BONE MARROW δ-AMINOLEVULINIC ACID SYNTHETASE ACTIVITY IN HEMATOLOGICAL DISORDERS

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ABSTRACT

An improved radiochemical method to measure the δ-aminolevulinic acid (ALA) synthetase activity in human bone marrow erythroblasts by incubating bone marrow cell homogenate with ^{14}C-succinate and succinyl CoA generating system has been reported. Studies were made to obtain the optimal conditions for the enzyme assay and to confirm the reliability of the assay procedures.

ALA-synthetase in erythroblasts was found to be decreased in all 3 cases of iron deficiency anemia and 3 cases of β-thalassemia minor. In 5 cases of idiopathic sideroblastic anemia, the enzyme activity was markedly decreased. A case of sideroblastic anemia, who restored normal erythropoiesis following the administration of pyridoxal phosphate (PLP) showed low PLP level in serum & blood as well as *in vivo* evidence of PLP deficiency and an *in vitro* stimulation of ALA-synthetase activity by PLP. Erythroblasts of 4 patients with erythroleukemia showed diminished bone marrow ALA-synthetase activity.

INTRODUCTION

The initial and rate-limiting step in the heme biosynthetic pathway is the enzymatic condensation of succinyl-CoA and glycine to form delta-aminolevulinic acid (ALA).[1] This condensation is mediated by the enzyme, delta-aminolevulinic acid synthetase (succinyl-CoA: glycine succinyltransferase). This enzyme was shown to be inducible in high level in the liver by a number of compounds which produced experimental porphyria.[2]-[3] Although the normal level of ALA-synthetase in bone marrow erythroblasts is too low to be measured by the colorimetric method, the recent development of sufficiently sensitive radiochemical assay method has made it possible to assay ALA-synthetase activity in human bone marrow cells with limited number of erythroblasts.[4]-[10] The assay procedure adopted herein is the method developed by Evert et al and has the advantage of being both simple and rapid.

Previous investigation on erythroleukemia, "refractory sideroblastic anemia", thalassemia and iron deficiency anemia have suggested the presence of a disturbance in hemoglobin formation in these conditions. The author reports the results of experimental studies on assay procedures of bone marrow ALA-synthetase activity in normal human subjects and patients with sideroblastic anemia, iron deficiency anemia, betathalassemia minor, and erythroleukemia in oder to elucidate the possible involvement of this enzyme in these disorders.

METERIALS AND METHODS

A.   Normal subjects and patients.

1. *Normal controls.*   Bone marrow ALA-synthetase activity was assayed in 6 healthy individuals with the age ranging from 19 to 24 years old.

2. *Sideroblastic anemia.*   Bone marrow ALA-synthetase activity was assayed in 5 patients with idiopathic sideroblastic anemia (ISA) at the time of diagnosis.   In 2 cases, the enzyme was assayed for 2 to 3 times after the treatment with pyridoxal phosphate. The diagnosis in each case was made on the basis of the following findings: moderate to severe dimorphic anemia, reticulocytopenia, normal or increased serum iron concentration, erythroid hyperplasia and the presence of ringed sideroblasts in the bone marrow aspirate, ineffective erythropoiesis by ferrokinetic studies, and the absence of recognizable underlying diseases.

3. *Beta-thalassemia minor.*   The diagnosis in each case was established on the basis of hypochromic anemia, normal or increased serum iron, increased Hb-A_2 content ranging from 4.2 to 8.2% and family study revealing the presence of inheritance of A_2-type beta-thalassemia.

4. *Iron deficiency anemia.*   The three patients investigated had hypochromic anemia, low serum iron and high unsaturated iron binding capacity of serum.   The cause of iron deficiency was bleeding from gastrointestinal tract in all cases.   The enzyme assay was performed before any treatment in all cases.

5. *Erythroleukemia.*   All three patients had very bizzare nucleated red blood cell morphology as well as numerous circulating nucleated red blood cells and erythroid hyperplasia of the bone marrow with megaloblastoid change.   They were on no therapy at the time of the study and were studied at their erythremic stage prior to their evolution to frank acute myelogenous leukemia.

