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MEASUREMENT OF AMINOLAEVULINATE SYNTHETASE ACTIVITY IN NORMAL MOUSE LIVER WITH [2-^{14}C]GLYCINE

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SUMMARY

An isotopic method for the estimation of aminolaevulinate synthetase is described. This utilises [2-^{14}C]glycine as a precursor, unfractionated homogenate as a source of enzyme, and is carried out in a small reaction volume. The aminolaevulinate product is separated by electrophoresis and estimated by scintillation counting. The method is more sensitive than conventional colorimetric techniques and capable of measuring activity changes in normal liver.

[^{14}C]Aminolaevulinate produced in the reaction is apparently stable, in contrast to the instability of [^{3}H]aminolaevulinate added to the reaction mixture.

The identity of the product has been confirmed by electrophoresis and chromatography.

The enzyme from adult mouse liver is stable for at least 4 h in the reaction mixture. The reaction proceeds at a maximal rate at pH 7.0 and shows little or no requirement for citric acid cycle intermediates. The K_m (glycine) is 4.2 mM. The observed range of mean activities of several different samples, 2–3 pmoles aminolaevulinate per min per mg protein, agrees with previous published reports.

INTRODUCTION

δ-Aminolaevulinate synthetase is important in the regulation of haem synthesis[1-4]. Although existing sensitive colorimetric techniques can be used with anaemic chicken erythrocytes[5,6], and porphyric liver[2,7,8], isotopic methods provide greater sensitivity[8,9]. In this microassay [2-^{14}C]glycine produces [5-^{14}C]aminolaevulinate which is isolated by electrophoresis and measured by scintillation counting.

MATERIALS AND METHODS

Preparation of enzyme extract

Freshly dissected mouse liver was homogenised for 2 min at 0°, in an equal amount of 0.06 M phosphate buffer (pH 7.0) containing 0.25 M sucrose, 0.02 M $MgCl_2$,

0.01 M EDTA, 4 mM mercaptoethanol, and 1 mM glycine (modified from refs. 10, 11). All cells were lysed though the condition of the mitochondria was not determined.

Enzyme assay

The reaction mixture contained 1 mM glycine, 12 μC/ml [2-^{14}C]glycine (Radiochemical Centre, Amersham; 21.8 mC/mM), 5 mM sodium malate, 2 mM $MgCl_2$, 0.4 mM pyridoxal phosphate, 0.05 M phosphate buffer (pH 7.0) in 20 μl *plus* 10 μl homogenate. The phosphate concentration (*cf.* refs. 3, 5, 11) is below 0.07 M, a level which may be inhibitory[2]. Incubations were carried out in sealed polystyrene tubes, 15 mm × 70 mm, immersed up to 2/3 of their height in a covered shaking water bath for 4 h at 37°. This produced minimal evaporation and adequate aeration[7]. 5 μl 15 mM aminolaevulinic acid were then added as carrier and 20 μl of the mixture transferred to 5 μl 1.5 M trichloroacetic acid in polypropylene microcentrifuge tubes (Beckman, Glenrothes) and centrifuged on a Beckman Microfuge for 2 min. The protein of the sediment was determined[12].

Separation of aminolaevulinic acid

5 μl of the deproteinised supernatant were applied to a 15 cm × 20 cm silica gel thin-layer sheet (Malinkrodt "Chromar 1000" glass fibre mat, impregnated with silica gel; total thickness 1 mm; Camlab, Cambridge) and electrophoresed for 1 h 50 min at 36 V/cm and 120–160 mA, at 3–5° in 0.05 M phthalate buffer (pH 4.0). It was then dried and stained with ninhydrin. The aminolaevulinic acid was eluted and counted in a scintillation counter. Recovery of aminolaevulinic acid was $\geq$ 95%.

RESULTS

Preparation of homogenate; solubility of enzyme and product

The normal homogenate gave reproducible yields while hypotonic lysis, ultrasonication, and freezing and thawing produced some variability. Omission of mercaptoethanol resulted in a loss of 90% of the activity. After centrifugation at 10 000 × *g* for 10 min the particulate fraction contained most of the activity, though less than the equivalent amount of homogenate (Table I).

When the reaction products were centrifuged 10 000 × *g* almost as much [^{14}C]-

TABLE I

PERCENTAGE ACTIVITY OF δ-AMINOLAEVULINATE IN SUBCELLULAR FRACTIONS

The homogenate was centrifuged at 10 000 × *g* for 10 min. The pellet was resuspended in fresh homogenate buffer and assayed for δ-aminolaevulinate as described in MATERIALS AND METHODS; homogenate and supernatant were assayed directly. Results are expressed as percentages derived from duplicate observations.

