Chloroplast-based *in vitro* translation systems.

A Thesis

presented to

The Office of Graduate Studies and Research

of

Lakehead University

by

Ivona Kozieradzki©

In partial fulfilment of the requirements

for the degree of

Master of Science

December 1987

ISBN 0-315-44783-4

ABSTRACT

Intact chloroplasts from 8-9 day old pea seedlings were capable of translating mRNA into proteins at high rates. Such metabolically active chloroplasts were lysed and fractionated further in order to develop a chloroplast-based *in vitro* translation system usable in the analysis of chloroplastic protein synthesis.

Three *in vitro* systems were utilized for these studies: 1) lysed chloroplasts, 2) thylakoid bound polysomes supplemented with post-ribosomal supernatant of stroma, 3) low speed supernatant (30,000xg) alone. The chloroplast lysates and low speed supernatant (30,000xg) were capable of translating polyurydilic acid at high rates. Relatively low rate of short-lived translation, comparable to published data, was observed when translation was driven by endogenous mRNA. Addition of mRNA from phage MS2 to the system significantly inhibited translation. Results are discussed in relation to possible lack of re-initiation or interaction with added RNA.

ACKNOLEDGMENTS

I am indebted to N.S.E.R.C. and Lakehead University for financial support, which made this thesis possible.

Most of all, I would like to extend my gratitude to my supervisor, Dr. L Malek, for his support and guidance during the course of the thesis. Dr. Malek's encouragement lead to the final completion of this research.

My committee, Dr. A. Jagendorf, Dr. A. Macdonald and Dr. P. Spare, I thank for their constructive criticisms.

The many individuals, who contributed with technical and laboratory assistance. Special thanks go to Mr. A. Mackenzie for helping with the electron microscopy, as well as, Mr. D. Jones and all the chemistry technicians for keeping my lab functional.

Thanks is extended to Ms. K. Knudsen who had suffered through messy writing and broken english while typing, and re-typing this thesis and my office mates, Ms. A. Karshti, Ms. J. Bourgeois, Mr. B. Day, and Mr. C. Mroz, who made my stay at Lakehead University enjoyable.

Special thanks goes to my son, Paul, who's smiles, dreams, and good behaviour made everything fall into perspective. For without him, I would not have had the will to finish this project.

I would like to excuse my readers while I make a special dedication to my mother for her love and support in her native language-polish.

Mamo-dziekuje Ci za to, ze zawsze bylas dla mnie wtedy kiedy potrzebowalam Ciebie najbardziej. Twoja inspiracja, poparcie, troska i milosc pozwolily mi zawsze wedrowac droga moich marzen.

Table of Contents

List of Figures

List of Tables

1 INTRODUCTION

1.1 Purpose of the study.

Biogenesis of the chloroplast includes the division and growth of the organelle. It is a highly organized and well regulated process which is controlled by both chloroplast and nuclear genomes (Smillie and Scott, 1969; Woodcock and Bogorad, 1971; Gilham, 1978, Parthier, 1979). Expression of genes coding for plastid proteins may be regulated at many levels from transcription to the assembly and integration of their protein products into the appropriate chloroplast compartment. Little is known of how changes in the environment or the physiological condition of the organism regulate the genes for plastid proteins. The aim of early studies of chloroplast protein synthesis was to determine the function of the chloroplast genetic system in terms of identification of polypeptide molecules made. The other reason for studying protein synthesis is to understand the details of the enzymatic reactions involved. Relatively little work in this area has been carried out perhaps because an exact equivalency to bacterial and animal systems was assumed. Recently, researchers are finding more differences between chloroplasts and other systems, with the major difference being the control of chloroplast development by nuclear products (for references see below).

In this work, chloroplast translation was studied on two different levels of organization, in organello (using intact chloroplasts) and in vitro (using lysed chloroplasts or chloroplast fractions). Only the in organello studies achieved the rate of translation close to in vivo (Nivison and Jagendorf, 1984). In order to study details of translation machinery effectively, the in vitro rate of initiation should be as close as possible to the in vivo rate. The main purpose of this work was further purification of translation components and optimization of incubation medium parameters. The ultimate goal was to develop an active translation system, where reinitiation may be confirmed and possible translational control factors identified. The following is a brief review of some facts of chloroplast biology relevant to this research.

1.2 Chloroplast gene expression.

All living eukaryotic cells are characterized by their multiple, compartmentalized genomes: nuclear, mitochondrial and the third, specific only to plants, plastid genome. In comparison to bacteria and nuclei, still little is known about plastid biogenesis and attendant gene expression. The following are, to our present knowledge, the components required to express chloroplast genetic information; DNA, DNA polymerase, RNA polymerase and a protein synthesizing system. During the development of a proplastid into a mature chloroplast, the accumulation of chlorophyll, thylakoid membranes, plastid mRNAs and protein synthesis can be detected (Buetow, 1982, 1986; Galling and

Michaels, 1986).

The involvement of chloroplast genome in chloroplast biogenesis has been indicated in numerous studies (Surzycki et al., 1970; Howell et al., 1975; Gillham et al., 1978). Chloroplastic DNA is transcribed at all stages of the cell cycle (Howell et al., 1977). This is based on the observation that 60% of the DNA from fully developed chloroplasts, from rapidly growing cells hybridizes at saturation with chloroplast RNA isolated from asynchronously grown cells. During the different stages of the cell cycle the transcription of chloroplast genome varied from 39% to 60% of total chloroplast DNA (Howell et al., 1977), suggesting regulation of chloroplastic gene expression at the transcriptional level.

Plastid encoded large subunit of ribulose bisphosphate carboxylase/oxygenase (Rubisco) was reported to be synthesized by stromal polyribosomes and this was presumably dependent on the availability of mRNA (Ellis et al., 1977. Recently, Minami and Watanabe, 1984; Hattori and Margulies, 1985; Grinsven et al., 1986, also observed post-transcriptional regulation of chloroplast gene expression. Hattori and Margulies (1985) and Minami and Watanabe (1984) observed a substantial amount of Rubisco mRNA also present on thylakoids but they could not detect an efficient translation in this membrane fraction. Bhaya and Jagendorf (1984) showed readout translation of the carboxylase large subunit by both thylakoid bound and soluble polysomes of pea chloroplasts. These results could indicate that the expression of the chloroplastic genome is regulated not only at the transcriptional but

also at translational level. Acording to this proposal, synthesis of particular protein would depend on the site where its mRNA became functional (Minami and Watanabe, 1984). Similar results were reported by Leu et al.(1984) who reported regulatory role of the thylakoid membranes in chloroplastic mRNA translation. Grinsven et al. (1986) concluded that chloroplast gene expression in Petunia hybrida depends on the need for de novo synthesis of the proteins. For example, in mature chloroplasts, all thylakoid membranes are already present, so there is no longer need for high levels of membrane proteins like the P700 apoprotein. Decreased amount of mRNA hybridizing to DNA (psa A gene) coding for the P700 apoprotein is then observed. A summary of the above reports indicates that the regulation of plastid gene expression takes place on at least two different levels: transcriptional and post-transcriptional, most likely translational control.

1.3 Integration of plastid genome with nuclear genome

Although chloroplasts contain all the components necessary for biological systems to be genetically autonomous, their DNA does not code for all of the chloroplast functional proteins (Ellis, 1976, 1981). The majority of the chloroplast proteins are nuclear coded and are synthesized on 80S cytosolic ribosomes (Wettstein, 1981) as precursor proteins (Apel and Kloppstech, 1978; Cerf and Kloppstech, 1982; Dobberstein et al., 1977; Meyer and Chartier, 1983). According to Chua and Schmidt (1978) about 70-80% of the chloroplast proteins are synthesized in the cytosol. For example, the small subunit of ribulose-1,5-bisphosphate carboxylase (Gray and Cashmore, 1976; Ray et

al., 1976), the delta subunit (part of CF_1) and subunit II(part of CF_0) of the H^+-translocating ATPase complex (CF_1-CF_0)(Mendiola-Morgenthaler et al., 1976; Bouthyette and Jagendorf, 1978), some of the proteins of chloroplast ribosomes (Margulies, 1975), chloroplast amino-acyl-tRNA synthetases (Weil et al., 1982), the apoprotein of the light-harvesting chlorophyll a/b protein complex (CPII)(Apel and Klopstech, 1978), most of the enzymes of the photosynthetic carbon pathway (Feierabend and Wildner, 1979) and likely others.

The transport of cytoplasmically synthesized proteins into chloroplast has been studied a) using isolated intact chloroplasts combined with identification of the translocation products (Chua and Schmidt, 1978; Highfield and Ellis, 1978; Schmidt et al., 1980) and b) using inhibitors specific for 70S and 80S ribosomes (Ellis, 1977; Strzalka and Kwiatkowska, 1985). The transport is energy dependent (Grossman et al., 1980) and because polysomes are not directly involved in this process it is called post-translational transport (Blobel and Dobberstein, 1975). The binding proteins capable of specifically selecting precursor proteins reside in the outer chloroplast envelope (Highfield and Ellis, 1978; Cline et al., 1985;Bitsch and Kloppstech, 1986).

The in vivo and in vitro studies suggest that the conversion of the precursor forms of some proteins into mature forms may involve as many as two processing steps. Robinson and Ellis (1984) and Mishkind et al (1985) have shown that the precursor of the ribulose bisphosphate carboxylase small subunit from pea is processed by chloroplasts in two

independent steps. Also, Schmidt et al. (1985) indicated that the maturation of Chlamydamonas protein L-18 in vivo involves at least two processing steps, one of which depends on a protein made by the chloroplast ribosomes.

According to Ellis (1977) some regulatory proteins also cross the chloroplastic envelope in order to balance the synthesis of the proteins made outside and inside the chloroplasts. In contrast with this observation, Strzalka and Kwiatkowska (1979) have shown that during inhibition of protein formation within plastids by chloramphenicol, the synthesis of protein in the cytoplasm and their transport into plastids can still proceed. There remains considerable work to be done to understand the integration of the plastid and nucleo-cytoplasmic systems. During the synthesis of ribulose bisphosphate carboxylase/oxygenase coordination of the two mRNA levels was observed (Sasaki et al., 1985). Contradicting findings about the coupled and uncoupled synthesis of these proteins may be due to the use of different plant species, conditions and methods.

Although most of chloroplast protein complexes contain proteins coded for by the nuclear genome and are translated on cytoplasmic 80S ribosomes, protein synthesis within the chloroplast is generally thought to resemble that of prokaryotes (Kozak, 1983). Bacterial and chloroplast ribosomes, for example, are similar in size (they dissociate into 30s and 50s subunit components, Trempe and Glitz, 1981), and r-protein composition (Capel and Bourque, 1981). Another similarity is that the initiator tRNA is formylated (Schwartz et al.,

1967). Amino acid incorporation in isolated chloroplast is strongly depressed by N^5-formyltetrahydrofolate, a competitive inhibitor of formylation reactions (Lucchini and Bianchetti, 1980). Also initiation and elongation factors from chloroplasts are active with bacterial ribosomes (Kraus and Spremuli, 1986; Sreedharan et al., 1985). Other features of chloroplastic translation system, however, appear to differ from the bacteria. Although chloroplast initiation factor 3 from Euglena gracilis has been effective in initiation complex formation with bacterial ribosomes (Kraus and Spremulli, 1986), chloroplast initiation factor 2 showed only little activity on E. coli 30S subunits (Gold et al., 1985). While there are many similarities between chloroplastic elongation factor-G , elongation factor-Ts, elongation factor-Tu and E. coli elongation factors they should not be treated as the same (Sreedharan et al., 1985). For example, under similar conditions, in the absence of GTP or elongation factor-Ts, the chloroplast elongation factor-Tu is more stable than E. coli elongation factor-Tu. Because the step-by-step assembly of translation complexes has not yet been studied in organelles in detail, more work is needed to understand this process.

After translation, the post-translational processing step occurs rapidly in chloroplasts (Edelman and Gallant, 1978). It is suggested that processing may be necessary for proper structural integration of the polypeptide. The precursor itself may allow initial recognition and insertion of the newly synthesized polypeptide into the thylakoid membrane. In maize this process was not confirmed in isolated chloroplasts, though it occurs normally in vivo. It has been suggested that this processing may be under nuclear control by production of a

processing factor, perhaps a specific protease (Grebanier et al., 1978).

Post-translational protein phosphorylation has been also demonstrated (Bennett, 1977). The role of protein phosphorylation in chloroplast membranes has been investigated by Bennett (1979). A post-translational physiological mechanism by which light energy can be efficiently divided between the two photosystems of electron transport under varying light intensity conditions has been suggested by this author .

1.4 Systems for study of chloroplastic translation.

Studies on chloroplast protein synthesis have used the following strategies: in vivo by labelling the cells in the presence of specific inhibitors (Ellis , 1969; Chua and Gillham, 1977), in organello using a suspension of isolated intact chloroplasts (Bottomley et al., 1974; Ellis, 1977 and 1981; Fish and Jagendorf, 1982; Bloom et al., 1983 and Nivison and Jagendorf, 1984), and in vitro using lysed chloroplasts (Bourque et al., 1985) or chloroplast subfractions (Michaels and Margulies, 1975; Ellis, 1977; Aschler et al., 1978; Bolli and Boshetti, 1981; Bhaya and Jagendorf, 1984; Herrin and Michaels, 1985; Hattori and Margulies, 1986). The systems used to study chloroplastic translation vary depending on the questions being asked. The following is a brief review of the approaches employed in the study of chloroplastic protein synthesis.

1.4.1 Studies *in organello*.

Suspensions of isolated chloroplasts have the advantage of maintaining structural organization and a decreased level of complexity (compared to intact cells). The limitations of this method are the possible loss of control by cytoplasmic components and difficulties in preparing undamaged, active chloroplasts. The first chloroplastic *in organello* protein translation system which produced discrete polypeptides using light energy was derived from *Pisum* (Blair and Ellis, 1973; Ellis, 1977). Since only intact chloroplasts can generate ATP from light this method allows the use of rapidly obtained crude chloroplast preparations. As long as the envelope around the chloroplast remains intact, the microenvironment around the polysomes is likely to be more normal than in the lysed systems.

Light driven translation by isolated chloroplasts was accomplished at very high rates, equivalent to those estimated *in vivo* (Nivison and Jagendorf, 1984). Low light intensity prolonged the time course of chloroplast protein synthesis, indicating possible damage from prolonged exposure to high light (Nivison and Jagendorf, 1984). Up to 100 nmoles of leucine was incorporated during 30 minute reaction periods by using an improved system. Rates of ATP-driven protein synthesis in darkness, by chloroplasts from spinach (Ramirez *et al.*, 1963; Bottomley *et al.*, 1974), pea (Blair and Ellis, 1973; Siddell and Ellis, 1975) and sorghum (Geetha and Gnanam, 1980) were quite high, but still lower than those found in light.

The addition of Mg^{2+} to ATP-driven protein synthesis increased the rate of translation to the rate reported when light was used as a source of energy (Fish and Jagendorf, 1982). The optimum translation can be obtained with equimolar concentrations of ATP and Mg^{2+}. An essential component of the medium for protein synthesis by isolated chloroplasts are potassium ions (Ellis, 1977). Substitution of potassium ions by sodium ions prevented light and ATP dependent protein synthesis. The optimization of amino acid concentration has allowed increased levels of protein synthesis (Nivison and Jagendorf, 1984). The optimization of labelled amino acid concentration has also improved the quality of translation products (Mullet et al., 1985). The system reported by Nivison and Jagendorf (1984) showed 39 membrane proteins and 60 soluble proteins which were radioactively labelled in isolated chloroplasts. This number of polypeptides approaches the theoretical protein coding capacity of the chloroplast genome (Ellis, 1981).

Isolated etioplasts were also used to study translation. It was demonstrated that etioplasts were capable of synthesizing protochlorophyllide (Daniell and Rebeiz, 1982,a) and chlorophyll (Daniell and Rebeiz, 1982,b) at rates higher than those observed in greening tissues in vivo (Daniell et al., 1986). Etioplasts developed in vitro showed extensive grana formation (Rebeiz et al., 1984). According to Daniell et al. (1986) they continued incorporation of amino acids into a protein fraction for 8 hours.

1.4.2 Studies in lysed chloroplasts (*in vitro*).

Resuspending the chloroplast pellets in a hypotonic solution breaks the chloroplast envelope. Protein synthesis can be measured in the presence of added ATP but not in the light (Ellis, 1977; Bottomley and Whitfeld, 1979). The chloroplast lysate system can be used to study regulatory factors involved in chloroplast gene expression. Regulatory events may be detected at transcriptional or translational levels, since *de novo* RNA or protein synthesis can be independently monitored in the chloroplast-derived *in vitro* systems. The system is homologous with respect to chloroplast gene expression. Aspects of transcription or translation can, therefore, be studied under conditions which might be similar to those found *in vivo*. Conditions required for optimal synthesis (salt concentrations, nucleoside triphosphates, etc.) may also be sought. The chloroplast lysate system should have the advantage over highly refined, fractionated systems in that it is subjected to a minimal amount of manipulation (Bard *et al.*, 1985). A chloroplast lysate could be an intermediate step in further purification and identification of specific regulators.

The identification of the translation products by using purified chloroplast fragments or mRNA or DNA is fundamental to the understanding of how plastids develop and function. One system that has been developed to transcribe DNA or to translate RNA, is a cell-free preparation from *E. coli* (Nirenberg, 1963; Zubay *et al.*, 1970; Gold *et al.*, 1971). The conditions of the Zubay system have been modified by Bottomley and Whitfeld (1979) and used to study transcription and

translation of chloroplast DNA with sufficient fidelity to allow the identification of the produced large subunit of ribulose bisphosphate carboxylase.

1.4.3 Heterologous *in vitro* systems.

Heterologous systems also have been utilized with chloroplasts DNA or RNA and *E. coli* RNA polymerase supplemented with rabbit reticulocyte lysate (Coen *et al.*, 1977). Also wheat germ preparations have been used to translate chloroplast RNA (Sagher *et al.*, 1976). Reported differences in efficiency of the *E. coli* and the wheat germ cell-free protein synthesizing systems with chloroplast mRNAs suggested that both should be tried initially when translating chloroplast mRNA. Bottomley and Whitfeld, (1978) found that prokaryotic system (*E. coli*) translated chloroplast RNA more efficiently than the eukaryotic system (wheat germ). Characterization of many of the radioactive products should be possible due to the fact that previously defined systems are more efficient and products of very high specific activity could be obtained. However, heterologous systems may differ from chloroplasts in regard to specific characteristics of the transcription and translation process as well as in regard to recognition of regulating and processing signals on chloroplast genes (Jolly and Bogorad, 1980). Using heterologous systems for expression of chloroplastic genes, a significant portion of low molecular weight polypeptides was synthesized *in vitro* (Bottomley and Whitfeld, 1979; Whitfeld and Bottomley, 1983). Therefore, to study the details of the chloroplast translation machinery, homologous systems are probably most suitable. Homologous

translation extracts should ideally transcribe and translate chloroplast genes with high fidelity, comparable to *in vivo* studies.

