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CHARACTERIZATION AND MEASUREMENT OF Δ-AMINOLAEVULINATE SYNTHETASE IN BONE MARROW CELL MITOCHONDRIA*

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SUMMARY

1 Δ-Aminolaevulinate synthesis was demonstrated in normal rabbit bone marrow and the measurement of Δ-aminolaevulinate synthetase (succinyl-CoA: glycine succinyltransferase) was accomplished in mitochondria obtained from marrow cells ruptured by sonication The micromethod used to quantify Δ-aminolaevulinate was sensitive to the range of 10^{-9} mole The optimal concentrations of glycine, α-ketoglutarate, CoA, pyridoxal phosphate and NAD^+ for maximum enzyme activity at pH 7.5 were identified Succinate and succinyl-CoA were less effective as substrates than α-ketoglutarate Neither $MgCl_2$ nor EDTA stimulated enzyme activity Inhibition of the enzyme by its product, Δ-aminolaevulinate, was observed Neither heme nor protoporphyrin inhibited the production of Δ-aminolaevulinate

2 Electronmicroscopic study of the mitochondrial preparation disclosed that the mitochondria were partially damaged. The isolated mitochondria contained succinate dehydrogenase (EC 1 3 99 1) and ferrochelatase (EC 4 99 1.1) activities. The soluble enzymes of the heme synthetic pathway were absent

3 The rate of production of Δ-aminolaevulinate observed in the present experiments approximates the rate which would be required *in vivo* by the normal rabbit for hemoglobin synthesis.

INTRODUCTION

The first step in the heme synthetic pathway is the formation of Δ-aminolaevulinate by condensation of glycine and succinyl-CoA[2-5] The enzyme for this reaction, Δ-aminolaevulinate synthetase (succinyl-CoA glycine succinyltransferase), has been shown to be a mitochondrial enzyme in guinea pig and rat liver[6,7] Previous studies of Δ-aminolaevulinate synthesis in microorganisms[8-14], avian erythrocytes[5,15-17] and

Abbreviations MAPP, the pyrrole made by the condensation of Δ-aminolaevulinate with acetyl acetone, *i e*, 2-methyl-3-acetyl-5-propionic acid–pyrrole, DMAP, the pyrrole made by the condensation of amino acetone with acetyl acetone, *i e*, 2,4-dimethyl-3-acetyl-pyrrole

* Part of this work has been published in abstract form[1]

mammalian liver[6,7,18] indicate this step to be rate-limiting in the heme synthetic pathway.

Although determinations of erythroid Δ-aminolaevulinate synthetase have been described in mitochondria isolated from rabbit and chicken reticulocytes in preliminary reports[19,20], measurement of the enzyme in red cell precursors prior to the reticulocyte stage has not been reported previously In the present studies a procedure for isolation of mitochondria from bone marrow cells is described A micromethod for measurement of Δ-aminolaevulinate synthetase in marrow cell mitochondria is elaborated Δ-Aminolaevulinate synthetase from normal rabbit marrow has been characterized and its activity in this tissue determined

MATERIALS AND METHODS

Δ-Aminolaevulinate hydrochloride, CoA, α-ketoglutaric acid, sodium succinate, and L-[2-^{14}C]glycine were purchased from Calbiochem, Δ-[4-^{14}C]aminolaevulinate from Volk, and [^{59}Fe]ferrous citrate from Squibb NAD$^+$ and ATP were obtained from Sigma and hemin from Eastman

Succinyl-CoA was prepared by the method of SIMON AND SHEMIN[21] and determined by reaction with hydroxylamine[22]

Protoporphyrin was prepared as the methyl ester by the method of GRINSTEIN[23]. Free protoporphyrin was obtained by hydrolysis of the methyl ester with 3 M HCl.

