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δ-AMINOLAEVULINIC ACID DEHYDRATASE IN TISSUE CULTURES OF *KALANCHOË CRENATA*

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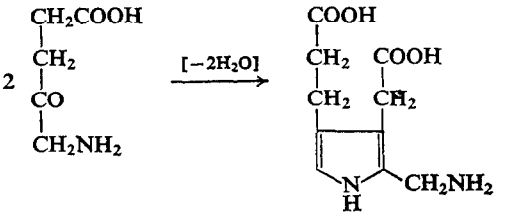
Abstract—δ-Aminolaevulinic acid-dehydratase activity increased with increasing chlorophyll content in greening tissue cultures of *Kalanchoë crenata*. The increase in enzyme activity was related to the maturation of the chloroplasts.

INTRODUCTION

ILLUMINATED callus tissue cultures of *Kalanchoë crenata* fail to synthesize chlorophyll in amounts equivalent to that found in mature leaves, although plastid carotenoids are present in relatively high concentrations before chlorophyll is detectable.[1] The enzyme, chlorophyllase, thought to be responsible for the phytylation of the chlorophyll precursor chlorophyllide a,[2] increases in activity in greening tissue cultures.[3] The diterpenoid alcohol, phytol, is produced in quantity in greening callus[4] and the results obtained might be taken to suggest that the availability of phytol does not limit the formation of chlorophyll a from chlorophyllide a. It seemed possible that the inability of these cultures to synthesize chlorophylls rapidly and in any quantity might be due to the absence or low activity of enzymes which are involved in the earlier stages of chlorophyll synthesis.

Reports exist[5, 6] concerning the presence of enzymes responsible for catalysing some of the biosynthetic steps leading from δ-aminolaevulinic acid (ALA) to chlorophyll in higher plants, but as yet no investigations have been carried out on the levels of activity of these enzymes in greening systems. The first compound synthesized with a pyrrole structure is porphobilinogen, a monopyrrole synthesized from two molecules of ALA, catalysed by the enzyme ALA-dehydratase.[7] It is the activity of this enzyme in greening callus tissue cultures of *K. crenata* that is reported here.



ALA PBG

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Abbreviations: ALA, δ-aminolaevulinic acid; PBG, porphobilinogen.

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RESULTS

Dark-grown callus tissues showed low levels of ALA-dehydratase activity and the activity did not differ by much from that of the first and second generation callus (Table 1). With the onset of chlorophyll synthesis there was a marked increase in enzyme activity in the third and fourth generation callus. The green callus, however, exhibited a higher activity on a protein basis than any of the other cultures. When the results were expressed on a chlorophyll basis the ALA-dehydratase activity increased from the third generation to fourth generation callus. Indeed, fourth generation callus contained more enzyme activity on a chlorophyll basis than did the clone of green callus.

TABLE 1. ALA-DEHYDRATASE ACTIVITY IN CALLUS CULTURES OF *Kalanchoë crenata*

Callus	Chlorophyll content mg per g acetone powder	Enzyme activity: μmoles PBG formed per mg protein in 10 min $\times 10^{-4}$	Enzyme activity: μmoles PBG formed per mg chlorophyll in 10 min $\times 10^{-4}$
Dark-grown	not detectable	16	0
1st generation	not detectable	21	0
2nd generation	not detectable	25	0
3rd generation	0·17	73	87
4th generation	0·30	192	129
Green	0·47	230	97

The intracellular distribution of ALA-dehydratase in these cultures was investigated using conventional fractionation procedures (see Materials and Methods). Assays were carried out on buffered extracts of acetone powders from 3000 *g*, 20,000 *g* fractions, and the final supernatants (Table 2). No ALA-dehydratase activity was detected in the 3000 *g* fractions from dark-grown or first and second generation callus, although pellets were always obtained in these fractions. However, the 20,000 *g* fractions and supernatants contained a small but detectable enzyme activity. No major differences in activity were detected in comparable fractions from these three types of callus. Third and fourth generation callus and green callus provided substantial 3000 *g* pellets which contained high enzyme activity,

TABLE 2. INTRACELLULAR DISTRIBUTION OF ALA-DEHYDRATASE IN CALLUS CULTURES OF *Kalanchoë crenata*

Callus	Enzyme activity in fractions expressed as μmoles PBG formed per mg protein in 10 min: 3000 *g* $\times 10^{-4}$	20,000 *g* $\times 10^{-4}$	supernatant $\times 10^{-4}$
Dark-grown	not detectable	19	16
1st generation	not detectable	29	12
2nd generation	not detectable	23	11
3rd generation	57	18	17
4th generation	148	39	21
Green	193	36	16

whereas the activity in 20,000 *g* fractions and supernatants did not differ markedly from that detected in these fractions of callus cultures which lacked chlorophyll.

