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Factors Permitting Prolonged Translation by Isolated Pea Chloroplasts[1]

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ABSTRACT

The following parameters were found to prolong the time-course of translation by isolated pea (*Pisum sativum*, cv Progress No. 9) chloroplasts: addition of other amino acids (an effect synergistic with sufficient free Mg^{2+}), use of lower light intensities, and additions of inorganic phosphate and ATP. In a chloroplast system which includes these parameters, active translation usually extends to almost an hour. The total amount of leucine incorporated is routinely 60 to 100 nanomoles/milligram chlorophyll and often 200 nanomoles/milligram chlorophyll. Accurate estimation of the amount of amino acid incorporated depends on supplying the labeled amino acid at a concentration sufficient to overcome isotope dilution effects from endogenous pools. Approximately 39 thylakoid and 60 stroma polypeptides were visible on autoradiographs after labeling with [^{35}S]methionine. Label in a few of the polypeptide bands was increased or decreased by specific changes in the reaction conditions. Due to the long period of activity and the large number of labeled products, this chloroplast system should be useful for future studies of chloroplast translation.

Isolated, intact chloroplasts have been used to identify products of chloroplast protein synthesis as well as to investigate posttranslational protein transport, processing, assembly, and insertion into membranes. The results of such studies may depend strongly on the characteristics and competence of the *in vitro* chloroplast systems. For example, in an early *in vitro* chloroplast system only one soluble product was detected (1), while in more recent work up to 39 thylakoid (13) and 80 soluble (10) polypeptide products have been reported. The movement across the envelope of cytoplasmically synthesized precursors to chloroplast proteins depends on an adequate supply of internal ATP (15). Processing of the chloroplast-synthesized, herbicide-binding protein (6) has been demonstrated to occur in isolated pea (9, 17) and lettuce (17) chloroplasts. However, in maize the processing has been seen *in vivo* but so far not *in organello* (14) perhaps because some necessary condition(s) has not been identified. Assembly of cytoplasmically synthesized small subunits and chloroplast-synthesized large subunits of the chloroplast enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase into a holoenzyme did not occur in isolated chloroplasts until sorbitol, rather than KCl, was used as an osmoticum (2, 8). Clearly, increasing the functionality of isolated chloroplasts will increase our ability to study and understand mechanisms of chloroplast biogenesis, and interactions between chloroplasts and other parts of the cell.

Previously, work in this laboratory led to the development of conditions under which pea chloroplasts showed high rates of light-driven translation (13). Leucine incorporation was routinely at the level of 20 to 60 nmol/mg Chl, and occasionally up to 100 nmol. However, the incorporation was most rapid during the first 5 min of the incubation and then continuously declined, stopping altogether by 15 to 30 min. Similar kinetic behavior was reported for other chloroplast systems (3, 7, 27). Thus, the optimal states in the chloroplasts were very short-lived, under the conditions used.

The work reported here was designed to test further parameters needed to extend the time-course for protein synthesis by isolated, intact chloroplasts. With the improvements resulting from this work, active translation can now be maintained for almost 1 h. In addition, we are now able to clarify the role of leucine concentration in determinations of leucine incorporation.

MATERIALS AND METHODS

Plant Material and Chloroplast Isolation. Peas (cv Progress No. 9, from Agway Corp) were sown in vermiculite and grown for 8 d on a 12-h light/12-h dark cycle as described earlier (12). The seedlings were kept in darkness for 13 to 16 h to remove starch, then illuminated for 30 to 45 min (about 250 μmol/m$^2\cdot$s) immediately before harvesting. Chloroplasts were isolated as before (13) except that after centrifugation on a Percoll gradient the intact chloroplast band was washed and resuspended in resuspension medium containing 375 mM sorbitol, 35 mM Hepes-KOH (pH 8.3), 0.96 mM DTT, and unless otherwise noted, 10 mM Na-phosphate.

Reaction Conditions. Protein synthesis incubations were carried out in 12 × 75 mm test tubes in a rack attached to the shaking arms of an Aminco illuminated Warburg apparatus. The temperature was 27°C, and the light intensity, supplied by 300-w incandescent flood lamps, was either 45 or 900 μmol/m$^2\cdot$s. For the lower intensity, a filter made of 40 layers of wet Miracloth sealed in clear plastic was attached to the bottom of the test tube rack. The basic incubation medium contained 350 mM sorbitol, 33 mM Hepes-KOH (pH 8.3), 0.9 mM DTT, (^{3}H)-leucine at 26.7 μCi/ml, from 0.11 to 46 μCi/nmol; or [^{35}S]methionine at 6.7 μCi/ml and 1.33 μCi/nmol; and chloroplasts at about 0.1 mg Chl/ml. During the course of these experiments, various additions were made to the basic medium (see "Results").

