Biochem. J. (1970) **116**, 111–124
Printed in Great Britain

Amino Acid Incorporation by Ribosomes and Polyribosomes from Wheat Chloroplasts

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(*Received* 16 *July* 1969)

Sucrose-gradient and analytical ultracentrifugation showed that chloroplast polyribosomes from 4-day-old seedlings had mono-, di-, tri-, tetra- and traces of penta-ribosomes, in contrast with those from 7-day-old seedlings in which only the mono-, di- and traces of tri-ribosomes were present. Without Mg^{2+} the polyribosomes dissociated into ribosomal subunits. The rate of L-[U-^{14}C]phenylalanine incorporation was threefold greater for preparations from 4- than from 7-day-old seedlings. Incorporation by the latter was stimulated by polyuridylic acid. The rates of incorporation were similar whether the reaction mixture contained chloroplast or wheat-germ transfer RNA and amino acid synthetases purified on methylated albumin-on-kieselguhr and Sephadex G-75 columns respectively. The cofactor requirement was the same as for isolated intact chloroplasts. Osmotic rupture of chloroplasts with and without Triton X-100 revealed the presence of free and bound ribosomes. Free single ribosomes isolated by osmotic shrinkage or prepared by pancreatic ribonuclease digestion of chloroplast polyribosomes had negligible incorporation activity. This activity was increased by washing or by polyuridylic acid, but was still only a fraction of that given by polyribosomes. A comparison of incorporation activity of chloroplast polyribosomes with those from the surrounding cytoplasm showed the former to be 20 times more active.

Intact isolated chloroplasts are able to synthesize protein. If this synthesis is of the normal type one may assume that chloroplasts contain template RNA, ribosomes, amino acid-activating enzymes and tRNA (Kirk & Tilney-Bassett, 1967). It was also demonstrated by Boardman, Francki & Wildman (1966) that single ribosomes sedimented from chloroplasts had an incorporating activity that was considerably less than that of ribosomes left in the soluble fraction of ruptured chloroplasts. Since analytical ultracentrifugation by them revealed the presence of polyribosomes in the soluble fractions we assumed that the chloroplast mRNA molecule was 'read' simultaneously by a number of ribosomes. If RNA molecules holding these particles together were mRNA, then for isolated polyribosomes it should be possible to obtain a protein-synthesizing system *in vitro* having its own template RNA.

Previously we isolated and characterized the chloroplast and cytoplasmic polyribosomes from wheat leaves (Mehta, Hadziyev & Zalik, 1968). The study has now been extended to investigate a protein-synthesizing system of chloroplast polyribosomes isolated from wheat seedlings at two early stages of growth. The system involved purified chloroplast tRNA and a partially purified preparation of chloroplast aminoacyl-tRNA synthetases or similar preparations from wheat germ. In addition the amino acid-incorporating activity of single ribosomes obtained from polyribosomes by ribonuclease digestion and by osmotic shrinkage of intact chloroplasts was studied.

MATERIALS AND METHODS

Chemicals. L-[U-^{14}C]Phenylalanine (315mCi/mmol) was obtained from Calbiochem (Los Angeles, Calif., U.S.A.) and U-^{14}C-labelled reconstituted protein hydrolysate (100μCi/ml) from Schwarz BioResearch (Orangeburg, N.Y., U.S.A.). Non-labelled amino acids, ATP, CTP, GTP, yeast nucleic acid, poly U and creatine phosphokinase (from rabbit muscle; 50 units/mg of protein) were obtained from Calbiochem. Bovine serum albumin (fraction V), orcinol, GSH, pancreatic ribonuclease (5× recrystallized) and streptomycin sulphate (U.S.P.) were obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.). Trypsin (crystalline) was purchased from C. F. Boehringer und Soehne G.m.b.H., (Mannheim, Germany), and Triton X-100 from Hartman Leddon Co., Philadelphia, Pa., U.S.A. Other chemicals were analytical grade.

The solutions were prepared with freshly distilled demineralized and sterilized water and were stored frozen in small batches.

Plant material. Seedlings of three varieties of *Triticum vulgare* Vill., Manitou, Selkirk and Thatcher and one variety of *Triticum durum* Desf., Stewart 63, were grown in sterilized soil in a growth chamber at 21°C, relative humidity 77% and continuous illumination at 1500ft-candles. The leaves were harvested on the fourth and seventh day after planting.

Chloroplast isolation. Fresh leaf material was ground in a mortar with buffer in a ratio 1:2 (w/v). The isolation procedure was as reported by Hadziyev, Mehta & Zalik (1969) and the following buffers were used: (I) 2.5% (w/v) Ficoll, 5% (w/v) dextran, 0.25M-sucrose, 25mM-tris–HCl buffer, pH7.8, 10mM-$MgCl_2$ and 4mM-2-mercaptoethanol; (II) 0.4M-sucrose, 25mM-tris–HCl buffer, pH7.8, 10mM-$MgCl_2$ and 4mM-2-mercaptoethanol; (III) 67mM-potassium phosphate buffer, pH7.6, containing 0.4M-sucrose.

Isolation of polyribosomes. The chloroplasts were isolated in buffer IV (0.4M-sucrose, 0.1M-tris–HCl buffer, pH7.8, 50mM-KCl, 10mM-$MgCl_2$ and 4mM-2-mercaptoethanol) and disrupted in the presence of 2% (w/v) Triton X-100 and 1mg of bentonite/ml in hypo-osmotic buffer V (10mM-tris–HCl buffer, pH7.6, 10mM-$MgCl_2$, 50mM-KCl and 4mM-2-mercaptoethanol). The polyribosomal pellet was resuspended in buffer VI (25mM-tris–HCl buffer, pH7.6, 10mM-$MgCl_2$, 50mM-KCl and 4mM-2-mercaptoethanol) by using the procedure described by Mehta *et al.* (1968), with the exception that the pellet was rinsed three times without detachment from the tube walls, first with 0.44M-sucrose, then with buffer VI, before the final centrifugation at 144000*g*. Since Triton X-100 is adsorbed reversibly on the surface of polyribosomes additional rinsing was used to remove the adsorbed detergent.

Centrifugation was on a Spinco model LC-265B centrifuge with Ti60 rotor. Polyallomer tubes and chromic acid-washed glassware were used in all other operations.

Preparation of ribonuclease-treated ribosomes. These were prepared by addition of enzyme in the amounts specified in the tables to a polyribosomal suspension in 45mM-tris–HCl buffer, pH7.5, containing 10mM-$MgCl_2$ and 50mM-KCl (medium 1). After incubation at 37°C for 20min in polyallomer tubes the ribosomes were sedimented at 144000*g* for 40min at 0°C. The pellet was rinsed without detachment with 0.44M-sucrose, then with medium 1, and finally was resuspended in the same medium by gentle homogenization.

Preparation of washed ribosomes. Ribonuclease-treated ribosomes suspended in medium 1 were recentrifuged at 144000*g* for 40min. The supernatant was removed by suction and the pellet was suspended in the same medium.

Isolation of chloroplast tRNA. Chloroplasts were isolated and washed three times in buffer I. The pellet was suspended in demineralized water and disrupted in the presence of bentonite by adding 100ml of buffer V. After 30min the suspension was centrifuged at 20000*g* and the supernatant was further centrifuged at 144000*g* for 2h. The amber-yellow supernatant was mixed with 1mg of bentonite/ml of solution and an equal volume of ice-cold aq. 80% (w/v) phenol, shaken gently for 30min and centrifuged at 17000*g* for 15min. The aqueous layer was collected and deproteinized three times with cold phenol. From the final aqueous layer RNA was precipitated by addition of 0.1vol. of 10% NaCl, then 2.5vol. of chilled ethanol, and was left overnight at −20°C. The precipitate was washed several times with ethanol, then dissolved in 50mM-tris–HCl buffer, pH7.8, and deproteinized by shaking three times in each of phenol and chloroform–pentanol (24:1, v/v). The RNA was then precipitated with ethanol and stored at −20°C. A portion of RNA was further purified to remove bound amino acids by incubation at 37°C for 40min in 10ml of 50mM-tris–HCl buffer, pH8.9. The mixture was adjusted to pH7.6 with 0.1M-HCl, dialysed overnight against water, concentrated by freeze-drying and stored at −20°C. Another portion of RNA, not incubated, was purified by fractionation on an MAK* column. Effluent from the appropriate tubes was combined, dialysed against water, freeze-dried, redissolved in water and stored at −20°C.

