Measurement of δ-Aminolevulinic Acid Synthetase Activity in Human Erythroblasts

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ABSTRACT A new, specific, and simple method for the determination of δ-aminolevulinic acid (ALA) synthetase activity in human bone marrow cells has been developed. ALA synthetase of erythroblasts was partially purified so as to permit the use of [^{14}C]succinyl-CoA as a substrate for this enzyme. In this enzyme preparation there were negligible activities of succinyl-CoA hydrolase, α-ketoglutarate dehydrogenase, and succinyl-CoA synthetase and there was no activity of ALA dehydrase.

The ALA formed from [^{14}C]succinyl-CoA has been isolated by column chromatography. Radioactivity in the eluate from the column has been proved by paper chromatography to be exclusively that of [^{14}C]ALA.

The entire assay can be completed within 4 h, and [^{14}C]succinyl-CoA was incorporated into [^{14}C]ALA on the order of several percent.

Moderate to marked decreases of ALA synthetase activity have been demonstrated in the erythroblasts of all cases of sideroblastic anemia. In the cases of iron deficiency anemia, on the other hand, normal or slightly elevated activity has been obtained.

INTRODUCTION

δ-Aminolevulinic acid (ALA)[1] synthetase, the enzyme which catalyzes the condensation of glycine and succinyl-CoA to form ALA, has been accepted as a rate-limiting enzyme in heme biosynthesis (1–3). This enzyme is inducible in the mitochondria of liver in experimental animals (4), and is elevated in the liver of patients with hepatic porphyria (5, 6). Properties of the enzyme have been mainly investigated in bacteria (7–9).

The importance of this enzyme activity in the regulation of heme synthesis has been well recognized. However, determination of its activity in human bone marrow erythroid cells has not been conducted because the activity in human erythroblasts is too low to measure by the conventional colorimetric method.

In recent years, radioisotopic methods for the measurement of ALA synthetase activity have been introduced. Irving and Elliott (10) developed a radioisotopic method using [^{14}C]succinate as a precursor. Ebert, Tschudy, Choudry, and Chirigos (11) used [^{14}C]succinate and [^{14}C]α-ketoglutarate, and Freshney and Paul (12) used [^{14}C]glycine as precursors. Takaku and Nakao (13) measured the enzyme activity in human erythroblasts by using [^{14}C]α-ketoglutarate as a precursor. However, in methods which use [^{14}C]succinate or [^{14}C]α-ketoglutarate as precursor, ALA synthetase activities are indirectly measured. [^{14}C]succinate or [^{14}C]α-ketoglutarate has to be metabolized to [^{14}C]succinyl-CoA before it is condensed with glycine to form [^{14}C]ALA. The possibility therefore exists that the pathways from succinate or α-ketoglutarate to succinyl-CoA may be influenced by various drugs or pathologic conditions. Although glycine is the direct precursor of ALA synthetase, its K_m is approximately 160 times higher than that of succinyl-CoA. Since mitochondrial fractions or cell homogenates were used as the enzyme solutions in previous methods it is also possible that succinate, α-ketoglutarate, or glycine could be metabolized into various products. Moreover, fluctuations in the activities of other metabolic pathways not related to ALA formation may influence the incorporation of these precursors into ALA. Contamination with these precursors and their metabolites during the separation of the newly formed ALA is another troublesome problem.

In the present investigation, ALA synthetase activity in the hemolyzed particles of erythroblasts was markedly increased by sonication. Extraction of the enzyme from the sonicated particles by deoxycholate also caused

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[1] *Abbreviation used in this paper:* ALA, δ-aminolevulinic acid.

the enzyme activity to increase (see Results). These facts imply that extraction of ALA synthetase from erythroblasts is necessary to obtain an accurate estimation of ALA synthetase activity in erythroblasts. We have previously purified the ALA synthetase of rabbit reticulocytes (14). In this study, we have modified this technique and partially purified the enzyme from human erythroblasts with a minimum loss of the activity. The partially purified enzyme solution contained negligible activities of succinyl-CoA hydrolase and succinyl-CoA synthetase, very low activity of α-ketoglutarate dehydrase, and no activity of ALA dehydrase. By measuring the incorporation of [^{14}C]succinyl-CoA into ALA in this enzyme solution, a direct and specific determination of ALA synthetase activity in human erythroblasts has become possible.

We have confirmed the specificity and reproducibility of our new method, and then measured ALA synthetase activities in erythroblasts from patients with several hematological disorders by this new method.