Preparation of mitochondria

Bone marrow was obtained from white New Zealand rabbits (2 5–3.2 kg) The long bones (femora, tibiae and humeri) were cracked open, and the bone marrow delivered into cold plasma obtained from the same animal A uniform suspension of marrow cells was prepared by passing the marrow three times through a stainless-steel sieve. The cell suspension was mixed with 10–20 ml of packed red cells. The use of plasma and red cells prevented clumping of nucleated cells in the preparation of the marrow cell suspension The plasma and any remaining fat were removed by centrifugation at 700 $\times$ g for 20 min and the cells suspended in 4 vol of phosphate-buffered 0 25 M sucrose (pH 7 4) A total normoblast count was performed on this suspension The preparation from one animal yielded $3\,6 \cdot 10^9$ nucleated cells and the erythroid content varied from 25 to 35%

Adequate yields of mitochondria or significant enzyme activity could not be obtained by lysis of the cells with hypotonic solutions or homogenization techniques. The cells were lysed effectively by sonication with the Bronwill high intensity ultrasonic probe in 10-ml aliquots at 4°. Mitochondria were isolated by a minor modification of the method of SCHNEIDER[24]. The mitochondria were washed with 0 25 M sucrose to remove any remaining hemoglobin and resuspended in 0 25 M sucrose containing 0 05 M phosphate buffer (pH 7 4) Protein was determined by the method of LOWRY *et al* [25]

Characterization of mitochondria

The mitochondrial preparation was examined by electron microscopy. The washed mitochondria were fixed with 1% osmium tetroxide (in 0.25 M sucrose and 0.05 M phosphate buffer, pH 7 5). The specimen was dehydrated in graded alcohol

solutions and imbedded in 80% butyl- and 20% ethylmethacrylate containing 3% benzoyl peroxide. Sections were cut using the Porter-Blum MT-1 and MT-2 ultramicrotomes and stained with 1% phosphotungstic acid in 15% alcohol and 0.5% uranyl acetate in water. The electronmicrographs were taken with the R.C.A.-3F electron microscope.

The respiratory activity of the mitochondria was assessed by measurement of succinate dehydrogenase (EC 1.3.99.1) activity employing the manometric method described by GREEN AND ZIEGLER[26]

Enzyme assay

Δ-Aminolaevulinate synthetase activity was measured by determining the quantity of Δ-aminolaevulinate formed after incubating the mitochondrial preparation with the substrates and cofactors in a final volume of 1.0 ml at 37° for 30 min. Enzyme activity was expressed as number of nmoles of Δ-aminolaevulinate formed per 30 min at 37°. The specific conditions of incubation in the various experiments are indicated under RESULTS and in the appropriate tables and figures. Incubations were terminated by addition of 0.25 ml of 30% trichloroacetic acid. The Δ-aminolaevulinate in the deproteinized supernatant was determined as the MAPP by the method of GRANICK[27] with the following modifications. In order to increase the sensitivity of the determination of Δ-aminolaevulinate the entire supernatant (1.0 ml) was adjusted to pH 4.6 with 0.16 ml of 2 M sodium acetate, 0.04 ml of acetyl acetone was used for condensation to form the pyrrole. For the formation of the color 0.6 ml of Ehrlich mercury reagent was added. 15 min after addition of the reagent the absorbance at 552 mμ was determined in a cell of 1-cm optical length. When compared with the previously reported method[27], this procedure allowed a 2.5-fold increase in the sensitivity of the measurement of Δ-aminolaevulinate in spite of a decrease in the molar absorbance from $5.7 \cdot 10^4$ to $3.8 \cdot 10^4$.

For microassays of Δ-aminolaevulinate synthesis in bone marrow cell mitochondria incubations were carried out at 37° in a final volume of 0.26 ml. After addition of 0.12 ml of 15% trichloroacetic acid, 0.3 ml of supernatant was obtained. In subsequent steps the following volumes were used: 2 M sodium acetate, 0.025 ml; 2 M sodium acetate buffer (pH 4.6), 0.015 ml, acetyl acetone, 0.020 ml, and Ehrlich mercury reagent, 0.15 ml. Under these conditions the molar absorbance was $5.3 \cdot 10^4$ and the sensitivity of the method was 9 times greater than as described by GRANICK[27].

