Amino Acid Incorporation by Wheat Chloroplasts[1,2]

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Received November 18, 1965.

Summary. Isolated chloroplasts from wheat leaves incorporate radioactive amino acids into protein. Both physiological and biochemical evidence show that contaminating bacteria are not responsible for the activity. Activity is best in plastids from 5-day-old or younger seedlings; a sharp drop usually occurs by day 6 or 7. The system requires added adenosine triphosphate, guanosine triphosphate and Mg^{++}, and is inhibited by ribonuclease, puromycin and chloramphenicol. Preliminary evidence is presented that polyribosomes are present in the young leaf chloroplast fraction. Half of the protein that is formed in a 20-minute incubation is released in soluble form.

The possible function of chloroplasts as self-contained units of reproduction is a matter of current experimental interest. Synthesis of their own proteins is one major aspect of this concept, and numerous reports of such activity have appeared. A possible difficulty in interpretation lies in the presence of living bacteria which will be collected when centrifuging cell particulates at low speeds. Warnings to this effect have been published with respect to animal mitochondria (15), wheat endosperm protein bodies (16) and spinach chloroplasts (2); but obviously do not apply to isolation of particulates from organisms such as *Euglena* grown in sterile culture (5, 6, 7).

In previous work from this laboratory, bacterial contamination of isolated chloroplasts was very severe due to the use of market spinach (2). Even after turning to wheat seedling plumules less than a week old and used immediately after harvest, some further difficulties were traced to bacteria present in reagents (even simple salt solutions such as $MgCl_2$) stored in the refrigerator between 3 and 10 days. Current procedure involves preparing reagents in freshly distilled water, then storing them in the frozen state.

Given these conditions closer to sterility we have found amino acid incorporation by wheat chloroplasts strongly dependent on the physiological state of the plant and on biochemical characteristics of the reaction mixture. These results support the concept that the activity is due to chloroplasts, not to any bacteria that might still be present; a conclusion previously reached in work with chloroplasts from tobacco (4, 8, 13), spinach (12) and *Euglena* (5, 6, 7). Some comparative aspects of the wheat chloroplast activity have also been examined.

Materials and Methods

Wheat seedlings, variety Selkirk, were grown in vermiculite at first under natural day length in the greenhouse, later in a controlled condition growth chamber under a 15-hour day. Tap water was used for subirrigation by an automatic system.

Essentially all the above-ground parts of the shoot, consisting primarily of leaf tissue, were harvested on the fourth or fifth day after planting. Homogenates were prepared by the hand-chopping method of Spencer and Wildman (13), using the Honda medium (Ficoll 2.5 %, dextran 5 %, sucrose 0.25 M, Tris 0.025 M pH 8.2 at 0°). The usual chloroplast fraction was obtained by centrifuging the homogenate for 10 minutes at 1000 × *g*; this fraction was then resuspended to a final chlorophyll concentration of 0.2 mg/ml in Tris 10 mM, Mg chloride 10 mM, mercaptoethanol 4 mM at pH 7.8.

For preparing isolated ribosomes, 2 procedures were used. In the first, chloroplasts were suspended in 0.10 M Tris-Cl pH 8.0, 0.06 M KCl, 0.01 M $MgCl_2$ and 3 % Triton. It was later found that the Triton disruption was not needed, and chloroplasts were simply broken in a hypotonic medium containing 0.01 M Tricine, pH 8.0, 0.01 M $MgCl_2$ and 0.004 M mercaptoethanol. In either procedure the broken chloroplasts were sedimented at 20,000 × *g* for 15 minutes, and ribosomes collected from the supernatant solution by centrifuging at 100,000 × *g* for 1 hour. The resulting pellet was suspended in 1 to 2 ml of Tris-Mg-KCl as above, without the Triton. This solution was further clarified of green particles by centrifuging 1 or 2 more times at 20,000 × *g* for 15 minutes.

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[1] Contribution number 470 from the McCollum-Pratt Institute.

[2] Supported in part by grant GB-1192 from the National Science Foundation, and by a Kettering Research Award.

