

# High Rates of Protein Synthesis by Isolated Chloroplasts<sup>1</sup>

Received for publication February 8, 1982 and in revised form June 4, 1982

LEONARD E. FISH AND ANDRE T. JAGENDORF<sup>2</sup>

*Plant Biology Section, Cornell University, Ithaca, New York 14853*

## ABSTRACT

Improvements are described in the preparation and *in vitro* conditions of an intact pea (*Pisum sativum* Progress No. 9) chloroplast system which provides high efficiency for translation of endogenous messenger RNA, using light as an energy source. High rates result in the incorporation into protein of up to 100 nanomoles tritiated leucine per milligram chlorophyll. These rates suggest extensive reinitiation, and repeated utilization of the messenger RNA that code for thylakoid proteins. Up to 39 radioactive thylakoid peptide bands were detected by fluorography after labeling with tritiated leucine.

Protein synthesis by intact chloroplasts isolated from several species of plants has been studied to a considerable extent, and was reviewed recently (10). Much of the work has had the goal of identifying which of the numerous soluble and membrane proteins of the chloroplast are synthesized within the organelle on its own 70S ribosomes. However, the rates of protein synthesis, and especially of polypeptide chain initiation, achieved in this '*in organello*' (32) synthesis must be adequate in order to meet this goal. This consideration is important because a low initiation rate can result in preferential translation of mRNA with high rate constants for initiation (19, 23). This would give a distorted picture of the synthetic capacities of the isolated chloroplasts. Thus, it is highly desirable, for studies in which translation products are examined, that the *in vitro* rate of initiation be as close as possible to the *in vivo* rate.

Toward this goal, and in conjunction with related studies on ribosome binding to the green, thylakoid membranes (12, 41), we have sought to optimize most of the parameters involved in isolation of intact chloroplasts and in their reaction conditions, in order to increase rates of protein synthesis. These studies have resulted in obtaining rates two orders of magnitude higher than those reported in previous papers. Examination of labeled membrane polypeptides after denaturing gel electrophoresis shows up to 39 products of '*in organello*' synthesis, or three to four times more than noted in earlier studies (9, 13–15, 40, 42).

## MATERIALS AND METHODS

**Plant Material.** Peas (Progress No. 9, Agway Corp.) were sown in vermiculite and grown for 8 d on a 12-h light/12-h dark cycle as described elsewhere (12). The plants were placed in darkness for 13 to 16 h before harvest to reduce the amounts of starch in the leaves, then illuminated for 15 to 30 min just before homoge-

nization. Shoots were cut in the light ( $275 \mu\text{mol m}^{-2} \text{s}^{-1}$ ), immediately immersed in ice water, then homogenized within 7 min.

**Isolation of Intact Chloroplasts.** Intact chloroplasts were isolated on 25% to 92% Percoll gradients. The procedure was based on that of Morgenthaler *et al.* (31); details for the Percoll gradients were provided by N.-H. Chua (personal communication). All manipulations were carried out at 4°C; centrifugations were at 0°C to 2°C.

In the final adopted procedure, shoot tissue was homogenized in two batches of 20 g each with 47 ml of grinding buffer containing 350 mM sorbitol, 50 mM Hepes-KOH (pH 8.3 at 4°C), 2 mM EDTA, 1 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, 0.5% BSA, 2 mM EGTA,<sup>3</sup> and 4 mM ascorbic acid using a Polytron homogenizer (Brinkmann Instruments) for 20 to 30 s, until all tissue was smaller than 1 mm<sup>2</sup>. The homogenate was filtered through eight layers of cheesecloth, and centrifuged for 3 min at 3,000 rpm in a Sorvall HB-4 rotor. The pellet was resuspended in a minimal volume (0.2–0.4 ml) of the grinding buffer and overlaid onto a 13-ml linear 25% to 92% Percoll gradient containing the same ingredients as the grinding buffer, 0.6 mM glutathione, and gradients of 0.7 to 2.7% (w/v) of polyethyleneglycol 4000 and 0.25 to 0.92% (w/v) of both BSA and Ficoll. The gradient tubes were centrifuged for 7 min at 9,000 rpm in the HB-4 rotor, and the lower green band of intact chloroplasts was collected and diluted with 30 ml of resuspension medium containing 375 mM sorbitol, 35 mM Hepes-KOH (pH 8.3 at 4°C), 2 mM EDTA, 1 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, 0.96 mM DTT. The suspension was centrifuged for 3 min at 5,000 rpm in the HB-4 rotor. The resulting pellet of intact plastids (about 92–96% refractile in the phase contrast microscope) was drained at 4°C, then resuspended in 50 to 100  $\mu\text{l}$  of added resuspension medium using a small artists' brush. Aliquots of 3 to 5  $\mu\text{l}$  were taken for measurement of Chl (2). The chloroplasts were diluted to 3.0 mg Chl/ml, and stored on ice.

**Incubation for Protein Synthesis.** Aliquots of 5  $\mu\text{l}$  of the chloroplasts (15  $\mu\text{g}$  Chl) were added to 135  $\mu\text{l}$  resuspension medium, 5  $\mu\text{l}$  [<sup>3</sup>H]leucine (4–5  $\mu\text{Ci}$  at a specific radioactivity of 1.7–13.3  $\mu\text{Ci/nmol}$ ) and 5  $\mu\text{l}$  of either water or other additive. The reaction mixtures were incubated for 5 min on ice, then transferred to an illuminated water bath at either 25°C or 27°C, and 800 to 1,200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  of light (400–700 nm) as measured with a Lambda Instrument Corp. model LI-185 quantum/radiometer/photometer, using 200 w movie lamps. The tubes were agitated manually every minute for 4 min, then once every 2 min for a total of 20 min incubation. The light was turned off, and the tubes were transferred to ice, with addition of 150  $\mu\text{l}$  10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub> (pH 7.4) at 4°C.

For measurement of total counts incorporated into chloroplast protein, 100  $\mu\text{l}$  of the diluted reaction mixture was transferred to a 1.5 ml microcentrifuge tube containing 25  $\mu\text{l}$  of 10% Triton X-100, then centrifuged for 5 min in an Eppendorf centrifuge to remove possible contaminating bacteria. Phase contrast microscopic examination of the solution near the bottom after centrif-

<sup>1</sup> Supported in part by Grant 5901-0410-9-0327-0 from the United States Department of Agriculture, Science and Education Administration, Competitive Research Grants Organization program in photosynthesis.

<sup>2</sup> To whom reprint requests should be sent.

<sup>3</sup> Abbreviations: EGTA, ethylene-bis (aminoethylether)-*N,N'*-tetraacetic acid; LiDodSO<sub>4</sub>, lithium dodecylsulfate.

ugation failed to show appreciable numbers of bacteria to be present. Duplicate 50  $\mu$ l aliquots of the solubilized chloroplasts were spotted onto 2.5 cm discs of 3 MM filter paper, and processed as described (25) for measurement of radioactivity in proteins.