For time-course experiments, 50 μl aliquots of incubation medium were removed from duplicate tubes at intervals and combined with 30 μl of a stopping solution containing 4% (w/v) Triton X-100 and 50 mM unlabeled leucine. The samples were centrifuged for 5 min in an Eppendorf micro-centrifuge to remove possible contaminating bacteria, and duplicate 25 μl aliquots of the solubilized chloroplasts were spotted onto disks of

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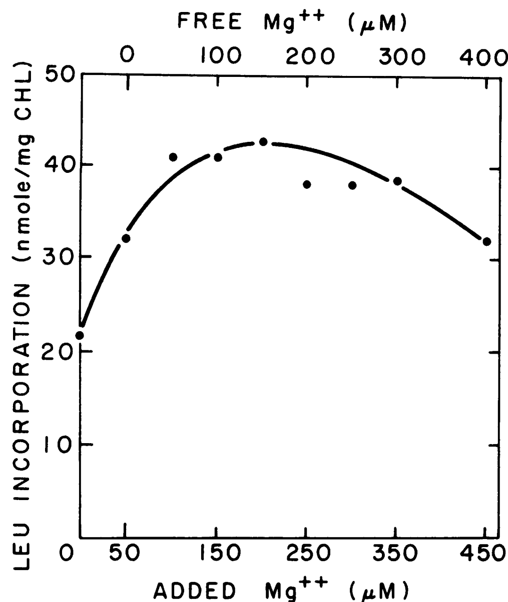

FIG. 1. Stimulation of chloroplast protein synthesis by low concentrations of $MgCl_2$. Intact chloroplasts were incubated with 250 μM [^{3}H] leucine, 50 μM EDTA, and from 0 to 450 μM $MgCl_2$ for 30 min, under a light intensity of 900 μmol/m^2·s. Incorporation of labeled leucine into protein was measured. The numbers on the bottom abscissa indicate concentration of added Mg^{2+}. The actual value for nonchelated (free) Mg^{2+} is listed at the top of the figure, taking into account the presence of 50 μM EDTA in each tube.

Whatman 3MM filter paper. The disks were processed as described in (23) for measurement of TCA-insoluble radioactivity.

In single time experiments, duplicate samples of 150 μl each had 100 μl of stopping solution added. These samples were centrifuged, and duplicate aliquots were spotted on paper disks and processed as described above.

Electrophoresis and Autoradiography. [^{35}S]methionine was used at 0.1 μCi/μl and a specific radioactivity of 25 μCi/nmol. Following the incubation the chloroplasts were lysed, stroma proteins collected, and the thylakoids washed 4 times in 10 mM Na-pyrophosphate (pH 7.4) as described earlier (13). The proteins were denatured by heating at 80°C for 2 min in 2% LDS,[3] then electrophoresed on 10 to 16% acrylamide gels containing 0.1% LDS. The gels were fixed, stained, and exposed to x-ray film as described before (13). The exposure time was 3 to 4 d for approximately 80,000 cpm loaded per well.

Materials. Percoll and Ficoll were from Pharmacia, ATP (Na salt, 99%) was from Sigma as were most other organic chemicals. 4,5-[^{3}H]Leucine (46 or 146 Ci/mmol) and [^{35}S]methionine (1010 to 1450 Ci/mmol) were purchased from Amersham.

RESULTS

Mg^{2+} and Amino Acids. Under conditions used here, light-driven protein synthesis in isolated intact chloroplasts was stimulated approximately 25% (Fig. 1) by free Mg^{2+} between 50 and 300 μM (*i.e.* Mg^{2+} concentrations above the 50 μM EDTA present in each reaction). This contrasts with a previous study (3) in which adding Mg^{2+} only inhibited protein synthesis; however, the lowest concentration tested in that case was 1.0 mM, which we also find to be inhibitory. In another case (13) apparent stimulation by 1.0 mM added Mg^{2+} was seen. However, since the medium also contained 2.0 mM EDTA and 1.0 mM Mn^{2+} (13), the optimum leucine incorporation occurred at 0 mM free (unchelated) Mg^{2+}. Again, the next higher concentration used was in the inhibitory range.

In recent work from this laboratory low levels of free Mg^{2+} were found to be essential for ATP-driven protein synthesis in the dark (11) and a low level also stimulated light-driven protein synthesis (5). The actual Mg^{2+} concentration in the medium increases significantly during incubation, due to leakage of Mg^{2+} from the stroma (5). Thus, in the present study, the dependence of protein synthesis on low levels of added Mg^{2+} was most apparent when EDTA was added, as in Figure 1. The chelator probably neutralizes the effect of released Mg^{2+} in tubes to which more $MgCl_2$ was not added. (Note the lower incorporation at 0 added Mg^{2+} in Figure 1, than when the EDTA and Mg^{2+} are balanced.)

Some amino acids are synthesized by intact chloroplasts, while others are made in the cytoplasm and must be imported for use in chloroplast protein synthesis (19, 25, 26, 29 among others). Thus, during *in vitro* chloroplast protein synthesis one or more of the amino acids might become limiting. When 18 of the other protein amino acids were added, both leucine and methionine incorporation were stimulated (Fig. 2). The stimulatory effect of the other amino acids saturated at 200 μM, resulting in this experiment (Fig. 2) in a 2- to 3-fold increase in incorporation of either methionine or leucine at the end of 30 min.

The response to these amino acids only occurred when free Mg^{2+} was also added (data not shown). While Mg^{2+} by itself (without the extra amino acids) stimulated incorporation about 25%, the effects of amino acids and Mg^{2+} were strongly synergistic. The dependence of the response to amino acids on Mg^{2+} probably did not involve chelation of Mg^{2+} by the amino acids. The optimum concentration of added Mg^{2+} in stimulating leucine incorporation was the same, whether the other amino acids were absent or present at 250 or at 800 μM. This would not have been the case if the function of Mg^{2+} was that of overriding the chelating effects of the added amino acids.

