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δ-Aminolevulinic Acid Formation from γ,δ-Dioxovaleric Acid in Extracts of *Euglena gracilis*[1]

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ABSTRACT

γ,δ-Dioxovaleric acid (DOVA) has been proposed as a precursor to heme and chlorophyll in plants and algae. DOVA transaminase activity was found in extracts of the unicellular green alga *Euglena gracilis* Klebs strain Z Pringsheim. Optimum conversion of DOVA to δ-aminolevulinic acid (ALA) occurred at pH 6.8. ALA formation was linear with time for at least 30 minutes at 37°C and was proportional to amount of cell extract in the incubation mixture. Boiled cell extract was inactive. DOVA transaminase from either wild-type or aplastidic derivative strain W_{14}ZNalL ran as a single band in agarose gel permeation chromatography, with a calculated molecular weight of 98,000 ± 3,000. L-Glutamic acid was the most effective amino donor. D-Glutamic acid was inactive. K_m values for L-glutamic acid and DOVA were 11 and 1.1 millimolar, respectively. Pyridoxal phosphate stimulated activity maximally at 30 micromolar, and (aminooxy)acetate was strongly inhibitory. Glyoxylic acid was a competitive inhibitor with respect to DOVA, with an inhibition constant of 0.62 millimolar. Wild-type and aplastidic cells yielded equal activity, 31 ± 1 nanomoles ALA per 30 minutes per 10^7 cells, whether grown in light or dark. DOVA transaminase could not be separated from glyoxylate transaminase activity by agarose gel permeation or diethylaminoethyl-cellulose column chromatography. In all fractions, glyoxylate transaminase activity was at least 75 times greater than DOVA transaminase activity. DOVA transamination appears to be catalyzed by glyoxylate transaminase, and not to be of physiological significance with respect to chlorophyll synthesis in *Euglena*.

The formation of ALA[3] is the first identified unique step in the biosynthesis of tetrapyrroles, a groups of molecules that includes hemes, corrins, bilins, and Chl (11). ALA formation in animals and bacteria, including photosynthetic bacteria, is catalyzed by the enzyme ALA synthase (succinyl-CoA:glycine C-succinyl transferase [decarboxylating] EC 2.3.1.37) (for review, see 1). The existence of ALA synthase had not been demonstrated in any oxygenic, photosynthetic organism, until recently, when it was shown that the enzyme is present in extracts of the unicellular green alga *Euglena gracilis* (3). Further studies on the regulation of ALA synthase in *Euglena* have indicated that the enzyme is probably not involved in the formation of ALA precursors to Chl, but serves instead to supply non-plastid tetrapyrrole precursors when the plastids are metabolically quiescent (9).

In plants, the major route to ALA formation utilizes the intact carbon skeleton of glutamate or α-ketoglutarate (5). This 5-carbon pathway has been shown to operate in the tissues of higher plants and in greening alga (2), including members of the *Cyanophycae*, *Rhodophycae*, *Chlorophycae*, and *Euglena* (24). Although the 5-carbon pathway has not yet been fully elucidated, it is considered to be responsible for providing precursors to Chl.

Two biochemical routes from glutamate or α-ketoglutarate to ALA have been proposed. One involves the intermediate formation of glutamate-1-semialdehyde, and the other, the intermediate formation of DOVA. Enzyme activity capable of transaminating DOVA to ALA has been detected in mammalian tissues, nonphotosynthetic and photosynthetic bacteria, higher plant tissues, and greening algae, including *Chlorella* (10) and *Euglena* (25).

Noguchi and Mori (18) have copurified DOVA:L-alanine aminotransferase and glyoxylate:L-alanine aminotransferase from bovine liver mitochondria, and they concluded that both activities are catalyzed by a single enzyme which exhibits much higher activity toward glyoxylate. Because of the structural similarity of DOVA and glyoxylate, it is likely that DOVA is accepted only as an artificial substrate and that DOVA transamination is of no physiological significance.

We report here the purification and some properties of DOVA:L-glutamate aminotransferase from wild-type and aplastidic strains of *E. gracilis*, and its probable identity with glyoxylate:L-glutamate aminotransferase.

MATERIALS AND METHODS

Cells and Culture Conditions. Cultures of *Euglena gracilis* Klebs strain Z Pringsheim and an aplastidic mutant derived from this strain, W_{14}ZNalL, were kindly provided by H. Lyman (State University of New York, Stony Brook). Cells were cultured in a glucose-based heterotrophic medium at 25°C with orbital shaking as described previously (3). Cells were grown either in light or complete darkness. The culture medium could support exponential growth up to a population density of approximately 10^7 cells·ml^{-1}.

Cell Growth Measurement. Cell population densities were determined with a Coulter Counter (model ZBI, Coulter Electronics).

DOVA Preparation. DOVA was prepared using a modification of the procedures of Kissel and Heilmeyer (15), and Dörnemann and Senger (8). This synthesis was also described in outline form (19).

Benzaldehyde (20 ml; 200 mmol) and levulinic acid (22 ml; 220 mmol) were dissolved in 200 ml of 95% ethanol, and 500 ml of boiling water was added. This was followed by the addition of 100 ml of 12% NaOH (in water) while stirring; the solution became clear yellow. After 5 to 7 min, the solution was chilled to 0 to 5°C, and benzylidene levulinic acid was precipitated from the stirred

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[3] Abbreviations: ALA, δ-aminolevulinic acid; DOVA, γ,δ-dioxovaleric acid; Caps, 3-(cyclohexylamino)-1-propane sulfonic acid; Taps, tris (hydroxymethyl) methylamino propane sulfonic acid; AOA (aminooxy)acetate.

solution by the dropwise addition of 6 N HCl; the final pH was about 1.5. The precipitate was filtered off and dried. The yellowish white solid was recrystallized three times from boiling water (2–3 g/l water) to obtain white, needle shaped crystals of benzylidene levulinic acid which had a melting point of 119 to 121°C (reported 124°C [8]). The yield of crude material was 24.68 g (60%) and that of the thrice-recrystallized material was 7.0 g (17%).

Benzylidine levulinic acid [5.0 g; 24.5 mmol] was dissolved in 70 ml of methanol and cooled to 0°C. Ozone was generated with a Welsbach model T-23 ozonator and introduced into the sample via a fretted glass bubbler tube, at 2,265 ml/min and 155 kPa for 20 min. Excess ozone was removed by flushing the solution with N_2, then 2.2 ml (30.0 mmol) of dimethyl sulfide (20) was introduced dropwise, and the solution was stirred overnight under N_2 as the temperature was allowed to rise gradually from 0°C to room temperature. Solvent, unreacted dimethyl sulfide, and dimethyl sulfoxide product were removed by rotary vacuum evaporation at room temperature. The residue was dissolved in 30 ml H_2O, and benzaldehyde was removed by extraction with five 30-ml portions of ether. The product was stored as 1.0-ml aliquots of the aqueous phase after ether extraction, at −20°C, where it was indefinitely stable. Yield of DOVA from benzylidine levulinic acid was 58%, determined as described below.

