SYNTHESIS OF CHLOROPLAST PROTEINS DURING GERMINATION AND EARLY DEVELOPMENT OF CUCUMBER (*CUCUMIS SATIVUS*)

BY

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I declare that this thesis has been composed by myself and that the experiments and results described therein are a product of my own work.

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Each night I still ask the Lord "Why?" and I haven't heard a decent answer yet.

Jack Kerouac (1922-1969)

Desolation Angels.

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Abbreviations

A	Adenosine base
A_{260}	Absorbance at 260 nm wavelength
ATP	Adenosine-5'-triphosphate
ATPase	Adenosine-5'-triphosphatase
AMPS	Ammonium Persulphate
bis-acrylamide	NN'-methylene-bisacrylamide
BMV	Brome Mosaic Virus
BSA	Bovine Serum Albumin
butyl-PBD	2-(4-tert-butyl phenol)-5-(4 biphenyl) -1,3,4 oxadiazone
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
cDNA	Complementary DNA
Ci	Curie (s)
cpm	Counts per minute
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra-acetic acid
g	Unit of gravitational force (average) or grams
GTP	Guanosine-5'-triphosphate
h	Hours
HEPES	N-2-hydroxyethylpiperazine N'-2-ethane sulphonic acid
log	Logarithm
LSu	Large subunit of Ribulose 1,5,Bisphosphate carboxylase
M	molar
mA	millampere (s)
mM	millimolar

M_r	relative molecular weight
mRNA	messenger RNA
nm	nanometer (s)
OD	optical density
PAS	4 amino-2-hydroxybenzoic acid (Sodium Salt).
PEP	Phospho-enol-pyruvate
pH	log hydrogen ion concentration
PK	Pyruvate Kinase
PMSF	Phenyl methyl sulphonyl fluoride
Poly $(A)^+$ RNA	RNA containing poly(A)
Poly $(A)^-$ RNA	RNA lacking poly (A)
PPO	2,5 diphenyl oxazole
PSSu	Precursor to the small subunit of Ribulose 1,5 Bisphosphate Carboxylase
RNA	Ribonucleic acid
RNAase	Ribonuclease
RuBPCase	D-Ribulose 1,5 Bisphosphate Carboxylase
S-30	Supernatant of a 30,000 x g centrifugation
SDS	Sodium Dodecyl Sulphate
SSu	Small subunit of Ribulose 1,5 Bisphosphate Carboxylase
T	Thymidine base
TCA	Trichloroacetic acid
TEMED	NNN'-N'-tetramethyl ethylene diamine
TMV	Tobacco Mosaic Virus
TNS	Tri-iso-propylnapthalene sulphonic acid (Sodium salt)
Tricine	N-tris (hydroxymethyl) methyl glycine
Tris	Tris (hydroxymethyl) amino methane
Triton-X-100	Octyl phenoxy polyethoxy ethanol

μCi	microcurie (s)
μg	microgram (s)
μl	microliter (s)
μm	micrometer (s)
v/v	volume per volume (as percentage)
w/v	weight per volume (as percentage)

Abstract

Cell-free protein-synthesizing systems have been prepared and characterised in order to study three developmental aspects of the synthesis of chloroplast proteins in the cotyledons of Cucumber seedlings grown in the light and dark.

i) The E. coli translation system was used to assay the accumulation of chloroplast mRNA. A major translation product of this system has been identified as the large subunit (LSu) of RuBPCase.

ii) The Wheat germ translation system was used to assay the accumulation of cytoplasmic mRNAs coding for chloroplast proteins. The precursor (PSSu) of the small subunit (SSu) of RuBPCase has been identified among the translation products of this system by immunoprecipitation. Tentative identification of the precursor of the chlorophyll a/b binding protein has also been made.

iii) Isolated plastids from light-grown cotyledons were used to study the capacity of the developing organelles to synthesize protein and also follow the spectra of polypeptides produced.

The results obtained with the in vitro techniques have been compared with the developmental changes in chloroplast proteins observed in vivo.

Results indicate that the initiation of synthesis of the mRNA coding for LSu and PSSu RuBPCase is light independent. The initial increase in the levels of LSu and SSu observed in vivo is positively related to the accumulation of LSu and PSSu mRNA. Although in vitro assays indicate that there are subtle qualitative

differences in the spectra of cytoplasmic mRNAs between light- and dark-grown tissue, etioplast and chloroplast mRNAs appear qualitatively similar. The light dependent initiation of synthesis of the presumptive mRNA for the chlorophyll a/b binding protein occurs a day after the initiation of synthesis of the RuBPCase mRNAs. This corresponds with the in vivo appearance of chlorcphyll and chlorophyll a/b binding protein.

