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PURIFICATION OF THE CHLOROPLASTIC VALYL-tRNA SYNTHETASE FROM EUGLENA GRACILIS

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Summary

Euglena gracilis chloroplast valyl-tRNA synthetase was purified 990 fold to a specific activity of about 1100 units/mg protein, by a series of steps including ammonium sulfate precipitation and chromatography on hydroxyapatite, DEAE-cellulose, Blue Dextran - Sepharose and Sephadex G200. The enzyme gives a single band upon polyacrylamide gel electrophoresis, appears to be a monomer with a molecular weight of 126,000 daltons and has Km values of 1.5 x 10^{-5} M for L-valine, 5 x 10^{-5} M for ATP, and 6 x 10^{-8} for $tRNA^{Val}$.

INTRODUCTION

It is well established that chloroplasts have their own protein synthesizing apparatus and contain chloroplast-specific aminoacyl-tRNA synthetases (E.C. 6.1.1...). Reger et al. (1) and Krauspe and Parthier (2) have shown that a number of Euglena chloroplast aminoacyl-tRNA synthetases differ from their cytoplasmic counterparts in their chromatographic behaviour on hydroxyapatite or DEAE-cellulose and in their substrate (tRNA) specificity.

In contrast to the chloroplast-specific tRNAs which are coded for by chloroplast DNA (3-5), the chloroplast-specific aminoacyl-tRNA synthetases appear to be coded for in the nucleus, synthesized on cytoplasmic ribosomes and imported into the chloroplasts (6, 7). But it is not known if the cytoplasmic and the chloroplastic enzymes, catalyzing the attachment of a given amino acid to the cognate tRNA, are coded for by the same or by two different genes and how much they really differ. This problem can be approached by comparing the structural and catalytic properties of a cytoplasmic and a chloroplastic enzymes (specific for the same amino acid), but this requires the purification of both enzymes.

Although aminoacyl-tRNA synthetases from a variety of sources (bacteria, yeasts, animal tissues) have been purified to homogeneity, only a few plant aminoacyl-tRNA synthetases have been purified so far (8-15), all from seeds or

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Abbreviations : chloro ValRS = chloroplastic valyl-tRNA synthetase
cyto ValRS = cytoplasmic valyl-tRNA synthetase

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embryos, thus avoiding the critical problem of enzyme degradation which is frequently encountered in enzyme purification from green tissues. There is only one report in the literature so far, describing the purification of a cytoplasmic aminoacyl-tRNA synthetase and of its chloroplastic counterpart : Locy and Cherry (16) have purified soybean cytoplasmic tyrosyl-tRNA synthetase from etiolated cotyledons and the chloroplastic enzyme from green cotyledons, but did not show any gel electrophoresis pattern to demonstrate the purity of the two enzymes and did not indicate the specific activity of the chloroplastic enzyme.

We have decided to compare Euglena gracilis cytoplasmic and chloroplastic valyl-tRNA synthetases and are reporting here the purification to apparent homogeneity of the chloroplastic enzyme, as well as results concerning its molecular weight and kinetic properties.

MATERIAL AND METHODS

Euglena gracilis Z. cells were grown on the medium described by Cramer (17) and modified by Padilla et al. (18) under continuous illumination at 25°C, and harvested by centrifugation at the end of the exponential growth phase.

Protein concentration was determined according to Ehresmann et al. (19), using bovine serum albumine as a standard.

Valyl-tRNA synthetase activity was determined by measuring the aminoacylation of Euglena, or E.coli, or yeast tRNA, in a 100 µl incubation mixture containing: Tris-HCl pH 8 20 mM, $MgCl_2$ 5 mM, β-mercaptoethanol 5 mM, ATP 2 mM, bovine serum albumin 0.1 mg/ml, ^{3}H L-valine (1.1 Ci/mM) 3.4 x 10^{-6} M when testing column fractions or ^{14}C L-valine (20 µCi/µM) 5.7 x 10^{-5} M when measuring enzyme specific activity ; tRNA 0.4 mg/ml. After incubation at 30°C for 5 min (when testing column fractions) or for various time intervals (when measuring enzyme specific activity), a 80 µl aliquot was put on a Whatman 3MM paper disc, which was washed according to Mans and Novelli (20) and counted in a liquid scintillation counter. E.coli tRNA (Schwartz-Mann, Orangeburg, N.Y.) was used to measure the activity of chloro ValRS, yeast tRNA (Boehringer, Mannheim) to measure cyto ValRS activity, and Euglena tRNA prepared according to Meissner et al. (21) was used to determine the Km of purified chloro ValRS.

