

Optimal Conditions for Translation by Thylakoid-Bound Polysomes from Pea Chloroplasts¹

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ABSTRACT

Polysomes bound to washed thylakoids from pea *Pisum sativum* cv Progress No. 9 chloroplasts are capable of protein synthesis when supplemented with amino acids, ATP and a regenerating system, GTP, and soluble factors required for translation. The extent of protein synthesis in previous reports, however, was quite low when compared to *in organello* translation. By systematic testing of parameters in the isolation of thylakoids and reaction mixture components we have been able to establish more optimal conditions. Incorporation of 2 to 10 nanomoles of leucine per milligram chlorophyll in a 20-minute reaction period is now possible, representing a 10- to 60-fold increase over amounts previously reported. Autoradiographs of solubilized, electrophoresed membranes show about 30 discrete labeled polypeptides which remain associated with the thylakoid membranes.

There are two populations of ribosomes within chloroplast—those free in the stroma, and those bound to the thylakoid membranes (20, 27). Further, it is known that a large fraction of the bound ribosomes are in the form of polysomes attached at least in part by nascent peptide chains (3, 20, 21, 27). These attached polysomes can continue protein synthesis when supplied with ATP, GTP, amino acids, and soluble factors (enzymes and tRNAs) for translation, although at quite low rates (3, 21) compared to those found *in organello* (12, 22). Before investigating the nature of polypeptides synthesized by attached polysomes, we felt it important to optimize the conditions for translation, since a distorted picture of the extent and relative abundance of specific polypeptides may result from nonoptimal reaction rates (17). Improved conditions have been achieved, and autoradiographs of labeled membrane peptides show up to 30 products ranging from 11 to 60 kD.

MATERIALS AND METHODS

Materials. Peas (cv Progress No. 9) were from Agway Corp., *Escherichia coli* strain K12 (ATCC No. 10798) and amino acids were from Miles Laboratories. Percoll and Ficoll were from Pharmacia and radioactive amino acids were from Amersham. Other reagents and inhibitors were from Sigma.

Chloroplast and Thylakoid Isolation. Growth conditions for pea seedlings were described in Fish and Jagendorf (11) and

isolation of intact chloroplasts from Percoll gradients in Fish and Jagendorf (12). The pea seedlings were harvested 8 to 9 d after planting, when all the leaflets were still folded, or the first pair of leaves had just begun to unfold. The intact chloroplast pellet from the third centrifugation step was resuspended in 15 ml of hypotonic breaking buffer containing: 20 mM Tris-HCl (pH 8.5 at 4°C), 15 mM Mg-acetate, and 20 mM K-acetate; then passed thrice through an 18-gauge canula to ensure chloroplast breakage. The thylakoid membranes were sedimented at 27,000 g for 4 min in a Sorvall SS-34 rotor, the supernatant carefully drained away, and the final pellet resuspended in breaking buffer. The passage through a canula and centrifugation were repeated twice. The final pellet was resuspended to 3 mg Chl/ml in membrane resuspension buffer containing: 20 mM Hepes-KOH (pH 7.6 at 25°C), 15 mM Mg-acetate, 20 mM K-acetate, and 8 mM 2-mercaptoethanol, and stored on ice. All steps were carried out between 0 and 4°C. Chl concentration was estimated according to Arnon (1).

Isolation of S-30 from *E. coli*. Extract (S-30) was prepared according to Alscher *et al.* (3), and desalted on a G-25 Sephadex column using an elution buffer which contained: 20 mM Hepes-KOH (pH 7.6 at 20°C), 120 mM KCl, 5 mM Mg-acetate, and 5 mM 2-mercaptoethanol. It was stored as beads in liquid N₂ at a concentration of 90 A₂₆₀ units/ml.

Isolation of Stroma S-30 from Chloroplasts. Intact chloroplasts at 4 to 5 mg Chl/ml were stored in liquid N₂ in a buffer containing: 35 mM Hepes-KOH (pH 8.3), 375 mM sorbitol, 2 mM EDTA, 1 mM MgCl₂, 1 mM MnCl₂, and 0.96 mM DTT. Freezing, then thawing, broke the plastid envelope; the broken plastids were diluted to 3 mg Chl/ml with membrane resuspension buffer (20 mM Hepes-KOH [pH 7.6], 15 mM Mg-acetate, 20 mM K-acetate, 8 mM 2-mercaptoethanol) and centrifuged for 10 min at 27,000 g (Sorvall SS-34 rotor) at 4°C. The supernatant was centrifuged 2 to 4 times more until it was no longer cloudy, then again at 30,000 g for 30 min. In early experiments it was incubated at 30°C for 20 min with additions required for protein synthesis, in order to promote runoff of polyribosomes present. In later experiments, this preincubation was omitted since it significantly reduced the ability of stroma S-30 to supplement the activity of thylakoid-bound polysomes. Both types of stroma extract were desalted by centrifuging through a Sephadex G-25 (fine) mini-column (23). Material in successive collections varied between 30 and 50 A₂₆₀ units starting with 1 mg Chl in the unbroken chloroplasts.