DOVA Determination. A variation of the method of Porra *et al.* (22) was employed. When samples were known to contain no α-ketoglutarate, coupling with 2,3-diaminonaphthalene to form 2(3-propionic acid)-benzoquinoxaline was accomplished by incubation for 15 min with a freshly prepared 1 mM solution of the diamine in 1 N acetic acid. It was found that, under these conditions, the reaction goes to completion within 10 min, compared to the many hours required with other conditions (22). Following the coupling reaction, an equal volume of 12 N HCl was added, and the *A* was read at 384 nm. At the final HCl concentration of 6 N, the molar absorption coefficient was 1.27×10^4, compared to the value of 6.37×10^3 in 1 N NaOH reported by Porra *et al.* (22). In addition to greater absorption in 6 N HCl, the blank value was considerably lower than in 1 N NaOH (Fig. 1). Linearity of A_{384} with final DOVA concentration was observed throughout the tested range of 0 to 85 μM.

For determination of the molar absorption coefficient, a sample of DOVA was reacted with 2,3-diaminonaphthalene as described above, then the product was extracted into diethyl ether, back extracted into 1 N NaOH, the aqueous solution was acidified with HCl and the product was reextracted into ether. After washing with water, the ether was dried, and the residue was dissolved in absolute ethanol and decolorized with Norit-A. The ethanol was evaporated to a small volume, mixed with water, and the product was allowed to crystallize overnight at 4°C, then it was dried *in vacuo* over $CaCO_3$. The melting point was 196°C (reported value 191–196°C [15]). Proton nuclear magnetic resonance spectroscopy in CD_3OD indicated that the product was essentially pure, and all of the proton peaks were assigned (Fig. 2). The net absorption coefficient of our product at 384 nm in 6 N HCl was 1.27×10^4, and at 365 nm in 1 N NaOH was 7.11×10^3, the latter being in good agreement with the reported value of 6.37×10^3 (22).

Extraction of Enzymes. All steps were performed at 0 to 5°C. Cells in exponential growth were harvested by centrifugation for 3 min at full speed in a table top centrifuge (International Equipment), washed once with cold water and once with extraction medium (50 mM phosphate, 5 mM EDTA, 0.1 mM pyridoxal phosphate, adjusted to pH 6.80 with NaOH), and finally resuspended in extraction medium at 10^8 cells·ml^{-1}. Cells were disrupted by sonication for two 15-s periods using a MSE Ultrasonic Power Unit. The broken cell suspension was then centrifuged at 16,000*g* for 5 min in a Sorvall model RC-5B refrigerated centrifuge, and the supernatant was used for the assays. In some experiments, where indicated, changes in the composition of the extraction medium were necessary. When column chromatography was employed, the broken cell suspension was centrifuged at 16,000*g* for 20 min, and the supernatant was filtered through a Millipore type HA 0.45 μm filter before application to the column. No detectable differences in activity were observed using this procedure.

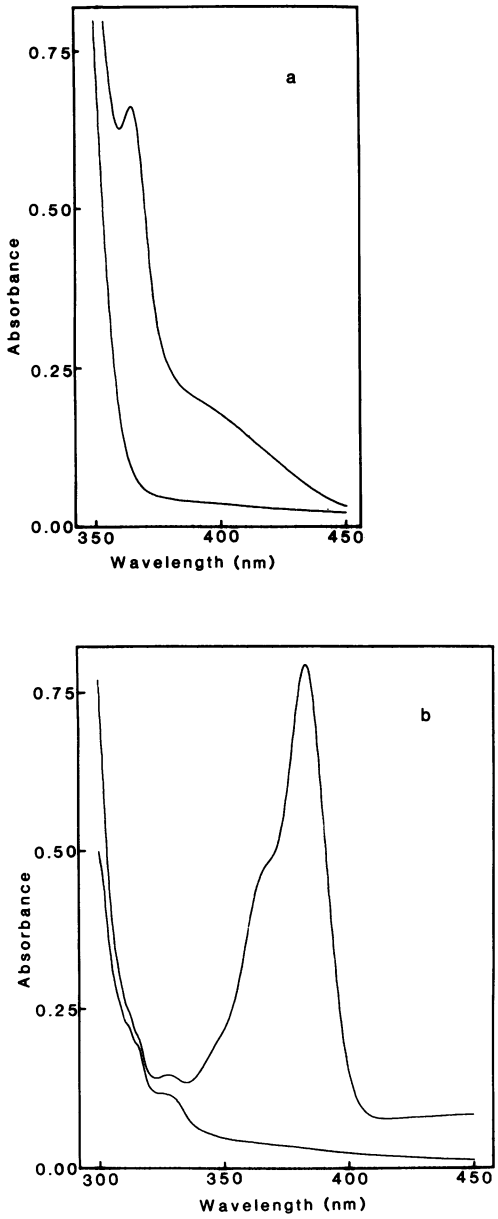

FIG. 1. Absorption spectra of 2-(3-propionic acid)-benzoquinoxaline. The compound was formed by the condensation of DOVA and 2,3-diaminonaphthalene as described in "Materials and Methods;" a, in 1 N NaOH; b, in 6 N HCl. In each part, the upper trace is from a solution containing DOVA, while the lower trace is from an identically treated sample without DOVA.

DOVA:L-Glutamate Aminotransferase Assay. Broken cell extract was incubated at 37°C for 30 min at pH 6.80, in 1 ml final volume of extraction medium containing 100 mM L-glutamate and 2.6 mM DOVA. Typically, extract from 2×10^6 cells was used for each 1-ml incubation. The enzyme reaction was terminated by addition of 50 μl 70% (w/v) $HClO_4$. Precipitate was removed by centrifugation at 13,000*g* for 1 min in an Eppendorf model 5412 microcentrifuge. Nine-tenths ml of the supernatant was titrated to pH 3.00 with approximately 350 μl of 1.0 M K_2HPO_4. Samples

