Phytochemistry, 1975, Vol. 14, pp. 2399–2402. Pergamon Press. Printed in England.

THE BIOSYNTHESIS OF δ-AMINOLEVULINIC ACID IN GREENING MAIZE LEAVES

ERNA MELLER, SHIMSHON BELKIN and EITAN HAREL
Botany Department, The Hebrew University, Jerusalem, Israel

(*Received* 24 *April* 1975)

Key Word Index—*Zea mays*; Gramineae; maize; biosynthesis; δ-aminolevulinic acid; porphyrins; chlorophyll.

Abstract—In greening maize leaves δ-aminolevulinic acid (ALA) was not formed from succinyl-CoA and glycine as shown by the incorporation of [^{14}C]-labeled precursors. The most effective precursor was α-ketoglutarate. [^{14}C]-Glycine hardly contributed to the labeling of ALA, although it was taken up by the tissue. There was no significant difference in the incorporation of label from [1-^{14}C] and [5-^{14}C] glutamate. Most of the label from [1-^{14}C] glutamate and from [1,4-^{14}C] succinate was found in [5-C] of ALA. It is suggested that the carbon skeleton of α-ketoglutarate or glutamate is incorporated intact into ALA, [1-C] of the precursors becoming [5-C] of ALA.

INTRODUCTION

δ-Aminolevulinic acid (ALA) is the first commited intermediate in the biosynthesis of porphyrins in plants as well as in other organisms[1]. Its formation has been demonstrated *in vivo* in relation to chlorophyll synthesis[2–4] but not *in vitro*, using cell-free systems from plants. In photosynthetic bacteria and in animal tissues, the formation of ALA from succinyl-CoA and glycine is catalyzed by ALA synthetase (succinyl CoA-glycine *C*-succinyltransferase, E.C.2.3.1.37)[1]. Beale and Castelfranco[5] recently showed that [^{14}C]-glycine and succinate were poor precursors of ALA in greening cucumber cotyledons, as compared with glutamate, glutamine and α-ketoglutarate (α-KG). They suggested that ALA is formed from α-KG via transamination of γ,δ-dioxovaleric acid (DOVA)* [6] or from glutamate by cyclization followed by oxygenation and reopening of the pyrroline ring at the original C–N bond. We studied the route by which ALA is synthesized in greening maize leaves by isolating the ALA formed from precursors labeled in various carbons and determining the radioactivity in carbons 1–4 and 5 of the product by periodate degradation.

* DOVA-γ,δ-dioxovaleric acid.

RESULTS AND DISCUSSION

The formation of [^{14}C] ALA from labeled precursors by greening maize leaves was determined after treating the leaves with levulinic acid (LA), which inhibits the utilization of ALA for chlorophyll synthesis[2, 3, 12]. The accumulated ALA was extracted and isolated by ion-exchange chromatography. Under the conditions of the procedure used, ALA was well separated from organic acids and from acidic and neutral amino acids which were eluted from the column ahead of it. Basic amino acids were removed from the resin only by raising the concentration and pH of Na citrate. This was established by column chromatography of non-radioactive ALA together with a mixture of labeled amino acids.

Figure 1A shows the isolation of ALA extracted from greening leaves which had been fed [5-^{14}C] α-KG in the presence and absence of LA. ALA determined colorimetrically and a corresponding peak in radioactivity were found only in LA treated leaves. Table 1 summarizes the incorporation of label from various precursors into ALA isolated by column chromatography and into the pyrrole prepared from it. In the case of [1-^{14}C] glutamate, [5-^{14}C] α-KG and [1,4-^{14}C] succinate, more than 80% of the radioactivity in the peak containing ALA was recovered in the pyrrole. This value corresponds

