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Preparation of Chloroplasts from *Euglena* Highly Active in Protein Synthesis[1]

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WILLIAM ORTIZ[2,3], ELLEN M. REARDON[2], AND CARL A. PRICE[2]
[2] *Waksman Institute of Microbiology, Rutgers University Piscataway, New Jersey 08854 and* [3]*Laboratoire de Biochimie, Université de Neuchâtel, 2000-Neuchâtel Switzerland*

ABSTRACT

Chloroplasts can be obtained by gentle lysis or mild shear of spheroplasts of vitamin B_{12}-deficient *Euglena gracilis* and then purified by isopycnic sedimentation on gradients of Ludox AM or Percoll. The chloroplasts appear compact and highly refractile by phase contrast or Hoffmann contrast microscopy. Upon incubation with [^{3}H]leucine or [^{35}S]methionine, the chloroplasts incorporate the amino acids into protein at rates that are 100-fold faster than we had previously observed with *Euglena* and up to 8-fold faster than with chloroplasts of spinach. *Euglena* chloroplasts prepared by the current procedure are thus qualitatively superior to those previously available from *Euglena* and at least as active in protein synthesis as chloroplasts from higher plants.

Although *Euglena gracilis* offers many advantages in the study of chloroplast development, a serious limiting factor in pursuing the molecular biology of this process has been the difficulty in isolating pure, intact, and functional plastids from *Euglena*. The pioneer studies of Eisenstadt and Brawerman (5) were based on crude fractions obtained by differential centrifugation and differential flotation in sucrose. Although the plastids obtained were extensively contaminated with other subcellular particles and the integrity of the organelles was never assessed, their isolation procedures were nonetheless followed by a number of other laboratories. We now realize that the high osmotic pressures required for flotation in sucrose gradients are incompatible with the integrity of chloroplasts.

Several investigators have used spheroplasts in an effort to isolate plastids under relatively mild conditions (3, 8, 11, 13), but the chloroplasts obtained lacked stroma and the envelopes were broken or lost.

We have described (18) a separation procedure in which the chloroplasts were separated by rate zonal centrifugation in isosmotic gradients of Ficoll. This method yielded chloroplasts that appeared intact by electron microscopy but were not functional. We then found (10) that pure, intact, and functional chloroplasts could be obtained from spinach by centrifugation in density gradients of the silica sol Ludox AM. Salisbury *et al.* (14) adapted the silica procedure to *Euglena* by combining Ludox gradients with the other components of the recipe of Vasconcelos *et al* (18). The chloroplasts obtained could carry out protein synthesis but were still photosynthetically inactive (*cf.* 12, 17). It became evident, moreover, that the integrity of the chloroplasts was imperfect; the plastids had less than the expected levels of ribulose-1,5-bisphosphate carboxylase and had lost most of their Cyt 552 (6, 7).

Tokunaga *et al.* (16) meanwhile returned to the use of spheroplasts and isolated, for the first time, mitochondria from *Euglena* with respiratory control. They found specifically that *Euglena* made deficient in vitamin B_{12} are especially susceptible to spheroplasting, presumably because the cells become considerably enlarged (4). More recently, the Osaka group (15) showed that their procedure yielded photosynthetically active chloroplasts. We have in turn adapted their method to the purification of chloroplasts in gradients and have examined the capacity of these chloroplasts to synthesize protein.

MATERIALS AND METHODS

Growth of Cells. *E. gracilis* (Klebs) z strain (Pringsheim) was grown through three transfers in a modified Hutner medium (18) in which the concentration of cyanocobalamin was maintained at 50 ng/l (Table I). As Carell had shown (4), cytokinesis in *Euglena* is strongly inhibited at this concentration of the vitamin, whereas Chl formation and cell mass are only moderately decreased from control levels. The cells consequently increase considerably in volume. We find that spheroplasts suitable for the isolation of chloroplasts can be obtained from cells whose median volume is about 3,500 μm^3 as measured with the Coulter counter.

