Isolation of Active Polyribosomes from the Cytoplasm, Mitochondria and Chloroplasts of *Euglena gracilis*

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1. A procedure is described for the isolation of intact polyribosomes from the cytoplasm, chloroplasts and mitochondria of *Euglena gracilis*. 2. All three polyribosomal preparations incorporated labelled amino acids in a system *in vitro*. The cytoplasmic system was inhibited by cycloheximide but not by chloramphenicol. Both the chloroplast and the mitochondrial systems, however, were inhibited by chloramphenicol but not by cycloheximide. It is shown that mitochondrial polyribosomes, like the polyribosomes from cytoplasm and chloroplasts, can participate directly in protein synthesis without supplementary mRNA being added to the synthesizing system, as in previously reported instances. 3. Sedimentation coefficients were measured for the ribosomes, ribosomal subunits, and rRNA of the cytoplasm, chloroplasts and mitochondria. 4. The G+C content was 55% for cytoplasmic rRNA, 50% for chloroplast rRNA, and 29% for mitochondrial rRNA. 5. The cytoplasmic ribosomal subunits contained a ribonuclease activity that was inhibited by heparin.

A considerable amount of information has been accumulated on the nucleic acid function and specificity of *Euglena gracilis* (see, e.g., Brawerman, 1968) and many of the nucleic acid components of these cells have been characterized. In spite of efforts by various groups of workers (see Table 1 for details), however, the sedimentation coefficients of *Euglena* cytoplasmic and chloroplast ribosomes and their RNA are still a matter of conflict and confusion (Table 1).

In contrast with some of the initial studies (Table 1), it is now generally accepted that, like all other cell types examined (Stutz & Noll, 1967), *Euglena* cells contain two cytoplasmic rRNA components (Rawson & Stutz, 1968; Loening, 1968; Schuit & Buetow, 1968; Portier & Nigon, 1968; Krawiec & Eisenstadt, 1970; Scott *et al.*, 1970; Heizman, 1970; Mendiola *et al.*, 1970; Spiess & Richter, 1970). Various sedimentation coefficients, however, have been reported for these two ribosomal RNA components (Table 1). This variation could be caused either by the high RNAase* activity in *Eug. gracilis* (Fellig & Wiley, 1960; Smillie *et al.*, 1963) or the harsh methods often used to rupture the tough cell pellicle of this organism; however, involvement of other factors is also possible.

Previous studies (Rawson & Stutz, 1969; Mendiola *et al.*, 1970) have shown the possibility of isolating polyribosomes from the chloroplasts of *Eug. gracilis*; however, these preparations did not contain the highly polymerized particles found in polyribosomes iso-

lated from plant chloroplasts (Stutz & Noll, 1967). Preston *et al.* (1970) could not isolate polyribosomes from chloroplasts, although they could isolate ribosomes and ribosomal subunits. The work of Harris & Eisenstadt (1971) indicated indirectly the existence of polyribosomes in chloroplasts on the basis of patterns of amino acid incorporation. A similar difficulty seems to exist in detecting ribonucleo-protein particles in *Euglena* mitochondria (Krawiec & Eisenstadt, 1970; Schiff, 1970; Hirvonen & Price, 1971).

In the present paper we describe an improved method for the isolation of active polyribosomes from the cytoplasm, chloroplasts and mitochondria of *Eug. gracilis*. Because of the increasing interest in mitochondrial protein-synthesizing systems, the results obtained with mitochondrial polyribosomes are discussed in detail.

Materials and Methods

Organisms and growth conditions

Organisms used were a streptomycin-bleached, chloroplast-lacking mutant of *Eug. gracilis* (strain SM-L1) and the photosynthetic forms, *Eug. gracilis* (*bacillaris* variety) and *Eug. gracilis* (strain Z). The cells were maintained as described previously (Schuit & Buetow, 1968; Schuit *et al.*, 1970). For all the experiments reported in the present paper, cells were grown under sterile conditions in a defined medium (Buetow, 1965) with 0.2M-ethanol as carbon source

*Abbreviation: RNAase, ribonuclease.

Table 1. Sedimentation coefficients reported for cytoplasmic and chloroplast ribosomes and their RNA components in *Euglena gracilis*

For details see the text.

Sedimentation coefficients (S)							References
Source	Cytoplasm			Chloroplast			
	Monomers	Subunits		Monomers	Subunits		
		Large	Small		Large	Small	
Ribosomes	70	50*	—	60	—	—	Brawerman & Eisenstadt (1964); Eisenstadt & Brawerman (1964)
	65	47	38	65	43	30	Gnanam & Kahn (1967)
	87	68	41	68	46	29	Rawson & Stutz (1968, 1969)
	88	67	46	70	50	30	Scott <i>et al.</i> (1970)
	70	50	30	—	—	—	Cline <i>et al.</i> (1968)
	80	—	—	—	—	—	Heizman (1970)
	89	64	46	70	55	30	Mendiola <i>et al.</i> (1970); Vasconcelos <i>et al.</i> (1971)
	—	—	—	68	45	29	Preston <i>et al.</i> (1970)
Ribosomal RNA		19*	—		19	14	Brawerman & Eisenstadt (1964); Eisenstadt & Brawerman (1964)
		19†	10†		19	14	Zeldin & Schiff (1967)
		27	23		—	—	Portier & Nigon (1968)
		22	17		—	—	Schuit & Buetow (1968)
		24	20		22	17	Rawson & Stutz (1968, 1969)
		24 (26)	20 (22)		23.5	16.5	Scott <i>et al.</i> (1970)
		25	19		—	—	Krawiec & Eisenstadt (1970)
		24	20		—	—	Spiess & Richter (1970)
		25	21		22	16	Heizman (1970)
		—	—		24‡	16‡	Preston <i>et al.</i> (1970)
		25	21–22		23–24	16	Mendiola <i>et al.</i> (1970)

* These results indicate the presence of only one type of ribosomal subunit and rRNA; however, for the purpose of presentation, we have classified them as large components.

† RNA species found in a 'ribosomal fraction'.

‡ 21S and 15S values for the chloroplast rRNA components were also reported when sucrose density gradients were used.

(Buetow & Padilla, 1963). The bleached strain was always grown in the dark. The photosynthetic strains were incubated either in the light or in the dark as required. Cultures, still in the exponential phase of growth (about 4×10^6 cells/ml), were used for the isolation of cytoplasmic polyribosomes, mitochondria and the S-150 fraction. Chloroplasts were isolated from photosynthetic cells grown to the end of the exponential phase (about 6×10^6 – 7×10^6 cells/ml) when the chlorophyll content was maximum (Chang & Kahn, 1970).