Protein Synthesis by Thylakoid-Bound Polysomes. Incubation was carried out in 50 μl for 20 min at 30°C. During the course of the work, many components were tested individually. The final optimized mixture used contained: 0.3 mg Chl/ml of washed thylakoids; 20 to 30 A₂₆₀ units/ml of S-30 from *E. coli* or chloroplast stroma; 25 μM each of 19 amino acids; [³H]leucine (about 50 Ci/mmol) at 0.72 μM and extra cold leucine to 8 μM; 1 mM ATP; 0.2 mM GTP; 100 mM K-acetate; 12.5 mM Mg-

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acetate; 1 mM EDTA; 6 mM creatine phosphate; 50 $\mu\text{g/ml}$ creatine kinase; 4.7 mM 2-mercaptoethanol; and 64 mM Hepes-KOH (pH 7.6 at 25°C). The EDTA, Mg-acetate, K-acetate, Hepes-KOH, ATP, GTP, creatine phosphate, creatine kinase, and amino acids were combined together at the appropriate concentrations so they could be added as a 15- μl aliquot. Washed thylakoids were added as a 10- μl aliquot, S-30 as a 15- μl aliquot, and the extra volume made up with more membrane resuspension buffer in which any other additives were dissolved. The reaction was started by addition of the thylakoid membranes and transfer to a 30°C water bath. The tubes were shaken at least once every 3 min; the reaction was stopped by transferring to an ice bath and adding 10 μl of 100 mM leucine.

After incubation, 30 μl of 5% Triton X-100 was added to 15 μl of the reaction mixture, and the dissolved membranes centrifuged for 5 min in an Eppendorf model 5412 centrifuge at 15,600 g to sediment any bacteria. In most later experiments, the centrifugation step was omitted, since experience had shown no measurable difference in leucine incorporated with or without centrifugation. Duplicate aliquots of 25 μl were spotted on 3 MM filter paper discs, processed as described by Mans and Novelli (19), and counted in a Packard liquid scintillation spectrometer. The counting efficiency was approximately 7%. All results are reported as nmol leucine incorporated/mg Chl, with the calculations based on this efficiency factor, the specific activity of total leucine in the reaction (labeled plus added unlabeled leucine) and appropriate dilution factors.

To separate thylakoid membranes from soluble products, 50 μl of 10 mM NaPPi (pH 7.4 at 25°C) were added to the remaining 25 μl reaction mixture, and centrifuged for 7 min in an Eppendorf centrifuge. Aliquots (25 μl each) of the supernatant were spotted on paper discs and the remaining supernatant removed by aspiration. The green pellet was solubilized in 2% Triton X-100 and spotted on paper discs, to be processed and counted for protein. The sum of acid-insoluble counts in the membranes plus those in the supernatant varied between 85 and 100% of the total counts in the reaction mixture.

The translation activity due to S-30 factors alone (since these often included some polyribosomes) was determined in every experiment; this background was subtracted before calculating the amount of incorporation by the membrane-bound polyosomes. Activity in the *E. coli* S-30 amounted to 5 to 9% of the total counts.

Gel Electrophoresis and Autoradiography. For analysis of polypeptide patterns by autoradiography, [^{35}S]methionine at 600 Ci/mmol was used instead of [^3H]leucine, with cold methionine omitted and leucine present at 25 μM . After incubation, the thylakoids were sedimented as described above, and proteins in the supernatant were precipitated with 4 volumes of acetone at -20°C. These were centrifuged, washed once with 80% (v/v) acetone, dried under a stream of N_2 , then redissolved for electrophoresis in 2% (w/v) LDS³, 50 mM Tris-HCl (pH 8.5 at 4°C), 50 mM DTT, and 12% (w/v) sucrose with heating for 2 min at 80°C.

Thylakoids from the first centrifugation were washed once more in 10 mM NaPPi (pH 7.4 at 25°C) and three times more in 100 mM Tris-HCl (pH 8.5 at 4°C), 100 mM DTT. They were then suspended in 2% (w/v) LDS, 12% (w/v) sucrose, and heated 2 min at 80°C.

Proteins were electrophoresed using the Laemmli buffer system (16) but substituting LDS for SDS. Four % acrylamide was used in the stacking gel and a 10 to 16% acrylamide gradient (also 5.5–11% sucrose) in the resolving gel. Gel dimensions were 0.5 \times 130 \times 140 mm.

Gels were fixed for 30 min in 15% (w/v) TCA, 50% (v/v)

methanol; then stained for 3 h in 0.2% Coomassie Brilliant Blue R, 0.1% Coomassie Brilliant Blue G, 7% (w/v) acetic acid, 50% (v/v) methanol. Destaining was carried out at 50°C in 7% (v/v) acetic acid, 20% (v/v) methanol. The gels were dried down on Whatman 3MM filter paper and exposed to Kodak XAR-5 x-ray film at -80°C.

M_r values for peptide bands were calculated from a linear standard curve of R_f versus log mol wt of marker thylakoid peptides and mol wt marker soluble proteins: myoglobin (17.6 kD), trypsin inhibitor (21.5 kD), carbonic anhydrase (30 kD), asparaginase (35 kD), creatine kinase (40.5 kD), and BSA (68 kD).

RNA content of thylakoids was estimated by the method of Fish and Jagendorf (10).