**Electrophoresis and Fluorography.** Thylakoid samples were prepared for polyacrylamide gel electrophoresis by washing the [ $^3$ H] leucine-labeled chloroplasts four times with cold 10 mM Na pyrophosphate (pH 7.4), washing once with 100 mM Tris-Cl/100 mM DTE (pH 8.5 at 4°C), resuspending to 2 mg Chl/ml in Tris/DTE, and then dissolving an aliquot with an equal volume of 4% (w/v) LiDodSO<sub>4</sub>/24% (w/v) sucrose. [ $^3$ H]leucine-labeled stroma protein was precipitated from the combined supernatants from the Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub> washes of the thylakoids by adding four volumes of acetone at -20°C. Adding the acetone at -20°C greatly facilitated redissolution of the protein. The precipitate was washed twice with 80% acetone at -20°C, dried under a stream of N<sub>2</sub> gas, then dissolved in 2% (w/v) LiDodSO<sub>4</sub>/50 mM Tris-Cl/50 mM DTE/12% (w/v) sucrose (pH 8.5) at 4°C. Electrophoresis was performed for 11.5 h at 1.1 w, constant power, or at 150 v, constant voltage (see figures for details), at 4°C on a 1.3 mm thick, 10% to 16% (w/v) polyacrylamide linear gradient resolving gel containing a 5.5% to 11.0% linear sucrose gradient. The stacking gel (4% [w/v], 13–15 mm long) was overlaid onto the resolving gel (120 mm length  $\times$  130 mm width) immediately after pouring of the resolving gel solution, resulting in concurrent polymerization of the two sections. The buffer system of Laemmli (21) was used, except for substitution of LiDodSO<sub>4</sub>. After electrophoresis, the gel was fixed 1 h in 15% (w/v) TCA/50% (v/v) methanol, stained 2 h at 55°C in a mixture of Coomassie Brilliant Blue R (0.2%, w/v) and G (0.1%, w/v) in 7% (v/v) acetic acid/50% (v/v) methanol, and then destained at 55°C in 7% acetic acid/20% methanol. For fluorography, the stained gel was soaked 45 min in Enhance and 45 min in water, dried onto Whatman 3 MM paper, and exposed to Kodak XAR-5 x-ray film at -80°C.

The  $M_r$  values for thylakoid peptide bands (Figs. 7 and 8) were calculated from a linear standard curve generated from the positions of marker thylakoid peptides. The  $M_r$  values for marker bands were determined by comparison to the distance of migration of lysozyme (14.3 kD), hemoglobin (15.0 kD), myoglobin (17.6 kD), trypsin inhibitor (21.5 kD),  $\alpha$ -chymotrypsinogen A (25 kD), carbonic anhydrase (30 kD), asparaginase (35 kD), creatine kinase (40.5 kD) and BSA (68 kD).

**Materials.** 4,5- $^3$ H-L-Leucine (55 Ci/mmol) was purchased from ICN Chemical and Radioisotope Division, Enhance from New England Nuclear, and Percoll and Ficoll from Pharmacia. Other organic chemicals were from Sigma.

## RESULTS

Using intact chloroplasts from Percoll gradients, the components and conditions of the resuspension medium were examined individually in a series of experiments. A broad pH optimum was found between pH 8.2 and 8.5 (data not shown). A strong requirement for added KCl (Fig. 1) at 20 to 40 mM was satisfied in the final adopted standard resuspension medium by using Hepes-KOH (pH 8.3) at 35 mM, inasmuch as this contains 30 meq/l of K<sup>+</sup>. As in previous studies (18), the medium contained 2 mM EDTA and 1 mM each of MgCl<sub>2</sub> and MnCl<sub>2</sub>. Omission of these three components led to slightly decreased amino acid incorporation rates as seen in the time-course experiment of Figure 2. If EDTA and MnCl<sub>2</sub> were present in a constant amount and the MgCl<sub>2</sub> concentration was varied, added MgCl<sub>2</sub> showed an optimum at 1 mM as in the original medium (Fig. 3). Complete omission of MgCl<sub>2</sub> led to slight (8%) inhibition, and 50% inhibition was caused by 5 to 8 mM MgCl<sub>2</sub> under these conditions. The fairly sharp optimum for sorbitol occurred at 340 to 350 mM (data not shown). Temperatures between 20°C and 30°C gave about equal amounts of incorporation into soluble peptides in the 20 min

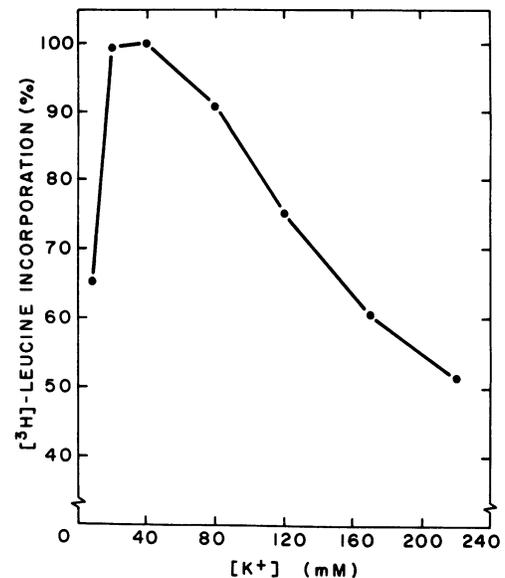


FIG. 1. Effect of KCl on protein synthesis. Intact chloroplasts were incubated for protein synthesis, in duplicate, in a 150  $\mu$ l reaction mix containing 178  $\mu$ g Chl/ml, 9 mM Hepes-KOH (pH 8.3 at 22°C), 327 mM sorbitol, 1.87 mM EDTA, 0.93 mM MgCl<sub>2</sub>, 0.93 mM MnCl<sub>2</sub>, for 20 min at 25°C. KCl was used to obtain final K<sup>+</sup> concentrations of 8 to 220 mM. Incorporation of 100% represents 6.98 nmol/mg Chl.

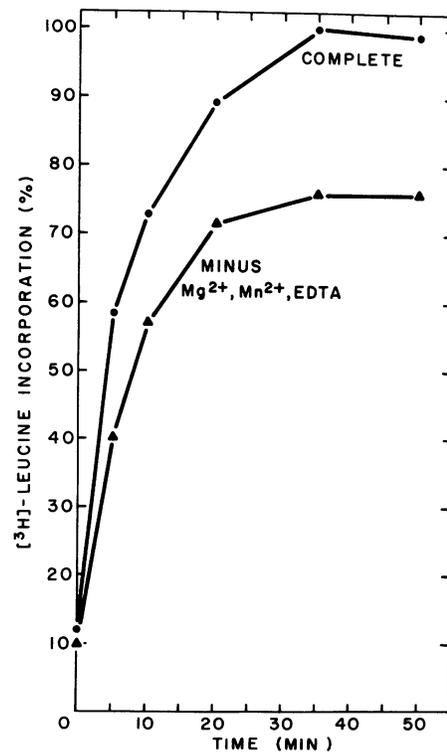


FIG. 2. Time course of protein synthesis. Intact chloroplasts were washed once with the complete resuspension medium or with one lacking MgCl<sub>2</sub>, MnCl<sub>2</sub> and EDTA, then incubated for protein synthesis at 27°C, using the same resuspension media. Incorporation of [ $^3$ H]leucine into total chloroplast protein was determined. One reaction mix was used for each time point. Incorporation of 100% represents 29.1 nmol/mg Chl.

reaction period, but incorporation into thylakoid proteins was 20% higher at 30°C than at 20°C. At 35°C, all protein synthesis was inhibited by about 30% (data not shown).