B.   Assay Procedure.

1. *Preparation of Succinyl-CoA Synthetase.*
   Crude succinyl CoA synthetase from *Rhodepseudomonas spheroides* ALA-requiring mutant H-5 (generously provided by Dr. June Lascelles of the University of California, Los Angeles) was used.   Cultures were grown at 30°C with aeration for 16 hrs.   The cells were then concentrated by centrifugation, washed in one half culture volume of 40 mH tris, pH 7.5 and sonicated.   The cells were spun at 42,000 rpm for 1 hour and the supernatant was assayed for succinyl CoA synthetase activity by the method of Kaufman.[11]   The crude preparation contained 28.6 U/ml of succinyl CoA synthetase.   One unit can catalize the formation of 1 micromole of succinyl CoA per hour.

2. *Preparation of bone marrow lysate.*
   Cells from 3 to 4 ml aliquot of a heparinized bone marrow aspirate were dispersed through a needle and washed twice with 0.15M NaCl.   The total nucleated cell count

and the percent of erythroid cells were determined on the cell suspension prior to the second wash. The washed cells were exposed to 2 volumes of water for 4 minutes, followed by addition of KCl to restore the isotonicity and then diluted to represent the desired erythroid cell content. To completely disrupt the cell membrane, the preparation was homogenized prior to assay.

3. *Enzyme assay procedure.*

ALA-synthetase was measured by quantifying ^{14}C-ALA formed after incubating the bone marrow lysate (final concentration ranging from 2.5 to 5% of the original cell volume) in the presence of the following substances: 0.08M tris-HCl buffer, glycine, sodium EDTA, sodium succinate containing a certain amount of 1-4 ^{14}C-succinate (specific activity 10.2 mCi/m mole), 10 mM $MgCl_2$, 1 mM ATP, 0.1 mM CoA and succinyl CoA synthetase prepared from *Rhodepseudomonas spheroides,* which could catalize the formation of approximately 3.0 micromoles of succinyl CoA.

For assessment of ALA utilization during the incubation, duplicate samples were incubated with ^{14}C-ALA instead of ^{14}C-succinate. The conditions of the incubation in various experiments are indicated under Results and in the appropriate figures and tables. Incubations were terminated by addition of 0.5 ml of 25% trichloracetic acid. ^{14}C-ALA was isolated from the trichloracetic acid supernatant as described by Evert *et al.* [5] with the following modifications: after the application of the sample to the resin, the column was washed successively with 20 ml of 0.1M acetate buffer, pH 3.9, 20 ml of methanol: acetate buffer pH 3.9 (4 : 1, v/v) and 5 ml of 0.01M HCl. ^{14}C-ALA was then eluted from the resin column with 3 ml of 0.1 M NaOH. One ml aliquot was added to Aquasol (New England Nuclear) and counted in a liquid scintilation spectrometer with the efficiency of greater than 90%.

RESULTS

1. *Experimental studies on ALA-synthetase assay procedures.*

A. *Determination of optimal condition for the enzyme assay.*

The results of experiments designed to determine the optimal condition for the measurement of ALA-synthetase activity are presented in figure 1, 2, 3, 4. The optimal pH was 7.5 in trisbuffer (fig. 1). Enzyme activity was maximal with 50 mM glycine (fig. 2-a),150 μM ^{14}C-succinate (fig. 2-d) and 1 mM Na-EDTA (fig. 2-b) and was not enhanced by PLP over a concentration range of 0.2 mM to 1.2 mM (fig. 2-c).