Cells, collected by flowing the culture through a Sharples Super centrifuge, were washed twice with the required buffer (described below) and finally were suspended in an equal volume of the same buffer. Freshly grown cells were used for the isolation of polyribosomes, whereas cells previously frozen at

–20°C were thawed and used for the isolation of ribosomes and rRNA.

Buffers used for the isolation of polyribosomes and ribosomes

All the buffers had the same concentrations of KCl (25mM) and tris-HCl (50mM, pH 7.5 at 25°C). They differed only in MgCl₂ content as follows: KT buffer, no MgCl₂; KTM2 buffer, 0.2mM-MgCl₂; KTM5 buffer, 5mM-MgCl₂; and KTM15 buffer, 15mM-MgCl₂.

Methods

Isolation of RNAase inhibitor from rat liver. A crude RNAase inhibitor was isolated from rat liver as described by MacGregor & Mahler (1969). The

only modification was to dissolve the final preparation in KTM15 buffer instead of a buffer containing EDTA. This fraction is referred to as 'rat liver protein fraction'.

Determination of RNA. The RNA content of a sample was measured by reading the E_{260} . For phenol-extracted RNA, 1 E_{260} unit was considered to be 40 $\mu\text{g}/\text{ml}$. For polyribosomes and ribosomes, 1 E_{260} unit was considered to be 50 μg of RNA/ml (Heywood *et al.*, 1968).

Isolation of polyribosomes from cytoplasm. Polyribosomes were isolated from the cytoplasm of *Eug. gracilis* by two methods and all the operations were done at 0–2°C.

Method I. Cells were suspended in KTM5 buffer containing 0.25M-sucrose (5g of cells/10ml of buffer). Heparin (500 μg ; sodium salt, grade I; Sigma Chemical Co., St. Louis, Mo., U.S.A.), rat liver protein fraction (0.3ml) and β -mercaptoethanol (0.055ml; Eastman Organic Chemicals, Rochester, N.Y., U.S.A.) were added to every 10ml of cell suspension. The suspension was put in an ice-cold French pressure cell (American Instrument Co., Silver Springs, Md., U.S.A.) and the cells were ruptured at 3000 lbf/in² (86.8 N/m²). The cell homogenate was centrifuged at 1000g for 10min to remove nuclei, intact cells and large debris. Unless specifically mentioned otherwise, all the g forces are average forces at the centre of the tube. The 1000g supernatant was then centrifuged at 15000g for 20min to remove mitochondria and other large particles. The post-mitochondrial supernatant was mixed with a 10% (w/v) solution of sodium deoxycholate (K & K Laboratories, Plainview, N.Y., U.S.A.) in water to obtain a final concentration of 1% deoxycholate. The treated supernatant (2.3ml) was layered over 1.0ml of 2.0M-sucrose in KTM5 buffer and centrifuged in the SB 405 rotor of an International model B-60 ultracentrifuge (0–1°C) for 2.5h at 60000rev./min (405000g_{max.}). The polyribosomal pellet was rinsed four times with KTM5 buffer to remove sucrose and was finally dissolved in KTM5 buffer at a final concentration of 2.0mg of RNA/ml.

Method II. This method was identical with method I except that the cells (10g/10ml of buffer) were ruptured by grinding in a porcelain mortar with a pestle. Cell rupture was facilitated by including an equal volume (cell suspension: glass beads) of acid-cleaned 'Superbrite' glass beads (Minnesota Mining and Mfg. Co., Minneapolis, Minn., U.S.A.) as described by Buetow & Buchanan (1964, 1965). About 5min of constant grinding resulted in 65–70% of the cells being ruptured as judged by light-microscopy. The cell homogenate was washed from the beads with 2–3 vol. of KTM5 buffer containing 0.25M-sucrose.

Isolation of chloroplasts and chloroplast polyribosomes. Chloroplasts were isolated from the green *Euglena* cells by a method modified from that of

Chang & Kahn (1970), which yields enzymically active chloroplasts. All steps leading to the isolation of chloroplasts and chloroplast polyribosomes were done at 0–2°C. Cells suspended in an equal volume of KTM15 buffer containing 0.25M-sucrose were ruptured by grinding with glass beads as described in method II for cytoplasmic polyribosomes. The lysate was further homogenized in a Dounce homogenizer (ten strokes) to facilitate the release of chloroplasts from partially broken cells. The resultant homogenate was centrifuged at 700g for 3min in a model PR2 International refrigerated centrifuge. The supernatant was centrifuged again at the same speed for 2min and then further centrifuged at 1200g for 10min. The pelleted crude chloroplasts were suspended in 2vol. of KTM15 buffer containing 0.25M-sucrose by gentle homogenization in a Dounce homogenizer (five strokes). The homogenate was centrifuged at 700g for 5min to sediment the contaminating nuclei and cell debris. The supernatant containing the chloroplasts was centrifuged at 1200g for 15min. The latter two steps were repeated three more times to obtain pure chloroplasts. The purified chloroplast pellet was suspended in 2vol. of KTM15 buffer containing 0.25M-sucrose and was used for the isolation of polyribosomes. Heparin (500 μg), rat liver protein fraction (0.3ml) and β -mercaptoethanol (0.055ml) were added to every 10ml of chloroplast suspension. Chloroplasts were lysed with 0.5% sodium deoxycholate and the lysate was centrifuged at 15000g for 20min to remove debris and membranes. The 15000g supernatant (2.3ml) was layered over 1.0ml of 2.0M-sucrose in KTM15 buffer and centrifuged at 60000rev./min (405000g_{max.}) for 2.5h at 0–1°C. The resultant polyribosomal pellet was rinsed with and suspended in KTM15 buffer at a final concentration of 2mg of RNA/ml.

Isolation of mitochondria and mitochondrial polyribosomes. Mitochondria were isolated from the bleached cells or cells grown in the dark to avoid contamination with chloroplast fragments. The method used was similar to that of Buetow & Buchanan (1964, 1965), which yields particles that show oxidative phosphorylation. Cells suspended in KTM15 buffer containing 0.25M-sucrose were ruptured as described above for the isolation of chloroplasts. Whole cells, debris and nuclei were removed by centrifuging the homogenate at 1000g for 10min. The supernatant was centrifuged at 15000g for 20min. The crude mitochondrial pellet was suspended in 2vol. of KTM15 buffer containing 0.25M-sucrose by gentle homogenization in a Dounce homogenizer (five strokes). The mitochondrial suspension was centrifuged at 1500g for 10min to eliminate any remaining large particles. Mitochondria were then sedimented as described above. The pelleted mitochondria were suspended in 2vol. of KTM15 buffer containing 0.25M-sucrose and were washed three

times by sedimenting at 7000g for 30min in the same buffer. The final pellet was suspended in 2vol. of KTM15 buffer containing 0.25M-sucrose and was used for polyribosome preparation.