RESULTS

1) Enzyme purification

Freshly harvested Euglena cells (300 g), suspended in 300 ml of Tris-HCl buffer pH 8, 50 mM, $MgCl_2$ 1 mM, β-mercaptoethanol 1 mM, EDTA 0,1 mM, L-valine 2 x 10^{-3} mM, propane 1-2 diol 10% (v/v), polyethyleneglycol 6000 (Merck) 2% (w/v), glycerol 10% (v/v), were disrupted in a French Press (Aminco) at 12,000 psi. The homogenate was centrifuged at 35,000 x g for 1 hr and ammonium sulfate was added to 70% saturation. The resulting precipitate was redissolved in buffer B : potassium phosphate pH 7.5 50 mM , $MgCl_2$ 1 mM, β-mercaptoethanol 1 mM, EDTA 0.1 mM, L-valine 2 x 10^{-3} mM, propane 1-2 diol 15% (v/v). The solution was

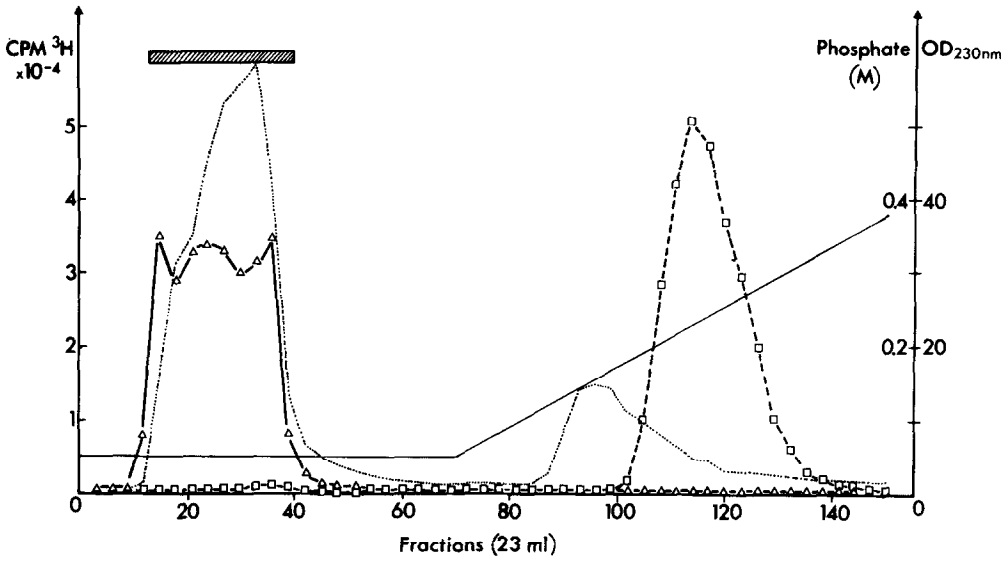


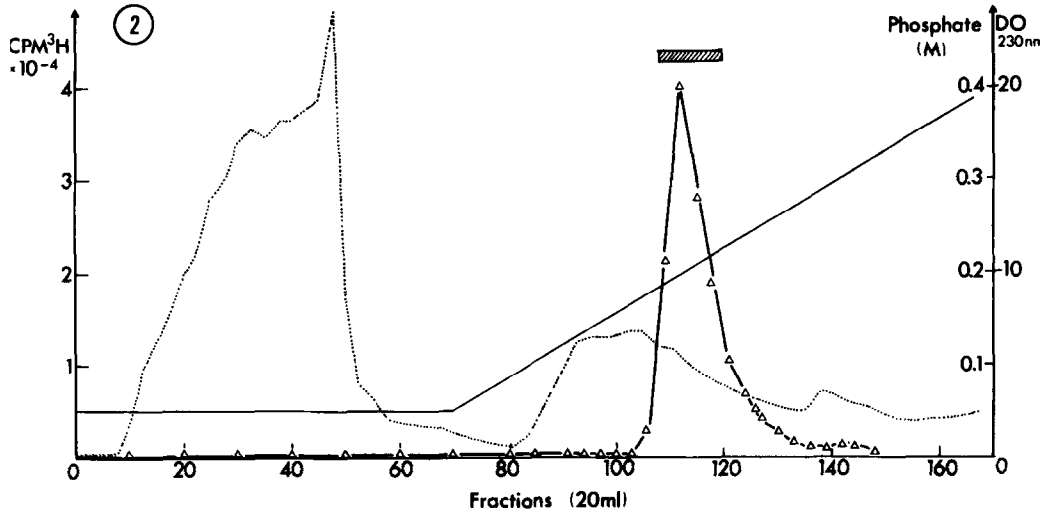
FIG. 1. Hydroxyapatite chromatography. The chloroplastic ValRS activity was measured using E.coli tRNA (Δ—Δ) and the cytoplasmic ValRS activity using yeast tRNA (□-·-□) ; (·····) $A_{230\ nm}$; (———) potassium phosphate concentration ; (▨) fractions collected and pooled for the next purification step.