Under the optimal condition of the substrate and EDTA, ALA generation was nearly linear for 30 minutes and was a direct function of enzyme concentration up to 4×10^6 erythroid cells per assay (fig. 3). Under the above conditions, ^{14}C-ALA utilization was not completely inhibited by EDTA. ^{14}C-ALA loss was proportional to the concentration of erythroid precursor cells as well as to mature red cells. However, ^{14}C-ALA utilization was linear with the concentration of ALA only at a final concentration of lysate of 10% or less (fig. 4). At greater lysate concentration, ALA utilization increased disproportionately with increasing ALA-concentration. For the assay of the enzyme as reported below, lysate and erythroid cell concentration were kept below 5% and 5×10^6, respectively.
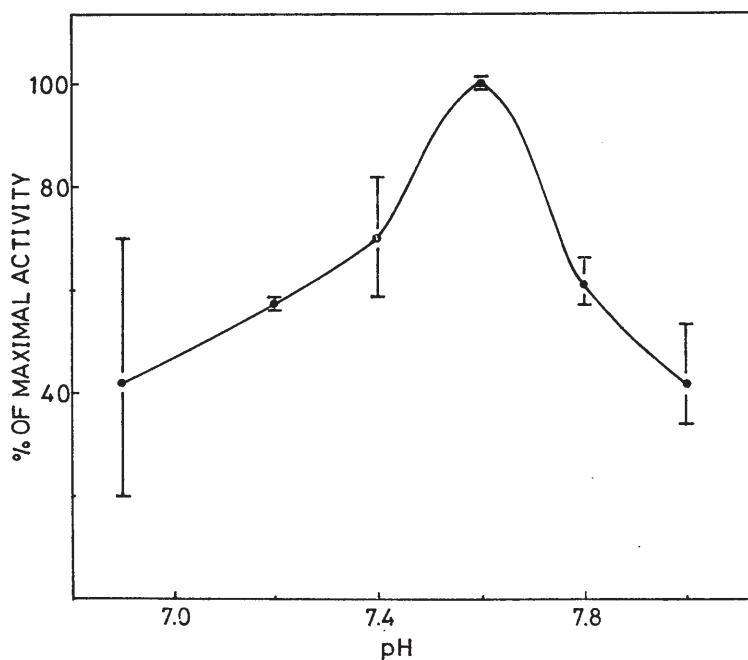
Fig. 1

Fig. 1.   Effect of pH on ALA-synthetase activity.
Incubation conditions were: bone marrow lysate, 1.0 ml; tris buffer, 80 mM; glycine 100 mM; ^{14}C-Na Succinate, 150 μM; NaEDTA, 1 mM; pyridoxal phosphate, 0.2 mM; MgCl$_2$, 10 mM; ATP, 1 mM; CoA, 0.1 mM.
Three units of partially purified succinyl CoA synthetase was added to each assay. Specific activity of 1-4 ^{14}C-succinate was 5.5 Ci/mole. Incubation was done at 37°C for 30 minutes. Incubation volume was 2 ml.
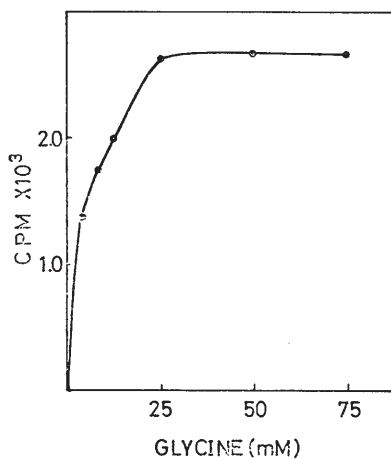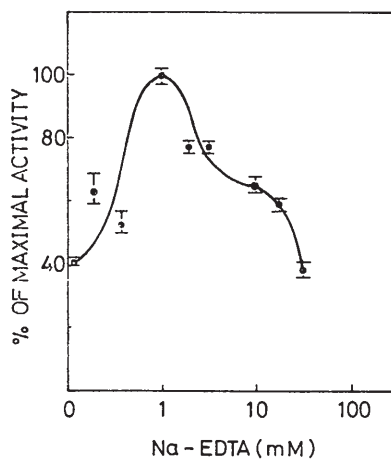

Fig. 2 (a)

Fig. 2 (b)