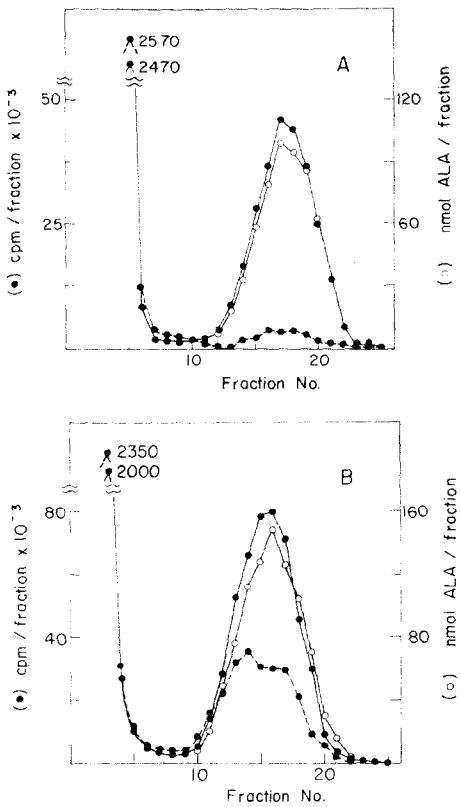

Fig. 1. Isolation by ion-exchange chromatography of [^{14}C] ALA from greening maize leaves which have been incubated with labeled precursors. Etiolated leaves were pre-illuminated for 1 hr and incubated for an additional 5 hr in light with the [^{14}C]-labeled precursor, in the presence or absence of 50 mM LA. Leaf extracts (4% CCl_3COOH) were chromatographed on Dowex 50W × 4 columns by elution with 0·2 M Na citrate buffer pH 4·25. Precursors: A—[5-^{14}C] α-ketoglutarate; B—[5-^{14}C] glutamate. —— leaves treated with LA; ----- no LA.

to the yield of the pyrrole prepared from commercially obtained [^{14}C] ALA.

A considerable difference was observed between the amount of label found in the ALA peak and that recovered in the pyrrole when [5-^{14}C] glutamate and [2-^{14}C] acetate were used. Figure 1B shows the ion-exchange chromatography of extracts from leaves which had been fed [5-^{14}C] glutamate, with or without LA. Radioactivity in the ALA-containing peak was present also in leaves not treated with LA. Paper chromatography of the peak suggested that a considerable part of the label in leaves treated with LA and most of that in non-treated leaves was in γ-aminobutyric acid. Decarboxylation of glutamate labeled in carbons 2–5 within the tissue could thus account for the observations with [5-^{14}C] glutamate and [2-^{14}C] acetate.

Comparison of the contribution of label from the various precursors shows that glycine was a poor precursor and that there was no preferential incorporation of the methylene carbon into ALA (Table 1). α-KG and glutamate were relatively efficient precursors with no significant difference between the extent of labeling by [1-^{14}C] and [5-^{14}C] glutamate. These results are in agreement with the report by Beale and Castelfranco[5] although we observed higher levels of incorporation, the incorporation from α-KG being particularly high. However, comparison of incorporation levels is difficult to interpret without detailed information on the internal pools of the metabolites used, their entry into the tissue, their

Table 1. The labeling of ALA produced after supply of various precursors by greening maize leaves

Precursor	Specific activity (μCi/μmol)	*cpm in peak containing ALA	*cpm in ALA pyrrole	†Specific activity of ALA (μCi/μmol)	‡Label in [5-C] of ALA (%)	§Label remaining in osmotic space of leaves (%)
[1-^{14}C] Glutamate	15·6	5343	5053	0·07	84	
[5-^{14}C] Glutamate	11·4	7700	4816	0·07	3	5·2
[5-^{14}C] α-Ketoglutarate	8·5	16797	14004	0·18	14	16·0
[1,4-^{14}C] Succinate	15·6	3506	3757	0·05	89	9·6
[2-^{14}C] Acetate	17·7	6012	2410	0·03	3	11·4
[1-^{14}C] Glycine	11·4	212	0			
[2-^{14}C] Glycine	11·4	0	0			25·1

* Expressed as the difference in cpm/10^6 cpm in extract/g fr. wt between leaves treated and not treated with LA. † Based on ALA-pyrrole. ‡ Expressed as % of label in ALA, as obtained by periodate degradation. § CCl_3COOH soluble radioactivity remaining in the tissue after preloading leaves with the precursor for 2·5 hr in light, followed by 3 hr wash-out-expressed as % of the label taken-up by leaves. Ten-day-old etiolated maize leaves were pre-illuminated for 1 hr with 860 lux of white fluorescent light and incubated with various [^{14}C]-labeled precursors for 5 hr in light, in the presence of 50 mM levulinic acid. ALA was isolated by ion-exchange chromatography and its pyrrole prepared as described in the Experimental. Each value is the mean of 2–3 experiments.

distribution and metabolic fate. Measurements of $^{14}CO_2$ evolution[5] are also inadequate since the precursors could also be metabolized on the cell surface.

To gain information about the compartmentation of the various precursors within the tissue we compared the course of exit of radioactivity from thin strips of leaves after pre-loading with the labeled precursors (Fig. 2). This approach[11] enables one to estimate the relative amount of the label originating from the various precursors which remained in the osmotic space of the tissue after a wash-out period. In the case of glycine, 25% of the label which had been taken up by the leaves during 2·5 hr pre-loading remained in the tissue as CCl_3COOH-soluble compounds after 3 hr wash-out (Table 1), while an additional 9% was found in the CCl_3COOH-insoluble fraction. About 20% of the CCl_3COOH-soluble label was still in glycine, as determined by paper chromatography. It seems therefore unlikely that glycine is a poor precursor of ALA because it does not enter the cells or due to its rapid metabolic conversion to other compounds.

