6-Aminolevulinic Acid Synthetase Activity in Human Plasma: Relation to Erythropoiesis and Evidence of Induction in Erythropoietic Porphyria

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Measurement of 6-aminolevulinic acid synthetase (ALA-S) activity in human plasma has been carried out with samples from normal individuals, cases of erythropoietic porphyria (EP) and erythropoietic protoporphyria (EPP), and of the three principal forms of hepatic porphyria—acute intermittent porphyria, variegate porphyria, and porphyria cutanea tarda. The method of measurement depends on formation of 14C-ALA when the plasma is incubated with 14C-succinic acid, succinyl-Co A synthetase, glycine, and other essential substances. The normal samples, as well as those from the hepatic porphyria cases, had small but significant activity of the same extent; those from the erythropoietic group showed consistently higher values, especially in the two cases of congenital type. A remarkably high value in one of these cases in which there was outspoken erythropoiesis was believed to be related to the presence of many fluorescing normoblasts in the peripheral blood. Following multiple transfusions these disappeared concomitantly with striking reduction of the porphyria. The plasma ALA-S activity declined to 1.4% of the pretransfusion value. These results are considered in respect to the question of induction of ALA-S in the developing red cells of the disease, special attention being given to the minor increase of ALA-S activity in the plasma of a nonporphyric individual whose peripheral blood contained large numbers of circulating normoblasts.

As is well known, ALA-S is a mitochondrial enzyme that initiates and limits the synthesis of Proto- and heme.\(^1,2\) Its presence has been demonstrated in nucleated red cells and reticulocytes,\(^3\) as well as liver cells.\(^2\) Current knowledge of porphyrin biosynthesis has been considered in some detail in recent reviews.\(^4,5\) In the context of the present study it will suffice to mention briefly the more significant information relating to the induction of ALA-S in the forms of human porphyria. In two of the three principal types of hepatic porphyria, i.e., AIP and VP, direct evidence of induction of the enzyme in the
Liver has been described in the third, PCT, the evidence is less convincing and, in fact, the most recent report based on direct assay of 14C-ALA formed fails to provide evidence of induction. The large amounts of PBG often noted in the urine in asymptomatic cases of AIP may indicate that there is induction of ALA-S even in clinical remission.

The suggestion was made in 1964 that the overproduction of types I and III porphyrins in EP might be due to a regulator constitutive mutation with relative deficiency of isomerase (uroporphyrinogen III cosynthetase) to account for the large amounts of type I Uro- and Copro- of the circulating red cells and excreta. More recently, Romeo and Levin have given particular emphasis to the isomerase deficiency, minimizing the significance of the very considerable net overproduction of type III porphyrins. This will be considered in more detail in a separate communication.

In the disease initially described as EPP, the greatly excessive free Proto- of the circulating red cells, plasma and feces, was believed to derive entirely from erythrocytes, both immature and mature. More recent studies, however, have pointed to an important formation of Proto- by the liver. Both direct and indirect evidence of an hepatic induction of ALA-S has been described together with other evidence of an hepatic participation in the disease. Thus, it has recently been termed “erythrohepatic.”

In the present study, the activity of ALA-S has been assayed in samples of plasma from cases of various types of porphyria, erythropoietic and hepatic, as well as nonporphyric controls, including a case in which there were many circulating normoblasts because of bone marrow metastases. The findings in this case were of special interest in comparison with those in one of the cases of EP in which there were also large numbers of circulating normoblasts that disappeared following multiple packed red cell transfusions, thus it was possible to study the plasma ALA-S serially, in relation to a rapid diminution in erythropoiesis. This was of special interest in view of earlier observations that “throttling” of the porphyria occurred following splenectomy, believed due to consequent decrease in erythropoiesis and that bleeding of bovine porphyries enhanced the porphyria, with prompt and marked increase of fluorescing normoblasts in the bone marrow. Quite in line with these observations, Haining and co-workers have recently shown in a human case of this type that repeated transfusions to produce mild plethora were followed by marked diminution of the excessive porphyrin production.