The increased incorporation with Mg^{2+} and amino acids arose from extension of the high rate phase of the time-course (Fig. 3), and did not change the initial rate of incorporation significantly. To find out if one or more specific amino acids had become limiting after the first 5 min, a series of experiments was carried out in which each amino acid (except leucine) was individually left out while the others were added at 200 μM. Leucine incorporation was depressed only when isoleucine or threonine was omitted (Table I). Furthermore, adding only isoleucine + threonine resulted in the same stimulation as that given by all 19 amino acids (Table I). Thus, we conclude that at least isoleucine

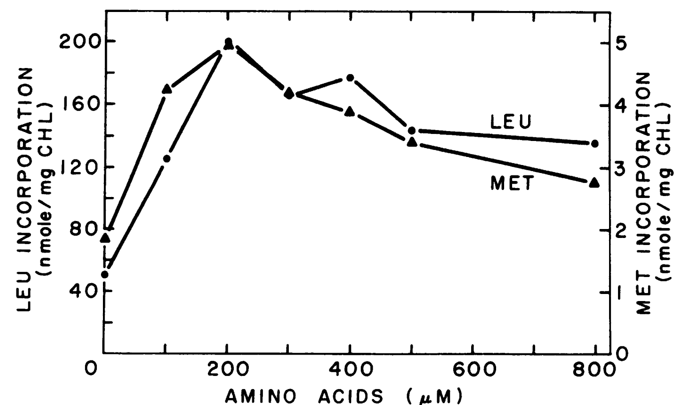

FIG. 2. Effect of other amino acids on incorporation of leucine and methionine. The incubation solutions contained 1 mM EDTA, 1.5 mM $MgCl_2$, 250 μM [^{3}H]leucine, and 5 μM [^{35}S]methionine, and from 0 to 800 μM of each of the other 18 common protein amino acids. The reactions ran for 30 min under white light at 900 μmol/m^2·s.

[3] Abbreviation: LDS, lithium dodecyl sulfate.

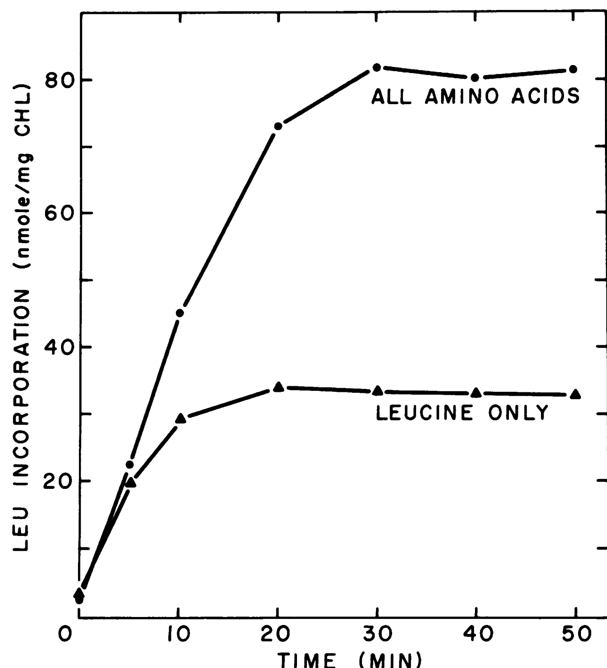

FIG. 3. Time course prolongation by adding the other amino acids. The incubation solutions included 200 μM [^{3}H]leucine, 200 μM $MgCl_2$, with or without the addition of 19 common protein amino acids at 200 μM each. Aliquots were removed for assay of leucine incorporation between 0 to 50 min, with a light intensity of 900 μmol/m^2·s.

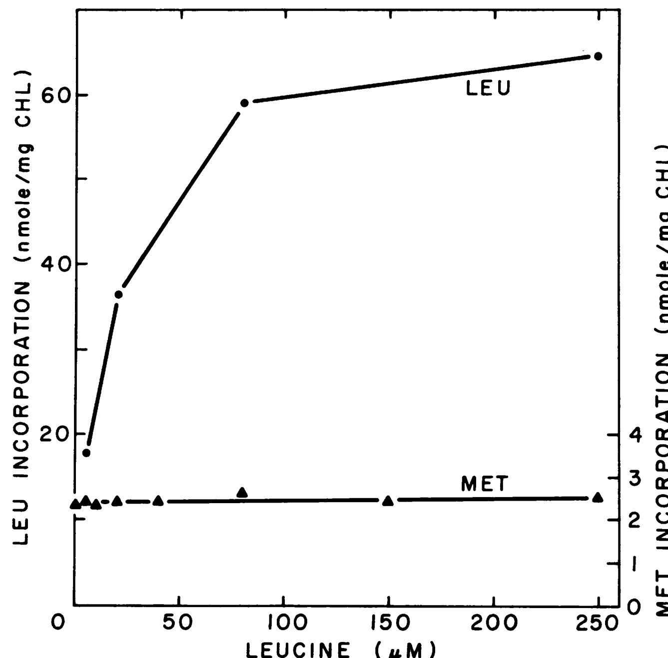

FIG. 4. Effect of leucine concentration on the incorporation of leucine or methionine. Chloroplasts were incubated for 30 min in white light at 900 μmol/m^2·s, in solutions containing 50 μM EDTA, 200 μM $MgCl_2$, 5 μM methionine, 0 to 250 μM leucine, and either [^{3}H]leucine or [^{35}S] methionine as shown.

Table I. *Amino Acid Requirement during Light-Driven Chloroplast Protein Synthesis*

All samples contained 200 μM $MgCl_2$ and 200 μM leucine. Individual samples also contained either no additional amino acids, all of the other common 19 amino acids (200 μM each), or the other amino acids except isoleucine or threonine or both omitted; or only isoleucine added (200 μM), only threonine added (200 μM), or both added (200 μM each). Incubations were at 900 μmol/m^2·s for 40 min.