Expt. No.	*Whole homogenate*	*Supernatant*	*Pellet*
1	100	7.0	52
2	100	2.5	15
3	100	N.D.*	67

* N.D. = not determined.

aminolaevulinic acid was recovered from the supernatant as from the uncentrifuged suspension (Table II). Hence the product is freely diffusable.

Identification of product

After electrophoresis the distribution of radioactivity corresponded exactly to the visible reflectance of stained carrier aminolaevulinic acid, using either [^{14}C]glycine or [2,3-$^{14}C_2$]succinate as precursor (Figs. 1a, 1b). Aminoacetone, synthesis of which is low in phosphate buffer[7] and undetectable by this method, migrated just in advance of glycine where contamination of aminolaevulinic acid was very unlikely.

TABLE II

DISTRIBUTION AND STABILITY OF PRODUCT

Duplicate incubations were carried out with 1.5 ml of reaction mixture in sealed 25-ml conical flasks. After 4 h, aliquots from each flask were terminated and the [^{14}C]aminolaevulinic acid determined as described under MATERIALS AND METHODS. The remaining reaction mixture was centrifuged at 10 000 × *g* for 10 min and the supernatant divided in two. One-half was terminated and the [^{14}C]aminolaevulinic acid determined directly. The other half was incubated (30 µl in polystyrene tubes) for a further hour at 37° and the amount of [^{14}C]aminolaevulinic acid again determined. The results are expressed as pmoles aminolaevulinic acid present per mg protein of the initial reaction mixture per min of the initial 4-h incubation.

Expt. No.	*Whole homogenate*	*Supernatant*	*Supernatant after 1 h incubation*
1	0.498	0.483	0.534
2	0.483	0.462	0.524

The product, converted to a pyrrole[13], was also identified as a peak of radioactivity corresponding to aminolaevulinic acid-pyrrole on chromatography (Fig. 1c).

There is no evidence of the presence of other metabolites in Fig. 1a and 1c which might interfere with the specificity of the assay.

Stability of product

Degradation of [$^{3}H_5$]aminolaevulinic acid (317 mC/mmole; Radiochemical Centre) used in place of [^{14}C]glycine, was reduced 3-fold by 5 mM EDTA (*cf.* ref. 7) and 6-fold by 10 mM unlabelled aminolaevulinic acid (Table III). This effect of EDTA in the presence of excess Mg^{2+} is not understood. In spite of the observed degradation, no activity was obtained in porphobilinogen, isolated by thin-layer chromatography on silica gel with chloroform–methanol–acetic acid (14:4:1, by vol.) with [^{14}C]glycine or [^{3}H]aminolaevulinic acid as precursor.

Differences in the amount of exogenous [^{3}H]aminolaevulinic acid degraded were not reflected in the amount of [^{14}C]aminolaevulinic acid synthesised; *e.g.* while estimates of activity in different samples fell within a narrow range (see below) the estimates of exogenous aminolaevulinic acid degradation varied enormously (0–95% in 1 h).

When the ^{14}C-labelled product was incubated for a further period in the absence of significant aminolaevulinic acid synthesis (achieved by removing the mitochondria from the reaction mixture in a 10 000 × *g* spin) no degradation was observed (Table