Isolation of Chloroplasts. Spheroplasts are formed by a procedure (15) similar to that described for the isolation of mitochondria (16): 6 g wet weight of cells are incubated at 0 C in 10 ml buffer containing 50 mM K-phosphate pH 7, 30 mM sorbitol, and 50 mg trypsin or 30 mg crude mixture of pancreatic proteases. The trypsin should not be too highly purified since a certain level of chymotrypsin may also be needed for digestion of the cell wall (pellicle). The course of digestion is monitored by light microscopy: thinning of the pellicle and rounding of the cells become visible in 15 to 30 min. Sufficient weakening of the pellicle is usually achieved in 1 h, whereupon the suspension is diluted 2-fold with the buffered sorbitol, centrifuged gently, and transferred to a mildly hypotonic mixture of 0.25 M sorbitol, 20 mM Hepes (pH 7.4), and 0.4 mM EDTA. Soybean trypsin inhibitor may also be added to a concentration of 1 mg/ml. The spheroplasts then are lysed by vigorous stirring with a magnetic stirrer for 10 min. Larger yields may be obtained by low speed homogenization in a Waring Blendor for 3 s or by passage through a French press at 90 kg/cm^3.

The cell brei is clarified by one spin at 1000 rpm for 3 min ($270g_{max}$) in 100-ml centrifuge tubes (IEC2806) and the chloroplasts were pelleted at 3,000 rpm for 5 min ($2400g_{max}$) in the same size tubes. The pellet, designated "crude chloroplasts," then is resuspended in a "gradient mix," which is slightly modified (12) from the "breaking mix" of Salisbury *et al.* (14), and layered on

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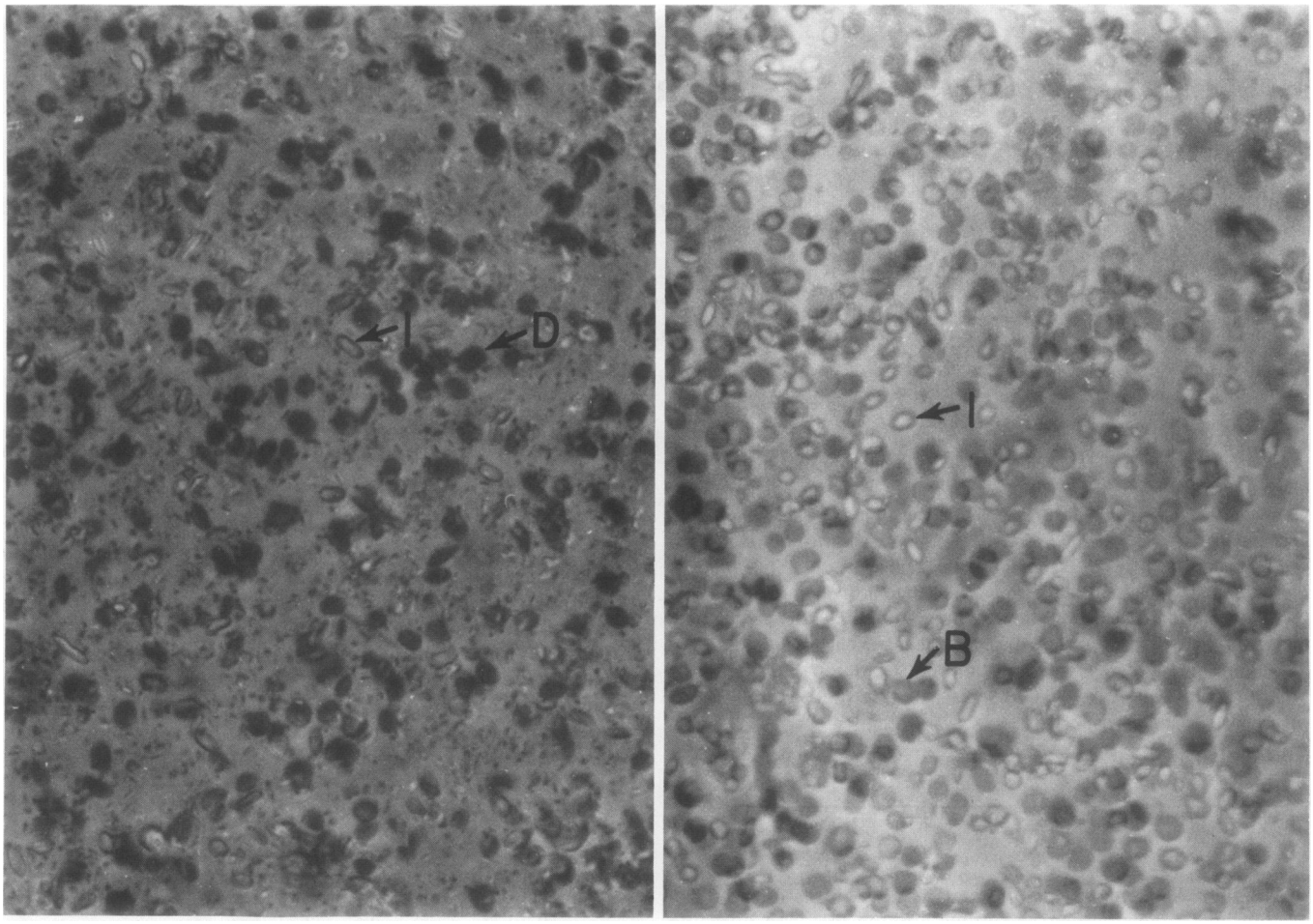

