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CATABOLISM OF δ-AMINOLAEVULINIC ACID IN ETIOLATED *EUGLENA GRACILIS*

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Key Word Index—*Euglena gracilis*; algae; δ-aminolaevulinic acid; chlorophyll biosynthesis; degradation; methyl phaeophorbide; methyl ethyl maleimide; acetate; propionate.

Abstract—[4-^{14}C]-δ-Aminolaevulinic acid (δ-ALA) was employed to study the specificity of δ-ALA incorporation into chlorophyll in etiolated *Euglena gracilis*. Degradation of chlorophyll *a* to acetate and propionate, obtained from ring B, indicated that, although some of the radioactivity was incorporated, it did not result from incorporation of intact δ-ALA.

INTRODUCTION

In the course of our research on chlorophyll biosynthesis, we required a biological system which would facilitate a high specific incorporation of δ-ALA into chlorophyll. It is well known that δ-ALA is the first biochemical intermediate unique to the tetrapyrrole pathway and its incorporation into chlorophyll has been demonstrated in higher plants such as wheat [1] and cucumber [2] and with some isolated chloroplast or etioplast preparations [3]. However, reports on the incorporation of δ-ALA into chlorophyll with the flagellate *Euglena gracilis*, which produces large quantities of chlorophyll *a* and chlorophyll *b*, are somewhat conflicting. Kirk reported [4] a three-fold increase in chlorophyll production upon addition of δ-ALA to greening cultures of *E. gracilis*. It has been doubted [5] that δ-ALA could penetrate the cells of *Euglena*, and Hovenkamp-Obema failed to repeat Kirk's findings, but reported [6] that δ-ALA could be incorporated into chlorophyll in etiolated cells of *E. gracilis*. In the latter work, dark-grown cells were kept in a resting medium for 10 days, treated with [^{3}H]-δ-ALA and after 2 hr exposed to light. The chlorophyll content in the cells, determined spectrophotometrically during the period of illumination, indicated that addition of δ-ALA resulted in a two-fold increase in chlorophyll concentration for a 10 mM δ-ALA solution and a 50% increase for a 1 mM solution. The radioactivity of the cells measured by direct planchet counting showed a steady increase during the period of illumination.

However, the presence of radioactivity detected in the cells does not necessarily imply that exogeneous δ-ALA was incorporated into chlorophyll. Moreover, if it is assumed that the radioactivity detected in the cells is present only in chlorophyll, a discrepancy is apparent: 20% of activity from a 10 mM δ-ALA solution was found in the cells, while even if all the increase in chlorophyll production were due to exogeneous δ-ALA, no more than 2% should have been incorporated.