Chromatography

For identification of the MAPP in the assay of Δ-aminolaevulinate synthetase incubations were carried out with L-[^{14}C]glycine as substrate (specific activity 0.3 mC/mmole). After condensation the MAPP was subjected to ascending chromatography as described by MAUZERALL AND GRANICK[28]. The labeled Δ-aminolaevulinate was localized by radioautography. The radioactive spot was cut out and counted in a Tracerlab Omniguard low background counting system (Model BLB 5020L) with a counting efficiency of 30%.

Incorporation of [^{14}C]glycine, [^{14}C]Δ-aminolaevulinate and ^{59}Fe into heme by bone marrow cell mitochondria

The incorporation of certain isotopically labeled precursors by the mitochon-

drial preparation into heme was studied in order to determine whether any significant quantity of Δ-aminolaevulinate which was formed in the assay system was further utilized in the biosynthesis of porphyrins and heme. The precursors which were included were [^{14}C]glycine and [^{14}C]Δ-aminolaevulinate. The incorporation of [^{59}Fe]ferrous citrate was determined to assess whether the mitochondrial preparation contained any ferrochelatase (EC 4.99.1.1) activity. Incubations were terminated by the addition of a hemoglobin solution (500 mg) and then a 1.4 solution of 2% strontium chloride in glacial acetic acid and acetone. Hemin was isolated by the method of LABBE AND NISHIDA[29]. The radioactivity of the isolated ^{59}Fe-labeled hemin was determined in a Nuclear Chicago automatic gamma well counting system (Model 4222) with a counting efficiency of 35%. The radioactivity of the isolated ^{14}C-labeled hemin was determined in the gas flow counter mentioned above.

RESULTS

Preliminary experiments were performed to determine the optimum time of sonication of the marrow cell suspension. Such an experiment is illustrated in Table I. A sonication time of 15 sec was selected for lysis of marrow cells for the subsequent isolation of mitochondria.

TABLE I

EFFECT OF SONICATION ON Δ-AMINOLAEVULINATE SYNTHETASE ACTIVITY

Identical aliquots of a marrow cell suspension were subjected to sonication for a given period of time. Mitochondria were isolated as described under MATERIALS AND METHODS. The conditions for incubation were as given in the legend to Fig. 3B.

Duration of sonication (sec)	*Per cent of maximum activity*
8	64
12	95
15	100
18	98
21	68
28	35

Electron microscopic examination of packed mitochondria fixed with osmium tetroxide showed somewhat damaged, relatively stroma-free mitochondria. A number of dark-staining particles could not be identified as mitochondria and probably represent lysosomes. Some mitochondria showed recognizable cristae, others were swollen and deformed.

The respiratory function of the mitochondrial preparation was assessed by determining its capacity to oxidize succinate. The rate of oxygen uptake was linear for 30 min (Fig. 1). The succinoxidase activity was found to be 83 μl of oxygen consumed per h per mg of protein.

The optimum conditions for the measurement of Δ-aminolaevulinate synthetase can be derived from the effects of variables presented in Figs. 2 and 3. As shown in