The reaction was strongly dependent on light, with a sharp

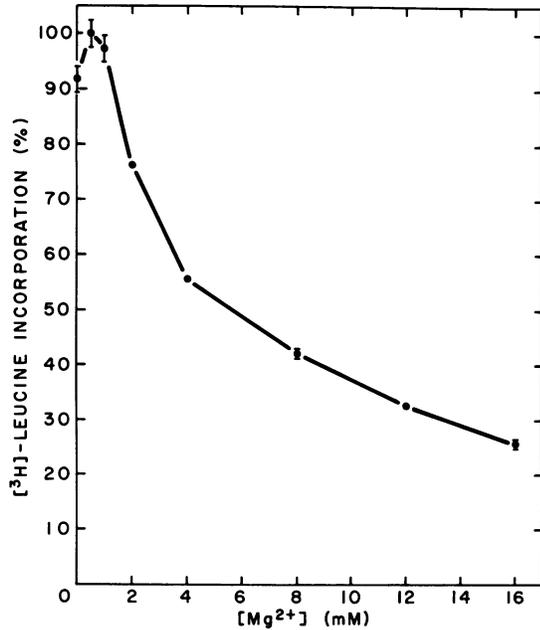


FIG. 3. Effect of MgCl on protein synthesis. Intact chloroplasts were incubated for protein synthesis, in duplicate, as described in "Materials and Methods," except at 25°C and without D. MgCl<sub>2</sub> was used to obtain final Mg<sup>2+</sup> concentrations between 0.033 and 16 mM. The concentration of [<sup>3</sup>H]leucine was 4.1 μM. After a 20 min incubation, incorporation of [<sup>3</sup>H]leucine into chloroplast protein was determined. Incorporation of 100% represents 6.67 nmol/mg Chl. Range bars are not shown where they would be obscured by the point.

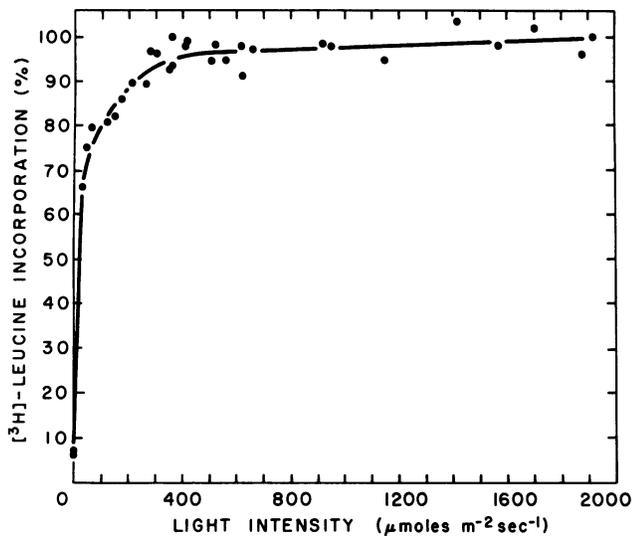


FIG. 4. Dependence of protein synthesis on light. Chloroplasts were incubated for protein synthesis in foil-wrapped tubes or with illumination attenuated by wire mesh. Data from two separate experiments (30–615 μmol m<sup>-2</sup> s<sup>-1</sup> and 350 to 1,920 μmol m<sup>-2</sup> s<sup>-1</sup>) were combined. After a 30 min incubation at 27°C, incorporation of [<sup>3</sup>H]leucine into total chloroplast protein was determined. Incorporation of 100% represents 36.7 nmol/mg Chl for the 30 to 615 μmol m<sup>-2</sup> s<sup>-1</sup> experiment and 19.9 nmol/mg Chl for the 350 to 1920 μmol m<sup>-2</sup> s<sup>-1</sup> experiment.

increment between 0 and 30 μmol m<sup>-2</sup> s<sup>-1</sup>, then a slower rise to a maximal rate at 400 μmol m<sup>-2</sup> s<sup>-1</sup> (Fig. 4). Under conditions tested so far, protein synthesis rates were maximal during the first 5 min, and had stopped entirely by 35 min (Fig. 2). Changing the medium components as in Figure 2, changed reaction rates but not the duration of protein synthesis.

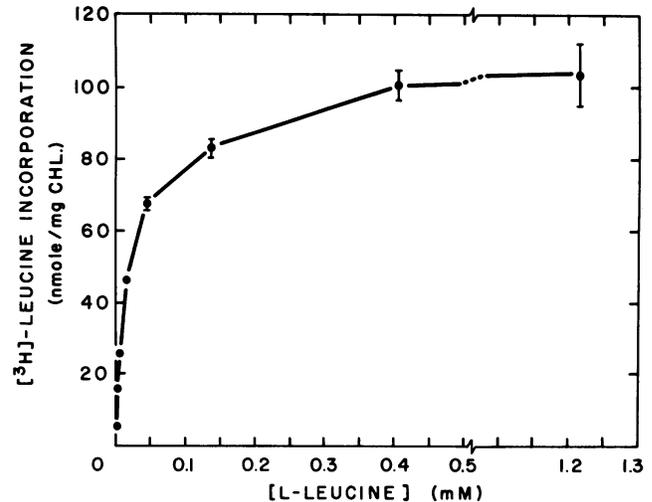


FIG. 5. Dependence of [<sup>3</sup>H]leucine incorporation on the external leucine concentration. Chloroplasts were incubated for protein synthesis, in duplicate, with 10 μCi [<sup>3</sup>H]leucine and different amounts of unlabeled leucine for 20 min at 27°C. Incorporation of [<sup>3</sup>H]leucine into total chloroplast protein was determined. Range bars are not shown where they would be obscured by the point.

Isolated intact pea chloroplasts were reported to contain leucine at about 0.36 mM in the stroma (28); hence added radioactive leucine will be subject to some isotope dilution, depending on the amount entering through the envelope. The response to increasing amounts of leucine is shown in Figure 5; saturation of the reaction appears to occur at 0.4 mM. Leucine was the last parameter tested in this rather lengthy series of optimization experiments. The rest of the work was performed using 5 to 15 μM leucine in the reaction mixture, and time did not permit repeating the experiments at higher concentrations.

Photosynthetic fixation of CO<sub>2</sub> by intact chloroplasts is often stimulated by adding one or another of the Calvin cycle intermediates. This is not the case for protein synthesis; substantial inhibition was caused by adding substrate levels of 3-P-glyceric acid, ribose-5-P, NaHCO<sub>3</sub> or α-ketoglutarate plus glutamine (Table I). Added Pi (0.2–1.2 mM) or methionine (0.05–1.0 mM) had essentially no effect on incorporation (data not shown).