Wheat-germ tRNA was isolated and purified as described by Glitz & Dekker (1963).

Preparation of aminoacyl-tRNA synthetases (pH5 *enzyme*). Chloroplasts were isolated in buffer I and ruptured by osmotic shock as described above. The chloroplast fragments were removed by centrifugation at 20000*g* for 30min and the supernatant was further centrifuged at 140000*g* for 2h. The resulting supernatant was decanted and adjusted to pH4.8 with 1M-acetic acid at 0°C. After 30min the precipitate was collected by centrifugation at 6000*g* for 10min and the pellet was resuspended in ice-cold water and adjusted to pH7.8 with 10% $KHCO_3$. The insoluble residue was removed at 6000*g* for 10min and the clear supernatant was acidified with acetic acid. The precipitate was collected by low-speed centrifugation, redissolved at pH7.8, again acidified and the above procedure repeated a second time. The final protein pellet was dissolved in 25mM-tris–HCl buffer, pH7.8, and subjected to further purification. The RNA was removed by precipitation with streptomycin sulphate (Melik-Sarkisyan, Kuznetsova, Avdeeva & Sissakian, 1967). A 5% (w/v) solution of streptomycin sulphate in the same buffer was added to the chilled protein to a final concentration of 1.5%. After 20min of continuous stirring the precipitate was removed by centrifugation at 10000*g* for 10min. The low-molecular-weight contaminants were removed by dialysis against 25mM-tris–HCl buffer, pH7.5, or by gel filtration on a 2.5cm × 25cm column of Sephadex G-75 with the same buffer. Elution was at a rate of 55ml/h and the effluent was monitored at 280nm. Fractions (5ml) were collected and tubes 7–14 were combined, made up to 10% (v/v) glycerol and stored at −20°C.

Aminoacyl-tRNA synthetases from wheat germ were isolated as follows: 20g of commercial wheat germ was blended in 60ml of 50mM-tris–HCl buffer, pH7.6, containing 0.5M-sucrose, 5mM-$MgCl_2$, 2mM-$CaCl_2$ and 6mM-2-mercaptoethanol. The slurry was pressed through ten layers of cheesecloth and centrifuged three times at 20000*g* for 20min, the sediment and the floating layer of starch and lipids being discarded each time. Finally, the supernatant was centrifuged at 144000*g* for 1h, the floating starch was removed and the yellow supernatant collected and dialysed overnight against 20mM-potassium phosphate buffer, pH7.6, containing 6mM-2-mercaptoethanol. The dialysed supernatant was applied to a

* Abbreviation: MAK, methylated albumin on kieselguhr.

2 cm × 5 cm DEAE-cellulose column (Cellex D, BioRad exchange capacity 0.96 mequiv./g that had been equilibrated with the same buffer and was then washed with 50 ml of the buffer. The synthetase fraction was eluted with the buffer containing 0.5 M-KCl (Allende & Bravo, 1966) at a flow rate of 55 ml/h and fractions (8 ml/tube) were collected. Tubes 3–6 were combined and further dialysed against 10 mM-tris–HCl buffer, pH 7.5, containing 1 mM-GSH. Finally, it was made up to 10% (v/v) glycerol and stored at −20°C.

ANALYTICAL PROCEDURES

Aminoacylation of tRNA. The reaction mixture consisted of 500 μg of tRNA, 0.1–1.0 mg of aminoacyl-tRNA synthetase preparation, 100 μmol of tris–HCl buffer, pH 8.0, 10 μmol of $MgCl_2$, 10 μmol of ATP, 0.3 μmol of CTP, 2 μmol of GSH, a mixture of 13 U-^{14}C-labelled L-amino acids (reconstituted protein hydrolysate) with a radioactivity of 450000 c.p.m./assay mixture in a final volume of 1 ml (Vold & Sypherd, 1968). Incubation was at 20°C and portions (50 μl) were pipetted on Whatman 3 MM filter-paper discs and, after adsorption of the sample, the reaction was terminated by dropping the discs into cold 10% trichloroacetic acid containing 13 non-labelled amino acids. The discs were prepared for scintillation counting as described by Mans & Novelli (1961).

Amino acid incorporation by polyribosomes. The incubation medium was essentially that for the wheat-embryo system used by Allende & Bravo (1966). It contained 50 μmol of tris–HCl buffer, pH 7.5, 25 μmol of KCl, 10 μmol of $MgCl_2$, 1.0 μmol of ATP, 0.5 μmol of GTP, 0.125 μmol each of 19 protein amino acids (except phenylalanine), 5 μmol of creatine phosphate, 10 μg of creatine phosphokinase, 150 μg of wheat chloroplast tRNA, 1 mg of chloroplast aminoacyl-tRNA synthetase protein, 150 μg of polyribosomal protein and 0.2 μCi of [U^{14}C]-phenylalanine in a final volume of 1.0 ml. The components were combined at 0°C and incorporation was started by adding labelled amino acid and warming the mixture to 30°C. After 30 min the reaction was terminated by cooling the tubes and adding 1 ml of 10% (w/v) trichloroacetic acid plus 0.5 ml of unlabelled 0.1 M-phenylalanine. The precipitate was further treated as described by Spencer & Wildman (1964). After being heated at 80°C for 30 min with 5% (w/v) trichloroacetic acid plus 0.05% unlabelled phenylalanine, the precipitate was collected and washed on a millipore filter type HA and the radioactivity determined by scintillation counting with standard Liquifluor mixture in dioxan.

Amino acid incorporation by intact chloroplasts. Incorporation studies were essentially as described for polyribosomes. Additional details are presented with the results.

Fractionation on MAK columns. Either the three-layered MAK column described by Mandell & Hershey (1960) as used previously (Hadziyev *et al.* 1969) or the simplified column suggested by Yamane & Sueoka (1963) was employed. To a boiled and chilled suspension of 6 g of kieselguhr in 30 ml of 50 mM-sodium phosphate buffer, pH 6.7, 1.5 ml of aq. 1% methylated albumin was added by continuous stirring. The suspension was packed in a 2 cm × 24 cm column to a depth of 7 cm. It was covered by a barrier layer prepared from 1 g of kieselguhr in the phosphate buffer containing 0.2 M-NaCl. The RNA in the effluent was monitored at 260 nm and the salt concentration with a Zeiss–Abbe refractometer.

Analytical ultracentrifugation. A Spinco model E ultracentrifuge with schlieren optics was used. The sedimentation coefficients were corrected for viscosity and density of water at 20°C. The runs for aminoacyl-tRNA synthetase preparations were carried out at 20°C and those for polyribosomes and their incorporating reaction mixtures at 4°C. Other analytical methods used are given in the Results section.

RESULTS

The wheat seedlings were grown under continuous illumination, as light was shown to inhibit particulate-bound ribonuclease activity (Udvardy, Farkas, Marre & Forti, 1967). In addition, bentonite was used as a ribonuclease inhibitor throughout the isolation procedure. As shown in Table 1 the yield of polyribosomes was higher from the younger seedlings and their E_{260}/E_{280} and E_{260}/E_{235} ratios were closer to 2.0 and 1.65 respectively, which characteristics may be taken to represent less-degraded polyribosomal preparations (Mehta *et al.* 1968). However, the RNA/protein ratios, which were close to 1.0, did not change with age.

Manitou wheat yielded consistently the highest amount of polyribosomes. Among the other varieties there was no essential difference between the *T. vulgare* sp. and the *T. durum*, which lacks a whole genome. Analytical ultracentrigufation of polyribosomal preparations showed mono-, di-, tri-, tetra- and occasionally penta-ribosomes. In addition to the monoribosomal subunits traces of protein with sedimentation coefficients 19S and 16S were present. In older leaves (7-day-old seedlings) the faster-moving particles were absent, and besides the enriched monoribosomes only di- and usually tri-ribosomes were present (Figs. 1*a* and 1*b*).