Treatment	Leu Incorporation	Stimulation
	nmol/mg Chl	*%*
−19 Amino acids	29	0
+19 Amino acids	53	83
+19 Amino acids −ile	44	52
+19 Amino acids −thr	44	52
+19 Amino acids −ile, −thr	35	21
+Ile	41	41
+Thr	47	62
+Ile, +Thr	53	83

and threonine can become limiting for translation in this chloroplast system.

Effect of Added Leucine. As reported earlier (13), the calculated incorporation of leucine increases dramatically with increasing concentrations of added leucine, saturating at 200 to 400 μM (Fig. 4). However, the higher levels of leucine do not increase the incorporation of [^{35}S]methionine. Similar observations were made by R. J. Ellis (personal communication). Higher levels of added [^{3}H]leucine must be needed to overcome an isotope dilution effect of endogenous nonlabeled leucine.

In the experiment shown, all samples contained 5 μM methionine. Other experiments (not shown) demonstrated increasing methionine incorporation with increasing methionine concentration, saturating at about 50 μM. The same insensitivity of methionine incorporation to leucine occurred using methionine concentrations as high as 100 μM. Therefore, the results shown in Figure 4 were not due to using limiting amounts of methionine.

Inorganic Phosphate. Resuspending and storing intact chloroplasts in a solution containing 10 mM Na-phosphate has been found to prolong the feasible storage time for expression of CO_2-dependent O_2 evolution activity, probably by preventing net Pi leakage from chloroplasts (28). In contrast, we found that washing, resuspending, and storing chloroplasts on ice in a solution containing 10 mM Na-phosphate had no effect on the gradual decay in ability to accomplish translation. After 90 min of storage on ice, 25% of the initial activity (when expressed under standard reaction conditions) had disappeared, whether or not the storage medium contained 10 mM Na-phosphate (data not shown).

However, including phosphate in the chloroplast resuspension medium did increase the total amount of leucine incorporation during a 40-min reaction period (Fig. 5). Addition of chloroplasts in their resuspension medium containing 10 mM Na-phosphate brought the concentration in the incubation medium to approximately 300 μM. If instead, the chloroplasts were prepared without Pi but the Pi was added to the incubation medium directly, there was a smaller stimulation of leucine incorporation (Fig. 5). As with Mg^{2+} + amino acids, the stimulation by Pi arose from extending the time-course, not from an increase in the initial rates of incorporation (data not shown). The mechanism of the phosphate stimulation is not understood.

Low and High Intensities and ATP. All of the experiments described so far were done using a light intensity of about 900 μmol/m^2·s. The effect of lower light intensity (from 20 to 180 μmol/m^2·s) on the time course of leucine incorporation was examined in a series of experiments in which all of the improvements that have been discussed so far were included. Results of one such experiment are shown in Figure 6. At lower light intensities the initial rates of leucine incorporation were lower, but incorporation continued for a longer time. The result of this pattern was that total leucine incorporation was greater at lower light intensities (Fig. 6, open symbols).

We thought the inhibitory effect of high light intensity might be due to damage to electron transport and ATP synthesis, and

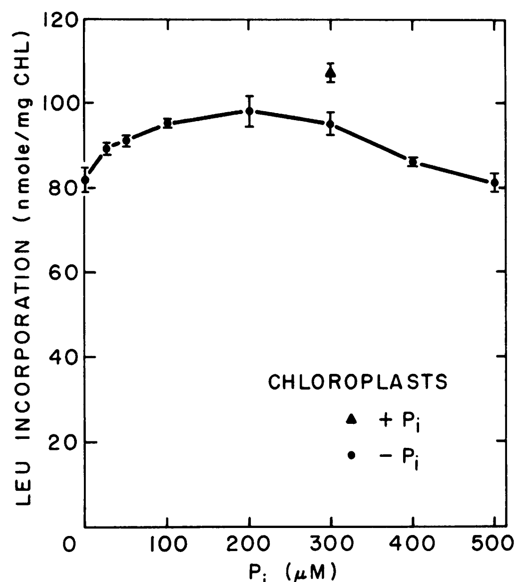

FIG. 5. Effect of inorganic Pi on protein synthesis. The intact chloroplast band from a Percoll gradient was collected and divided into 2 aliquots. These were washed and resuspended in a medium with or without 10 mM Na-phosphate. Chloroplasts from the tube without Pi were incubated for [^{3}H]leucine incorporation in duplicate with 0 to 500 μM Na-phosphate added as shown (O). Chloroplasts from the tube with Na-phosphate were incubated in duplicate without further Pi additions (▲). The concentration of Pi in the reaction in the latter case was approximately 300 μM. All incubations included 200 μM $MgCl_2$ and 200 μM of each of 20 amino acids; and were run under 900 μmol/m^2·s of white light for 40 min. Range bars are not shown where they would be obscured by the point.

