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δ-AMINOLAEVULINIC ACID FORMATION IN GREENING *AVENA* LAMINAE

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Key Word Index—*Avena sativa*; Gramineae; δ-aminolaevulinic acid; chlorophyll; phaeophorbide; pyrophaeophorbide; maleimide.

Abstract—[1,5-^{14}C]-citrate was employed to indicate complete five carbon incorporation into the chlorophylls from the labelling pattern to be found in degraded chlorophylls and the maleimides. This provides additional evidence that there may be an alternative pathway to that involving δ-aminolaevulinic acid (δ-ALA) synthetase in higher plants.

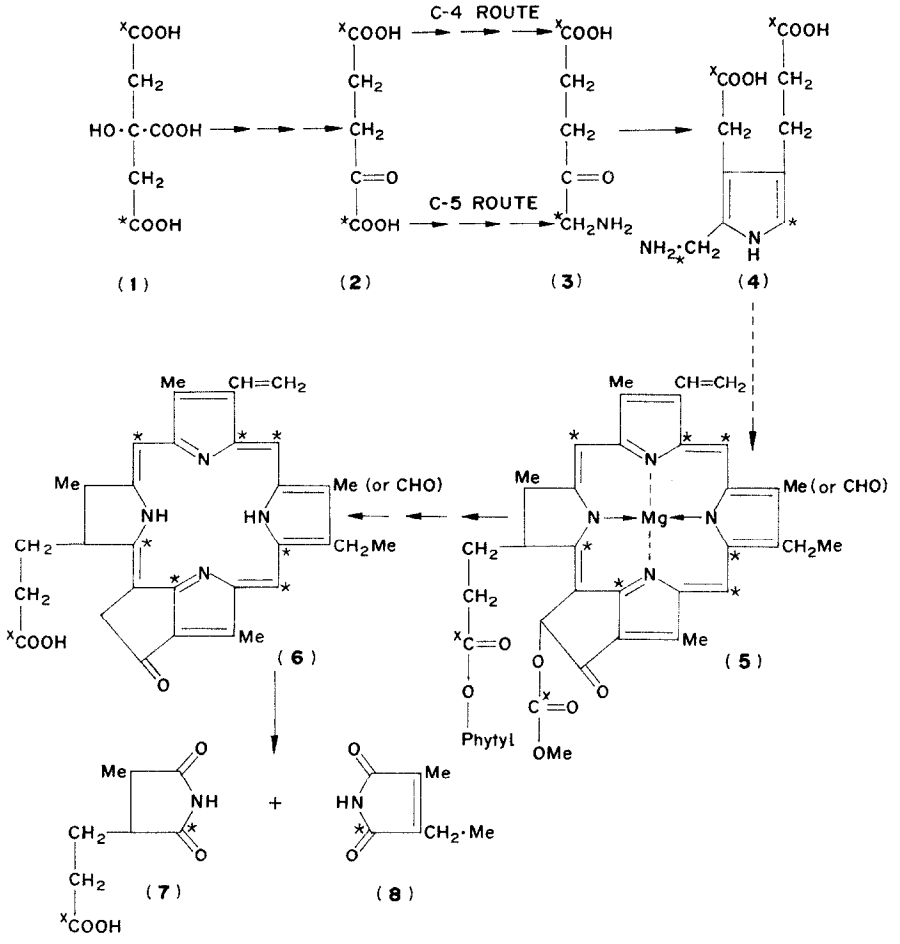
INTRODUCTION

Alternative routes of δ-ALA formation were first suggested by work on *Chlorella* [1] but until recently the pathway for the formation of δ-aminolaevulinic acid (δ-ALA) in plants by a C-4 route involving succinyl-CoA-glycine transferase or δ-ALA synthetase has not been seriously challenged. However, Beale and Castelfranco [2,3] have indicated that in the presence of laevulinic acid, an inhibitor of δ-ALA dehydratase, the most effective labelled precursors of δ-ALA were glutamate, glutamine and α-ketoglutarate; ^{14}C-labelled glycine and succinate were poorly incorporated and there was little difference in the specificity of incorporation between labelled carboxyl and methylene carbon atoms of glycine. From this they concluded that alternative routes of δ-ALA formation directly from C-5 carbon skeletons of glutamate and α-ketoglutarate exist which by-pass the requirement for δ-ALA synthetase in higher plants.