1.5 Goals of this study.

The goal was to obtain an effective translation of endogenous mRNA using an *in vitro* homologous chloroplastic system which could be used to further study regulation of chloroplastic translation. The following objectives were set in order to achieve this:

- -purify further the lysed system
- -determine optimal parameters of translation medium
- -find conditions effectively inhibiting hydrolytic enzymes which may interfere with protein synthesis
- -find limiting factors of *in vitro* chloroplastic translation
- -decrease endogenous mRNAs but with other translation components such as tRNAs, ribosomes, translation enzymes remaining active
- -translate exogenous synthetic and natural mRNAs.

2 MATERIALS AND METHODS

2.1 Materials.

The 4,5-[^{3}H]-Leucine (58 Ci/mmol) and Enlightening were purchased from Du Pont NEN Research Products (Boston, USA), phenylalanine, [side-chain ^{3}H] 21 Ci/mmol was from ICN Radiochemicals (Canada), RNase inhibitor from human placenta and ribonucleic acid from phage MS2 (lyophylisate) were from Boehringer Mannheim GmbH (West Germany), Tris, Hepes (Research Organics, Cleveland, OH), mercaptoethanol from Aldrich Chemical Company, Inc. (USA), ammonium persulphate from BDH Ltd (England), trichloroacetic acid (TCA) from Fisher Scientific (New Jersey, USA). Other biochemicals were from Sigma (St. Louis, USA).

2.2 Isolation of intact chloroplasts.

Chloroplasts were isolated from pea shoots (Pisum sativum L. Cv.Spring, Asgrow Seed Company; Brantford, Ont.), grown for 8 days on 8-hour light (120 W/m^{2})/16-hour dark cycle at 25^{o}C. The isolation method was based on that of Fish and Jagendorf (1982) with some modifications. All steps were at a temperature near 4 ^{o}C. In the final procedure, 30g of shoot tissue was homogenized in a Tissuemizer (Tekmar

Model SDT 1810) in 100ml of grinding buffer (50mM HEPES-KOH, pH 8.3 at $4^{o}C$, 350mM sorbitol, 3mM $MgCl_2$, 1mM $MnCl_2$, 2mM EDTA, 2mM EGTA, 0.5% w/v bovine serum albumin (BSA) and 5mM ascorbic acid) until the pieces were less than 1mm. The homogenate was filtered through 8 layers of cheesecloth and centrifuged for 4 minutes at 2,500xg. The pellets were resuspended in a small volume of resuspension buffer (35mM HEPES-KOH, pH 8.3 at $4^{o}C$., 375mM sorbitol, 1mM $MgCl_2$, 1mM $MnCl_2$, 2mM EDTA, 1mM dithiothreitol) and overlayed on a 25-95% Percoll gradient (50mM HEPES-KOH, pH 8.3 at room temperature, 350mM sorbitol, 1mM $MgCl_2$ and gradients of 0.25-0.92%(v/w) BSA and Ficoll and 0.7-2.7% (w/v) of polyethylene glycol 6000). The gradients were centrifuged for 7 minutes at 11,800xg. The lower green band was collected, washed of the Percoll with 30ml resuspension buffer (see above) and centrifuged for 3 minutes at 5,000xg to get chloroplast pellet.

2.3 Isolation of stroma S-100 from chloroplasts.

Intact chloroplasts at approximately 5mg chlorophyll/ml (Arnon, 1951) were stored in liquid nitrogen in a buffer containing 35mM Hepes-KOH, pH 8.3, 1mM Mg-acetate, 1mM DTT. After thawing and passing three times through a 20-gauge canula, broken chloroplasts were centrifuged at 100,000xg for two hours. Supernatants (S-100) were collected and used in translation systems as untreated S-100. Ultrafiltered S-100 was obtained by ultrafiltration (Amicon YM 10,000 m.wt. cut off) and dissolved in the same buffer as before, with an addition of 19mM Mg-acetate and 167mM K-acetate.

2.4 Thylakoid isolation.

The intact chloroplast pellet was resuspended in buffer containing 35mM Hepes-KOH (pH 8.3), 15mM Mg-acetate, 20mM K-acetate, 1mM DTT, than passed three times through 20-gauge canula and spun down at 30,000xg. The pellet was washed twice more and the final pellet was resuspended to 0.5mg chlorophyll/ml in stroma (S-100). Chlorophyll was extracted in 80% acetone and measured according to Arnon (1951).

2.5 Preparation of chloroplast lysates.

Intact chloroplasts at approximately 0.3mg chlorophyll/ml were stored at -20^{o}C, in buffer containing 35mM Hepes-KOH (pH 8.3), 1mM Mg-acetate, 1mM DTT. After thawing, chloroplasts were lysed on ice with three 10 sec ultrasound pulses at an output control setting at 6, at 50% duty cycle, using the microprobe of Sonifier Cell Disruptor W-350 (Branson Sonic Power Company; Danbury, USA) or passed three times through a 20-gauge canula.

2.6 Isolation of stroma (S-30) from chloroplasts.

Sonified plastids (Sect. 2.5) were centrifuged at 30,000xg for 5 minutes. Supernatants (S-30) were collected, ultrafiltered (10,000 M. wt cut off) and dissolved , to get 4mg protein/ml, in a buffer (35 mM Hepes-KOH, pH 8.3, 1mM DTT, 19 mM Mg-acetate and 167mM K-acetate). For some experiments supernatant (S-30) was centrifuged

further at 100,000xg for 2 hours. Resulting pellet (P-100) was dissolved in above buffer, to get 1mg protein/ml. Stroma (S-100) was ultrafiltered (10,000 m. wt. cut off) and dissolved in 35 mM Hepes-KOH, pH 8.3, 1 mM DTT, 19 mM Mg-acetate and 167 mM K-acetate buffer.

2.7 Protein determination.

The proteins were assayed using the Bio-Rad dye reagent and a procedure described by Bradford (1976), against bovine serum albumin standard. Absorbance was measured on Beckman DU-50 Spectrophotometer (Beckman Instruments, Inc.; USA).

2.8 In vitro protein synthesis incubation conditions.

All preparatory steps, except incubation, were done at approximately $4^{o}C$. The glassware and the buffers were autoclaved to minimize bacterial contamination. Protein synthesis incubations were carried out at $25^{o}C$ in 12x75 mm flint glass test tubes in Shak-R-Bath (Lab-Line Instruments,Inc.; Melrose Park, USA) at a rate of rotation 150 rpm.

2.9 Protein synthesis by isolated intact chloroplasts.

Incubation medium, of a total volume 100ul, contained 5ul of the isolated chloroplasts (15ug chlorophyll/reaction mix) resuspended in buffer (35mM Hepes-KOH at pH 8.3, 375mM sorbitol, 1mM $MgCl_2$, 2mM EDTA and 1mM DTT),5ul [^{3}H]-leucine (5uCi, at specific radioactivity of 58.4

Ci/mmol), 200uM each of 19 amino acids, 1mM DTT, 10mM ATP, 12mM $MgCl_2$, 10mM K-phosphate. Reaction tubes were illuminated (200 W/m^2) or wrapped in aluminum foil. After incubation the tubes were transfered to an ice bath, with the addition of 50ul of 60 mM cold leucine.

2.10 Protein synthesis by lysed chloroplasts.

Basic incubation medium (50ul) contained:lysed chloroplasts at 300ug protein, 0.16mM each of 19 amino acids, 1.72uM [^{3}H]-phenylalanine (5uCi, at specific radioactivity of 21 Ci/mmol), 5mM ATP, 6mM creatine phosphate, 50ug/ml phosphocreatine kinase, 0.2mM GTP and 1.4mM DTT, 12mM Mg-acetate, 100mM K-acetate and 35mM Hepes-KOH, pH 8.3. Reaction was stopped with 50ul of 10mM $Na_4P_2O_7$.

2.11 Protein synthesis by polysomes bound to the washed thylakoids.

The standard incubation mixture (50ul) contained 0.5mg chlorophyll/ml of washed thylakoids resuspended in 30ul of S-100 (200ug protein). In addition, 20ul aliquots containing 0.16mM each of 19 amino acids, 1.72uM [^{3}H]-leucine (58.4 Ci/mmol), an extra 0.72mM cold leucine, 5mM ATP, 6mM creatine phosphate, 50ug/ml phosphocreatine kinase, 0.2 mM GTP, 1.4mM DTT and 5uM ampicillin, were added. Reaction was stopped with 50ul of 10mM $Na_4P_2O_7$.

2.12 Protein synthesis using S-30 system.

The standard incubation mixture contained in 50ul: (300ug

protein) chloroplastic stroma only (S-30) or stroma supernatant (S-100, 200ug protein) and pellet (P-100, 50ug protein), 0.16mM each of 19 amino acids, 1.72uM [^{3}H]-phenylalanine (5uCi, at specific radioactivity of 21 Ci/mmol), 5mM ATP, 6mM creatine phosphate, 50ug/ml phosphocreatine kinase, 0.2mM GTP and 1.4mM DTT. Total volume was 50ul. After incubation 50 ul of 10mM $Na_4P_2O_7$ was added to reaction medium.

2.13 Measurement of radioactivity in protein.

The method of Mans and Novelli, (1961) was modified as follows. Duplicate, 30ul aliquots of reaction mix, after incubation, were spotted on 2cm^2 pieces of Whatman 3MM chromatography paper (Whatman Ltd.; Maidstone, England) and dried at room temperature. Proteins were precipitated by boiling the dried discs for 10 minutes, in 5% (w/v) trichloroacetic acid (TCA). TCA-soluble label was washed out of discs, by gentle shaking, for 10 minutes twice, in cold 5% (w/v) TCA, 5 minutes in absolute ethanol and 5 minutes in anhydrous ethyl ether. Washed and dried discs were placed into 20ml scintillation vials (Fisher Scientific Company; Canada), with an addition of 5 ml of 0.5% (w/v) diphenyloxazole (PPO) in toluene. Samples were counted in RackBeta Liquid Scintilation Counter (LKB Wallac; Finland).

2.14 Electron microscopy.

Intact chloroplast pellet (sect. 2.2) or about 3mm^2 leaf pieces were fixed with 6% (w/v) gluteraldehyde, in 0.1M phosphate buffer at pH 7, for 3 hours at 5C. Then washed 3 times with 0.1M phosphate

buffer, pH 7 and fixed with 1% (w/v) osmium tetroxide, in 0.1M phospate buffer, pH 7 at room temperature. After washing 3 times in water, specimens were dehydrated with graded acetone series: 50%, 70%, 80%, 100% (v/v), in water, for 30 minutes each. Spurr's epon was used as a embedding medium (O'Brien et al., 1981). Specimens were slowly infiltrated in a mixture of resin in acetone (1:2, 2:1, 3:0 parts) for 1 hour each. After 12 hours of polymerisation at 70°C, bloks of resin were trimmed and sectioned on the Porter-Blum MT2-B ultra-microtome. Sections mounted on 300 mesh grids were stained for 15 minutes with 1% (w/v) uranyl acetate (Whatson, 1958), followed by Reynolds (1963) 18mM lead citrate. Observations were made on the Philips EM-300 Transmission Electron Microscope.

3 RESULTS

3.1 Chloroplast Morphology

Highly purified, intact chloroplasts were obtained using Percoll gradient and conditions described earlier by Fish and Jagendorf (1982). Based on electron microscope studies, most of the plastids remained intact (Fig. 1). Each plastid remained surrounded by a double membrane; grana discs and the stroma lamellae were well preserved. Some of the chloroplasts appeared swollen in comparison to chloroplasts observed in leaf tissue (Fig. 2).

3.2 Protein synthesis by isolated chloroplasts.

Components and conditions optimal for <u>in organello</u>, light driven protein synthesis were investigated. Amino acid incorporation in the light (200 W/m^2) was about 40 fold greater than in the dark, with a rapid rate of synthesis up to 15 minutes and total [^{3}H]-leucine incorporation of 7×10^7 CPM x mg^{-1}chlorophyll after 30 minutes of incubation (Fig. 3).

In the dark, the addition of 10 mM ATP and 12 mM $MgCl_2$ resulted in a significant stimulation of amino acid incorporation, which

was about 20 fold over the dark control. The rate of synthesis was increased over

about 15 minutes with a significant decrease in the total radioactivity thereafter (Fig. 3). Similar kinetics of incorporation were observed when ATP and $MgCl_2$ were added to chloroplasts incubated in the light. The total incorporation in the light with added ATP and $MgCl_2$ was 4 x 10^7 CPM x mg^{-1} chlorophyll (Fig. 3). The addition of 19 common amino acids at 0.3 mM each resulted in only a slightly stimulatory effect, but two-fold increase of [^{3}H]-leucine incorporation was observed, when isoleucine and threonine at 0.3 mM concentration of each were added. This system was active for 60 minutes and over and up to 14 x 10^7 CPM x mg^{-1}chlorophyll was incorporated into chloroplastic proteins.

The addition of KCl did not have a significant effect as in previous studies (Fish and Jagendorf, 1982). The rate of traslation in 10 mM KCl was comparable to the control without KCl added, whereas 50mM KCl slightly increased amino acid incorporation rates.

Protein synthesis <u>in organello</u> was also tested in the presence of cyloheximide (CHX), an inhibitor of translation on 80S ribosomes and chloramphenicol, an inhibitor of translation on 70S ribosomes (Ellis <u>et al.</u>, 1973). Cycloheximide in a commonly used range up to 1 mg/ml did not have any significant effect, but at 2.5 mg/ml inhibited chloroplastic protein synthesis completely. About 70% inhibition of translation was observed when 0.4 mM chloramphenicol was added.

3.3 Protein synthesis by lysed chloroplasts.

As the first step in the development of an in vitro translation system, intact plastids were disrupted as follows: after freezing and thawing, the chloroplasts were resuspended in a lytic buffer (Sect. 2.5) and lysis was aided by passage through a needle or by brief sonication. Both disruption methods resulted in ATP-dependent translation activity with maximal rates occuring in the first 15 minutes (Fig. 4a and b). Incorporation of [^{3}H]-phenylalanine was at similar rates in both types of preparation when endogenous message was used. Addition of 30 ug poly U to 50ul reaction mixture resulted in higher rates of translation, especially by sonicated chloroplasts and a fast drop after 15 minutes of incubation. Ribonucleic acid from phage MS2 1 unit or about 40 ug per 50 ul inhibited protein synthesis in both systems, but higher inhibition was observed when chloroplasts were broken by passage through a needle (Fig. 4).

3.4 Protein synthesis by thylakoid bound polyribosomes.

Homologous translation was carried out using polyribosomes bound to the washed thylakoid membranes, supplemented with stromal fractions (S-100). Thylakoids and S-100 have been tested in order to see if linear kinetics comparable to those measured in vivo could be obtained.

Further purification of soluble factors (compared to that of Bhaya and Jagendorf, 1984) was attempted. Instead of 30,000xg

supernatant (100 ug protein /50ul incubation mixture) 100,000xg supernatant (100 ug protein /50ul incubation mixture) was used, which was found to be more active (data not shown). Further concentration of S-100 by ultrafiltration on 10,000 m.wt. cut-off Amicon YM-10 membrane stimulated total amino acid incorporation by 50% (Fig. 5). To economize on the amount of S-100 required, the ultrafiltered stroma was subsequently diluted twice to get appoximately 50 ug protein/ml. Table 1 shows little difference between twice diluted and undiluted stroma, whereas the dilution of the ultrafiltered stroma by a factor of ten decreased incorporation by about 50%.

With all major components present, up to 10 nmoles of leucine were incorporated per mg chlorophyll during the initial 10-15 minutes of incubation with little additional incorporation beyond that time (Fig. 5 and 6). Kinetic of the reaction might not been linear between 0-15 min. and probably closely approximated the kinetics shown in Fig. 10.

The addition of amino acids did not improve the duration of translation or total incorporation (Table 1) when using untreated stroma . With ultrafiltered and diluted chloroplast stroma, addition of 19 amino acids became necessary (Table 1). However, extra 1 mM tRNA (from wheat germ, Sigma, St. Louis) added to the incubation mixture had no effect on incorporation of leucine or appeared slightly inhibitory.

Capacity for leucine incorporation by components of the mixture was tested by eliminating one component at a time from the

incubation medium. The absence of thylakoid membranes with attached polyribosomes resulted in negligible [^{3}H]-leucine incorporation into protein. Slight leucine incorporation without S-100 or without amino acids plus energy system was observed (Fig. 6).

Optimal concentration of membranes (i.e. polyribosomes) measured on chlorophyll basis was 15 ug chlorophyll per 50 ul incubation mixture. Increasing the membrane concentration 2x inhibited translation by 50%.

The possibility that radioactive substrate may become limiting after 15 minutes of incorporation was tested by doubling its concentration. This approximately doubled the apparent total incorporation in a range of 0.043 to 0.086 nmoles substrate but did not extend the duration of incorporation (Fig. 7). Additional label provided at 15 minutes of incubation was not incorporated into TCA precipitate.

In the presence of 36 nmoles cold leucine and 0.086 nmoles of [^{3}H]-leucine, an extra addition of 0.086 nmoles radioactive substrate after 15 minutes of incubation had only a slight effect on incorporation into protein expressed in CPM x mg^{-1} chlorophyll. But incorporation expressed in nmoles x mg^{-1}chlorophyll showed a dramatic decrease after addition of an extra 0.086 nmoles of [^{3}H]-leucine (Fig. 8). Further lack of correlation between substrate incorporation expressed in CPM x mg^{-1}chlorophyll and nmoles x mg^{-1}chlorophyll is seen in Figure 9. Addition of 36 nmoles cold leucine to incubation mix decreased

incorporation expressed as CPM x mg^{-1}chlorophyll and increased apparent incorporation in terms of nmoles x mg^{-1}chlorophyll (Fig. 9), whereas increasing concentration of a radioactive leucine increased CPM values and decreased the nmole values.