The data obtained with the cell-free protein-synthesizing systems would indicate that in the light approximately 48 hours after the emergence of the cotyledons above the soil there is a decline not only in the levels of mRNAs coding for chloroplast proteins but also in the capacity of the isolated plastids to synthesize proteins. This decline is not reflected in the rate of accumulation of native RuBPCase which continues to be maintained at the same rate during this period.

Chapter 1. Introduction

(I) The Chloroplast as a Semi-Autonomous Organelle

The chloroplast, which in higher plants has the unique function of carrying out photosynthesis, is a semi-autonomous organelle. This being said, the chloroplast, like the mitochondrion (reviewed in Schatz and Mason, 1974; Borst and Grivell, 1978) contains all the components theoretically required for complete autonomy. This has been discussed extensively in books (Kirk and Tilney-Bassett, 1967; Miller, 1970; Sager, 1972; Grun, 1976) and reviews (Levine and Goodenough, 1970; Tewari, 1971; Walles, 1971; Woodcock and Bogorad, 1971; Gillham, 1974; Ellis, 1976; Adams et al., 1976; Schmidt and Lyman, 1976; Ellis, 1977; Kung, 1977; Gillham et al., 1978) hence only a brief description of the genetic machinery of the chloroplast will be presented here.

Although a wealth of genetic evidence suggested that in Chlamydomonas reinhardi many genes that control chloroplast functions were located in the nucleus (Surzycki et al., 1970), the demonstration that chloroplasts contain DNA (Ris and Plaut, 1962) and their own complement of ribosomes (Lyttleton, 1962) stimulated interest in the possible genetic autonomy of chloroplasts.

The replication of chloroplast DNA was first demonstrated in Chlamydomonas reinhardi (Chiang and Sueoka, 1967) and what are thought to be replicative intermediates of chloroplast DNA in higher plants have been studied by electron microscopy (reviewed in Bedbrook and Kolodner, 1979). Complementing these studies came the demonstration of DNA polymerase activity in the

chloroplasts isolated from Spinach (Spencer and Whitfeld, 1967) and Tobacco (Tewari and Wildman, 1967), and the partial purification of a chloroplast DNA polymerase from Euglena gracilis (Keller et al., 1973). An RNA polymerase has also been partially characterised from Maize chloroplasts (Bottomley et al., 1971a, 1971b).

Chloroplasts contain their own protein synthesizing machinery. 70S ribosomes are located in the chloroplast whereas 80S ribosomes are present in the cytoplasm. The presence of chloroplast polysomes was first described by Clark (1964). The differences between the chloroplast and cytoplasmic ribosomes are reflected in their differential sensitivity to inhibitors of protein synthesis and, as will be described later, this characteristic has been exploited extensively. That chloroplast ribosomes contain RNA that is distinguishable from cytoplasmic ribosomal RNA was first demonstrated by Stutz and Noll (1967) while Loening and Ingle (1967) showed that the major RNAs of the chloroplast ribosomes were 23S and 16S. Chloroplast ribosomes also contain low molecular weight RNAs, a 5S RNA (Dyer and Leech, 1967; Payne and Dyer, 1971) and a 4.5S RNA (Whitfeld et al., 1976; Dyer et al., 1977). The 23S, 5S and 4.5S RNAs are components of the 50S chloroplast ribosomal subunit and the 16S is a component of the 30S subunit (Dyer and Bedbrook, 1979).

DNA-RNA hydridization studies demonstrate that chloroplast DNA contains sequences that are complementary to chloroplast ribosomal RNAs (Tewari and Wildman, 1968), and quantitative hybridization studies showed that in several species of plants chloroplast DNA contained two 16S and two 23S ribosomal RNA

cistrons (Thomas and Tewari, 1974). Interestingly, several reports appeared showing the chloroplast ribosomal RNA hybridized to nuclear DNA whereas cytoplasmic ribosomal RNA did not hybridize to chloroplast DNA (Tewari and Wildman, 1968; Ingle et al., 1970). This observation has not been explained satisfactorily but it raised the possibility that chloroplast ribosomal genes are located in the nucleus as well as in the chloroplast.

Chloroplast tRNAs are also found to hybridize to chloroplast DNA with a level of complementarity equivalent to a coding capacity of 20-30 tRNAs (Tewari and Wildman, 1970). The same authors found that tRNAs did not compete with ribosomal RNAs for sites of hybridization. They also calculated that the cistrons for both tRNA and ribosomal RNAs were contained in 2% of the chloroplast DNA. More recent data would suggest that this figure is closer to 7% (Dyer and Bedbrook, 1979; Burkard et al., 1979).