RESULTS

Cation concentrations were found to be critical for protein synthesis by polysomes of washed thylakoids. A sharp optimum at 100 mM was found for K^+ (Fig. 1) with the rate 33% lower at 42 mM, and progressive inhibition above 100 mM. The lowest concentration tested was 42 mM because this was the amount brought in with the *E. coli* S-30 preparation, plus the Hepes-KOH of the thylakoid resuspension buffer. Polyamines (2) did not substitute for K^+ , and both spermidine and putrescine were inhibitory at all concentrations tested (Table I). The optimum Mg^{2+} concentration was between 9 and 11 mM with inhibition seen both above and below this narrow range (Fig. 2). The effect of other cations and anions was not examined, although the KCl and MgCl in the original recipe for breaking buffer (3) were replaced here by the corresponding acetate salts, because Cl^- had been reported to inhibit protein chain initiation (26).

Increasing the leucine concentration from 0.72 to 8 μM caused a 3-fold increase in the calculated amount of leucine incorporated, but higher concentrations (25 μM) had no effect (data not shown). The system appears to be quite efficient for reacting with leucine, however, since when its concentration was 0.72 μM , 97% of the label was incorporated into protein.

Varying the volume of added S-30 from 0 to 15 $\mu\text{l}/50 \mu\text{l}$ reaction mix caused a progressive increase in the rate of protein

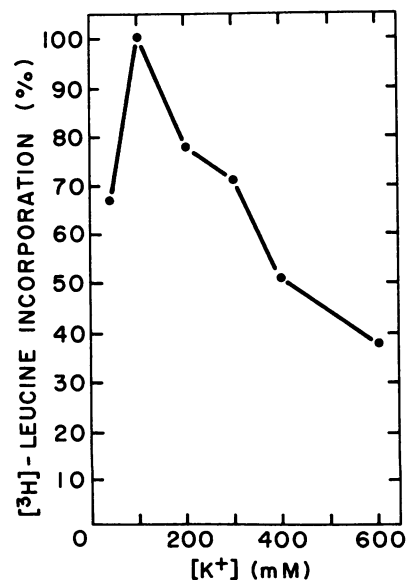


FIG. 1. Protein synthesis as a function of K^+ concentration. The basal level of 42 mM K^+ was brought into the reaction by thylakoid resuspension buffer and S-30 from *E. coli*; concentrations above that level were achieved by added K-acetate. 100% incorporation in this experiment was 5.94 nmol [^3H]leucine/mg Chl in a 20-min reaction period.

³ Abbreviations: LDS, lithium dodecylsulfate; ATA, aurintricarboxylic acid.

Table I. Effect of Polyamines on Protein Synthesis by Bound Ribosomes

Thylakoids were incubated for protein synthesis for 20 min.

Compound	Concn.		Leucine Incorporation	
	mm	nmol/mg Chl	% of control	% in thylakoids
Putrescine	0.0	10.08	(100)	63
	0.5	7.94	79	56
	4.0	6.47	64	57
	8.0	4.88	48	52
	16.0	2.81	28	50
Spermidine	0.0	1.35	(100)	63
	0.1	1.24	92	67
	0.5	1.24	92	63
	1.0	0.54	40	97
	8.0	0.35	26	100

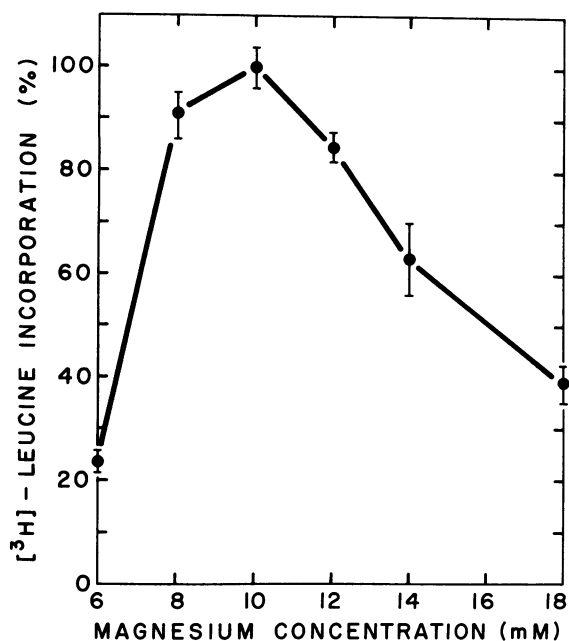


FIG. 2. Protein synthesis as a function of Mg^{2+} concentration. Optimized conditions were used, except for variable Mg^{2+} levels. The basal level of 6 mM was brought into the reaction mixture with the *E. coli* S-30 elution buffer and the thylakoid membrane resuspension buffer; extra Mg^{2+} was added as Mg-acetate. 100% incorporation in this experiment was 10.4 nmol/mg Chl in the 20-min reaction period.

synthesis without reaching apparent saturation (data not shown). However, it was not practicable to add more than 15 μ l, and this amount (usually 1.1–1.5 A_{260} units) was used routinely.