Changes in the concentration of 19 amino acids in a range of 0-160 uM had no dramatic influence on label incorporation (Table 2).

In an attempt to find factors most affected and presumably becoming limiting during incubation, components of the medium were incubated for 15 minutes at 25°C, prior to their addition into the complete reaction mixture. Preincubation of the thylakoid polyribosomes resuspended in stroma S-100 resulted in the highest inhibition of [^{3}H]-leucine incorporation (about 50%) as compared to 30% decrease when both components were preincubated separately (Fig. 10). Preincubation had little effect on the initial rates of incorporation (over 5 minutes), but affected the time at which "saturation" was reached and, therefore, the absolute level of incorporation (Fig. 10).

To limit possible degradation of messenger RNA as well as other ribonucleic acids, human placental RNase inhibitor (Blackburn, et al., 1977) was used at 30 or 40 units in 50 ul of incubation mixture (Fig. 11). Total levels of incorporation were increased by its inclusion, but the short 5-10 minute linear phase of incorporation, with no further activity beyond was still observed.

The addition of 2mM iodoacetamide, a cysteinyl protease

inhibitor, did not affect translation. Increasing the concentration to 5mM inhibited amino acid incorporation by 25% and 10mM by 50%. Serine protease inhibitor, phenylmethylsulfonyl fluoride, was without any effect in the 0.4 mM to 1.6 mM range.

Bacterial contamination was likely negligible since ampicillin, bacterial (gram negative) wall synthesis inhibitor, added routinely to buffers and incubation mixtures did not have any inhibitory effect at 5-10 ug/ul concentration.

3.5 Protein synthesis by 30,000xg supernatant (S-30 system).

The addition of exogenous mRNA (3 to 30 ug poly U per 50 ul total incubation medium) did not have any effect on translation by membrane bound polysomes supplied with S-100 (data not shown). Therefore, 30,000xg supernatant (S-30) only was tested, which possibly contained additional free ribosomes available for translation of a new message. In the S-30, thylakoid fraction (pellet 30,000xg) was discarded. Furthermore, in some experiments 30,000xg supernatant was divided into two components: 100,000xg pellet and 100,000xg supernatant (see Methods, sect. 2.6).

A sharp peak of maximal activity was observed at 50 ug pellet protein per 50 ul incubation with 200 ug of stromal (S-100) proteins (Fig. 12). Conversely, varying the concentration of added 100,000xg supernatant from 0 to 300 ug protein in 50 ul reaction with 50 ug thylakoid protein caused a progressive increase in the total

incorporation of [^{3}H]-phenylalanine, nearly reaching saturation at 300 ug protein (Fig. 13). Both sets of data (Fig. 12 and 13) were obtained with 30 ug poly U added to the standard incubation mixture (Methods).

Protein synthesis showed optimum rates for about 30 minutes with 3 x 10^{5} CPM [^{3}H]-phenylalanine x mg^{-1}protein incorporation (Fig. 14). Addition of 3 ug poly U stimulated incorporation slightly. Excluding 100,000xg pellet or 100,000xg supernatant from the medium prevented translation completely (Fig. 14).

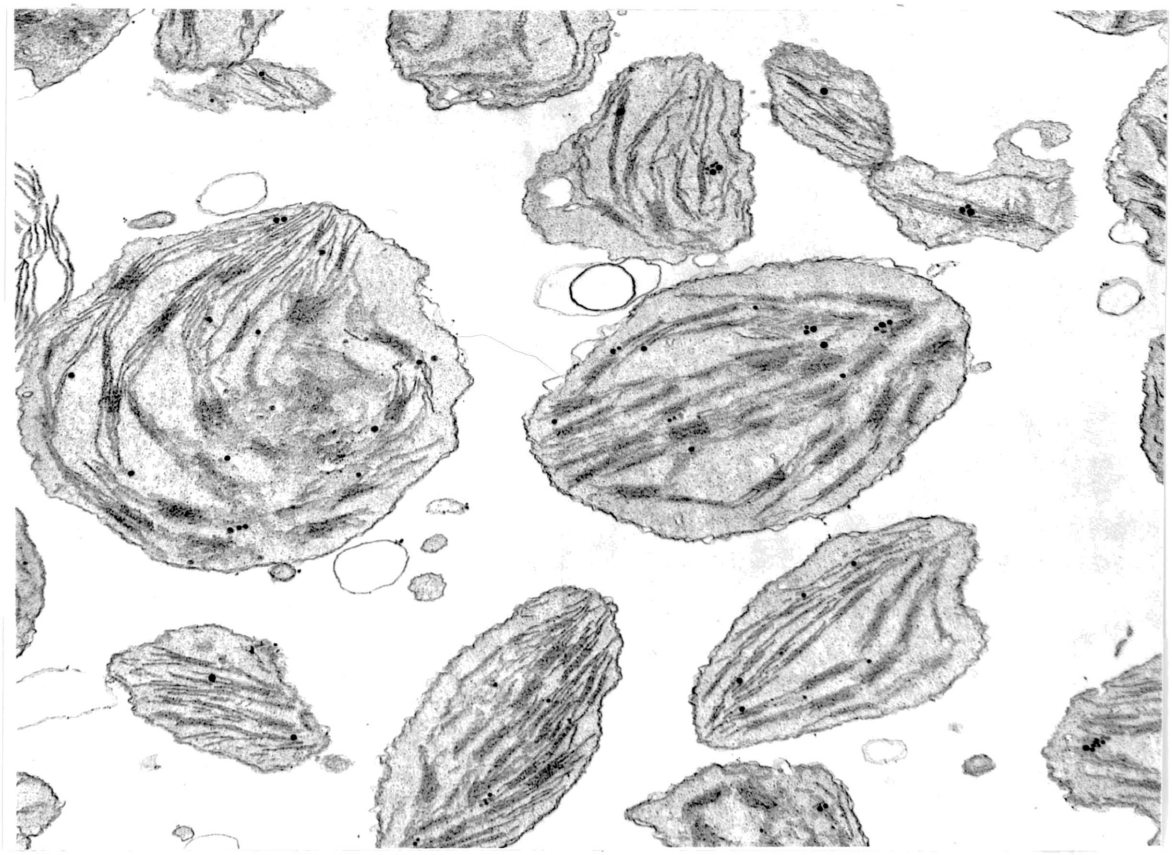
An increase in phenylalanine incorporation was found to correspond to increasing concentrations of poly U (Fig. 15). A slight increase was observed by adding 3 ug poly U and a 2 fold increase by adding 30 ug poly U to the reaction mixture. The incorporation of [^{3}H]-phenylalanine stimulated by poly U was more affected when the other amino acids were excluded from the translation medium. A 25% increase of total counts was observed (data not presented). Furthermore, it did not appear necessary to separate the 30,000 x g supernatant into the high speed supernatant and pellet, with incorporation comparable to that in Figures 14 and 15.

Bacteriophage MS2 RNA added to the incubation mixture inhibited amino acid incorporation by 50% at 4 ug and by 75% at 40 ug per 50 ul of the mixture (Fig. 16). The labelled products of poly U and MS2 translation were examined by PAGE electrophoresis followed by fluorography. Poly U driven translation resulted in a broad high

molecular weight band superimposed on endogenous chloroplastic proteins. MS2 products were not observed and labelling of all endogenous products was totally suppressed (data not shown).

Figure 1. Transmission electron micrograph of chloroplasts. Chloroplasts were obtained from Percoll gradient, fixed in gluteraldehyde and osmium tetroxide. (x 10,000)

Figure 2. Transmission electron micrograph of leaf section. Material was fixed in gluteraldehyde and osmium tetroxide. (x 30,000)



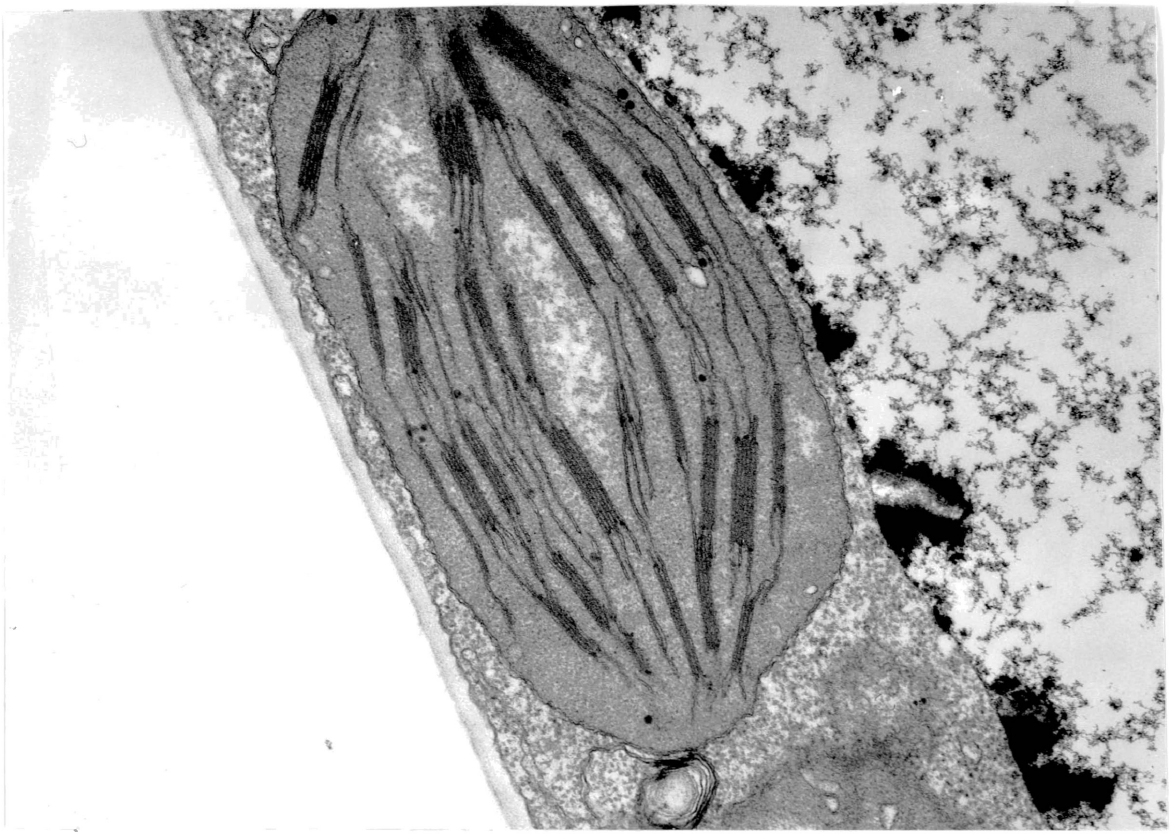


Figure 3. Incorporation of [^{3}H]-leucine by intact chloroplasts. Reaction tubes were illuminated (open symbols) or wrapped in aluminum foil (solid symbols); no additions (circles), plus 10 mM ATP and 12 mM $MgCl_2$ (squares). Each point represents three experiments seen in duplicate assays, only maximal S.E.M. is shown.

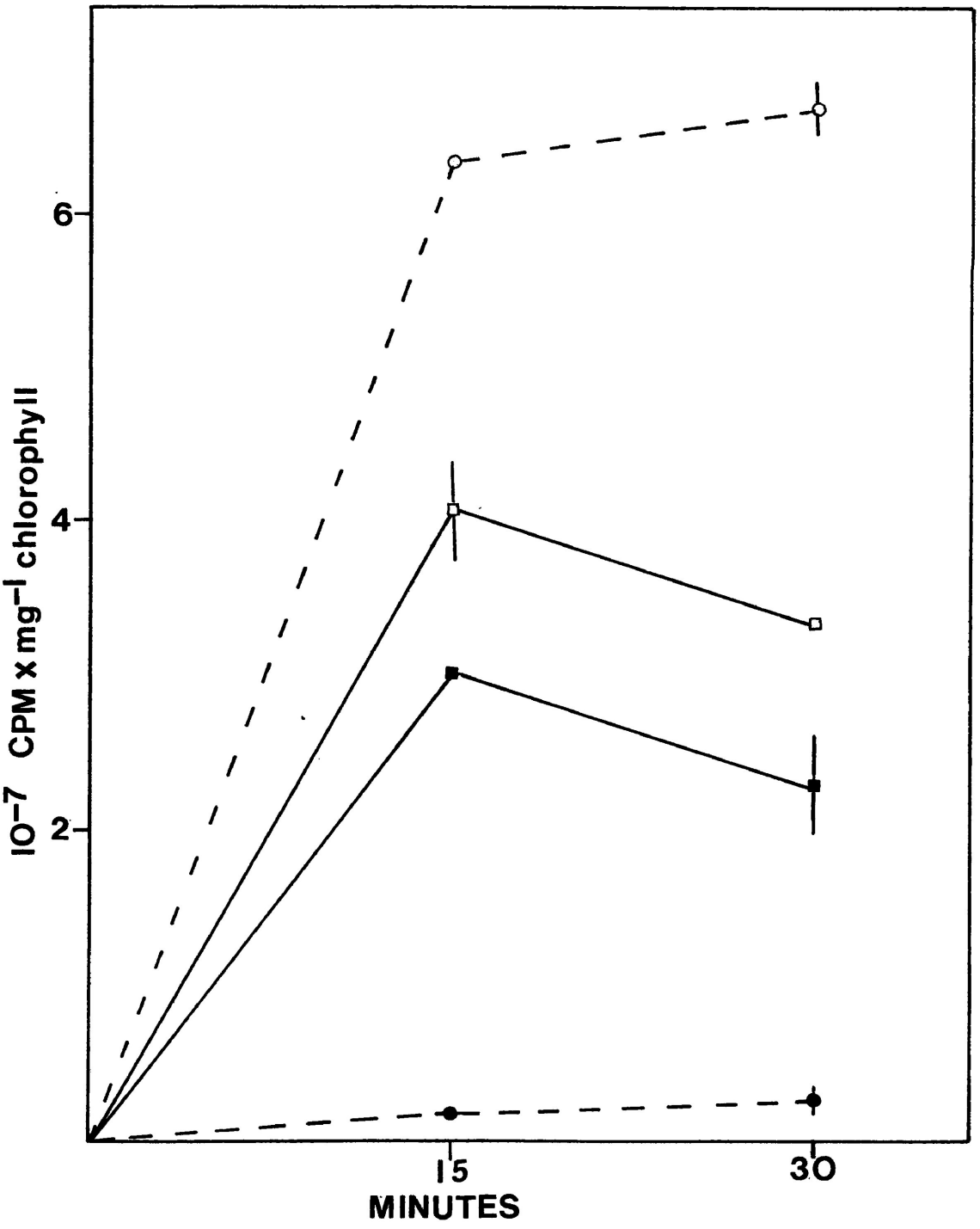

6
4
2
10^{-7} CPM x mg^{-1} chlorophyll
15
30
MINUTES

Figure 4. Comparison of [^{3}H]-phenylalanine incorporation using chloroplast lysates prepared by different methods. Lysate prepared by passing through a 20 guage needle three times (left panel) and lysed by sonication with three 10 second ultrasound pulses (right panel); endogenous activity (circles), 30 ug of poly U added (squares) and 1 unit of MS2 (crosses). The average of duplicate assays for each time point is shown.

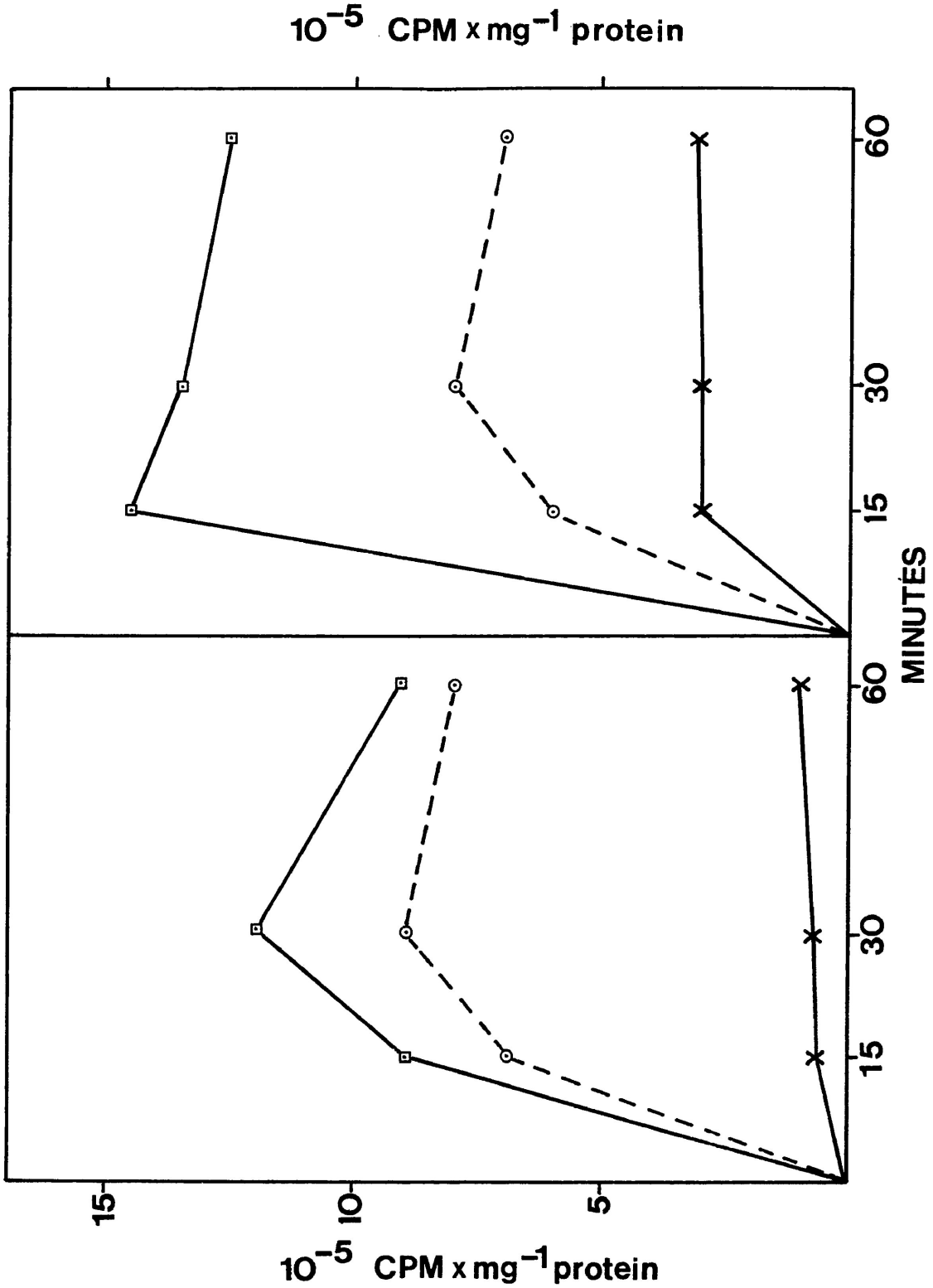

10^{-5} CPM x mg^{-1} protein
60
30
15
MINUTES
60
30
15
15
10
5
10^{-5} CPM x mg^{-1} protein

Figure 5. Time course of [^{3}H]-leucine incorporation by stroma suplemented membrane bound polysomes. High speed (100,000xg) stroma added without further purification (solid circles) or ultrafiltered (10,000 M.Wt. cut-off)(open circles). Data from two experiments run in duplicate $\pm$ S.E.M.