Amino acid incorporation was markedly affected by the age of the seedlings (Table 2). The polyribosomal preparation from young seedlings was about three times as active as that from older seedlings. The addition of 50 μg of poly U resulted in a twofold increase in incorporation by polyribosomal preparations from 7-day-old seedlings, whereas the incorporation by the preparation from 4-day-old seedlings was increased by only 15%. Additional amounts of poly U did not result in a marked further increase.

The incorporation was strongly dependent on ATP and less dependent on GTP, whereas there was essentially no incorporation when the aminoacyl-tRNA synthetase (pH 5 enzyme preparation) was omitted from the reaction mixture.

Poly U-induced stimulation of incorporation by the extract from 7-day-old seedlings was not surprising, since these extracts contained large amounts of free monoribosomes. However, there was no

Table 1. *Yield, spectral characteristics, RNA and protein content of chloroplast polyribosomes from wheat*

The polyribosome yield is given in E_{260} units of chloroplasts isolated from 100g of fresh leaves. RNA was determined spectrophotometrically by the method of Spirin (1958). Washings of polyribosomes with cold 0.2M-$HClO_4$ to remove acid-soluble nucleotides decreased the apparent RNA content by an average of 27.7 μg/E_{260} unit. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) on samples of polyribosomes dissolved in buffer VI without 2-mercaptoethanol with crystalline bovine plasma albumin as a standard.

Variety	polyribosome yield (E_{260} units)	Spectral characteristics E_{260}/E_{280}	Spectral characteristics E_{260}/E_{235}	RNA (μg/E_{260} unit)	Protein (μg/E_{260} unit)	RNA/protein ratio
		4-day-old seedling				
Manitou	124	2.00	1.66	37.4	39.9	0.94
Selkirk	62	1.98	1.64	42.7	42.8	1.00
Stewart 63	57	1.99	1.63	44.4	39.9	1.11
Thatcher	57	1.97	1.64	46.4	40.7	1.14
		7-day-old seedling				
Manitou	67	1.72	1.55	44.6	39.2	1.14
Selkirk	30	1.87	1.57	47.8	43.0	1.11
Stewart 63	41	1.63	1.40	41.3	42.5	0.97
Thatcher	42	1.85	1.56	37.4	41.8	0.90

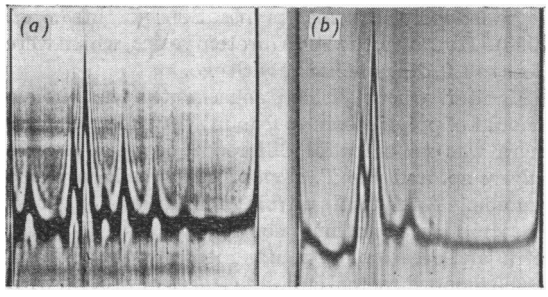


Fig. 1. Analytical-ultracentrifuge pattern of chloroplast polyribosomes of 4-day-old (*a*) and 7-day-old seedlings (*b*) of Manitou wheat. Polyribosomes isolated from five-times washed chloroplasts were in 25mM-tris–HCl buffer (pH 7.6)–50mM-KCl–10mM-$MgCl_2$–4mM-2-mercaptoethanol. The direction of sedimentation is from left to right. Pictures were taken 8min after a speed of 39460rev./min was reached at a bar angle of 50° with schlieren optics. The concentration of polyribosomal preparations was 35 E_{260} units for (*a*) and 70 E_{260} units for (*b*).

stimulation in extracts from 4-day-old seedlings, which also contained a significant amount of monoribosomes. The increase in [^{14}C]phenylalanine incorporation in the presence of poly U may have been due to poly U programming a number of monoribosomes to form polyribosomes, to which protein synthesis is confined. However, analytical ultracentrifugation of such preparations from 7-day-old seedlings did not reveal any newly formed peak nor any increase of existing peaks.

Sedimentation profiles of chloroplast polyribosomes determined by sucrose-gradient centrifugation are presented in Fig. 2. Besides the heavy-polyribosome region and the diribosome peak, there were peaks corresponding to single ribosomes and their subunits. In contrast with 4-day-old seedlings the polyribosomes from older seedlings had a large amount of monoribosomes, diribosomes and the peak coinciding with triribosomes. In both cases the extinction and radioactivity curves coincided with those obtained for incubation mixtures without exogenous template, but higher radioactivity was obtained with poly U in the monoribosome region of 7-day-old seedlings.

As shown in Table 2 the incorporation by polyribosomes was extremely sensitive to pancreatic ribonuclease. Sucrose-gradient centrifugation showed that treatment of the polyribosomes with ribonuclease at 0°C caused a shift of extinction units to the monoribosome region. Additionally a small increase in diribosomes appeared as a small subpeak as a shoulder on the monoribosome peak. When the polyribosomes were treated with a mixture of trypsin and ribonuclease (each at 2μg/ml of incubation mixture) at 0°C the diribosomes were completely fragmented into single ribosomes. Trypsin alone did not result in complete fragmentation (Figs. 2*a* and 2*b*). Similar results were obtained even without trypsin if the incubation was done with ribonuclease at 37°C for 20min.

Amino acid-incorporating activity coincided with the polyribosomal peaks (Fig. 2*c*). There was no apparent concentration of incorporation activity on any of the polyribosomal peaks. With ribonuclease-treated polyribosomes radioactivity was confined to monoribosomes, whereas in the presence of trypsin

Table 2. *Effect of age, energy donor and regenerating system, poly U and ribonuclease on amino acid incorporation by polyribosomes from chloroplasts of Manitou wheat*

The polyribosomes were isolated in the presence of bentonite and Triton X-100 and suspended in buffer VI. The results are corrected for bacterial contamination and non-specific (zero-time) adsorption (values for bacterial contamination were obtained by adding polyribosomes to the incubation mixture at the end of the incubation period before addition of trichloroacetic acid, whereas non-specific (zero-time) adsorption was measured by adding [U-^{14}C]phenylalamine at the end of the incubation period after the addition of trichloroacetic acid.) The chloroplast tRNA used was purified on a MAK column and the chloroplast aminoacyl-tRNA synthetase was purified on a column of Sephadex G-75 followed by streptomycin sulphate treatment.

	Amino acid incorporation			
	4-day-old seedling		7-day-old seedling	
	(c.p.m./mg of polyribosomal protein)	(%)	(c.p.m./mg of polyribosomal protein)	(%)
Complete system				
Incubation 15 min	12500			
30 min	35500	100	10520	100
45 min	37400	(control)		
Complete system				
Incubation 30 min				
−(creatine phosphate, creatine phosphokinase)	26625	75		
−(ATP, creatine phosphate, creatine phosphokinase)	2735	8		
−GTP	21300	60		
−(aminoacyl-tRNA synthetase)	151			
Complete system				
Incubation 30 min				
+50 μg of poly U	40825	115	19995	190
+100 μg of poly U	48280	136	22013	209
Complete system				
Incubation 30 min				
+5 μg of ribonuclease	4450	12	1367	13
+50 μg of ribonuclease	1280	4		

even monoribosomes lacked radioactivity, all of it being found at the top of the gradient.

As found by Lyttleton (1960) ribosomes from wheat germ were effectively preserved in the presence of 1 mM-Mg^{2+}, 0.2 mM-Mn^{2+} and 0.5 mM-Ca^{2+}. In the absence of Mg^{2+} or Ca^{2+} complete dissociation of wheat-germ ribosomes was not prevented by addition of Mn^{2+}. To determine the extent to which the findings for wheat embryonic tissue are reflected by wheat chloroplast polyribosomes their response to these ions was checked.

The polyribosomes at a concentration of 1 mg of polyribosomal protein/ml were dialysed for 5 h against a few changes of buffer VI from which K^+ and Mg^{2+} ions had been omitted, and the dialyzed sample was examined in the ultracentrifuge. Large amounts of ribosomal subunits with sedimentation coefficients 50S and 35S, and a small amount of intact ribosomes (66S), were obtained, whereas the amount of undissociated 80S ribosomes remained essentially the same. However, among the dialysed products there were no polyribosomal particles. It might therefore be expected that lack of Mg^{2+} in the suspending medium for polyribosomes should affect the incorporation activity of these particles. To test this effect polyribosomes were sedimented in the presence of Mg^{2+} in buffer VI as usual, then resuspended in medium containing Mg^{2+}, Ca^{2+} or Mn^{2+}. The resulting incorporation is presented in Table 3.