**MATERIALS AND METHODS**

ALA-S activity in the plasma was assayed by the following modification of the method of Scholnick et al. This was done by determining the 14C-ALA formed in an incubation mixture having a total volume of 4.0 ml, of which 2 ml is plasma. This mixture also contained in μmoles: glycine, 400; disodium succinate, 20; succinic acid 2, 3-14C, 1.7 μmoles = 10μCi (specific activity 5.84 mCi/mmol); magnesium chloride, 40; glutathione, 4; EDTA, 40; pyridoxal phosphate, 2; ATP, 20; coenzyme A, 10; Tris buffer pH 7.4, 200; and succinyl-Co A synthetase prepared from *Rhodopseudomonas spheroides* strain H-5, sufficient to generate at least 8 μmoles succinyl-Co A in 30 min. (We are grateful to Prof. June Lascelles, Department of Bacteriology, University of California, for a gift of a culture of the *R. spheroides* strain.) After 1-hr incubation in air at 37°C at 100 shakes/min, the reaction was stopped by adding 0.5 ml of 50% TCA solution. The supernate and two subsequent
washes of the precipitate with 6% TCA solution were applied together with 100 μmoles of carrier ALA on a Dowex-50 (H⁺) resin column 3 × 1 cm, which was then washed with 500 ml of distilled water. After elution of ALA, conversion to pyrrole, and further washing, the aminoacetone fraction was separated from ALA-pyrrole by TLC. For the TLC, a thin layer of silica gel G, precoated on an aluminum plate (Merck-Darmstadt), was used. The area of the 14C-ALA-pyrrole was then removed and it was eluted from the silica gel four times with 2.0-ml portions of absolute alcohol. This was dried in a scintillation counting vial, redissolved in 0.2 ml of 100% ethanol, and mixed with Fluorite liquid scintillation fluid (Beckman). Ethanol in this proportion did not affect the efficiency of counting. The activity was counted with a Beckman liquid scintillation counter LS-200. As a control, an incubation mixture of the same composition was used in each instance, except that the reaction was stopped at zero time of incubation. The value obtained was subtracted from that recorded after incubation. These control values ranged from 50.5 to 84.2 DPM, mean 60. As a further control, the incubation mixture without plasma but with the same amount of distilled water was also incubated in the same manner and this gave values within the above range. This was also true of solutions of individual plasma proteins (albumin and globulin, Sigma grade III), nor was any activity found when the proteins of EP plasma were first precipitated with TCA, the supernate adjusted to pH 7.4, 2 ml then being subjected to the above assay method.

The possibility that the presence of transaminases might contribute to the formation of amino acids difficult to separate completely from ALA was excluded by means of the above chromatographic procedures. Cystine, isoleucine, leucine, methionine, phenylalanine, threonine, and tryptophan were separated at the outset by the column chromatography. Alanine, arginine, aspartic acid, cystine, glutamic acid, histidine, lysine, proline, serine, tyrosine, and valine were separated from ALA-pyrrole by the above method of TLC (Fig. 1).

Under the assay condition described, the formation of 14C-ALA was time and enzyme dependent (Fig. 2).

Samples of plasma assayed were from 28 individuals of which 14 were cases of porphyria, two cases of nonporphyrin excessive erythropoiesis, and 12 normal controls. Essential laboratory data for the porphyric cases are given in Table 1. The erythrocyte porphyrin values were not determined in the cases of hepatic porphyria included in this Table.
Fig. 2.—Dependence of plasma ALA-S assay on time and volume of plasma. 1.9 μCi of 14C-succinic acid (sp. act. 5.84 mCi/mmol) was incubated with EP plasma under above assay condition, with varied time and plasma volumes.

