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δ-Aminolevulinic acid (ALA) synthetase
erythrocyte
globin
heme
hemoglobin Köln disease
β-thalassemia

Heme Synthesis in Hereditary Hemolytic Anemias: Decreased δ-Aminolevulinic Acid Synthetase in Hemoglobin Köln Disease

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Extract

The activities of δ-aminolevulinic acid (ALA) synthetase and ALA dehydratase in cord blood erythrocytes of newborn infants and peripheral blood red cells of patients with β-thalassemia major, β-thalassemia intermedia, hemoglobin Köln (Hb Köln) disease, sickle cell anemia, and pyruvate kinase deficiency were studied. The activity of ALA dehydratase did not vary appreciably with the number of immature RBC (reticulocytes and nucleated red blood cells) or the severity of the hemolytic anemia except in pyruvate kinase deficiency. The activity of ALA synthetase was linearly correlated with the number of immature RBC ($r = 0.974$, $p < 0.001$). The ALA synthetase activity was significantly decreased in the RBC of Hb Köln ($p < 0.01$) when compared with the activity in immature RBC of newborns and of patients with pyruvate kinase deficiency, sickle cell anemia, and thalassemia intermedia.

Speculation

The decreased activity of ALA synthetase activity in thalassemia major and Hb Köln is postulated to be secondary to feedback inhibition of heme. The site of feedback inhibition is not at the RBC membrane but could be directly on ALA synthetase itself or at its synthetic step.

Heme has a key role in the synthesis of globin and is involved in the initiation and assembly of globin chains. Heme also controls its own synthesis by inhibiting the utilization of glycine at the ALA synthetase step and thus regulates the synthesis of both the heme and globin moieties of the hemoglobin molecule. Disorders in the formation of heme will be reflected in disturbed synthesis and assembly of globin; disorders in the synthesis of globin will affect the production of heme.

In hemoglobin Köln disease, an unstable hemoglobin hemolytic anemia is caused by a structural defect of the β-globin chains ($\beta^{98Val\ Met}$). Recent studies have indicated that the synthesis of Hb Köln tetramer is defective (5, 38) and that $\beta^{Köln}$-globin chains are synthesized at one-fifth the rate of β^{A}-chains (5). In β-thalassemia major decreased synthesis of structurally normal β-globin chains has been demonstrated (36) and in earlier, indirect studies, impaired heme synthesis was suggested (2, 3, 7, 28, 34, 36). Recently, decreased ALA synthetase activity has been demonstrated in β-thalassemia (32) using a direct assay. Similar studies in Hb Köln disease have not been reported.

The rate-determining step in the synthesis of heme involves the formation of ALA from succinyl coenzyme A and glycine. This reaction is catalyzed by the enzyme, ALA synthetase, which is present in the mitochondria of erythroid precursors but not in mature red blood cells. For this reason, most assays of ALA synthetase require large amounts of peripheral blood (4, 8, 11, 17) or bone marrow samples (1, 33–35). In 1970, Strand *et al.* (29) developed a microassay method for the determination of ALA synthetase. Using 100 μl packed red blood cells, they found increased ALA synthetase activity in patients with congenital or acquired hemolytic anemia.

Applying this micromethod, we assayed the activity of ALA synthetase in peripheral blood reticulocytes in neonates and in patients with thalassemia syndromes, unstable hemoglobin hemolytic anemias, and other heriditary hemolytic anemias. The purpose of this study was to substantiate directly the earlier observations (2, 3, 7, 24, 30, 34) of decreased levels of ALA synthetase in β-thalassemia major, and to determine whether the decreased rate of globin chain synthesis in Hb Köln disease is associated with decreased activity of ALA synthetase. The activity of ALA dehydratase, the subsequent reaction in heme synthesis in which 2 mol ALA are converted to porphobilinogen, was also measured in these disorders.