As shown, Mn^{2+} or Ca^{2+} alone did not effectively restore the initial activity of polyribosomes obtained in the presence of 10 mM-Mg^{2+}. With both ions the activity increased and exceeded the activities observed for each separately. Finally, 1 mM-Mg^{2+} plus Ca^{2+} and Mn^{2+} was not as effective as 5 mM-Mg^{2+} alone, a concentration that we found to repress effectively monoribosome dissociation. Thus the Mg^{2+} requirement for maximum amino acid incorporation appears higher than that required to prevent dissociation of chloroplast monoribosomes. The additional Mg^{2+} requirement might be involved in the binding of mRNA or tRNA to monoribosomes, polyribosomes or both.

A series of fractionations was conducted to isolate

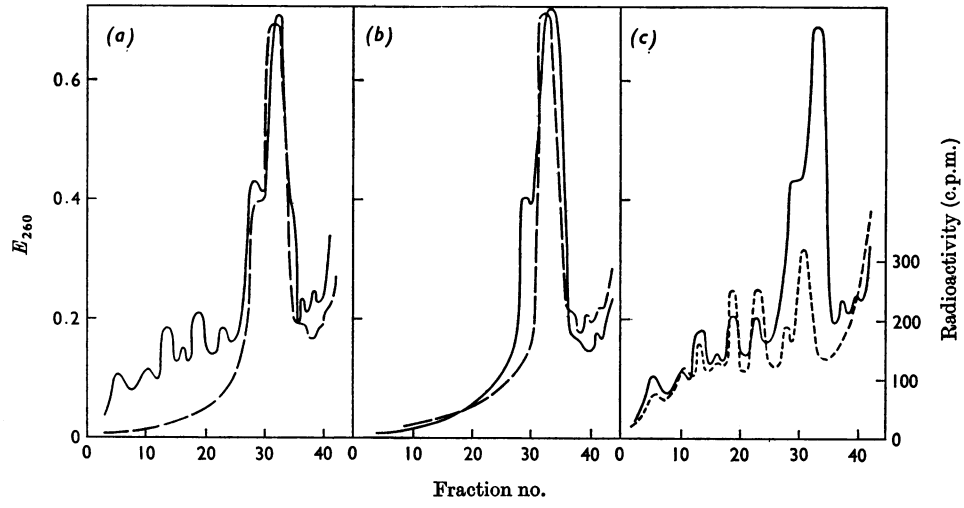


Fig. 2. Sucrose-density-gradient sedimentation of chloroplast polyribosomes from Manitou wheat. (*a*) Polyribosomes (20 E_{260} units) suspended in buffer VI were layered over a linear 10–34% (w/v) sucrose gradient in the same buffer prepared as described by Britten & Roberts (1960) and centrifuged for 2½h at 27000rev./min in a Spinco SW-27 rotor. The bottoms of the tubes were punctured and fractions (6 drops) were collected, diluted to 3ml with buffer VI and the extinction was read at 260nm. For ribonuclease-treated polyribosomes portions (15 E_{260} units) were incubated with 2μg of ribonuclease/ml at 0°C for 1h (——), control; – – –, ribonuclease-treated; (*b*) Polyribosomes treated with ribonuclease, without (——) and with (– – –) added trypsin each at 2μg/ml at 0°C for 1h; (*c*) distribution of labelled amino acid in polyribosomes. A portion (15 E_{260} units) of polyribosomes was incubated with [U-^{14}C]phenylalanine in incorporation medium at 37°C for 10min, chilled on ice for 20min, layered on the sucrose gradient and centrifuged as above. Fractions (6 drops) were collected in scintillation vials, mixed with 5ml of a standard Liquifluor mixture in dioxan and the radioactivity was determined. ——, E_{260}; – – –, radioactivity.

Table 3. *Effect of bivalent ions on amino acid incorporation by polyribosomes isolated from chloroplasts of 4-day-old seedlings of Manitou wheat*

The polyribosome pellet isolated in buffer VI as usual was washed and resuspended in the same buffer from which KCl and $MgCl_2$ were omitted.

	Amino acid incorporation (c.p.m./mg of polyribosomal protein)	(%)
Complete system (incubation 30min)	35550	100 (control)
0mM-Mg^{2+}	2300	6
5mM-Mg^{2+}	12913	36
0.5mM-Ca^{2+}	2675	8
0.2mM-Mn^{2+}	1968	6
1mM-Mg^{2+}, 0.2mM-Mn^{2+}	5980	17
0.5mM-Ca^{2+}, 0.2mM-Mn^{2+}	6633	19
1mM-Mg^{2+}, 0.5mM-Ca^{2+}, 0.2mM-Mn^{2+}	9517	27

and characterize the functional tRNA from chloroplasts. MAK-column fractionation of total wheat-leaf nucleic acids showed several peaks in the tRNA region (Fig. 3). The first main peak had a second as a shoulder, and the third and fourth appeared as distinct peaks followed by a fifth as a shoulder on the DNA peak. Chloroplasts from the same leaf material gave an elution profile in the tRNA region with the first two peaks corresponding to those found in whole leaf. The third peak was markedly increased in amount and had a subpeak shoulder. When purified chloroplasts were ruptured by osmotic shock and the fragments and soluble fraction were analysed separately, elution profiles as shown in Fig. 4 were obtained. The RNA from the fragments showed extensive enzymic degradation of their rRNA and accumulation of degradation products in the region of tRNA. On the other hand, the soluble fraction showed an elution pattern like that from the whole leaf.

The elution patterns of RNA did not change when the phenol–sodium dodecyl sulphate procedure was replaced by extraction with 6% (w/v) sodium 4-aminosalicylate and aq. 80% phenol with or without 1mg of bentonite/ml, or by aqueous phenol containing 0.1% 8-hydroxyquinoline and 0.5% naphthalene-1,5-disulphonic acid.

For the soluble fraction collected after centrifugation at 105000*g* for 2h the elution profile had the

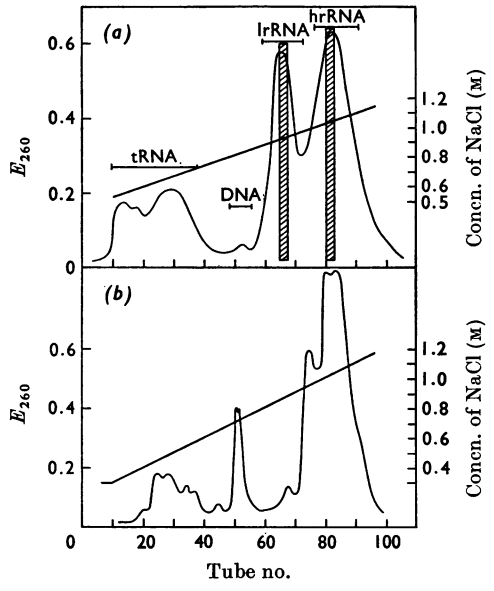


Fig. 3. MAK elution profiles of nucleic acids from leaves and chloroplasts of 4-day-old seedlings of Thatcher wheat. The nucleic acids, dissolved in 50mM-sodium phosphate buffer, pH6.7, containing 0.3M-NaCl and 10mM-$MgCl_2$, were adsorbed on a column (2cm×7cm) prepared as described by Yamane & Sueoka (1963) and eluted with 300ml of buffer containing a linear concentration gradient of NaCl. (*a*) Chloroplast nucleic acids. The NaCl gradient was 0.4–1.6M. A total of 50 E_{260} units was adsorbed on the column and fractions (2ml/tube) were collected at an elution rate of 0.8ml/min, the shaded area represents combined effluents used for the assay of nucleotide composition. lrRNA (16S) and hrRNA (23S) represent light and heavy ribosomal RNA. (*b*) Total leaf nucleic acids. The gradient NaCl was 0.3–1.2M. A total of 30 E_{260} units was adsorbed and fractions were collected as described for (*a*). Similar elution patterns were obtained with the three-layered column of Mandell & Hershey (1960).