METHODS

ALA-HCl and ATP(99% pure) were obtained from Sigma Chemical Co., St. Louis, Mo. CoA(85–95% pure) was purchased from Boehringer Mannheim Corp., New York. Pyridoxal-5′-phosphate was a generous gift of Wakamoto Pharmaceutical, Tokyo. [4-^{14}C]ALA (53.0 mCi/mmol), [1,4-^{14}C]succinic anhydride (10.8 mCi/mmol), and [2,3-^{14}C]succinic acid(18.0 mCi/mmol) were the products of Daiichi Pure Chemicals, Tokyo. [5-^{14}C]α-ketoglutarate (9.33 mCi/mmol) was obtained from New England Nuclear, Boston, Mass.

Preparation of [^{14}C]succinyl-CoA. 0.1 ml of [1,4-^{14}C]-succinic anhydride (10.8 mCi/mmol, 0.1 mCi/ml in benzene) was combined with 0.1 ml of CoA(10 mg/ml in water) in 2 ml of 0.1 M $KHCO_3$ as described by Simon and Shemin (15) and the amount of the succinyl-CoA formed was checked by the method of Lipmann and Tuttle (16). Aliquots of this isotopic solution were frozen immediately after preparation and used within 1 wk.

Pretreatment of Dowex resins. Dowex 50 × 8(200–400 mesh, Dow Chemical Co., Midland, Mich.) was washed first with 10 vol of 2 N sodium hydroxide, then with 10 vol of water, and further with 10 vol of 2 N hydrochloric acid. This procedure was repeated 5 times. It was then washed twice with 10 vol of water. The pH of the resin suspension was 2–3. Dowex 1 × 8(200–400 mesh) was treated similarly, and the pH was adjusted to 6–7.

Preparation of enzyme solution. 3–5 ml of bone marrow was aspirated into a heparinized syringe from sternum or iliac bone. After the bone marrow was well mixed, the nucleated cell count and percent of erythroblasts were calculated. Volumes that contained 5×10^6–5×10^7 of erythroblasts were used to determine the ALA synthetase activities. The cellular fraction which was obtained by centrifuging the bone marrow suspension at 3,000 *g* for 10 min was hemolyzed with 4 vol of water for 10 min. After restoring isotonicity by adding 1.15% KCl solution, the hemolysate was centrifuged at 10,000 *g* for 10 min. The precipitate was washed once with 1.15% KCl solution containing 0.01 M potassium phosphate buffer (pH 7.2). The precipitate suspended in 5 vol of 1.15% KCl solution containing 0.01 M potassium phosphate buffer (pH 7.2) was sonicated for 1 min in ice water (20 kcycle/s, 120 W, model UR-150P, Tomy, Tokyo), and then centrifuged at 20,000 *g* for 20 min. 5 vol of 0.2% sodium deoxycholate solution was added to the pellet. It was kept in ice for 10 min with frequent agitation, and centrifuged at 20,000 *g* for 10 min. The same extraction procedure was conducted twice. The supernates of the centrifugations were collected to be used as the enzyme solution.

Radioassay of ALA synthetase. The incubation mixture contained [^{14}C]succinyl-CoA, 50 nmol (0.5 μCi); glycine, 90 μmol; pyridoxal phosphate, 20 nmol; potassium phosphate buffer (pH 7.2), 22.5 μmol, and 0.3 ml of enzyme solution (equivalent to approximately 1.7×10^6–1.7×10^7 of erythroblasts, 0.3–1.2 mg protein) to a final vol of 0.5 ml. After 30 min of incubation at 37°C, the reaction was terminated by adding 0.5 ml of 5% trichoroacetic acid. After the addition of 0.5 ml of 0.1 M sodium succinate, the incubation mixture was centrifuged at 3,000 *g* for 10 min. 2 ml of 2% trichloroacetic acid was added to the sediment which was mixed and recentrifuged at 3,000 *g* for 10 min. Both supernates were combined.

Isolation of [^{14}C]ALA. 0.2 ml of 0.1 M ATP was added to the supernate. It was then applied to the top of a Dowex 50 × 8 (200–400 mesh) column, 0.5 × 1.0 cm. [^{14}C]-ALA was eluted with 3 ml of 2 M pyridine acetate (pH 6.2) after the column was washed with 100 ml of water. The [^{14}C]succinyl-CoA and like substances which arose from the reaction between [^{14}C]succinic anhydride and nucleic acid contaminations in CoA were removed from the column by this washing. ATP was thought to accelerate this removal.

Counting by liquid scintillation counter. The eluate from the Dowex column was mixed with 15 ml of dioxane scintillator containing 2,5-diphenyloxazole, 4 g/liter; 1,4-bis[2-(5-phenyloxazolyl)]benzene, 0.4 g/liter; and naphthalene, 60 g/liter to be counted by a liquid scintillation counter. The counting efficiency was approximately 75%.