FIG. 1. Phase contrast photomicrograph of chloroplasts from *E. gracilis* (135/31). Left: crude chloroplast suspension. Field includes highly refractile, intact chloroplasts (I), dark and rather swollen chloroplasts (D), and numerous smaller particles. Right: chloroplasts purified by sedimentation in a density gradient; lower band collected. Nearly all of the particles appear to be biconvex discs: they show as bright ovals (I) on edge or as darker, but compact discs (B) when seen flat.

Table I. *Growth Medium for E. gracilis*

Medium is based on that of Vasconcelos *et al.* (18). Components are added as stock solutions in the order indicated. Final solution is titrated to pH 3.5 with 6 N HCl and diluted to volume with deionized water.

Stock	Component	Final Concentration
10 μg/l	Cyanocobalamin[a]	50 ng/l
178 mM	Thiamine·HCl	0.178 mM
1 M	Potassium phosphate buffer (pH 6.0)	0.6 mM
6 N	HCl	10 mM
1 M	$Ca(NO_3)_2$	0.1 mM
1 M	$MgSO_4$	1.6 mM
1 M	DL-Malic acid	2 mM
1 M	KCl	2 mM
10 mM	$MnSO_4$[b]	0.9 μM
10 mM	$ZnSO_4$[b]	1 μM
20 mM	$FeSO_4$[b]	40 μM
1 M	NH_4 glutamate[c]	20 mM
	Glucose	100 mM

[a] Stock solution of cyanocobalamin is a complete medium containing 10 μg cyanocobalamin/l.

[b] Prepared in 0.1 N HCl.

[c] Glutamic acid was adjusted to pH 6.0 with NH_4OH.

Table II. *Yields of Chloroplasts Isolated from Spheroplasts of E. gracilis*

Yields are calculated from the Chl contents of the several fractions and expressed on the basis of 1 liter of culture. Spheroplasts were disrupted by either gentle lysis ("Lysis") or by passage through a French press ("Press"). "Crude Chloroplasts" and "Purified Chloroplasts" refer to fractions obtained by differential centrifugation and density gradient centrifugation, respectively.

Experiment No.	Method of Breakage	Chlorophyll Content				
		Cells	Crude Chloroplasts		Purified Chloroplasts	
		mg	*mg*	*% of cells*	*mg*	*% of cells*
115/31	Lysis	10.9	1.64	15	0.38	3.5
131/58	Lysis	31.5	3.83	12.2	1.31	4.2
131/59	Lysis	60.0	10.9	18.2	0.13	0.2
136/1	Lysis	7.135			0.025	0.4
136/4	Lysis	11.05			0.79	7.2
136/4	Press	11.05			1.84	16.7
136/6	Lysis	10.4			0.032	0.3
136/6	Press	10.4	4.35	20.8	0.667	3.2

gradients essentially identical to those of Salisbury *et al.*, except that the concentration of Ludox AM is 5 to 50% (v/v). Gradients of 10 to 80% (v/v) Percoll with a cushion of 80% (v/v) Percoll may be substituted for the purified Ludox AM. The gradients are