The tRNAs and the aminoacyl-tRNA synthetases that are contained in the chloroplast are different from those found in the cytoplasm (Burkard et al., 1970, 1979). It has been found that the chloroplast ribosomes from Euglena, like E. coli, could translate RNA from bacteriophage f2 into viral coat protein containing N-formyl methionine (Schwartz et al., 1967). Later it was shown that Bean chloroplasts contain N-formyl-methionine-tRNA (Burkard et al., 1972) and that chloroplast methionyl-tRNA is formylatable in vitro by a chloroplast transformylase (Merrick and Dure, 1971). This evidence indicates that the initiation of protein synthesis by chloroplasts is similar to that in procaryotes in that it uses a formylated methionyl-tRNA, but distinct from that in the cytoplasm.

which uses an unformylated methionyl-tRNA (Ellis, 1976).

The elongation factors of chloroplast protein synthesis in Chlorella also appear to be distinct from those found in the cytoplasm or mitochondria (Ciferri and Tiboni, 1973).

Thus these diverse studies have indicated that the chloroplast contains DNA and the ability to replicate and transcribe it as well as its own complement of ribosomes and the capacity to synthesize proteins. However, although these studies show that the chloroplast contains the apparatus to allow it genetic autonomy it is now well established that the chloroplast is not an autonomous organelle. This conclusion has been drawn from a number of experiments that are reviewed below.

(a) Studies with Genetic Mutants

Many of these studies have been carried out with Chlamydomonas and have been extensively reviewed (Surzycki et al, 1970; Sager and Ramanis, 1970; Levine and Goodenough, 1970; Gillham, 1974; Bogorad, 1975; Bogorad et al. 1977).

In Chlamydomonas many genes concerned with chloroplast structure and function have been found to be inherited in a Mendelian fashion and are thus thought to be located in the nucleus. Others have been found to be inherited in a uniparental fashion and so are thought to be located in the chloroplast. Uniparental inheritance in Chlamydomonas would be unexpected because sexual reproduction involves the fusion of 2 gametes each containing a chloroplast. Chloroplast fusion has been observed (Cavalier-Smith, 1970), suggesting that both copies of parental chloroplast DNA are present in the daughter cell. Two models

have been proposed to explain uniparental inheritance, as discussed by Adams et al. (1976). Sager and Ramanis (1974) and Burton et al. (1979) have suggested that a modification and restriction mechanism, analogous to that found in bacteria, may be active in the chloroplast thus removing the chloroplast DNA of the male gamete. This removal has also been postulated to take place as a result of a mechanism based on the principle of competitive exclusion (Gillham et al., 1974). Those mutations that are inherited in a Mendelian fashion include 7 different genes which control steps in chloroplyll synthesis, one involving the synthesis of phosphoribulose kinase and at least 5 genes concerned with the synthesis of components of the chloroplast electron transport chain (Surzyki et al., 1970).

Chlamydomonas has been used extensively for the study of ribosome biogenesis primarily because Chlamydomonas chloroplast ribosomal function is dispensible, the cells can be grown heterotrophically using acetate as a source of fixed carbon (for reviews see Harris et al., 1974; Bogorad, 1975; Harris et al., 1976; Bogorad et al., 1977 and 1978).

20 genes concerned with chloroplast ribosomal biogenesis have now been identified through the use of appropriate assembly-defective or antibiotic resistant or dependent mutants. Results have indicated that there are a number of genes present both in the chloroplast and the nucleus that are involved in chloroplast ribosomal biogenesis (Harris et al., 1976). Unfortunately, to date, only 2 specific mutations have been found to alter a particular protein of a given ribosomal subunit. One has been found to be

coded by the nuclear genome, the other by the genome of the chloroplast (Bogorad et al., 1978). Although the majority of the mutations have not been specifically identified their phenotypes are similar to those bacterial mutations that are known to alter ribosomal proteins (Gillham et al., 1978). However, it is conceivable that some nuclear and chloroplast genes identified by antibiotic resistance mutations may act directly on ribosomal RNA, its processing, or possibly, the processing of a ribosomal protein.