DISCUSSION

The synthesis of chlorophylls and the development of chloroplasts in callus cultures of *Kalanchoë crenata* was paralleled by a substantial increase in ALA-dehydratase activity. A similar situation was reported for the bacterium, *Rhodopseudomonas spheroides* by Lascelles[8] who correlated an increase in ALA-dehydratase activity with increasing bacteriochlorophyll levels. The enzyme activity, expressed on a protein basis, increased with time in greening callus and approached that of the green callus clone. It is known that an increase in protein in greening seedlings is attributed largely to the synthesis of chloroplast protein[9] and it seems likely that the increase in ALA-dehydratase activity in the greening callus cultures is due to a synthesis of new protein rather than an activation of existing protein. The increased enzyme activity is due clearly to the maturation of plastids as most activity was recovered in the 3000 *g* fractions of those callus tissues which contained detectable amounts of chlorophyll. The callus was fractionated in an aqueous medium and it is of interest that the ALA-dehydratase activity remained associated with the 3000 *g* fraction and was only solubilized after treatment of the fraction with acetone. This possibly indicates that the enzyme is intimately associated with the membranes of the chloroplast and its production is closely linked to the development of thylakoids and normal grana which are found only in chlorophyll-containing callus.

When ALA-dehydratase activity was expressed on a chlorophyll basis, the fourth generation callus contained more enzyme activity than the green callus clone. It would seem that this is a reflexion of the lower chlorophyll content of fourth generation callus. This result also might suggest that factors other than the production of ALA-dehydratase limit the production of chlorophylls in the greening callus. When incubated with ALA, etiolated barley seedlings accumulated protochlorophyllide, the precursor of chlorophyllide.[10] Granick[11] suggested that ALA-synthetase is the key enzyme controlling porphyrin synthesis, steps after ALA being catalysed by enzymes present in non-limiting amounts. In a growth experiment with the green callus clone 0·01 per cent (w/v) ALA was supplied in the medium. After 1 month's growth in 16-hr days, the chlorophyll content of the treated green callus (17 μg chlorophyll/g fresh weight) was double that of the normal green callus (9·5 μg chlorophyll/g fresh weight). Thus it would seem profitable to investigate the production of ALA-synthetase in greening callus, as low amounts of this enzyme might be one of the factors limiting the production of chlorophylls in the callus. However ALA-synthetase has yet to be demonstrated in higher plants and until it can be, conclusions regarding the control of chlorophyll synthesis in the greening callus remain conjectural.

MATERIALS AND METHODS

The growth medium and culture conditions for these tissue cultures have been reported elsewhere.[12] The cultures under investigation in this work are designated as follows:

1. Dark-grown callus: callus cultures which have been grown for 4 years in the dark with regular monthly subculturing.

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2. First, second, third and fourth generation callus: callus which has been subcultured from dark-grown tissue and then grown under a 16-hr day for 4 weeks is termed first generation callus. Subcultures from this, after a further month's growth in the same conditions of illumination provide second generation callus from which third and fourth generation callus were derived by subculturing at monthly intervals.
3. Green callus: a clone of callus tissue which has been growing under 16-hr day conditions for 3 years with subculturing at monthly intervals.

Acetone powders of these cultures were prepared by the method of Loomis[13] and the protein extracted and solubilized in small volumes of 0·1 M phosphate buffer, pH 8, containing 10 μmoles β-mercaptoethanol per 3 ml. Protein concentrations were determined using the Folin-Ciocalteu reagent[14] and chlorophyll was estimated spectrophotometrically in diethyl ether[15] from the organic solvent extracts obtained in the preparation of the acetone powders.

Fractionation Procedures

The callus tissues were first washed with buffer (0·35 M NaCl, 0·01 M $K_2H\,PO_4$, pH 7·4) and then ground in a mortar with an equal volume of acid washed sand and more precooled buffer. After filtering through 8 layers of cheesecloth[16] the filtrate was centrifuged for 5 min at 200 *g* to remove cell debris. The supernatants were centrifuged at 3000 *g* for 7 min, and finally at 20,000 *g* for 20 min on a Spinco Model L Ultra-centrifuge at 0°. Acetone powders of the 3000 *g* and 20,000 *g* fractions were prepared in the usual way. Protein in the final supernatant was precipitated with large volumes of cold acetone and collected by centrifugation.

ALA dehydratase was assayed according to the method of Shemin.[7] The reaction mixtures contained 15 μmoles ALA and 10 μmoles β-mercaptoethanol, previously neutralized with $NaHCO_3$, and then made up to the necessary volume with 0·1 M phosphate buffer, pH8. After the addition of the protein extract the reaction was allowed to proceed for 10 min at 34° and then stopped by the addition of 1 ml 10 per cent (w/v) trichloroacetic acid which was 0·1 M with respect to $HgCl_2$. An equal volume of modified Ehrlichs solution was added to the supernatant and the optical density read at 555 nm after standing for 8 min. Corrections were always made for tetrapyrroles synthesized from PBG during the reaction. PBG concentrations were calculated using a molar extinction coefficient of $6{\cdot}2 \times 10^4\ m^{-1}$.

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