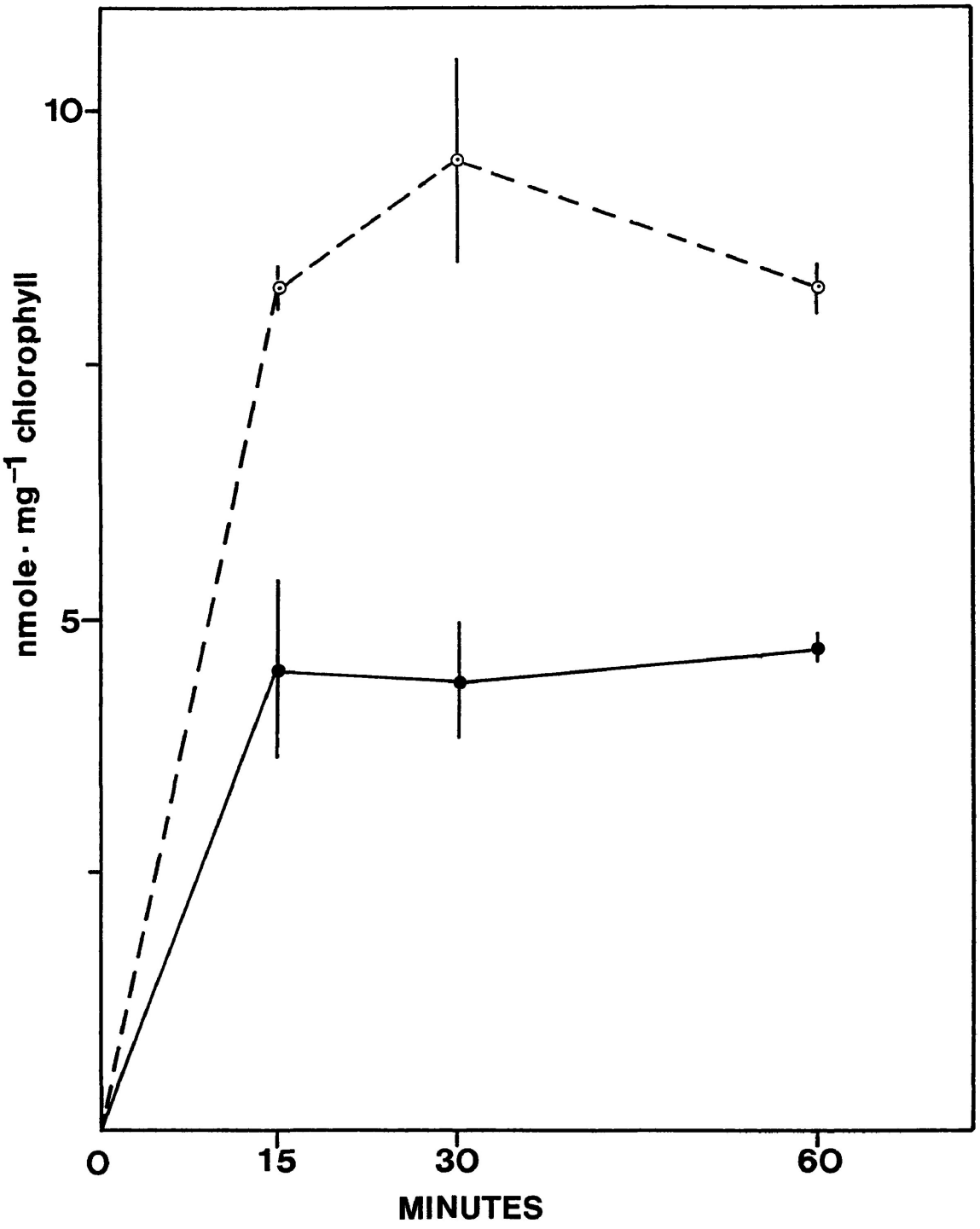

10
5
nmole · mg⁻¹ chlorophyll
0
15
30
60
MINUTES

Figure 6. Time course of [^{3}H]-leucine incorporation by membrane bound polysomes. At concentration of 1.72 uM hot leucine and 0.72 mM cold leucine was added to a) complete incubation medium (circles), b) minus S-100 (open triangles), minus amino acids, GTP, ATP (solid triangles), minus thylakoid polyribosomes (squares). Each point is the average of two experiments run in duplicate $\pm$ S.E.M.

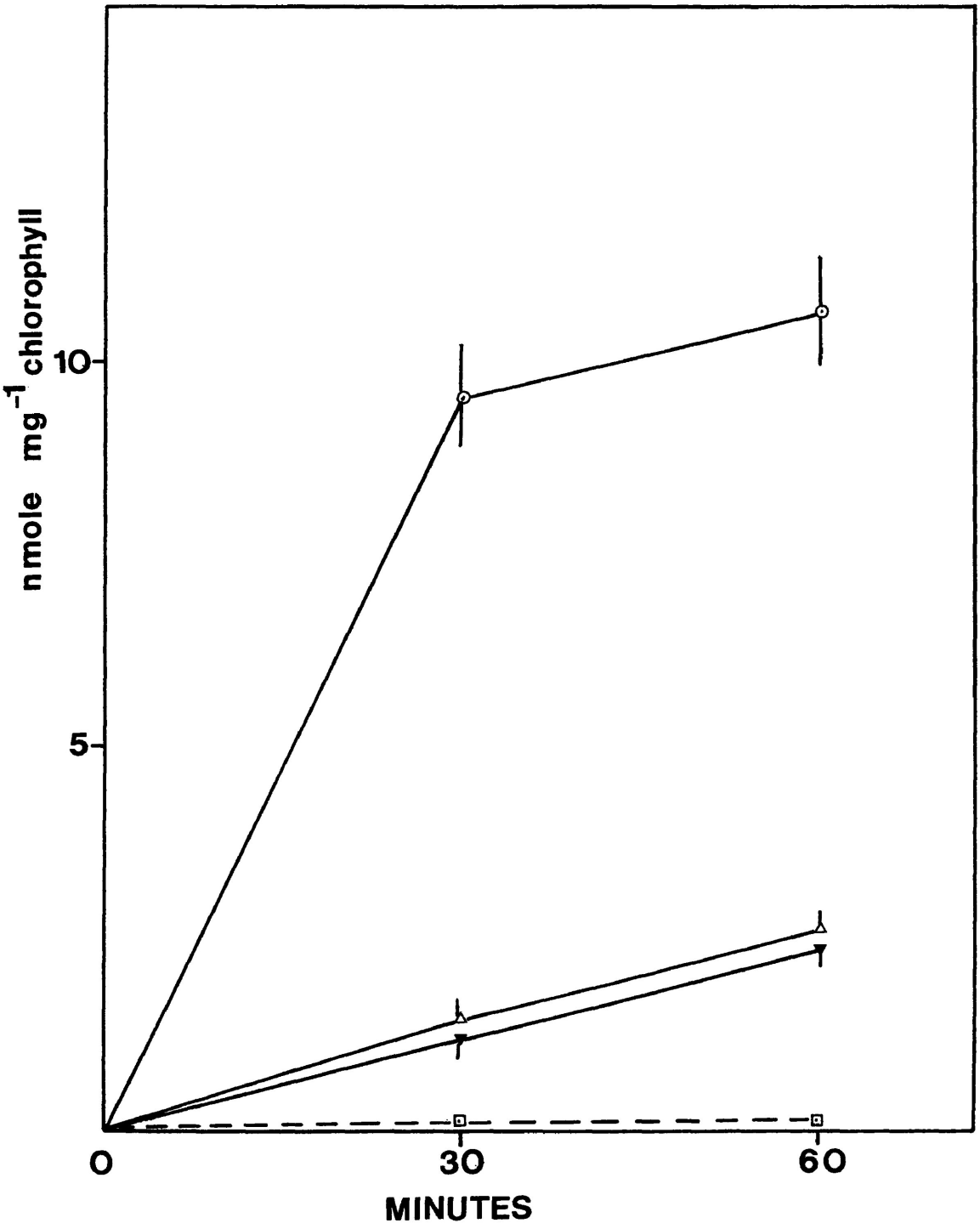

nmole mg^{-1} chlorophyll
10
5
0
30
60
MINUTES

Figure 7. Incorporation of [^{3}H]-leucine into protein by thylakoid polyribosomes. Incubation mixture contained 0.043 nmoles (open circles) and 0.086 nmoles (open squares) of [^{3}H]-leucine. After 15 minutes an extra 0.043 nmoles (solid circles) and 0.086 nmoles (solid circles) of [^{3}H]-leucine were added. Average of two experiments run in duplicate $\pm$ S.E.M. is shown.

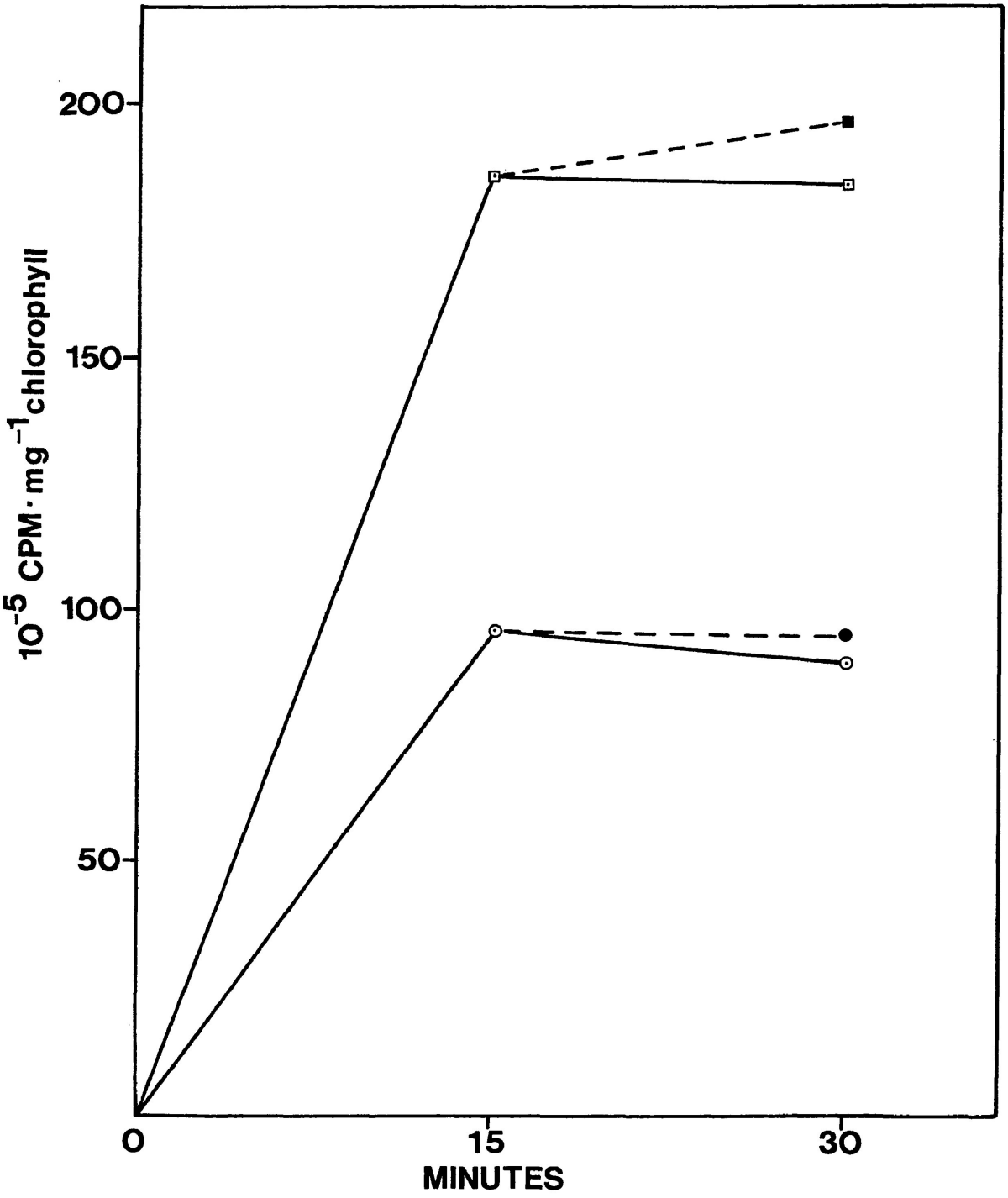

200
150
100
50
10^{-5} CPM · mg^{-1} chlorophyll
0
15
30
MINUTES

Figure 8. Incorporation of [^{3}H]-leucine by thylakoid polyribosomes; in cpm and recalculated as nmole x mg^{-1} chlorophyll. CPM x mg^{-1} chlorophyll (left panel) and recalculated as nmole x mg^{-1} chlorophyll (right panel) Standard incubation mixture contained 0.086 nmoles [^{3}H]-leucine (open circles). An extra 0.086 nmoles [^{3}H]-leucine were added after 15 minutes incubation (solid circles). All reaction mixtures contained 36 nmoles of cold leucine. Each point is the average of two experiments run in duplicate.

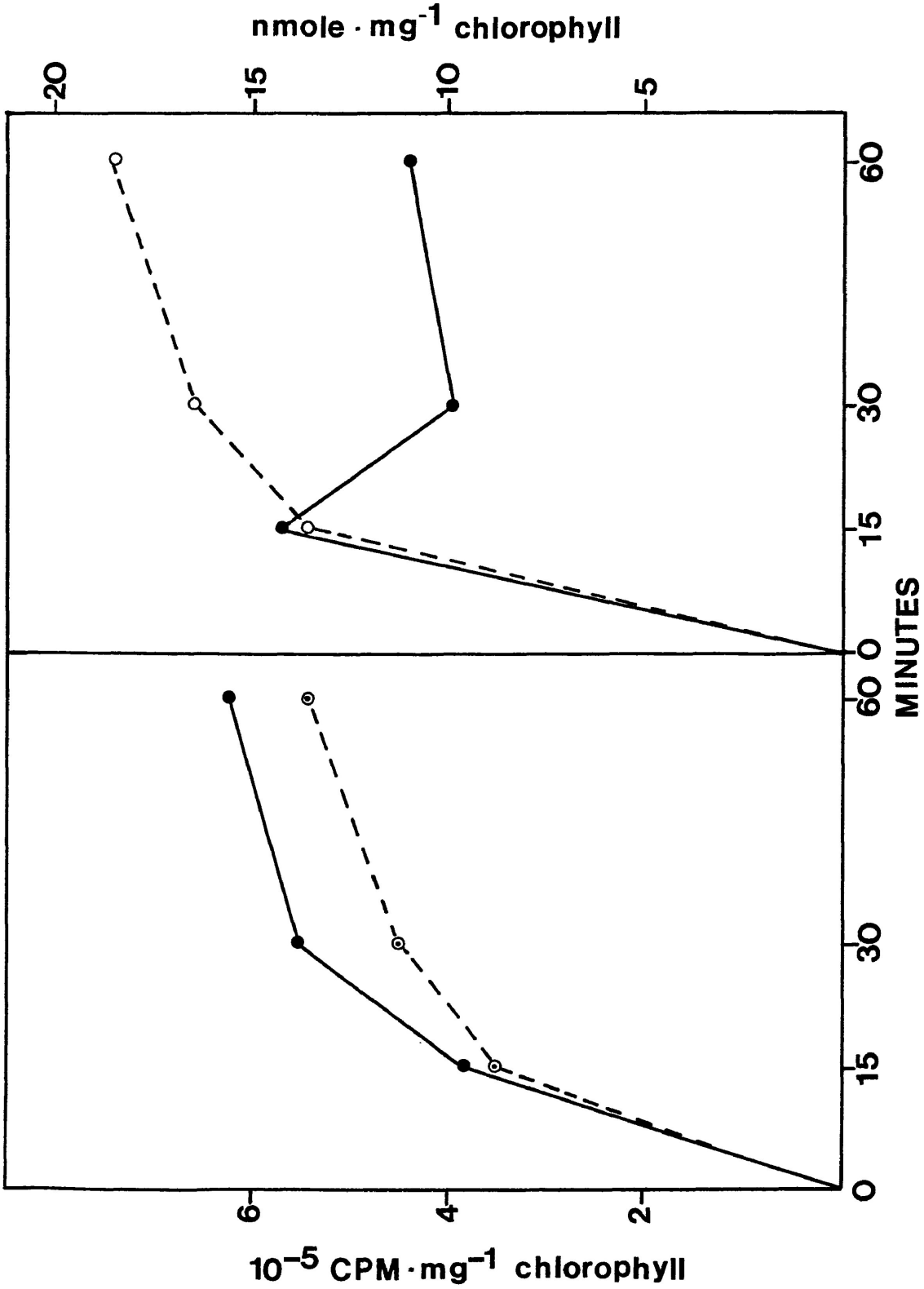

nmole · mg⁻¹ chlorophyll
20
15
10
5
60
30
15
0
60 0
MINUTES
0
15
30
60
6
4
2
10⁻⁵ CPM · mg⁻¹ chlorophyll

Figure 9. Incorporation of [H]-leucine into chloroplastic protein; effect of label dilution. Without (open symbols) and with added cold leucine (36 +nmoles in 50 ul mixture, solid symbols). Data are presented as CPM x mg^{-1} chlorophyll (left panel) and as nmoles x mg^{-1} chlorophyll (right panel). Radioactive [^{3}H]-leucine was added at 0.043 nmoles (circles), 0.086 nmoles (triangles) and 0.172 nmoles (squares). Each point is the average of three experiments run in duplicate.