Table I also shows the effects of some nonmetabolites. The experiment with Triton X-100 was included to test for activity of bacteria, which would not be affected (33). The degree of inhibition—in this case 96%—represents the minimum proportion of activity that can be ascribed to chloroplasts, whose membranes are dissolved by the Triton. The very low concentration of 0.25 μM DCMU caused a 15% stimulation of protein synthesis. To determine if activity of the carbon reduction pathway reduces protein synthesis, an inhibitor of the Calvin cycle, DL-glyceraldehyde (39), was tested; it did not stimulate, but inhibited amino acid incorporation slightly. Unlike CO<sub>2</sub> fixation, which is stimulated by exogenous catalase (37), [<sup>3</sup>H]leucine incorporation was not affected by a mixture of superoxide dismutase and catalase; and neither zeatin (10<sup>-9</sup> to 10<sup>-6</sup> M) nor gibberellic acid A<sub>3</sub> (10<sup>-9</sup> to 5 × 10<sup>-6</sup> M) had any effect (data not shown).

**Effect of Inhibitors of Protein Synthesis.** Chloramphenicol at a saturating concentration (0.4 mM) inhibited incorporation 97% (I<sub>50</sub> 13 μM); cycloheximide had no effect (data not shown). Lincomycin is a potent inhibitor of chloroplast ribosomes (11), acting shortly after initiation on ribosomes bearing a short nascent chain (7). At a saturating concentration, lincomycin inhibited total protein synthesis 72%, and incorporation into peptides of the thylakoid membrane 83% (data not shown). Folic acid, a competitive inhibitor for the <sup>10</sup>N-tetrahydrofolate-mediated formylation of initiator met-tRNA, has been used as a specific inhibitor

Table I. Effect of Added Metabolites and Other Compounds on Protein Synthesis

Chloroplasts were incubated for protein synthesis with 600 pmol [<sup>3</sup>H]leucine (experiments 1, 4 and 6), or 735 pmol (experiments 2, 3 and 5). All tubes in experiment 6 contained 0.17 mM ethanol. Incorporation of [<sup>3</sup>H]leucine into chloroplast protein was determined.

Expt.	Compound Added	Concentration	[ <sup>3</sup> H]Leucine	% of Control
			Incorporated	
			<i>nmol/mg Chl ± range/2</i>	
1	None		6.3 ± 0.2	(100)
	3-Phosphoglyceric acid	5.0 mM	4.6 ± 0.1	73
	Ribose-5-P	0.5 mM	5.3 ± 0.0	84
		2.0 mM	4.3 ± 0.1	68
2	None		16.3 ± 0.0	(100)
	NaHCO <sub>3</sub>	0.25 mM	15.8 ± 0.0	97
		2.0 mM	14.9 ± 0.2	91
		4.0 mM	13.4 ± 0.2	83
		8.0 mM	12.2 ± 0.3	75
3	None		18.5 ± 0.2	(100)
	α-Ketoglutarate + glutamine	1.0 mM each	10.3 ± 0.1	56
4	None		9.9 ± 0.4	(100)
	Triton X-100	0.01%	4.5 ± 0.3	45
		0.08%	0.4 ± 0.0	4
5	None		16.8 ± 0.1	(100)
	DL-Glyceraldehyde	8.0 mM	14.7 ± 0.2	88
6	None		12.2 ± 0.4	(100)
	DCMU	0.25 μM	13.9 ± 0.3	114
		1.0 μM	12.6 ± 0.2	103

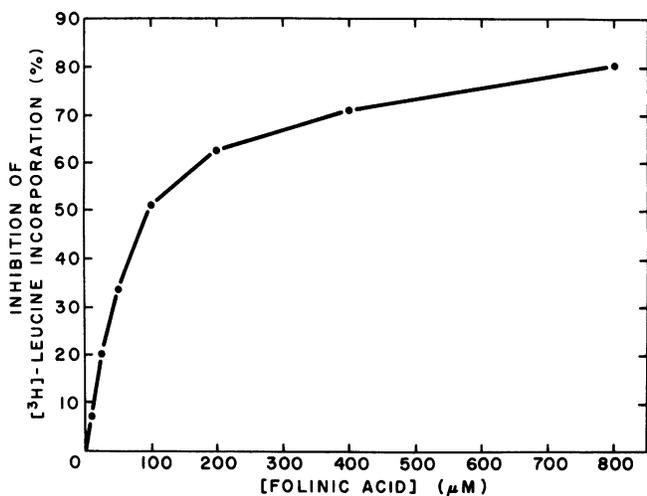


FIG. 6. Inhibition of protein synthesis by folic acid. Chloroplasts were incubated for protein synthesis in duplicate with folic acid (0–800 μM) for 20 min at 27°C. Incorporation of [<sup>3</sup>H]leucine into total chloroplast protein was determined. Incorporation for the control, without folic acid, was 49.4 nmol/mg Chl.

of peptide chain initiation in isolated chloroplasts and mitochondria (24). We found substantial inhibition by folic acid (Fig. 6), but the steady increase in inhibition with higher concentrations may indicate a second site of action for this compound. Edeine, a pentapeptide which inhibits initiation specifically (34) had no effect (data not shown), probably because of its inability to penetrate the chloroplast envelope.

**Products of Protein Synthesis: Thylakoid Peptides.** Figure 7 slots 1 and 2 show a fluorograph of labeled polypeptides made under conditions for high [<sup>3</sup>H]leucine incorporation, and slots 3

and 4 show the stained thylakoid bands from other slots on the same gel. Thirty-nine labeled bands are visible in slot 1, but in the original fluorograph, 9 additional faint bands can be seen, making a total of 48 labeled bands. As in earlier work, the most heavily labeled band has an *M<sub>r</sub>* (34.8 kD), corresponding to that of peak D (10) which has been identified as the precursor of the azido-atrazine binding peptide associated with PSII (38). We have identified band 3 as a component of Chl/protein complex I by reelectrophoresis of the dissociated radioactive complex and note that bands 6 and 7 correspond to stained bands of the α and β subunits of CF<sub>1</sub>, and band 33 (17.4 kD) to a stained peptide associated with PSI (data not shown). Lanes 5 and 6 in Figure 7 show the labeled bands of the stroma and thylakoid fractions, respectively, and are included to show the expected difference in the patterns of labeled bands.

Lanes 3 and 4 of Figure 7 were photographed, prior to fluorography, with unfortunately suboptimal exposures. Following treatment with Enhance and drying down of the gel, the dye patterns are altered and are not suitable for photographing again. Hence, Figure 8, a photograph of a different gel, is included, to show the full range of polypeptides visible following one-dimensional electrophoresis. Up to 50 stained bands can be seen in the photograph, and about 59 in the original gel.