Fig. 2.   Effect of the concentration of glycine (a), NaEDTA (b), pyridoxal-phosphate (c), and Na-succinated (d) on ALA-synthetase activity. Incubation condition was the same as in the legend to fig. 1.
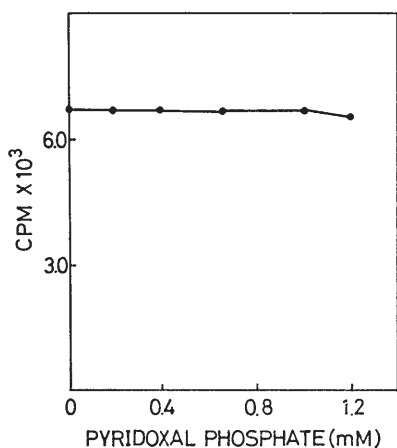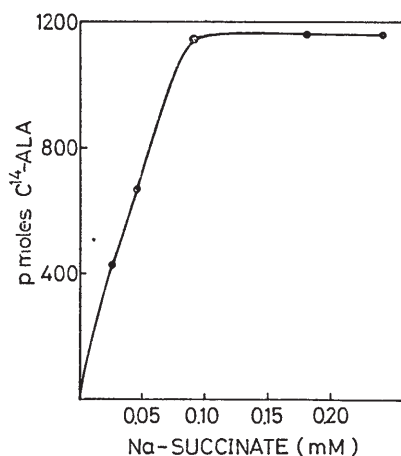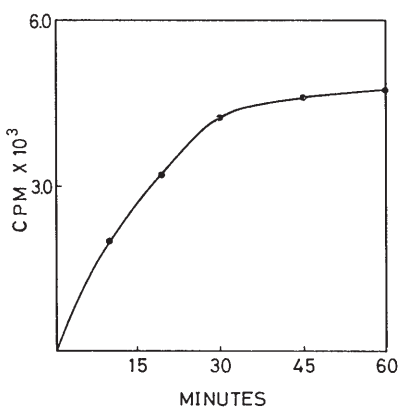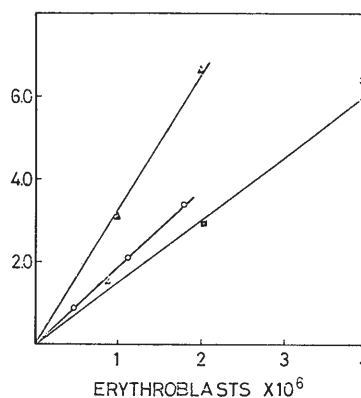
Fig. 2 (c)

Fig. 2 (d)

Fig. 3 (a)

Fig. 3 (b)

Fig. 3. Effect of time (a) and enzyme concentration (b) on ALA-synthetase activity. Incubation condition was the same as in the legend to fig. 1. The vertical axis is the CPM of ^{14}C-ALA formed during incubation.

B.   *Identification of ALA.*

Column eluate (ALA-fraction) resulting from an incubation of normal bone marrow with ^{14}C-succinate as well as ^{14}C-ALA added to a "killed" lysate were subjected to an ascending paper chromatography. ALA in the eluate was first converted to pyrrole by boiling with acetylaceton; then concentrated to dryness and the residue was dissolved in a small amount of methanol. This solution was subjected to paper chromatography. Fig. 5 shows that the radioactivity of the eluate consisting mostly (above 90%) of ^{14}C-ALA-pyrrole.

2.   *ALA-synthetase activity of marrow cells in normal subjects, idiopathic sideroblastic anemia, iron deficiency anemia, beta-thalassemia minor and erythroleukemia patients.*

Table 1 depicts the values of ALA-synthetase activity in bone marrow erythroblasts of 6 normal subjects and those of 5 patients with ISA. In all patients with ISA, the
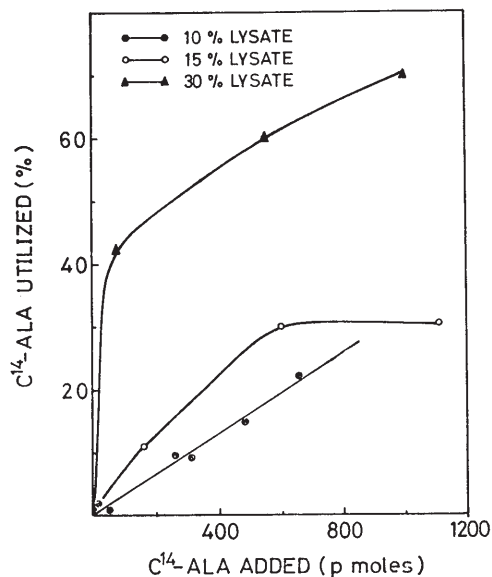
Fig. 4