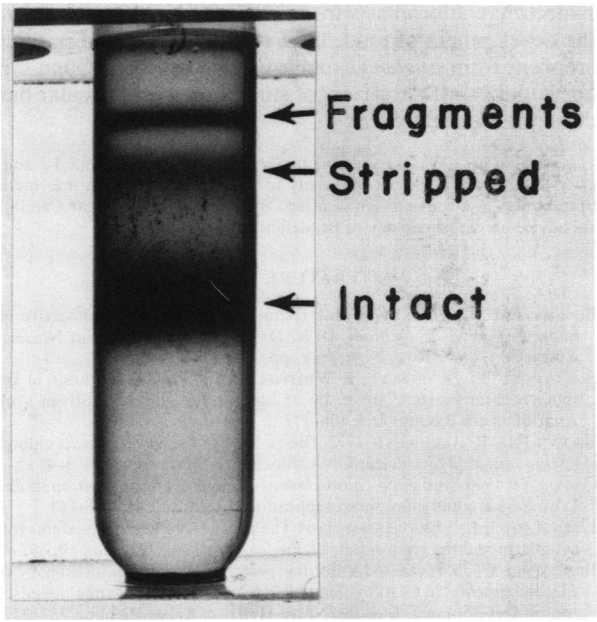

FIG. 2. Separation of *Euglena* chloroplasts by sedimentation in gradients of silica sol (115/38). Crude chloroplasts centrifuged into gradient of 5 to 50% v/v in Ludox AM. Three green zones were seen: an upper zone consisting mostly of thylakoid fragments, a middle zone containing stripped chloroplasts, and a lower zone containing intact chloroplasts.

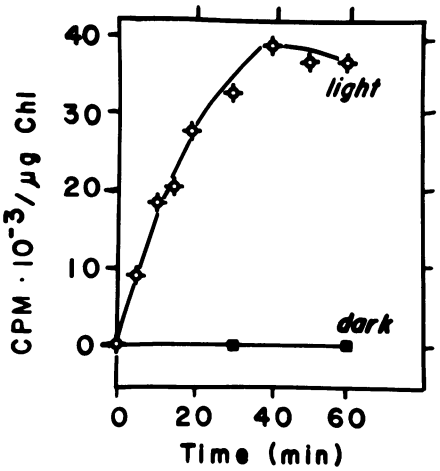

FIG. 3. Time course of protein synthesis by intact *Euglena* chloroplasts. Intact chloroplasts isolated as described under "Materials and Methods" were incubated at a concentration of 44 μg Chl/ml at 20 C with 50 nM [^{35}S]methionine, 1,240 Ci/mmol. Samples from tubes shaken in red light or in the dark were taken at intervals and the radioactivity incorporated into protein determined by the method of Bollum (1).

centrifuged at 7,000 rpm for 20 min ($8600g_{max}$). The isopycnically banded chloroplasts then are recovered from the gradient, washed, and resuspended in sorbitol-Tricine (2).

Protein Synthesis. The translational activity of the isolated chloroplasts is determined as described by Morgenthaler and Mendiola-Morgenthaler (9) with all solutions and containers sterilized by autoclave or sanitized with 70% v/v ethanol.

RESULTS

Photoheterotrophic *Euglena* are made deficient in vitamin B_{12} and harvested when the cell volume reaches a maximum. Spheroplasts are prepared by incubating the cells with proteolytic

Table III. *Protein Synthesis by Chloroplasts of E. gracilis Isolated from Spheroplasts*

Chloroplasts were purified by density gradient centrifugation and incubated with [^{35}S]methionine or [^{3}H]leucine for 30 min. Incorporation into trichloroacetic acid-insoluble protein was measured by the method of Bollum (1).

Experiment No.	Labeled Amino Acid			Rate of Incorporation per μg Chl	
	Species	Specific Activity	Concentration		
		Ci/mmol	*nM*	*cpm*	*fmol*
131/59	[^{35}S]Met	1240	50	34,400	13.9
136/4	[^{35}S]Met	1240	50	6,500	2.6
136/6	[^{35}S]Met	1022	50	27,200	13.3
136/8	[^{35}S]Met	1022	50	27,600	13.5
135/29	[^{35}S]Met	1022	50	24,100	11.8
115/31	[^{3}H]Leu	145	207	6,036	46.9
WON	[^{3}H]Leu	125	160	15,481	139.0

Table IV. *Sensitivity to Inhibitors of Protein Synthesis by Isolated Chloroplasts of E. gracilis*

Chloroplasts were isolated from spheroplasts and purified on density gradients. Each ml of the incubation mixture contained 67 μg of Chl and 20 μCi of [^{3}H]leucine (125 Ci/mmol) in sorbitol-Tricine. Protein synthesis was measured by incorporation of ^{3}H into hot trichloroacetic acid-insoluble protein by the method of Bollum (1). CAP, CHX, and RNase refer to D-threochloramphenicol, cycloheximide, and ribonuclease, respectively.