Heparin, rat liver protein fraction and β -mercaptoethanol were added to the mitochondrial suspension as with chloroplast and cytoplasmic polyribosome preparations. Mitochondria were lysed by adding sodium deoxycholate to a final concentration of 0.3%. The lysate was centrifuged at 15000g for 10min. The supernatant was layered over 1.0ml of 2.0M-sucrose in KTM15 buffer and centrifuged as described for the cytoplasmic and chloroplast preparations. The final polyribosomal pellet was rinsed and was dissolved in KTM15 buffer as described above. All steps were done at 0–2°C.

Preparations of cytoplasmic, chloroplast and mitochondrial ribosomes. The polyribosome pellets obtained from the cytoplasm, chloroplasts and mitochondria as described above were suspended in KTM2 buffer containing 0.25M-sucrose and 50 μ g of heparin/ml and were incubated on ice for 30min. After 30min, most of the polyribosomes were dissociated to monoribosomes and subunits. The ribosomes were pelleted by centrifugation at 105000g for 1h and were suspended in KTM2 buffer at a final concentration of 2.0mg of RNA/ml. These ribosomal preparations could be stored at –20°C for at least 4 weeks without any noticeable change in sedimentation coefficients.

Preparation of rat liver and Escherichia coli ribosomes. *Esch. coli* A19 cells were grown in a casein-hydrolysate medium as described by Clark *et al.* (1965). Packed cells were washed and suspended in an equal volume of KTM5 buffer containing 0.25M-sucrose. Cells were lysed with 0.4% egg-white lysozyme and Brij 58 as described by Friedman *et al.* (1969). The lysate was treated with 20 μ g of bovine pancreatic deoxyribonuclease (RNAase-free; Worthington Biochemical Corp., Freehold, N.J., U.S.A.) for 10min at 0–2°C and was centrifuged at 10000g for 10min. Polyribosomes were isolated from the 10000g supernatant as described for *Euglena* cytoplasmic polyribosomes. The polyribosomes were dissociated into ribosomes and subunits by decreasing the Mg^{2+} concentration as in the *Euglena* preparations described above.

About 10g wet wt. of 0.9% NaCl-washed rat livers were minced and homogenized in 20ml of KTM5 buffer containing 0.25M-sucrose with a Teflon pestle electrically driven at 500rev./min. The homogenate was centrifuged at 15000g for 20min and the supernatant was aspirated off. Sodium deoxycholate was added to the supernatant at a final concentration of 2%. After incubation at 0–4°C for 5min, the mixture was centrifuged at 15000g for 10min and 2.3ml of the resulting supernatant was centrifuged over 1.0ml of 2.0M-sucrose in KTM5

buffer as described above for *Euglena*. Ribosomes were prepared as described above for *Euglena*.

Preparation of rRNA. Polyribosomal pellets were washed and suspended in KT buffer containing 0.25M-sucrose, 50 μ g of heparin/ml and 0.03ml of rat liver preparation/ml and were kept at 0–4°C for 30min. The ribosomal subunits thus produced were collected by centrifugation in an SB 405 rotor at 60000rev./min (405000g_{max.}) for 30min at 0–1°C. The subunit pellets were suspended in 5mM-tris-HCl buffer (pH 7.4 at 25°C) containing 2% (w/v) sodium dodecyl sulphate and 50 μ g heparin/ml and were used directly for the analysis of rRNA on sucrose gradients (described below) or for the isolation of the rRNA by phenolic extraction. In the latter case, the ribosomal subunits were suspended in 5mM-tris-HCl buffer (pH 7.4 at 25°C) containing 50 μ g of heparin/ml and mixed with 2vol. of 88% (w/v) phenol (Fisher Scientific Co., Fair Lawn, N.J., U.S.A.). After constant mixing for 10min, the mixture was centrifuged at 10000g for 10min. The aqueous phase was aspirated off and extracted repeatedly with cold diethyl ether to remove residual phenol. Diethyl ether was removed from the aqueous phase by a flow of N_2 . In cases where the RNA content was too low to recover adequately after precipitation with 2vol. of chilled ethanol as described by Schuit *et al.* (1970), the diethyl ether-free aq. phase was directly used for gradient centrifugation or base analysis.

Zone-velocity sedimentation. Polyribosomes. Linear (10–34%, w/v) sucrose density gradients (10ml vol.) were prepared over a 2.0ml 'cushion' of 40% (w/v) sucrose. The gradients contained KTM5 buffer. Polyribosome preparations equivalent to 100 μ g of RNA in 0.1ml were layered over the gradients. The tubes were centrifuged in an SB 206 rotor of the International model B-60 ultracentrifuge for 2h at 35000rev./min (210000g_{max.}) at 0–1°C. Gradient patterns were determined by upward displacement with a 55% (w/v) sucrose solution by using an ISCO gradient fractionator. The E_{260} was recorded in a Gilford model 2000 automatic recording spectrophotometer.

Ribosomes. Approx. 0.1ml of ribosomes (in KTM2 buffer) representing 45–50 μ g of RNA were layered over a 3.4ml linear gradient of 10–34% sucrose in KTM2 buffer. Centrifugation was done in the SB 405 rotor of an International model B-60 ultracentrifuge for 2h at 50000rev./min (280000g_{max.}) at 0–1°C. The gradients were analysed as described above.

rRNA. About 70–75 μ g of rRNA in 5.0mM-tris-HCl buffer (pH 7.4) were layered over a 10ml linear 5–25% (w/v) sucrose gradient prepared over a 2.0ml 'cushion' of 30.5% (w/v) sucrose. The gradients were centrifuged in the SB 206 rotor for 11h at 35000rev./min (210000g_{max.}) at 0–1°C. The gradients were

analysed as described above. Base composition was determined by the method of Katz & Comb (1963).

Preparation of the S-150 fraction from *Euglena*. All the operations were done at 0–2°C. Cells of photo-synthetically grown *Eug. gracilis* (strain Z) were suspended in an equal volume of BRS medium (40mM-tris-HCl buffer, pH 7.8, 12.5mM-magnesium acetate, 40mM-KCl, 10mM-NH₄Cl, 10mM- β -mercaptoethanol). Heparin and rat liver protein fraction were added as described for the isolation of polyribosomes. The cells were ruptured in a French pressure cell at 18000 lbf/in² (521 N/m²). The lysate was centrifuged at 900g for 5 min. The 900g supernatant containing the chloroplasts and mitochondria was treated with sodium deoxycholate at a final concentration of 0.5%. The lysate was then centrifuged at 15000g for 20 min and the top 75% of the supernatant was aspirated off and further centrifuged at 150000g_{max} for 2 h. The clear supernatant was pipetted out and dialysed against 3 litres of cold BRS medium (three changes of 1.0 litre each over a period of 12 h) to remove free amino acids, bases and the deoxycholate. The non-diffusible material was finally clarified at 10000g for 10 min and stored as small portions (i.e. the S-150 fraction) in liquid N₂.