Fig. 4.   Effect of ALA concentration on ALA-utilization.
               Incubation condition was the same as in the legend to fig. 1.
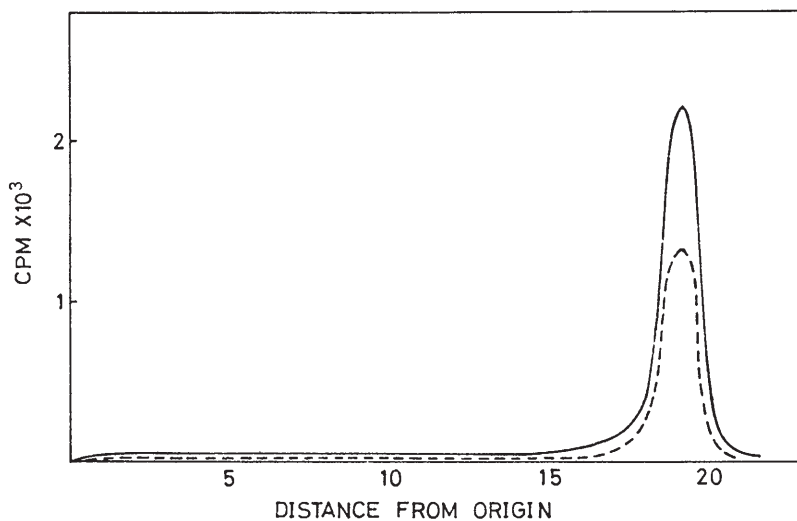
Fig. 5

Fig. 5.   Identification of ^{14}C-ALA in the eluate.
          ^{14}C-succinate (1 uCi, 0.045 μ mole) was incubated with normal bone marrow lysate and then chromatographed on column as described in materials and method. ^{14}C-ALA in the presence of trichloracetic acid -treated tissue homogeneite was not incubated, but was treated similarly.
          ^{14}C-ALA in the column eluate was converted to the pyrrole form, spotted on paper, and then subjected to ascending chromatography, using n-butanol-acetic acid -water (4 : 1 : 8). Chromatographs were sprayed with ninhydrin to observe aminoacid contamination and with Ehrlich reagent to localize the pyrrole spot. No ninhydrin-positive spot attributable to amino-acid was seen.

Table 1. Bone marrow ALA-synthetase activity in normal subjects and patients with idiopathic sideroblastic anemia.

Case	p moles ^{14}C-ALA formed/10^6 erythroblasts/30 minutes	
	PLP** (−)	PLP** (+)
1. Normal	220	226
2. Normal	325	337
3. Normal	320	320
4. Normal	257	262
5. Normal	226	229
6. Normal	337	342
Normal Range	281 ±53	286 ±54
7. ISA*	132	137.5
8. ISA*	60.5, 53.5	65.5, 50
9. ISA*	88	75
10. ISA* − before PLP therapy	52	290
	68	190
− after PLP therapy	78	108
− 3 months after PLP therapy	243	253
11. ISA* − before PLP therapy	58	50
	38	50
− after PLP therapy	60	58

*ISA = Idiopathic sideroblastic anemia
**PLP = Pyridoxal phosphate

Table 2. Bone Marrow ALA synthetase activity in β thalassemia, iron deficiency anemia, and erythroleukemia.

case	disorder	age	p moles of ^{14}C-ALA formed /10^6 erythroblasts /30 minutes *
1.	β-thalassemia minor	23	103
2.	β-thalassemia minor	26	112
3.	β-thalassemia minor	18	104
4.	Iron deficiency anemia	42	121
5.	Iron deficiency anemia	28	71
6.	Iron deficiency anemia	61	82
7.	Erythroleukemia	61	177
8.	Erythroleukemia	64	80
9.	Erythorleukemia	42	56
10.	Erythroleukemia	38	41

* Assayed with PLP in the incubation mixture

ALA-synthetase activity was significantly diminished. In case 10, who was unresponsive to pyridoxine treatment, addition of pyridoxal phosphate (PLP) (0.2 mM) to the enzyme assay restored the enzyme activity to normal. Because his blood PLP concentration was low, the patient was given parenteral PLP. A gradual and nearly normal complete erythropoietic response followed this therapy. After the initial reticulocytosis, bone marrow ALA-synthetase activity remained low and was no longer stimulated by PLP *in vitro*. After the full erythropoietic response, enzyme activity re-

turned to normal. In the remaining 4 cases, PLP failed to improve the enzyme activity *in vitro*.

