinogen oxidase. Previous studies with rat liver coproporphyrinogen oxidase show that harderoporphyrinogen is a competitive inhibitor of the decarboxylation of coproporphyrinogen (18). The results (Table VI) confirm this finding and show that, reciprocally, coproporphyrinogen competitively inhibits the decarboxylation of harderopor-

phyrinogen. In addition, coproporphyrin III, a competitive inhibitor of coproporphyrinogen decarboxylation (Fig. 5), equally affects the decarboxylation of harderoporphyrinogen. When considered together, these results confirm the hypothesis that only one active site exists for the two decarboxylations catalyzed by coproporphyrinogen oxidase. Moreover, the data presented in Fig. 6 are in agreement with the idea that during the sequential decarboxylation of coproporphyrinogen to protoporphyrinogen, most of the intermediate harderoporphyrinogen stays on the enzyme surface before being decarboxylated. Consistent with this hypothesis, coproporphyrinogen oxidase from harderoporphyric patients had a similarly increased $K_m$ for both substrates: coproporphyrinogen and harderoporphyrinogen (Table IV). Due to the reduced affinity of the abnormal coproporphyrinogen oxidase, harderoporphyrinogen may leave the enzyme surface more easily and this may account for its accumulation in patients.



FIGURE 6 Biosynthesis of harderoporphyrin by rat liver coproporphyrinogen oxidase. The ratio of [3H]harderoporphyrin over [3H]harderoporphyrin plus [3H]protoporphyrin was plotted against [3H]coproporphyrinogen concentration. ●, without harderoporphyrinogen; ▲, in the presence of harderoporphyrinogen.

We previously described a homozygous case of coproporphyria with a very low activity of coproporphyrinogen oxidase. This patient excreted almost only coproporphyrin (6). In contrast to the cases reported here, residual coproporphyrinogen oxidase had an apparently normal $K_m$ for coproporphyrinogen and normal thermosensitivity (data not shown). It seems therefore logical to attribute the different porphyrin excretion pattern in the present patients to a different coproporphyrinogen oxidase abnormality.

The parents of the harderoporphyric patients were clinically asymptomatic, although they showed slightly abnormal porphyrin excretion in urine and a decreased activity of coproporphyrinogen oxidase in their lymphocytes. These biological features are identical to those usually encountered in subjects with clinically latent coproporphyria (4). Therefore, it is attractive to speculate that our patients with harderoporphyria may be homozygous for a gene that causes hereditary coproporphyria in some families. This hypothesis would imply a genetic heterogeneity in coproporphyria because, in the homozygous case previously reported (6), porphyrin excretions as well as properties of the defective enzyme were clearly different. Alternatively, harderoporphyria may be due to a mutation never encountered previously.

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