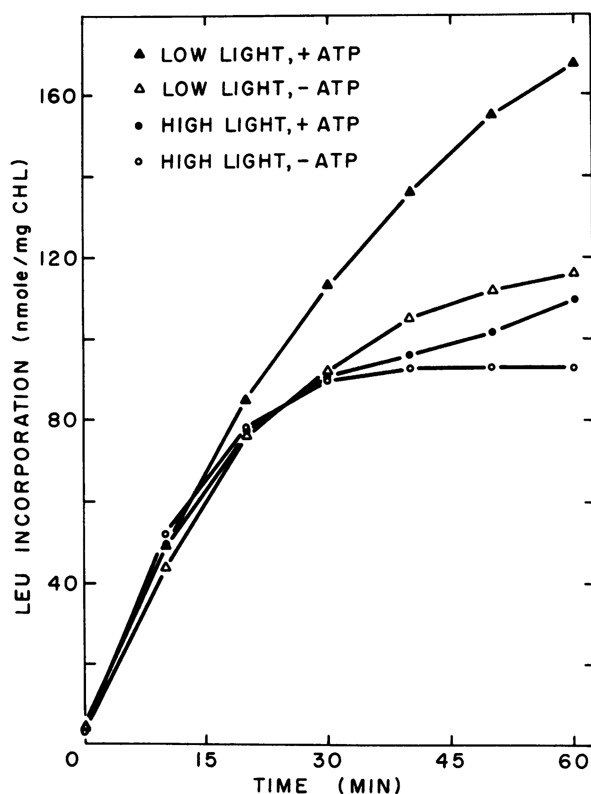

FIG. 6. Time course effects of low *versus* high light intensities, and of ATP addition. All samples contained 200 μM each of 20 amino acids, including [^{3}H]leucine, and either 0 or 9.5 mM ATP. The $MgCl_2$ concentration was 200 μM in reactions lacking ATP, and 12.5 mM in reactions containing ATP. The high light intensity was 900 μmol/m^2·s, low light intensity was 45 μmol/m^2·s.

hence might be reversed by adding more ATP. Since ATP is a chelator of Mg^{2+}, in preliminary experiments the optimum concentration of Mg^{2+} in the presence of ATP had to be determined. With 9.5 mM ATP present in the current reaction mixture, the optimum concentration for leucine incorporation in the light was 12.5 mM, while the dark optimum was 11.5 mM (Fig. 7). These concentrations of ATP and Mg^{2+} were used in all subsequent experiments which included ATP additions.

Adding ATP extended the time course of leucine incorporation at both low and high light intensities, but the effect was greater at low light (Fig. 6). In this experiment, addition of ATP stimulated leucine incorporation 45% at low light intensity and 18% at high light, after 60 min. Since the addition of ATP did not reverse the inhibition of incorporation by high light (Fig. 6), the damage could not have been restricted to the biochemistry of ATP synthesis.

To determine whether low light intensities also cause some inhibition, the time courses of leucine incorporation were compared at low light and in darkness, both with ATP added (data not shown). Low light intensity increased both the initial rate of leucine incorporation, and the extent of the time course. At all time points the extent of incorporation was 50 to 60% greater in low light than in darkness.

Previous *versus* Current System. In the previous sections Mg^{2+}, amino acids, Pi, and ATP additions, as well as light intensity, have been explored in relation to the time-course of chloroplast protein synthesis. These parameters were optimized one by one, as they were identified. In the final system for prolonged translation, the basic procedures were as described in "Materials and Methods", with the following changes: (a) 10 mM Na-phosphate was added to the resuspension medium used in preparing and storing chloroplasts; (b) to the basic reaction mixture were added 9.5 mM ATP, 12.5 mM $MgCl_2$, and 200 μM each of leucine, isoleucine, and threonine; and (c) the reactions were run in white light at 45 μmol/m^2·s, not 900. If translation is to be driven in darkness by ATP (9.5 mM), the $MgCl_2$ concentration should be 11.5 mM. If ATP is omitted and the reaction driven by light only, the $MgCl_2$ concentration should be 200 μM.

The net result of the combined modifications is shown in Figure 8 where light driven translation in the previous system is compared to that in the improved system, including 9.5 mM ATP in the light. The initial reaction rates are similar in both systems (an apparently slightly faster initial rate in the previous system was not repeated in other experiments). However, with the previous system incorporation stopped after 10 (or sometimes 20 to 30) min, while in the current improved system it continues for 60 min or longer. Thus, the total capacity of the chloroplasts to synthesize proteins *in vitro* has been increased 3- to 4-fold.

Proteins Synthesized during the Different Treatments. Figure 9 shows an autoradiograph of separated stroma and thylakoid polypeptides, labeled with [^{35}S]methionine during incubations that included different combinations of the optimized parameters. In all of the treatments, at least 39 thylakoid and 60 stroma polypeptides became labeled, most of which corresponded with stained bands (stained gels not shown).

In general, the labeling patterns in the different samples were strikingly similar. However, increases or decreases in the relative intensities of some of the labeled bands did occur, and a number of these are marked by arrows in the figure. The greatest number of differences in labeling occurred when isoleucine and threonine were added (compare lane 3, without, to lane 4, with the additions). Three thylakoid bands and 6 stroma bands decreased in intensity, while 3 thylakoid and 2 stroma bands increased in labeling. The most prominent of these changes was in the thyla-