Preparation of the S-150 fraction from *Esch. coli* and rat liver. Cell homogenates of *Esch. coli* A19 and rat liver were prepared as in the procedure for isolating ribosomes from these cells except that BRS medium was used instead of KTM5 buffer. The cell homogenates were centrifuged at 15000g for 20 min and then at 150000g for 2 h. The S-150 fractions were dialysed and clarified as described above for *Euglena*.

Cell-free protein synthesis. Assays of protein synthesis *in vitro* were modified from the methods of Clark *et al.* (1965) and Avadhani & Buetow (1970). The total volume of the system was 0.1 ml and included 40mM-tris-HCl buffer (pH 7.8), 12.5mM-magnesium acetate, 40mM-KCl, 10mM- β -mercaptoethanol, 10mM-NH₄Cl, 6.0mM-phosphoenolpyruvate (Sigma Chemical Co.), 3.0mM-ATP (Sigma Chemical Co.), 1.5mM-GTP (Sigma Chemical Co.), 0.5 μ Ci of algal hydrolysate containing fifteen ¹⁴C-labelled amino acids (New England Nuclear Corp., Boston, Mass., U.S.A.; 1.8 μ Ci/mg), 40 μ M each of the following unlabelled L-amino acids (asparagine, glutamine, tryptophan, methionine and cysteine), 2.5 μ g of crystalline pyruvate kinase (Sigma Chemical Co.), 0.02 ml of the S-150 fraction from *Euglena*, *Esch. coli* or rat liver equivalent to 2.5 mg of protein and polyribosomes equivalent to 0–80 μ g of RNA. Unless stated otherwise, reactions were incubated at 37°C for 30 min. The reaction was stopped by adding 5 ml of 5% (w/v) trichloroacetic acid containing 0.05% casein hydrolysate (Difco Laboratories, Detroit, Mich., U.S.A.). The tubes were heated at 90°C for 10 min and cooled in ice. The precipitate was collected on Millipore filter discs (0.45 μ m pore size) and

washed five times with 3–4 ml of 5% trichloroacetic acid. The filter discs were dried at 90°C and counted for radioactivity as described previously (Avadhani & Buetow, 1970).

Results

Cytoplasmic polyribosomes

Typical patterns of cytoplasmic polyribosomes isolated by methods I and II are shown in Figs. 1(a) and 1(b). The polyribosomes isolated by the glass-bead grinding technique (Fig. 1b) appeared to be more intact and more polymerized than the polyribosomes isolated by the French-pressure-cell technique (Fig. 1a). Treatments such as sonication and pressure to rupture the cell pellicle frequently yield broken polyribosomes (Marcus & Halvorson, 1967). In the initial experiments, the post-mitochondrial supernatant was mixed with 1.0% deoxycholate to solubilize membranes. In the later experiments, however, the deoxycholate treatment was eliminated and no difference in the polyribosome pattern was observed. This result followed as *Euglena* cells contain predominantly endoplasmic reticulum of the smooth type rather than the granular type (Brandes *et al.*, 1964; Buetow, 1968). The cytoplasmic polyribosomes were readily degraded by EDTA and RNAase (Figs. 1c and 1d). EDTA treatment produced comparatively more subunits than RNAase treatment. The former is known to dissociate the polyribosomes into ribosomal subunits, whereas the latter breaks down the high-molecular-weight polyribosomes to monomeric ribosomes (Gierer, 1963).

Chloroplasts and chloroplast polyribosomes

The concentration of Mg²⁺ in the isolation medium for chloroplasts and chloroplast polyribosomes was 15 mM, as suggested by Boardman *et al.* (1965), Rawson & Stutz (1969) and Scott *et al.* (1970). Fig. 2(a) shows the polyribosome profile from the chloroplasts. The present technique appeared to yield an improved chloroplast polyribosomal profile compared with previous reports (Rawson & Stutz, 1969; Mendiola *et al.*, 1970). The chloroplast polyribosomes, however, appeared to contain fewer highly-polymerized particles as compared with the cytoplasmic (Fig. 1b) and mitochondrial polyribosomes (Fig. 3a). Increasing the concentrations of Mg²⁺ and KCl up to 50 mM each did not further improve the chloroplast polyribosome pattern. Possibly, the chloroplast polyribosomes were the more sensitive to shear forces during the isolation steps. An extreme sensitivity of chloroplast ribosomes from *Euglena* to Mg²⁺ concentration and also to centrifugal force was observed by Scott *et al.* (1970). These observations

Fig. 1. Sucrose-density-gradient patterns of cytoplasmic polyribosomes from *Eug. gracilis* (SM-L1)

(a) Polyribosomes isolated by method I (see the Materials and Methods section); (b) polyribosomes isolated by method II; (c) polyribosomes isolated by method II and treated with 5 mM-EDTA at 0–4°C for 10 min before being layered on the gradient; (d) polyribosomes isolated by method II and treated with bovine pancreatic RNAase (deoxyribonuclease-free; Worthington Biochemical Corp.). RNAase treatment was done at a concentration of 5 μ g of RNAase/2.0 mg of RNA at 0–4°C for 10 min. Details of polyribosome isolation and sucrose-density-gradient centrifugation were as described in the Materials and Methods section. Similar polyribosomal preparations were obtained from *Eug. gracilis* strain Z.

might explain the reason for failure to obtain polyribosomes from isolated *Euglena* chloroplasts in some cases (Preston *et al.*, 1970).

Figs. 2(b) and 2(c) show the effect of EDTA and RNAase on the chloroplast polyribosomes. Both agents degraded the polyribosomes into typical ribosomes and ribosomal subunits.

Isolation of mitochondrial polyribosomes

The mitochondrial polyribosome pattern is shown in Fig. 3(a). In addition to the two small peaks at the top of the gradient representing the subunits, nine or ten peaks of highly polymerized particles were seen. The mitochondrial polyribosomes, like the cytoplasmic and chloroplast polyribosomes, were sensitive to EDTA and to RNAase (Figs. 3b and 3c).

Sedimentation coefficients of ribosomes and subunits

The sedimentation coefficients for the ribosomal subunits were calculated as described by Martin & Ames (1961) with *Esch. coli* A19 ribosomes and rat liver ribosomes used as standards. The sedimentation coefficients assigned for various peaks were the mean of at least four independent estimations (Table 2). When the cytoplasmic, mitochondrial and chloroplast polyribosomes were suspended in a low-Mg²⁺ KTM2 buffer, the highly polymerized particles dissociated to monomeric ribosomes and subunits (Figs. 4a, 4b and 4c).