Table 1 also shows the percent of label found in [5-C] of ALA after feeding leaves with labeled

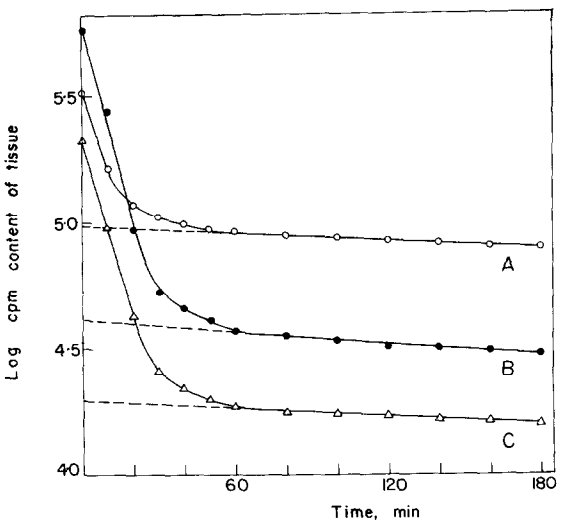

Fig. 2. The course of exit of radioactivity from maize leaves which have been pre-loaded with some [^{14}C]-labeled precursors into solutions of the unlabeled precursors. Etiolated leaves were pre-illuminated for 1 hr and incubated with the various precursors (5 μCi/1·5 g fr. wt of leaf-sample, 2·5 mCi/mmol) for 2·5 hr in light. The exit of radioactivity from 0·8 mm leaf-strips (0·2 g samples) into 2·5 mM solns of the unlabled precursors was then followed at 26°. A—[2-^{14}C] glycine; B—[5-^{14}C] glutamate; C—[1,4-^{14}C] succinate.

precursors and degradation of the ALA with periodate. Most of the label found in ALA following feeding with [1-^{14}C] glutamate or [1,4-^{14}C] succinate was recovered in [5-C] of ALA, while [5-^{14}C] glutamate and [2-^{14}C] acetate contributed little, if any label to [5-C]. The reliability of the degradation procedure was examined on commercially obtained [4-^{14}C] and [5-^{14}C] ALA. When [5-^{14}C] ALA was used, 91% of the label was recovered in the formaldehyde-dimedone precipitate and 8% in the filtrate, and the succinic acid isolated from the latter was unlabeled. The precipitate obtained after degradation of [4-^{14}C] ALA was unlabeled while the only labeled compound in the filtrate was succinic acid.

Several conclusions can be drawn from the results presented in Table 1. ALA is not synthesized from succinyl-CoA and glycine in greening maize leaves. This is shown by the negligible contribution of label from glycine, the almost exclusive incorporation of label from [1,4-^{14}C] succinate into [5-C] of ALA and the almost equal contribution by [1-C] and [5-C] of glutamate. ALA is apparently formed from a 5 carbon precursor, possibly α-KG or glutamate, whose carbon skeleton is incorporated intact, as was suggested by Beale and Castelfranco[5]. [1-C] of glutamate becomes [5-C] of ALA, [5-C] of glutamate or α-KG apparently becomes [1-C] of ALA, although there is no direct proof for this in our results. The contribution of label from succinate to ALA apparently results from its conversion to α-KG. The stereospecificity of citrate synthetase, which brings about the formation of [1-^{14}C] α-KG from [1,4-^{14}C] succinate[13] is the most likely explanation for the fact that label contributed by the latter enters [5-C] of ALA. This is also compatible with the observations that [2-^{14}C] acetate does not label [5-C] of ALA and that 14% of the label contributed by [5-^{14}C] α KG appears in [5-C] of the product. [5-^{14}C] α KG is probably converted to [1,4-^{14}C] succinate and part of the label reappears in [1-C] of α-KG. Our observations also show that ALA is not formed by cyclization of glutamate to Δ^1-pyrroline-5-carboxylic acid followed by oxygenation and reopening of the ring at the original C–N bond[5]. If this were the case, [5-C] of glutamate should have labeled [5-C] of ALA.

It seems likely that ALA in greening maize leaves is synthesized from α-ketoglutarate through its conversion to γ,δ-dioxovaleric acid followed by transamination to ALA [6, 14].