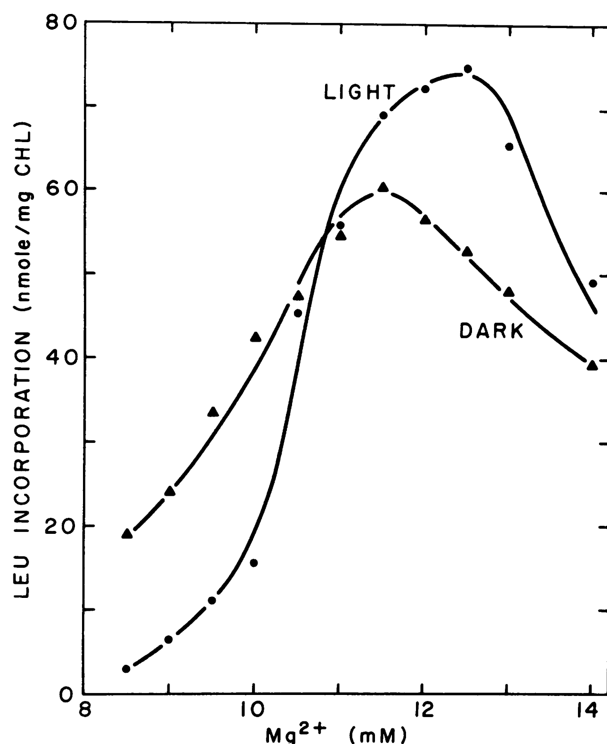

FIG. 7. $MgCl_2$ optima in reactions containing ATP. Incubation mixtures contained 200 μM each of 20 amino acids as well as [^{3}H]leucine, 9.5 mM ATP, and 8.5 to 14.0 mM $MgCl_2$ as shown. Reaction tubes were either wrapped in aluminum foil for darkness, or illuminated with light at 900 μmol/m^2·s, for 40 min.

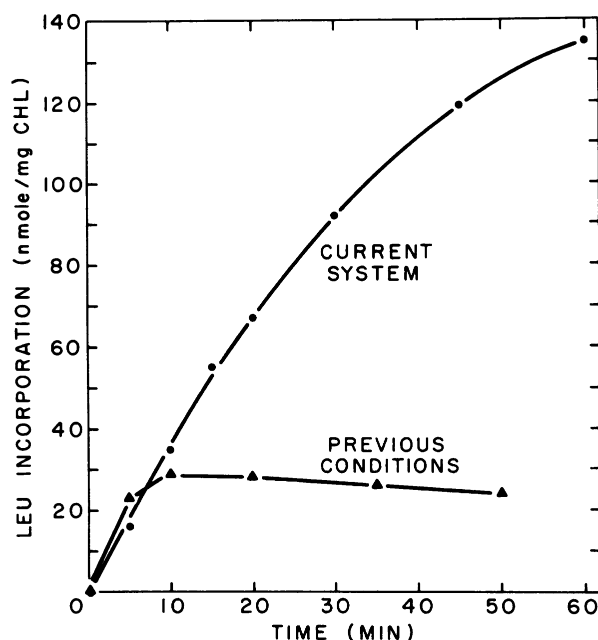

FIG. 8. Comparison of protein synthesis time courses under previous *versus* current conditions. Previous incubation conditions included 1.87 mM EDTA, 0.93 mM $MgCl_2$, 0.93 mM $MnCl_2$, 250 μM leucine, no Pi in the chloroplast resuspension medium, and light at 900 μmol/m^2·s. The current system included 200 μM each of 20 amino acids, 9.5 mM ATP, 12.5 mM $MgCl_2$, 10 mM Na-phosphate in the chloroplast resuspension medium, and light intensity of 45 μmol/m^2·s. Note that of the 20 amino acids included here, only leucine, isoleucine, and threonine at 200 μM each are needed (see Fig. 4 and Table I).

koid 64 kD region, close to where the apoproteins of the P_{700} Chl protein complex ('CPI') are found. Adding 17 more amino acids caused almost no further changes (lane 5, with other amino acids; lane 4 with only isoleucine + threonine).

No differences were noted between high light (lane 5) and low light intensity (lane 6). However, three stroma polypeptides were affected by adding ATP in low light (lane 6, no ATP; lane 7 + ATP). Furthermore, four stroma polypeptides were affected by low light (lane 7) compared to darkness (lane 8), with ATP present in both.

DISCUSSION

In this study the following parameters were found to extend the time course of protein synthesis by isolated pea chloroplasts: (a) Mg^{2+} in the medium at optimal concentrations; (b) additional amino acids, especially isoleucine and threonine; (c) inorganic phosphate at 100 to 300 μM; (d) low light intensity instead of bright light; and (e) adding ATP, even in the light. While the Mg^{2+} and amino acid effects were seen to be synergistic, it is quite likely that all of the factors are interdependent.

With the current system, time-courses routinely extend to 50 or 60 min. The improvements noted above did not increase initial rates of incorporation, but this is not surprising since as discussed previously (13) these rates are quite probably close to *in vivo* rates of translation by chloroplasts. However, it should be pointed out that in different experiments we did observe variability in initial rates, and hence in the total amount of radioactive amino acid incorporated. We attribute this variability to differences in maturity of the plants and, therefore, in the Chl content of the isolated chloroplasts. The plants were used at an age when rapid changes in Chl content were occurring, probably more rapid than changes in ribosome or mRNA content. Since the incorporation data are expressed per milligram Chl, higher apparent rates may result from using less mature plants. Because of this variation in rates, experiments were designed so that the effects of treatments could be compared within experiments, rather than between different experiments.

The stimulation of light driven protein synthesis (no ATP present) by low concentrations of added Mg^{2+} (Fig. 1) was similar to a result reported recently (5). This effect has been shown to involve the prevention of Mg^{2+} leakage from chloroplasts during incubation.