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The standard assay for amino acid incorporation was that described by Spencer and Wildman (13) but omitting the mixture of unlabeled amino acids and adding ammonium sulfate. The final reaction mixture volume was 0.5 ml, containing the 1000 × *g* fraction with about 80 μg of chlorophyll (i.e. about 0.75 mg of protein) and Tris pH 7.8 at 25°, 2.3 μmoles; $MgCl_2$ 2.3 μmoles, ATP 0.2 μmole; P-enolpyruvate 1.25 μmole, pyruvate kinase 10 μg, GTP 0.01 μmole; ammonium sulfate 12 μmoles. Either 1.6 mμmoles of uniformly labeled leucine-^{14}C, specific activity 155 mc/mmole, or 1.0 mμmole of phenylalanine-^{14}C, specific activity 252 mc/mmole were added per reaction. Unless otherwise specified the reactions were started by adding the plastid fraction to the other components, and the mixture was incubated at 32° for 10 minutes. After incubation the tubes were rapidly cooled, then 1 ml of 10 % trichloroacetic acid plus 0.5 ml of a 0.1 M solution of the unlabeled amino acid corresponding to the labeled one were added. Two kinds of controls were used routinely. In one, ^{14}C amino acid was added only after the addition of trichloracetic acid. This served as a check on nonspecific adsorption of the labeled amino acid. In the other, the chloroplast suspension was added at the end of the incubation period, just before addition of trichloroacetic acid; this served as a check for bacterial contaminants in the reaction mixture reagents.

The treated reaction mixtures were centrifuged at 10,000 × *g* for 10 minutes and the supernatant discarded. The pellets were frozen in a dry-ice and ethanol bath for 10 minutes, then resuspended in 5 % trichloroacetic acid again containing 0.05 % of the nonradioactive amino acid. This mixture was heated to 80° for 30 minutes, then filtered on Whatman No. 542 filter paper discs and washed 5 times with 5 ml of the trichloroacetic-amino acid solution. Control experiments showed no decrease in counts incorporated due to use of hot ethanol-ether or ether extractions; hence these steps were routinely omitted. The discs were cemented to planchets and counted with a Nuclear Chicago gas flow counter. The efficiency of counting was 10 %, and 34 cpm of ^{14}C leucine or 49 cpm of the ^{14}C phenylalanine corresponded to 1 μμmole, respectively.

Samples were always assayed in duplicate, and the results were averaged to give the figures shown. Duplicates from the same chloroplast suspension generally agreed to within ± 10 %.

Protein concentration was measured with a modified Lowry method (9) and chlorophyll was estimated by the method of Arnon (3). In a few experiments the weight ratio of protein to chlorophyll in the crude chloroplast fraction was found to be 10:1. For routine assays thereafter only chlorophyll was measured, the protein concentration being estimated by use of this ratio. Radioactivity in N-terminal amino acids was measured by the procedure of Sanger (11). The hot TCA precipitate was dissolved in a minimal volume of 0.1 % $NaHCO_3$, with the addition of concentrated NaOH to effect complete solution. The final protein solution, at 10 mg/ml, was treated with a 1 % solution of 1-fluoro-2, 4-dinitrobenzene (FDNB) in ether. After 2 hours at room temperature the solution was acidified; and untreated FDNB extracted with ether. The dinitrophenylated protein was hydrolyzed by refluxing with 6 N HCl for 6 hours, and the complexed amino acids extracted with ether. The combined ether extracts were evaporated, the residue dissolved in 1 ml of acetone and aliquots of this and of the aqueous phase were tested for radioactivity.

The numbers of viable bacteria in aliquots of the reaction mixture were counted by plating on nutrient agar plates after serial dilution. Bacterial protein was determined by the Lowry method, and this was correlated with the number of bacteria as measured by plate counts. Viable colonies were counted after 24 hours.

Radioactive amino acids were obtained from the Nuclear Chicago corporation, pyruvate kinase from the California Biochemical Corporation, and other biochemical reagents from the Sigma Corporation. All reagents and buffer solutions were frozen, stored at −20° immediately after preparation, and thawed just before use.

Results

Nature of the Active Fraction. The cell-free homogenate from wheat leaves was fractionated by centrifugation. Fractions sedimenting in 10 minutes at 1000 × *g*, 20 minutes at 20,000 × *g* and 90 minutes at 100,000 × *g* were resuspended in buffer containing Tris, $MgCl_2$ and mercaptoethanol. When assayed for incorporation of radioactive leucine, (table I) the 1000 × *g* fraction was found to be the most active. Apparent lower activity of the whole homogenate could be due to dilution of the radioactive amino acid with cold leucine.