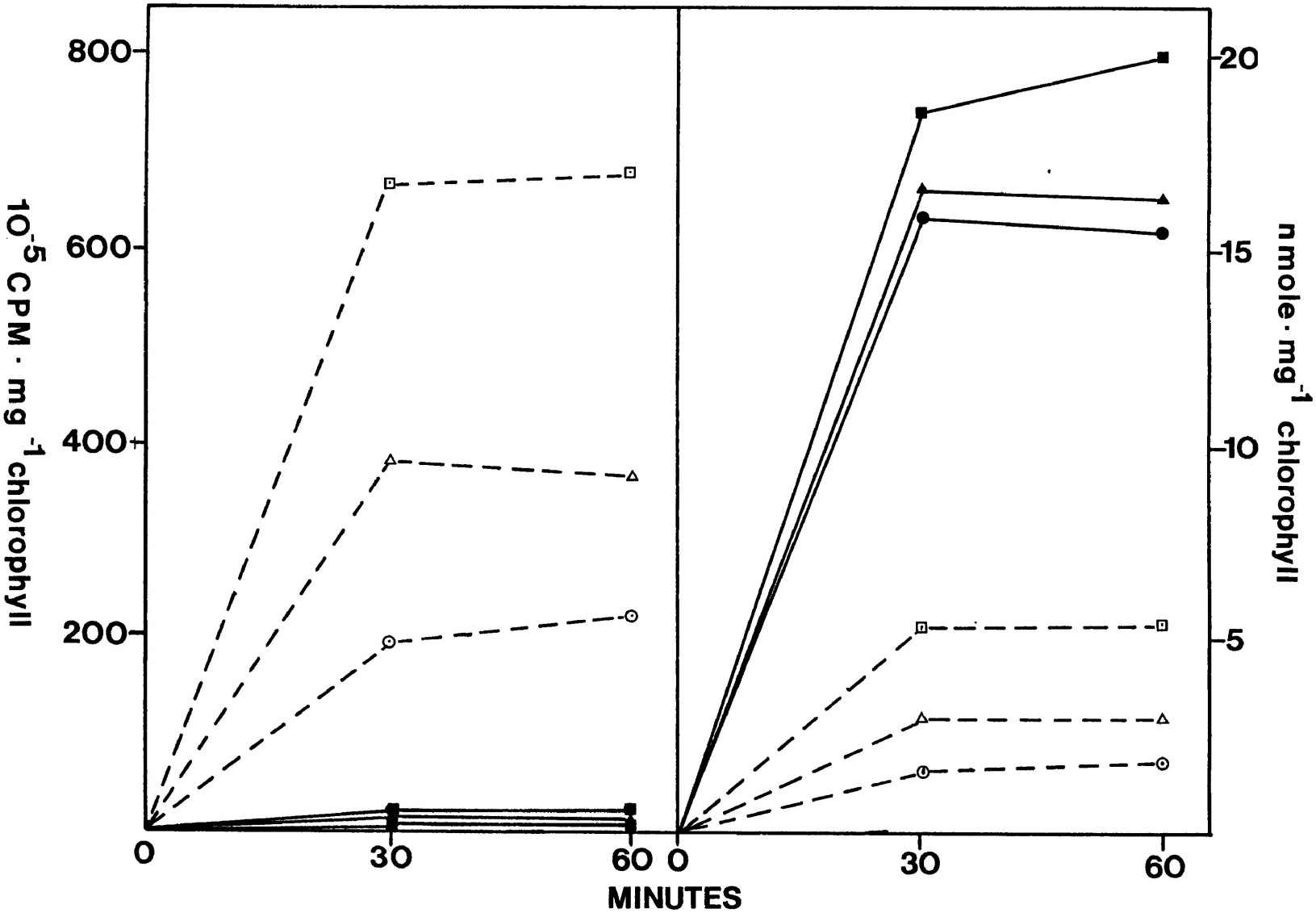

800
600
400
200
0
30
60
MINUTES
10^{-5} CPM · mg $^{-1}$ chlorophyll
20
15
10
5
0
30
60
n mole · mg^{-1} chlorophyll

Figure 10. Time course of [^{3}H]-leucine incorporation using pre-incubated components. Cold leucine was not included in incubation medium. Standard conditions (no pre-incubation, circles), S-100 and thylakoid polyribosomes were preincubated for 15 minutes at 25^{o}C: separately (triangles), together (squares). Each point is the average of three experiments run in duplicate.

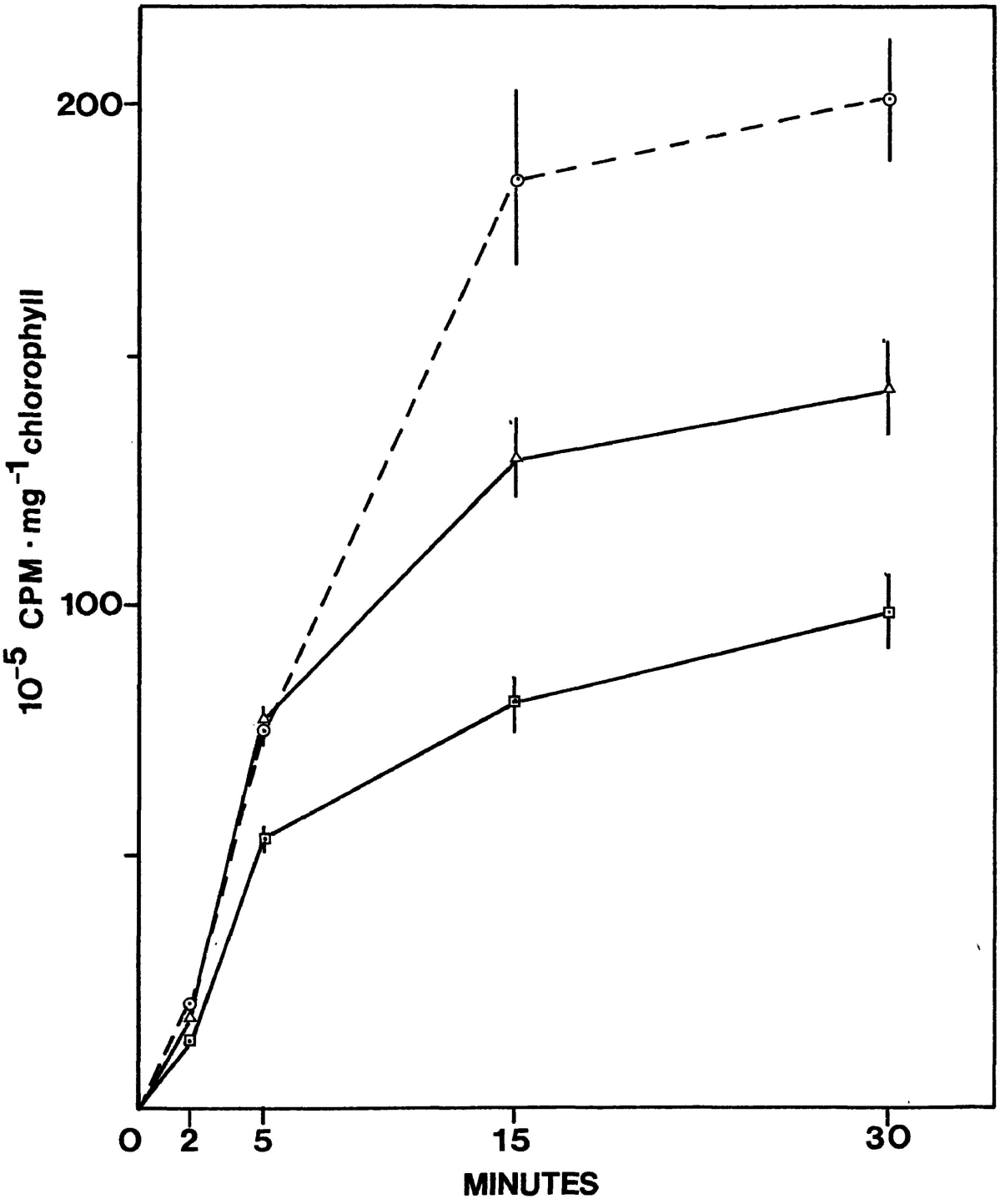

200
100
10^{-5} CPM · mg^{-1} chlorophyll
0
2
5
15
30
MINUTES

Figure 11. Effect of human placental RNase inhibitor on [^{3}H]-leucine incorporation, by membrane bound polysomes. Inhibitor not added (circles), 30 units (open squares), 40 units (solid squares). Each point is the average of two experiments run in duplicate, $\pm$ S.E.M.

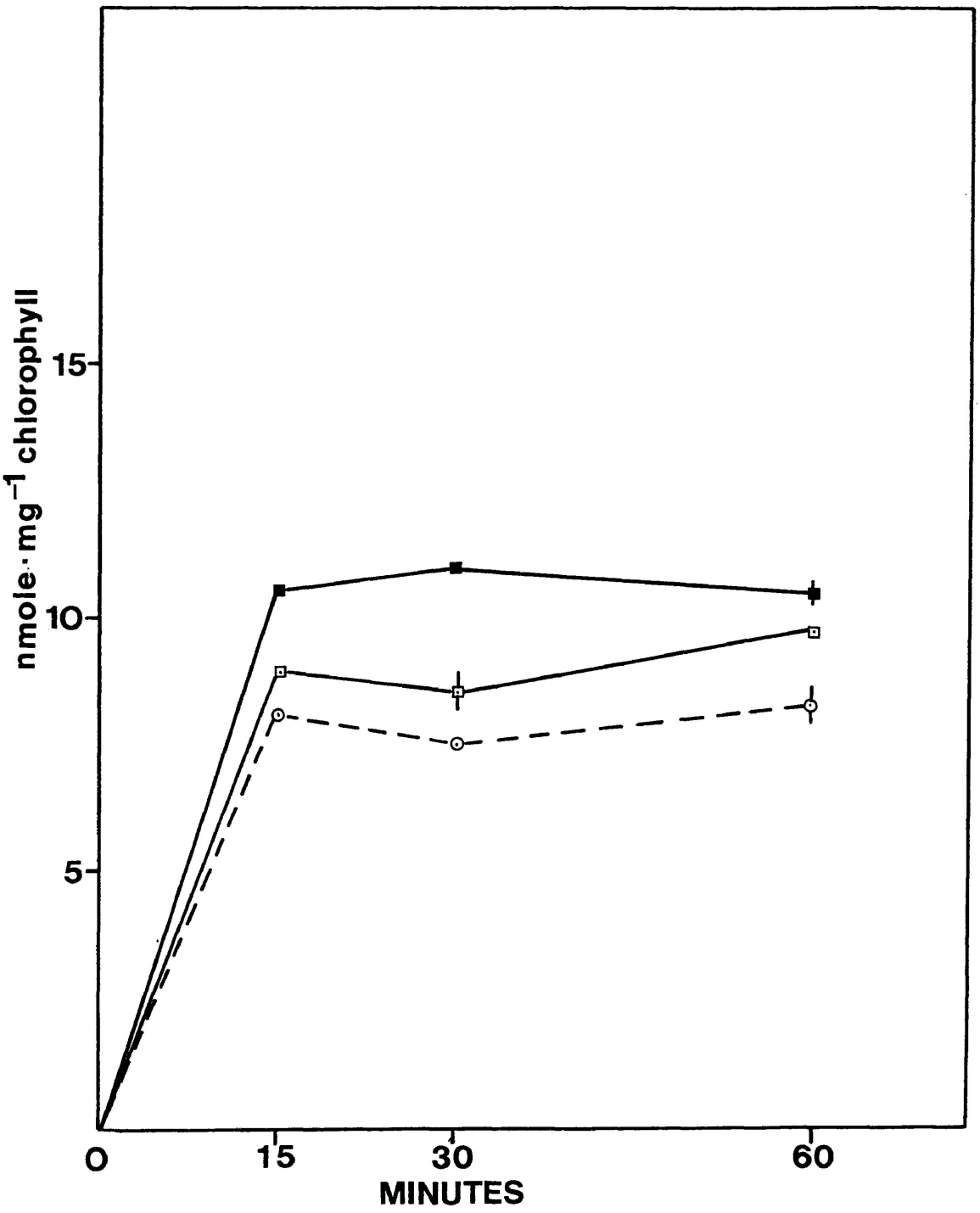

nmole · mg⁻¹ chlorophyll
15
10
5
0
15
30
60
MINUTES

Figure 12. Effect pellet 100,000xg (P-100, lacking thylakoids) concentration on incorporation of [^{3}H]-phenylalanine. Incubation was done for 60 minutes with 30 ug poly U and 200 ug stroma (S-100) proteins. For details of purification, see Materials and Methods. Data from two experiments run in duplicate.

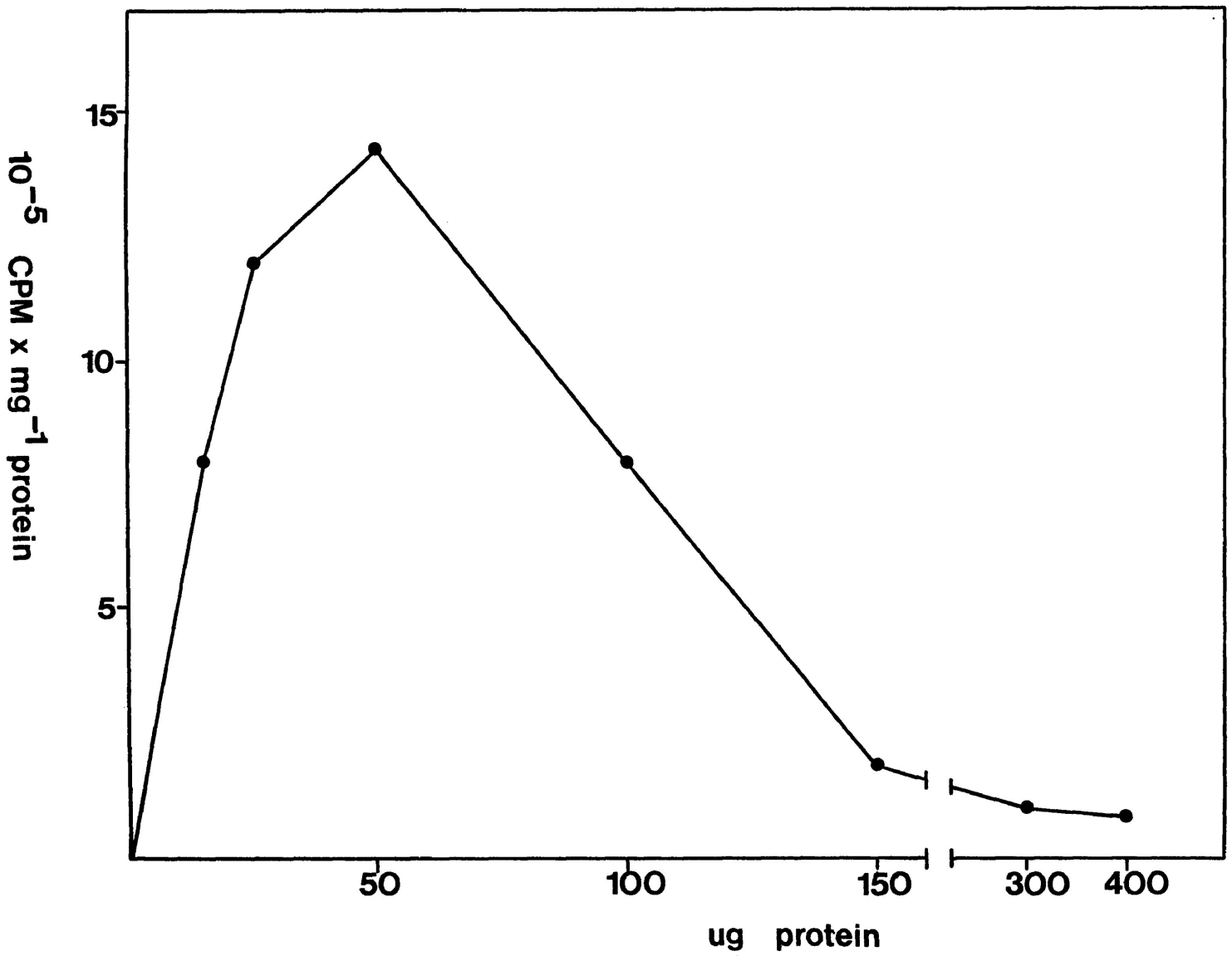

15
10
5
10^{-5} CPM x mg^{-1} protein
50
100
150
300
400
ug protein

Figure 13. Effect of varied stroma 100,000xg (S-100) concentration on incorporation of [^{3}H]-phenylalanine. Incubation was done for 60 minutes with 30 ug poly U and pellet 100,000xg (P-100, lacking thylakoids) at 50 ug protein concentration. See Methods for details. Data from two experiments run in duplicate.