## DISCUSSION

In the current work we have reexamined most of the parameters of potential importance for protein synthesis by isolated, intact pea chloroplasts, with the major goal of optimizing the conditions to obtain maximal rates. In this we have probably succeeded, inasmuch as under the best conditions up to 100 nmol [<sup>3</sup>H]leucine were incorporated per mg of Chl in a 20-min reaction period. In the time-course experiment (Fig. 2), the initial (5-min) rate was 2.4 times faster than the average for 20 min; hence, we can infer initial rates at optimum leucine concentrations of 720 nmol mg<sup>-1</sup>

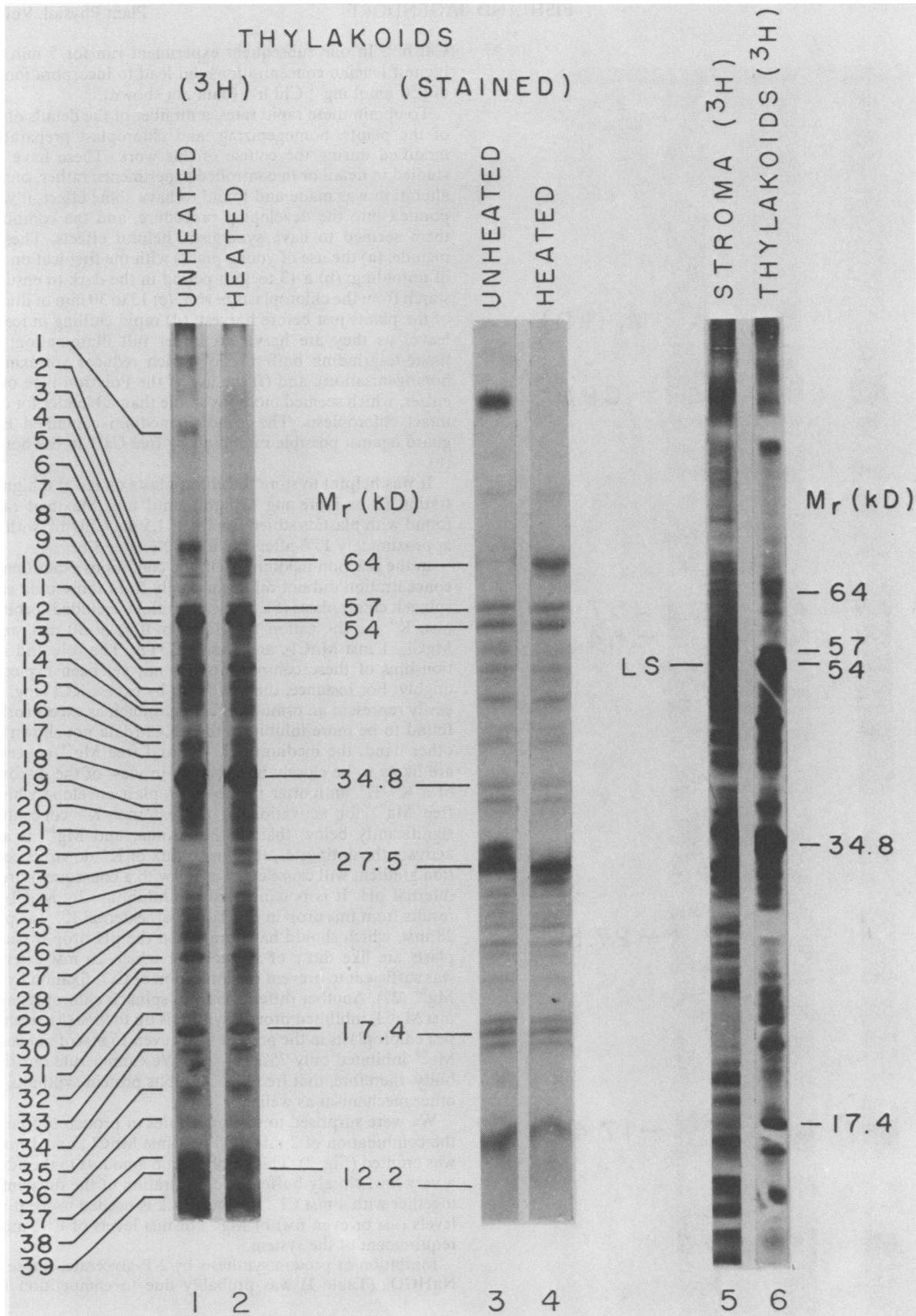
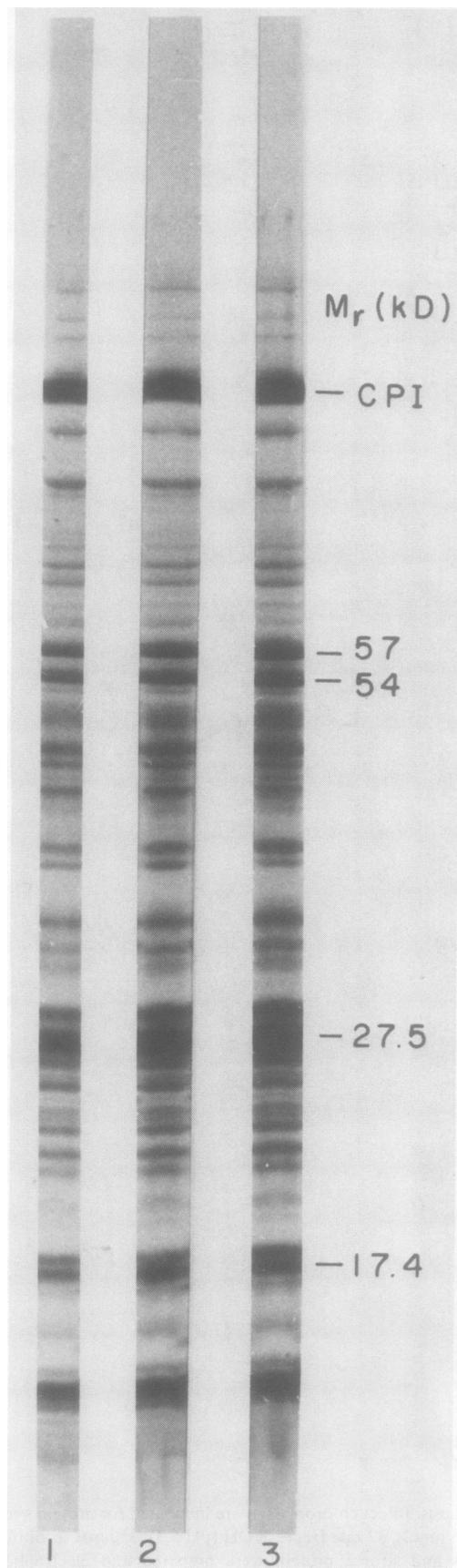


