^{14}C INCORPORATION FROM EXOGENOUS COMPOUNDS INTO δ-AMINOLEVULINIC ACID BY GREENING CUCUMBER COTYLEDONS

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SUMMARY--Greening cucumber cotyledons accumulate δ-aminolevulinic acid when treated with levulinic acid. A variety of specifically labelled compounds were applied to the tissue and label was measured in the δ-aminolevulinic acid. Glutamate, glutamine and α-ketoglutarate were found to be incorporated into δ-aminolevulinic acid to a much greater extent than were glycine and succinate. A new route of δ-aminolevulinic acid biosynthesis is proposed wherein the carbon skeleton of α-ketoglutarate is incorporated intact.

Although the pathway of tetrapyrrole biosynthesis has been elucidated in considerable detail (7), the first committed step of this sequence, the formation of δ-aminolevulinic acid (ALA), has not been directly demonstrated in extracts of green plants. In mammalian and avian heme-forming systems and in bacteriochlorophyll-producing micro-organisms, the key reaction has been shown to be the condensation of succinyl CoA and glycine to yield ALA, CO_2 (from the carboxyl carbon of glycine) and free CoA. This reaction is catalyzed by the pyridoxal phosphate requiring enzyme ALA synthetase (succinyl CoA-glycine succinyl transferase) (6,9). Such enzymatic activity has not been found in extracts of green plants. Furthermore, studies with chlorophyll synthesizing tissues, using exogenous labeled glycine have failed to reveal a significant preferential incorporation of the methylene over the carboxyl carbon into the phorbin moiety of chlorophyll, when compared to differential incorporation into other cellular components (4,13,14,15). This preferential incorporation pattern has been well established in the case of avian heme synthesis (11).

Recently, it has become possible to isolate quantities of ALA from greening plant tissues by treating them with levulinic acid which competitively inhibits the enzyme ALA dehydratase (2). In this study we have measured the incorporation of ^{14}C from a variety of exogenous substrates into ALA formed by greening cucumber cotyledons. Our results are not easily reconciled with the conventional hypothesis; the possibility of an alternative route of ALA formation which is consistent with our data is proposed.

Methods

Cucumber seeds (*Cucumis sativus*, L. var. Alpha green) were germinated in moist vermiculite in the dark at 25 C for 6 days. Cotyledon pairs were excised with hypocotyl hooks attached, and placed in petri dishes at 28 C under 240 ft. c. of light supplied by cool-white fluorescent tubes. Levulinic acid was added in an aqueous solution so as to coat thoroughly but not submerge the cotyledons. In labeling experiments 100 cotyledon pairs per covered petri dish (10 cm diameter) were incubated for 4 hr in the light. The covers were removed and 5 ml of a solution containing 0.1 M levulinic acid, 10% dimethylsulfoxide (DMSO) and 10 μc of the ^{14}C-labeled test compound were added. The dishes were incubated in the light for an additional 3 hr.

The tissue was rinsed thoroughly in cold water and homogenized in 15 ml of 5% $HClO_4$. After centrifugation, the precipitate was discarded, and the supernatant was adjusted to pH 4.5 with 10 M KOH. The $KClO_4$ precipitate was discarded, and the supernatant was passed through a small Dowex-50-H^+ column. After washing the column thoroughly with H_2O, the ALA was eluted with 1 M ammonium acetate.

The ALA was condensed with ethyl aceto acetate (10) to yield 2-methyl-3-carbethoxy-4-(3-propionic acid) pyrrole (ALA pyrrole) which was purified by extraction into ether at pH 3. ALA pyrrole was determined by the method of Mauzerall and Granick (10). Radioactivity was measured by liquid scintillation counting. Radio chemical purity was established by chromatography on

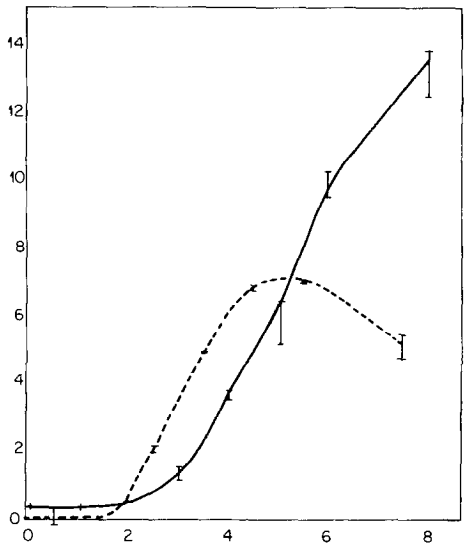

Fig. 1. Development of chlorophyll and ALA synthesis in etiolated cucumber cotyledons during continuous exposure to 240 ft.-c. of cool-white fluorescent light at 28°C. Chlorophyll is expressed as total nanomoles accumulated per cotyledon pair (——). ALA values refer to the nanomoles accumulated during one hour of treatment in the light with 100 mM levulinic acid. For each point, the treatment began one-half hour before and ended one-half hour after the time indicated (----). Abscissa: hours of illumination.

α-ketoglutarate. A second group of label contributors includes metabolically central compounds such as succinate, glyoxylate and acetate. These compounds are all rapidly metabolized in a tissue which converts triglycerides to carbohydrates via the glycolytic, glyoxylate and tricarboxylic acid pathways. The third group which contributes the least, includes proline, formate and glycine.

In experiments similar to the above, labeled glycine, glyoxylate, glutamate, succinate, and α-ketoglutarate were applied to the greening cotyledons and the respired CO_2 was collected. All of these compounds were found to be respired at comparable rates.