Application of the eluate from Dowex column to paper chromatography. In an attempt to demonstrate that the radioactivity of the eluate from the Dowex column was in [^{14}C]ALA, the eluate was applied, together with cold ALA (1 μmol), to the top of a Dowex 1 × 8 (200–400 mesh) column, 2 × 1 cm, to remove pyridine, and then 5 ml of water was added to the column. The eluate from the Dowex 1 × 8 column was coupled with acetylacetone; i.e., to 8 ml of the eluate was added 8 ml of the solution which contained 1 part of acetylacetone and 10 parts of 1 M acetate buffer (pH 4.8). The mixture was heated for 10 min in boiling water. After the mixture was cooled, it was evaporated to dryness by a rotary evaporator, and the residue was then dissolved in 15 drops of methanol. This methanol solution was then utilized for paper chromatography (Whatman 51 A, 2 × 30 cm). Separation was made by an ascending system using *n*-butanol-NH_4OH-water (50:1:50). The chromatogram was scanned by a gas flow counter, and then sprayed with Ehrlich's reagent.

Measurement of other enzyme activities. ALA dehydrase activity was measured by following the method of Wada et al. (17).

Succinyl-CoA hydrolase activity was measured in the incubation mixture which contained succinyl-CoA, 1 μmol; potassium phosphate buffer (pH 7.5), 50 μmoles; and enzyme to a final vol of 3 ml. It was incubated at 37°C for 15–30 min. After conversion to succinyl-hydroxamate, the

TABLE I

ALA Synthetase Activities in Each Step of Fractionation Procedure

Enzyme solution	Counts incorporated	Vol	Protein	Specific activity	Total activity
	cpm/tube*	*ml*	*mg/ml*	*units‡/mg protein*	*units*
Subject 1 (normal)					
Hemolyzed precipitate	250	20	7.1	8.15	1,160
Sonicated solution	1,210	19	7.1	39.5	5,330
Sonicated supernate	580	18	6.5	20.7	2,420
Deoxycholate-extracted supernate	41,000	2	1.5	6,330	19,000
Deoxycholate-extracted precipitate	4,200	2	1.0	972	1,940
Subject 2 (sideroblastic anemia)					
Hemolyzed precipitate	140	20	8.1	4.0	648
Sonicated solution	680	19	8.1	19.4	2,990
Sonicated supernate	410	18	7.5	12.7	1,710
Deoxycholate-extracted supernate	24,800	2	2.0	2,870	11,500
Deoxycholate-extracted precipitate	2,500	2	1.1	526	1,160

* Zero time cpm, which was always between 300 and 400, was subtracted.

‡ 1 unit is defined as the enzyme activity which forms 1 pmol of ALA per 30 min.

remaining part of succinyl-CoA was measured according to the method of Lipmann and Tuttle (16).

ALA synthetase activity was measured with [^{14}C]α-ketoglutarate as substrate in the incubation mixture which contained glycine, 90 μmol; [^{14}C]α-ketoglutarate, 0.5 μmol (4.68 μCi); potassium phosphate buffer (pH 7.2), 22.5 μmol; pyridoxal phosphate, 20 nmol; NAD, 0.5 μmol; and 0.3 ml of enzyme solution to a final vol of 0.5 ml. After 30 min of incubation at 37°C the reaction was terminated by addition of 0.5 ml of 5% trichloroacetic acid. Then 0.5 ml of 0.1 M α-ketoglutarate was added. Isolation of formed ALA was performed by the method used for ALA synthetase activity measurement with [^{14}C]succinyl-CoA as substrate except that ATP was not added in this case.

Measurement of the formation of [^{14}C]ALA from [^{14}C]-succinate was also conducted by the same method used for ALA synthetase activity measurement with [^{14}C]-succinyl-CoA as substrate, except that the incubation mixture contained ATP (0.8 μmol) and 0.3 μmol (5.4 μCi) of [^{14}C]succinate in place of [^{14}C]succinyl-CoA.

The colorimetric method for the determination of ALA synthetase activity was conducted as follows. The incubation mixture contained glycine, 40 μmol; succinyl-CoA, 50 nmol; pyridoxal phosphate, 20 nmol; potassium phosphate buffer (pH 7.2), 22.5 μmol; and 0.3 ml of enzyme solution to a final vol of 0.5 ml. After incubating for 30 min at 37°C the reaction was terminated by addition of 0.5 ml of 0.5% trichloroacetic acid. ALA formed was determined by reaction with modified Ehrlich's reagent.