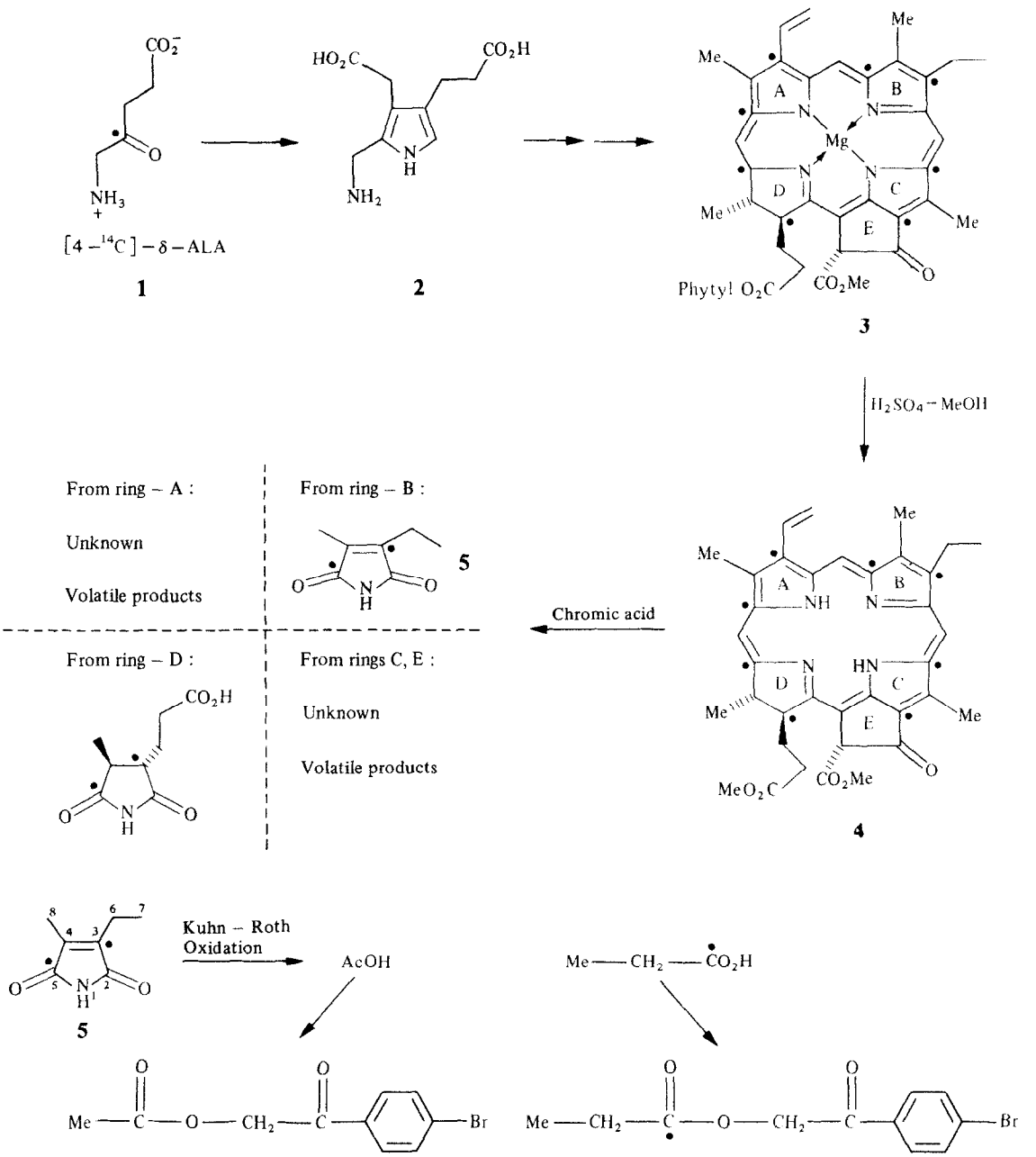
A critical test of whether or not δ-ALA was incorporated into chlorophyll would be achieved by feeding [4-^{14}C]-δ-ALA. If δ-ALA is specifically incorporated, then label from [4-^{14}C]-δ-ALA via porphobilinogen (**2**), uroporphyrinogen III and other intermediates in chlorophyll biosynthesis will appear as indicated in the tetrapyrrole ring of chlorophyll *a* (**3**). Appropriate degradation of the biosynthetic chlorophyll allows verification of the specificity of δ-ALA incorporation. This approach is best shown diagrammatically by Scheme 1.

RESULTS AND DISCUSSION

Etiolated cells of *E. gracilis* which were kept for 10 days in a resting medium were treated with [4-^{14}C]-δ-ALA and exposed to light. After 70 hr of illumination, the deep green cells were harvested and chlorophylls *a* and *b* were extracted and derivatized by methanolysis into the corresponding methyl phaeophorbides. The considerably more stable methyl phaeophorbide *a* (**4**) was purified by silica gel chromatography and recrystallization. Its radioactivity indicated that reasonable incorporation of total label (0.33%) into chlorophyll *a* was achieved.

However, the presence of activity in methyl phaeophorbide *a* does not in itself confirm incorporation of intact exogeneous δ-ALA. It is necessary to prove that **4** has the correct labelling pattern. This was done by two successive degradations [7]. Destructive oxidation of **4** with chromic acid afforded only one neutral species, methyl ethyl maleimide (**5**), which is derived from ring B only. This was diluted with synthetic material [8] and purified by sublimation and recrystallization to constant specific activity (Table 1).