FIG. 7. Thylakoid and stroma polypeptides labeled during light-driven protein synthesis. Intact chloroplasts were incubated for protein synthesis for 30 min at 27°C in a 0.8 ml reaction mix containing 160  $\mu$ g Chl, 139  $\mu$ Ci [<sup>3</sup>H]leucine (2.5 nmol), 33 mM Hepes-KOH (pH 8.3), 350 mM sorbitol, 1.87 mM EDTA, 0.93 mM MgCl<sub>2</sub>, 0.93 mM MnCl<sub>2</sub>, and 0.90 mM DTT. Thylakoid samples and stroma protein were prepared and electrophoresed on polyacrylamide gels. Slots 1 to 4 are from one gel, and slots 5 and 6 from a second gel. The gels were stained and then prepared for fluorography. Slots 1, 2, 5, and 6 show fluorograph bands, and slots 3 and 4 show stained bands. The stacking gel is not shown in the figure. Slot 1, Dissolved <sup>3</sup>H-labeled thylakoids (1.3  $\mu$ g Chl) were loaded without heating; slot 2, dissolved <sup>3</sup>H-labeled thylakoids (1.3  $\mu$ g Chl) were heated 3 min at 80°C; slot 3, dissolved thylakoids (10  $\mu$ g Chl) were loaded without heating; slot 4, dissolved thylakoids (10  $\mu$ g Chl) were heated 3 min at 80°C; slot 5, <sup>3</sup>H-labeled stroma protein equivalent to 5.2  $\mu$ g Chl was loaded; slot 6, dissolved <sup>3</sup>H-labeled thylakoids (1.3  $\mu$ g Chl) were loaded without heating.



Chl  $h^{-1}$ . In one subsequent experiment run for 5 min only, the highest leucine concentrations did lead to incorporation at a rate of  $950 \text{ nmol mg}^{-1} \text{ Chl h}^{-1}$  (data not shown).

To obtain these rapid rates, a number of the details of handling of the plants, homogenizing, and chloroplast preparation were modified during the course of this work. These have not been studied in detail or in controlled experiments; rather, once a given alteration was made and found to have some effect, it was incorporated into the developing procedure, and the combination of them seemed to have synergistic helpful effects. These factors include: (a) the use of young plants with the first leaf on the verge of unfolding; (b) a 13 to 16 h period in the dark to ensure loss of starch from the chloroplasts *in vivo*; (c) 15 to 30 min of illumination of the plants just before harvest; (d) rapid chilling in ice water of leaves as they are harvested under full illumination; (e) high tissue-to-grinding buffer ratio (which reduces vortexing during homogenization); and (f) the use of the Polytron type of homogenizer, which seemed more favorable than a blender for obtaining intact chloroplasts. The grinding medium contained EGTA to guard against possible inhibition by free  $\text{Ca}^{2+}$  in the homogenate (6).

It was helpful to store the chloroplasts on ice at a high concentration (3 or more mg Chl/ml) until use. Maximal rates were found with plastids stored for 1 h at 1.5 mg Chl/ml; with losses of approximately 15% after 2 h, and 30% after 3 h.

In the reaction medium itself, the conditions other than leucine concentration did not differ markedly from those used earlier for spinach chloroplasts (5). These conditions included sorbitol at 350 mM,  $\text{K}^+$  (as the cation for Hepes buffer) at 30 mM, and 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{MnCl}_2$ , and 2 mM EDTA. The role and interrelationships of these components has not yet been explored thoroughly. For instance, the inhibition by excess KCl (Fig. 1) might easily represent an osmotic effect, inasmuch as excess sorbitol was found to be more inhibitory than KCl (data not shown). On the other hand, the medium pH,  $\text{K}^+$ , and free  $\text{Mg}^{2+}$  concentrations are likely to be closely interrelated, in view of the discovery (17) of a  $\text{K}^+/\text{H}^+$  antiporter in the chloroplast envelope which needs free  $\text{Mg}^{2+}$  for activation. If the external  $\text{K}^+$  concentration is significantly below that in the stroma, and  $\text{Mg}^{2+}$  is added to activate the antiporter, then net efflux of  $\text{K}^+$  down its concentration gradient will cause entry of  $\text{H}^+$  with a consequent drop of the internal pH. It is possible that the inhibition by  $\text{MgCl}_2$  (Fig. 3) results from this drop in pH. However, external  $\text{K}^+$  was present at 28 mM, which should have prevented the pH drop if pea chloroplasts are like those of spinach, for which 20 mM external KCl was sufficient to prevent the inhibition of  $\text{CO}_2$  fixation by external  $\text{Mg}^{2+}$  (27). Another difference from spinach chloroplasts is that 5 mM  $\text{MgCl}_2$  inhibited protein synthesis up to 95% (5), whereas with pea chloroplasts in the present study even 15 mM of noncomplexed  $\text{Mg}^{2+}$  inhibited only 75% (Fig. 3). We cannot rule out the possibility, therefore, that free  $\text{Mg}^{2+}$  inhibits protein synthesis by some other mechanism as well.

We were surprised to see lower rates of protein synthesis when the combination of 2 mM EDTA, 1 mM  $\text{MnCl}_2$ , and 1 mM  $\text{MgCl}_2$  was omitted (Fig. 2). This combination amounts to the addition of a very low, closely buffered concentration of the divalent cations together with 4 mM  $\text{Cl}^-$ . Further work is needed to see if very low levels ( $\mu\text{M}$  or even nM) of  $\text{Mg}^{2+}$ , or mM levels of  $\text{Cl}^-$ , might be a requirement of the system.

Inhibition of protein synthesis by 2-P-glycerate, ribose 5-P and  $\text{NaHCO}_3$  (Table I) was probably due to competition for ATP

FIG. 8. Thylakoid samples were prepared and electrophoresed as described in "Materials and Methods" for 15 h at 150 v with constant voltage and 2 h at 10 w with constant power. The gel was stained and destained as described. The slots were loaded with dissolved, unheated thylakoid samples containing 10  $\mu\text{g}$  Chl (slot 1), 13  $\mu\text{g}$  (slot 2) or 15  $\mu\text{g}$  Chl (slot 3).

generated by photophosphorylation. Adding DL-glyceraldehyde should have inhibited the residual traces of Calvin cycle activity (39) and for that reason might have stimulated protein synthesis. However, it only inhibited incorporation of radioactive leucine. The combination of  $\alpha$ -ketoglutarate plus glutamine, which should stimulate electron flow through ferredoxin to glutamate synthase (22), also inhibited.

The stimulation caused by a low concentration of DCMU (Table I) was similar to that reported earlier for spinach chloroplasts (36). Its origin might be a poisoning effect needed for vigorous cyclic electron flow and ATP synthesis, inasmuch as in the absence of a net electron acceptor, such as  $\text{CO}_2$ , cyclic electron flow and photophosphorylation are inhibited by the unavailability of oxidized electron carriers (3). However, protein synthesis was not stimulated by  $\text{NaHCO}_3$ , even at the low concentration of 0.25 mM (Table I), suggesting that enhancement of cyclic electron flow by DCMU may not be the complete answer.