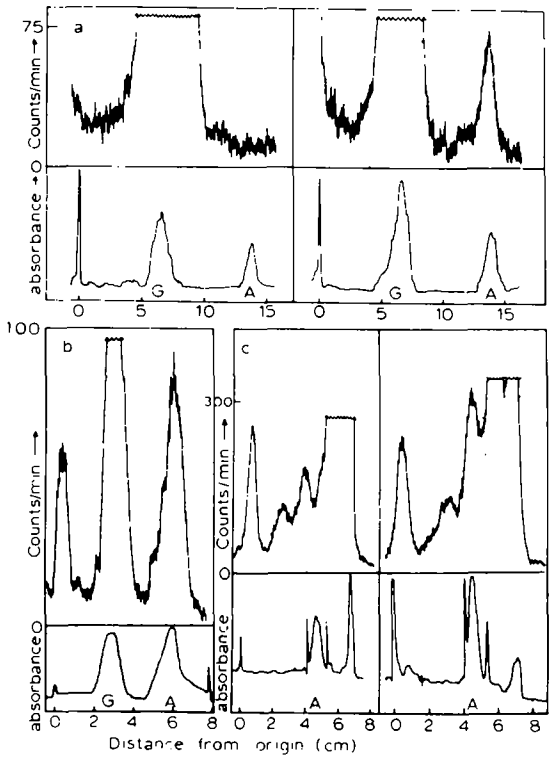


Fig. 1. Identification of products of reaction by chromatography and electrophoresis. Assays were performed as described in MATERIALS AND METHODS except that in b 6 μC/ml [2,3-$^{14}C_2$]succinate was substituted for [^{14}C]glycine, 1 mM succinate for malate, and the glycine concentration was raised to 0.1 M. In each diagram the top trace is radioactivity and the lower absorbance. A, aminolaevulinic acid; G, glycine. a. A total of 15 μl deproteinised supernatant were electrophoresed as described in MATERIALS AND METHODS, and scanned for absorbance and radioactivity. The left-hand pair of traces is from a blank assay. b. 30 μl deproteinised supernatant were applied to a thin-layer sheet (Eastman "Chromogram"; silica gel) and electrophoresed in 1.4 M formic acid 0.5 M acetic acid (pH 1.9) for 1 h. The dried sheet was scanned for absorbance and radioactivity. A blank is not shown. c. The reaction products from 1.5 ml incubations were converted to pyrroles[8] and an ethyl acetate extract chromatographed on silica gel thin layer, with methyl acetate–isopropanol–25% NH_4OH (9:7:4, by vol.). After staining with Ehrlich's reagent the chromatograms were scanned as above. The sharp peaks on either side of the aminolaevulinic acid–pyrrole absorbance peaks correspond to the limits of ultraviolet fluorescence, which were marked in pencil. They are connected to the upper trace to aid comparison. The left-hand pair of traces is a blank. In both traces "A" indicates aminolaevulinic acid–pyrrole, staining pink in Ehrlich's reagent. Remaining peaks derive from glycine.

II). Moreover, exogenous [^{3}H]aminolaevulinic acid was not degraded either under those conditions. However, when fresh 10 000 × g supernatant reaction mixture was added, degradation of exogenous [^{3}H]aminolaevulinic acid did occur, but there was no change in endogenous [^{14}C]aminolaevulinic acid. Hence the product of the reaction is apparently stable and its concentration unrelated to variation in the rate of exogenous aminolaevulinic acid degradation.

These results suggest that (1) degradation of exogenous [^{3}H]aminolaevulinic acid is not a useful measurement of degradation of the endogenous product, and (2) the product of the reaction is not free aminolaevulinic acid and perhaps does not

TABLE III

EFFECT OF EDTA AND EXCESS AMINOLAEVULINIC ACID ON SYNTHESIS AND DEGRADATION OF AMINOLAEVULINIC ACID

Synthesis of aminolaevulinic acid was measured as in MATERIALS AND METHODS with the alterations noted in the table. Degradation was measured in the same conditions by replacing [^{14}C]glycine with [^{3}H]aminolaevulinic acid. Increases were made in the $MgCl_2$ concentration equivalent to increases in EDTA, maintaining a 1.3 mM excess of $MgCl_2$. Activities in Expts. 1 and 2 are means of duplicates and in Expt. 3 are single observations.

Expt. No.	*Unlabelled aminolaevulinic acid (mM)*	*EDTA (mM)*	*pmoles aminolaevulinic acid produced/min per mg protein*	*% [^{3}H]aminolaevulinic acid degraded*
1	0	1.67	0.477	52
	10	1.67	0.494	8
2	0	5.0	0.453	22
	10	5.0	0.218	6
3	0	0	0.319	95
	0	1.67	0.557	60
	0	5.0	0.585	35

become such until after treatment with trichloroacetic acid. When the products of the reaction mixture were treated with 1.5 M trichloroacetic acid in the same manner as for normal termination, and then neutralised with NaOH, reincubation for a