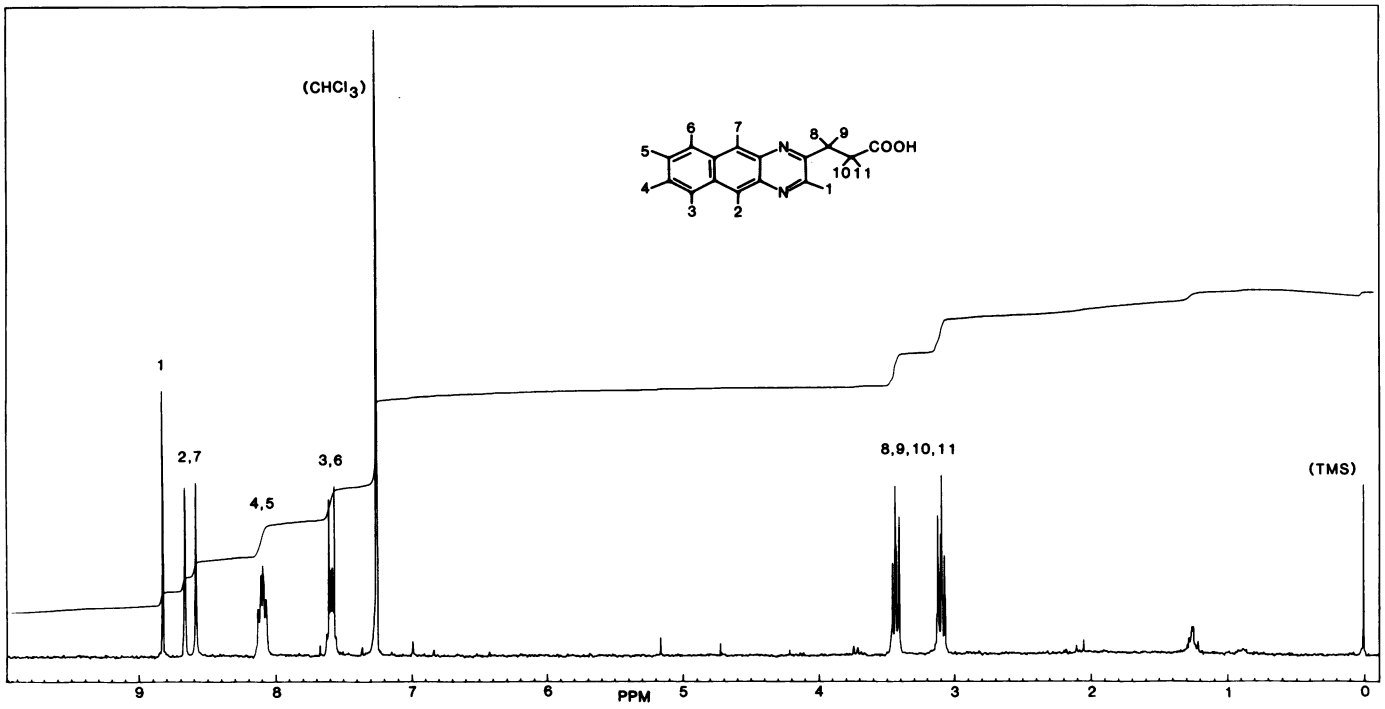

FIG. 2. Proton nuclear magnetic resonance spectrum of purified 2-(3-propionic acid)-benzoquinoxaline. Spectroscopy was performed in $CDCl_3$.

were then cooled to 0 to 5°C for 1 h before centrifugation at 13,000*g* for 1 min in the microcentrifuge, to remove $KClO_4$ precipitate. To avoid interference by DOVA during the ALA assay, DOVA was separated from the ALA product by applying 1.2 ml of the supernatant to a 3.5 cm column of Dowex-50X8(Na^+) 100–200 mesh cation exchange resin prepared in a pasteur pipet. The column was then washed with 1.0 ml water to remove DOVA, and the bound ALA was eluted with 50 mM $Na\text{-}PO_4$ (pH 6.80). Fractions containing ALA were collected and titrated to pH 6.80 with 0.5 M Na_3PO_4. Then 20 μl of ethylacetoacetate was added, and the solutions were heated to 95°C for 15 min to form 2-methyl-3-carbethoxy-4-(3-propionic acid) pyrrole (17). After cooling to room temperature, an equal amount of Ehrlich-Hg reagent (28) was added, and the light *A* at 552 nm was measured after 5 min, using a Cary model 219 Spectrophotometer (Varian Instruments). Standards containing different amounts of authentic ALA were prepared with each assay, for purposes of identification and quantitation, and to assure linearity of the color yield. The calculated molar absorption coefficient for the Ehrlich-Hg reagent-pyrrole complex derived from authentic ALA was approximately 8×10^4. Activity of the enzyme is expressed as nmol ALA·30 $min^{-1} \cdot 10^7$ $cells^{-1}$. Nonenzymic DOVA to ALA conversion was observed in each incubation (4). It was therefore necessary in each assay to incubate samples which contained all the components of the incubation mixture except cell extract. The calculated activity of samples containing cell extract was then corrected for nonenzymic activity. During normal incubations, nonenzymic ALA formation did not exceed 10% of the enzymic rate.

Glyoxylate:L-Glutamate Aminotransferase Assay. The incubation procedure was the same as that employed in the DOVA:L-glutamate aminotransferase assay, except that 10 mM glyoxylate was substituted for 2.6 mM DOVA. The incubation was terminated by addition of 50 μl 100% (w/v) TCA in water, and the precipitate was removed by centrifugation at 13,000*g* for 1 min in the microcentrifuge. Glycine was determined by the method of Klein and Linser (16). Nine-tenths ml of the supernatant was titrated to pH 8.00 with approximately 350 μl of 1.0 M K_3PO_4. Then 350 μl of 100 mM *o*-phthaldialdehyde in 95% ethanol was added. The solution was allowed to react for 2 min at room temperature before 1.35 ml of acidified 95% ethanol (14.3% H_2SO_4[v/v]) was added. The colored glycine derivative was then extracted into 1.35 ml $CHCl_3$ and the lower layer was pipeted into a 10 × 75 mm test tube. Light *A* at 570 nm was determined in the $CHCl_3$ phase after 20 min, using the Cary spectrophotometer. Standards containing known amounts of glycine were prepared with each assay, for purposes of quantitation and to assure linearity of the color yield. The calculated molar absorption coefficient of the glycine derivative was approximately 3.0×10^2. Enzyme activity is expressed as μmol glycine·30 $min^{-1} \cdot 10^7$ $cells^{-1}$. Correction for nonenzymic activity was applied as in the DOVA:L-glutamate aminotransferase assay. Nonenzymic glycine formation was normally less than 8% of the enzymic formation.

Protein Determination. Protein concentrations were determined by the dye-binding method of Bradford (6), using BSA as a standard.