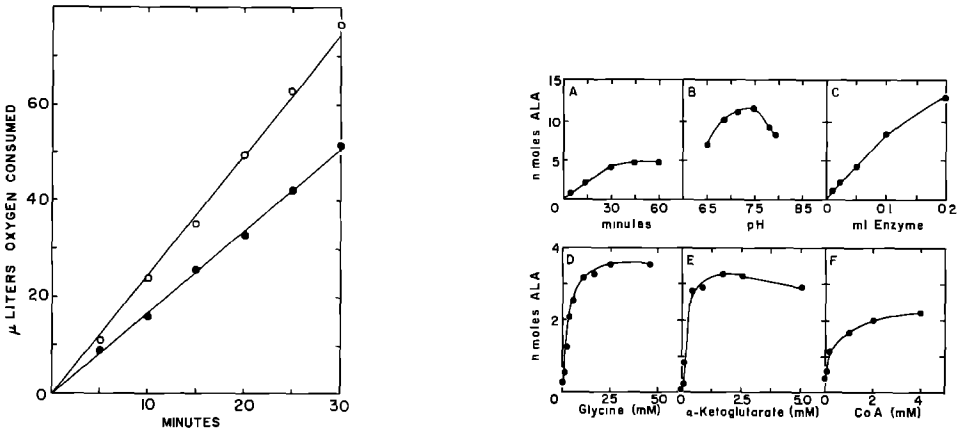

Fig 1 Expt 1 (●—●) and Expt 2 (○—○) Each Warburg flask contained 0 2 ml of 0 5 M potassium phosphate buffer (pH 7 4), 0 2 ml of 1% cytochrome *c*, in Expt 1, 0 2 ml of the mitochondrial suspension in 0 25 M sucrose, 2 4 ml of water, in Expt 2, 0 3 ml of the mitochondrial suspension and 2 3 ml of water The center well contained 0 2 ml of 6 M KOH After equilibration (38°) 0 2 ml of 1 M sodium succinate was tipped in from the sidearm and readings begun

Fig 2 A Effect of time on Δ-aminolaevulinate (ALA) synthetase reaction B Effect of pH on Δ-aminolaevulinate synthetase reaction C Effect of enzyme concentration on Δ-aminolaevulinate synthetase reaction D Effect of glycine concentration on Δ-aminolaevulinate synthetase reaction E Effect of α-ketoglutarate concentration on Δ-aminolaevulinate synthetase reaction F Effect of CoA on Δ-aminolaevulinate synthetase reaction Incubation conditions in A and C to F were mitochondrial suspension, 0 2 ml, glycine, 15 mM, α-ketoglutarate, 1 7 mM, CoA, 4 mM, pyridoxal phosphate, 0 48 mM, NAD^+, 38 4 mM, $MgCl_2$, 5 mM, and NaEDTA, 5 mM, in a final volume of 0 26 ml Incubation conditions in B were the same except for mitochondrial suspension, 0 9 ml, pyridoxal phosphate, 0.12 mM, NAD^+, 9 6 mM, and in a final volume of 1 0 ml.

Fig 2A there is a constant rate of production of Δ-aminolaevulinate by rabbit marrow mitochondria for 30 min which drops off at 45 min The pH optimum is 7 2 to 7.5 in potassium phosphate buffer (Fig 2B). Tris–HCl buffer reduced the amount of Δ-aminolaevulinate formed by 20% Δ-Aminolaevulinate production is a linear function of enzyme concentration from 0 0125 to 0 2 ml of mitochondrial suspension (containing 23 mg of protein per ml) (Fig. 2C), 0 05 ml of mitochondrial suspension was used in most of the studies described here.

Δ-Aminolaevulinate synthetase in rabbit marrow mitochondria is maximal with 25 to 45 mM glycine (Fig 2D), 1.7 mM α-ketoglutarate (Fig 2E) and approaches a maximum with 4 mM concentration of CoA (Fig 2F) The K_m values for these substrates are about 5 mM for glycine, 0 1 mM for α-ketoglutarate and 0 6 mM for CoA The K_m of Δ-aminolaevulinate synthetase for glycine and α-ketoglutarate is similar to that found by LAVER, NEUBERGER AND UDENFRIEND[15] and BROWN[16] for the enzyme in chicken erythrocyte particles The optimal pyridoxal-phosphate concentration in this system is 0 48 mM, and higher concentrations are inhibitory (Fig. 3A). The highest concentration of NAD^+ which enhances enzyme activity is 38 4 mM (Fig. 3B)