Condition	Incorporation at 40 Min	Light Control
	cpm/μg Chl	%
Light	15,481	100
Dark	188	1.2
Light + CAP, 50 μg/ml	729	4.7
Light + CHX, 10 μg/ml	14,529	102
Light + RNase, 30 μg/ml	15,847	102

enzymes, the spheroplasts are disrupted, and the chloroplasts are isolated by a combination of differential and density gradient centrifugation as described above. When we examined the crude chloroplast suspension by phase contrast microscopy, we saw numerous refractile, biconvex discs of approximately 5 μm in diameter (Fig. 1), looking similar to the chloroplasts of higher plants, in addition to many smaller particles and some debris. The low-speed centrifugation removes contaminating spheroplasts and intact cells. The density gradient step yields two green bands (Fig. 2). By the criterion of refractility, the lower band appears to consist almost exclusively of intact chloroplasts and the upper band mostly of stripped thylakoids. By Hoffman modulation contrast microscopy the intact chloroplasts appear smooth and compact, whereas the stripped chloroplasts appear to have numerous projections or to contain concentric or overlapping layers of membranes.

If the spheroplasts are lysed by the gentle method of magnetic stirring, nearly all of the chloroplasts are recovered in the lower zone. If, however, the spheroplasts are broken in the French press, only about one-third of the total Chl is recovered in the lower zone. The yields of chloroplasts are relatively low, varying from 0.2 to 7% (Table II), although it is not difficult to obtain intact chloroplasts corresponding to 3 mg of Chl from a liter of culture if the spheroplasts have been broken in the French press. The lowest yields correspond to poor spheroplast formation and to the aggregation of chloroplasts with cell debris prior to density gradient centrifugation.

We collected the intact chloroplasts from the lower band of the

gradients and tested their ability to synthesize protein. The chloroplasts were incubated in the light with [^{35}S]methionine over the course of 1 h. Incorporation into hot trichloroacetic acid-insoluble material occurs rapidly during the first 30 to 40 min (Fig. 3). The rates of incorporation vary from 6,000 to over 30,000 cpm/μg Chl in 30 min (Table III). These rates are greater than those we have observed previously with *Euglena* by factors of several hundred. Incorporation requires light, is insensitive to cycloheximide and ribonuclease, but is sensitive to chloramphenicol (Table IV). These properties are characteristic of protein synthesis by isolated, intact chloroplasts.

DISCUSSION

We find that *Euglena* chloroplasts isolated from spheroplasts will incorporate in excess of 15,000 cpm (139 fmol) [^{3}H]leucine/μg Chl in 30 min. These rates compare to less than 100 cpm (1.8 fmol) for chloroplasts that we prepared by the method of Salisbury *et al.* (14). Such large quantitative differences imply a qualitative difference in the state of the organelles. These rates of protein synthesis are, moreover, among the highest ever recorded for protein synthesis *in organello*. In comparison to the rate of 34,000 cpm or 14 fmol [^{35}S]methionine/μg Chl reported here, we have found up to about 4,000 cpm and 2 fmol/μg Chl with chloroplasts of field-grown spinach tested under similar conditions. *Euglena* thus becomes an organism of choice for studying translation quite apart from its suitability for the study of development.

Two lines of evidence indicate that the observed protein synthesis is accountable to the chloroplasts and not to contaminating bacteria: (*a*) incorporation in the dark is indistinguishable from that at zero time (*cf.* Fig. 3), and (*b*) the set of translation products detected by SDS-gel electrophoresis and fluorography is similar to the set of polypeptides stainable with Coomassie blue (Reardon and Price, in preparation).

The bright, refractile quality of the chloroplasts in phase-contrast microscopy, their content of Cyt 552 (Reardon and Price, in preparation), their activity in CO_2 fixation (15), and their extraordinary activity in protein synthesis are all evidence that these chloroplasts are fully intact and functional. It is nonetheless evident from the low and variable yields that the present isolation procedure is still suboptimal.

Our isolation procedure departs from that of Shigeoka *et al.* (15) in our use of isosmotic gradients based on silica sols. They report that *Euglena* chloroplasts can be resolved from mitochondria and ER on a linear sucrose gradient, but Shigeoka *et al.* (15) do not claim that chloroplasts recovered from sucrose gradients retain the ability to fix CO_2. Since chloroplasts of higher plants invariably lose both photosynthetic and protein synthetic activities following isopycnic sedimentation in sucrose gradients (*cf.* ref. 12), we expect that chloroplasts from *Euglena* would fare no better.