Gel Filtration Chromatography. For determination of mol wt, cells were broken in elution medium containing 50 mM phosphate, 8 mM $MgCl_2$, 5 mM EDTA, and 0.1 mM pyridoxal phosphate, adjusted to pH 6.80 with NaOH. Cell extracts and protein standards of known mol wt were applied to a 1.50 × 83 cm column of Bio-Gel A-1.5m agarose (Bio-Rad). Typically, the extract from 5 × 10^7 cells, or 1 mg of protein standard, was applied to the column. The void volume was determined using blue dextran, and the low mol wt limit was measured using $K_2Cr_2O_7$. Relative elution volumes were calculated as the ratio $K_d = (V - V_o) \cdot (V_c - V_o)^{-1}$, where V is the elution volume of a protein standard (or enzyme) peak, V_o is the void volume, and V_c is the elution volume of $K_2Cr_2O_7$. The flow rate was approximately 8 $ml \cdot cm^{-2} \cdot h^{-1}$, and 2 ml fractions were collected. Eluted fractions of cell extract were assayed for DOVA and glyoxylate transaminase activities, respectively, in elution buffer containing 100 mM L-glutamate and 2.6 mM DOVA or 10 mM glyoxylate at pH 6.80, for 30 or 60 min at 37°C. Glycine was measured by the standard assay described above. ALA was detected using a modified assay in which the cation exchange chromatography step was omitted. In this procedure, one-half ml of the incubation product was titrated directly to pH 6.80 with approximately 180 μl of 0.5 M Na_3PO_4, and pyrrole formation and color development were performed as described in the standard

assay. Eluted fractions of protein standards were assayed for protein content by the Bradford method as described above.

In some experiments, where indicated, cell extract was passed through a column of Sephadex G-25 (50–150 μm) to remove low mol wt components.

DEAE-Cellulose Anion Exchange Chromatography. Cells were broken in elution buffer containing 10 mM Tris, adjusted to pH 8.00 with HCl. Sephadex G-25-purified extract was applied to a 2.50 × 10.5 cm column of DEAE-cellulose anion exchange material (Whatman), at pH 8.00. The column was then washed with three bed volumes of elution buffer before a 200-ml linear gradient from 0 to 210 mM NaCl, in elution buffer at pH 8.00, was applied to the column. The flow rate was 6 $ml \cdot cm^{-2} \cdot h^{-1}$, and 2-ml fractions were collected. Eluted fractions were assayed for both DOVA:L-glutamate and glyoxylate:L-glutamate aminotransferase activities. One-half ml of each fraction was combined with 0.5 ml of double-strength incubation medium, and incubated at 37°C for 60 min at pH 6.80. The final concentrations of components in the incubation mixture were 100 mM L-glutamate, 50 mM phosphate, 5 mM Tris, 2.6 mM DOVA or 10 mM glyoxylate, 2 mM EDTA, and 0.1 mM pyridoxal phosphate. Glycine was detected by the assay described above. ALA was measured by the modified assay used in the mol wt determination. The NaCl concentration of each fraction was determined with a conductivity meter (Radiometer, type CDM 2f).

Chemicals. Protein mol wt standards were purchased from Boehringer Mannheim. All other chemicals and reagents were obtained from Fisher or Sigma.

RESULTS

Optimal Assay Conditions for DOVA:L-Glutamate Aminotransferase. For determination of the pH optimum, light-grown wild-type cells were extracted in medium containing 50 mM L-glutamate, 25 mM phosphate, and 5 mM EDTA, adjusted to pH 6.80 with NaOH. Two incubation media were prepared which had the same composition as the extraction medium, plus 25 mM citrate, 25 mM Caps, and 25 mM Taps; one was adjusted to pH 4.00 and the other to pH 10.00, with NaOH. Incubation mixtures containing varying proportions of the pH 4.00 and pH 10.00 buffers were incubated for 30 min with cell extract at 37°C. The pH of each sample was determined at 37°C during the incubation. Maximal ALA formation was observed at pH 6.80 (Fig. 3). At pH 6.80, identical activity was observed with phosphate, citrate, Caps, or Taps serving as buffer (data not shown).

Activity was measured at seven different temperatures, ranging from 20°C to 80°C. Temperatures were recorded as the mean of measurements taken at the beginning and end of the incubation. Maximum enzymic product yield occurred at 50°C. Because a large nonenzymic activity was observed at this temperature, 37°C was employed in all other experiments. Cell extract that had been heated to 100°C for 10 min showed no enzymic DOVA:L-glutamate aminotransferase activity upon subsequent assay at 37°C.

Using extract from light-grown wild-type cells, the amount of ALA formed was observed to be a linear function of time for the first 30 min of incubation, and then fell off slowly for the next 90 min. The rate of ALA formation was directly proportional to the volume of cell extract used in the incubation mixture, within the range of extract from 0 to 1.25 × 10^7 cells/ml of incubation mixture.

Extractable enzyme activity was measured as cells approached late log phase of growth and entered stationary phase of growth. A series of six flasks of aplastidic cells were grown in the light so that, at the time of harvest, their population densities ranged from 8 × 10^5 cells·ml^{-1} to 9 × 10^6 cells·ml^{-1}, varying in approximately equal increments. Protein concentration of the cell extracts was determined as described in "Materials and Methods." Extractable DOVA:L-glutamate aminotransferase activity remained constant at 35.8 ± 2.3 nmol ALA·30 $min^{-1} \cdot 10^7$ $cells^{-1}$ until a cell density of about 8 × 10^6 cells·ml^{-1} was reached. Enzyme activity diminished somewhat at higher cell densities, and at 9 × 10^6 cells·ml^{-1}, the activity was 28.0 nmol ALA·30 $min^{-1} \cdot 10^7$ $cells^{-1}$. When cells were kept in stationary phase of growth for 24 h, the activity dropped to 22.8 nmol ALA·30 $min^{-1} \cdot 10^7$ $cells^{-1}$. The changes in level of enzyme activity were closely paralleled by changes in the protein concentration of the extracts. Specific activity remained constant at 16.7 ± 1.6 nmol ALA·30 min^{-1}·mg $protein^{-1}$ into stationary phase of growth. After 24 h at stationary phase, the specific activity dropped to 14.9 nmol ALA·30 min^{-1}·mg $protein^{-1}$.