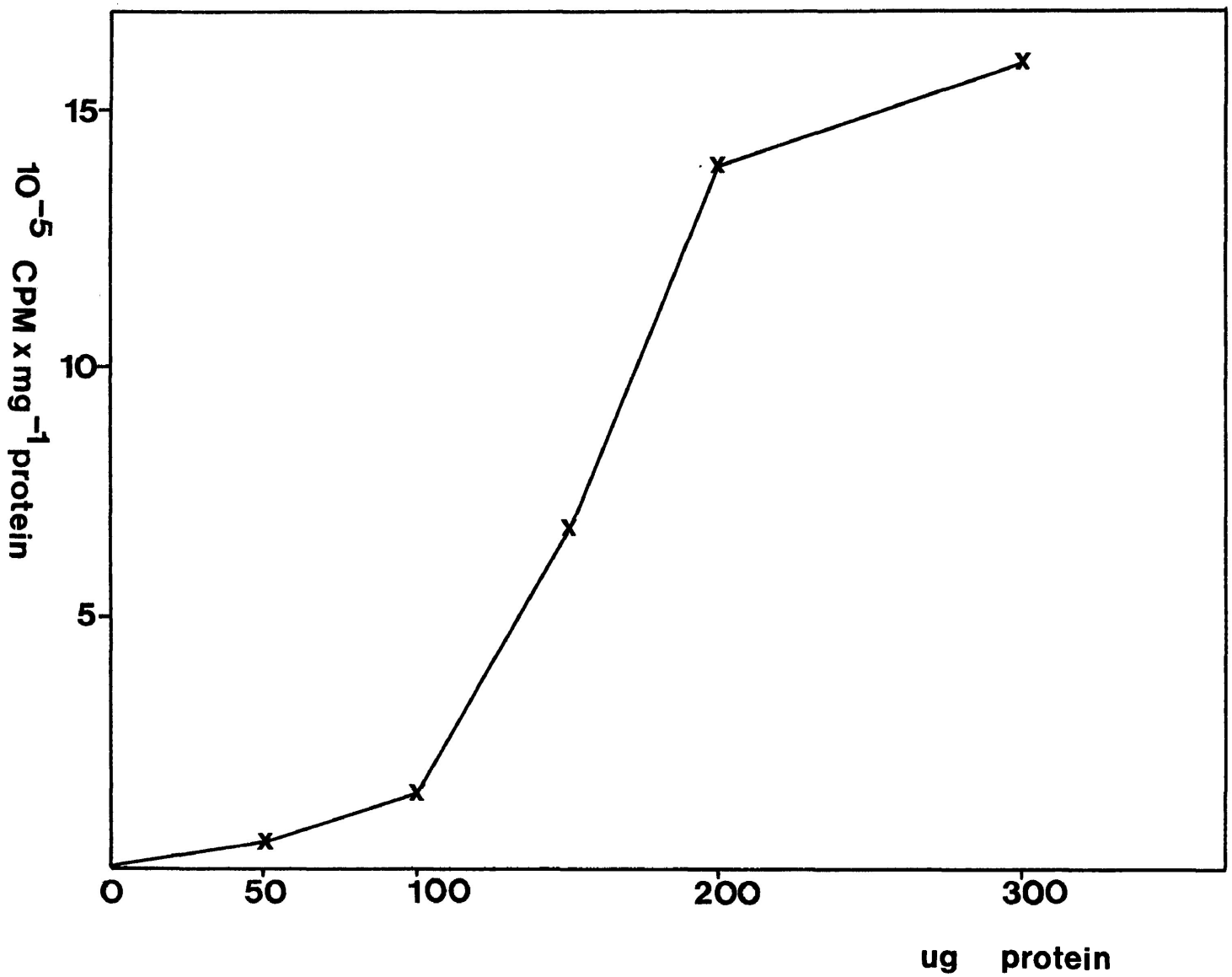

15
10
5
10^{-5} CPM x mg^{-1} protein
0
50
100
200
300
ug protein

Figure 14. Time course of [^{3}H]-phenylalanine incorporation using S-30 system. Incubation medium contained 300 ug of stromal protein (S-100) and 50 ug of pellet protein (P-100) (circles) plus 30 ug of poly U (squares), minus S-100 (triangles) and minus P-100 (solid triangles). Data from two experiments run in duplicate.

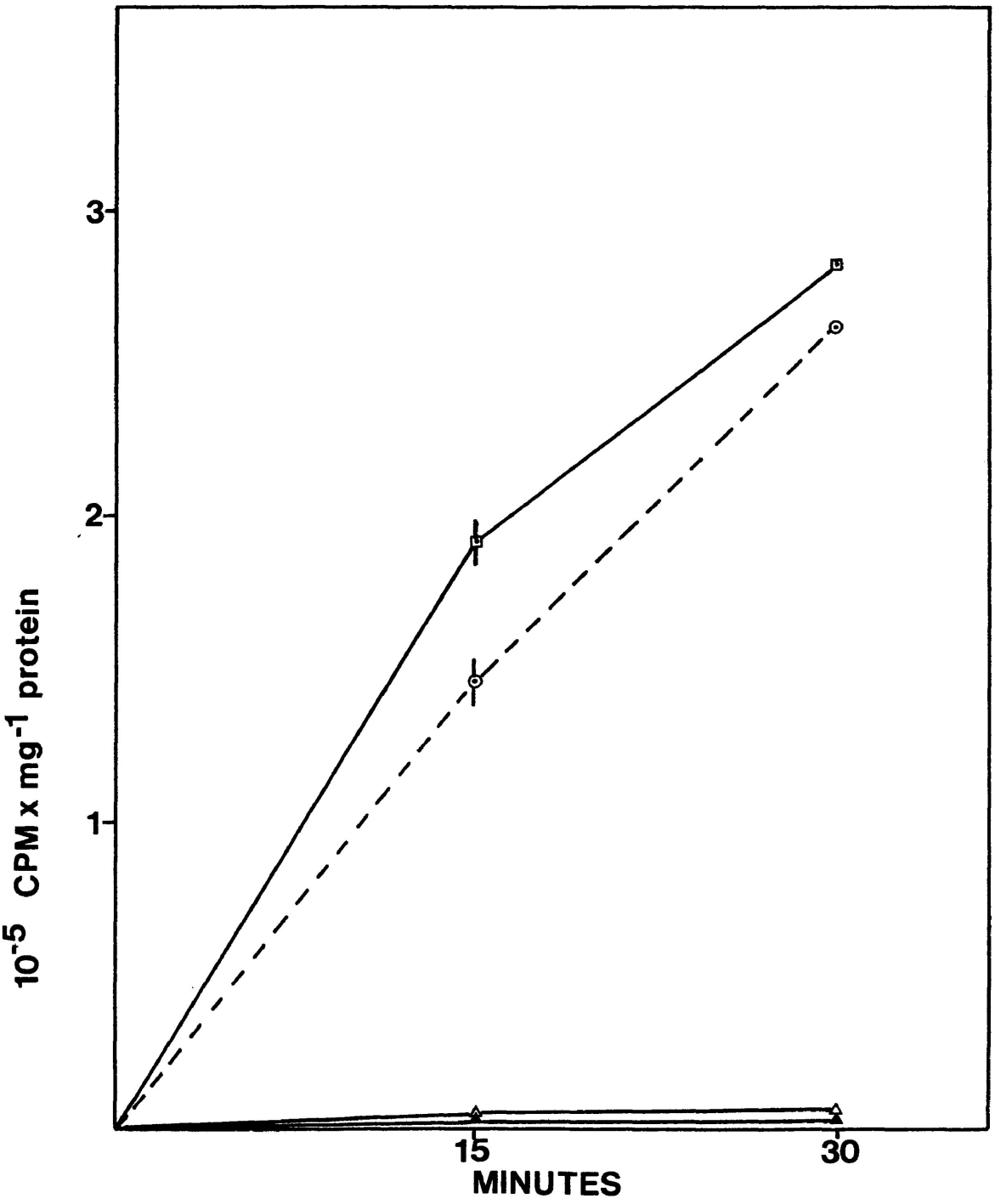

3
2
1
10^{-5} CPM x mg^{-1} protein
15
30
MINUTES

Figure 15. Extended time course of [^{3}H]-phenylalanine incorporation by adding poly U to S-30 system. Protein concentration of S-30 (30,000 x g supernatant) was 300 ug ; endogneous activity (circles), 3 ug poly U per 50 ul incubation mixture (triangles) and 30 ug poly U per 50 ul incubation medium (squares). Data from two experiments run in duplicate.

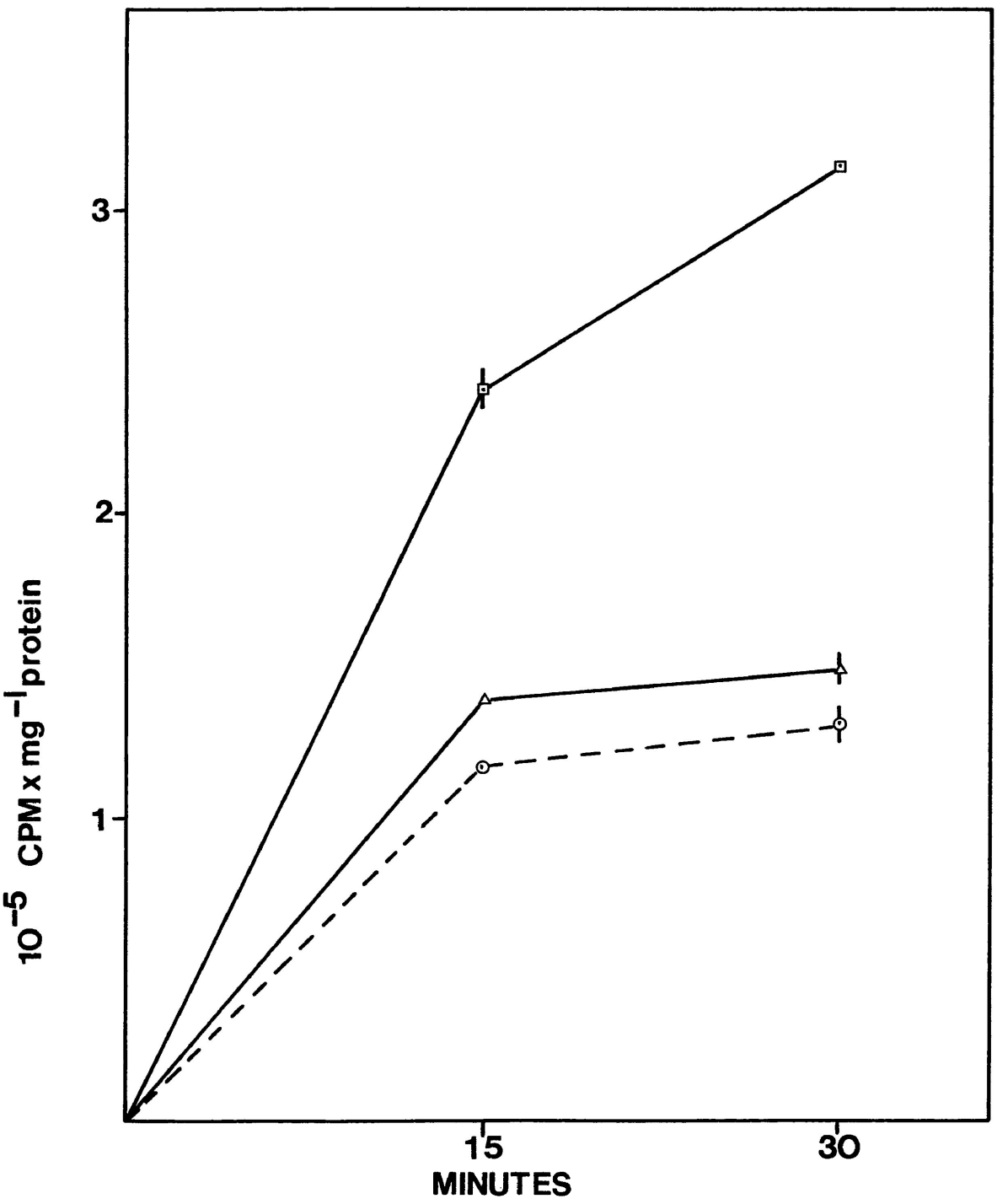

3
2
1
10^{-5} CPM x mg^{-1} protein
15
30
MINUTES

Figure 16. The effect of MS2 phage RNA on incorporation of [^{3}H]-phenylalanine into protein. The stroma (S-30) was incubated without MS2 (solid circles), with 0.1 unit MS2 (solid triangles) and with 1 unit MS2 (solid squares). Each point is the average of two experiments run in duplicate.

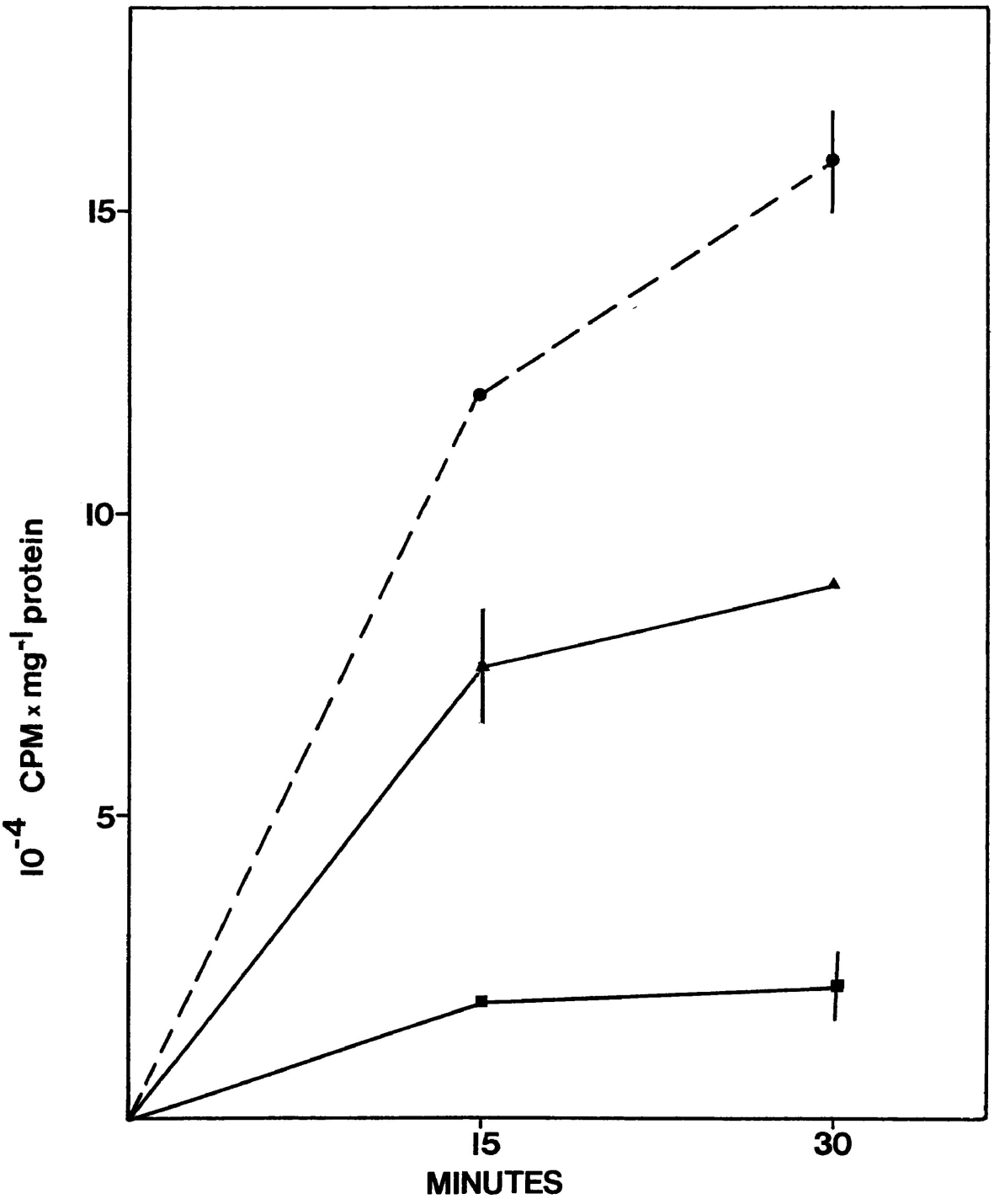

15
10
5
10^{-4} CPM x mg^{-1} protein
15
30
MINUTES

Table 1: The effect of S-100 concentration on the incorporation of[^{3}H]-leucine by membrane bound polysomes with and without 0.3 mM each of 19 amino acids added to the incubation mixture. Data expressed as nmole x mg^{-1}chlorophyll. Stroma S-100 without dilution contained approximately 1 mg/ml protein.

	nmoles leucine x mg^{-1}chlorophyll x 30 min^{-1}			
	untreated S-100		ultrafiltered S-100	
Amino Acids	+		+	
Stroma (S-100)				
without dilution	10.4	10.5	17.0	7.8
diluted 2x	7.2	5.1	15.4	7.1
diluted 10x	4.8	3.4	7.2	3.7

Table 2: Effect of varying the concentration of cold leucine and 19 amino acids on the incorporation of [^{3}H]-leucine into protein by membrane bound polysomes. Data expressed in nmole x mg^{-1}chlorophyll. The concentration of [^{3}H]-leucine was 1.72 uM (0.086 nmoles per reaction mixture).

Concentration of additional cold leucine(uM)	0	7.2	72	720
Concentration of 19 amino acids (uM each)	(nmole x mg^{-1}chlorophyll x 0.5 hr^{-1})			
160	0.28	5.6	12	18
16	0.38	4.6	9	16
0	0.47	3.3	10	10

4 DISCUSSION

Intact chloroplasts were separated from less dense chloroplast fragments and mitochondria by using centrifugation medium of appropriate density (Miflin and Beevers, 1974). Percoll was consider to be suitable for the control of medium density since it has high density, yet low viscosity, low osmolality and is non-toxic to organelles (Mills and Joy, 1980). The suitability of Percoll gradients in isolation of intact chloroplasts was shown by Bhaya and Jagendorf (1984), using marker enzymes such as catalase and cytochrome c oxidase described earlier by Mills and Joy (1980). The lack of contamination with active 80S ribosomes in the chloroplastic preparations was confirmed by the addition of cycloheximide, an inhibitor of protein synthesis in eukaryotic cytoplasm in the 0-1 mg/ml range. The complete inhibition of organellar protein synthesis by 2.5 mg/ml cycloheximide could be interpreted as a non-specific secondary effect of high concentration of the inhibitor on the chloroplastic system (Ellis, 1977). The inhibition of translation by chloramphenicol was expected in view of previously reported sensitivity to this inhibitor (Ellis, 1969; Kirk and Tilney-Basset, 1978).

Some parameters of <u>in organello</u> protein synthesis systems were reinvestigated. An essential component of the medium for protein

synthesis by both intact and lysed chloroplasts are potassium ions (Ellis, 1977). Replacement of potassium ions by sodium prevented protein synthesis in intact chloroplasts driven by light and ATP-dependent protein synthesis by free ribosomes. Fish and Jagendorf (1982) used potassium ions in chloroplastic translation medium. A sharp optimum between 20-50 mM was found. Inhibition by excess K^+ could be interpreted as an osmotic effect. Similar results were obtained by using an excess of sorbitol (Fish and Jagendorf, 1982). In our studies potassium ions at 50 mM were added routinely to the intact chloroplast system and at 100 mM to *in vitro* chloroplastic systems as an osmoticum and cofactor of translation since it was found to stimulate protein synthesis (Kaizer *et al.*, 1980; Fish and Jagendorf, 1982). Addition of free Mg^{2+} to the medium was found to be related to potassium ions and pH (Huber and Maury, 1980). Free Mg^{2+} may be needed to activate the K^+/H^+ antiporter in the chloroplast envelope . Deficit or excess of magnesium ions could change the pH of the medium and thus inhibit translation (Fish and Jagendorf, 1982).