The large amount of leucine incorporation, 100 nmol/mg Chl, was obtained when the external concentration was raised to the saturating level of 400  $\mu\text{M}$  or above (Fig. 5). Isolated chloroplasts of *Vicia faba* were reported to be unable to synthesize leucine (20). If this is the case for pea chloroplasts as well, then protein synthesis must depend on the stored pool of this amino acid, on internal protein turnover, or on entry of new leucine from the medium. The endogenous stored leucine was previously reported to be 9 nmol/mg Chl in isolated pea chloroplasts (28) for a stroma concentration of 360  $\mu\text{M}$ . This is 9% of the total leucine incorporated in our experiments. Thus, the increasing amount of incorporation as external leucine concentration was raised to 400  $\mu\text{M}$  (Fig. 5) cannot be ascribed simply to overcoming the dilution effect of unlabeled endogenous leucine, but must represent a definite increase in protein synthesis. Both isotope dilution and limitation of protein synthesis by leucine may be in part responsible for the previously reported (28, 30, 35, 36) maximal amounts of radioactive leucine incorporation by isolated intact chloroplasts, which did not exceed 1.2 nmol/mg Chl. Even with lower incorporation rates it was demonstrated recently (29) that continuing internal synthesis of some of the amino acids (e.g. methionine) is needed for protein synthesis. Also, some of the other amino acids are present at concentrations lower than that of leucine (28). Thus, in the present system, it seems possible that a shortage of one or more of the other amino acids may limit the total amount, or even the initial rates, of protein synthesis.

As a practical matter the highest leucine concentrations led to low counts on the filter paper discs, due to isotope dilution, when using reasonable levels of tritium. For routine experiments we have used 12 to 15  $\mu\text{M}$  leucine in the reaction, and, in repeated experiments, between 30 and 60 nmol were found to be incorporated in 20 min.

With a few assumptions based on results from the literature, and some additional measurements, it is possible to calculate roughly the doubling time for thylakoid proteins represented by the most rapid incorporation, given 400  $\mu\text{M}$  leucine (Fig. 5). We measured 6 mg of protein per mg Chl in washed thylakoids by the method of Markwell *et al.* (26), and assumed, as noted earlier (8) that 35% of the thylakoid proteins are made inside of the chloroplasts. We used 9.7 mol % as an average value for the leucine content of the thylakoid proteins, as found by others for three of its major proteins (1, 4). The calculated increase in thylakoid protein turned out to be 1.8% for the 20-min reaction period, corresponding to a doubling time of 18.5 h. The estimated increase in the first 5 min was 1.05%, for a doubling time of 7.9 h.

To compare these rates with those occurring *in vivo*, we measured the rate of Chl synthesis in seedlings at this stage of growth. Shoot samples were collected several times during the first 7 h of illumination on day 8, and analyzed for total Chl content. The data (not shown) indicated a linear rate of increase with a doubling

time of 12 h. Although it is likely that the protein/Chl ratio of the thylakoids is roughly constant over this interval, the doubling time for thylakoid protein should be the same as that for Chl. Thus, our estimated *in organello* protein doubling time (7.9 h) based on the first 5 min of the most rapid reaction is somewhat faster than those actually sustained *in vivo*; and overall, it seems certain that the goal of reaching maximal rates of protein synthesis by isolated chloroplasts has been achieved.

From examination of fluorographs such as those in lanes 1, 2 and 6 of Figure 7, we can estimate the average mass of the newly synthesized thylakoid membrane polypeptide to be about 35,000 D. We have previously (12, 41) established the amount of RNA, and therefore the number of ribosomes bound to pea chloroplast thylakoids. We can for the moment assume that only these thylakoid-bound polysomes make thylakoid proteins, and that, as for the stroma polysomes (16), the average polysome is only a trimer. Using these assumptions we are able to provide rough estimates of the rate of handling of amino acids by each active ribosome, and the numbers of reinitiations per mRNA chain. At the rates obtaining during the first 5 min of the reaction, each ribosome is estimated to process 10.6 amino acids/s, and each mRNA goes through 5.5 initiations/min. These estimates are close to those made for *Chlamydomonas* cells *in vivo* (16) where each ribosome was reported to process 5 amino acids/s.

The stained  $\text{LiDodSO}_4$  gels of the thylakoids show up to 59 discrete polypeptides, of which about 50 are visible in Figure 8, and examination of the original fluorographs indicates up to 48 different labeled bands, with 39 bands that are visible after photography (Fig. 7). This is a striking increase compared to results from earlier studies, where between 9 and 15 radioactive thylakoid membrane products were observed (9, 13–15, 40, 42). The large number in the present studies can not be ascribed to inadequate washing of the thylakoids prior to analysis, since no inordinate amount of stain or label was found at an  $M_r$  of 54,000 D, where the large subunit of ribulose bisP carboxylase/oxygenase, the most abundant stroma ribosome product, is located. The overall dissimilarity in the pattern of labeled bands found for stroma and thylakoid fractions (Fig. 7) also argues against significant contamination by stroma proteins.

The most interesting possibility is that the larger number of labeled bands in the present work results from translation of mRNA species which would be translated only infrequently under conditions favoring a low rate of initiation. The current conditions permitting high rates of protein synthesis may allow successful competition by these mRNA for free ribosomes.

However, it is also possible that many of these minor protein bands were synthesized in earlier studies, but not detected because of lower resolution on the electrophoresis gel. The high specific radioactivity of the thylakoids obtained here permitted use of a small amount of protein for electrophoresis, providing a degree of resolution not otherwise possible. For example, slots 1 and 2 in Figure 7 were loaded with 4.8  $\mu\text{g}$  protein, which is about 8- to 12-fold less than that needed to provide adequate protein for visualizing the approximately 59 bands stained by the combination of Coomassie Brilliant Blue G and R used in this work (Fig. 8). At the high protein load needed to detect the faintest stained bands, the major peptides are broadened, and overlap with some of the minor bands. Minor bands tend to become broadened and distorted, and would be confused with 'background' radioactivity. The use of appropriate two-dimensional gels, in conjunction with a high rate of protein synthesis, should help to establish more clearly the number and identity of polypeptide products.

A final possibility is that some of the labeled bands represent premature termination products of a defective translation system. Currently, however, we have no reason to think this is more likely for high rate conditions than for low rate conditions. Further work is needed to assess this possibility, and to determine the identity

of the larger number of thylakoid proteins that now appear to be synthesized inside isolated chloroplasts.