The development of procedures for the isolation of pure, intact chloroplasts from *Euglena* capable of high rates of protein synthesis opens the door to a variety of studies on the molecular biology of chloroplast development.

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LITERATURE CITED

1. Bollum FJ 1966 Filter paper disc techniques for assaying radioactive macromolecules. *In* G. L. Cantoni, D. R. Davies, eds, Procedures in Nucleic Acid Research. Harper Row, New York, pp. 296–300
2. Bottomley W, D Spencer, P Whitfield 1974 Protein synthesis in isolated spinach chloroplasts: comparison of light-driven and ATP-driven synthesis. Arch Biochem Biophys 164: 106–117
3. Brown RD, R Haselkorn 1972 The isolation of *Euglena gracilis* chloroplasts uncontaminated by nuclear DNA. Biochim Biophys Acta 259: 1–4
4. Carell EF 1969 Studies on chloroplast development and replication in *Euglena*. I. Vitamin B_{12} and chloroplast replication. J Cell Biol 41: 431–440
5. Eisenstadt JM, G Brawerman 1964 The protein-synthesizing systems from the cytoplasm and the chloroplasts of *Euglena gracilis*. J Mol Biol 10: 392–402
6. Freyssinet G, G Harris, JA Schiff 1978 Synthesis of cytochrome *c*-552 in *Euglena gracilis*. *In* G Akoyunoglou, JH Argyroudi-Akoyunoglou, eds. Chloroplast Development. Elsevier/North-Holland, Amsterdam, pp 267–270
7. Freyssinet G, G Harris, M Nasitir, JA Schiff 1979 Events surrounding the early development of *Euglena* chloroplasts. 14. Biosynthesis of cytochrome c-552 in wild type and mutant cells. Plant Physiol 63: 908–915
8. Knight GJ, CA Price 1968 Measurements of S-ρ coordinates during the development of *Euglena* chloroplasts. Biochim Biophys Acta 158: 283–285
9. Morgenthaler JJ, L Mendiola-Morgenthaler 1976 Synthesis of soluble, thylakoid, and envelope membrane proteins by spinach chloroplasts purified from gradients. Arch Biochem Biophys 172: 51–58
10. Morgenthaler JJ, CA Price, M Robinson, M Gibbs 1974 Photosynthetic activity of spinach chloroplasts after isopycnic centrifugation in gradients of silica. Plant Physiol 54: 532–534
11. Price CA, ME Bourke 1966 "Spheroplasts" prepared from *Euglena gracilis* by proteolysis. J. Protozool 13: 474–477
12. Price CA, M Bartolf, W Ortiz, EM Reardon 1979 Isolation of chloroplasts in silica-sol gradients. *In* E Reid, ed, Plant Organelles. Methodological Surveys in Biochemistry Vol 9. Ellis Horwood, Ltd, Chichester, pp 25–33
13. Rawson JR, E Stutz 1969 Isolation and characterization of *Euglena gracilis* cytoplasmic and chloroplast ribosomes and their ribosomal RNA components. Biochim Biophys Acta 190: 368–380
14. Salisbury JL, AC Vasconcelos, GL Floyd 1975 Isolation of intact chloroplasts of *Euglena gracilis* by isopycnic sedimentation in gradients of silica. Plant Physiol 56: 399–403
15. Shigeoka S, A Yokota, Y Nakano, S Kitaoka 1980 Isolation of physiologically intact chloroplasts from *Euglena gracilis* z. Bull Univ Osaka Pref, Ser B 32: 37–51
16. Tokunaga M, Y Nakano, S Kitaoka 1976 Preparation of physiologically intact mitochondria from *Euglena gracilis* Z. Agric Biol Chem 40: 1439–1440
17. Vasconcelos A 1976 Synthesis of proteins by isolated *Euglena gracilis* chloroplasts. Plant Physiol 58: 719–721
18. Vasconcelos A, M Pollack, LR Mendiola, HP Hoffmann, DH Brown, CA Price 1971 Isolation of intact chloroplasts from *Euglena gracilis* by zonal centrifugation. Plant Physiol 47: 217–221