In searching for factors which might enhance enzyme activity further the effect of certain substances on Δ-aminolaevulinate synthetase were studied. Glutathione had no effect at $1 \cdot 10^{-3}$ to $1 \cdot 10^{-2}$ M but was inhibitory at $2\,5 \cdot 10^{-2}$ M concen-

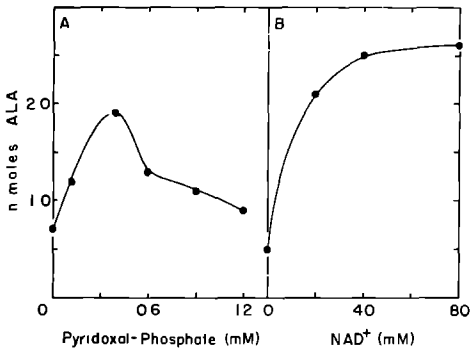

Fig 3 A Effect of pyridoxal-phosphate concentration on Δ-aminolaevulinate (ALA) synthetase reaction B Effect of NAD^+ on Δ-aminolaevulinate synthetase reaction The conditions were the same as given in the legend to Fig 2A

tration or greater This inhibition may be a result of Δ-aminolaevulinate utilization by Δ-aminolaevulinate dehydratase (EC 4 2 1 24) which is enhanced by glutathione However, the isotope tracer studies presented below indicated no significant utilization of Δ-aminolaevulinate for heme synthesis in the standard assay system Ferrous iron in concentrations ranging from $1\,7\ 10^{-5}$ to $1\,7\ 10^{-3}$ M had no significant stimulatory effect on Δ-aminolaevulinate synthetase activity This differs from the observation of VAVRA[20] in chicken reticulocyte mitochondria in which iron enhanced Δ-aminolaevulinate synthetase activity The requirement of ferrous iron in Δ-aminolaevulinate synthesis may depend on the type of enzyme preparation[30] or may vary with the species studied Mg^{2+} and EDTA had no effect on enzyme activity This is in contrast to the findings of GIBSON, LAVER AND NEUBERGER[17] in chicken erythrocyte particles and the stimulatory effect of EDTA on Δ-aminolaevulinate synthetase activity in liver homogenate[31]

The effect of other known substrates for Δ-aminolaevulinate synthesis are shown in Table II Succinate, at the concentration studied (12 mM), was 46% as effective as α-ketoglutarate ATP (0 5 mM), known to enhance Δ-aminolaevulinate synthesis from succinate[16,17], increased Δ-aminolaevulinate production 20%. Succinyl-CoA was utilized as well as succinate indicating that the fresh mitochondrial

TABLE II

EFFECT OF VARIOUS SUBSTRATES ON Δ-AMINOLAEVULINATE SYNTHETASE ACTIVITY

The conditions for incubation were as given in the legend to Fig 2A except that when succinate was used α-ketoglutarate and NAD^+ were omitted In the presence of added succinyl-CoA, CoA was also omitted

Substrate	*Δ-Aminolaevulinate formed (nmoles)*
α-Ketoglutarate, 1 7 mM	9 8
Succinate, 12 mM	4 5
Succinate, 12 mM + ATP, 0 5 mM	5 4
Succinyl-CoA, 2 mM	5 3

preparation is permeable to succinyl-CoA as has been reported for mammalian liver[7]

To determine what fraction of the Ehrlich reactant in the assay system was true Δ-aminolaevulinate and what fraction was amino acetone, the pyrrole solution obtained from a typical mitochondrial incubation mixture was adjusted to pH 7.0 to 7.5 and then extracted with equilibrated ether[27] As determined with Ehrlich mercury reagent, 97.7% of total pyrrole was detected in the aqueous phase as compared with 92.5% recovery of pure MAPP Recovery of added Δ-aminolaevulinate from the mitochondrial mixture was 100% Using [^{14}C]glycine as substrate in the incubation mixture, the ^{14}C-labeled pyrrole, when chromatographed, was detected in the region of MAPP (R_F 0.20) and no contamination with DMAP (R_F 0.80) was observed Thus essentially all of the pyrrole formed during incubation appeared to be the pyrrole derivative of Δ-aminolaevulinate