The cytoplasmic ribosomes sedimented as 87S particles and contained 46S and 67S subunits (Fig. 4a). The cytoplasmic ribosomes sedimented faster than the 80S rat liver ribosomes. These values for *Euglena* ribosomes and ribosomal subunits are close

Fig. 2. Sucrose-density-gradient patterns of chloroplast polyribosomes from *Eug. gracilis* (strain Z)

(a) Untreated polyribosomes; (b) polyribosomes treated with EDTA; (c) polyribosomes treated with RNAase. Details of EDTA and RNAase treatments were as given in Fig. 1. Other details were as described in the Materials and Methods section. Similar polyribosomes were isolated from *Eug. gracilis* variety *bacillaris*.

Fig. 3. Sucrose-density-gradient pattern of mitochondrial polyribosomes from *Eug. gracilis* (SM-L1)

(a) Untreated polyribosomes; (b) EDTA-treated polyribosomes; (c) RNAase-treated polyribosomes. Details of EDTA and RNAase treatments were as given in Fig. 1. For other details, see the Materials and Methods section. Similar polyribosomal preparations can also be obtained from *Eug. gracilis* (strain Z).

to those reported (Table 1) by Rawson & Stutz (1968, 1969), Scott *et al.* (1970), Mendiola *et al.* (1970) and Vasconcelos *et al.* (1971).

Fig. 4(b) shows a typical pattern of chloroplast ribosomes and subunits. Chloroplast ribosomes sedimented as 69S peaks similar to those of *Esch.*

coli ribosomes. When *Esch. coli* and chloroplast ribosomes were mixed and run on the same gradient, both sedimented as a single peak. The chloroplast ribosomes were dissociable to two subunits of 29S and 50S (Fig. 4b). These results agree closely with the

Table 2. *Sedimentation coefficients of ribosomes and rRNA*

Cytoplasmic, chloroplast and mitochondrial ribosomes and rRNA were isolated from *Euglena* (variety *bacillaris*) as described in the Materials and Methods section. Sedimentation coefficients $\pm$ s.d. were calculated on the basis of four independent determinations. The values given in parentheses are the corresponding values for various ribosomes and rRNA from *Euglena* (strain Z).

Type of particle or molecule	Sedimentation coefficients (S)		
	Cytoplasm	Chloroplast	Mitochondria
Ribosome			
Monomeric	86.9 $\pm$ 0.5 (86.3)	69.4 $\pm$ 0.9 (69.2)	71.3 $\pm$ 0.9 (71.7)
Large subunit	67.2 $\pm$ 0.9 (67.4)	49.8 $\pm$ 0.7 (50.1)	50.1 $\pm$ 0.6 (50.4)
Small subunit	45.9 $\pm$ 0.6 (50.2)	29.3 $\pm$ 0.5 (29.4)	32.4 $\pm$ 0.7 (32.2)
Ribosomal RNA			
Large subunit	24.4 $\pm$ 0.5 (24.6)	22.1 $\pm$ 0.5 (22.4)	21.4 $\pm$ 0.6 (21.3)
Small subunit	20.1 $\pm$ 0.4 (19.9)	16.5 $\pm$ 0.6 (16.8)	15.9 $\pm$ 0.5 (16.3)

values published (Table 1) by Rawson & Stutz (1969), Scott *et al.* (1970), Mendiola *et al.* (1970), Preston *et al.* (1970), and Vasconcelos *et al.* (1971). Some variance is noted, however, particularly in the sedimentation coefficient of the large subunit (Table 1). We always found that the larger subunit from the chloroplast sedimented exactly like the *Esch. coli* 50S subunit.

The mitochondrial ribosomal pattern is shown in Fig. 4(c). Monomeric ribosomes sedimented as 71S particles and the ribosomal subunits sedimented as 32S and 50S particles. The sedimentation values for these particles were the same for *Euglena* variety *bacillaris* and strain Z (Table 2).

Ribosomal RNA

As the sedimentation coefficients reported for the cytoplasmic rRNA are conflicting (Table 1), utmost care was taken to isolate the intact RNA molecules. The cytoplasmic ribosomes of *Euglena* are known to contain a ribonuclease activity that becomes functional only when the ribosomes dissociate to subunits (Heizman, 1970). The ribosomal subunits were therefore sedimented quickly in the presence of heparin, an RNAase inhibitor (Palmiter *et al.*, 1970). Whenever the ribosomes were suspended in 5 mM-tris-HCl buffer (pH 7.4), heparin was always present. Further, in our experience, both sodium dodecyl sulphate treatment and phenol extraction gave the same results. The former method, however, was convenient since it could be done quickly and also was successful with samples of RNA as small as 150 μ g.

As shown in Fig. 5(a), the cytoplasmic rRNA gave major peaks at 20S and 24S with a small peak at the 5S region. These values agree with those found by Rawson & Stutz (1968, 1969), Scott *et al.* (1970) and Spiess & Richter (1970), and are very near

to the values reported by Mendiola *et al.* (1970), Krawiec & Eisenstadt (1970) and Heizman (1970). The 26S and 22S RNA species also observed by Scott *et al.* (1970) could be contaminating RNA species. The values of 22S and 17S for the cytoplasmic rRNA reported from this laboratory (Schuit & Buetow, 1968) were the result of a difficulty in accounting fully for the changing viscosity of the sucrose gradient under the conditions used. In the present study this difficulty was overcome through the use of an ISCO gradient fractionator (see the Materials and Methods section).

The chloroplast rRNA had 16S and 22S components and a 5S component (Fig. 5b). Similar values (Table 1) were published by Rawson & Stutz (1969) and Heizman (1970). Slightly higher *s* values (Table 1) were reported for the larger RNA component in some cases (Scott *et al.*, 1970; Preston *et al.*, 1970; Mendiola *et al.*, 1970). This variance may be due to the use of polyacrylamide gels in the latter cases, as indicated in the experiment of Preston *et al.* (1970).

The mitochondrial ribosomes contained 16S and 21S components and a small 5S component (Fig. 5c). Similar components were shown in the mitochondria from various sources (Wintersberger & Viehhauser, 1968; Rogers *et al.*, 1967; Leon & Mahler, 1968; Fauman *et al.*, 1969; Kuntzel & Noll, 1967; Swanson & Dawid, 1970; Chi & Suyama, 1970). Our values, however, are higher than the values (11S and 14S) published for mitochondrial RNA from *Eug. gracilis* (Krawiec & Eisenstadt, 1970). The above-mentioned sedimentation coefficients for various ribosomal RNA components were the same for both *Euglena* variety *bacillaris* and strain Z (Table 2).