An optimum concentration of 1.0 mM was found for ATP (Fig. 3). In this experiment, 4 mM inhibited 30%; in some other experiments the inhibition by excess ATP was even more severe. Surprisingly, 40% (in other experiments 25%) of the maximum incorporation occurred without adding any ATP at all. The concentration curve for GTP showed an even sharper optimum at 0.2 mM, and here 1.6 mM inhibited 100% (Fig. 4). Again, an absolute requirement for GTP was not demonstrated. With creatine kinase present at 20 μ g/ml, adding creatine phosphate between 0 and 6 mM increased the incorporation by 30% (data not shown), indicating the need for some ATP regeneration in order to achieve maximal synthesis in this cell-free system.

To determine a pH curve, the pH was adjusted for all components except for the S-30. The final pH of the complete reaction mixture was then measured separately at each point. Protein synthesis showed optimum rates between pH 7.6 and

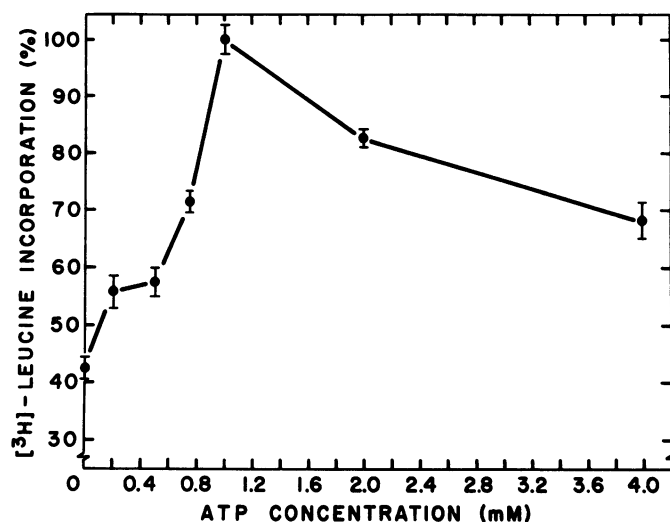


FIG. 3. Protein synthesis as a function of ATP concentration. Washed thylakoids were incubated as described in "Materials and Methods." The GTP concentration was 0.2 mM, creatine phosphate at 6 mM, and Mg^{2+} at 12.5 mM. 100% incorporation in this experiment amounted to 3.9 nmol [³H]leucine/mg Chl in the 20-min reaction period.

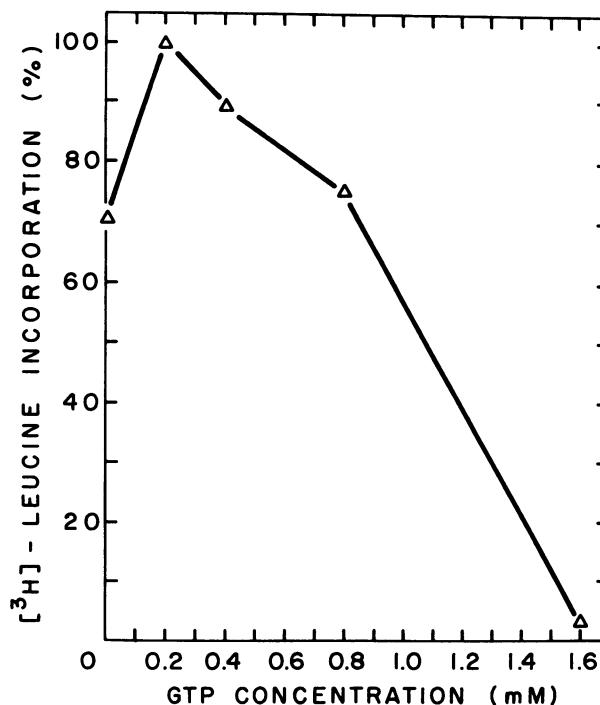


FIG. 4. Protein synthesis as a function of GTP concentration. As in Figure 5, with ATP at 1.0 mM, creatine phosphate at 6 mM, and Mg^{2+} at 12.5 mM. 100% incorporation amounted to 1.32 nmol [³H]leucine/mg Chl in the 20-min reaction.

7.9, with considerable inhibition above and below these values (data not shown). Fortunately, earlier experiments in the series had been performed at pH 7.6, in the optimum pH range.

This series of experiments led to an optimized 'recipe' for thylakoid polysome translation using *E. coli* S-30, whose components are listed in "Materials and Methods." A typical time course of incorporation into both soluble products and thylakoid-associated proteins is shown in Figure 5. Incorporation was nearly linear for the first 10 min, then increased at a slower rate up to 40 min, especially for the thylakoid-bound products. The rate in the first 10 min was 0.3 nmol of leucine/mg Chl · min (18