MATERIALS

The radionuclide, [5-^{14}C]ALA-HCl (specific activity 0.156 mCi/mg) was purchased from New England Nuclear; Amersham-Searle supplied 2,3-^{3}H-succinic acid (specific activity 250 mCi/mmol). Other chemicals were obtained from Sigma Chemical Company. Polyethylene columns (1 by 4 cm) were purchased from Bio-Rad and filled with Dowex 1X-8 acetate (100–200 mesh and 200–400 mesh) and Dowex 50W-X4 acid (100–200 mesh).

PATIENTS

Twenty-five patients with hereditary hemolytic anemias and 10 normal newborns were evaluated. Previously published methods were used to confirm the diagnoses of pyruvate kinase (PK) deficiency (21), Hb Köln disease (20), and sickle cell anemia. These patients had not received blood transfusions in the 6 months prior to testing. Five patients with thalassemia major were transfusion-dependent and received frozen or washed red blood cell transfusions every 2–3 weeks. The five patients with thalassemia intermedia had homozygous β-thalassemia, had undergone splenectomy, but did not require transfusions of red blood cells to maintain a level of hemoglobin above 7–8 g/100 ml. Healthy, nonanemic adults served as normal control subjects. Informed consent was obtained from all subjects and/or their mothers.

METHODS

PREPARATION OF ERYTHROCYTES

The erythrocytes from the normal, full term newborn infants were obtained from cord blood and collected in heparin-containing tubes (39). From all other subjects, blood was collected from the antecubital vein into similar tubes, separated into two fractions, and cooled to 4° immediately. The plasma and leukocytes were removed from one of the fractions after centrifugation at 4° and 1,000 × *g* for 10 min. The erythrocytes were washed twice in 0.05

M phosphate buffer, pH 7.4, which contained 0.1 M KCl, 0.005 M $MgCl_2$, and 0.5 mM EDTA. The leukocyte-free red cells were then twice alternately frozen in an acetone-Dry Ice bath and thawed for utilization in the assay for ALA synthetase. Whole blood from the second fraction was used in the assay of ALA dehydratase. Packed cell volume, hemoglobin, red blood cell, and reticulocyte counts were determined by standard methods.

δ-ALA DEHYDRATASE ASSAY

The assay is a modification of the method of Mauzerall and Granick (19) in which a buffered substrate disc containing citrate buffer and δ-ALA (Bio-Rad) was added to a test tube containing 1 ml doubly distilled water and then incubated for 15–30 min. Whole blood (0.1 ml) was added to a second tube containing 1.5 ml doubly distilled water which was then incubated for 5 min. The contents of the two tubes were mixed to initiate the reaction which was stopped after 60 min by the addition of 1.0 ml trichloracetic acid (TCA) reagent (Bio-Rad). Equal volumes of the supernatant from the reaction mixture and Ehrlich's reagent were mixed and, after 8–12 min, the absorbance was measured at 555 nm in a Beckman Acta III spectrophotometer. The porphobilinogen produced was determined using the extinction coefficient $E_{555} = 6.1 \times 10^4\ M^{-1}\ cm^{-1}$ (19).

δ-ALA SYNTHETASE ASSAY

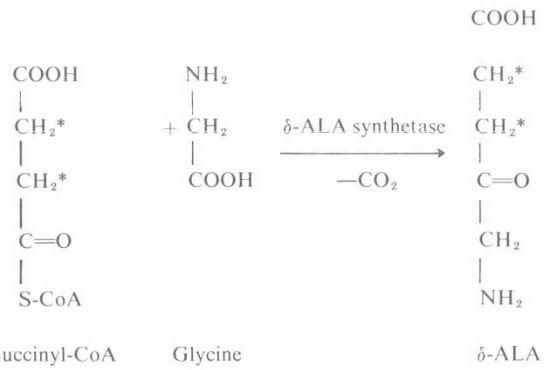
The incubation mixture was that described by Strand *et al.* (29) and contained inhibitors of the tricarboxylic acid cycle and other pathways to prevent utilization of succinyl-CoA. Succinyl-CoA was formed from succinic acid and CoA using *Escherichia coli*-derived succinyl-CoA synthetase (1.7 U). Succinyl-CoA synthetase was purified by the method of Ramalay *et al.* (23) from *E. coli* grown in a 13 liter fermenter. Succinyl-CoA synthetase was assayed as described by Ramalay *et al.* (23). The appearance of the thioester bond of succinyl-CoA at 230 nm was monitored in a Gilford recording spectrophotometer and calculation of succinyl-CoA formation was based upon the E_{230} of $4.5 \times 10^3\ M^{-1}$, cm^{-1} as determined by Stadtman (25).