TABLE II

Formation of ALA from Various Precursors

Substrates	ALA formed
	pmol/30 min/tube
[^{14}C]α-ketoglutarate	150
[^{14}C]succinate	8
[^{14}C]succinyl-CoA	1,080
Succinyl-CoA	1,190*

* Colorimetric method was employed, but the value is considered to be not very accurate because the reading of optical density in this case was very low (about 0.02).

RESULTS

As shown in Table I, ALA synthetase activity is markedly increased by sonication and deoxycholate extraction. An approximately 4–5-fold increase of the activity was achieved by sonication. Moreover, an about threefold increase of the activity, as compared with that of the sonicated mixture, was obtained by extraction with sodium deoxycholate. These facts indicate that disruption of the mitochondria by sonication as well as the extraction with deoxycholate enhances the activity of the ALA synthetase. Table I also shows that only a little activity was lost into the sonicated supernate and deoxycholate-extracted sediment, as compared with the activity contained in the deoxycholate-extracted supernate (20–30% of the deoxycholate extracted supernate).

ALA synthetase in the bone marrow obtained from the patients with sideroblastic anemia is fractionated in the same manner as that of normal individual (Table I). The same fractionation pattern was also seen with the enzyme obtained from other patients with iron deficiency anemia.

Purity of the enzyme. Table I also shows the results of partial purification of the enzyme. ALA synthetase activity was most markedly demonstrated in the deoxycholate-extracted supernate. In this partially puri-

fied ALA synthetase solution, negligible or very small amounts of [^{14}C]ALA were formed from [^{14}C]succinate or [^{14}C]α-ketoglutarate as shown in Table II. These facts indicate that this enzyme solution contains only negligible activity of succinyl-CoA synthetase and very low activity of α-ketoglutarate dehydrogenation complex.

ALA dehydrase activity was not detected in the enzyme solution.

Disappearance of succinyl-CoA in the incubation mixture was followed as shown in Fig. 1. Succinyl-CoA in the deoxycholate-extracted supernate was slightly more stable than that in the sonicated mixture or sonicated supernate. This may be considered to be due to the low succinyl-CoA hydrolase activity of the deoxycholate-extracted supernate as compared with that of sonicated mixture or sonicated supernate. The rate of disappearance of succinyl-CoA in the deoxycholate-extracted supernate was almost as low as that by the spontaneous degradation in the absence of the enzyme. Succinyl-CoA hydrolase activity in the erythroblasts is much lower than that in the liver (18). The low succinyl-CoA hydrolase activity in the erythroblasts and elimination of the enzyme activity during the process of ALA synthetase preparation enabled us to use [^{14}C]succinyl-CoA as the precursor in the measurement of ALA synthetase activities of the erythroblasts. As shown above, the added succinyl-CoA was hydrolysed approximately 50%

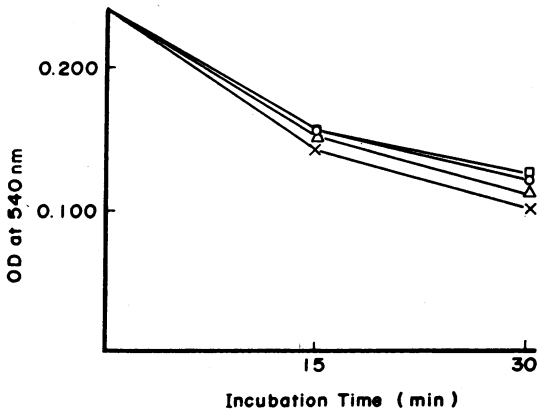

FIGURE 1 Disappearance curves of succinyl-CoA at 37°C. 1 μmol of succinyl-CoA was incubated at 37°C in potassium phosphate buffer (pH 7.2), 50 μmoles and enzyme solution to final vol of 3 ml. After conversion to succinylhydroxamate, the remaining succinyl-CoA was measured according to the method of Lipmann and Tuttle (16). ○——○, Deoxycholate-extracted supernate; △——△, sonicated solution; ×——×, sonicated supernate; □——□, water was added instead of enzyme solution.

within 30 min. However, enough of this substrate was added to the incubation mixture so that it would not be the rate-limiting factor for the reaction (see Fig. 3).