The addition of 12 mM $MgCl_2$ to the medium did not change the total rates of incorporation in our work. It would seem likely that in intact chloroplasts the localized concentration of Mg^{2+} is adequate to support protein synthesis. When ATP and $MgCl_2$ was added to incubation mixtures considerable protein synthesis by intact chloroplasts was observed (Fig. 3). We were not able to obtain rates of translation driven by ATP comparable to light driven synthesis, as reported by Fish and Jagendorf (1982). Inhibition of light driven amino acid incorporation with a significant drop of total counts after 15 minutes

of incubation and/or after adding ATP-$MgCl_2$ could be caused by increased activity of endogenous proteases. Liu and Jagendorf (1984) and Malek et al. (1984) have found 25-40% degradation of newly labelled proteins by isolated chloroplasts. They showed that ATP stimulated proteases could play a role.

In eukaryotic cells incubation with Mg ATP caused the inactivation of the amino acyl tRNA synthetase complex (Damuni et al., 1982). The phosphorylation by ATP could also affect chloroplastic proteins which are active during translation process. In the dark, protein synthesis driven by ATP showed little stimulation when equimolar amount of Mg^{2+} was added to incubation mixture (Fish et al., 1982, Fig. 3). Each of these two factors, when present in excess, inhibited translation. From Heldt's (1979) studies it was found that stroma pH rose from 7.0 in the dark to 7.8 in the light. Hurewitz and Jagendorf (1987) concluded that stimulation of protein synthesis by light compared to protein synthesis driven by ATP (found also in our studies, Fig. 3) is related to the increase in stroma pH caused by electron transport, rather than to increase in stroma ATP levels. The same authors found 24 to 74% more RNA was bound to the thylakoids at pH 8.3 that at pH 7. Therefore, the effect of light on ribosome binding in vivo may be due to increased stroma pH. Incorporation of [^{3}H]-leucine was proportional to the amount of mRNA bound to the thylakoid membranes (Hurewitz and Jagendorf, 1987)

There was a surprisingly low stimulation of protein synthesis by the addition of amino acids. This could be an evidence of high

intactness of the chloroplast membranes. Mills and Joy (1980) already reported that Percoll and fast preparative methods in plastid isolation prevented major leakage of amino acids from chloroplasts. Another explanation could be that during translation, different amounts of each of the amino acids are utilized for translation. At equal concentration there would be a significant excess of some of them, which would compete for available tRNAs and cause increased misreading by the ribosomes. Ruusala et al. (1982) and Thomson and Dix (1982) synthesized abnormal proteins and showed increased amount of short peptides using poly U

directed translation system with excess of leu-tRNA in cell-free E. coli system. Earlier, Goldberg (1972) presented evidence of increased proteolytic activity against abnormal proteins and unfinished peptides in E. coli. The presence of discrete sizes of partially completed translation products was observed using isolated intact chloroplasts pulse labelled with [^{35}S]-methionine (Mullet et al., 1985). The same authors reported increased proteolytic activity when one of the amino acids ([^{35}S]-methionine) became limited. Under these conditions several lower molecular-mass- translation products were observed, which did not comigrate with polypeptides labelled within chloroplasts in vivo. This effect was overcome after increasing concentration of substrate methionine. Further work is needed on the role of amino acid and amino acyl-tRNA availability in order to find more suitable amino acid ratios for chloroplastic protein synthesis. Stimulation of [^{3}H]-leucine incorporation by addition of isoleucine and threonine observed here (data not shown) and reported previously by Nivison and Jagendorf (1984)

is probably due to inhibition of leucine synthesis, making labeled leucine accessible to charging of tRNA.

The rate of *in organello* protein synthesis (Fig. 3) was generally in keeping with published data (Nivison and Jagendorf,1984) and suggested that preparations were of adequate quality to use as a starting material for further *in vitro* work.

The capacity for *in vitro* translation based on chloroplastic preparations was tested using the following systems: 1) chloroplast lysates, 2) thylakoid bound polysomes supplemented with high speed supernatant (S-100) and 3) low speed supernatant (S-30) only. The translation was driven by an external ATP supply. Studies using endogenous mRNA activity as well as external mRNAs (poly U and MS2) were carried out. Parameters and conditions of *in vitro* translation were examined in order to optimize these systems, using conditions reported by Bhaya and Jagendorf (1984) as a starting point. Since in the previous studies the *in vitro* chloroplastic systems were incapable of reinitiation, with mostly "run off" from endogenous mRNA taking place, our work was focused on the potential achievement of re-initiation on exogenous mRNAs.

Disruption of the membranes caused by mechanical breakage usually changes the conditions around the polysome complexes (Schmidt *et al.*, 1985). Correct initiation, elongation, termination and release of macromolecular chains could, therefore, be different from that *in vivo*. Also, lysed systems are more likely to have different concentrations of

all components (broken chloroplasts cannot use light as a source of energy because of the dilution of ferredoxin that occurs after breakage, Ellis and Hartley, 1982) than intact plastids. Disruption of the membranes appeared to increase lipid peroxidation which had been demonstrated to have an inhibitory effect on protein synthesis, probably by releasing toxic products which may react with and inactivate some components of the protein synthesizing complex (Dhindsa, 1982). Mechanical methods could also release or activate proteases which under *in vivo* conditions are separated from their substrates in different compartments of the chloroplasts. Under normal conditions they can become effective only during physiological changes or during chloroplast development (Miller and Muffaker, 1981; Watanabe and Kondo, 1984; Malek *et al.*, 1984; Liu and Jagendorf, 1984). Preincubation of thylakoid fraction and soluble fraction (S-100) showed the lowest protein synthesis when both of them were preincubated together (Fig. 10). A proteolytic interaction between both of the components could explain this effect. Addition of proteolytic enzyme inhibitors resulted in an effect contrary to that expected, especially in the case of iodoacetamide, which significantly inhibited translation (data not presented). This might be a secondary effect of the inhibitor on translation not mediated by its effect on sulfhydryl proteases. Alternately, proteolysis may be a normal requirement in the protein synthesis reactions of chloroplasts.

Low rates of *in vitro* translation compared to *in organello* may have been partialy due to the degradation of endogenous message by RNase. Activity of this enzyme has been observed *in vitro* in wheat germ

and reticulocyte systems (Scheele and Blackburn, 1979) and was thought to be present as a contaminant among the translation components. RNase would be expected to affect the translation of large mRNAs more, because they present larger targets. Some mammalian tissues contain a neutral RNase inhibitor (eg. human placental RNase inhibitor). Slight improvement with respect to the thylakoid bound polysome system were achieved by the use of this inhibitor (Fig. 11). Blackburn (1977) and Scheele and Blackburn (1979) showed that polysomes isolated in the presence of this inhibitor were intact and longer proteins were translated in a wheat germ extract. The human placental RNase inhibitor was unable to prolong the time course of translation in our system. This could be an indication that there are other factors limiting translation than degradation of RNA or synthesized proteins.

Another suggestion explaining the instability of the in vitro system came from pulse chase assays using intact chloroplasts (Mullet et al., 1985). It was shown by these authors that many of the polypeptides observed during in vitro studies are incomplete translation products. This appears to be as a result of ribosome pausing at discrete points along chloroplast mRNAs due to a deficit of a single amino acid. In our studies the system was saturated with radioactive leucine and the addition of an extra label had no effect on the total incorporation (CPM per mg chlorophyll, Fig. 8). Addition of non-radioactive leucine, however, and recalculation into nmoles of leucine per mg chlorophyll always resulted in an apparent increase in the incorporation without reaching a saturation point (Fig. 9). These results indicate that the level of translation errors can be increased by using abnormal

concentrations of particular amino acids and formation of noncognate amino acyl-tRNAs in in vitro studies (Thomson and Dix, 1982). Another dimension was added to this problem by the studies of Spirin and co-workers (1973). They demonstrated that mRNA-programmed ribosomes can carry out polypeptide synthesis without the aid of elongation factors and GTP. This means that there might be more than one kinetic pathway over which an amino acyl tRNA species can elongate a polypeptide chain on the ribsome. In the in vitro experiments one pathway might be favoured over another. Also, Thomson et al. (1981) found that a GTP-dependent proof-reading pathway is required for the accurate selection of amino acyl-tRNA species on the ribosome. It is possible that under abnormal conditions (a high concentration of one of the amino acids) incorporation of these amino acids may overcome the editing system during the elongation of the polypeptide chain.

The first step in the further purification of the thylakoid translation system was exclusion of high speed pellet P-100 (free polysomes) from the medium since they had a slightly inhibitory or no effect at all on the level of translation. The possibility of increased competition for ribosomes and soluble translation factors by the addition of an extra message to an already saturated system could not be excluded. Further improvements of the system came from eliminating (10,000 m.wt. cut off) small molecules such as: endogenous amino acids, dinucleotides and other small molecules from high speed supernatant. The absence of amino acids, especially leucine, increased the apparent recalculated incorporation of [^{3}H]-leucine (Fig. 5). Filtration of the stroma caused an increased sensitivity of the system to exogenous amino

acids, (Table 1). As reported by Galili et al. (1986), dinucleotides inhibited protein synthesis by having an effect on the initiation of polypeptide synthesis. Dinucleotides complementary to the initiation codon were able to compete with the initiation tRNA during the initiation of protein synthesis. These may have been eliminated by ultrafiltration.

Washing the thylakoid membranes and ultrafiltration did not purify the system completely from low molecular weight factors. While the addition of GTP, ATP and ATP regenerating system or inclusion of stroma components should have been a requirement for translation, it was not possible to demonstrate total lack of translation in their absence (Fig. 6). It could be assumed that small amounts of the above factors, amino acids and soluble translation enzymes might have been trapped within the membranes. By decreasing the amount of thylakoid membranes and using low speed supernatant (S-30) we were able to obtain more purified translation system. In this case, it was not possible to show translation activity in the absence of one of the fractions (Fig. 13).

Testing the availability of individual components of translation mixtures such as amino acids (Table 2), radioactive label (Fig. 7), tRNAs and ATP (data not presented) showed that they were at saturating concentrations. Furthermore, increasing concentration of thylakoid membranes (data not presented) or polyribosomal fraction (100,000xg pellet, Fig. 12) did not have any beneficial effect on total incorporation or duration of incorporation. The optimal concentration of active polyribosomes in these fractions was important only when extra

message (poly U) was added. At high concentration of polyribosomes (up to 400 ug protein), poly U was translated very poorly, but at low concentration of polyribosomes (50 ug protein) the addition of 30 ug poly U increased the total phenylalanine incorporation significantly (Fig. 12). At high concentration of endogenous mRNA, competition for ribosomes and translation factors may be possible. Some of the limiting factors might be in the soluble fraction (S-100) since it had not saturated at the maximal possible concentration of addition (300 ug protein, Fig. 13). Furthermore, since poly U directed translation is known not to reguire initiation factors (Gold et al. 1971), this measured increased incorporation may not reflect an improvement in initiation efficiency of the system.

Synthetic mRNA (poly U) was very poorly translated on thylakoid-bound polysomes probably because this system was already saturated with endogenous mRNA and the extra message would have to compete for ribosomes and translation factors. Chloroplast lysates and especially the S-30 system was found to be more useful in these studies since it presumably had more free ribosomes and fewer endogenous polyribosomes (Fig. 4). Poly U was translated more effectively in the S-30 system (Fig. 15) than in the chloroplast lysate system (Fig. 4). Using the time course experiments we could detect a rapid burst of elongation with linear kinetics up to 15 to 30 minutes (Fig. 15). Free chloroplastic ribosomes may have the ability to bind to a new message and proceed with the elongation step. The poly U product was identified as a high molecular weight polyphenylalanine, using electrophoresis and

autoradiography.

Significant inhibition of translation was observed using chloroplast lysate (Fig. 4) or the S-30 system (Fig. 16) when ribonucleic acid from phage MS2 was used to drive protein synthesis. The inhibitory effect of adding an extra natural message might be explained by the saturation of the system with endogenous mRNA. It is possible that phage mRNA has complementary sequences to chloroplastic mRNA which can selectively prevent its translation. Similar results were observed by Melton (1985) in Escherichia coli. The initiation factor requirements for binding of natural mRNAs are more complex than those for binding of the single codon poly U. The lack of reinitiation on natural mRNA could then be due to a limitation in some of the initiation factors or ribosomal proteins which may be lost or degraded during chloropolast preparation or during incubation. It was found that the requirements for some initiation factors as well as ribosomal proteins were abolished when denatured "straightened" mRNA was used as a template (Berissi et at., 1971; Vermeer et al., 1973; Van Dieijen et al., 1976; Sonenberg et al., 1981). Also ribosomes might be slowed by hairpin loops found in the naturally occuring mRNA. In vitro conditions could increase formation of such loops, thus decreasing translation of particular mRNAs. Effectivenes of denaturation of MS2 mRNA may be tested in the chloroplasts in the future.

Using the four different systems, intact chloroplasts, chloroplast lysates, membrane bound polyribosomes and S-30, we were able to obtain active homologous systems. The reinitiation of translation

was shown only after the addition of a synthetic message, poly U. The endogenous mRNA may have competed with added RNA for ribosomes and factors required for translation. In studies on exogenous mRNA translation it is advantageous to reduce the concentration of endogenous mRNA by a treatment with micrococcal nuclease (Scheele and Blackburn, 1979). This approach was not successful, as any incubation inactivated this system. Another possible approach may be the isolation of free ribosomes and testing their ability to translate certain messenger RNAs, if possible chloroplastic mRNAs. In the future special consideration should be given to all enzymes involved in translation especially the initiation and amino-acylation reactions. These two reactions are most likely the weak points of this system. Purification of the protein synthesis initiation factors should became one of the primary goals. The availability of the amino acids and of cognate amino acyl tRNAs to the system should be tested further since abnormal concentrations of these were found to increase translation errors (Thomson and Dix, 1982). The duration of active incorporation should be at least 60 minutes. Longer reaction periods often result in decreased net incorporation of radioactive amino acids into the proteins presumably due to the degradation of the newly synthesized polypeptide chains, i.e the proteolytic enzymes need to be characterised and controlled. Using endogenous protease inhibitors should be more succesful in decreasing degradation of the translation products (Ryan, 1973; Baumgartner and Chrispels, 1976). The optimal temperature of reaction should also be chosen. At higher temperatures protein synthesis proceeds faster but terminates sooner which also decreases the overall rate of incorporation (Ellis, 1981). RNase activity should be inhibited rutinely by using

placental RNase inhibitor which was shown to be effective in the thylakoid translation system. With these considerations the in vitro homologous chloroplastic systems could become a useful tool for analysis of chloroplast gene expression and regulation.

5 REFERENCES

Alscher R, R Patterson, AT Jagendorf, 1978. Activity of thylakoid-bound ribosomes of pea chloroplasts. Plant Physiol 62: 88-93.

Apel V, K Kloppstech, 1978. Light-induced appearance of mRNA coding for the apoprotein of the light-harvesting chlorophyll of a/b protein. Eur J Biochem 85: 581-588.

Apel V, 1979. Phytochrome-induced appearance of mRNA activity for the apoprotein of the light-harvesting chlorophyll a/b protein of barley (Hordeum vulgare L.) Eur J Biochem 97: 183-185.

Arnon D, 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris. Plant Physiol 24: 1-15.

Baumgartner B, M J Chrispels, 1976. Partial characterization of a protease inhibitor which inhibits the major endopeptidase present in the cotyledons of mung beans.Plant Physiol 58:1-6.

Bennet J 1977. Phosphorylation of chloroplast membrane polypeptides. Nature 269:344-346.

Bennet J 1979. Chloroplast phosphoproteins. Phosphorylation of

polypeptide of the light-harvesting chlorophyll protein complex. Eur J Biochem 99:133-137.

Berissi H, Y Groner, M Revel 1977. Effect of a purified initiation factor F3 on the selection of a ribosomal binding sites on phage MS2 RNA. Nature 243:44-47.

Bhaya D, A T Jagendorf 1984. Optimal conditions for translation by thylakoid-bound polysomes from chloroplasts. Plant Physiol 75:832-838.

Bhaya D, A T Jagendorf 1984. Synthesis of the alfa and beta subunits of coupling factor 1 by polysomes from pea chloroplasts. Arch Biochem Biophys 237:217-223.

Bitsch A, K Kloppstech 1986. Transport of proteins into chloroplasts. Reconstitution of the binding capacity for nuclear-coded precursor proteins after solubilization of envelopes with detergents. Eur J Cell Biol 40:160-166.

Blackburn P, G Wilson, S Moore 1977. Ribonuclease inhibitor from human placenta. J Biol Chem 252:5904-5910.

Blair G E, R J Ellis 1973. Protein synthesis in chloroplasts. Light-driven synthesis of the large subunits of fraction 1 protein by isolated pea chloroplasts. Bioch Biophys Acta 319:223-234.

Blobel G, B Dobberstein 1975. Transfer of proteins accross membranes. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. J Cell Biol 67:835-851.

Bolli R, L Mendiola-Morgenthaler, A Boschetti 1981. Isolation and characterisation of polysomes from thylakoid membranes of Chlamydomonas reinhardtii. Biochim Biophys Acta 653:276-287.

Bloom M V, P Millos, H Roy 1983. Light dependent assembly of ribulose-1,5-bisphosphate carboxylase. Proc Natl Acad Sci, U.S.A. 80:1013-1017.

Bottomley W, D Spencer, P R Whitfeld 1974. Protein synthesis in isolated spinach chloroplasts: comparison of light-driven and ATP-driven synthesis. Arch Biochem Biophys 164:106-117.

Bottomley W, P R Whitfeld 1979. Cell-free transcription and translation of total spinach chloroplast DNA. Eur J Biochem 93:31-39.

Bourque D P, J Bard, M Hildebrand, D Zaitlin 1985. In vitro expression of chloroplast genes in lysates of higher plant chloroplasts. Proc Natl Acad Sci, U.S.A. 82:3983-3987.

Bouthyette P Y, A T Jagendorf 1982. Calcium inhibition of amino acid incorporation by pea chloroplasts and the question of loss

activity with age. Akoyunoglou G (ed) Proceedings of the Fifth International Congress on Photosynthesis Vol. V Balaban International Science Service, Philadelphia 599-609.

Bradford M M 1976. A rapid and sensitive method for the quantities of protein-dye binding. Analyt Biochem 72:248-254.