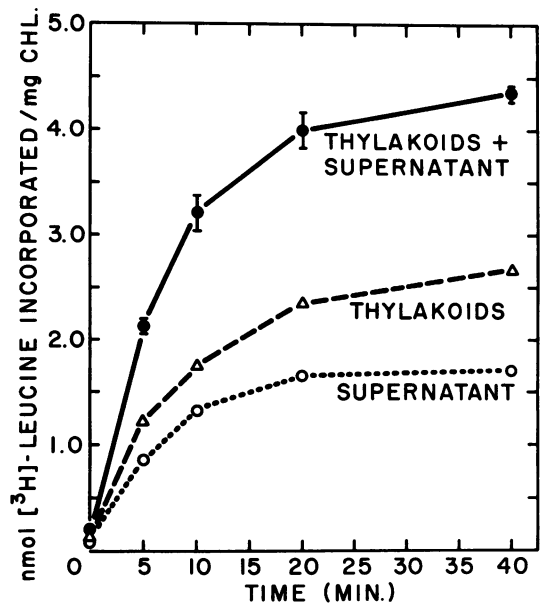


FIG. 5. Time course for incorporation of [^3H]leucine by washed thylakoids. Incubation was under standard conditions with S-30 factors from *E. coli*. Incorporation was measured in proteins of the soluble and thylakoid phases, separated after the incubation was over; the total incorporation is the arithmetic sum of these two. Each point is the average of duplicate tubes.

nmol/mg Chl·h). The total incorporated was 4.4 nmol/mg Chl. Sixty to 65% of the total TCA-precipitable radioactivity remained associated with the thylakoids in spite of extensive washes, and the rest was in the supernatant. Rates of protein synthesis may be converted to a μg RNA basis since thylakoids at this stage of development have about 400 μg bound RNA/mg Chl (11, 12). Thus, the initial rate might also be expressed as 0.75 nmol leucine/mg RNA·min (or 45 nmol leucine/mg RNA·h).

Effects of Inhibitors. The RNase inhibitor, heparin, stimulated leucine incorporation as much as 50% at 0.2 to 0.5 mg/ml (data not shown). However, it also caused a somewhat lower percentage of the products to remain associated with the thylakoids, and thus was not used in the optimized system. The RNase inhibitor from human placenta (9) became available at the end of this work. In one or two experiments it stimulated translation up to 35% without changing the proportion of thylakoid-bound products, and so should be useful in further studies with this system. These results indicate the presence of at least a low level of RNase in the reaction mixture.

Protein synthesis inhibitor effects are shown in Table II. Cy-

cloheximide was without effect, while chloramphenicol at 250 μM inhibited 75%. Four compounds were used at concentrations reported to inhibit initiation. Of these, ATA (24), edeine (24), and folic acid (18) had no effect, and lincomycin (7) inhibited 18%. However, lincomycin is also known to affect early peptide bond function (7). Finally, 100% inhibition resulted from adding RNase at 1 $\mu\text{g}/\text{ml}$. These data show that (a) there is no substantial contamination by active 80S cytoplasmic ribosomes; (b) most if not all of the activity is due to 70S ribosomes, (c) bacterial contamination is negligible, since RNase would not prevent bacteria from incorporating radioactive leucine, and (d) most of the activity represents completion of preexisting peptide chains, with little or no reinitiation occurring under these conditions.

LDS-PAGE of Labeled Products Synthesized by Thylakoid-Bound Polysomes. The labeled soluble and thylakoid-bound products of optimized thylakoid translation were examined by LDS-PAGE (Fig. 6). Approximately 30 labeled bands were visible in track 2 containing polypeptides from washed thylakoids. Only some of the labeled bands in track 2 correspond to visible, stained bands in track 1. A number of the labeled bands must represent very minor proteins which do not appear in the stained pattern. However, on the original gel as many as 40 discrete stained bands could be seen in track 1; the fainter bands do not show up in the published photograph. There was very little radioactivity in thylakoid proteins at M_r values at or below 20 kD.

A large number of labeled bands are found in the combined translation supernatant and pyrophosphate washes (Fig. 6, track 4). The pattern of labeled bands in this fraction is very different than that from the thylakoids (track 2). It seems to include more material at the low mol wt region, and much less between 17 and 45 kD, than is found in the thylakoids. Both thylakoid and stroma fractions have a large amount of label in the 52 to 54 kD region where one would expect to see the large subunit of ribulose biphosphate carboxylase. The *E. coli* S-30 by itself (tracks 5 and 6) produced very little labeled protein, all of it apparently in one faint band at about 52 kD.

Use of Stroma-Derived S-30 Factors. *E. coli* S-30 is used extensively for protein synthesis by procaryotic systems, but experience with it in connection with chloroplast ribosomes is less extensive (3, 21). Since thylakoid-bound polysomes might be translated more efficiently in a homologous system, we investigated the use of S-30 from pea chloroplast stroma for translation. It was as active as the S-30 from *E. coli* (Fig. 7); although translation appeared to cease sooner with the stroma S-30 (at 5 min) than with *E. coli* S-30 (8 min in this experiment). The total incorporation was very similar with either source for S-30; up to 9 nmol/mg Chl in a 20-min incubation. A higher proportion of the radioactive proteins remained associated with thylakoids (70%) using stroma S-30 than with *E. coli* S-30 (53% in this experiment).

The thylakoid-bound products of the bound polysomes, seen

Table II. Effect of Protein Synthesis Inhibitors on Protein Synthesis by Thylakoid-Bound Ribosomes
Protein synthesis inhibitors were added to the 50- μl reaction mix at the start of the 20-min incubation period with [^3H]leucine. Inhibition is expressed as a percentage of the control amount (*i.e.*, without added inhibitor).