Stability of [^{14}C]succinyl-CoA. [^{14}C]succinyl-CoA, when stored at − 20°C, was very stable for at least 1 wk,

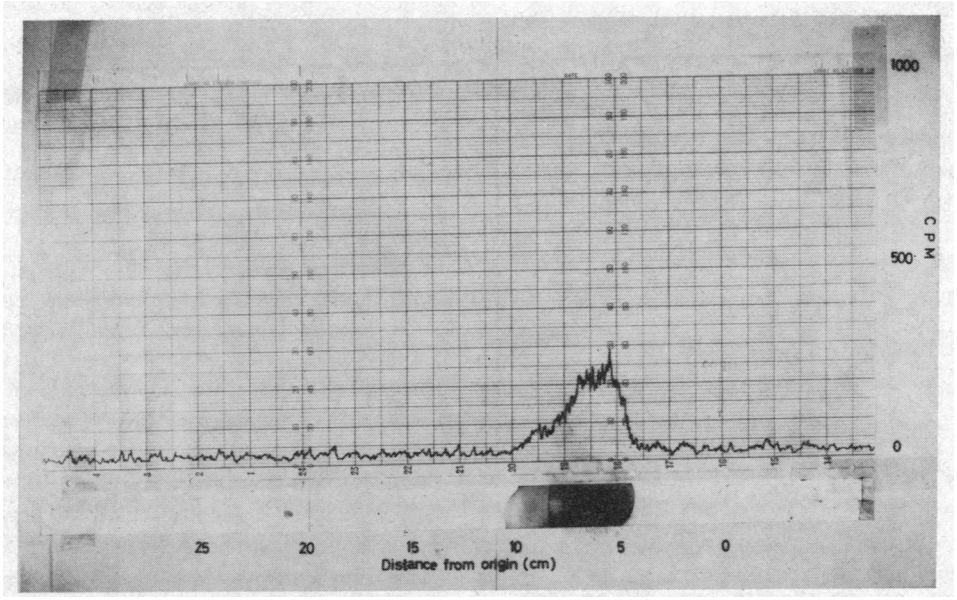

FIGURE 2 Paper chromatogram of the eluate from a Dowex 1 × 8 column. The eluate from the Dowex 1 × 8 column, which was coupled with acetylacetone to form ALA-pyrole, was evaporated to dryness and the residue was then dissolved in a small amount of methanol. This solution was applied to paper (Whatman 51A, 2 × 30 cm). Separation was made by an ascending system using *n*-butanol-NH_4OH-water (50:1:50). Chromatogram was scanned by a gas flow counter and sprayed with Ehrlich's reagent.

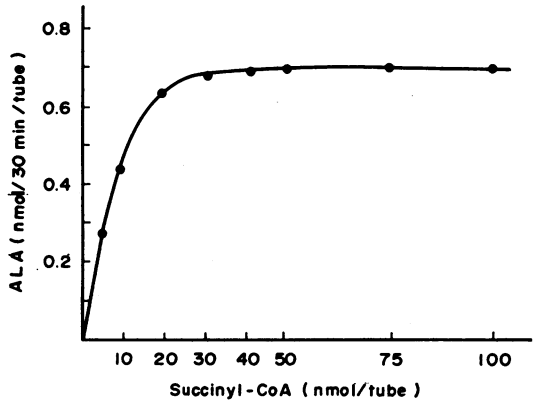

FIGURE 3 Effect of concentration of [^{14}C]succinyl-CoA on the enzyme activity. Incubation mixture contained glycine, 90 μmol; pyridoxal phosphate, 20 nmol; potassium phosphate buffer (pH 7.2), 22.5 μmol; enzyme, 0.3 ml; and [^{14}C]succinyl-CoA varied as indicated. Incubated for 30 min at 37°C.

and significant destruction did not occur when checked by the method of Lipmann and Tuttle (16).

Identification of ALA. The eluate from the Dowex 50 × 8 column was applied to the top of a Dowex 1 × 8 column to remove pyridine which causes tailing of ALA-pyrrole on paper chromotography and then coupled with acetylacetone to form ALA-pyrrole. The solution was concentrated to dryness, and the residue was dissolved in a small amount of methanol. This methanolic solution was then subjected to paper chromatography. Fig. 2 shows that the radioactivity of the eluate consisted mostly (above 90%) of [^{14}C]ALA-pyrrole. Negligible activity (below 5%) was found in the Dowex 1 × 8 resin which was washed with 5 ml of water.

Determination of optimum substrate and cofactor concentrations. To obtain the optimum conditions for

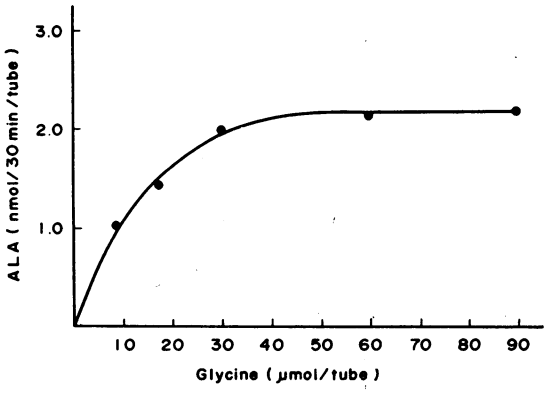

FIGURE 4 Formation of [^{14}C]ALA as a function of glycine concentrations. Incubation conditions are shown in Fig. 3, 50 nmol of [^{14}C]succinyl-CoA added. Amount of glycine varied as indicated.