#### LITERATURE CITED

1. APEL K 1977 The light harvesting chlorophyll *a/b* protein complex of the green alga *Acetabularia mediterranea*. Isolation and characterization of two subunits. *Biochim Biophys Acta* 462: 390-402
2. ARNON DI 1949 Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol* 24: 1-15
3. ARNON DI, HY TSUJIMOTO, BD MCSWAIN 1967 Ferredoxin and photosynthetic phosphorylation. *Nature* 214: 562-566
4. BINDER A, A JAGENDORF, E NGO 1978 Isolation and composition of the subunits of spinach chloroplast coupling factor protein. *J Biol Chem* 253: 3094-3100
5. BOTTOMLEY W, D SPENCER, PR WHITFIELD 1974 Protein synthesis in isolated spinach chloroplasts: comparison of light-driven and ATP-driven synthesis. *Arch Biochem Biophys* 164: 106-117
6. BOUTHYETTE PY, AT JAGENDORF 1982 Calcium inhibition of amino acid incorporation by pea chloroplasts and the question of loss of activity with age. *In G Akoyunoglou, ed, Proceedings of the Fifth International Congress on Photosynthesis*, Vol V. Balaban International Science Service, Philadelphia, pp 599-609
7. BURNS DJW, E CUNDLIFFE 1973 Bacterial protein synthesis: a novel system for studying antibiotic action *in vivo*. *Eur J Biochem* 37: 570-574
8. CHUA NH, NW GILLHAM 1977 The sites of synthesis of the principal thylakoid membrane polypeptides in *Chlamydomonas reinhardtii*. *J Cell Biol* 74: 441-452
9. ELLIS RJ 1977 Protein synthesis by isolated chloroplasts. *Biochim Biophys Acta* 463: 185-216
10. ELLIS RJ 1981 Chloroplast proteins: synthesis, transport, and assembly. *Annu Rev Plant Physiol* 32: 111-137
11. ELLIS RJ, MR HARTLEY 1971 Sites of synthesis of chloroplast proteins. *Nat New Biol* 233: 193-196
12. FISH LE, AT JAGENDORF 1982 Light-induced increase in the number and activity of ribosomes bound to pea chloroplast thylakoids *in vivo*. *Plant Physiol* 69: 814-824
13. GEETHA V, A GNANAM 1980 Synthesis of soluble thylakoid and envelope polypeptides by isolated chloroplasts of *Sorghum vulgare*. *Biochim Biophys Acta* 608: 427-434
14. GREBANIER AE, KE STEINBACK, L BOGORAD 1979 Comparison of the molecular weights of proteins synthesized by isolated chloroplasts with those which appear during greening in *Zea mays*. *Plant Physiol* 63: 436-439
15. GREEN BR 1980 Protein synthesis by isolated *Acetabularia* chloroplasts. *Biochim Biophys Acta* 609: 107-120
16. HOWELL S, P HEIZMAN, S GELVIN 1976 Properties of the mRNA and localization of the gene coding for the large subunit of ribulose biphosphate carboxylase in *Chlamydomonas reinhardtii*. *Colloq Int Cent Natl Rech Sci* 261: 313-318
17. HUBER SC, W MAURY 1980 Effects of magnesium on intact chloroplasts. *Plant Physiol* 65: 350-354
18. JENSEN RG, JA BASSHAM 1966 Photosynthesis by isolated chloroplasts. *Proc Natl Acad Sci USA* 56: 1095-1101
19. KABAT D, MR CHAPPELL 1977 Competition between globin messenger ribonucleic acids for a discriminating initiation factor. *J Biol Chem* 252: 2684-2690
20. KIRK PR, RM LEECH 1972 Amino acid biosynthesis by isolated chloroplasts during photosynthesis. *Plant Physiol* 50: 228-234
21. LAEMMLI UK 1970 Cleavage of structural proteins during assembly of phage T4 head. *Nature* 227: 680-685
22. LEA PJ, BJ MIPLIN 1974 Alternative route for nitrogen assimilation in higher plants. *Nature* 251: 614-616
23. LODISH HF 1976 Translational control of protein synthesis. *Annu Rev Biochem* 45: 39-72
24. LUCCHINI G, R BIANCHETTI 1980 Initiation of protein synthesis in isolated mitochondria and chloroplasts. *Biochim Biophys Acta* 608: 54-61
25. MANS RJ, GO NOVELLI 1961 Measurement of the incorporation of radioactive amino acids into proteins by a filter-paper disk method. *Arch Biochem Biophys* 94: 48-53
26. MARKWELL MAK, SM HAAS, LL BIEBER, NE TOLBERT 1978 A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* 87: 206-210
27. MAURY WJ, SC HUBER, DE MORELAND 1981 Effects of magnesium on intact chloroplasts. II. Cation specificity and involvement of the envelope ATPase in (sodium) potassium/proton exchange across the envelope. *Plant Physiol* 68: 1257-1263
28. MILLS WR, KW JOY 1980 A rapid method for isolation of purified physiologically active chloroplasts, used to study the intracellular distribution of amino acids in pea leaves. *Planta* 148: 75-83
29. MILLS WR, KG WILSON 1978 Effects of lysine, threonine and methionine on light-driven protein synthesis in isolated pea (*Pisum sativum* L.) chloroplasts. *Planta* 142: 153-160
30. MORGENTHALER JJ, L MENDIOLA-MORGENTHALER 1976 Synthesis of soluble, thylakoid, and envelope membrane proteins by spinach chloroplasts purified from gradients. *Arch Biochem Biophys* 172: 51-58
31. MORGENTHALER JJ, CA PRICE, JM ROBINSON, M GIBBS 1974 Photosynthetic activity of spinach chloroplasts after isopycnic centrifugation in gradients of silica. *Plant Physiol* 54: 532-534
32. ORTIZ W, EM REARDON, CA PRICE 1980 Preparation of chloroplasts from *Euglena* highly active in protein synthesis. *Plant Physiol* 66: 291-294
33. PARENTI F, MM MARGULIES 1967 *In vitro* protein synthesis by plastids of *Phaseolus vulgaris*. I. Localization of activity in the chloroplasts of a chloroplast containing fraction from developing leaves. *Plant Physiol* 42: 1179-1186
34. PESTKA S 1977 Inhibitors of protein synthesis. *In H Weissbach, S Pestka, eds, Molecular Mechanisms of Protein Biosynthesis*. Academic Press, New York, pp 468-553
35. PRICE CA 1976 Protein synthesis by spinach and *Euglena* chloroplasts isolated in gradients of silica sols. *Colloq Int Cent Natl Rech Sci* 261: 473-480
36. RAMIREZ JM, FF DEL CAMPO, DI ARNON 1968 Photosynthetic phosphorylation as energy source for protein synthesis and carbon dioxide assimilation by chloroplasts. *Proc Natl Acad Sci USA* 59: 606-611
37. SLOVACEK RE, G HIND 1980 Energetic factors affecting carbon dioxide fixation in isolated chloroplasts. *Plant Physiol* 65: 526-532
38. STEINBACK KE, L MCINTOSH, L BOGORAD, CJ ARNTZEN 1981 Identification of a triazine receptor protein as a chloroplast gene product. *Proc Natl Acad Sci USA* 78: 7463-7467
39. STOKES DM, DA WALKER 1972 Photosynthesis by isolated chloroplasts. Inhibition by D,L-glyceraldehyde of carbon dioxide assimilation. *Biochem J* 128: 1147-1157
40. VASCONCELOS AC 1976 Synthesis of proteins by isolated *Euglena gracilis* chloroplasts. *Plant Physiol* 58: 719-721
41. YAMAMOTO T, J BURKE, G AUTZ, AT JAGENDORF 1981 Bound ribosomes of pea chloroplast thylakoid membranes: location and release *in vitro* by high salt, puromycin and RNase. *Plant Physiol* 67: 940-949
42. ZIELINSKI RE, CA PRICE 1980 Synthesis of thylakoid membrane proteins by chloroplasts isolated from spinach. Cytochrome *b559* and P700-chlorophyll *a*-protein. *J Cell Biol* 85: 435-445