Compound	Site of Inhibition	Concn.	Inhibition	Control
			%	Incorporation
				nmol/mg Chl
Cycloheximide	Elongation on 80S ribosomes	250 μM	5	1.5
Chloramphenicol	Elongation on 70S ribosomes	250 μM	75	1.5
RNase	Hydrolyses RNA	1 $\mu\text{g}/\text{ml}$	99	1.1
Lincomycin	Initiation on 70S ribosomes	1 $\mu\text{g}/\text{ml}$	18	0.5
ATA	Initiation on 70S ribosomes	50 μM	0	1.6
Edeine	Initiation on 70S ribosomes	5 μM	0	0.6
Folic acid	Initiation on 70S ribosomes	100 μM	0	4.7

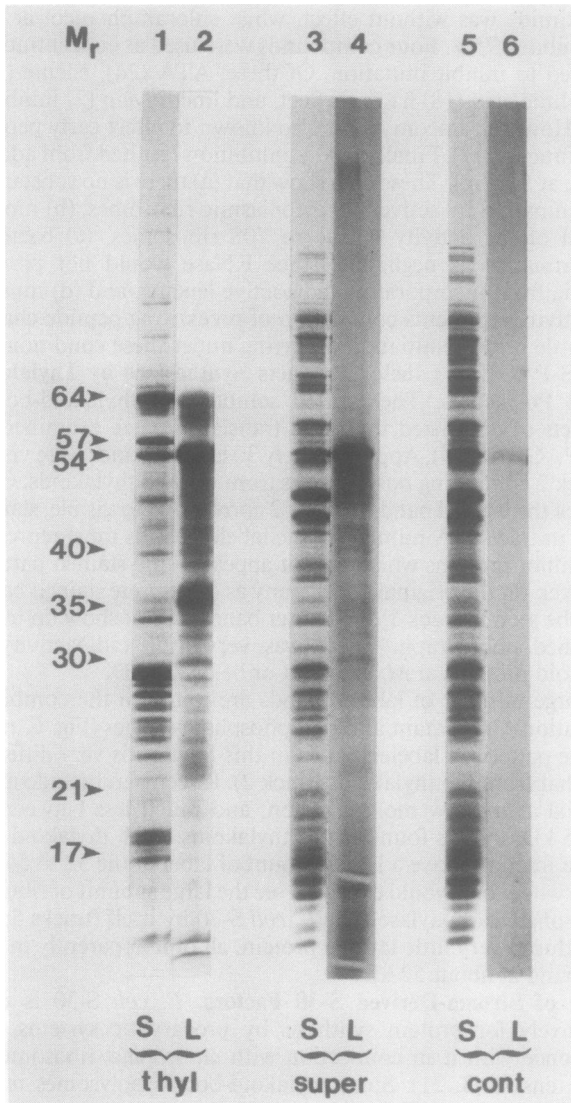


FIG. 6. Products synthesized by thylakoid-bound ribosomes supplemented with S-30 factors from *E. coli*. Washed thylakoids were incubated for protein synthesis with [35 S]methionine, processed, electrophoresed, and exposed to x-ray film for 48 h as described in "Materials and Methods." Track 1, Heated thylakoids (Thyl) equivalent to 5 μ g Chl (8×10^4 cpm), stained (S) with Coomassie Blue. Track 2, Autoradiograph of the labeled proteins (L) of track 1. Track 3, Stained bands of thylakoid translation supernatant (super), with 7.2×10^4 cpm. Track 4, Autoradiograph of track 3. Track 5, Stained bands of total reaction mixture incubated without thylakoids. This track was loaded with the same amount of proteins in track 3, and served as its control (cont). Track 6, Autoradiograph of track 5. M_r values derived from standard proteins are shown at the extreme left.

on autoradiography of electrophoretic gels, are almost identical whether the S-30 came from *E. coli* or from chloroplast stroma (Fig. 8, compare lanes 1 and 2). The major difference is a more prominent label in a few polypeptides below 17 kD, and less prominent label in those at 64 kD or above, when using stroma S-30. About 30 labeled bands appear in each case, all of them corresponding between the two lanes.

When *E. coli* S-30 was used, the thylakoid-bound polysomes synthesized a large number of peptides that were not integrated into the membrane, some of them in the low mol wt end but many more between 40 and 57 kD (Fig. 6, track 4 and Fig. 8, track 3). It was not possible to distinguish soluble polypeptides