When ^{59}Fe was included in the assay system some synthesis of heme could be detected (Table III) Heme synthesis was increased 8-fold if protoporphyrin was

TABLE III

COMPARISON OF THE INCORPORATION OF VARIOUS LABELED PRECURSORS INTO HEME BY RABBIT BONE MARROW MITOCHONDRIA

Incubation conditions were mitochondrial suspension, 0.4 ml, glycine, 15 mM, α-ketoglutarate 1.7 mM, CoA, 4 mM, pyridoxal phosphate, 0.12 mM NAD^+, 9.6 mM, $MgCl_2$, 5 mM, NaEDTA, 5 mM, and ferrous chloride, 36 mM, in a final volume of 1.0 ml Incubation was for 30 min at 37° In Tubes 1 and 2, the ferrous chloride was replaced by [^{59}Fe]ferrous citrate, in Tube 3, glycine was replaced by [^{14}C]glycine, in Tube 4, glycine was replaced by [^{14}C]Δ-aminolaevulinate The specific activities for the isotopic precursors were [^{14}C]glycine, 2.6 mC/mmole, [^{14}C]Δ-aminolaevulinate, 29.8 mC/mmole, and [^{59}Fe]ferrous citrate, 4.6 mC/mg The labeled hemin was isolated as described in MATERIALS AND METHODS

Tube No	*Isotopic precursor in reaction*	*Radiohemin synthesized* (pmoles)*
1	[^{59}Fe]Ferrous citrate, 36 nmoles	72.5
2	[^{59}Fe]Ferrous citrate, 36 nmoles + protoporphyrin (38 μM)	557.0
3	[^{14}C]Glycine, 15 nmoles	0
4	[^{14}C]Δ-Aminolaevulinate, 330 nmoles	8.4

* Calculated from the counts/min of isotope added and counts/min in isolated hemin

added, indicating that ferrochelatase was present in the mitochondrial preparations [^{14}C]Glycine incorporation into heme could not be detected. Only 11.6% of heme synthesis was observed with Δ-aminolaevulinate as the labeled precursor when compared with that found with [^{59}Fe]ferrous citrate This degree of Δ-aminolaevulinate utilization represents approx 0.02% of Δ-aminolaevulinate which is formed in the mitochondrial assay system for Δ-aminolaevulinate synthetase.

There was a 75% loss of Δ-aminolaevulinate synthetase activity when the mitochondrial suspension was heated to 56° for 10 min, and after 45 min there was nearly a total loss of enzyme activity (Fig 4) Similarly, the enzyme was rapidly inactivated after 30 min at 25° and 37° (Table IV) but retained full activity for at least 1 month when stored at −20°

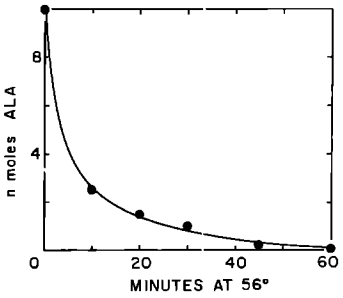

Fig 4 The effect of preincubation of the mitochondrial suspension for varying periods of time at 56° on the Δ-aminolaevulinate (ALA) synthetase reaction After preincubation, the mitochondrial suspension, 0 9 ml, was cooled to 4° and assayed for activity employing the conditions as given in the legend to Fig 2B