DOVA:L-Glutamate Aminotransferase Kinetics. The K_m values for DOVA and L-glutamate were determined by measuring activity *versus* concentration of each substrate. Enzyme extracted from light-grown wild-type cells was incubated with varying amounts of DOVA. A double reciprocal plot of the results extrapolated to a K_m value of 1.1 mM for DOVA (Fig. 4). For determination of the K_m for L-glutamate, extract from light-grown aplastidic cells was first passed through a Sephadex G-25 column to remove low mol wt components before assay. The purified cell extract was

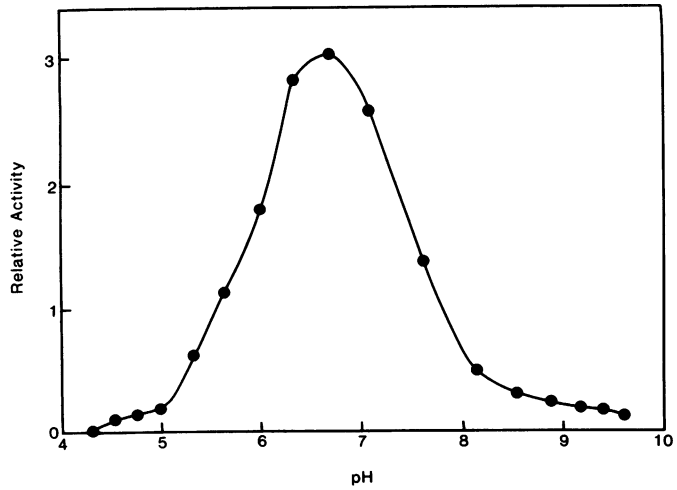

FIG. 3. pH optimum of DOVA:L-glutamate aminotransferase. Extract was obtained from light-grown wild-type cells. Relative yield of ALA from incubation mixture is plotted *versus* pH of the incubation medium.

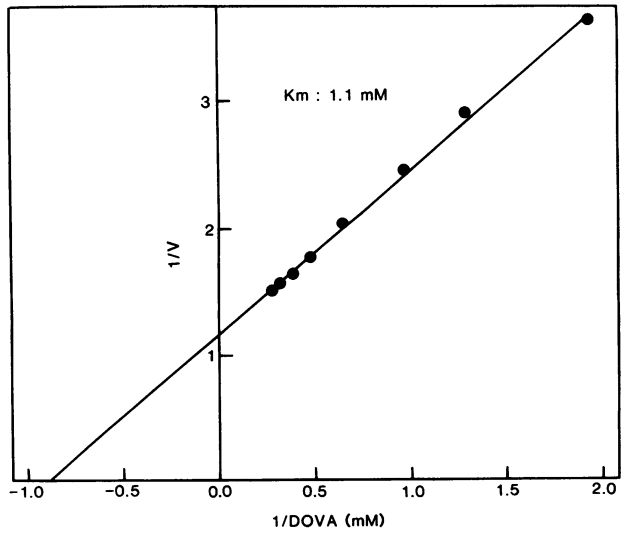

FIG. 4. Double reciprocal plot of DOVA concentration *versus* relative yield of ALA. Calculated K_m for DOVA is 1.1 mM.

then assayed for DOVA:L-glutamate aminotransferase activity in the presence of varying amounts of L-glutamate. A double reciprocal plot of the results yielded a K_m value of 11.3 mM for L-glutamate (Fig. 5).

Amino Donor Specificity of Enzymic DOVA Transamination. Broken cell extract from light-grown aplastidic cells was passed through Sephadex G-25 in extraction medium containing 50 mM phosphate and 5 mM EDTA, adjusted to pH 6.80 with NaOH. Purified cell extract was assayed in the presence of each of the 22

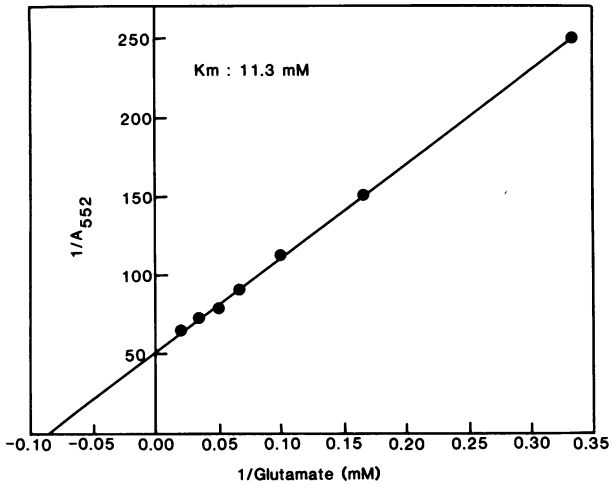

FIG. 5. Double reciprocal plot of L-glutamate concentration *versus* relative yield of ALA. Calculated K_m for L-glutamate is 11.3 mM.

Table I. *Amino Donor Specificity for DOVA Transamination*

Cell extract from light-grown aplastidic cells was passed through Sephadex G-25, and then incubated for 30 min at 37°C in 50 mM phosphate buffer at pH 6.8, with 5 mM EDTA, 2.6 mM DOVA, 0.1 mM pyridoxal phosphate plus one of the indicated amino donors at the indicated concentrations. ALA was assayed as described in "Materials and Methods."

Amino Donor	Relative Activity at 100 mM	Relative Activity at 10 mM
	%	
L-Glutamic acid	100	100
L-Alanine	24	8
L-Glutamine	24	11
L-Phenylalanine	23[a]	18
L-Tryptophan	16[a]	11[a]
L-Asparagine	15	13
L-Aspartic acid	10	13
L-Methionine	10	11
L-Lysine	9	1
Glycine	8	4
L-Leucine	7	9
L-Cystine	7[a]	4[a]
Taurine	7	8
β-Alanine	6	6
Hydroxy-L-proline	6	1
L-Arginine	5	13
L-Cysteine	4	1
L-Histidine	4	5
D-Glutamic acid	4	4
L-Valine	2	2
L-Serine	2	4
L-Isoleucine	1	3
L-Tyrosine	1[a]	4[a]
L-Threonine	0	1
L-Proline	0	0

[a] Not soluble at indicated concentration.

protein amino acids plus several naturally occurring non-protein amino acids. In each incubation, 10 and 100 mM amino donor concentrations were used. For those amino acids that are not soluble at these concentrations, saturated solutions were used. At both 10 and 100 mM amino donor concentrations, maximal enzymic DOVA to ALA conversion was observed with L-glutamate (Table I). L-Alanine, L-glutamine, and L-phenylalanine at 100 mM were the next best amino donors. D-Glutamate showed only trace amounts of activity at either concentration.

Pyridoxal Phosphate Requirement of DOVA:L-Glutamate Aminotransferase. Stability of the enzyme during the extraction procedure was independent of the presence of pyridoxal phosphate in the extraction medium. Light-grown aplastidic cells were broken in pyridoxal phosphate-free extraction medium and passed through Sephadex G-25. Purified cell extract was assayed for DOVA:L-glutamate aminotransferase without pyridoxal phosphate. Addition of pyridoxal phosphate to the incubation mixture caused stimulation of activity (Fig. 6). Maximal enhancement of activity occurred at 30 μM pyridoxal phosphate. Higher concentrations yielded activity only slightly less than maximal. Without added pyridoxal phosphate, the enzyme retained about 65% of its maximal activity.