Washing the 1000 × *g* fraction in the hypotonic Tris-Mg-mercaptoethanol solution always led to large losses in activity, without significant restoration by the supernatant from which ribosomes had been removed by centrifuging for 2 hours at 100,000 × *g*.

Table I. *Distribution of Amino Acid Incorporating Activity in Wheat Leaf Extracts*

Leaves (31.6 g) were chopped in Honda medium, and the final extract made to a volume of 31 ml. Various centrifugal pellets were resuspended in a small volume, and the activity from aliquots of these suspensions was calculated back to that expected from the full 31 ml volume. Leucine was the radioactive amino acid.

Fraction	cpm/homogenate
Homogenate	2250
1000 × *g* pellet	5130
20,000 × *g* pellet	344
100,000 × g pellet	278

Further analysis of components needed for activity has not yet been attempted.

Various preparations of ribosomes extracted from the chloroplast fraction were examined in the analytical ultracentrifuge. Figure 1 shows one of these experiments. The bottom curves were from a cell containing ribosomes extracted from the chloroplasts by simple hypotonic breakage. Four peaks are visible, with calculated $S_{20,w}$ values of 51, 66, 79 and 100 S respectively. In other experiments an additional component having a 123 S value was also visible. These sedimentation constants include corrections for temperature and viscosity of solvents but are not exact, since they are not corrected for protein concentration. The top curve is a duplicate aliquot of the ribosome suspension, treated for 5 minutes prior to the run with 10 μg of ribonuclease; this causes the disappearance of the faster moving components, the 2 remaining peaks having sedimentation constants of 51 and 68 S respectively. In other experiments the 2 fastest moving peaks were entirely absent from chloroplast extracts obtained from 8-day-old plants, but were always present in those taken from 4-or 5-day-old plants.

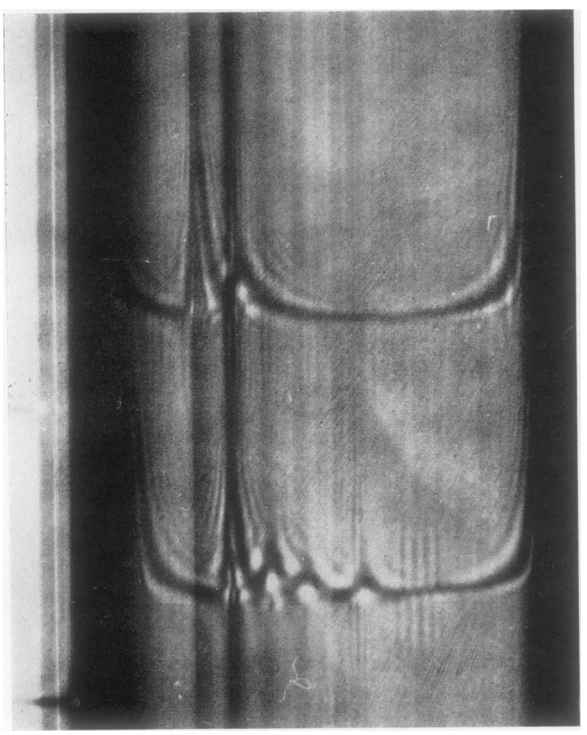

FIG. 1. Diagram of an analytical ultracentrifuge pattern of isolated wheat chloroplast ribosomes, obtained by hypotonic breakage of the chloroplast fraction from 5-day-old wheat seedlings. The protein concentration was 11 mg/ml. The picture shown here was taken 16 minutes after the start of the centrifugation at 39,460 rpm and rotor temperature of 2.0°. The bar angle was 50°, and direction of sedimentation is left to right. The lower curves are from the control cell, upper curves from a duplicate aliquot pretreated with ribonuclease. The high density close to the meniscus (far left) appears to be lipoprotein material, with which the preparation was contaminated.

Activity in the standard assay was very much dependent on the age of the plant (table II shows a representative experiment). Active preparations were obtained on the fourth and fifth day after planting; but usually on the sixth and always on the seventh and eighth days the activity had dropped very strongly. This change seemed to be correlated with the condition of the leaves; they were still more or less rolled up on the fourth and fifth days, but usually uncurled by the sixth day after planting the seeds. It was also noted that activities were better when seedlings were harvested on bright, sunny days, than under cloudy conditions. Consistently good activities are also found from seedling grown at 1000 ft-c of fluorescent light.