Buetow D E 1982. Molecular biology of chloroplasts. Govindjee (ed) Photosynthesis, Vol. II Academic Press, New York 43-88.

Buetow D E 1986. Chloroplast development. Akoyunoglou G, H Senger (eds) Plant Biol, Vol. 2 Liss A R Inc. New York 427-432.

Capel M S, D P Bourque 1982. Characterisation of Nicotiana tabacum chloroplast and cytoplasmic ribosomal proteins. J Biol Chem 257:7746-7755.

Cerf R, K Kloppstech 1982. Structural diversity and differential light control of mRNAs coding for angiosperm gluteraldehyde-3-phosphate dehydrogenases. Proc Natl Acad Sci, U.S.A. 79:7624-7628.

Chua N-H, N W Gillham 1977. The sites of synthesis of the principal thylakoid membrane polypeptides in Chlamydomonas reinhardtii. J Cell Biol 74:441-452.

Chua N-H, G Schmidt 1978. In vitro synthesis, transport and assembly of ribulose bisphosphate carboxylase subunits. Photosynthetic

Carbon Assimilation, New York/London: Plenum 325-347.

Cline K, M Werner-Washburne, T H Lubben, K Keegstra 1985. Precursors to two nuclear-encoded chloroplast proteins bind to the outer envelope membrane before being imported into chloroplasts. J Biol Chem 260:3691-3696.

Coen D M, J R Bedbrook, L Bogorad, A Rich 1977. Maize chloroplast DNA fragment encoding the large subunit of ribulosebisphosphate carboxylase. Proc Natl Acad Sci, U.S.A. 74:5487-5491.

Damuni Z, F B Caudwell, P Cohen 1982. Regulation of the aminoacyl-tRNA synthetase complex of rat liver by phosphorylation/dephosphorylation *in vitro* and *in vivo*. J Biol Chem 257: 7730-7735.

Daniel H, C A Rebeiz 1982 a. Chloroplast culture. A new effect of kinetin in enchancing the synthesis and accumulation of protochlorophyllide *in vitro*. Biochem Biophys Res Commun 104:837-843.

Daniel H, C A Rebeiz 1982 b. Chloroplast culture. Chlorophyllide *a* biosynthesis *in vitro* at rates higher than *in vivo*. Biochem Biophys Res Comm 106:466-470.

Daniel H, K Krishnan, U Uma Bai, A Gnanan 1986. An efficient and prolonged *in vitro* translational system from isolated cucumber etioplasts. Biochem Biophys Res Comm 135:248-255.

Dhindsa R S 1982. Inhibition of protein synthesis by products of lipid peroxidation. Phytochemistry 21:309-313.

Dobberstein B, G Blobel, N-H Chua 1977. In vitro synthesis and processing of a putative precursor for the small subunit of ribulose-1,5-bisphosphate carboxylase of Chlamydomonas reinhardtii. Proc Natl Acad Sci, U.S.A. 74:468-473.

Edelman P, J Gallant 1978. Mistranslation in E. coli. Cell 10:131-137.

Ellis R J 1969. Chloroplast ribosomes: stereospecificity of inhibition by chloramphenicol. Science 163:477-478.

Ellis R J 1976. The search for plant messenger RNA. Perspectives in Experimental Biology. Sunderland N (ed) 2:238-298.

Ellis R J 1977. Protein synthesis by isolated chloroplasts. Biochem Biophys Acta 463:185-215.

Ellis J R 1981. Chloroplast proteins: synthesis, transport and assembly. Ann Rev Plant Physiol 32:111-137.

Ellis R J, M R Hartley 1982 Preparation of higher plant chloroplasts active in protein and RNA synthesis Edelman M et al. (eds) Methods in Chloroplasts Molecular Biology. Elsevier Biomedical

Press 560-568.

Feierabend J, G Wildner 1979. Formation of the small subunit in the absence of the large subunit of ribulose-1,5-bisphosphate carboxylase in 70 S ribosome-deficient rye leaves. Arch Biochem Biophys 186:283-291.

Fish L E, A T Jagendorf 1982. High rates of protein synthesis by isolated chloroplasts. Plant Physiol 70:1107-1114.

Galili G, E Kawata, R Cuellar, L D Smith, B Larkins 1986 Synthetic oligonucleotides inhibit in vitro and in vivo translation of SP6 transcripts of maize zein cDNA clones. Nucl Acid Res 14:1511-1523

Galling G, A Michaels 1986 Chloroplast development in algae and higher plants: a general survey. Akoyunoglou G, H Senger (eds) Plant Biology Vol. 2, Liss A R, Inc. New York 433-441

Geetha V, A Gnanam 1980 An in vitro protein-synthesizing system with isolated chloroplasts of Sorghum vulgare. J Biol Chem 255:492-497.

Gillham N W, J E Boyton, N-H Chua 1978 Genetic control of chloroplast proteins. Curr Advan Bioen 9:211-260.

Gold L, D Pribnow, T Schneider, S Shinedling, B Singer, G Stormo 1971 Translational initiation in procaryotes. Annu Rev Microb

35:365-403.

Gold J C, L Spremuli 1985 Euglena gracilis Chloroplast Initiation Factor 2. J Biol Chem 260:14897-14900.

Goldberg A L 1972. Degradation of abnormal proteins in Escherichia coli. Proc Natl Acad Sci, U.S.A. 69:422-426.

Gray R E, A R Cashmore 1976 RNA synthesis in plant leaf tissue: the characterisation of messenger RNA species lacking and containing polyadenylic acid. J Molec Biol 108:595-608.

Grebanier A E, D M Cohen, A Rich, L Bogorad 1978. Membrane proteins synthesised but not processed by isolated maize chloroplasts. J Cell Biol 78:734-746.

Grinsven M Q J M, J J L Gielen, J L A Zethof, H J J Nijkamp, A J Kool 1986. Transcriptional and post-transcriptional regulation of chloroplast gene expression in Petunia hybrida. Theoret Appl Genet 73:94-101.

Grossman A, S Bartlett, N-H Chua 1980. Energy-dependent uptake of cytoplasmically synthesized polypeptides by chloroplasts. Nature 285:625-628.

Hattori T, M M Margulies 1985. Synthesis of large subunit of ribulosebisphosphate carboxylase by thylakoid-bound polyribosomes

from spinach chloroplasts. Arch Biochem Biophys 244:630-640.

Herrin D, A Michaels 1985. In vitro synthesis and assembly of the periferal subunits of coupling factor CF_1 (alpha and beta) by thylakoid-bound ribosomes. Arch Biochem Biophys 237:224-236.

Heldt H W 1979. Light dependent changes of stromal H^+ and Mg^{2+} concentrations controling CO_2 fixation. Photosynthetic Carbon Metabolism and Related Processes. Gibs M, E Latzko (eds), Springer-Verlag, New York 202-207.

Highfield P E, R J Ellis 1978. Synthesis and transport of the small subunit of chloroplast ribulose bisphosphate carboxylase. Nature 271:420-424.

Howell S H, W J Blascho, C M Drew 1975. Inhibitor effects during cell cycle in Chlamydomonas reinhardtii. J Cell Biol 67:126-135.

Howell S H, P Heizmann, S Gelvin, L L Walker 1977. Identification of the messenger RNA activity in Chlamydomonas reinhardtii coding for the large subunit of ribulose-1,5-bisphosphate carboxylase. Plant Physiol 59:464-470.

Huber S C, W Maury 1980. Effects of magnesium on intact chloroplasts. Evidence for activation of (sodium) potassium/proton exchange across the chloroplast envelope. Plant Physiol 65:350-354.

Hurewitz J, A T Jagendorf 1987. Further characterization of ribosome binding to thylakoid membranes. Plant Physiol 84:31-34.

Joy K W, R G Ellis 1975. Protein synthesis in chloroplasts. Polypeptides of the chloroplast envelope. Bioch Biophys Acta 378:143-151.

Jolly S O, L Bogorad 1980. Preferential transcription of cloned maize chloroplast DNA sequences by maize chloroplast RNA polymerase. Proc Natl Acad Sci, U.S.A. 77:822-826.

Kaizer W M, W Urbach, H Grimmler 1980. The role of monovalent cations for photosynthesis of isolated intact chloroplasts. Planta. 143:170-175.

Kirk J T O, R Tilney-Bassett 1978. The plastids: Their Chemistry, Structure, Growth and Inheritance. Amsterdam/New York/Oxford: Elsevier/North Holland 235-240.

Kozak M, D Nathans 1983. Translation of the genome of a ribonucleic acid bacteriofage. Rev Bacteriol 36:110-119.

Kraus B L, L L Spremulli 1986. Chloroplast Initiation Factor 3 from Euglena gracilis. J Biol Chem 261:4781-4784.

Leu S, L Mendiola-Morgenthaler, A Boschetti 1984. In organello protein synthesis by Chlamydomonas reinhardtii chloroplasts.

Adv Photosyn Res 4:541-544.

Liu X, A T Jagendorf 1984. Roles for ATP-dependent and ATP-independent proteases of pea chloroplasts in regulation of the plasmid translation products. Physiol Veg 23:749-755.

Luccini G, R Bianchetti 1980. Initiation of protein synthesis in isolated mitochondria and chloroplasts. Bioch Biophys Acta 608:54-61.

Malek L, L Bogorad, A R Ayers, A Goldberg 1984. Newly synthesized proteins are degraded by an ATP-stimulated proteolytic process in isolated pea chloroplasts. FEBS Lett 166:253-257.

Mans R J, D J Novelli 1961. Measurement of the incorporation of radioactive amino acids into proteins by a filter-paper disk method. Arch Biochem Biophys 98:48-53.

Melton D A 1985. Injected anti-sense RNAs specifically block messenger RNA translation in vivo. Proc Natl Acad Sci, U.S.A. 82:144-148.

Mendiola-Morgenthaler L R, J J Morgenthaler, C A Price 1976. Synthesis of coupling factor CF_1 protein by isolated spinach chloroplasts. FEBS Lett 62:96-100.

Meyer J, Y Chartier 1983. Long-lived and short-lived heat shock proteins in tabacco mesophyll protoplasts. Plant Physiol 62:26-32.

Michaels A, M M Margulies 1975. Amino acid incorporation into protein by ribosomes bound to chloroplast thylakoid membranes: formation of discrete products. Biochem Biophys Acta 390:352-362.

Miflin B J, H Beevers 1974. Isolation of intact plastids from a range of plant tissues. Plant Physiol 53:870-874.

Miller B L, R C Huffaker 1981. Partial purification and characterization of endoproteinases from senescing barley leaves. Plant Physiol 49:64-71.

Mills W B, K W Joy 1980. A rapid method for isolation of purified physiologically active chloroplasts and its use to study the intracellular distribution of amino acids in pea leaves. Planta 148:75-83.

Minami E I, A Watanabe 1984. Thylakoid membranes: the translational site of chloroplast DNA-regulated thylakoid polypeptides. Arch Biochem Biophys 235:562-570.

Mishkind M L, S R Wessler, G W Schmidt 1985. Functional determinants in transit sequences: import and partial maturation by vascular plant chloroplasts of the ribulose-1,5-bisphosphate carboxylase small subunit of Chlamydomonas. J Cell Biol 100:226-234.

Mullet J E, E M Orozko, N-H Chua 1985. Multiple transcripts for higher

plant rbc L and atp B genes and the localization of the transcription initiation site of the rcb L gene. Plant Mol Biol 4:39-54.

Mullet J E, R R Klein, A R Grossman 1986. Optimization of protein synthesis in isolated higher plant chloroplasts. Identification of paused translation intermediates. Eur J Biochem 155:331-338.

Nirenberg M W, P Leder 1963. The effect of trinucleotides upon the binding of sRNA to ribosomes. Science 145:1399-1407.

Nivison H, A T Jagendorf 1984 Factors permitting prolonged translation of isolated pea chloroplasts. Plant Physiol 75:1001-1008.

O'Brien T P, M E McCully 1981. The study of plant principles and selected methods. Termacarphi PTY LTD, Melbourne Australia.

Parthier B 1979. The role of phytohormones (cytokinins) in chloroplast development. Biochem Physiol Pflanzen 174:173-214.

Ramirez J M, F F Campo, D J Arnon 1968. photosynthetic phosphorylation as energy source for protein synthesis and carbon dioxide assimilation by chloroplasts. Proc Natl Acad Sci, U.S.A. 59:606-612.

Ray P M, R Eisinger, D G Robinson 1976. Organelles involved in cell wall polysaccharide formation and transport in pea cells. Biochem

Physiol Pflanzen 89:126-146.

Rebeiz C A, A Montazer-Zouhour, H Daniel 1984. Structure function and regulation of the photosynthetic apparatus. Ohad I, S Klein (eds) Chloroplast Development. The Weizman Science Press of Israel, Israel 225-235.

Reisfeld A, M Edelman 1982. Translation of chloroplast poly A^- mRNA in the wheat germ system. Edelman M et al. (eds.) Methods in Chloroplast Molecular Biology 605-614.

Reynolds E S 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J Cell Biol 17:208-211.

Robinson C, R J Ellis 1984. Transport of proteins into chloroplasts: the precursor of small subunit of ribulose bisphosphate carboxylase is processed to the mature size in two steps. Eur J Biochem 142:343-346.

Ruusala T, M Ehrenberg, C G Kurland 1982. Is there proofreading during polypeptide synthesis? EMBO J 1:746-745.

Ryan C A 1973. Proteolytic enzymes and their inhibitors in plants. Annu Rev Plant Physiol 24:173-196.

Sagher D, H Grosfeld, M Edelman 1976. Large subunit of ribulosebisphosphate carboxylase messenger RNA from Euglena

chloroplasts. Proc Natl Acad Sci, U.S.A. 73:722-725.

Sasaki Y, Y Tomoda, H Tomi, T Kamikubo, K Schinozaki 1985. Synthesis of ribulosebisphosphate carboxylase in greening pea leaves. Coordination of mRNA level of two subunits. Eur J Biochem 152:179-186.

Scheele G, P Blackburn 1979. Role of mammalian RNase inhibitor in cell-free protein synthesis. Proc Natl Acad Sci, U.S.A. 76:4898-4902.

Schmidt R, N Gilham, J Boynton 1985. Processing of the precursor to a chloroplast ribosomal protein made in the cytosol occurs in two steps, one of which depends on a protein made in the chloroplast. Molec Cell Biol 5:1093-1099.

Schwartz J H, R Meyer, J M Eisenstadt, G Braverman 1967. Involvement of N-formylmethionine in initiation of protein synthesis in cell-free extracts of Euglena gracilis. J Molec Biol 25:571-574.

Siddel S G, R J Ellis 1975. Protein synthesis in chloroplasts. Characteristics and products of protein synthesis in vitro in etioplasts and developing chloroplasts from pea leaves. J Biochem 146:675-685.

Smille R M, N S Scott 1969. Organelle biogenesis: The chloroplast. Hann F E (ed) Progress in Molecular and Subcellular Biology.

Springer-Verlag Berlin 136-140.

Sonenberg N, D Guerlin, D Cleveland, H Trachsel 1981. Probing the function of the eucaryotic 5′ cap structure by using a monoclonal antibody directed against cap-binding proteins. Cell 27:563-572.

Spirin A S 1973. Enzymatic and nonenzymatic translation. Kenney F T, T Hamkalo (eds) Gene Expression and Its Regulation. Plenum Press, New York 311-325.

Sreedharan S P, C M Beck, L L Spremuli 1985. Euglena gracilis chloroplast elongation factor Tu. J Biol Chem 260:3126-3131.

Strzalka K, M Kwiatkowska 1979. Transport of proteins from cytoplasm into plastids in chloramphenicol-treated bean leaf discs. Autoradiographic evidence. Planta 146:393-398.

Strzalka K, M Kwiatkowska 1985. Transport bialek z cytoplazmy do plastydow w komorkach traktowanych chloramfenikolem. Zeszyt Probl Post Nauk Roln 271:253-255.

Surzycki S J, U W Goodenough, P R Levine, J J Armstrong 1970. Nuclear and chloroplast control of chloroplast stucture in Chlamydomonas reinhardtii. Miller P J (ed) Control of Organelle Development. Cambridge University Press 13-17.

Thomson R C, D Dix, B Gerson, A Karim 1981. Effect of Mg^{2+}

concentration, polyamines, streptomycin and mutations in ribosomal proteins on the accuracy of the two-step selection of amino acyl-tRNAs in protein biosynthesis. J Biol Chem 256:6676-6681.

Thomson R C, D Dix 1982 Accuracy of protein biosynthesis. J Biol Chem 257:6677-6682.

Trempe M R, D G Glitz 1981. Chloroplast ribosome structure. Electron microscopy of ribosomal subunits and localization of N^6,N^6-dimethyladenosine by immunoelectronmicroscopy. J Biol Chem 256:11873-11879.

Van Dieijen G, P Van Knippenberg, T Van Duin 1976. The specific role of ribosomal protein S'1 in the recognition of native phage RNA. Eur J Biochem 64:511-518.

Vermeer C, W Van Alphen, P Van Knippenberg, L Bosh 1973. Initiation factor-dependent binding of MS2 RNA to 30S ribosomes and recycling of IF-3. Eur J Biochem 40:295-308.

Watanabe T, N Kondo 1983. The change in leaf protease and protease inhibitor activities after supplying various chemicals. Biol Plant 25:100-109.

Weil J H, M Mubumbila, M Kuntz, M Keller, E J Crouse, G Burkard, P Guillemaut, R Selden, L McIntosh, L Bogorad 1983. Comparative studies on tRNA and aminoacyl-tRNA synthetases from various

photosynthetic organisms. Cifferi O, L Dure (eds) Life Sciences. Plenum Press, New York 63:167-180

Wettstein D 1981. Chloroplast and nucleus: interplay between genomes of different cell organelles. Schweiger H (ed) International Cell Biology. Springer-Verlag, Berlin, Heidelberg, New York 250-272.

Whatson M L 1958. Staining of tissue sections for electron microscopy with heavy metals. Biophys Biomed Cyt J 4:475-478.

Whitfield P R, W Bottomley 1983. Organisation and structure of chloroplast genes. Annu Rev Plant Physiol 34:279-310

Woodcock C L F, L Bogorad 1971. Nucleic acid and information processing in the chloroplast. Gibbs M (ed) Structure and Function of Chloroplasts. Springer--Verlag, Berlin 89-101.

Zubay G, D A Chambers, L S Cheong 1970. The Lac Operon. Beckwith J, S Siper (eds) Cold Spring Harbor Laboratories New York 375-391.