TABLE III
Effects of Several Compounds on ALA Synthetase Activity in Our Enzyme Solution

Additions	Concentration	ALA synthetase activity
	M	%
None		100
$MgCl_2$	10^{-4}	97
$MgCl_2$	10^{-3}	71
Iodoacetamide	10^{-4}	92
Iodoacetamide	10^{-3}	52

ALA formation, the effects of substrate concentrations, incubation time, and pH on the enzyme activity were examined. Fig. 3 shows the effect of concentration of [^{14}C]succinyl-CoA on the enzyme activity. ALA formation became constant beyond 25 nmol of [^{14}C]succinyl-CoA per tube. ALA formation as a function of glycine is shown in Fig. 4. Optimum pH was in a range of 7.0–7.4 in potassium phosphate buffer. ALA synthesis as a function of enzyme content is shown in Fig. 5. Fig. 6 shows ALA formation as a function of incubation time. From these results, the assay system described in the Methods section was designed.

Table III shows the effects of $MgCl_2$ and iodoacetamide on ALA synthetase activity. These two substances inhibited ALA synthetase activity in this enzyme solution. Omission of pyridoxal phosphate decreased the activity by approximately half.

Recovery of ALA. In order to examine the recovery of ALA, 0.05 μCi of [^{14}C]ALA was added to the incubation mixture with 1 ml of 5% trichloroacetic acid, after which isolation was carried out as described for ALA synthetase assays. The results indicated that the recovery was above 95%.

Comparison with colorimetric method. As shown in Table II, the value of ALA synthetase activity obtained by the colorimetric method is similar to that determined by the radioisotopic method using [^{14}C]succinyl-CoA as substrate, although optical density in colorimetric method is so low that the value of the enzyme activity obtained by this method is not considered very accurate.

Replicate analysis. ALA synthetase activity was always measured in duplicate. Differences were always within 5%. When the enzyme activity in the erythroblasts from a hematologically normal person was determined twice within 1 wk, results of two measurements did not differ significantly (below 5%).

ALA synthetase activity in the erythroblasts of primary sideroblastic anemia. Table IV shows the ALA synthetase activity in the erythroblasts of patients with

TABLE IV

ALA Synthetase Activities in Erythroblasts of Patients with Primary Sideroblastic Anemia

Patients			
Age	Sex	Type	ALA formed
			nmol/10⁸ erythroblasts/30 min
69	Male	Acquired	5.7
34	Female	"	16.4
77	Male	"	5.4
74	Male	"	1.4
73	Male	"	9.3
54	Female	"	2.2
61	Male	"	12.6
34	Male	"	6.6
66	Male	"	15.7
51	Female	"	15.4
38	Female	"	3.6
59	Female	"	5.0
60	Male	"	12.3
38	Male	"	1.4
60	Male	"	3.5
39	Female	"	12.3
33	Male	Congenital	1.3
21	Male	Pyridoxine responsive	13.0
13	Male	" "	1.1
		(Mean±SEM)	7.6±1.1
Normal (n = 9)		(Mean±SEM)	30.5±1.7

primary sideroblastic anemia. These patients were diagnosed on the basis of hypochromic anemia, erythroid hyperplasia in the bone marrow, presence of a large number of ring sideroblasts (above 30%), hyperferremia, and absence of causative diseases. Diagnosis of a case of congenital sideroblastic anemia was given on the basis of the patient's sex, early occurrence of anemia, and family history of having a brother with sideroblastic anemia.

Nine cases of acquired type showed marked decreases of ALA synthetase activity as shown in this table, while four cases showed moderate decreases and three cases had values which were approximately half of normal.

The case of congenital sideroblastic anemia showed a marked decrease of this enyme activity.

Two cases of pyridoxine responsive anemia showed decreases in the enzyme activity; one showed a marked decrease, and the other a moderate decrease.

The mean value of ALA synthetase activities in erythroblasts of patients with primary sideroblastic anemia is significantly below that of normal ($P < 0.001$).

Addition of α, α'-dipyridyl(10^{-4} M) into the incubation mixture did not affect the enzyme activity in cells from normal subjects or patients with sideroblastic anemia.

ALA synthetase activity of iron deficiency anemia. ALA synthetase activity was measured in eight cases of iron deficiency anemia. They all showed hypoferremia, increased iron binding capacity, and excellent response to iron treatment. Three cases showed slightly elevated activity, while five cases had normal activity (Table V).