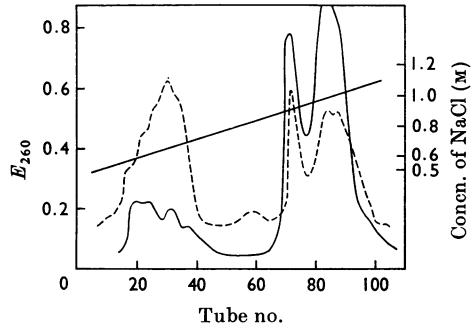


Fig. 4. Nucleic acid elution profiles of chloroplasts ruptured by osmotic shock. The chloroplasts were isolated in buffer III and ruptured with buffer V. The fragments (----) were sedimented at 20000*g* and the supernatant (——) was collected after centrifugation at 105000*g* for 30min. The nucleic acids were extracted in the presence of bentonite by the phenol–sodium dodecyl sulphate procedure. Other details were as described in Fig. 3 (*b*).

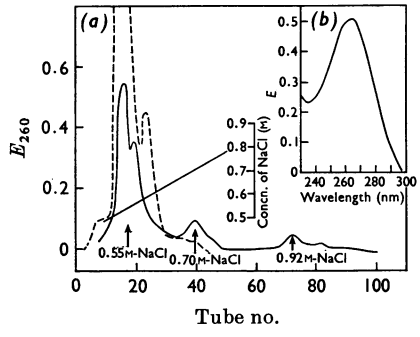


Fig. 5. MAK elution profiles of chloroplast and wheat-germ tRNA. (*a*) Chloroplast tRNA from 4-day-old Manitou wheat seedlings was isolated from the supernatant of ruptured chloroplasts at 105000*g* for 2h by the phenol–sodium dodecyl sulphate procedure. When a 144000*g* supernatant was used the peaks eluted at 0.7M- and 0.92M-NaCl were absent. The elution technique applied was as described in Fig. 3(*a*). (*b*) Ultraviolet absorption spectrum of chloroplast tRNA. The tRNA was dissolved in 50mM-sodium phosphate buffer, pH6.7, containing 0.3M-NaCl.

first peak and an associated subpeak as main peaks (Fig. 5). Since this centrifugation did not remove all of the ribosomes, the low-molecular-weight RNA eluted at 0.7M-sodium chloride after the two peaks could be of ribosomal origin. When the soluble fraction was centrifuged at 144000*g* for 2h, all the ribosomes sedimented, hence the MAK elution profile gave the first two peaks whereas the third was absent. In contrast the elution profile for chloroplast mono- and poly-ribosomes lacked the first two peaks but contained the third, low-molecular-weight rRNA (Fig. 6).

These results indicate that for aminoacyl-tRNA transfer reactions the chloroplast soluble fraction obtained at 144000*g* is a reliable source for isolation of functional tRNA, whereas whole chloroplasts, their fragments and even the soluble fraction obtained at 105000*g* are not adequate sources.

A further assessment of the purity of a tRNA preparation can be obtained from the nucleotide composition. As shown in Table 4 the tRNA had a high content of guanylic acid (Gp) and cytidylic acid (Cp). The high Gp content is typical also for 17S

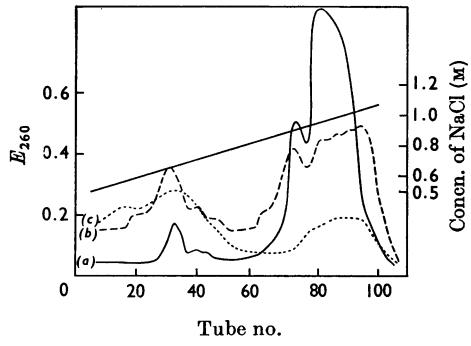


Fig. 6. MAK elution profiles of polyribosomal RNA. The RNA (75 E_{260} units) was adsorbed on a column (2.2 cm × 15 cm) prepared as described by Mandell & Hershey (1960) and eluted with 500 ml of 50mM-phosphate buffer with 0.4–1.6M gradient of NaCl. Fractions (3 ml/tube) were collected at an elution rate of 0.8 ml/min. (*a*) (——) RNA extracted from the purified polyribosome pellet by the phenol–sodium dodecyl sulphate procedure in the presence of 1 mg of bentonite/ml with mild shaking at 0°C. (*b*) (-----) As above, but deproteinization done with stronger shaking. The bulk of the degradation products were eluted at 0.65M-NaCl. (*c*) (....) Deproteinization done by vigorous shaking. Large amounts of degradation products were washed out by 0.4M-NaCl, whereas the rest was eluted in the range 0.5–0.85M-NaCl.

and 23S rRNA. But the ratio (Ap+Up+ψp)/(Gp+Cp) for tRNA is consistently lower than these ratios for 17S and 23S rRNA. The latter differ among themselves, the 23S rRNA having a higher (Gp+Cp) content and consequently lower Ap, Up and ψp contents than the 17S rRNA. However, any slight increase in the ratio of 0.75 for tRNA indicates the presence of fragments due to enzymic degradation of one or both species of rRNA. Such contamination of tRNA by rRNA degradation products was extensive even in the presence of bentonite (Fig. 4). Moreover, contamination of tRNA from rRNA of purified polyribosomes and ribosomes resulted when the phenol–sodium dodecyl sulphate deproteinization was done by vigorous shaking (Fig. 6).

On the basis of the above findings chloroplast tRNA isolated from the 144000*g* soluble fraction followed by purification on MAK columns and depletion of endogenous amino acids was used throughout the incorporation studies. The acceptor ability of the purified chloroplast tRNA compared with tRNA isolated from wheat germ is presented in Table 5.

Previously we found (Hadziyev *et al.* 1969) that in young wheat seedlings up to 37% of the total RNA is a template-like rapidly metabolizable RNA. During isolation and osmotic rupture of chloroplasts the bulk of this type of RNA may yield fragments as

Table 4. *Nucleotide composition of tRNA and rRNA from wheat chloroplasts*

The ribosomal low-molecular-weight and high-molecular-weight RNA samples collected from a MAK column (Fig. 3*a*) were prepared by phenol–sodium dodecyl sulphate extraction of the purified polyribosomes in the presence of bentonite, and the tRNA sample was obtained from the 144000*g* supernatant of the soluble fraction released by osmotic shock from intact chloroplasts. The nucleotide analyses were performed on the freeze-dried samples previously purified on MAK columns and dialysed against water. Portions of RNA were hydrolyzed in 1 ml of aq. 0.3M-KOH by incubation at 37°C for 18 h. The neutralized hydrolysates were run on a column (5 mm × 7 cm) of Dowex 1 (X10; formate form; 200–400 mesh) with an elution rate of 0.4 ml/min. tRNA isolated from commercial wheat germ had a nucleotide composition (mole percentage): Cp, 24.1; Ap, 24.4; Up, 20.4; ψp, 3.0, Gp, 31.1.