Reaction Conditions. Table III shows that Mg, ATP and a generating system, GTP and ammonium ions are all needed for optimal activity. The Mg^{2+} requirement was greater given a higher activity in the complete system (expt 2) and an excess was inhibitory.

Adding a mixture of ^{12}C amino acids was often found to be somewhat inhibitory (expts 1 and 3 of table IV). This was probably due to the use of a suboptimal concentration of added leucine; either traces of leucine, or competitive amino acids in the cold mixture, could then decrease the amount of labeled leucine incorporated. On increasing the added leucine to a 5-fold higher level the apparent rate of incorporation was increased some 60 %, and adding the cold amino acids no longer inhibited (expt 3 of table III).

Effective inhibitors of amino acid incorporating activity included ribonuclease at 0.2 μg (86 % inhibition), chloramphenicol at 20 μg (77 % inhibition) and puromycin at 10 μg (79 % inhibition) per 0.5 ml reaction mixture. High concentrations of deoxyribonuclease (10 and 100 μg/ml) inhibited up to 40 %.

At 25° or 30° amino acid incorporation ceased by 20 minutes, and showed linearity for the first 5 minutes (fig 2). At 37° there was less total incorporation, and the reaction continued for only 3 to 5 minutes.

Nature of the Protein Formed. Amino acid incorporated into the central part of protein chains was checked by forming the DNP derivatives, and measuring the proportion of radioactive leucine in the N-terminal position. Only 7 % of the radioactivity in the protein was detected as the DNP derivative, solubilized by hydrolysis and present in the ether extract. 72 % of the radioactivity was still found in the ether-insoluble portion; thus most of the leucine must have been incorporated in internal positions.

Distribution between particulate and supernatant proteins was estimated by diluting the reaction mixture, after various time periods, with 10 mM $MgCl_2$

and centrifuging at 100,000 × *g*. The percentage of the counts recovered in the particulate fraction (presumably ribosomes) decreased from 81 % at 2 minutes to 46 % after 20 minutes (table IV).

Labeled chloroplasts were treated with 0.1 % Triton, then recentrifuged at 1000 × *g*. Virtually all of the chlorophyll was in the supernatant solution, indicating a large disruption of chloroplast structure; whereas neither bacteria, nor nuclei (13) would be expected to be solubilized by this treatment. Of the total radioactivity incorporated, only 3.5 % remained with the 1000 × *g* pellet after Triton treatment

Table II. *Activity of 1000 × g Fraction in Amino Acid Incorporation from Plants of Different Ages*

Days after planting	1000 × *g* fraction		Leucine incorporation	
	Protein	Chlorophyll	cpm/mg chlor	μμmoles/mg prot
	mg*	mg*		
4	0.96	0.095	4150	12.1
5	1.29	0.139	3570	11.3
6	1.25	0.182	700	3.0
7	1.47	0.147	720	2.1

* Yield in the 1000 × g fraction from 1 g (fr wt) of original leaf tissue.

Table III. *Cofactor Requirements for Amino Acid Incorporation by the Chloroplast Fraction*

Expt	reaction mixture	cpm/mg chlor	μμmoles/mg chlor	% of Control
1	Complete	1116	30.8	100
	$-Mg^{2+}$	360	10.6	35
	$-NH_4^+$	560	16.5	53
	−ATP, PEP and pyruvate kinase	140	4.1	13
	−GTP	268	7.9	25
	+Amino acid mix*	650	19.2	62
2	0 Mg^{2+}	412	12.1	11.5
	0.25 μmole Mg^{2+}	688	20.3	19.5
	2.5 μmole Mg^{2+} (Complete)	3550	104	100
	25.0 μmole Mg^{2+}	350	10.4	10
3	Complete	3000	88	100
	" + Amino acid mix*	2500	74	84
	" + ^{12}C leucine**	940	139	100
	" + Amino acid mix and ^{12}C leucine	978	144	104

* 20 nonradioactive amino acids, excluding leucine, each one at 12.5 mμmoles per reaction mixture.
** 6.4 mμmoles of added ^{12}C leucine, with 1.6 mμmoles of the labeled compound already present, to effect a one-fifth dilution.