Base composition of rRNA

The cytoplasmic, chloroplast and mitochondrial rRNA molecules differed considerably in their base

Fig. 4. Sucrose-density-gradient patterns of ribosomes and ribosomal subunits

(a) *Euglena* cytoplasmic ribosomes from strain SM-L1; (b) chloroplast ribosomes from *Eug. gracilis* variety *bacillaris*; (c) mitochondrial ribosomes from *Eug. gracilis* (strain SM-L1). Ribosomes were prepared and centrifuged on 10–34% linear sucrose gradients as described in the Materials and Methods section. Sedimentation coefficients were measured with rat liver and *Esch. coli* A19 ribosomes as standards.

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Fig. 5. Sedimentation profiles of rRNA

(a) Cytoplasmic rRNA from *Eug. gracilis* (strain SM-L1); (b) chloroplast rRNA from *Eug. gracilis* variety *bacillaris*; (c) mitochondrial rRNA from *Eug. gracilis* (strain SM-L1). The rRNA species were prepared and centrifuged on 5–25% linear sucrose gradients as described in the Materials and Methods section. Sedimentation coefficients were measured with rat liver and *Esch. coli* A19 rRNA as standards.

compositions (Table 3). The overall trend in the base ratios of the cytoplasmic and chloroplast rRNA molecules was similar to the results of Brawerman (1963) and Rawson & Stutz (1969). The G+C content of the cytoplasmic rRNA was 55.7% and that of the chloroplast rRNA was 50.0%. The mitochondrial rRNA, in contrast, contained only 29.8% G+C, as expected (Rifkin *et al.*, 1967; Küntzel & Noll, 1967; Chi & Suyama, 1970). No significant strain differences were observed in the base composition of the various rRNA species.

Table 3. *Base composition of rRNA species*

Results are averages of two determinations. Details of RNA hydrolysis and base analysis are described in the Materials and Methods section.

Source of rRNA	Content (mol/100 mol)				
	U	C	G	A	G+C
Cytoplasm	22.1	24.7	31.2	22.0	55.7
Chloroplast	23.5	20.6	29.4	26.5	50.0
Mitochondria	30.2	14.0	15.8	40.0	29.8

Evidence for RNAase activity associated with the cytoplasmic ribosomes

The presence of an RNAase activity in the cytoplasmic ribosomal subunits of *Euglena* was reported by Heizman (1970). Heparin was an inhibitor of this activity as shown in Figs. 6(a) and 6(b). Samples incubated without heparin showed considerable degradation in the 24S region and some degradation in the 20S region (Fig. 6b). Chloroplast and mitochondrial subunits, whether incubated with or without heparin, did not show any significant degradation. It appears either that the latter subunits did not contain the RNAase activity or that the RNAase was not active under the conditions tested.

Cell-free protein synthesis

The polyribosomes from the cytoplasm, chloroplasts and mitochondria (from both *Euglena* variety *bacillaris* and strain Z) incorporated amino acids into the hot-trichloroacetic acid-insoluble fraction (Table 4). Omission of the *Euglena* S-150 fraction or polyribosomes from the system decreased incorporation by 1100 and 610 c.p.m. respectively. Kinetic experiments showed that the rate of incorporation was dependent on the time of incubation and on the concentration of polyribosomes. Protein synthesis by all three types of polyribosomes required the presence of amino acids, ATP and an ATP-generating system (Table 4). RNAase and EDTA inactivated the system. These results are consistent with the characteristics of a typical system for protein synthesis *in vitro* (Nirenberg & Matthaei, 1961; Noll *et al.*, 1963; Heywood *et al.*, 1968; Coleman, 1969).

Chloramphenicol, a potent inhibitor of bacterial protein synthesis, inhibited the mitochondrial and chloroplast systems *in vitro* by 52–53%, but had little effect (9% inhibition) on cell-free synthesis by the cytoplasmic system. Cycloheximide, which is effective only in eukaryotic systems (Bennet *et al.*, 1964), inhibited the cytoplasmic system by 78%, but had a negligible effect on the mitochondrial and chloroplast cell-free systems (Table 4).

Fig. 6. Effect of heparin on the ribosomal RNAase activity as judged by the rRNA sedimentation patterns

(a) Cytoplasmic ribosomes from *Eug. gracilis* (strain SM-L1) were incubated with 50 µg of heparin/ml for 15 min at 0–4°C; (b) cytoplasmic ribosomes incubated without heparin. RNA was isolated from these ribosomes and centrifuged on 5–25% linear sucrose gradients as described in the Materials and Methods section.

Table 4. Requirements for protein synthesis by polyribosomes

Protein-synthesis assays were done in a final volume of 0.1 ml at 37°C for 30 min as described in the Materials and Methods section. The complete system included cytoplasmic, chloroplast, or mitochondrial polyribosomes (equivalent to 40 µg of RNA), the S-150 fraction from *Euglena*, and other components as described in the Materials and Methods section. The cytoplasmic, chloroplast and mitochondrial polyribosomes incorporated 12000, 8900 and 9400 c.p.m., respectively. Bovine pancreatic RNAase (deoxyribonuclease-free; Worthington Biochemical Corp.) was added at a concentration of 20 µg/tube; EDTA was added to a 10 mM concentration. Chloramphenicol (Parke, Davis, Detroit, Mich., U.S.A.) and cycloheximide (Sigma Chemical Co.) were added at a concentration of 25 µg/tube. All the additions were made at zero time of incubation. The percentage activity was calculated on the basis of control values, which were considered to be 100% active.

Addition or omission	Activity (% of control)		
	Cytoplasm	Chloroplast	Mitochondria
None	100	100	100
–Five unlabelled L-amino acids	45	37	41
–ATP	9	7	8
–Phosphoenolpyruvate	16	21	21
+RNAase	29	30	28
+EDTA	20	24	25
+Chloramphenicol	91	48	47
+Cycloheximide	22	93	90

As compared with the *Euglena* S-150 fraction, the S-150 fraction from *Esch. coli* was only 72% efficient in stimulating protein synthesis in the *Euglena* cytoplasmic system, but was 130–140% efficient in the chloroplast and mitochondrial systems. The S-150 fraction from rat liver was only 58% efficient in the *Euglena* cytoplasmic system and was less efficient in the chloroplast and mitochondrial systems (15–18%). The *Euglena* cytoplasmic system contains formyl-methionyl-tRNA (N. G. Avadhani & D. E. Buetow, unpublished work) in addition to some eukaryotic-type tRNA (N. G. Avadhani & D. E. Buetow, unpublished work). This may account for the fact that both the *Esch. coli* and the rat liver fractions gave only fractional activities in the *Euglena* cytoplasmic system.