passed through a Sephadex G50 column (7 x 40 cm) equilibrated with the same buffer to eliminate the ammonium sulfate, and then applied to a hydroxyapatite column (6 x 12 cm) prepared according to Siegelman et al. (22).

As reported by Krauspe and Parthier (2), two peaks of ValRS activity were observed upon hydroxyapatite chromatography (Fig. 1) : The first one, which was not retained on the column in our conditions, can aminoacylate E.coli tRNA (but not yeast tRNA) and corresponds to the chloroplastic enzyme. The second peak, which was eluted by 0.2 M phosphate, can charge yeast tRNA (but not E.coli tRNA) and corresponds to the cytoplasmic enzyme.

The first peak, containing the chloro ValRS, was applied directly to a DEAE-cellulose (DE-52 Whatman) column (5 x 13,5 cm) equilibrated with buffer B. Elution with a (2 x 1000 ml) linear gradient of potassium phosphate from 50 mM pH 7.5 to 400 mM pH 6.5 yielded one peak of enzymatic activity (Fig. 2).

Fractions 108-120 from the DEAE-cellulose column were pooled and dialysed for 6 hrs against buffer C : potassium phosphate pH 7.0 20 mM, $MgCl_2$ 1 mM, propane 1-2 diol 15% (v/v). The $MgCl_2$ concentration was raised to 4 mM and the solution was applied to a Blue Dextran-Sepharose 4B column (5 x 18 cm) prepared according to Ryan and Vestling (23) and equilibrated with buffer D (identical to buffer C, but 4 mM $MgCl_2$). After extensive washing of the column with buffer D,



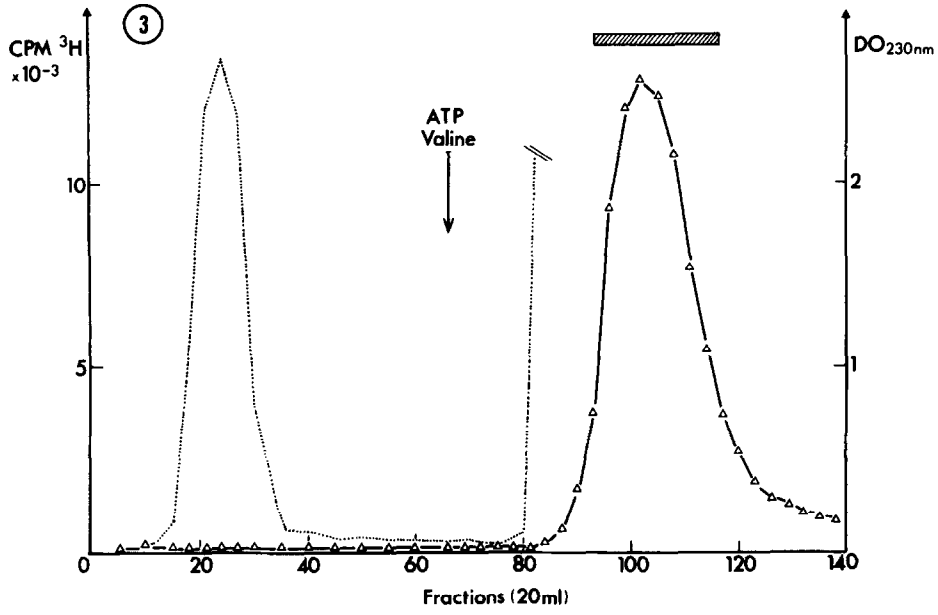


FIG. 2. DEAE-cellulose chromatography. (Δ—Δ) chloro ValRS activity ; (.....) $A_{230\ nm}$; (——) potassium phosphate concentration ; (▨) fractions collected and pooled for the next purification step.