Using 0.30 mM Na_2 succinate with tritium at the 2 and 3 positions, radioactive ALA was produced from the following reactions.

Reaction 1

$$\text{Succinic acid} + \text{CoA} \xrightarrow[\text{synthetase}]{\text{succinyl CoA}} \text{succinyl CoA}$$

Reaction 2



The incubation mixture contained the necessary cofactors for both succinyl-CoA synthetase and δ-ALA synthetase. The reaction was stopped after 30 min with 0.1 ml 25% TCA.

ISOLATION OF ALA

The isolation procedure was that described by Strand *et al.* (29) in which the incubation mixture was run through an anion exchange resin at pH 7 to remove unreacted radioactive succinic acid. The eluate from the first column was then passed directly onto a cation exchange column at pH 4.6 after treatment with Ehrlich's reagent. The chromatography and isolation procedures were monitored by using [5-^{14}C]ALA added to the incubation mixture to assess recovery using double label scintillation counting. The recovery was 70–80%. The tritiated product moved consistantly with [5-^{14}C]ALA.

SCINTILLATION COUNTING

Eluted samples were mixed with 10 ml Aquasol from New England Nuclear and counted in a Hewlett-Packard or Beckman LS-250 liquid scintillation spectrometer. The counts obtained were corrected for counting efficiency, percentage of recovery, and the activity of the succinyl-CoA synthetase blank.

RESULTS

The blood of most patients was analyzed for both ALA synthetase and ALA dehydratase activity, and attempts were made to correlate the activity with the relative youth of the peripheral red blood cells.

ALA DEHYDRATASE

The data in Table 1 show that little change in the overall activity of ALA dehydratase occurs despite the tremendous variation in reticulocytosis. With the extreme reticulocytosis seen in the patients with PK deficiency, a 1.8-fold rise in the activity of ALA dehydratase is observed. However, the activity within each group did not show a correlation with the actual reticulocyte counts.

ALA SYNTHETASE

The red cells of patients with PK deficiency are not known to have a defect in heme synthesis. The patients with PK deficiency evaluated in this study had undergone splenectomy and all had reticulocyte counts of greater than 30%. The values for patients should give a reasonable estimate of the "normal" ALA synthetase activity in an extremely young population of erythrocytes. The assays of ALA synthetase in normal individuals with reticulocyte counts of 1.0% or less were subject to considerable error. This was related to the presence of some ALA synthetase activity in the succinyl-CoA synthetase blank which was equivalent to the activity observed in samples containing 1.0% or fewer reticulocytes. Therefore, determinations of ALA synthetase were accurate only when the reticulocyte count was greater than 1%. Because of the low reticulocyte counts occurring in infants between 1 week and 3 months of age, accurate measurement of the activity of ALA synthetase during this period of physiologic anemia was not feasible in small samples of peripheral blood.