AOA was employed to test the pyridoxal phosphate dependence of DOVA:L-glutamate aminotransferase. This compound is a potent inhibitor of pyridoxal phosphate requiring transaminases (23). Light-grown aplastidic cells were extracted in 50 mM phosphate and 5 mM EDTA, adjusted to pH 6.80 with NaOH, and passed through Sephadex G-25. The purified cell extract was assayed in incubation medium containing varying amounts of AOA. Increasing inhibition of enzyme activity occurred as the concentration of AOA in the incubation mixture was raised (Table II). Greater than 99% inhibition was observed at 10 mM AOA.

Effects of Cycloheximide and Chloramphenicol on DOVA:L-Glutamate Aminotransferase Activity. The *in vivo* stability of DOVA:L-glutamate aminotransferase was studied by blocking protein synthesis in intact cells with cycloheximide or chloramphenicol. Cycloheximide is a potent inhibitor of protein synthesis on cytoplasmic ribosomes in many eukaryotic organisms, including *Euglena* (27). Chloramphenicol has been shown to inhibit protein synthesis on 70S-type ribosomes and has been used specifically as an *in vivo* inhibitor of plastid protein synthesis in *Euglena* (21). Cycloheximide and chloramphenicol were admin-

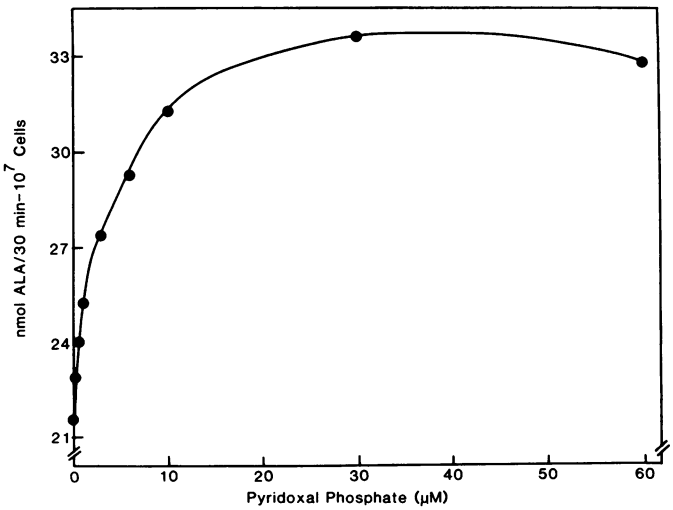

FIG. 6. Stimulation of DOVA:L-glutamate aminotransferase activity by added pyridoxal phosphate. Extract from light-grown aplastidic cells was passed through Sephadex G-25 before assay to remove low mol wt components. Yield of ALA is plotted *versus* concentration of pyridoxal phosphate added to the incubation medium.

Table II. *Effect of AOA on DOVA Transamination*

Extract from light-grown aplastidic cells was passed through Sephadex G-25, and then incubated for 30 min at 37°C and at pH 6.8 in 50 mM phosphate buffer, 5 mM EDTA, 2.6 mM DOVA, and 100 mM L-glutamate plus the indicated concentration of AOA. ALA was assayed as described in "Materials and Methods."

AOA	DOVA Transamination	
M	*nmol ALA · 30 min^{-1} · 10^7 cells^{-1}*	*% of control*
0	23.0	100
10^{-5}	5.8	25
10^{-4}	4.8	21
10^{-3}	3.4	15
10^{-2}	0.1	0.4

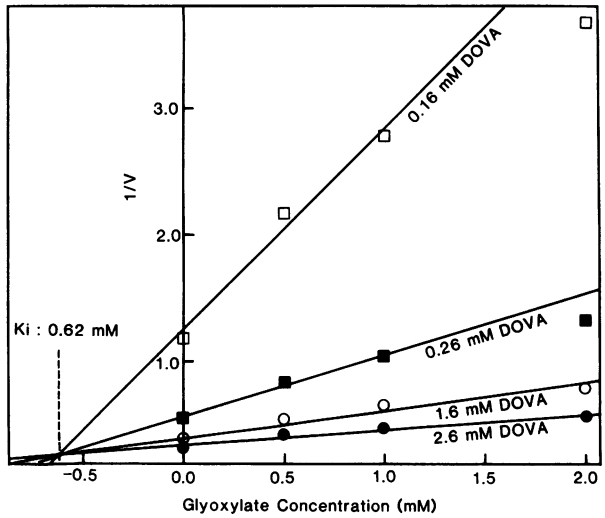

FIG. 7. Inhibition of DOVA:L-glutamate aminotransferase activity by glyoxylate. Extract from light-grown wild-type cells was passed through Sephadex G-25, and then placed in incubation medium containing one of four DOVA concentrations and one of three glyoxylate concentrations. Reciprocal ALA yield is plotted *versus* glyoxylate concentration at each concentration of DOVA. The calculated K_i for glyoxylate is 0.62 mM.

istered at 43 μM and 6.2 mM, respectively, to separate flasks of light-grown aplastidic cells during exponential growth. Cycloheximide was added in the form of a concentrated aqueous solution, and chloramphenicol was added as a dried powder. After 12 h of incubation with the inhibitors, the treated cultures and an untreated control culture were harvested and assayed. Extracts from cultures treated with cycloheximide, chloramphenicol, and untreated cells had activities of 36.2, 35.7, and 39.3 nmol ALA·30 min^{-1}·10^7 cells^{-1}, respectively.

Competitive Inhibition of DOVA:L-Glutamate Aminotransferase Activity by Glyoxylate. Cell extract from light-grown wild-type cells was passed through Sephadex G-25, and the purified extract was assayed for DOVA:L-glutamate aminotransferase activity in the presence of glyoxylate. Four DOVA concentrations and three glyoxylate concentrations were used in the assay. Inhibition of DOVA:L-glutamate aminotransferase activity by glyoxylate was observed at each DOVA concentration; reciprocal enzyme velocities extrapolated to a K_i of 0.62 mM for glyoxylate (Fig. 7).

Repetitive glyoxylate:L-glutamate aminotransferase assays were performed to determine a K_m for glyoxylate, but results did not yield a linear double reciprocal relationship. Attempts were also made to demonstrate DOVA inhibition of glyoxylate:L-glutamate aminotransferase activity. Although inhibition by DOVA was observed, no particular kinetic category of inhibition could be discerned.

Activity of DOVA:L-Glutamate Aminotransferase and Glyoxylate:L-Glutamate Aminotransferase in Extracts from Wild-Type and Aplastidic Cells. Cultures of light- and dark-grown wild-type and aplastidic cells were harvested during exponential growth, and both enzyme activities were measured in extracts. DOVA:L-glutamate aminotransferase activity was nearly equal in extracts from both cell types, grown either in the light or dark (Table III). The levels of glyoxylate:L-glutamate aminotransferase activity were nearly equal in extracts from light- and dark-grown aplastidic cells. Activity from light- and dark-grown wild-type cells was slightly lower (Table III). In all cases, glyoxylate:L-glutamate aminotransferase activity was at least 180 times greater than DOVA:L-glutamate aminotransferase activity.