FIG. 3. Blue Dextran - Sepharose chromatography. (Δ—Δ) chloro ValRS activity; (.....) $A_{230\ nm}$; (▨) fractions collected and pooled for the next purification step.

the chloro ValRS was eluted with buffer E : potassium phosphate pH 7.0 20 mM, $MgCl_2$ 3 mM, ATP 2 mM, L-valine 2 x 10^{-3} mM, propane 1-2 diol 15% (v/v), as shown on Fig. 3.

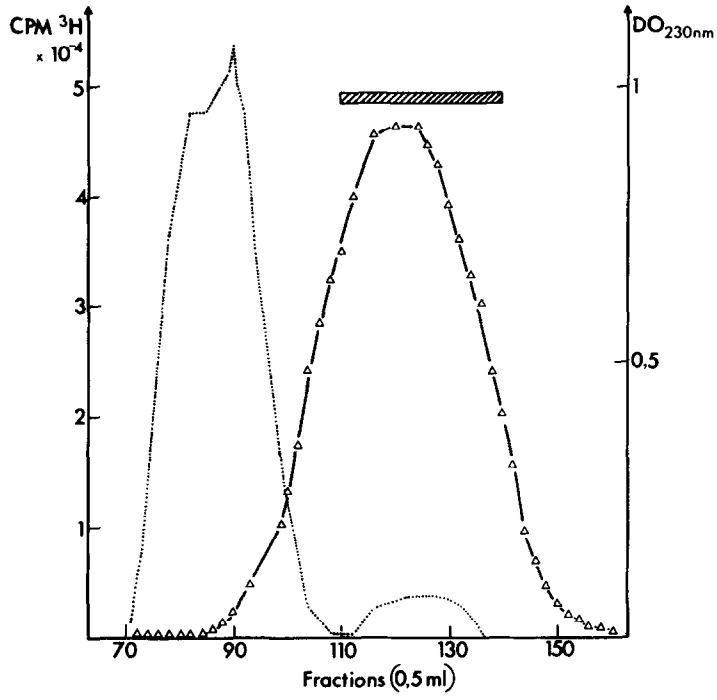


FIG. 4. Sephadex G200 chromatography. Symbols are as in FIG. 3.

Fractions 93-117 from the Blue Dextran-Sepharose column were pooled and the total volume reduced to 1.8 ml by filtration through a Diaflo membrane PM 10 (Amicon) under a nitrogen pressure of 3 atm. and applied to a Sephadex G200 column (1.7 x 47 cm) previously equilibrated with a potassium phosphate buffer pH 7.0 50 mM, $MgCl_2$ 1 mM, L-valine 2 x 10^{-3} mM, NaCl 100 mM, propane 1-2 diol 15% (v/v). Fractions 110-140 (Fig. 4) containing the pure chloro ValRS were pooled and used either for gel electrophoresis analysis or for Km determinations.

The purified enzyme can be stored at 4°C in the above-mentioned buffer and keeps at least 80% of its activity after one month. The complete purification procedure is summarized in Table I. The most efficient step of this procedure is chromatography on Blue Dextran-Sepharose.

2) Control of enzyme purity

The purified ValRS does not appear to be contaminated by any other aminoacyl-tRNA synthetases, as judged from the fact that no significant aminoacylation was observed upon addition of 15 other amino acids.

As shown on Fig. 5a, polyacrylamide gel electrophoresis in non-denaturating conditions of the purified chloro ValRS yields a single protein band (as revealed by Coomassie blue staining) which coincides with ValRS activity.