Figure 1 shows the correlation of the ALA synthetase activity

Table 1. *Activity of δ-aminolevulinic acid (ALA) dehydratase in erythrocytes*

No. of patients	Diagnosis	Reticulocyte count, % range	ALA dehydratase activity, nm/ml RBC/hr (mean ± SD)
8	Normal	0.5–1.5	720 ± 200
21	Newborns	1.0–7.0	860 ± 220
12	Thalassemia major	0.2–10	905 ± 210
5	Thalassemia intermedia	6–16	840 ± 380
4	Sickle cell disease	4.7–34	840 ± 130
5	Hemoglobin Köln disease	8–16	912 ± 270
4	Pyruvate kinase deficiency	37–76	1,300 ± 90

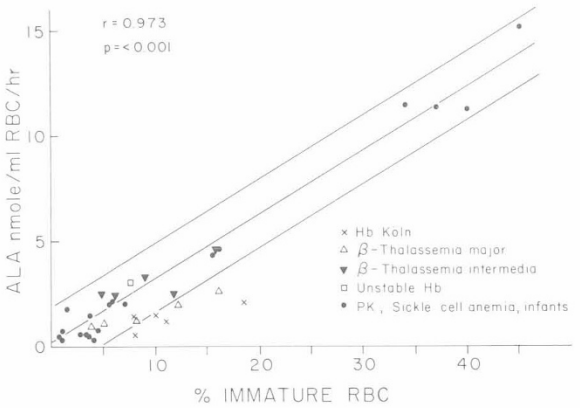


Fig. 1. Regression line for plot of δ-aminolevulinic acid (*ALA*) synthetase activity, expressed as nanomoles per ml RBC/hr, *vs.* percentage of reticulocytes and nucleated RBC in peripheral blood. Symbols for the various types of hereditary hemolytic anemias studied are given in the key. The symbol represents the mean of triplicate determinations for each patient. The correlation coefficient, $r = 0.973$, is highly significant ($p < 0.001$). PK: pyruvate kinase.

with immature red blood cells (immature cells included reticulocytes and nucleated red blood cells). The linear correlation is similar to that observed by Feldman and Lichtman (8). The correlation coefficient was 0.973 ($p < 0.001$) for the 25 patients in six categories (neonates, PK deficiency, sickle cell anemia, thalassemia intermedia, hereditary hemolytic anemia, and unstable hemoglobinopathy). As can be seen in Figure 1, a number of determinations fall below the regression line. These points correspond to patients with β-thalassemia major and Hb Köln disease. Table 2 shows the data presented as the ratio of ALA synthetase activity to immature RBC, the ALA synthetase or biosynthetic index for all patients studied. The ALA synthetic indexes (mean ± SE) of the different groups of patients are presented in Figure 2. The mean ALA synthetase index was significantly lower in Hb Köln disease ($p < 0.01$) and in β-thalassemia major ($p < 0.02$) than in the other hereditary hemolytic anemias or in newborns with equivalent reticulocytosis. The difference in means between the patients with Hb Köln disease and β-thalassemia major was not statistically significant nor were there any statistically significant differences between any of the other groups.

DISCUSSION

ALA DEHYDRATASE

The lack of variation in the ALA dehydratase activity despite a 150-fold change in the reticulocyte count is important for the utilization of the ALA dehydratase assay in the diagnosis of lead poisoning (37). The enzyme is extremely sensitive to lead ion but the reticulocytosis which often accompanies lead poisoning will not cause raised levels of ALA dehydratase activity.

The increase in ALA dehydratase activity occurring in pyruvate kinase deficiency associated with profound reticulocytosis is probably attributable to the fact that the population of red blood cells is much younger and has had less time for catabolism of the enzyme. The fact that the ALA dehydratase activity in human newborns and normal adults is the same may be contrasted to the situation in the mouse, in which the ALA dehydratase activity in the fetal liver is initially twice that found in the adult liver, decreases to well below adult levels in the days before delivery, and then slowly returns to the adult level (6).