Discussion

The accumulation of ALA in response to the administration of levulinic acid has now been demonstrated in a variety of plant tissues (2,3,8,12), and

Whatman No. 1 paper in a solvent consisting of 1-butanol : 1-propanol : 5% NH_4OH (2:1:1) followed by scanning for radioactivity in a Packard strip counter and locating the ALA pyrrole by spraying with Ehrlich's reagent. Chlorophyll was determined by the method of Arnon (1).

Results

Accumulation of ALA in the presence of levulinic acid.

When greening cucumber cotyledons are treated with levulinic acid, they accumulate ALA. The rate of ALA accumulation in levulinic acid-treated tissues parallels the rate of chlorophyll accumulation in the control tissue. In fact, the maximum rate of ALA formation in the treated tissue and the maximum rate of chlorophyll synthesis in the untreated both occur between 5 and 6 hours of illumination (Fig. 1). One hundred mM levulinic acid was found to inhibit chlorophyll formation by 50%. Under these conditions, the chlorophyll accumulated in the treated tissue, plus 8 X the ALA accumulated in the same equaled the chlorophyll accumulated in the controls.

Incorporation of label from exogenous compounds.

Table 1 is a summary of results from five separate experiments. It shows that there are clear differences in incorporation of label from the various sources into ALA. Within each experiment, the data are directly comparable, whereas some caution must be exercised when comparing data between experiments.

The general trend of the results is that the family of metabolically related compounds glutamate, α-ketoglutarate and glutamine contribute a similar, high amount of label to ALA, even when the label is in C-1 of glutamate, which would be lost as CO_2 on conversion to succinyl CoA via the ALA has been shown to be related to chlorophyll synthesis in *Chlorella*, *Euglena*, and higher plants.

In this study, levulinic acid has been used as a tool allowing us to

Table 1

Incorporation of ^{14}C into ALA. Excised 6 day old etiolated cucumber cotyledons were exposed to 240 ft.-c. of cool white fluorescent light at 28°C for 7 hours. At hour 4, each sample of 100 cotyledon pairs was treated with 5 ml containing 100 mM levulinic acid, 10% DMSO and approximately 10 μc of the ^{14}C-labeled compound. Abbreviations: Ac-1,2-^{14}C, acetate-1,2-^{14}C; Pro-U-^{14}C, proline-U-^{14}C; For-^{14}C, formate-^{14}C; Suc-1,4-^{14}C, succinate-1,4-^{14}C; Gly-1-^{14}C, glycine-1-^{14}C; Gly-2-^{14}C, glycine-2-^{14}C; Glx-1-^{14}C, glyoxylate-1-^{14}C; Glx-2-^{14}C, glyoxylate-2-^{14}C; α-KG-U-^{14}C, α-ketoglutarate-U-^{14}C; Gln-U-^{14}C, glutamine-U-^{14}C; Glu-1-^{14}C, glutamate-1-^{14}C; Glu-3,4-^{14}C, glutamate-3,4-^{14}C.

		cpm in purified ALA fraction									
		per 10^5 cpm in tissue extract					per 10^6 cpm in label supplied				
Experiment #		1	2	3	4	5	1	2	3	4	5
Label	mc/mmole										
Ac-1,2-^{14}C	47.5	361					965				
Pro-U-^{14}C	260	151					376				
For-^{14}C	52.5		195					203			
Suc-1,4-^{14}C	20.4			380					278		
Gly-1-^{14}C	46.5					41					69
Gly-2-^{14}C	36.7		67			44		73			91
Glx-1-^{14}C	7.42			285					281		
Glx-2-^{14}C	7.44			843					810		
α-KG-U-^{14}C	200		1,480	1,060				1,610	1,330		
Gln-U-^{14}C	45	1,040					2,350				
Glu-1-^{14}C	25				2,040	1,050				2,530	1,350
Glu-1-^{14}C	5.34					1,130					1,340
Glu-3,4-^{14}C	55.5	1,220	1,320		985		2,850	1,520		1,790	
Glu-3,4-^{14}C	14.2					1,070					2,215

isolate ALA from greening tissue fed specifically labelled possible ALA precursors. The results of this study are inconsistent with the succinyl transferase (ALA synthetase) route of ALA formation for the following reasons:

1) Glycine was incorporated into ALA only to a very small extent.
2) The carboxyl carbon of glycine was incorporated to the same extent as the methylene carbon, whereas it would not be incorporated at all via the succinyl transferase reaction.
3) General metabolic intermediates such as succinate, glyoxylate, acetate and formate were incorporated more efficiently into ALA than was glycine, implying that glycine is not on the pathway from general metabolic intermediates to ALA.
4) The metabolically closely related compounds α-ketoglutarate, glutamate and glutamine were all incorporated into ALA with the highest efficiency of any of the compounds tested.
5) C-1 of glutamate was incorporated to the same degree as C-3 and C-4. This implies that the route of incorporation is not: glutamate ⟶ α-ketoglutarate ⟶ succinyl CoA ⟶ ALA, for in this route C-1 would be lost during succinyl CoA formation.

On the basis of the evidence at hand, we propose as a working hypothesis that ALA is formed in this tissue by reductive amination of the C-1 of α-ketoglutarate. This might be accomplished in a concerted process or in two discrete steps: reduction of C-1 to form α-ketoglutaraldehyde (dioxovaleric acid) followed by transamination. The latter step has been reported in extracts of the green alga *Chlorella* (5).

The possible influence of specific permeability barriers on the low level of glycine incorporation has not yet been examined; however, all the compounds tested were actively respired to a similar extent. The similar degree of incorporation of label from α-ketoglutarate, glutamate, and glutamine--metabolically closely related but of differing ionic character--

and their superiority to succinate, glyoxylate and acetate in labeling the ALA suggest to us that these compounds stand metabolically closer to ALA than do glycine and succinate.

The detailed steps in the conversion of the 5-carbon dicarboxylic intermediates to ALA are now being investigated.

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