TABLE I

Purification of chloroplastic ValRS

	Protein (mg)	Specific activity (Units/mg)	Units*	Purification (fold)	Yield (%)
35,000 x g supernatant	9100	1.1	10,010	1	100
Ammonium sulfate precipitate	7400	0.9	6,660	0.8	66
Hydroxyapatite	4170	1.4	5,838	1.3	58
DEAE-cellulose	218	15.8	3,444	14.3	34
Blue Dextran - Sepharose	3.8	500.0	1,900	454.0	19
Sephadex G200	0.6	1090.0	654	990.0	6

* One unit of enzyme catalyses the aminoacylation of 1 nmole of E.coli tRNA in 1 min at 30°C under the above-described conditions.

Upon polyacrylamide gel electrophoresis in urea-SDS, the denatured enzyme also yields one single band (Fig. 5b).

3) Molecular weight of the enzyme

The mobility of the denatured chloro ValRS upon polyacrylamide gel electrophoresis in urea-SDS was compared to that of E.coli RNA polymerase (the β and β' subunits have a M.W. of 160,000 daltons, the α subunit has a M.W. of 39,000 daltons), yeast ValRS (M.W. = 126,000 daltons), bovine serum albumin (M.W. = 68,000 daltons), soybean trypsin inhibitor (M.W. = 21,500 daltons). Chloro ValRS was found to have a M.W. of 126,000 daltons. A similar value was obtain for the M.W. of the non-denatured enzyme upon Sephadex G200 filtration on a column (1 x 42 cm) calibrated with catalase (M.W. = 240,000 daltons), alcool dehydrogenase (M.W. = 150,000 daltons), yeast ValRS (M.W. = 126,000 daltons), bovine serum albumin (M.W. = 68,000 daltons), thus suggesting that chloro ValRS has a monomeric structure.

4) Kinetic parameters of the enzyme

The Km values of chloro ValRS have been determined for various substrates and are summarized in Table II. These Km values have been determined with respect to the aminoacylation reaction (not to the pyrophosphate exchange reaction).

DISCUSSION

Throughout this work, commercially available E.coli tRNA was used as a substrate to test chloro ValRS activity, rather than Euglena chloroplast tRNA, but