ALA SYNTHETASE

The reliability of assays for ALA synthetase using radioactive succinate has been questioned recently by Aoki *et al.* (1). These investigators have developed a new method for measuring the activity of ALA synthetase in red blood cell precursors using [^{14}C]succinyl-CoA, which they claim is more accurate than other assay systems. In related incorporation systems, the activity of ALA synthetase was 100-fold greater when [^{14}C]succinyl-CoA was used than when [^{14}C]succinate was the substrate. However, Aoki's method was not compared with that of Strand and coworkers (29). Aoki *et al.* (1) found that the activity of ALA synthetase in erythroblasts (300 nmol/10^9 erythroblasts/30 min) was 100 times greater than the activity of the enzyme measured in reticulocytes. In our study a value of 5 nmol/10^9 reticulocytes/30 min was calculated using estimates of mean corpuscular volume, known reticulocyte count, and the normal range of ALA synthetase activity. This value is 1/60 of the activity of ALA synthetase in erythroblasts as measured by Aoki and would indicate the activity of ALA synthetase in reticulocytes as determined by the method of Strand *et al.* is comparable. The patients in this study with appreciable normoblastemia have been splenectomized. Their cells, although nucleated, would not be expected to have the same activity as bone marrow erythroid precursors since the cells in the

Table 2. *δ-Aminolevulinic acid (ALA) synthetase activity in newborns and in hereditary hemolytic anemias*

No. of patients or patient's initials	Reticulocyte count, %	Diagnosis	ALA synthetase index[1] (mean ± SE)
10	1.0–7.0	Newborns	296 ± 36
4	37–75	Pyruvate kinase deficiency	249 ± 44
4	8–34[2]	Sickle cell anemia	235 ± 84
RC	6.1	Thalassemia intermedia	395
EL	16.2[2]	Thalassemia intermedia	276
CG	6	Thalassemia intermedia	345
JS	12	Thalassemia intermedia	155
FP	8.8	Thalassemia intermedia	330
AG	5.3	Thalassemia major	168
JG	17[2]	Thalassemia major	152
JI	5	Thalassemia major	227
AN	6.2	Thalassemia major	100
MD	1.6	Thalassemia major	210
RM, Jr.	18.7	Hb Köln	102
RM, Sr.	8.0	Hb Köln	67
AM	8.0	Hb Köln	169
AM	11.0	Hb Köln	103
MM	10.0	Hb Köln	142
SD	4.7	Hereditary hemolytic anemia (uncharacterized)	442
JV	7.6	Unstable hemoglobinopathy	410

[1] ALA synthetase index = mean ALA synthetase activity (nmol/ml RBC/hr)/reticulocyte count + nucleated RBC count.

[2] Patients had 1–2% nucleated RBC.

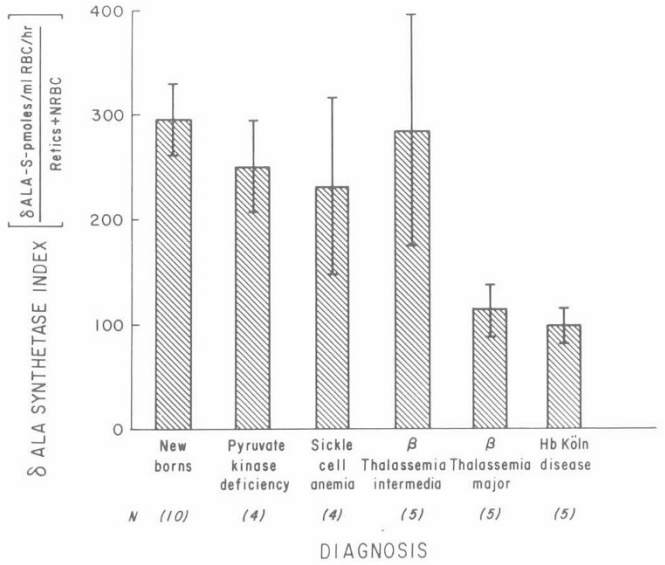


Fig. 2. δ-Aminolevulinic acid (*ALA*) synthetase index (biosynthetic index) in newborns, patients with Hb Köln disease, and other patients with hereditary hemolytic anemias. The bars and brackets represent the mean ± SE for each group of patients studied.

peripheral blood are more mature orthochromic normoblasts, in which the activity of δ-ALA synthetase is increased but is probably closer to that of the reticulocyte.