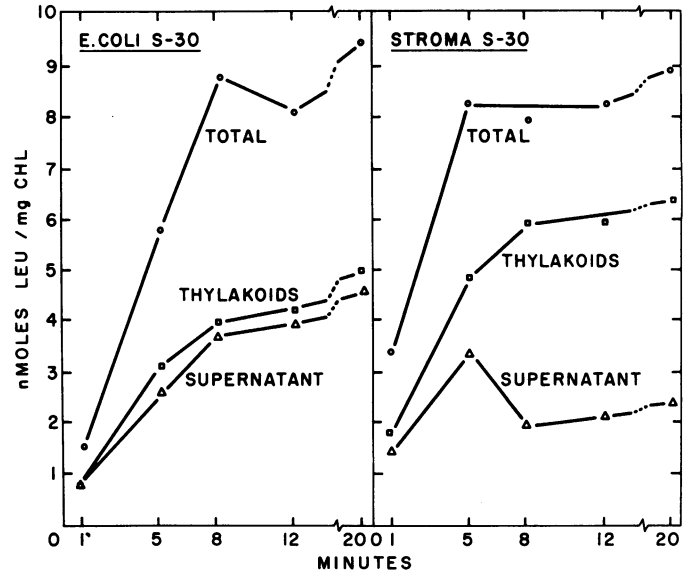


FIG. 7. Comparison of time courses using either *E. coli* or chloroplast stroma S-30 factors. Washed thylakoids were incubated for protein synthesis under optimized conditions, using S-30 from *E. coli* (left panel) or from chloroplast stroma (right panel). The thylakoids and soluble proteins were separated after the reaction was over, and processed separately for counting. Total incorporation (Total) is the arithmetic sum of the two. The results show the average of duplicate test tubes for each time point.

made by the thylakoid-bound polysomes from those made when using stroma S-30 itself, however, since the latter was very active in protein synthesis (Fig. 8, compare tracks 5 and 6).

DISCUSSION

By examining systematically one parameter at a time, a set of optimized conditions for translation by thylakoid-bound polysomes of pea chloroplasts has been established. Some of the improvements may result from the use of intact, highly active chloroplasts from young plants, and also a rapid (1.5–2 h) isolation procedure. These parameters were not compared to alternative procedures, however. Michaels and Margulies (21) required a 15-h centrifugation in the presence of chloramphenicol, to obtain washed thylakoids from *Chlamydomonas*, a procedure which may well have been damaging.

As in previous work with broken spinach chloroplasts (6), cation concentrations were highly critical for maximal translation rates. The optimum for K^+ at 100 mM (Fig. 1) was remarkably sharp, and corresponds well with the 80 mM needed by broken spinach chloroplasts (6). Herrin *et al.* (13) used 70 mM K^+ together with 40 mM NH_4^+ for translation by thylakoid-bound ribosomes of *Chlamydomonas*, with the total close to the 100 mM optimum we find, but did not calculate absolute rates of leucine incorporation. Alscher *et al.* (3) and Margulies and Michaels (20) used 120 and 50 mM KCl, respectively, far enough away from the optimum, to account, in part, for the lower rates of incorporation they reported.

The Mg^{2+} optimum at 9 mM (3, 6; Fig. 2) is striking, and is expected because Mg^{2+} is required to preserve procaryotic ribosome structure (14). The precise optimum for added Mg^{2+} could vary, however, if ATP concentrations were raised (since ATP chelates Mg^{2+}) or at higher levels of thylakoids with their intrinsic divalent cation binding capacity (4). The causes of inhibition by excess K^+ or Mg^{2+} have not been defined.

While additions of ATP, GTP, and an ATP-regenerating system should have been absolute requirements for translation by