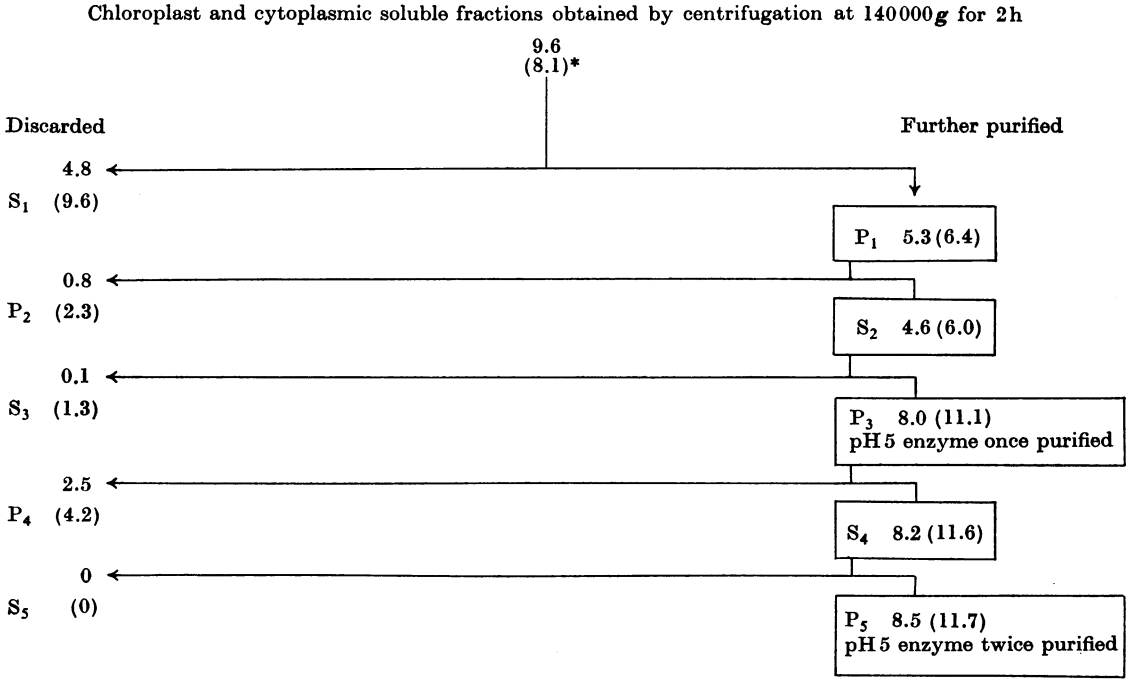
		rRNA		
Nucleotide (mixture of 2′- and 3′-acids)	tRNA	Low-molecular-weight	17S*	23S*
Amount of RNA hydrolysed (mg) ...	0.44	0.40	0.34	0.35
Mole percentage				
Cp†	25.4	25.7	22.5	23.5
Ap	21.2	21.1	21.7	22.1
Up	19.2	23.2	23.5	21.5
ψp	2.6	0	1.8	1.7
Gp‡	31.6	30.0	30.5	31.2
Purine/pyrimidine [(Ap+Gp)/(Cp+Up+ψp)]	1.12	1.04	1.09	1.14
6-Amino/6-oxo bases [(Ap+Cp)/(Gp+Up+ψp)]	0.87	0.88	0.79	0.84
(Ap+Up+ψp)/(Gp+Cp)	0.75	0.79	0.89	0.83

* Sedimentation coefficients obtained by analytical ultracentrigation.
† Does not include the non-cytidylic acid-like components.
‡ Includes GDP (Gpp).

Table 5. *Effect of aminoacyl-tRNA synthetases on the acceptor ability of tRNA isolated from chloroplasts and wheat germ*

Enzymes partially purified on Sephadex G-75 were used immediately or were frozen in 10% glycerol at −20°C and used within 3 days.

Enzyme preparation	Protein (mg/ml of reaction mixture)	Specific activity (c.p.m./mg of tRNA)	
		Chloroplast tRNA	Wheat germ tRNA
Chloroplast			
Crude supernatant factor	0.1	3850	5560
	0.5	4200	5600
	1.0	4360	5920
pH 5 enzyme preparation, once purified	0.1	2080	1810
	0.5	2200	1980
	1.0	2560	2210
pH 5 enzyme preparation, twice purified	0.1	1120	780
	0.5	1520	1010
	1.0	1640	1080
Wheat germ			
	0.1	5160	9720
	0.5	5200	11800
	1.0	5720	12360



Scheme 1. Endogenous ribonuclease activity during purification of pH 5 enzyme from shoots of 4-day-old seedlings of Thatcher wheat. The enzyme assay medium consisted of 0.125 M-cacodylic acid, 4 mg of dialysed high-molecular-weight yeast RNA/ml adjusted with imidazole to pH 5.8. After incubation at 37°C for 30 min the reaction was terminated by uranyl–perchloric acid reagent. Values are given as enzyme units/mg of protein (those in parentheses are for enzyme preparation from cytoplasm). S, supernatant; P, precipitate obtained by acetic acid and $KHCO_3$ treatments (see the Materials and Methods section).

a result of endogenous ribonuclease activity and these fragments are found in the supernatant. To avoid the effect of these fragments in incorporation studies, partially purified pH 5 enzyme preparations were used rather than the crude supernatant.

As presented in Scheme 1 successive precipitation

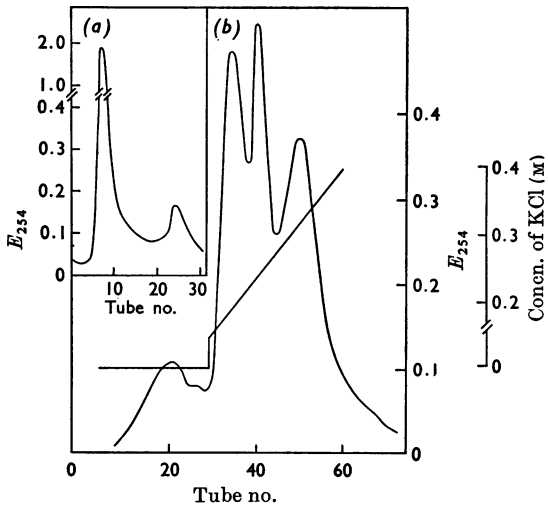


Fig. 7. (*a*) Sephadex G-75 column chromatography of the crude pH 5 enzyme from chloroplasts of Manitou wheat. Enzyme protein applied (140 mg) was eluted with 25 mM-tris–HCl buffer, pH 7.8, at 3°C at a flow rate of 55 ml/h and fractions (5 ml) were collected. (*b*) DEAE-cellulose column chromatography of the dialysed pH 5 enzyme sample collected from the first peak of the Sephadex column. The sample dissolved in 10 mM-potassium phosphate buffer, pH 7.0, was eluted by a linear 0.1–2.0 M gradient of KCl in the same buffer.

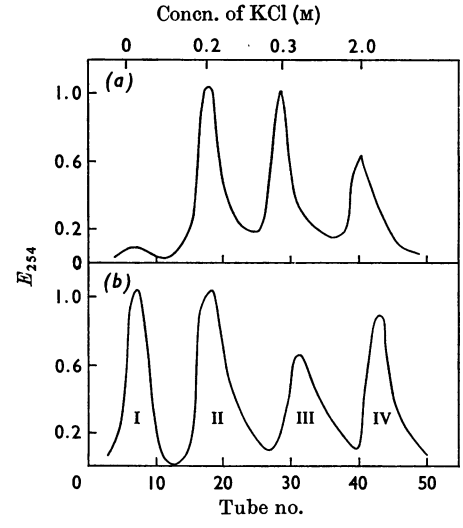


Fig. 8. DEAE-cellulose column chromatography of the pH 5 enzyme from Manitou-wheat chloroplasts (*a*) and cytoplasm (*b*) previously purified on a Sephadex column. A sample of protein (100 mg) dissolved in 10 mM-potassium phosphate buffer, pH 7.0, was loaded on a column (2 cm × 10 cm) of DEAE-cellulose, rinsed with buffer and the proteins were eluted successively by 0.2 M-, 0.3 M- and 2.0 M-KCl in the same buffer. Fractions (5 ml/tube) were collected.

of enzyme at pH 4.8 and solubilization at pH 7.8 resulted in a twice-purified pH 5 enzyme. Despite this purification the enzyme was still regarded as a crude preparation since it contained a high activity of endogenous ribonuclease relative to that initially found in the soluble fraction of ruptured chloroplasts. During purification in the cold activity increased. It was found earlier (Hadziyev *et al.* 1969) that the increase of activity by cold-treatment was specific for the ribonuclease B associated with chloroplasts, and not for ribonuclease A present in cytoplasm. The cytoplasmic pH 5 enzyme preparation showed a similar increase in activity, which suggests contamination by protein components from chloroplasts.

When a twice-purified enzyme preparation was subjected to purification by gel filtration on Sephadex G-75 two peaks were obtained, with all the proteins and residual RNA being eluted in the first peak (Fig. 7*a*). Chromatography of the fraction corresponding to this peak on a DEAE-cellulose column with continuous or stepwise elution (Figs. 7*b* and 8) by 0.2 M-, 0.3 M- and 2 M-potassium chloride revealed the presence of a number of protein components. The chloroplast preparation, in comparison with the cytoplasm, had a negligible amount of peak I, whereas peaks II, III and IV were present in both preparations (Fig. 8).

The chloroplast and cytoplasmic proteins purified on Sephadex columns by streptomycin sulphate treatment were studied with an analytical ultracentrifuge. There was one major fast-moving component with sedimentation coefficient 19S for cytoplasm and 16S for chloroplasts, followed by a minor peak with an average sedimentation coefficient 4S. When the removal of RNA by streptomycin sulphate was omitted a few additional faster-moving minor components were observed (Fig. 9).