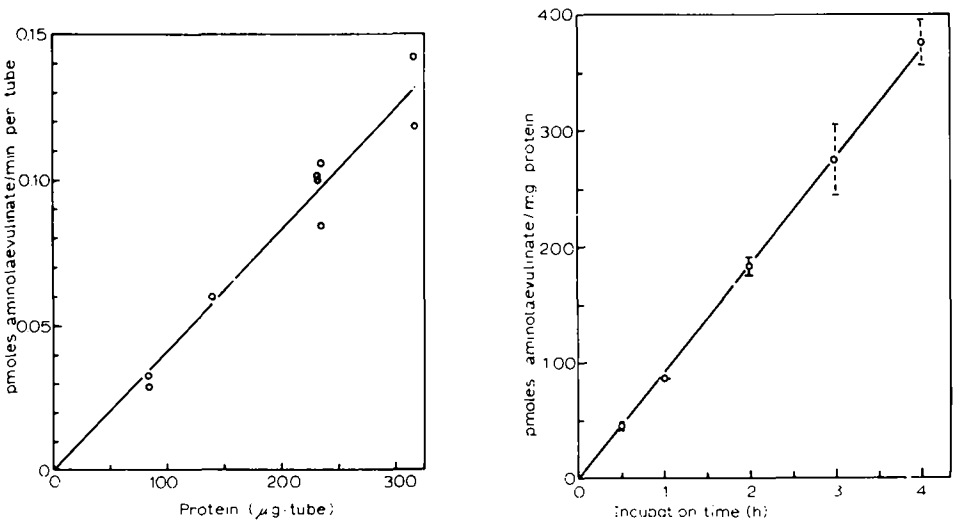


Fig. 2. Rate of aminolaevulinic acid production by different concentrations of homogenate. Incubation mixture contained 0.67 mM glycine, 6 μl/ml [^{14}C]glycine, 2 mM $MgCl_2$, 1.3 mM sodium succinate, 0.4 mM pyridoxal phosphate, 0.05 M phosphate buffer (pH 7.0) *plus* 10 μl homogenate. The 4-h incubation was terminated with 10 μl chloroform. The liver was homogenised in a constant proportion of the sucrose-buffer detailed in the MATERIALS AND METHODS, with distilled water substituted for liver where the liver concentration was less than 50%. Total pmoles aminolaevulinic acid produced per tube are plotted as a function of the total protein in the reaction mixture. Each point is a single observation.

Fig. 3. Formation of [^{14}C]aminolaevulinic acid from [^{14}C]glycine as a function of time. Conditions were as in MATERIALS AND METHODS with the incubation time varied as indicated. Points and vertical bars represent means and duplicate observations.

further hour in the presence of fresh 10 000 × *g* supernatant reaction mixture showed degradation of 35% compared to a blank without fresh supernatant. The implications of these findings will be discussed below.

Kinetics of reaction. Enzyme concentration and reaction time

The rate of aminolaevulinic acid synthesis was found to be proportional to the amount of homogenate present from 100 to 500 mg wet weight per ml (Fig. 2), and linear up to 4 h (Fig. 3).

Effect of pH

Maximum δ-aminolaevulinate synthetase activity was obtained at pH 7.0 (Fig. 4). The sharp decline in activity above pH 7.0 may be due to precipitation of

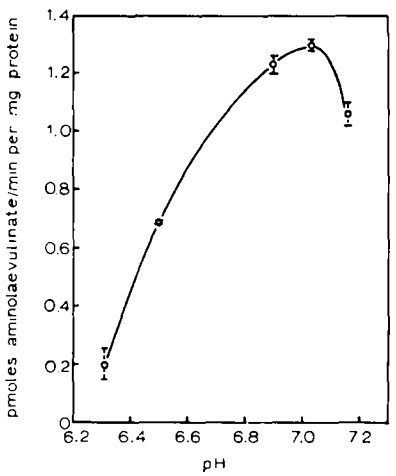


Fig. 4. Effect of pH on aminolaevulinic acid synthesis. The reaction mixture was as in Fig. 2 with the pH varied as shown. The reaction was terminated with 5 µl 1.5 M trichloroacetic acid.

Mg^{2+} as $Mg_3(PO_4)_2$. When Tris buffer was employed to avoid this, 88% of enzyme activity was lost.

Concentration of substrates

The K_m (glycine) derived from a Woolf plot (Fig. 5) was 4.2 mM. When [2,3-$^{14}C_2$]succinate was used instead of [^{14}C]glycine no increase in incorporation occurred between 1 and 0.1 M glycine.

1 mM α-ketoglutarate, citrate and malate gave higher activity than 1 mM succinate (Table IV). However α-ketoglutarate showed progressive inhibition with increased concentration (Fig. 6) in contrast to its effect on the chicken erythrocyte enzyme[5]. No inhibition and little stimulation was observed with up to 5 mM malate.