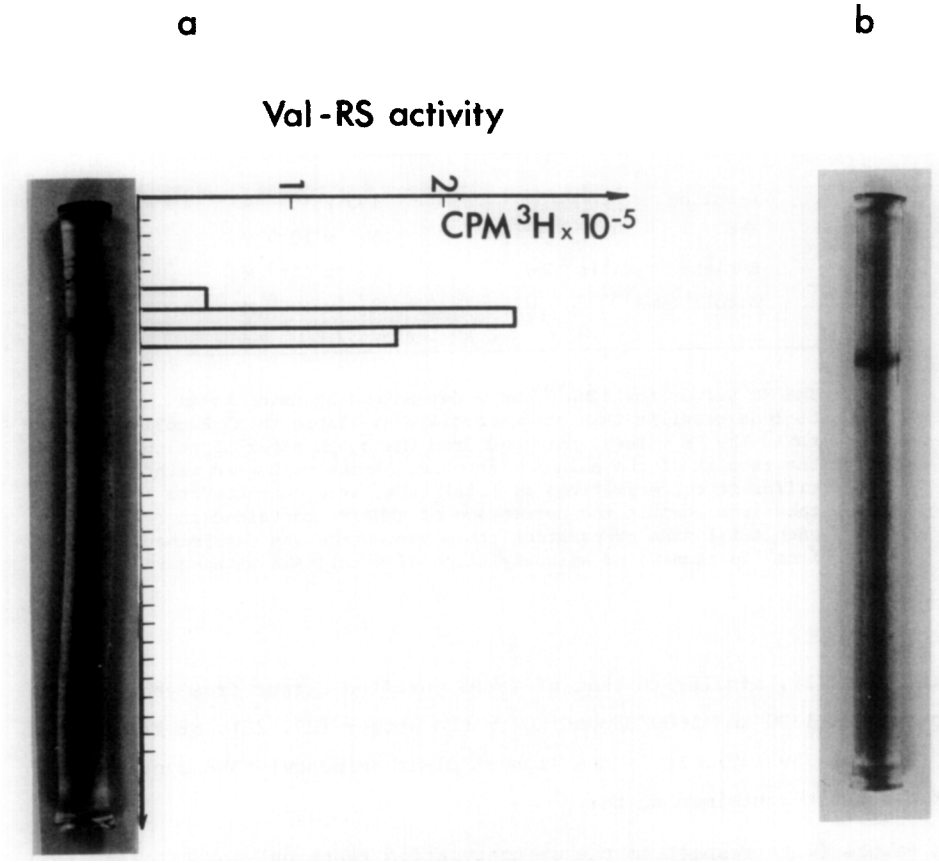


FIG. 5. Polyacrylamide gel electrophoresis of the purified chloro ValRS

a) Under non-denaturating conditions. The electrophoresis was performed on 15 µg of purified enzyme according to Davis (24), simultaneously on two 7.5% gels. One of the gels was stained with Coomassie Brillant Blue ; the other was cut into 1.7 mm long fragments each of which was incubated for 12 hrs at 4°C in electrophoresis buffer containing bovine serum albumin (0.1 mg/ml) before measuring the ValRS activity as described under Material and Methods.

b) Under denaturating conditions. The electrophoresis was performed on 15 µg of purified enzyme (denatured by incubation at 100°C for 4 min in the presence of 0.1% SDS, urea 4 M, β-mercaptoethanol 5%) on a 7.5% polyacrylamide gel containing the same concentrations of the above-mentioned denaturating agents, according to Laemli (25). After electrophoresis the gel was stained with Coomassie Brillant Blue.

this is justified by i) the fact that cross-aminoacylation reactions are often possible between chloroplastic and prokaryotic tRNAs and aminoacyl-tRNA synthetases (for a review see 26) and ii) the fact that in this particular case E.coli tRNA is as good a substrate for chloro ValRS as Euglena tRNA, as judged from their almost identical Km values (Table II).

Euglena chloro ValRS has been purified to apparent homogeneity. Its specific activity (with respect to the aminoacylation reaction) is about 1100 units/mg

TABLE II

Km values of chloroplastic ValRS for various substrates

Substrate	Km
L-valine	1.5×10^{-5} M
ATP	5×10^{-5} M
Euglena gracilis $tRNA^{Val}$	6×10^{-8} M
E.coli $tRNA^{Val}$	8×10^{-8} M

The Km values for $tRNA^{Val}$ were determined by using total Euglena gracilis tRNA or commercialy available total E.coli tRNA. The Km values, obtained from the graph after plotting the results of the aminoacylation reactions performed with different concentrations of total tRNA, were corrected to take into account the percentage of $tRNA^{Val}$ contained in each total tRNA preparation (this percentage was determined from the plateau of aminoacylation of total tRNA with valine).

protein (Table I), similar to that of ValRS purified either from yeast which varies between 1300 and 1600 depending in the author (27, 28), or from E.coli which is about 950 (29). It is the highest plant aminoacyl-tRNA synthetase specific activity obtained so far.

The Km values (with respect to the aminoacylation reaction) for ATP and L-valine are close to those found for ValRS from other species (3, 27, 28, 30), but the affinity of chloro ValRS for $tRNA^{Val}$ seems greater (Km = 10^{-8} M) than that observed for other ValRS (usually between 10^{-6} and 10^{-7} M).

Chloro ValRS appears to be a monomer with a M.W. of 126,000 daltons, a value similar to that obtained for ValRS purified from other sources (27-31).

So far very few studies have been devoted to the purification of chloroplast aminoacyl-tRNA synthetases and to the comparison between cytoplasmic and chloroplastic aminoacyl-tRNA synthetases. Krauspe and Parthier have compared some properties of Euglena cytoplasmic and chloroplastic leucyl-tRNA synthetases, which did not require a complete purification of the two enzymes (2). The only purification work reported in the literature is that of Locy and Cherry who have purified soybean cytoplasmic and chloroplastic tyrosyl-tRNA synthetases (16) ; they have found Km values similar to those we have observed, including a high affinity for the cognate tRNA (Km about 10^{-8} M), but it is difficult to estimate the degree of enzyme purification they have achieved, as they do not show any gel electrophoretic pattern and do not indicate the specific activity of their chloroplastic enzyme.

The cytoplasmic ValRS from Euglena is presently being purified in our laboratory, so that it should soon be possible to compare the structural features of the chloroplastic and cytoplasmic enzymes, and to see whether they have a common origin.

Acknowledgments

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