Erythropoietic Porphyria and Nonporphyric, Heightened Erythropoiesis

EP: The two cases studied have been described in detail previously: D.S., née D.H., a 26-yr-old female20,26 and M.M., a 37-yr-old female.11,27 In D.S., the first sample assayed was obtained on September 30, 1969 when the patient was in the sixth month of her second pregnancy. The second sample was obtained on January 23, 1970, 3 days after a normal delivery at term. At this time the urine and fecal porphyrin values were about twice those of the earlier period. There was no anemia at either time. At the first assay but one normoblast/100 leukocytes was observed, hemoglobin 10.8, reticulocytes 4.9%, and at the second study there were 12 normoblasts/100 leukocytes, hemoglobin 12.9, reticulocytes 2.4%. In the second case, M.M., there was hemolytic anemia (hemoglobin 9.9) with increased fecal pigments: bilirubin 212 mg/100 g and urobilinogen 472 mg/100 g. Circulating normoblasts numbered 150/100 leukocytes. The normoblasts were of various stages of maturity. By means of locating fluorescing cells in unstained smears in the fluorescence microscope, then staining with Wright’s stain, it was possible to identify individual fluorescing normoblasts and to determine that red fluorescence of cytoplasm and nucleus was first evident in polychromatophilic forms. A Reichert-Biozet unit on a modified Reichert fluorescence base with special optical filters was used.21 One or more fluorescing normoblasts were included in a small circle with a microscopic diamond marker and their geography within the circle was noted. Extrusion of porphyrin laden nuclei was repeatedly observed. As previously documented with the microfluorospectrophotometer, the nuclear porphyrin is mainly Uro-,28 most of which has been shown to be type I isomer.27 After preliminary observations including measurement of plasma ALA-S activity, the patient was given a series of four packed fresh red blood cell transfusions over a period of 7 days. The hemoglobin rose to 15.7 g/100 ml, and the red cell, serum, urinary, and fecal uroporphyrin declined to 5.1%, 17.8%, 9.4%, and 27.1%, respectively, of the pretransfusion values; the normoblasts disappeared from the circulating blood. The plasma ALA-S was assayed a second time when the normoblasts had diminished to 41/100 leukocytes, and a third time when they had disappeared. This patient is still under observation and further details on the remarkable “repression” of her porphyria by transfusion are to be described separately.

EPP: Samples from three typical cases (J.D., D.K., and B.B.) were assayed. The data for
Table 1.—Essential Data for Porphyric Cases in Which Plasma ALA-S Was Assayed

<table>
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<tr>
<th>Erythrocyte Group</th>
<th>Initials</th>
<th>Sex</th>
<th>Age</th>
<th>Diagnosis</th>
<th>Urine Porphyrins</th>
<th>Fecal Porphyrins</th>
<th>Erythrocyte Porphyrins</th>
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<td>Uro- (µg/day)</td>
<td>Copro- (µg/g dry wt)</td>
<td>Uro- (µg/100 ml)</td>
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<td>Uro- (µg/day)</td>
<td>Copro- (µg/g dry wt)</td>
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<td>17.4–69</td>
<td>44.6–182</td>
<td>1023–2126</td>
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plasma ALA-S and reticulocyte and normoblast percentages are given in Table 2. Normoblast data are lacking for case J.D.

**Nonporphyrin Erythropoiesis:** C.R., a 54-yr-old female with extensive skeletal metastases from breast carcinoma was studied: reticulocytes, 12.5%; normoblasts, 50/100
leukocytes: total serum bilirubin, 7.4 mg/100 ml; fecal urobilinogen, 73 mg/100 ml. D.M., a 51-yr-old female, Hb 10.3, reticulocytes 18.7%, and normoblasts 0/100 leukocytes, was also studied.

Hepatic Porphyria

VP: Two cases were studied: M.J., a 51-yr-old female, a member of a large kindred, including a number of entirely latent cases. M.J. had only photocutaneous symptoms, often rather severe. Her mother, H.W., an 80-yr-old woman had mild but typical photocutaneous manifestations. She is the other of the two cases presently studied (see Table 1 for essential porphyrin data). It should be noted that M.J.’s niece, never seen by us, died of an acute neurologic relapse with respiratory paralysis that followed pentothal anesthesia. The urine on the day before death was found to contain a large amount of PBG.

AIP: Four cases were all studied repeatedly over a period of years and were in clinical remission at the time examined. However, each was excreting a large excess of urinary PBG (Table 1).

PCT: Three typical cases were studied, the range of essential data for porphyrins and precursors being given in Table 1.

Normal Control Group

Twelve normal individuals (Table 2) were studied.

RESULTS

Results are given in Table 2. Values for duplicate runs and averages are included. It is evident that in all of the cases of both types of erythropoietic porphyria (Group 1, A and B) and in the case of nonporphyric, heightened erythropoiesis (Group 1, C) there was a significant increase of ALA-S activity, as compared either with the data for hepatic porphyria (Group 2, A, B, C) or that for the normal control group (Group 3). By far the greatest increase was in M.M., the case of congenital erythropoietic type in which there were large numbers of fluorescing and nonfluorescing normoblasts in the circulating blood (see above).