Discussion

In the present paper we describe a procedure for the isolation of active polyribosomes from the cytoplasm, chloroplasts and mitochondria of *Eug. gracilis*. Rupturing the cells by grinding with glass beads appears to be a milder treatment than rupturing the cells with a French pressure cell, at least as regards the pattern observed for cytoplasmic polyribosomes in Fig. 1. The glass-bead technique can be used also for the isolation of mitochondrial and chloroplast polyribosomes (Figs. 2 and 3).

Besides the technique used to disrupt the cells, another critical requirement for the isolation of intact organelles and active polyribosomes from *Euglena* was the careful maintenance of the pH of

the buffer. When the cells are lysed in a 10 mM-tris buffer, the pH of the lysate falls to 6.3. At this pH, RNAase of *Euglena* is quite active (Fellig & Wiley, 1960). In these studies, therefore, the concentration of tris buffer was increased to 50 mM. This concentration is sufficient to maintain the pH above 7.0 (Buetow & Buchanan, 1964). Finally, three different RNAase inhibitors were included in the isolation medium. In the early experiments, when the polyribosomes were prepared without any added RNAase inhibitors, the preparations contained only up to trimeric aggregates, as analysed in the sucrose gradients. Various RNAase inhibitors were therefore tried in this system. The combination of heparin and rat liver protein fraction gave the best results. Heparin was used successfully in the isolation of polyribosomes from oviducts (Palmiter *et al.*, 1970). The cytosol of rat liver is known to contain a strong RNAase inhibitor (Bont *et al.*, 1965; MacGregor & Mahler, 1969). In addition to these two inhibitors, β -mercaptoethanol was also used.

As a result of all these precautions, the present technique yields polyribosomes, in contrast with previous techniques that used a French pressure cell to break the cells (Rawson & Stutz, 1969; Heizman, 1970; Mendiola *et al.*, 1970). Polyribosome preparations obtained by the present procedure had an E_{260}/E_{280} ratio of 1.6–1.7 for different preparations. These preparations retained both their typical pattern in sucrose gradients and their protein-synthesizing ability for several months when quickly frozen and stored in liquid N₂. Compared with the

chloroplast and mitochondrial polyribosomes (Figs. 2b and 3b), however, the cytoplasmic polyribosomes (Fig. 1c) appeared more resistant to EDTA treatment. This could be due to a comparatively higher sensitivity of chloroplast (Scott *et al.*, 1970) and mitochondrial (Küntzel, 1969b,c; Chi & Suyama, 1970) ribosomes towards low Mg^{2+} concentrations.

Mitochondrial ribosomes and their associated RNA have been investigated in various cells (Ashwell & Work, 1970). Generally, the sedimentation coefficient for the mitochondrial ribosomes is between 55S and 72S. The mitochondrial ribosomes from *Eug. gracilis* are similar as they are composed of 71S monomers, which dissociate into 32S and 50S subunits (Table 2). The latter ribosomes contain 16S and 21S RNA components (Table 2, Fig. 5d). Previous attempts at isolating the mitochondrial ribosomes from *Eug. gracilis* were unsuccessful, though 11S and 14S RNA components with a low G+C content could be isolated (Krawiec & Eisenstadt, 1970). These low *s* values suggest that a breakdown of the RNA occurred in the latter experiments. Our preparations are free of cytoplasmic components, as they do not contain any 87S particles (Fig. 4b). Also, a mitochondrial preparation from the bleached, proplastid-lacking strain SM-L1 is free of chloroplast components.

The mitochondrial rRNA contains about 29% G+C (Table 3). Such a low G+C content has been observed before and has been used as a criterion for differentiating mitochondrial rRNA from cytoplasmic rRNA (Küntzel & Noll, 1967; Chi & Suyama, 1970; Küntzel, 1969a).

Isolated mitochondria and extracts from lysed mitochondria are known to incorporate amino acids (Das *et al.*, 1964; Kroon, 1963; Lamb *et al.*, 1968; Sebald *et al.*, 1969; Avadhani *et al.*, 1971). Also, the presence of tRNA and formylmethionyl-tRNA in mitochondria (Smith & Marcker, 1968; Galper & Darnell, 1969; Epler *et al.*, 1970) strongly indicate the existence of an autonomous protein-synthesizing system in these organelles. This view was further supported by the characterization of a poly(U)-directed cell-free protein synthesis by mitochondrial ribosomes (Küntzel, 1969a). While the present work was in progress, the initiation of protein synthesis by yeast mitochondrial ribosomes, and ribosomal washes with viral RNA (Scragg *et al.*, 1971) and poly(U) (Grivell *et al.*, 1971) templates was reported. Together with a previous study (Avadhani & Buetow, 1972), the present results show that mitochondrial polyribosomes can participate directly in protein synthesis. This result provides direct evidence for the presence of an active protein-synthesizing system in mitochondria. The results with antibiotics (Table 4) give further emphasis to the idea that an independent biosynthetic system is present in this organelle (Ashwell & Work, 1970).