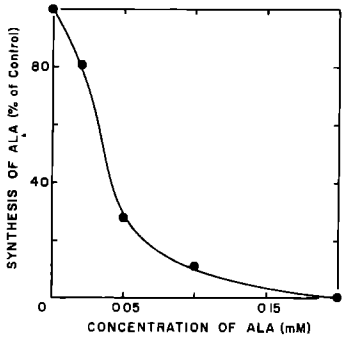

Fig 5 The effect of Δ-aminolaevulinate (ALA) on the Δ-aminolaevulinate synthetase reaction. The conditions were the same as given in the legend to Fig 2B except that mitochondria were added in 0 6 ml and the various concentrations of Δ-aminolaevulinate in 0 3 ml For each sample incubated with added Δ-aminolaevulinate, an unincubated sample served as a control for measurement of the added Δ-aminolaevulinate

The presence of Δ-aminolaevulinate in the assay system inhibited Δ-aminolaevulinate formation (Fig. 5) At 0 025 mM there was a 20% inhibition, and inhibition increased at progressively higher concentrations of Δ-aminolaevulinate The product inhibition of Δ-aminolaevulinate synthetase by Δ-aminolaevulinate is consistent with a similar observation of LASCELLES[13] in *Rhodopseudomonas spheroides*. Δ-Aminolaevulinate synthetase in the mitochondrial preparation was not inhibited by protoporphyrin or hemin in concentrations ranging from 1 10^{-5} to 1 10^{-3} M

The normal activity of Δ-aminolaevulinate synthetase in rabbit mitochondria under the optimal conditions described above in 7 animals ranged from 26 to 81 pmoles Δ-aminolaevulinate formed per 10^6 erythroid precursors or 950 to 2800 pmoles of Δ-aminolaevulinate formed per mg of mitochondrial protein The average Δ-aminolaevulinate produced from the total tibial, femoral and humeral marrow samples was 55 nmoles/30 min

TABLE IV

HEAT INACTIVATION OF Δ-AMINOLAEVULINATE SYNTHETASE

The mitochondrial suspension, 0 9 ml, was incubated for 30 min at a given temperature At the end of incubation period, the enzyme was cooled to 4° and assayed for activity employing the conditions as given in the legend to Fig 2B

Temperature	*Activity (nmoles Δ-aminolaevulinate)*	*Per cent inactivation*
Unheated	10 0	0
25°	5 5	45
37°	2 0	80
56°	1 0	90
100°	0	100

DISCUSSION

Recent studies have shown that Δ-aminolaevulinate synthetase is not only rate-limiting in heme biosynthesis but an increased rate of Δ-aminolaevulinate production[32] and hemin[33] also stimulate globin production Measurement of Δ-aminolaevulinate synthetase in red cell precursors in bone marrow could be a particularly useful tool in the investigation of the control of normal erythropoiesis and of various erythropoietic defects in man The present studies indicate that Δ-aminolaevulinate synthetase can be quantitated in normal mammalian bone marrow cells. Although the mitochondrial preparation used in these studies included mitochondria from cells other than erythrocyte precursors it may be assumed that nearly all the Δ-aminolaevulinate synthetase activity measured was from the erythroid cells since very large amounts of heme are normally synthesized for hemoglobin production in contrast to the amount of heme needed for the various heme enzymes

Using methods usually employed for preparation of mitochondria from solid tissues, such as liver, it has been difficult to rupture nucleated blood cells in suspensions and to preserve mitochondrial structure GUGGENHEIM *et al.*[34] have prepared functioning reticulocyte mitochondria by homogenization of clotted blood Our attempts to apply this procedure to bone marrow were unsuccessful The present studies indicate that a rate and duration of sonication can be selected at which nucleated cell membranes are ruptured but mitochondria are only partially destroyed. Although the mitochondrial preparation is contaminated with other particulate fraction(s) of the cell, it has been possible to remove the soluble enzymes of the heme synthesis pathway to permit measurement of Δ-aminolaevulinate production Without exogenous protoporphyrin the mitochondrial preparation contains sufficient ferrochelatase activity to utilize 0 17% of the Δ-aminolaevulinate formed The isotopic studies indicate that only 0 02% of the Δ-aminolaevulinate formed in the assay is converted for heme synthesis. Utilization of Δ-aminolaevulinate by other pathways such as the succinate–glycine cycle[3] or for synthesis of purines[35] has not been ruled out by these studies. DRESEL AND FALK[35] observed that 90% of the Δ-aminolaevulinate added to hemolyzed chicken erythrocytes is converted to porphyrins and it was suggested that once Δ-aminolaevulinate is formed in erythrocytes little of it is oxidized.