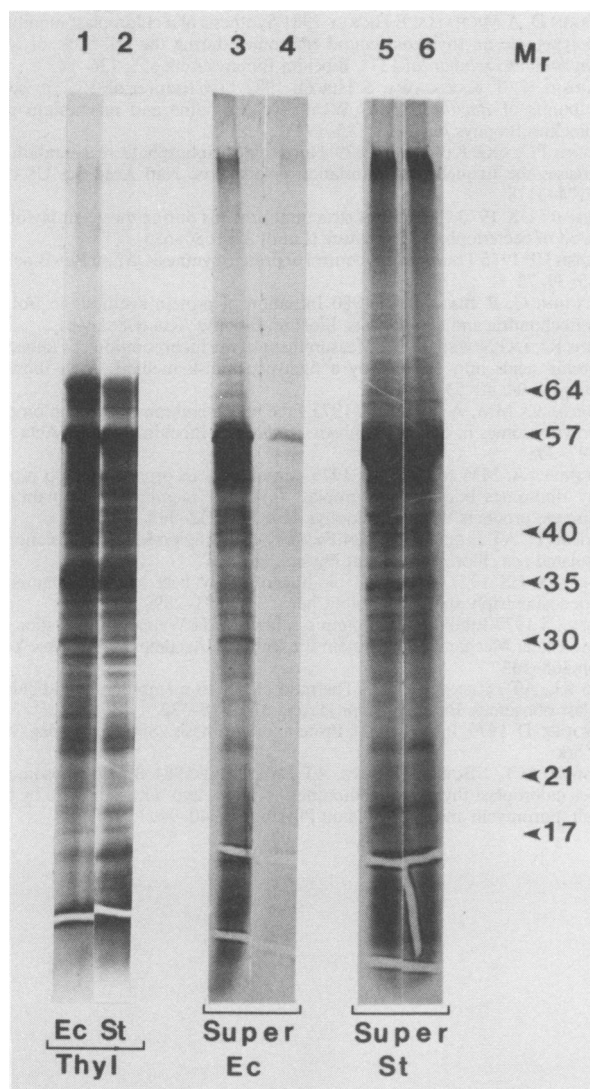


FIG. 8. Labeled products made when using either *E. coli* or chloroplast stroma S-30 factors. Conditions as in Figure 7, except for alternative use of S-30 from either *E. coli* (Ec) or stroma (St). Thylakoid membranes (Thyl) were separated from soluble proteins (Super) after the incubation. Only autoradiographs are shown. Tracks 1 and 2, Thylakoid proteins derived from 6 μg Chl with 8×10^4 cpm, after incubation with S-30 from *E. coli* (track 1) or stroma (track 2). Track 3 shows the autoradiograph of soluble products (with 7×10^4 cpm) made by the mixture of thylakoids and *E. coli* S-30; track 4 is that from incubation of the *E. coli* S-30 alone, with the same amount of protein as in track 3. Track 5 shows soluble products (6×10^4 cpm) from thylakoids incubated with stroma S-30; track 6 indicates the products made by the stroma S-30 alone, with the same amount of protein.

washed thylakoids in darkness, it was not possible to demonstrate zero activity in their absence (Figs. 5 and 6). We have to assume that the very small amounts actually needed came in as contaminants, especially with the S-30 which was only briefly desalted. The optima at 0.2 mM for GTP and 1.0 mM for ATP are very close to concentrations used in previous work (3, 15, 21). The intriguing inhibitions by excess GTP (100% at 1.6 mM) or ATP (30% in this experiment, 90% in another) have not yet been explored. The 30% stimulation by creatine phosphate indicates a possible need, as with *E. coli* ribosomes (15) to maintain a high ATP/[ADP + AMP] ratio, rather than an absolute requirement for more ATP, to maintain both high rates and greater accuracy in translation.

The absence of any effect of peptide chain initiation inhibitors (Table II) agrees with two earlier reports of the lack of inhibition of ATA (3, 13). Bolli *et al.* (5) reported 35% inhibition of protein synthesis by washed thylakoids from *Chlamydomonas* due to 100 μM ATA; but this concentration is likely to inhibit elongation as well (26). Thus, there is as yet no convincing evidence of chain reinitiation by polysomes attached to thylakoid membranes. This could be due to a lack of appropriate initiation factor activity, or to degradation of mRNA.

The original impetus for this work had been the discrepancy between low reported rates of thylakoid translation, (3, 21, 27) and much more rapid ones with intact chloroplasts (12). Thus with thylakoids, leucine incorporation in nmol/mg Chl·h were 0.06 (3) (recalculated from the value per μg RNA assuming 400 μg RNA/mg Chl with these chloroplasts), 0.40 (21), and 0.30 (27). The corresponding rate with intact pea chloroplasts was up to 300 (12, 22). Even assuming that only a third of the ribosomes in intact chloroplasts are attached to the thylakoids, the washed membrane polysomes still seemed to be 300-fold less active than they may have been inside the intact chloroplast. Under such nonoptimal conditions, it was conceivable that a distorted picture would be obtained of the role and synthetic capacity of the thylakoid-bound ribosomes.

As a result of the current work, initial (5 min) rates of translation can be seen from 0.4 (Fig. 1) to 1.0 (Fig. 7) nmol leucine incorporated/min, or 24 to 60 nmol/mg Chl·h. The current maximal rates are thus 60- to 150-fold greater than those seen earlier, and are only 5-fold less than those observed with intact plastids. Further improvements with washed thylakoids are probably possible, by using human placenta RNase inhibitor, and by using a more concentrated and possibly purified form of the active components in S-30 preparations, whose effects had not saturated at the maximal feasible volume of addition. It should also be noted that at least half (8) or more (25) of the chloroplast ribosomes are free in the stroma, so no more than half of the protein synthesis by intact chloroplasts is likely to occur on thylakoid-bound polysomes. Another advantage of the intact chloroplasts lies in their extensive chain reinitiation, with 80 to 90% inhibition by appropriate inhibitors (12). The absence of initiation in the washed thylakoid system might affect even the initial rate of leucine incorporation to some extent, and certainly severely limits the total amount.

Another measure of the usefulness of the washed thylakoid system will be the demonstration that specific thylakoid proteins are synthesized under these improved conditions. Numerous labeled polypeptides could not be washed out of thylakoids, following incubation (Figs. 6 and 8), many of them corresponding to the position of stained proteins. Both the rates of translation (Fig. 7) and positions of labeled bands (Fig. 8) agreed very closely whether S-30 was used from *E. coli* or from pea chloroplast stroma. Thus, our first conjecture that the low rates of translation reported in the literature might have been due to an inefficient heterologous cell-free system, was not correct.

From 53 to 65% of the labeled protein remained attached to thylakoids when using *E. coli* S-30, and 70% when using stroma S-30. While these figures are lower than the 90% reported earlier (3), it is difficult to make a direct comparison because the amount of incorporation was so low in the earlier experiments. It is of some importance to find out if the soluble proteins that are formed represent only premature termination products, or are actual soluble proteins of the stroma made in part by bound polysomes. That the latter is possible is indicated by the intensity of label in the region expected for the large subunit of ribulose biphosphate carboxylase (Fig. 6, track 4 and Fig. 8, track 3) and this has been demonstrated using antibodies by M. Margulies (personal communication). Further work is in progress to attempt positive identification of some of the thylakoid proteins

synthesized by the bound polysomes.

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