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References

- Ashwell, M. A. & Work, T. S. (1970) *Annu. Rev. Biochem.* **39**, 251
- Avadhani, N. G. & Buetow, D. E. (1970) *Nature (London)* **228**, 242
- Avadhani, N. G. & Buetow, D. E. (1972) *Biochem. Biophys. Res. Commun.* **46**, 773
- Avadhani, N. G., Lynch, M. J. & Buetow, D. E. (1971) *Exp. Cell Res.* **69**, 226
- Bennet, L. L., Jr., Smithers, D. & Ward, C. T. (1964) *Biochim. Biophys. Acta* **80**, 60
- Boardman, N. K., Francki, R. I. B. & Wildman, S. G. (1965) *Biochemistry* **4**, 872
- Bont, W. S., Rezelman, G. & Bloemendal, H. (1965) *Biochem. J.* **95**, 15c
- Brandes, D., Buetow, D. E., Bertini, F. & Malkoff, D. B. (1964) *Exp. Mol. Pathol.* **3**, 583
- Brawerman, G. (1963) *Biochim. Biophys. Acta* **72**, 317
- Brawerman, G. (1968) in *The Biology of Euglena* (Buetow, D. E., ed.), vol. 2, p. 97, Academic Press, New York
- Brawerman, G. & Eisenstadt, J. M. (1964) *J. Mol. Biol.* **10**, 403
- Buetow, D. E. (1965) *J. Cell. Comp. Physiol.* **66**, 235
- Buetow, D. E. (1968) in *The Biology of Euglena* (Buetow, D. E., ed.), vol. 1, p. 168, Academic Press, New York
- Buetow, D. E. & Buchanan, P. J. (1964) *Exp. Cell Res.* **36**, 204
- Buetow, D. E. & Buchanan, P. J. (1965) *Biochim. Biophys. Acta* **96**, 9
- Buetow, D. E. & Padilla, G. M. (1963) *J. Protozool.* **10**, 121
- Chang, I. C. & Kahn, J. S. (1970) *J. Protozool.* **17**, 556
- Chi, J. C. H. & Suyama, Y. (1970) *J. Mol. Biol.* **53**, 531
- Clark, J. M., Jr., Chang, A. Y., Spiegelman, S. & Reichmann, M. E. (1965) *Proc. Nat. Acad. Sci. U.S.A.* **54**, 1193
- Cline, G. B., Whitson, G. L. & Levedahl, B. H. (1968) *Biochem. Biophys. Res. Commun.* **30**, 534
- Coleman, G. (1969) *Biochim. Biophys. Acta* **182**, 180
- Das, H. K., Chatterjee, S. K. & Roy, S. C. (1964) *J. Biol. Chem.* **239**, 1126
- Eisenstadt, J. M. & Brawerman, G. (1964) *J. Mol. Biol.* **10**, 392
- Epler, J. L., Shugart, L. R. & Barnett, W. E. (1970) *Biochemistry* **9**, 3575
- Fauman, M., Rabinowitz, M. & Gets, G. S. (1969) *Biochim. Biophys. Acta* **182**, 355
- Fellig, J. & Wiley, C. E. (1960) *Science* **132**, 1835
- Friedman, H., Lu, P. & Rich, A. (1969) *Nature (London)* **223**, 909
- Galper, J. B. & Darnell, J. E. (1969) *Biochem. Biophys. Res. Commun.* **34**, 205
- Gierer, A. (1963) *J. Mol. Biol.* **6**, 148
- Gnanam, A. & Kahn, J. S. (1967) *Biochim. Biophys. Acta* **142**, 493
- Grivell, L. A., Reijnders, L. & Borst, P. (1971) *Biochim. Biophys. Acta* **247**, 91

- Harris, E. H. & Eisenstadt, J. M. (1971) *Biochim. Biophys. Acta* **232**, 167
- Heizman, P. (1970) *Biochim. Biophys. Acta* **224**, 144
- Heywood, S. M., Dowben, R. M. & Rich, A. (1968) *Biochemistry* **7**, 3289
- Hirvonen, A. P. & Price, C. A. (1971) *Biochim. Biophys. Acta* **232**, 696
- Katz, S. & Comb, D. G. (1963) *J. Biol. Chem.* **238**, 3065
- Krawiec, S. & Eisenstadt, J. (1970) *Biochim. Biophys. Acta* **217**, 132
- Kroon, A. M. (1963) *Biochim. Biophys. Acta* **72**, 391
- Küntzel, H. (1969a) *FEBS Lett.* **4**, 140
- Küntzel, H. (1969b) *Nature (London)* **222**, 142
- Küntzel, H. (1969c) *J. Mol. Biol.* **40**, 315
- Küntzel, H. & Noll, H. (1967) *Nature (London)* **215**, 1340
- Lamb, A. J., Clark-Walker, G. D. & Linnane, A. W. (1968) *Biochim. Biophys. Acta* **161**, 415
- Leon, S. A. & Mahler, H. R. (1968) *Arch. Biochem. Biophys.* **126**, 305
- Loening, U. E. (1968) *J. Mol. Biol.* **38**, 355
- MacGregor, R. R. & Mahler, H. R. (1969) *Biochemistry* **8**, 3036
- Marcus, L. & Halvorson, H. O. (1967) *Methods Enzymol.* **12**, 498
- Martin, R. G. & Ames, B. N. (1961) *J. Biol. Chem.* **236**, 1372
- Mendiola, L. R., Kovacs, A. & Price, C. A. (1970) *Plant Cell Physiol.* **11**, 335
- Nirenberg, N. W. & Matthaei, J. H. (1961) *Proc. Nat. Acad. Sci. U.S.* **47**, 1588
- Noll, H., Staehelin, T. & Wettstein, F. O. (1963) *Nature (London)* **198**, 632
- Palmiter, R. D., Christensen, A. K. & Schimke, R. T. (1970) *J. Biol. Chem.* **245**, 833
- Portier, C. & Nigon, V. (1968) *Biochim. Biophys. Acta* **169**, 540
- Preston, J. E., III, Harris, E. H. & Eisenstadt, J. M. (1970) *Bacteriol. Proc.* 48a
- Rawson, J. R. & Stutz, E. (1968) *J. Mol. Biol.* **33**, 309
- Rawson, J. R. & Stutz, E. (1969) *Biochim. Biophys. Acta* **190**, 368
- Rifkin, M. R., Wood, D. D. & Luck, J. L. (1967) *Proc. Nat. Acad. Sci. U.S.* **58**, 1025
- Rogers, P. J., Preston, B. N., Titchener, E. B. & Linnane, A. W. (1967) *Biochem. Biophys. Res. Commun.* **27**, 405
- Schiff, J. A. (1970) *Symp. Soc. Exp. Biol.* **24**, 295
- Schuit, K. E. & Buetow, D. E. (1968) *Biochim. Biophys. Acta* **166**, 702
- Schuit, K. E., Avadhani, N. G. & Buetow, D. E. (1970) *Arch. Mikrobiol.* **71**, 79
- Scott, S. N., Munns, R. & Smillie, R. M. (1970) *FEBS Lett.* **10**, 149
- Scragg, A. H., Morimoto, H., Villa, V., Nekhorocheff, J. & Halvorson, H. O. (1971) *Science* **171**, 908
- Sebal, W., Schwab, A. J. & Bucher, Th. (1969) *FEBS Lett.* **4**, 243
- Smillie, R. M., Evans, W. R. & Lyman, H. (1963) *Brookhaven Symp. Biol.* **16**, 89
- Smith, A. E. & Marcker, K. A. (1968) *J. Mol. Biol.* **38**, 241
- Spiess, E. & Richter, G. (1970) *Arch. Mikrobiol.* **75**, 37
- Stutz, E. & Noll, H. (1967) *Proc. Nat. Acad. Sci. U.S.* **57**, 774
- Swanson, F. R. & Dawid, I. B. (1970) *Proc. Nat. Acad. Sci. U.S.* **66**, 117
- Vasconcelos, A. C., Pollack, M., Mendiola, L. R., Hoffmann, H. P., Brown, D. H. & Price, C. A. (1971) *Plant Physiol.* **47**, 217
- Wintersberger, E. & Viehhauser, G. (1968) *Nature (London)* **220**, 699
- Zeldin, M. H. & Schiff, J. A. (1967) *Plant Physiol.* **42**, 922