Mol Wt Determinations. The mol wt of DOVA:L-glutamate aminotransferase and glyoxylate:L-glutamate aminotransferase were measured by gel filtration analysis on a calibrated column of Bio-Gel A-1.5m agarose. Calibration was achieved by passing protein standards of known mol wt through the column. The standards (and mol wt) were chymotrypsinogen A (25,000), ovalbumin (45,000), BSA (68,000), rabbit muscle aldolase (158,000), beef liver catalase (240,000), and ferritin (450,000). Activity of DOVA:L-glutamate aminotransferase from light-grown wild-type cells ran as a single peak in the elution field; no aggregates or subunits were detected. Glyoxylate:L-glutamate aminotransferase activity ran as a broad band in the elution field, the low mol wt side of the band coinciding with DOVA:L-glutamate aminotransferase activity (Fig. 8). Identical results were obtained for both enzymes using light-grown aplastidic cell extract.

A plot of relative elution volume *versus* the logarithm of mol wt, for the protein standards, yielded a straight line. The peak fraction of DOVA:L-glutamate aminotransferase corresponded to a mol wt of 98,000 ± 3,000; the precision of the measurement was estimated using a least squares analysis. Because the broad elution band observed for glyoxylate:L-glutamate aminotransferase activity was probably due to multiple overlapping peaks, a single mol wt was not assigned to the enzyme(s) which catalyze this function.

DEAE-Cellulose Anion Exchange Chromatography. Light-grown aplastidic cells were harvested and the extract was passed through Sephadex G-25. Fractions containing DOVA:L-glutamate and glyoxylate:L-glutamate aminotransferase activities were pooled and applied to a column of Whatman DEAE-cellulose anion exchange material. Eluted fractions were assayed for both the DOVA and glyoxylate transaminase activities. Nonenzymic rates of ALA and glycine formation ranged from 0 to 1.5 nmol and 0 to 0.25 μmol, respectively. Enzymic DOVA:L-glutamate aminotransferase activity occurred between 32 and 98 ml, and showed two peaks at 50 and 74 ml (Fig. 9). Enzymic glyoxylate:L-

Table III. *Effect of Light or Dark Growth, and Presence or Absence of Plastids, on Extractable DOVA and Glyoxylate Transaminase Activities*

Extract from light- and dark-grown wild-type and aplastidic cells was incubated at 37°C for 30 min at pH 6.8 in 50 mM phosphate, 5 mM EDTA, 0.1 mM pyridoxal phosphate, 100 mM L-glutamate, plus either 2.6 mM DOVA or 10 mM glyoxylate. ALA and glycine were assayed as described in "Materials and Methods."

Cell Type	Growth Conditions	Transaminase Activities	
		nmol ALA · 30 min^{-1} · 10^7 cells^{-1}	*μmol glycine · 30 min^{-1} · 10^7 cells^{-1}*
Wild-type	Light	30.0	7.12
Wild-type	Dark	31.2	5.84
Aplastidic	Light	30.4	8.12
Aplastidic	Dark	32.3	8.40

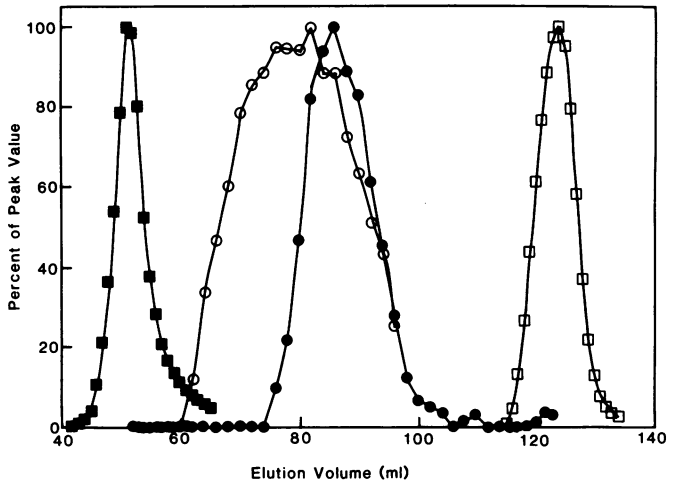

FIG. 8. Elution profiles of DOVA:L-glutamate (●) and glyoxylate:L-glutamate (○) aminotransferase activities from a column of Bio-Gel A 1.5m agarose. Also shown are elution profiles of void volume marker blue dextran (■) and low mol wt marker $K_2Cr_2O_7$ (□). Column dimensions were 1.50 × 83 cm, and fraction volumes were 2.0 ml.

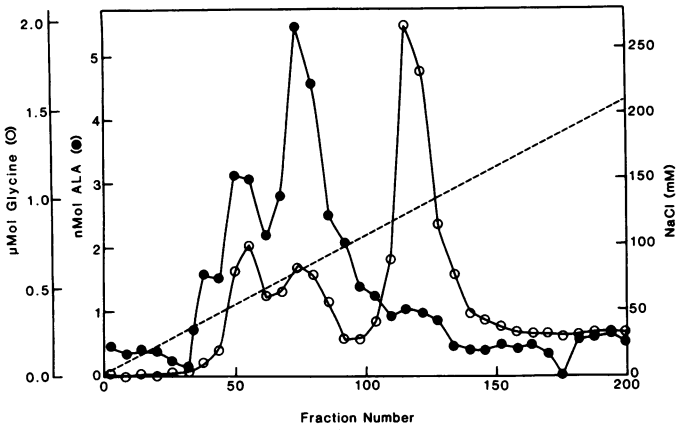

FIG. 9. Elution profiles of DOVA:L-glutamate (●) and glyoxylate:L-glutamate (○) aminotransferase activities from a column of DEAE-cellulose. Concentration of NaCl in the elution gradient is indicated on the right ordinate. Relative activities of the two aminotransferases are plotted *versus* elution fraction number. Column dimensions were 2.5 × 10.5 cm, and fraction volumes were 2.0 ml.

glutamate aminotransferase activity was observed between 38 and 158 ml elution volume, with peaks occurring at 56, 74, and 116 ml. At 74 ml elution volume, enzymic glyoxylate transamination was approximately 78-fold greater than enzymic DOVA transamination, and at 50 ml elution volume, it was about 173-fold greater. DOVA:L-glutamate aminotransferase activity was not observed between 98 and 158 ml elution volume, where the major peak of glyoxylate:L-glutamate aminotransferase activity occurred.