ALA-synthetase activity in iron deficiency anemia and beta-thalassemia minor are shown in table 2. All 3 patients with iron deficiency anemia had a diminished activity in bone marrow erythroblasts. The enzyme activity was also depressed in patients with beta-thalassemia minor. Table 2 also shows the enzyme activity in the bone marrow erythroblasts of erythroleukemia. The enzyme activity in this disorder was also quite low.

DISCUSSION

The radiochemical assay methods of ALA-synthetase have been reported by several authors in recent years. Irving and Elliot [4] described a radiochemical method employing ^{14}C-succinate as precursor. Evert [5] used ^{14}C-succinate and ^{14}Cα-ketoglutarate. Freshney and Paul [6] used ^{14}C-glycine as a precursor. Aoki *et al.* [9] developed an assay method using ^{14}C-succinyl CoA in bone marrow erythroblasts. Strand *et al.* [8] and Ohashi *et al.* [10] established the method which used succinyl-CoA synthetase as the source of succinyl CoA. The author modified Evert's method by employing succinyl-CoA generating system (succinic thiokinase from the ALA-synthetase lacking mutant of *Rhodepseudomonas spheroides*). The advantage of this method is that it uses ^{14}C-succinyl CoA as a direct precursor of ALA-synthetase and that it does not employ the tedious procedure of partially purifying enzyme solution from bone marrow. In comparison to existing radiochemical assay, the sensitivity of this present assay has been increased by an improved method employing ^{14}C-succinyl CoA generation and bone marrow lysate as enzyme solution without any loss of enzyme activity in the sonicated supernate. The assay method using succinyl CoA generating system increased the sensitivity of assay 2.5 fold as compared with that of the assay without succinyl CoA generating system (unpublished data). The result confirms that methods which do not employ a succinyl CoA generating system understimate total ALA-synthetase activity.[8] The ALA-synthetase activity in the erythroblasts of normal bone marrow was 281 ±53 p moles/10^6 erythroblasts/30 minutes. This value is quite close to the value of 305 ±17 p moles of ALA generated by partially purified enzyme solution from normal bone marrow hemolysate mitochondria reported by Aoki.[9] This fact suggests that the assay of enzyme activity is not interfered by the low permeability of the substrate or inhibitor contained in the crude hemolysate.

The activity and the systhesis of ALA-synthetase is partially controlled by end product inhibition of heme.[12] – [13] Similar mechanism affecting heme synthetase may limit the formation of heme from protoporphyrin 1X.[14] This is the first report of diminished ALA-synthetase activity in the bone marrow erythroblasts in β-thalassemia. Although the true mechanism for the reduced ALA-synthetase in thalassemia is not clear, there is some evidence that heme synthesis is controlled to some extent by globin synthesis: when protein synthesis in reticulocytes is inhibited by cyclohexamide, heme synthesis is also rapidly and reversibly reduced. Vavra and Poff [15] reported that heme synthesis is abnormally reduced in thalassemia reticulocytes, presumably as a result of the primary decrease in globin synthesis in this disorder. The data of the present study and the Takaku's report [7] suggest that the reduced activity of ALA-synthetase is mediated by the feedback inhibition of free heme present in the erythroblasts of thalassemic bone marrow.

Experimental results on the effect of iron on ALA-synthetase are controversial. Morrow

et al.[16] observed that ferrous iron depressed the activity of the enzyme in particles of avian hemolysate. Aoki[9] reported an inhibitory effect of ferrous iron on partially purified ALA-synthetase from rabbit reticulocytes. However, in chicken reticulocytes mitochondria, iron had an enhancing effect on ALA-synthetase activity,[17] and in rabbit bone marrow mitochondria, ferrous iron had no effect on this enzyme in concentration ranging from 1.7×10^{-6} M to 1.7×10^{-3} M.[18]

Our present study indicated that ALA-synthetase activity is depressed in iron deficient bone marrow erythroblasts. This data is consistent with Brown's observation[19] that the formation of ALA from glycine is decreased in iron deficient duck erythrocytes. It is suggested that the decreased activity of ALA-synthetase in this condition is due to the feedback inhibition of accumulated protoporphyrin in erythroblasts.