Table IV. *Distribution of Radioactivity between Soluble and Particulate Proteins*

Aliquots of the reaction mixture were assayed for ^{14}C amino acid incorporation; then the remainder was diluted with 10 mM $MgCl_2$ and centrifuged at 100,000 × *g* for 60 minutes. The pellets were resuspended, precipitated with trichloroacetic acid and assayed for incorporation of radioactivity into protein.

Time min	Expt no.	Original 1000 × *g* fraction cpm	Particulate proteins cpm	% of total in particulates %
2	1	81	64	79
	2	57	48	84
5	1	235	155	66
	2	285	190	67
10	1	531	301	57
	2	577	256	44
20	1	763	339	44
	2	721	352	49

(table V). Some 47 % of the activity could still be sedimented at 100,000 × *g*, and could represent either labeled ribosomes, or membrane fragments.

Comparison with Bacteria. On various occasions aliquots were taken of the final reaction mixtures, and plated out on nutrient agar to estimate the number of viable bacteria present. These tests showed no more than 10^4 bacteria to be present per 0.5 ml of reaction mixture. Bacteria were collected by scraping the surface of these plates, and tested in the usual amino acid incorporating mixture. The bacterial system was neither ribonuclease sensitive, nor dependent on any of the cofactors for amino acid incorporation. After Triton treatment, the label found in the bacteria could to a large extent be sedimented at 1000 × *g* (see table V), again differing from the results with chloroplast suspensions. In a mixture of Triton-treated bacteria and chloroplasts, the counts due to bacteria were sedimented, whereas those due to chloroplast activity remained in the supernatant after a 1000 × *g* centrifugation. Finally, as might be expected, the time course of incorporation by bacteria was linear up to and beyond 20 minutes, both at 25° and 37° (fig 2).

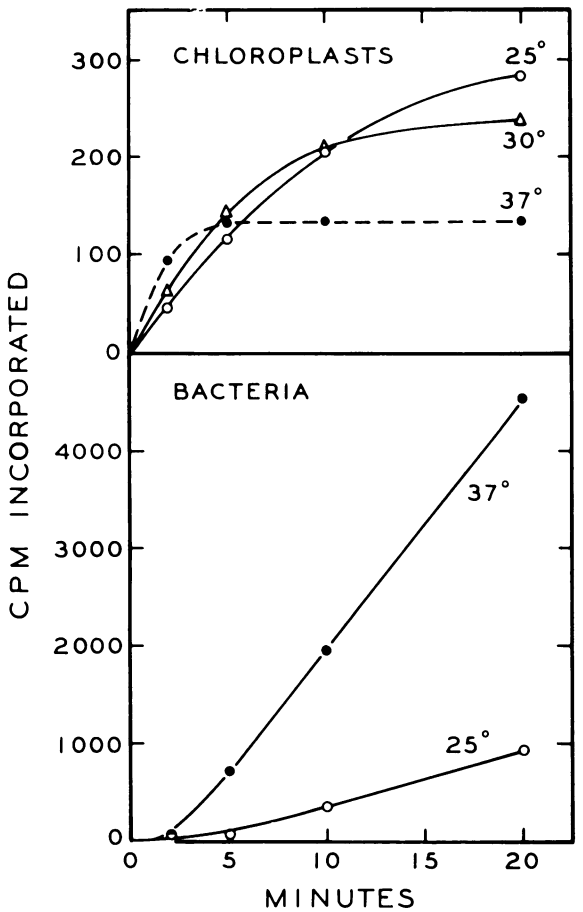

FIG. 2. Time courses for incorporation of radioactive leucine by wheat leaf chloroplast fraction at 3 different temperatures, and for contaminating bacteria after growth on nutrient agar as described in the text.

Discussion

Participation of bacteria in the activity measured here may be ruled out by criteria similar to those used recently for spinach (12) or tobacco (13) chloroplasts. These include the high sensitivity to ribonuclease, strong dependence on ATP and a generating system, on GTP and on Mg additions (table IV); a limited time course for incorporation especially at higher temperatures (fig 2), and disruption of the final product by Triton (table V). None of these characteristics are shared by whole bacteria incorporating radioactive amino acids under similar conditions (see table V and fig 2). In addition, the apparent number of bacteria present, some 10^4 per reaction, is too small to account for the observed results. Thus, both in present experiments, and by calculation from previous results (2) 1 × 10^4 bacteria would incorporate approximately 0.1 μμmole in the 10-minute incubation period used standardly here. This contrasts with the 6 to 12 μμmoles of incorporation actually observed with active chloroplast preparations per 0.5 ml assay. As can be seen from these estimates, 10^6 bacteria would invalidate the results obtained, and even 10^5 would begin to interfere seriously.