DISCUSSION

Of the possible 5-carbon routes to ALA synthesis in plants, a favored mechanism involves the intermediate formation of DOVA. *In vitro* enzymic DOVA transamination has been clearly demonstrated in many organisms, including a variety of higher plant tissues and algae (10, 25, 27). DOVA, on the other hand, has not been detected in any of these organisms except possibly when intracellular ALA levels are artificially elevated by levulinic acid administration (8).

We have measured *in vitro* enzymic DOVA to ALA conversion during incubations with extracts from *E. gracilis*. It was important to establish the enzymic nature of the reaction in view of the known nonenzymic reaction of DOVA (4). The enzyme dependence of the reaction is established by the lack of activity during incubations with boiled cell extract, optimal conditions of temperature and pH for the assay appropriate to an enzyme-catalyzed reaction, specificity with respect to amino donor, and the saturation kinetics of the reaction. Furthermore, the pyridoxal phosphate stimulation of the enzyme and inhibition by AOA suggest that *in vitro* DOVA to ALA conversion occurs specifically via a transamination type reaction.

Studies have shown that isolated chloroplasts from higher plant tissues and algae, including *Euglena*, contain all of the enzymes necessary for the formation of ALA by way of the 5-carbon plant pathway (13, 24). We have found that extracts from wild-type *Euglena* cells, whether greening in the light or not greening in the dark, have levels of extractable DOVA transaminase activity equal to those in extracts from light- or dark-grown aplastidic mutant cells. Because the mutant cells are totally devoid of all chloroplast material, including chloroplast DNA (26), these results strongly suggest that the observed enzyme activity is not associated with the synthesis of ALA precursors to Chl, and that functional chloroplasts are not required for expression of DOVA transaminase activity.

Salvador has reported the extraction of DOVA:L-alanine aminotransferase activity from *Euglena* (25). The level of enzyme activity from dark-grown cells in resting medium increased upon exposure of the cells to light. The change in activity paralleled the change in rate of Chl synthesis upon illumination. $MgCl_2$ and NADPH were reported to stimulate DOVA transamination, but pyridoxal phosphate did not. It was proposed that DOVA transamination may be a rate-limiting step to ALA synthesis.

The cells used in Salvador's report were held for 3 d in resting medium (25). The cells used in our experiments were harvested during exponential growth under conditions which are optimal for heterotrophic growth (5, 9). In our preparations, we observed stimulation of activity by pyridoxal phosphate, but no stimulation occurred when either NADPH or $MgCl_2$, or both reagents, were added to the incubation mixture. We also observed that extractable enzyme activity began to decline when the cells were kept at stationary phase of growth for 24 h. This decline is probably caused by protein degradation in the absence of *de novo* protein synthesis. Light-stimulated activity in resting cells may reflect an increase in protein synthesis related to the induction of plastid differentiation. Stimulation of non-plastid protein synthesis upon dark-to-light transition of resting cells has also been observed by Horrum and Schwartzbach (12).

The DOVA transaminating enzymes from other organisms have been reported to function at near maximal rates with L-alanine, L-glutamate, L-phenylalanine, or L-valine serving as amino donor (10). Because of the apparent relaxed specificity with respect to amino donor, there is a possibility that, in these organisms, more than one species of enzyme exists which is capable of catalyzing the transamination of DOVA to ALA. Our results indicate that this may be the case in *Euglena*, in part because DOVA transamination does not have a stringent amino donor specificity. Although L-glutamate was the preferred amino donor, considerable DOVA transamination activity was observed when either L-alanine or L-phenylalanine was used in the assay. Additional evidence for the existence of multiple DOVA transaminating enzymes in *Euglena* comes from the results of DEAE-cellulose chromatography of cell extract: at least two peaks of DOVA:L-glutamate aminotransferase activity were clearly resolved using this technique.

DOVA:L-alanine aminotransferase activity has been detected in bovine liver mitochondria at a level that is greater than that of ALA synthase (29), the enzyme generally considered to be responsible for providing ALA precursors to tetrapyrroles in animal

tissues. Although the investigators proposed that the DOVA transaminating enzyme in that tissue functions to synthesize ALA, Noguchi and Mori have copurified bovine liver mitochondrial DOVA:L-alanine aminotransferase with a species of glyoxylate:L-alanine aminotransferase (18). They concluded from their results that both transamination reactions are catalyzed by a single enzyme, which has a mol wt of about 110,000. The glyoxylate transamination activity of this enzyme was observed to be 5- to 100-fold greater than its DOVA transamination activity.

Because the structure of glyoxylate closely resembles the dioxo portion of the DOVA molecule, and because the mol wt of DOVA:L-glutamate aminotransferase from *Euglena* is comparable with the DOVA:L-alanine aminotransferase from bovine liver, it was reasonable to hypothesize that the DOVA transaminating enzyme from *Euglena* might also function as a glyoxylate transaminase, which could accept DOVA as an artificial substrate. Glyoxylate:L-glutamate aminotransferase exists in the peroxisomes of higher plant tissues and algae (7, 14), where it plays a role in the metabolism of glycolate during the photorespiration process. The enzyme has been detected in *Euglena* and has been shown to exist in several subcellular locations, including the microbodies and the mitochondria (7, 30). It has not been reported whether this enzyme, in higher plants and algae, is also capable of DOVA transamination activity.

Euglena DOVA:L-glutamate aminotransferase could not be separated from glyoxylate:L-glutamate aminotransferase activity by agarose gel filtration or DEAE-cellulose column chromatography. The elution profiles of both transamination activities are identical in the region of the elution field where enzymic DOVA transamination occurs. Furthermore, the glyoxylate transamination activity is much greater than DOVA transamination activity in the coinciding peaks of glyoxylate:L-glutamate aminotransferase and DOVA:L-glutamate aminotransferase. These results strongly suggest that in *Euglena*, as in bovine liver mitochondria, both transamination activities are associated with the same protein species, and that glyoxylate transamination is the primary function in the cell. Competitive inhibition of DOVA:L-glutamate aminotransferase by glyoxylate supports the hypothesis that both transamination activities are catalyzed by the same protein species. The lack of competitive kinetics for the inhibition of glyoxylate:L-glutamate aminotransferase by DOVA can be explained by the possibility that not all species of glyoxylate:L-glutamate transaminase present can accept DOVA as a substrate. This is supported by the results of DEAE-cellulose chromatographic fractionation; that is, where there is DOVA transamination activity, there is also glyoxylate transamination activity, but not vice versa.

We conclude that DOVA:L-glutamate transamination is catalyzed by one or more species of glyoxylate:L-glutamate aminotransferase in extracts from *E. gracilis*. DOVA transamination probably occurs exclusively *in vitro* when DOVA is the only available substrate. This reaction therefore is of no physiological significance and does not function *in vivo* as a part of the 5-carbon pathway to ALA synthesis which provides precursors to Chl.

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