To check the C-5 route proposed by Beale and Castelfranco, a different approach was devised involving the use of [1,5-^{14}C]-citric acid. This is best shown diagrammatically by Scheme 1. If a route of C-5 formation of δ-ALA exists then label from [1,5-^{14}C]-citric acid (**1**) via [1,5-^{14}C]-α-ketoglutaric acid (**2**) might appear in both carbon atoms 1 and 5 of δ-ALA (**3**) and thence as indicated to porphobilinogen (**4**) and so to the chlorophylls (**5**) in both the tetrapyrrole ring, the carboxyl carbon of the esterified succinyl group of ring IV and the carboxyl of the carboxymethoxy of C-6e. If however a route from a α-keto-glutaric acid involves the C-4 or succinyl CoA plus glycine mechanism, then the labelling of C-5 of δ-ALA would be lost and no significant radioactivity should appear in the tetrapyrrole ring of the chlorophylls. To verify this a series of degradation via phaeophytins and phaeophorbides to the pyrophaeophorbides (**6**) would result in loss of magnesium, phytol and the carbomethoxy of C-6e respectively. Destructive oxidation of the pyrophaeophorbides with chromic acid would release the maleimides. The presence of label in the methyl ethyl maleimide (**8**) from chlorophylls would be good evidence of labelling in the tetrapyrrole ring of the chlorophylls and that a C-5 pathway may be involved in the formation of δ-ALA.

RESULTS AND DISCUSSION

This suggested procedure was carried out and the accumulated results are shown in Table 1, with the theoretical number of labelled atoms of δ-ALA that might be expected to appear in each degradation product assuming total C-5 incorporation. Reasonable incorporation of total label (0·52%) into the chlorophylls was achieved and very little



Scheme 1. Expected fate of label from [1,5-^{14}C]-citric acid during the biosynthesis and chemical degradation of the chlorophylls. (* and x-carbon atoms labelled by routes involving the C-5 and C-1 of δ-ALA respectively). N.B. chlorophyll *b* does not form a methyl ethyl maleimide (8).

radioactivity was left in the uptake solution after 24 hr. The results in Table 1 show that the incorporated label is specifically associated with the chlorophylls and some of this label remained in the methyl ethyl maleimide produced by degradation of chlorophyll *a*. Chlorophyll *b* does not give rise to a methyl ethyl maleimide but the results of these degradations of chlorophyll b are useful to provide a check on the pattern of labelling of the chlorophyll *a* degradations. The specific activities are similar at all stages.

It seems clear from these results that δ-ALA may have an alternative mode of formation in higher plants. Supporting evidence for this conclusion is indicated by examination of the amounts of label lost during the degradation and the specific activities of each derivative. If, for example, only the carboxyls of δ-ALA were labelled by a C-4 route involving α-ketoglutaric acid dehydrogenase and δ-ALA synthetase; then the amounts of label lost by the conversion of both the phaephorbides to their respective pyrophaeophorbides would have been *ca* half and the specific activities changed accordingly.

The alternative routes by which δ-ALA is formed have recently been discussed [3] and further speculation at this stage would be premature. The elucidation of a detailed C-5 pathway and its enzymology in higher plants is now a prime priority. Undoubtedly progress in this direction will be made with cell-free systems but there are many problems with their use to be solved.

Table 1. Fate of label incorporated into chlorophylls from [1,5-^{14}C]-citrate

Derivative	Expt. no.	Total act (dpm × 10^6) 1	2	Sp. act. (dpm × 10^4 per mg) 1	2	Theoretical number of labelled atoms of δ-ALA C-5	C-1
Chlorophyll *a*		1·85	1·73	3·91	3·69	7	2
Phaeophorbide *a*		1·73	1·70	3·93	3·80	7	2
Pyrophaeophorbide *a*		1·41	1·37	3·75	3·68	7	1
Haematinic imide (from chlorophyll *a*)		0·27	0·23	5·06	4·37	1	1
Methyl ethyl maleimide (from chlorophyll *a*)		0·15	0·15	3·64	3·69	1	—
Chlorophyll *b*		1·04	0·93	3·96	3·54	7	2
Phaeophorbide *b*		0·88	0·81	3·75	3·45	7	2
Pyrophacophorbide *b*		0·61	0·50	3·56	2·93	7	1
Haematinic imide (from chlorophyll *b*)		0·09	0·10	4·50	4·69	1	1

Illuminated excised etiolated *Avena* laminae were allowed to imbibe [1,5-^{14}C]-citrate (500 μc) in phosphate buffer (pH 7·0) for 24 hr. Labelled chlorophylls were extracted and degraded by methods described in Experimental.