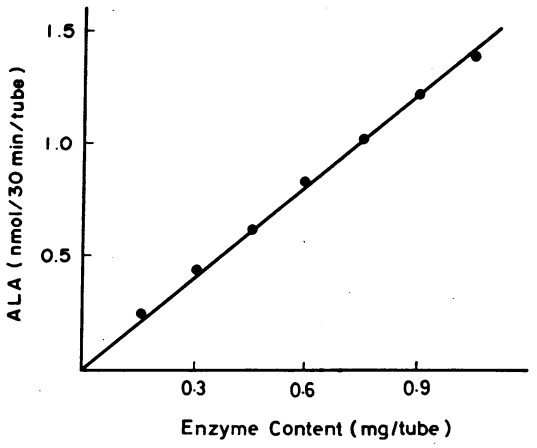

FIGURE 5 ALA formation as a function of enzyme content. Experimental conditions are described in Fig. 4, 90 μmol of glycine added. Enzyme content varied as indicated.

DISCUSSION

The colorimetric method has been sufficient to determine ALA synthetase activity in porphyric liver, in the reticulocytes of experimental animals, or in *Rhodopseudomonas spheroides* which contain high enzyme activity. However, this method is not sufficient to measure the low activity in the normal liver or human bone marrow erythroid cells. This is the reason why several radioisotopic methods have been introduced, using [^{14}C]-succinate, [^{14}C]α-ketoglutarate, or [^{14}C]glycine as precursors. However, the methods using these precursors have several disadvantages (see Introduction).

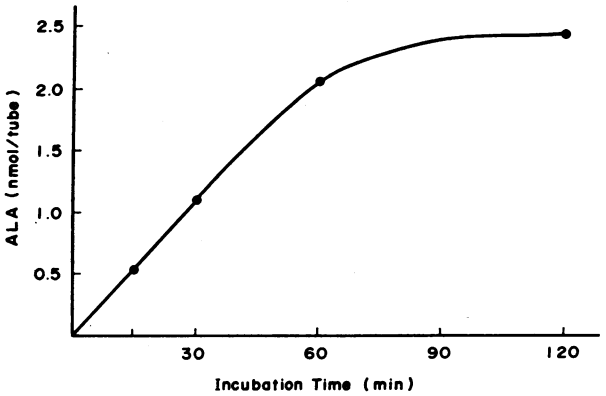

FIGURE 6 ALA formation as a function of incubation time. Experimental conditions are described in Fig. 4, 90 μmol of glycine added. Incubation time varied as indicated.

TABLE V

ALA Synthetase Activities in Erythroblasts of Patients with Iron Deficiency Anemia

Patients			
Age	Sex	Causative diseases	ALA formed
			nmol/10^8 erythroblasts/30 min
40	Female	Idiopathic	30.9
40	Female	Bleeding from gastric ulcer	34.3
25	Female	Idiopathic	37.6
15	Male	Idiopathic	25.4
23	Female	Idiopathic	40.0
19	Female	Idiopathic	26.0
45	Male	Bleeding from gastric ulcer	40.0
60	Female	Bleeding from gastric erosion	52.0
		(Mean±SEM)	35.8±3.0
Normal (n = 9)			(Mean±SEM) 30.5±1.7

Our enzyme solution contains negligible activity of succinyl-CoA hydrolase. This is due to the fact that erythroid tissue contains very low succinyl-CoA hydrolase activity, as compared with liver, and also to the fact that partial purification of the enzyme eliminated succinyl-CoA hydrolase activity. These facts enabled us to use [^{14}C]succinyl-CoA as a precursor of ALA synthetase. We presume that this enzyme solution contains only negligible activity of succinyl-CoA synthetase and very low activity of α-ketoglutarate dehydrogenation complex, because negligible or very few counts were incorporated into [^{14}C]ALA from [^{14}C]succinate or [^{14}C]α-ketoglutarate, in spite of the fact that very high counts were incorporated into [^{14}C]ALA from [^{14}C]-succinyl-CoA by using the present enzyme solution.

ALA synthetase activity in our partially purified enzyme solutions is considered to reflect the activity in erythroblasts, although 20–30% of the total activity is lost into the sonicated supernate and sodium deoxycholate-treated sediment. However, there was no difference in this loss of enzyme activity between normal and pathologic erythroblasts. ALA synthetase activity of erythroblasts markedly increases when hemolysed precipitates are sonicated. This phenomenon is considered attributable to the fact that disruption of mitochondria by sonication changes the permeability of the substrate and the structure surrounding the enzyme. Moreover, the enzyme activity is further enhanced by extraction from sonicated precipitates with sodium deoxycholate. These facts indicate that partial purification and extraction of the enzyme are necessary to obtain maximal values of ALA synthetase activity in erythroblasts. If cell homogenate or hemolysate is used as the enzyme solution for ALA synthetase, the enzyme activity may be interfered with by the low permeability of the substrate or inhibitor contained in the crude homogenate or hemolysate.