However, the exact concentration of free Mg^{2+} in the medium needed for optimal translation shows puzzling variations depending on other components present. An optimum at 50 to 300 μM occurred here (Fig. 1) and from 100 to 250 μM previously (5). These values are consistent with data shown in (3) and (13). In all of these studies sorbitol was used as an osmoticum. But if spinach chloroplasts were suspended in 200 mM KCl (3), the optimum Mg^{2+} concentration rose to 2 to 3 mM, and very little inhibition showed up at higher levels. When using 9.5 mM ATP in the current system, the optimum concentration for free Mg^{2+} apears to be 3.0 mM in the light and 2.0 mM in the dark, even with sorbitol as an osmoticum. In the previous report of protein synthesis in the dark (11) Mg^{2+} equimolar to the 10 mM ATP was optimal, and 2.5 mM excess Mg^{2+} was slightly inhibitory (but intermediate concentrations were not checked). These differences may have to do with alterations in the electrochemical activity gradient for Mg^{2+} between the stroma and the medium, depending on the presence and incompletely understood effects of other components in the medium. Alternatively, the externally supplied Mg^{2+} may itself have other effects in addition to simply preventing leakage of the stroma Mg^{2+}.

As discussed in "Results", the synergistic response to Mg^{2+} and amino acids was not related to chelation of Mg^{2+} by the amino acids. The simplest explanation for the synergism is that

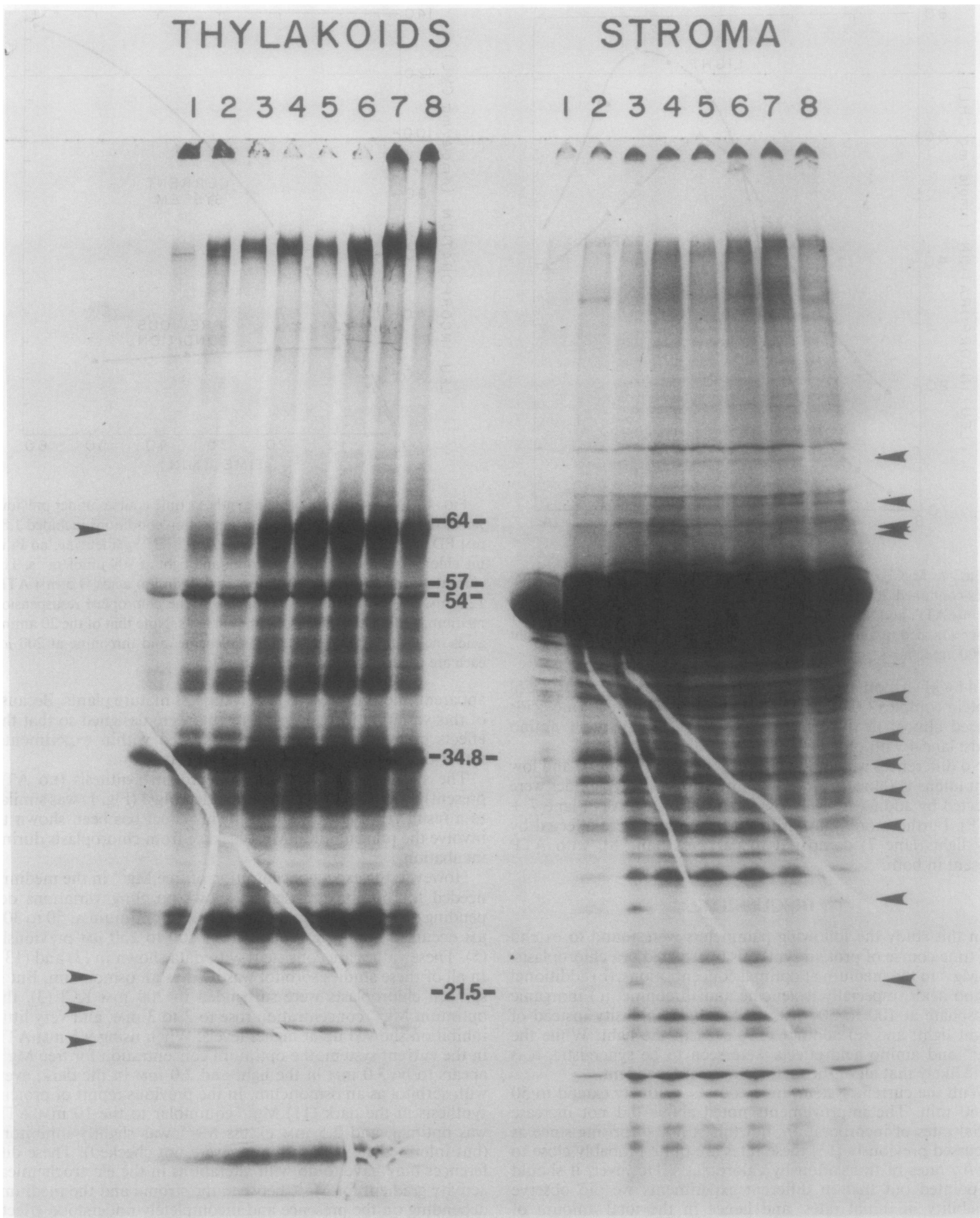

FIG. 9

it was a consequence of removing limiting factors. As long as Mg^{2+} was limiting, no further stimulation was possible. Thus, the response to added amino acids would be seen only after the Mg^{2+} requirement had been satisfied.

Low light intensity prolonged the time course of chloroplast protein synthesis (Fig. 5), indicating possible damage from prolonged exposure to high light. It may be relevant that these pea plants were grown under relatively low light intensities (200 to 250 $\mu mol/m^2 \cdot s$), a treatment shown to change the levels of Chl protein complexes and electron transport complexes (20), making isolated chloroplasts more susceptible to damage by high light. This may have especially been a problem in the absence of added $NaHCO_3$ sufficient to take care of photochemically produced reducing power, which otherwise can generate O_2 radicals and other damaging species.