The present study demonstrates that the erythroblasts of patients with ISA had invariably diminished ALA-synthetase activity. This data confirms previous observation of Tanaka *et al.*[20] and Aoki *et al.*[9] that bone marrow ALA-synthetase is diminished in ISA. The fact that 4 patients did not respond to PLP *in vitro* could be explained by the heterogeneity of ISA: pyridoxine responsive and non-responsive cases. The reported findings of PLP deficiency provide basis for the low ALA-synthetase activity in certain cases of this disorder. Although the impaired heme synthesis in ISA has been postulated by the presence of hypochromic anemia, the presence of ineffective erythropoiesis on ferrokinetic study and the accumulation of iron in the mitochondria of erythroblasts, Tanaka *et al.*[20] have shown the decreased activity of ALA-synthetase in this condition. The decreased PLP concentration in blood and plasma was shown in experimental sideroblastic anemia in guinea pig given INH and cycloserine.[21] Complete understanding of the pathogensis of ISA must take into acount other apparent abnormalities in heme biosynthesis, such as the accumulation of large amount of protoporphyrin and the possible disturbance of ferrochelatase and moreover the as yet unexplained mechanism for the massive accumulation of mitochondrial iron in erythroblasts in this disorder. However, the alteration in the activity of ALA-synthetase can be a factor in the pathogenesis of anemia and mitochondrial change in ISA, since this enzyme is one of the mitochondrial, rate limiting enzyme in heme biosynthesis.

Defective heme and globin synthesis have been reported in erythroleukemia. Steiner *et al.*[22] and Necheles and Dameschek[23] demonstrated lowered activities of ALA-dehydrase and heme synthetase in erythroleukemia. In the studies of globin synthesis in erythroleukemia, Pagnier *et al.*[24] found a very unusual large pool of β_2 dimer, which is not a precursor of normal hemoglobin. They reported that the presence of Hb-H was always due to unbalanced synthesis of α and β chains, large excess of β chain, which was caused either by a defect in α chain synthesis or to an excess of β chain synthesis.

Tanaka *et al.*[25] previously reported the decreased ALA-synthetase activity in bone marrow erythroblasts in erythroleukemia. In that report ALA-synthetase activity was assayed by the method using ^{14}C-αketoglutarate, which did not use succinyl CoA generating system.

The present report confirms our previous data by the assay method using succinyl CoA generating system. If, as Damescheck[26] suggested, sideroblastic anemia is a preleukemic condition finally ending with leukemia, decreased ALA-synthetase activity might be a common pathologic phenomenon of erythroleukemia and sideroblastic anemia.

It is possible with the impaired heme synthesis does not represent the primary defect in erythroleukemia and sideroblastic anemia, but it would conceivably be a reflection of a

more basic underlying disturbances in proliferation and maturation of stem cells which is suggested by the presence of the chromosomal abnormalities in this disorder. However, the similarity of the biochemical, cytochemical and clinical manifestation between erythroleukemia and sideroblastic anemia suggests that they may constitute a group of related changes due to an aquired and probably neoplastic disturbances in proliferation and differentiation of stem cells. The activity of ALA-synthetase was uniformly depressed in erythroleukemia, whereas in sideroblastic anemia, the depression of this enzyme activity was not uniform. The reduction of bone marrow ALA-synthetase has thus been revealed in various hematological disorders, although the mechanism of the reduction might be diverse.

SUMMARY

A sensitive and simple radiochemical assay method was established and employed for the measurement of ALA-synthetase in human bone marrow erythroblasts. Low ALA-synthetase activity has been documented in the bone marrow of five patients with idiopathic sideroblastic anemia. One patient showed an *in vitro* stimulation of bone marrow ALA-synthetase activity by PLP as well as *in vivo* response of the anemia by PLP treatment. In iron deficiency anemia, β-thalassemia minor and erythroleukemia, bone marrow ALA-synthetase activity was all depressed. Although the mechanism of the lowered enzyme activity was considered to be diverse in each disorder, the pathogenetic significance of the diminished enzyme activity in the development of anemia of these disorders were discussed.

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