In the two nonporphyric cases, C.R. and D.M., exhibiting many normoblasts or reticulocytes in the circulating blood, the activity was 14.4 and 13.9 mmoles ALA formed/100 ml of plasma per hour. The possible significance of this in relation to the data in the cases of erythropoietic porphyria is discussed in the following.

In the cases of hepatic porphyria there was no increase above the normal range of values despite the fact that in these cases induction of ALA-S in the liver was evident in terms of the marked increases of porphyrins or their precursors in the excreta.

DISCUSSION

The foregoing results, especially the remarkably increased value for plasma ALA-S in case M.M. in which erythropoiesis was stimulated in the greatest degree, clearly indicate that the porphyria normoblasts provide the enzyme to the circulating plasma. Whether this was entirely derived from the circulating normoblasts that were present in such large numbers or partly from those in the bone marrow that had not yet entered the circulation cannot be determined. In case D.S., also one of EP but in whose blood there was but
one normoblast per 100 leukocytes, in striking contrast to M.M., the value for ALA-S was much smaller though significantly increased. In the second assay of D.S. when there were 12 normoblasts (3 days after delivery), the activity was considerably increased. There was no evidence of increased hemolysis. It is interesting to contrast her values with those of M.M. (EP with hemolytic anemia) and C.R., the case of bone marrow metastases exhibiting many circulating normoblasts and reticulocytes. The latter finding suggests that C.R. had greater erythropoietic activity than D.S., especially in view of the fact that D.S. had been splenectomized 21 yr previously and would have been expected to exhibit many more circulating nucleated red cells and Jolly bodies had there been a more active erythropoiesis at this time. In this connection M.M. had also been splenectomized 12 yr ago but she presented obvious evidence of mild hemolytic anemia with the very great increase of circulating normoblasts as noted. Considering these points in relation to the values for plasma ALA-S activity in the two cases, it is reasonable to believe that there was induction of ALA-S in the developing red cells in M.M. and perhaps in much lesser degree in D.S., especially in period 2, while in the nonporphyric case C.R., with so many circulating normoblasts, the slight increase of ALA-S activity may simply represent escape of physiologic minute amounts of enzyme from young red cells in which ALA-S had not been induced, as in the porphyric individuals. It is of much interest to note the remarkable decline of plasma ALA-S in M.M., in relation to the transfusion. The drop from 455 to 14 μmoles ALA formed at a time when the normoblasts still numbered 41 and the reticulocytes 9.4% together with the further decline in period 3, strongly points to induction of ALA-S during period 1.

The values in the three cases of EPP (or erythrohepatic) are about double those of the controls or of the hepatic porphyria cases. In the absence of appreciable evidence of increased erythropoiesis this may represent a mild induction of the enzyme in the young red cells of the bone marrow, although other possibilities are not excluded.

The plasma of the cases of hepatic porphyria showed no greater ALA-S activity than in the normal control group, although there is little reason to doubt that ALA-S was induced in the liver, at least in the cases of AIP and VP. The lack of increase in the plasma may well relate to the long life span of the mammalian liver cell, which is measured in months or years in contrast to a few days for the normoblast. The half life of ALA-S in liver cells has been estimated to be from 67 to 72 min, the enzyme presumably reentering the liver protein pool at the end of its life span without loss, at least as ALA-S, to the plasma. This line of reasoning, together with the present data in Table 2 showing that the normal control values are essentially the same as those of the hepatic porphyria cases, indicates that the values in both groups are probably related to ALA-S of erythropoietic rather than hepatic origin. A daily loss of enzyme related to nuclear extrusion by the maturing normoblasts reasonably accounts for the small amounts of activity in these two groups. The variation that is noted is unexplained.

Although the amount of ALA-S determined in the plasma was small, it is conceivable that if in erythropoietic porphyria of either type sufficient amounts
were liberated into plasma (as in case M.M.) these might gain entrance to other cells such as the liver giving rise to porphyrin biosynthesis or, presuming the presence of succinyl-Co A in plasma, the possibility of a humoral synthesis, at least of minute quantities of ALA, might be entertained.

ACKNOWLEDGMENT

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