The pH 5 enzyme preparations purified by gel filtration showed aminoacyl-tRNA transfer activity. As given in Table 5 the freshly prepared crude supernatant factor had higher activity than the partially purified preparations. Though the higher activity might be attributed to presence of tRNA in the supernatant, instability of the enzyme cannot be excluded. This is indicated by the lower activity of the preparation subjected to repeated purification.

When wheat-germ aminoacyl-tRNA synthetase was fractionated on a DEAE-cellulose column, with discontinuous elution, four protein components were obtained. The first was lowest in amount and corresponded to the first cytoplasmic component. The other three components corresponded to those obtained for proteins of cytoplasm and chloroplasts. Unlike the chloroplast preparations, the wheat-germ

synthetase had negligible ribonuclease activity and a higher aminoacyl-tRNA transfer activity.

To compare the incorporation by isolated purified polyribosomes with their capacity *in situ*, the incorporation capacity of intact chloroplasts was determined. The chloroplast preparations synthesized protein and like polyribosomes required Mg^{2+}, energy donor and a regenerating system and GTP for optimum activity (Table 6). The synthesis was higher by 13% for chloroplasts isolated in buffer I than in buffer II, the difference being more pronounced for chloroplasts from older leaves. Only 24% of the activity found for younger seedlings was obtained for chloroplasts of 7-day-old seedlings. The fivefold increase in endogenous ribonuclease activity associated with chloroplasts of older leaves (from 10 units for 4-day-old to 50 units for 7-day-old leaves) might have been the cause of low incorporation and the observed polyribosomal pattern (Fig. 1*b*) may have been a consequence of this activity.

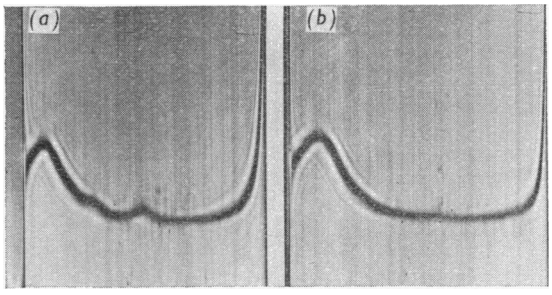


Fig. 9. Analytical-ultracentrifuge pattern of chloroplast aminoacyl-tRNA synthetase. The enzyme preparation was in 25mM-tris–HCl buffer (pH 7.6)–50mM-KCl–4mM-2-mercaptoethanol. The direction of sedimentation is left to right. Pictures were taken 16min after a speed of 47660rev./min was reached, at a bar angle of 50° with schlieren optics. The concentration of enzyme protein was 7mg/ml. (*a*) Before and (*b*) after purification on the Sephadex column and streptomycin sulphate treatment. Similar patterns were obtained for enzyme preparations isolated from wheat germ and cytoplasm.

Chloroplasts purified on a discontinuous sucrose gradient had a lower incorporating activity than the crude preparation. This finding suggested that during purification various factors, ribosomes or both, were lost. To determine if ribosomes were lost as a result of osmotic shrinkage during purification, chloroplasts were isolated from 4-day-old seedlings of Manitou wheat and washed with buffer III. They were resuspended and kept at 0°C in the buffer containing 10mM-magnesium chloride and 2M-sucrose. After 30min the sucrose was adjusted to 0.4M with the

Table 6. *Amino acid incorporation by intact chloroplasts*

The reaction mixture contained 50mM-tris–HCl buffer, pH 7.8, 7.5mM-$MgCl_2$, 25mM-KCl, 1mM-ATP, 0.5mM-GTP, 5mM-creatine phosphate, 10μg of creatine phosphokinase, 100μl of chloroplast suspension (88μg of chlorophyll assayed by the method of Arnon 1949) and 20μl of a uniformly labelled mixture of 13 amino acids (reconstituted protein, radioactivity 265000c.p.m.) in a final volume of 1ml. After incubation at 30°C for 30min one replicate of each sample was treated with 3% Triton X-100, centrifuged at 6000*g* and the radioactivity of the pellet substracted from that of the untreated sample. Incorporation is expressed as hot-trichloroacetic acid-insoluble radioactivity.

	Amino acid incorporation		
	Before purification		After purification†
Reaction mixture	(c.p.m./mg of chlorophyll)	(%)	(c.p.m./mg of chlorophyll)
	4-day-old seedling		
Complete (buffer I)	3560		2850
Complete (buffer II)‡	3080	100 (control)	2150
1μmol of Mg^{2+}	1295	42	
No Mg^{2+}	300	10	
–(ATP, creatine phosphate, creatine phosphokinase)	308	10	
–(ATP, creatine phosphate, creatine phosphokinase, GTP)	312	10	
–GTP	1045	34	
	7-day-old seedling		
Complete (buffer I)	850		640
Complete (buffer II)	625		585

* As an average the protein/chlorophyll weight ratio in the crude chloroplast was 9:1.

† Chloroplasts were purified on a discontinuous sucrose gradient containing 7ml each of 1.0M-, 1.5M-, 2.0M- and 2.5M-sucrose in buffer I or II.

‡ Isolated chloroplasts were washed, resuspended and used for incorporation assay in the same medium, with sucrose omitted and with or without $MgCl_2$.

Table 7. *Spectral characteristics, RNA and protein content, and amino acid-incorporation activity of ribosomes obtained from chloroplast polyribosomes by ribonuclease treatment at 0°C and 37°C*

The polyribosomal suspension in medium I (4mg of polyribosomal protein/ml.) was treated with 10μg of pancreatic ribonuclease and either washed or unwashed ribosomes were used (see the Materials and Methods section). Amino acid incorporation was assayed with 1mg of ribosomal protein/ml of incubation mixture as specified for polyribosomes. Endogenous ribonuclease activity (units/mg of protein) in polyribosomes was before treatment 2.1, and those of ribosomal preparations, unwashed or washed, were 3.6–4.2.

	Incubated at 0°C for 1h	
	Unwashed ribosomes	Washed ribosomes
Spectral characteristics		
E_{260}/E_{280}	1.91	1.70
E_{260}/E_{235}	1.38	1.34
Content (μg/E_{260} unit)		
RNA	42.2	36.3
Protein	57.0	47.2
RNA/protein ratio	0.74	0.77
Specific radioactivity (c.p.m./mg of protein)	70	180
Ribosomes+50μg of poly U	120	290
	Incubated at 37°C for 20min	
	Unwashed ribosomes	Washed ribosomes
Spectral characteristics		
E_{260}/E_{280}	1.89	1.62
E_{260}/E_{235}	1.30	1.23
Content (μg/E_{260} unit)		
RNA	35.5	34.0
Protein	35.8	41.6
RNA/protein ratio	0.99	0.82
Specific radioactivity (c.p.m./mg of protein)	41	600
Ribosomes+50μg of poly U	60	890

buffer. The chloroplasts were sedimented at 40000*g* for 10min and the supernatant was centrifuged at 144000*g* for 40min. The green ribosomal pellet was clarified by resuspension in buffer IV and centrifuged at 20000*g* for 15min. The supernatant had an RNA/protein ratio 0.86 and the spectral characteristics were E_{260}/E_{280} 2.01 and E_{260}/E_{235} 1.40, similar to values for monoribosomes prepared from polyribosomes by ribonuclease treatment (Table 7). The yield of free ribosomes, representing leakage due to osmotic shrinkage of chloroplasts, was 24.2 E_{260} units/100g fresh wt. These ribosomes showed amino acid-incorporation activity of 320c.p.m./mg of protein, which activity was increased to 420 and 860c.p.m./mg in the presence of 50 and 100μg of poly U respectively.

The above finding posed the question whether the ribosomes remaining within the intact chloroplasts were free or membrane-bound. Therefore the chloroplast pellet after release of free ribosomes was subjected to osmotic rupture in hypo-osmotic tris–hydrochloric acid buffer at pH 7.3, 7.5 and 7.8. There was ready release of ribosomes when Triton X-100 was added to the media and only a slow release when the chloroplast fragments were repeatedly washed with buffer lacking detergent.