Kuhn–Roth oxidation of the imide (**5**) afforded acetic acid and propionic acid, which were separated by thick-layer chromatography as their *p*-bromophenacyl esters and recrystallized to constant specific activity. Both esters appeared to be labelled. These results, as summarized in Table 1, are contrary to what was expected from the incorporation of intact δ-ALA into chlorophyll. The imide **5** was expected to carry the label at C-3 and C-5. Acetic acid, which is produced from C-4 and C-8 and partly from C-6 and C-7 of **5**, was therefore expected to be radio-inactive, while propionic acid, which is produced from C-3, C-6 and C-7 of **5**, was expected to have half of the specific activity of methyl ethyl maleimide.



Scheme 1. (●) Expected fate of label from [4-^{14}C]-δ-ALA during the biosynthesis and chemical degradation of chlorophyll *a*.

The inappropriate distribution of the label in the degradation products derived from the biosynthetic chlorophyll *a* clearly indicates that the [4-^{14}C]-δ-ALA has not been incorporated in the expected manner. Rather, the figures are close to what would be expected if the label had been randomly distributed, at least in ring B.

This provides powerful evidence that the administered δ-ALA is not utilized intact by etiolated cells of *E. gracilis*, but suffers extensive breakdown before incorporation. A similar case of enzymatic breakdown of δ-ALA, through transamination and further oxidation via the tricarboxylic acid cycle, has recently been reported by Duggan *et al.*, who studied the catabolism of [4-^{14}C]- and [5-^{14}C]-δ-ALA by etiolated barley leaves [9]. As a consequence of these findings, it may turn out that the results of Hovenkamp-Obema [6] could be re-interpreted.

EXPERIMENTAL

Radioactive counting involved the use of a Packard liquid scintillation counter, model 526, using [^{14}C]hexadecane as internal standard. Samples of Me phaeophorbide *a* were bleached before counting by treatment with benzoyl peroxide [10]. TLC was carried out on commercially prepared plates coated with Merck Kieselgel GF_{254}, 0.25 mm thick. Prep. TLC was undertaken with plates (20 cm × 20 cm) coated with the same silica gel,

Table 1. Fate of label incorporated into chlorophyll* from [4-^{14}C]-δ-ALA

Degradation product	Total act. (dpm)	Sp. act.† (10^6 dpm/mmol)	Theoretical number of labelled atoms from [4-^{14}C]-δ-ALA
Methyl phaephorbide *a*	200000	20.17	8
Methyl ethyl maleimide	20000	4.45	2
p-Bromophenacyl acetate	1630	1.21	—
p-Bromophenacyl propionate	1520	1.69	1

*Labelled chlorophyll *a* was extracted and degraded by the methods described in the Experimental.

†An average from three successive recrystallizations after correcting for dilution with radio-inactive material.

1 mm thick. CC on silica gel was carried out on 'short, fat columns' (height = diameter) on Merck Kieselgel H60 material. Organic solns which had been in contact with H_2O were dried over dry Mg_2SO_4 prior to evapn at 25 Torr. All solvents were redistilled, $CHCl_3$ and CH_2Cl_2 being kept in the dark after distillation from dry K_2CO_3.