EXPERIMENTAL

Eleven-day-old dark grown *Avena sativa* (var. Mostyn) seedlings (300 weighing 9·0 g) grown in moist peat at 20° were plucked from the cotyledonous tubes and dipped into a soln of 0·05 M potassium phosphate buffer (pH 7·0) before the laminae were re-cut under the soln just below the yellowing blades of the laminae. [1,5-^{14}C]-citric acid monohydrate (500 μc, Amersham, 20·2 mCi/mM, 98% purity) was added to the uptake soln in similar buffer and the laminae were left in the light (6000 lx) for 24 hr at 28°. The uptake soln was topped up at intervals with unlabelled buffer over the 24 hr period. Later the laminae were homogenized in an Ultraturrax for 30 s in 80% Me_2CO and the whole lipid extracts were extracted (× 3) with Et_2O. The ethereal extracts were washed repeatedly with H_2O, dried and blown to dryness under N_2. PLC using Si gel G (600 μ) on 40 × 20 cm plates in the long dimension followed by a reversed phase partition method [4] gave excellent separations of pigments, particularly of chlorophylls *a* and *b* (R_f 0·25, 0·33 respectively). Total amounts of chlorophyll *a* and *b* isolated were measured spectrophotometrically in EtOH to be 4·5 mg and 2·5 mg respectively. Twenty times as much cold chlorophyll *a* and *b* previously isolated from greening were added to each to act as carriers (total chlorophyll *a* = 94·50 mg; chlorophyll *b* = 52·5 mg) and each chlorophyll soln was equally divided for separate degradations.

Hydrolysis of the chlorophyll *a* and *b* to their respective phaeophorbides, rechromatography on the cellulose system [4] (R_f: phaeophorbide *a* 0·30, phaeophorbide *b* 0·43) and spectrophotometric assay was followed by boiling in pyridine to form the pyrophaeophorbides [5]. Rechromatography on cellulose (R_f: pyrophaeophorbide *a* 0·76, pyrophaeophorbide *b* 0·85) and spectrophotometric assay preceded the degradation to the maleimides by a microversion of the method of Finken *et al.* [6]. The PC, system using *n*-BuOH and the visualization system using Cl_2 and 1% (w/v) starch described in this paper were tried with moderate success but better separation of the maleimides was achieved using Si gel H coated TLC plates and petrol (bp 60–80°)–EtOAc–*n*-PrOH (44:5:1) [7]. Van Urk's reagent was subsequently found to be a better visualizing agent and in the case of the maleimides from chlorophyll *b* only one component, haematinic imide, showed as a purple spot (R_f 0·30) whilst the maleimides from chlorophyll a showed as two spots, the haematinic imide and methyl ethyl maleimide (R_fs 0·30 and 0·85 respectively). Similar separations were achieved using the acidic maleimide separation technique of Ellsworth and Aronoff [8] (methyl ethyl maleimide, R_f 0·90, haematinic acid, R_f 0·60).

The bulk of the maleimides were chromatographed as lines on the Si gel H system and Van Urk's reagent was used to visualize the ends of the developed bands and a Panax windowless radiochromatogram scanner and a Pullan spark chamber used to confirm the chromatographic behaviour of the maleimides. The areas on the chromatogram between the stained markers were scraped off, eluted with Me_2CO and taken to dryness before weighing. Samples taken at all stages of the degradation for estimation of radioactivity were bleached under UV light in scintillation vials, 10 ml of Bray's soln [9] plus 1 mg of benzoyl peroxide was added to each vial and left for 2 days before counting in a Packard 3375 liquid scintillation counter against appropriate external and internal standards using preset counting characteristics for ^{14}C.

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