Table 8. *Comparison of amino acid-incorporation capacities of intact chloroplasts and their isolated polyribosomes from Manitou wheat*

Yield of chloroplasts (mg of chlorophyll/100 g of fresh leaves)	4-day-old seedling	7-day-old seedling
Buffer I	70.0	47.9
Buffer II	68.7	43.6
Calc. incorporation capacity (c.p.m.)	245000	36640
Yield of polyribosomes*		
As E_{260} units/chloroplasts from 100g of fresh leaves	124	67
As E_{260} units/mg of chlorophyll	1.8	1.5
As mg of protein/chloroplasts from 100g of fresh leaves	5.2	2.8
Calc. incorporation capacity (c.p.m.)	183535	29351

* Chloroplasts were isolated in buffer II.

A comparison of the incorporation capacities of intact chloroplasts and their isolated polyribosomes from Manitou wheat is given in Table 8. The purified polyribosomes had about 75–80% of the incorporation capacity of the corresponding intact chloroplasts.

Table 9. *RNA and protein losses from polyribosomes digested by pancreatic ribonuclease*

Polyribosomes (2mg of protein/ml in medium I) were incubated with pancreatic ribonuclease. The ribosomes were sedimented at 144000*g*.

	Recovery (%)	
Ribonuclease (mg/ml)	RNA	Protein
Incubated at 37°C for 20min		
0	68.5	72.2
0.005	53.2	49.5
0.05	51.8	52.0
2	51.5	54.8
10	51.0	55.4
Incubated at 0°C for 1h		
0.005	81.5	78.5
10	51.7	50.8
10*	51.2	50.9
2+Trypsin (2mg/ml)	42.3	39.0

* With ribonuclease dissolved in 1mM-HCl heated at 100°C for 10min and used after cooling to 0°C.

Digestion of polyribosomal preparations with pancreatic ribonuclease resulted in considerable loss of polyribosomal protein and RNA (Table 9). As shown in Table 7 the loss of RNA resulted in a decrease in the RNA/protein ratio and was accompanied by a marked decrease in amino acid-incorporating activity compared with that of polyribosomes. Washing the ribosomes resulted in further loss of protein and RNA at both incubation temperatures and corresponding changes in the RNA/protein ratios. The amino acid incorporation at 0°C increased with washing to about the value obtained with free ribosomes obtained from intact chloroplasts by osmotic shrinkage. At 37°C the effect of washing was more marked. The addition of poly U to the washed ribosomes increased the activity to a value similar to that obtained for free ribosomes. However, the activity represented only a fraction of that obtained for their polyribosomal preparations.

Unless otherwise stated the tabular results and figures for other varieties were similar to those shown.

DISCUSSION

It is known that intact chloroplasts, as well as the soluble fraction from ruptured chloroplasts, can synthesize protein (Bamji & Jagendorf, 1966; Boardman *et al.* 1966) and it appears that this synthesis is of the normal type. It has also been reported that in imbibing wheat germ (Marcus & Feeley, 1966; Marcus, Feeley & Volcani, 1966) and in root tissue of certain plants (Leaver & Key, 1967) a given mRNA molecule is 'read' simultaneously by a number of ribosomes, resulting in the polyribosomal complex.

In the present study it was found that chloroplasts from 4-day-old wheat seedlings isolated in the presence of bentonite and disrupted by osmotic shock with Triton X-100 released polyribosomes ranging from di- to penta-ribosomes. On the other hand, chloroplasts from 7-day-old seedlings, which had a fivefold higher endogenous ribonuclease activity, yielded mainly single ribosomes along with di- and traces of tri-ribosomes. It appears that the endogenous ribonuclease was releasing single ribosomes by degrading the mRNA that held the polyribosomes together.

The amino acid incorporation by polyribosomal preparations from 4-day-old seedlings was threefold higher than that for 7-day-old seedlings. The activity of both preparations was increased by addition of poly U, and as expected this increase was higher for the preparations from the older seedlings, which had a higher content of free ribosomes. Incorporation was similar whether the mixture contained tRNA and pH5 enzyme obtained from chloroplasts or wheat germ. Replacement of Mg^{2+} by Mn^{2+} or Ca^{2+} alone or together resulted in lower activity. Addition of pancreatic ribonuclease to the incorporation mixture decreased the incorporation markedly. In view of the foregoing results, the differences in amino acid incorporation by polyribosomal preparations from the 4- and 7-day-old seedlings may be attributed to the amount of undegraded mRNA in the protein-synthesizing system.

The preparation of single ribosomes by enzymic digestion of polyribosomes was accompanied by considerable loss of protein and RNA. Such ribosomes had negligible amino acid-incorporating activity. Washing these ribosomes resulted in further loss of protein and RNA, but their amino acid incorporation increased. Therefore the removal of protein by washing may have exposed fragments of mRNA that remained attached to ribosomes and that could still programme amino acid incorporation. Attachment of poly U to *Escherichia coli* ribosomes in a way that left fragments 27 nucleotides long, even after ribonuclease treatment, has been demonstrated by Takanami & Zubay (1964) and is supported by other work (Raacke & Fiala, 1965; Brentani, Brentani, Raw, Cunha & Wrotschincky, 1968).

Free ribosomes obtained by osmotic shrinkage of intact chloroplasts had similar amino acid-incorporating activity to the washed single ribosomes obtained from polyribosomes. Hence some of the free ribosomes are assumed to have exposed fragments of mRNA attached to them. The low yield of free ribosomes and the difficulty in releasing polyribosomes from the disrupted chloroplasts even at pH7.8, without detergent, suggests that the bulk of these particles are lamellae-bound. Similar findings for ribosomes from pea chloroplasts were reported by Filippovich, Spandaryan, Svetailo & Sissakian

(1967). The occurrence of free and membrane-bound ribosomes has also been reported by Payne & Boulter (1969). They found that developing seeds of *Vicia faba* contained these particles, which were not interchangeable and which might even synthesize different groups of proteins.

The cofactor requirement for optimum amino acid incorporation by intact chloroplasts was similar to that of the isolated polyribosomes. The incorporation was consistently fourfold higher for chloroplasts from 4- than from 7-day-old seedlings. The difference is attributed to the differences in endogenous ribonuclease activity. Part of this enzyme activity, to the extent of 2 units, was confined to chloroplast polyribosomes of 4- and 7-day-old seedlings. As shown by MAK-column chromatography of polyribosomal RNA, this endogenous ribonuclease, which appeared to be in a latent form, had its activity intensified by vigorous shaking during deproteinization of the polyribosomes. This activity was not suppressed by bentonite. The bulk of the ribonuclease was located in the stroma and on the chloroplast lamellae and much of it could be leached from the chloroplasts. The activity of this enzyme was increased by cold-treatment and this activity could be suppressed by bentonite. In view of the fact that this enzyme accumulates rapidly with age the lower incorporation by intact chloroplasts from older seedlings might reflect degradation of polyribosomes *in situ*.

Intensive incorporation by polyribosomal preparations from chloroplasts supports the view that at least some of the RNA molecules holding the polyribosomes together are chloroplast mRNA. Single ribosomes prepared either from the pool of free ribosomes of intact chloroplasts, or by enzymic digestion of polyribosomes, which presumably retained fragments of mRNA resistant to ribonuclease, had very low incorporation. Even the addition of exogenous template did not raise the extent of incorporation to that of polyribosomes.

Analytical ultracentrifugation and sucrose-gradient sedimentation have shown that polyribosomal preparations contain single ribosomes as well as ribosomal subunits. Parenti-Rosina, Eisenstadt & Eisenstadt (1969) reported that native subunits released from the polyribosomal complex carry at least three initiating factors for protein synthesis. Hence the amino-acid incorporating efficiency by purified polyribosomes might be attributed not only to the presence of mRNA but also to the presence of initiating factors.

In a parallel study on the amino acid-incorporating activity of the polyribosomes from cytoplasm of the same wheat tissue (Mehta, Hadziyev & Zalik, 1969) we found that chloroplast polyribosomes had 20-fold higher activity than those from the cytoplasm. This finding strongly suggests that the green-plant cell has two different protein-synthesizing systems and that the one confined to the chloroplasts, because of its high efficiency, may have a unique role.

This work was supported by a grant to S. Z. from the National Research Council of Canada. D. H. was formerly a University of Alberta Post-doctoral Fellow, and is now Professor in the Department of Food Science.

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