β-THALASSEMIA MAJOR AND Hb KÖLN DISEASE

The activity of ALA synthetase in the red cells of patients with β-thalassemia major is decreased and is consistent with the findings reported in other studies (30). Other investigators have detected decreased ALA synthetase utilizing indirect methods (2, 3, 5, 28, 33, 34). The decreased activity of ALA synthetase has been attributed to a direct feedback inhibition of ALA synthetase by heme (2, 14, 17, 18, 27, 36) and to an inhibitory feedback of globin on heme synthesis (27).

It is clear from this study that the patients with thalassemia intermedia do not have a significant decrease in ALA synthetase despite having homozygous β-thalassemia. Of the five patients studied with thalassemia intermedia, only one patient (*JS*) had a level of ALA synthetase appreciably below average (ALA synthetase index 155). The patients with thalassemia intermedia are reasonably well compensated hematologically and do not have as great an imbalance in the production of globin chains (36). The fact that most patients with thalassemia intermedia have normal ALA synthetase activity may be an indication that the β-globin chain production, although decreased in rate, may be sufficient to prevent the accumulation of enough excess heme to inhibit ALA synthetase activity appreciably. The fact that two of five patients with thalassemia major have ALA synthetase activity values within the normal limits and that one of five patients with thalassemia intermedia has decreased ALA synthetase activity level may indicate that there is some overlap in the rate of β-globin chain production in patients with thalassemia intermedia and thalassemia major.

In Hb Köln disease, ineffective erythropoiesis, and an abnormal rate of β-chain production (38) have been described, but information on iron utilization and heme synthesis is not available. The serum iron and total iron-binding capacity of patients with Hb Köln disease are normal and in this respect they differ from patients with thalassemia major and sideroblastic anemias. The molecular defect in Hb Köln ($\beta^{98Val \cdot Met}$) is characterized by dissociation of heme from the structurally abnormal β-chains and oxidative denaturation of globin (5, 38). The released heme and the decreased β-Köln production may act in concert to produce levels of heme which inhibit ALA synthetase activity.

There is no evidence that globin has a feedback inhibitory effect on ALA synthetase activity. Experiments by Kappas and Granick (12) in cultures of liver cells showed that heme may have a repressor effect on ALA synthetase before translation. In Hb Köln disease and β-thalassemia, the observed decrease in ALA synthetase activity may be secondary to an earlier repression by heme of the synthetic machinery for ALA synthetase. The amount of heme present early in hemoglobin synthesis in more immature red blood cells may be a function of the instability of the abnormal hemoglobin and the magnitude of the defect in globin chain synthesis. Although Ponka and Neuwirt (22) have proposed that heme exerts an effect on its own synthesis by inhibiting the membrane transport of iron and glycine, this mechanism cannot explain our findings which are based upon as assay performed on hemolysates.

SUMMARY

Decreased ALA synthetase activity, the rate-limiting step in the synthesis of heme, has been observed in patients with Hb Köln disease, an unstable hemoglobin hemolytic anemia. In patients with other hereditary hemolytic anemias and in newborns the acitivty of ALA synthetase correlated with the number of reticulocytes and nucleated red blood cells.

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Corrigendum

On p. 367 of the April 1976 issue of this journal, included in the Program and Abstracts of the Annual Meeting of the American Pediatric Society and the Society for Pediatric Research, St. Louis, Missouri, April 28 30, 1976, appears abstract No. 395 entitled "Origin of Chromosomal Abnormalities: Evidence for Delayed Fertilization in Meiotic Nondisjunction." Dr. John B. Mailhes, listed as one of the authors, had earlier requested his name be removed from these data. As this information was not made available to the publisher in time to delete his name, Dr. Mailhes now requests members of the medical and scientific community to dissociate his name from the above cited abstract.