The major factor in obtaining active preparations from wheat leaves has turned out to be the age

Table V. *Triton Disruption of ^{14}C Labeled Chloroplasts and Failure to Disrupt Bacteria*

Fraction	cpm	% of total
Whole chloroplasts	200	100
Whole after Triton–1000 × *g* pellet	7	3.5
Whole after Triton–100,000 × *g* pellet	94	47
Bacteria	245	100
Bacteria after Triton–1000 × *g* pellet	193	79
Bacteria after Triton–100,000 × *g* pellet	45	18
Whole chloroplasts + bacteria	410	100
after Triton–1000 × *g* pellet	193	48
after Triton–100,000 × *g* pellet	140	34

of the plants. The chloroplasts must be taken from plants less than 7 days old. A probably analogous phenomenon was seen with spinach (12) or tobacco (13) chloroplasts, which had to be from young plants, no more than a few weeks old. The loss of activity is correlated in our work with the disappearance of the 2 or 3 heavier components observed in the analytical ultracentrifuge. Since it is likely that the faster peaks represent polyribosomes, their disappearance would be a sufficient cause for the drop in activity. However it should be noted that good activity in amino acid incorporation was seen with spinach chloroplasts where the largest ribosomes were of the 70 S variety (12) and in tobacco, with ribosome components no larger than 80 S (4). Rigorous proof of the cause of drop in activity of chloroplasts with wheat leaf age requires further experimentation, therefore.

The absence of dependence on added unlabeled amino acids for the incorporation of radioactive leucine was also seen with *Euglena* chloroplasts (5, 6); and even with tobacco (13) or spinach (12) dependence on adding other amino acids was far from complete. The reason may probably be ascribed to an internal pool of amino acids. Direct analysis of the 1000 × *g* fraction from wheat by the ninhydrin method (14) showed the presence of 1.25 μmoles of amino acids and alcohol-soluble peptides per mg chlorophyll in chloroplasts from 4-day-old leaves, and 1.86 μmoles/mg chlorophyll in those from 8-day-old leaves. In a second experiment the corresponding figures were 2.4 and 2.6 μmoles per mg chlorophyll. Since this pool was likely to have included some endogenous leucine, the specific activity of the actual precursor pool in our experiments was not known with precision. This fact makes comparisons between experiments difficult; although the results within any 1 experiment are still valid. The tabular results of μμmoles of leucine incorporated/mg protein are based on the known specific activity of the added leucine, and are therefore likely to be underestimates. These values, in the range of 3 to 12 μμmoles/mg total protein, are consistent with those found in other systems, especially since the active ribosomes could not represent more than a small fraction of the total protein present.

Wheat chloroplasts resemble those from spinach (12) but differ from those from tobacco (13) or *Euglena* (6) in having a strong dependence on added GTP, and in being inhibited by a relatively low concentration (40 μg/ml) of chloromphenicol. Inhibition of tobacco or *Euglena* chloroplasts needs 5 times as much of the latter reagent, and *Euglena* cytoplasmic ribosomes (7) are completely insensitive.

Wheat chloroplasts may be unique in the high proportion (up to 50 %) of labeled protein which is soluble, rather than attached to either ribosomes or membranes. This probably represents formation of protein chains which are released when complete, since the proportion of soluble counts increases with time of incubation (table IV). Another feature of the wheat chloroplast system is the appearance of ribosome peaks having apparent sedimentation constants of 80S and 100S. Ribosome components obtained from spinach chloroplasts have not been heavier than 65 to 70S, (1, 10, 12), and those from tobacco included 70 and 80 S peaks (4). In the present case, the 80 and 100 S components were destroyed by brief pretreatment with ribonuclease, a phenomenon not seen even for the largest component in tobacco chloroplast extracts. It seems most likely that these larger components are polyribosomes in view of this destruction; however a more precise definition of their nature, extent and function remain objectives of future work with this system.

Acknowledgments

This work was performed with the assistance of Miss Natalie Burroughs, and of Mrs. Ofra Ziv. We thank Professor W. Harrington for providing facilities and Miss Trudy Karr for technical aid in the ultracentrifugation experiments.

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