The response to added ATP, even in the light, may be an indication of leakage of nucleotides from these young pea plant chloroplasts. Measurements of retained adenine nucleotides during the course of these incubations are needed to assess this possibility. Another possibility is that ATP (as well as other factors tested) may prolong the uptake of [^{3}H]leucine from the medium.

In a previous paper from this laboratory (13) it was reported that adding higher concentrations of leucine to the medium stimulated net leucine incorporation as much as 10-fold. However, all calculated values for leucine incorporated are based on the specific activity of added leucine. If the added [^{3}H]leucine is significantly diluted by unlabeled leucine present (or generated) in the stroma, the calculations give an underestimate of the true incorporation rate. That this was probably the case for lower external leucine concentrations is seen from the fact that exogenous leucine does not stimulate the incorporation of methionine (Fig. 2). According to this interpretation, the rate of translation is just as fast at low levels of added leucine, but dilution of the isotope by internal unlabeled leucine makes the calculated rate appear too slow. Accurate estimates of rates require adding enough [^{3}H]leucine so that the dilution effect becomes trivial. A similar effect occurs with [^{35}S]methionine, where saturation of its own incorporation occurs at 50 μM but this has no effect on the incorporation of leucine (data not shown). Thus, our previous (13) and present estimates of absolute rates of leucine incorporation are correct; but earlier values, calculated from experiments with low concentrations (5–10 μM) of added [^{3}H]leucine were almost certainly underestimates.

With our current conditions the total amount of leucine incorporated can reach 200 nmol/mg Chl or more. Since the same incorporation may very well go on in chloroplasts to which very little exogenous leucine is added (see above), the question arises as to the source of the incorporated leucine. For instance, at 5 μM external leucine (= 5 nmol/ml) and Chl at 0.10 mg/ml, a total of 50 nmol/mg Chl is available from the medium. The free leucine pool in 9- to 12-d-old pea (cv Little Marvel) chloroplasts was reported as 9 nmol/mg Chl (24), and using similar methods we have measured 7 nmol/mg Chl in the chloroplasts we use. Adding this internal pool, 7 nmol/mg Chl, still does not achieve the total of 200 nmol incorporated/mg Chl in a 60-min reaction. Leucine is apparently not synthesized in chloroplasts (19); however, protein degradation does occur in isolated chloroplasts (2, 4) and could supply the required amounts. Recently ATP-dependent protein degradation was observed in isolated pea chloroplasts under conditions similar to those used here (21, 22).

The isotope dilution consideration does not affect results with added isoleucine and threonine, since these stimulated the incorporation of either leucine or methionine (Fig. 2; Table I). Since they extended the time-courses for protein synthesis (Fig. 3), it is possible that these two amino acids in particular became limiting during the course of the reaction. This was not expected, since they are on the list of those shown to be synthesized from precursors by isolated pea chloroplasts (25, 26, 30). The five specific polypeptides whose synthesis was stimulated at 10 min by isoleucine and threonine, before their entire effect on total incorporation was apparent, might be especially rich in these amino acids. However, specific regulatory effects might also be involved, especially in the case of the nine polypeptides whose early synthesis seemed to be suppressed by these two amino acids. For instance, the presence or absence of a single amino acid has been found to affect the transcription of specific genes in yeast (16).

We could not demonstrate that translation is dependent on simultaneous transcription. Streptolydigin and cordycepin, two specific inhibitors of transcription (18) did not inhibit leucine incorporation when used at concentrations which in parallel experiments resulted in 65% inhibition of [^{3}H]uridine incorporation (data not shown). However some transcription may have occurred during the reaction period, and it could have been required for the continued synthesis, or more vigorous synthesis, of a few specific proteins. Further work is needed to see if the changes in labeling of a few products due to changes in reaction conditions or components (Fig. 9) resulted from transcriptional or from translational events.

FIG. 9. Autoradiograph showing the thylakoid and stroma polypeptides labeled during 10 min of protein synthesis under varying incubation conditions. Chloroplasts were incubated with [^{35}S]methionine; thylakoid and stroma protein separated as in "Materials and Methods", heated for 2 min at 80°C, electrophoresed and autoradiographed. Special conditions for each lane are indicated in the table below:

Lane No.	Light Intensity	$MgCl_2$	ATP	Pi	Ile + Thr	20 Amino Acids
	$\mu mol/m^2 \cdot s$	*mM*	*mM*	*μM*	*μM each*	*μM each*
1	900	0	0	0	0	0
2	900	0.2	0	0	0	0
3	900	0.2	0	300	0	0
4	900	0.2	0	300	200	0
5	900	0.2	0	300	—[a]	200
6	45	0.2	0	300	—	200
7	45	12.5	9.5	300	—	200
8		11.5	9.5	300	—	200

[a] Ile and Thr are included in the mix of 20 amino acids.

The arrows point to labeled bands whose relative intensities varied in response to one or more of these treatments. The M_r values are shown in the center, taken from accompanying standard proteins.

A major product which was synthesized in all of the treatments had an M_r value close to that expected for the 32-kD atrazine binding protein (6, 17). Two heavily labeled bands can be seen in this region of the autoradioagraph (Fig. 9) at about 32 to 35 kD. It appears that the figure may show both the 34.5-kD precursor (9, 17) and the final product at 32 kD. While both of these bands were labeled after protein synthesis incubations of 10 min, only one band was apparent after a 30-min incubation period (data not shown). These preliminary observations suggest that processing of the 32-kD protein precursor occurs in the current system. If this observation is confirmed, it will be a further indication that the improved reaction conditions permit isolated chloroplasts to synthesize and process proteins in a mode close to that which occurs *in vivo*.

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