Separation of [^{14}C]ALA by column chromatography is difficult if contamination with compounds other than [^{14}C]succinate is significant (10). However, [^{14}C]succinyl-CoA was scarcely metabolized to substances other than [^{14}C]ALA in our enzyme solution. Therefore, separation of [^{14}C]ALA is very simple in our assay system. Only a 4-h period is required until the value of the ALA synthetase activity is obtained.

Since ALA synthetase activity in reticulocytes is very low when compared with that of erythroblasts (approximately 1/100), for the sake of simplicity, ALA synthetase activity in reticulocytes was neglected in the calculation of the enzyme activity in erythroblasts. Therefore, higher activity per erythroblast may be obtained by our method in the case of severe reticulocytosis. ALA synthetase activity in the reticulocytes in various hematological disorders will be published elsewhere.

In the present study, erythroblasts of patients of primary sideroblastic anemia invariably showed decreased ALA synthetase activity. Disturbance of heme synthesis has been considered one of the causes of sideroblastic anemia (19). However, its precise mechanism has not been elucidated. Takaku and Nakao (13) reported a case of acquired sideroblastic anemia which showed very low ALA synthetase activity of erythroblasts using [^{14}C]α-ketoglutarate as a precursor. However, the method using [^{14}C]α-ketoglutarate as a precursor has several problems concerning accuracy and simplicity as mentioned earlier. Our result confirmed their report by a more specific assay method, and also showed that the decrease of ALA synthetase activity in erythroblasts is a universal finding in primary acquired sideroblastic anemia, although the degree of decrease differs in each

case. Our results also showed that ALA synthetase activity in erythroblasts is also decreased in congenital sideroblastic anemia and pyridoxine responsive anemia.

Clinical features of sideroblastic anemia include hypochromic anemia, elevated serum iron level, ineffectiveness of iron therapy, erythroid hyperplasia in the bone marrow, and presence of large number of ring sideroblasts. It is possible to consider that these characteristics are caused by the primary or secondary decrease of ALA synthetase activity in erythroblasts.

The presence of ring sideroblasts is one of the most important features of sideroblastic anemia. Siderotic granules in the ring sideroblasts are due to the deposition of iron in the mitochondria (19–21) where ALA synthetase exists (1). We showed that purified ALA synthetase in the rabbit reticulocytes was inhibited by iron in the concentrations of 10^{-4}–10^{-5} M (14). If reduced ALA synthetase activity is an invariable finding in sideroblastic anemia, inhibition of ALA synthetase activity by the iron accumulated in the mitochondria may be important as the pathogenesis of this anemia. However, (*a*) the case of primary acquired sideroblastic anemia which showed iron deficiency but developed ring sideroblasts after blood transfusion (22), (*b*) the fact that the enzyme solutions used to determine ALA synthetase activity are considered free from iron because of the extraction from sonicated precipitate, and (*c*) the fact that addition of α, α'-dipyridyl to the incubation mixture did not affect the enzyme activity suggest that a primary decrease of ALA synthetase activity may be the cause of sideroblastic anemia.

In pyridoxine responsive anemia, administration of large amounts of pyridoxine is necessary to restore normal hemoglobin level. Considering the cases (23) that showed iron deficiency after phlebotomy but required pyridoxine to prevent anemia, inhibition of ALA synthetase activity by the accumulated iron does not seem to be the sole cause of pyridoxine responsive anemia. ALA synthetase needs pyridoxal phosphate as a cofactor (7, 14). It is, therefore, possible that pyridoxal phosphate in erythroblasts plays some role in the synthesis or degradation of ALA synthetase in erythroblasts. Low activity of pyridoxal kinase has been suggested to explain why large amounts of pyridoxine are required to prevent anemia in patients with pyridoxine responsive anemia.

Erythroblasts of iron deficiency anemia have been shown to contain normal or slightly elevated ALA synthetase activity, although the elevation of the mean value is not significant statistically ($P > 0.1$). These results have not been anticipated from the report that reticulocytes of iron deficient ducks have low ALA synthetase activities as compared with normal (24). However, in that report, ALA synthetase activity was measured by the colorimetric method using sodium citrate and glycine as substrate. This discrepancy of the result on ALA synthetase activity in erythroid cells of iron deficiency, therefore, might be due to the difference of the method used for the enzyme measurement or to species difference.

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