Reproducibility and variation

The results of seven identical assays on the same liver homogenate using 10 mM glycine gave an activity of δ-aminolaevulinate synthetase in adult mouse liver of

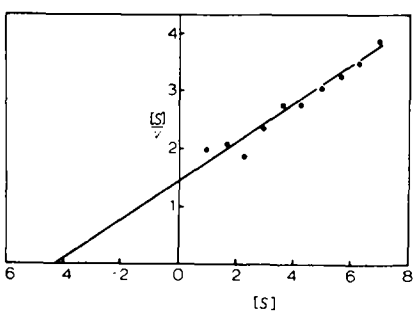
TABLE IV

EFFECT OF VARIOUS CITRIC ACID CYCLE INTERMEDIATES IN SYNTHESIS OF AMINOLAEVULINIC ACID

Assays were performed with 6 μC/ml [^{14}C]glycine and 1 mM malate or other citric acid cycle intermediates as indicated. Other conditions were as in MATERIALS AND METHODS. Activities are means of duplicates.

Citric acid cycle intermediate	*pmoles aminolaevulinic acid/ min per mg protein*
Succinate	0.608
α-Ketoglutarate	0.701
Citrate	0.777
Malate	1.04

2.19 ($\pm$ 0.21)* pmoles aminolaevulinic acid per mg protein. A comparison of seven different samples of liver assayed under similar conditions but at different times gave a range of mean activities of 2.19–3.08. These values are comparable with those of MARVER *et al.*[7] for rat liver.



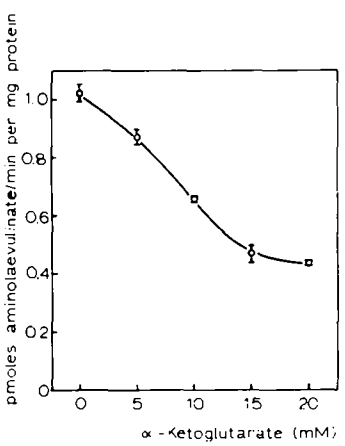


Fig. 5. Effect of glycine concentration on the rate of aminolaevulinic acid synthesis. Incubation conditions as in MATERIALS AND METHODS with glycine concentration varied over the range indicated. K_m (glycine) is obtained from intercept on x-axis with sign changed. The line through the points, which are single observations, was plotted by regression analysis.

Fig. 6. Aminolaevulinic acid synthesis as a function of α-ketoglutarate concentration. The incubation mixture contained 6.7 mM glycine 6 μC/ml [^{14}C]glycine, 2 mM $MgCl_2$, 0.4 mM pyridoxal phosphate, 0.05 M phosphate buffer (pH 7.0) and 10 μl homogenate in a final volume of 30 μl. α-Ketoglutarate was varied as indicated. The reaction was terminated after 3 h with 20 μl chloroform.

DISCUSSION

The results reported here imply that while there is utilization or degradation of exogenous aminolaevulinic acid, that made in the reaction is stable. This raises two important questions: (1) is the product of the assay really [^{14}C]aminolaevulinic

* Standard error of the mean.

acid? (2) If it is, why does it not behave in the same manner as exogenous aminolaevulinic acid?

The evidence for the identity of the product may be summarised thus: (1) electrophoretic mobility is identical to carrier aminolaevulinic acid. (2) Product synthesised from [2,3-$^{14}C_2$]succinate shows the same mobility. (3) Chromatographic mobility of pyrrole matches that of aminolaevulinic acid-pyrrole. (4) The K_m (glycine) of the enzyme is similar to that reported by HAYASHI *et al.*[14] for rat liver δ-aminolaevulinate synthetase.

Variations in the assay procedure, *e.g.* the excess of Mg^{2+} or the higher homogenate concentration may explain differences in exogenous aminolaevulinic acid degradation between this and other reports[7,9], while differences in exchangeability of ^3H and ^{14}C atoms or in the relative binding of exogenous and endogenous aminolaevulinic acid to other molecules within the cell may answer question (2).

The direct participation of [^{14}C]glycine in aminolaevulinic acid synthesis makes it a more suitable choice than [^{14}C]succinate for the assay. Where labelled succinate is used exogenous succinyl-CoA synthetase must be supplied in order that this step is not rate limiting[8]. This was confirmed by our failure to stimulate [^{14}C]succinate incorporation into aminolaevulinic acid by increasing the glycine concentration. IRVING AND ELLIOTT[8] observed considerable interference by other succinate metabolites during separation of aminolaevulinic acid by chromatography.

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