EXPERIMENTAL

Maize (*Zea mays*, cv. Neve Yaar 170) seedlings were grown in the dark as previously described [3]. Leaves were detached from 10-day-old plants, preilluminated for 1 hr with 860 lux of white fluorescent light. Their bases were recut under H_2O and then put in front of a fan, with their bases in isotope solns (10 μCi per leaf-sample of 3·0–3·5 g fr. wt) with or without 50 mM LA at pH 6·0. After 5 hr in light, the leaves were ground in 4% cold CCl_3COOH and the supernatant after centrifugation at 10000 xg for 15 min was used for further analysis. ALA was isolated from the leaf extracts by chromatography on Dowex 50W × 4 (100–200 mesh) columns (17 cm height, 0·9 cm in diam) equilibrated with 0·2 N Na citrate buffer pH 4·25. It was eluted from the column with the same buffer and determined after condensation with acetylacetone according to Mauzerall and Granick [7]. The labeling of carbons 1–4 and 5 of ALA was determined after periodate degradation according to Shemin *et al.* [8]. 20 μmol of $NaIO_4$ were injected through a rubber capped tube into the sample containing labeled ALA and 5 μmol carrier in Na–Pi buffer at pH 8·0. After 30 min at room temp. 0·1 ml ethylene glycol were added to destroy excess periodate followed by 19 ml 0·4% dimedone. pH was adjusted to 4·5 with HCl and the dimedone derivative of formaldehyde allowed to precipitate overnight at 2°. The ppt. was collected on Millipore filters (0·45 μ), washed with 1 mM Na succinate pH 4·5 and dissolved in EtOH after which its radioactivity was determined. Succinic acid in the filtrate was identified by PC (EtOH–NH_3–H_2O 8:2:1 and *n*-BuOH–HCO_2H–H_2O, 7:3:12) after removal of phosphate and iodate [9]. The radiopurity of ALA isolated from leaf-extracts was determined by PC (*t*-BuOH–MeCOEt–HOAc–H_2O, 10:6:9:9 and *n*-BuOH–HOAc–H_2O, 25:4:10) and by preparing and isolating the pyrrole according to Irving and Elliot [10]. γ-Aminobutyric acid and glycine were identified by PC. An estimate of the amounts of labeled precursors which had been taken up into the osmotic space was obtained by pre-loading leaves for 2·5 hr in light and following the course of exit of radioactivity from 0·8 mm leaf-strips into 2·5 mM solutions of unlabeled precursor according to Shtarkshall *et al.* [11]. Radioactivity was determined by liquid scintillation counting or by radiochromatogram scanning. [1-^{14}C] and [2-^{14}C] glycine, [2-^{14}C] acetate, [1,4-^{14}C] succinate and [5-^{14}C] α-KG were purchased from The Radiochemical Centre, Amersham; [4-^{14}C] and [5-^{14}C] ALA from New England Nuclear; [1-^{14}C] and [5-^{14}C] glutamate from Schwarz/Mann, Orangeburg, N.Y.; Dimedone (5,5 dimethylcyclohexane-1,3-dione) was purchased from NBCo.

Acknowledgement—This work was supported by a grant from the Israel Commission for Basic Research to S. Klein and E. Harel. The paper incorporates part of the M.Sc. thesis of E. Meller, to be submitted to the Hebrew University.

REFERENCES

1. Granick, S. and Sassa, S. (1971) in *Metabolic Regulation* (Vogel, H. J., ed.) **5**, p. 77. Academic Press, New York.
2. Beale, S. I. (1970) *Plant Physiol.* **45**, 504.
3. Harel, E. and Klein, S. (1972) *Biochem. Biophys. Res. Commun.* **49**, 364.
4. Richard, F. and Nigon, V. (1973) *Biochim. Biophys. Acta* **313**, 130.
5. Beale, S. I. and Castelfranco, P. A. (1974) *Plant Physiol.* **53**, 297.
6. Turner, J. M. and Neuberger, A. (1970) *Methods in Enzymology* (Tabor, H. and Tabor, C. W., eds.) **17A**, p. 188, Academic Press, New York.
7. Mauzerall, D. and Granick, S. (1956) *J. Biol. Chem.* **219**, 435.
8. Shemin, D., Russel, C. S. and Abramsky, T. (1955) *J. Biol. Chem.* **215**, 613.
9. Greenberg, D. M. and Rothstein, M. (1957) *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds.) **4**, p. 715, Academic Press, New York.
10. Irving, E. A. and Elliot, W. H. (1969) *J. Biol. Chem.* **244**, 60.
11. Shtarkshall, R. A., Reinhold, L. and Harel, H. (1970) *J. Exp. Botany* **21**, 915.
12. Fluhr, R., Harel, E., Klein, S. and Ne'eman, E. (1974) in *Proc. 3rd International Congress on Photosynthesis* (Avron, M., ed.) p. 2097, Elsevier, Amsterdam.
13. Englard, S. and Hanson, K. R. (1969) *Methods in Enzymology* (Lowenstein, J. M., ed.) **13**, p. 567, Academic Press, New York.
14. Gassman, M., Pluscec, J. and Bogorad, L. (1968) *Plant Physiol.* **43**, 1411.