The optimal conditions for the assay of Δ-aminolaevulinate synthetase in rabbit bone marrow cell mitochondria are very similar to those observed for the avian erythrocyte enzyme in regard to pH optima and glycine and α-ketoglutarate concentrations[15–17] In contrast to fresh chicken erythrocyte particles[15], succinyl-CoA was utilized as a substrate in the present system, perhaps because the mitochondria were sufficiently damaged to remove a permeability barrier However, mammalian liver mitochondria are permeable to succinyl-CoA[7] In lyophilized chicken erythrocyte particles[17] succinyl-CoA was utilized and nearly restored the Δ-aminolaevulinate synthetase activity to the level observed in fresh preparations with α-ketoglutarate as substrate GIBSON *et al.*[17] further observed that α-ketoglutarate was not effective in lyophilized chicken erythrocyte particles unless α-ketoglutarate dehydrogenase and NAD^+ were added, while NAD^+ had little enhancing effect on Δ-aminolaevulinate formation in their fresh preparation Since NAD^+ enhanced Δ-aminolaevulinate synthetase activity 5-fold in the present studies it could again

be inferred that the partially damaged mitochondria no longer possess the full capacity to generate oxidized NAD^+, the known hydrogen acceptor in this reaction[36,37]. Similarly, the effect of pyridoxal phosphate was considerably greater in a lyophilized avian erythrocyte preparation than in a fresh preparation[15,17] and in these studies, pyridoxal phosphate enhanced enzyme activity greater than 100%

Δ-Aminolaevulinate synthetase activity in liver can be altered by certain chemical compounds and the carbohydrate content of the diet[6,7,27]. The studies of GRANICK AND KAPPAS[38] in chick embryo liver cells suggest that the enzyme is also affected by certain steroids In addition, negative feedback inhibition of Δ-aminolaevulinate synthetase by the end products of heme biosynthesis (protoporphyrin and hemin) have been demonstrated in *R spheroides*[39] and in a crude lysate of rabbit reticulocytes[40]. Similar findings were described by WELLAND AND SCHWARTZ[19] in rabbit reticulocyte mitochondria but are in contrast to the recent observations by VAVRA[20] which indicated that globin exerts a negative control on Δ-aminolaevulinate synthetase and hemin may influence an earlier step in Δ-aminolaevulinate synthesis, such as succinyl-CoA synthetase GRANICK[27] found no inhibition of liver Δ-aminolaevulinate synthetase by heme and suggested that heme may be the corepressor in the control of the enzyme *In vivo* studies by WAXMAN, COLLINS AND TSCHUDY[41] indicated that hemin represses the production of the enzyme in rat liver In the present studies, the failure of protoporphyrin or hemin to inhibit Δ-aminolaevulinate synthetase activity may be the result of the absence of a negative feedback inhibition of this step by heme or may result from incomplete solubilization of the enzyme[41].

Hemoglobin concentration in peripheral blood may in part be dependent on the rate of production of Δ-aminolaevulinate Based on previous determinations of the daily production of hemoglobin in the rabbit (3.2 kg) as being about 29 g/day[42–44], the requirement of Δ-aminolaevulinate production would be about 3600 nmoles of Δ-aminolaevulinate per day If the figures of marrow distribution in the rabbit as determined by DIETZ[45] are used, the amount of Δ-aminolaevulinate produced by the animal as measured by the present method would be about 7000 nmoles/day Different substrate and cofactor concentrations than those employed in the present assay as well as the operation of certain control mechanisms on Δ-aminolaevulinate synthesis in the intact animal, as discussed above, may account for the differences obtained by these calculations

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