Five-day-old dark-grown cells of *E. gracilis* (1 l.) were re-suspended in 400 ml resting medium (as described in ref. [6]) to a final cell concn of *ca* 8 × 10^6 cells/ml. After 10 days in the dark, the cell suspension was treated with [4-^{14}C]-δ-ALA hydrochloride (0.4 mmol, 50 μCi (Amersham) in 10 ml H_2O neutralized to pH 7) and exposed to light for 70 hr. After 6 hr of illumination, the culture turned light green and then gradually turned deep green. The following method was developed for the extraction and *in situ* derivatization of chlorophylls *a* and *b*. The cells were collected by centrifugation on a continuous rotor head at 2500 rpm, and after being washed with H_2O, were centrifuged again and frozen at −17° for 2 days. The cells were thawed overnight at room temp., and the dark-green slurry was centrifuged at 12000 rpm for 30 min. The orange supernatant was decanted, and the green ppt. was mixed with Me_2CO (50 ml) and left in the dark, with occasional stirring for 1 hr. The mixture was filtered through a 2 cm bed of diatomaceous earth (Hiflo Supercel 545), which was washed with more Me_2CO (100 ml). The dark-green filtrate was evapd at 40°, and toluene (10 ml) was added twice and evapd each time (to remove any remaining H_2O azeotropically). The residual dark-green oil was dissolved in MeOH (9.5 ml)/H_2SO_4 (0.5 ml) and left at room temp. overnight. H_2O was added (10 ml) and after 3 min $CHCl_3$ (10 ml), followed by sufficient $NaHCO_3$ soln to bring the aq. layer to neutrality. The $CHCl_3$ layer was separated and the aq. layer extracted with more $CHCl_3$ (3 × 10 ml). The combined $CHCl_3$ extracts were dried and evapd to a dark-green oil (*ca* 100 mg). Total amounts of Me phaeophorbides *a* and *b* were measured spectrophotometrically in EtOH to be 6.2 and 1.5 mg, respectively. 14 mg 'cold' Me phaeophorbide *a* and 10 mg of 'cold' Me phaeophorbide *b* were added to act as carriers and the mixture was chromatographed on silica gel, Merck Kieselgel H 60 (10 g). Elution with 2% MeOH in CH_2Cl_2 afforded first a yellow product (R_f 0.9), then traces of a red compound followed by two separated green fractions (R_f 0.65, Me phaeophorbide *a* and R_f 0.55, Me phaeophorbide *b*). Elution with 5% MeOH in CH_2Cl_2 gave a dark-yellow (R_f 0.35) and a green (R_f 0.25) fraction, respectively. The R_f 0.65 green fraction, which contained all the Me phaeophorbide *a*, was evapd to a dark-green gum (19.2 mg, *ca* 200000 dpm) and the product was recrystallized from MeOH–CH_2Cl_2 to a constant sp. act. of 10400 dpm/mg.

The foregoing Me phaeophorbide *a* was dissolved in 50% H_2SO_4 (4 ml) and stirred for 2 hr at 20° to hydrolyse the ester. The deep-green soln was treated with ice (5 g), cooled to −10°, and CrO_3 (220 mg) in H_2O (2 ml) was added during a period of 40 min with stirring. The soln was stirred for 1 hr at 0°, then for a further 2 hr at 20°, and then all the acidic and neutral species were continuously extracted with Et_2O for 15 hr. Evapn of the extract afforded a yellow oil whose soln in H_2O (10 ml) was adjusted to pH 10 ($NaHCO_3$), and continuously extracted with Et_2O for a further 15 hr, giving the neutral fraction, methyl ethyl maleimide (*ca* 2 mg, *ca* 20000 dpm). This was mixed with radio-inactive material (15 mg), sublimed (80°, 0.1 Torr) and recrystallized from CH_2Cl_2–C_6H_{14} to a constant sp. act. of 1050 dpm/mg.

The foregoing product (10 mg, *ca* 11000 dpm) was heated under reflux for 2.5 hr with chromic acid [30 ml of a mixture, made by dissolving CrO_3 (33.6 g) in water (200 ml) and conc. H_2SO_4 (40 ml)]. Steam distillation was then carried out until 300 ml distillate had been collected. The steam distillate containing the volatile acids was titrated with 0.1 M NaOH (carbonate-free) and the soln adjusted until just alkaline to phenolphthalein and evapd to dryness. The residue was dissolved in H_2O (1.4 ml) and treated with *p*-bromophenacyl bromide (*ca* 3 equiv.) in EtOH (12 ml), and the mixture heated under reflux for 2.5 hr. The residue obtained by evapn was chromatographed in 10% EtOAc in C_6H_6 on thick-layer silica plates to yield *p*-bromophenacyl acetate, R_f 0.40 (6 mg, 30%) and *p*-bromophenacyl propionate, R_f 0.45 (4 mg, 20%). Crystallization of the products from petrol to constant sp. activity afforded *p*-bromophenacyl acetate (155 dpm/mg) and *p*-bromophenacyl propionate (204 dpm/mg).

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