Similar elegant experiments have been carried out to investigate the mode of inheritance of the Large and Small subunits (LSu and SSu) of RuBPCase using interspecific hybrids of Nicotiana (reviewed in Wildman et al., 1973). In these experiments the inheritance of a characteristic LSu or SSu was analysed by tryptic peptide mapping of the protein subunits present in the plants of the F1 generation. It was found that LSu was inherited in a uniparental fashion indicating that it was coded by the chloroplast genome (Chan and Wildman, 1972), whereas SSu was inherited in a Mendelian fashion and hence coded by the nuclear genome (Kawashima and Wildman, 1972). This work has been complemented by studies involving isoelectric focussing of carboxymethylated RuBPCase and comparison of the isoelectric variants of LSu and SSu (Sakano et al., 1974). The application of this has produced similar results in Wheat (Chen et al., 1975) and indicated that ferredoxin (Kwanyuen and Wildman, 1975) and aldolase (Anderson and Levin, 1970) are both coded for by nuclear genes. The tryptic peptide mapping technique has been used to show that the apoprotein of the light harvesting complex (chlorophyll a/b

binding protein) (Kung et al., 1972), and 2 proteins of the large subunit of chloroplast ribosomes are coded by nuclear genes (Bourque and Wildman, 1973). However, in the latter case the results are not entirely unambiguous. The experiments of Wildman and co-workers have been criticised (Gillham et al., 1978) because the inheritance of interspecific differences beyond the F1 generation was not investigated due to the sterility of Nicotiana hybrids, but these experiments do have the advantage of being concerned with the differences in tryptic peptides or isoelectric variants, and not with the presence or absence of a polypeptide which may be caused by other factors (Ellis, 1976).

Indeed to date no fully convincing proof exists that any chloroplast mutation actually results from an alteration in chloroplast DNA. The best evidence has been provided by Wildman's group. These workers described a white plastome mutant of Tobacco, inherited in a uniparental fashion which resulted in variegated leaves (Wildman et al., 1973). The chloroplast DNA of the plastome mutant had differing thermal denaturation characteristics from that of the wild-type (Wong-Staal and Wildman, 1973). Analysis of heteroduplex mixtures of mutant and wild-type DNA suggested that the mutant contains a nonhomologous region consisting of 50-100 base pairs. With the recent advances in recombinant DNA techniques and physical mapping of DNA, more chloroplast mutations are amenable to characterisation (for example, see Frankel et al. (1979)).

Extensive studies have been carried out using mutants which appear to be variegated or albino due either to impaired expression (Hagemann and Börner, 1978) or total lack of chloroplast DNA

(Schiff, 1978). Interesting work has also been carried out exploiting the fact that when certain higher plants are grown at elevated temperatures (32°-34°C) they become 'heat bleached' (Feierabend and Mikus, 1977). This effect is similar to temperature sensitive mutations in bacteria and the plants lack functional chloroplast ribosomes (Feierabend and Mikus, 1977). Although these types of mutations are useful in indicating the extent of interaction between the genetic systems of the chloroplast and the nucleus, for example, see Hagemann and Börner (1978) and Feierabend (1978), the primary effect of these mutations remains unknown.

(b) Nuclear Transplantation in Acetabularia

With the giant alga Acetabularia nuclear transplantation has been carried out using various species to elucidate the possible role of the nucleus in coding for chloroplast proteins. Nucleate rhizoids of one species were transplanted onto enucleate fragments of another species and it has been shown that the species specific protein pattern changes under the influence of the nucleus. In this way it was shown that malic dehydrogenase (Schweiger et al., 1967), lactate dehydrogenase (Reuter and Schweiger, 1973), and chloroplast membrane proteins (Apel and Schweiger, 1972) were coded for in the nucleus.

(c) Antibiotic Inhibition of Protein Synthesis

It is now well established that chloroplast protein synthesis is inhibited by some antibiotics which inhibit procaryotes (Boulter et al., 1972). These antibiotics include spectinomycin, lincomycin,

erythromycin and the D-threo isomer of chloroamphenicol (Ellis, 1969, 1970). Whereas ion uptake, oxidative phosphorylation and photophosphorylation are inhibited by all four stereoisomers of chloramphenicol, inhibition of protein synthesis by isolated chloroplasts is specific to the D-threo isomer (Ellis, 1969). This allows a check on the non-specific action of chloramphenicol on other cellular processes. On the other hand 80S ribosomes are inhibited by cycloheximide (Ennis and Lubin, 1964) which does not affect the activity of chloroplast ribosomes (Ellis, 1976). Whereas the aforementioned experiments indicated which genome codes for chloroplast polypeptides, the differential sensitivity of the ribosomes of the cytoplasm and the chloroplast to inhibitors has, in the absence of specific mutants, allowed tentative identification of the polypeptides synthesized on each type of ribosome.

In Chlamydomonas, where chloroplast ribosomal function is blocked by antibiotics (e.g. spectinomycin) deficiencies are found in the activities of RuBPCase, photosystem II and electron transport, and characteristic alterations are seen in the lamellar organisation of the chloroplast (Surzycki et al., 1970). Using concentrations of inhibitors which do not inhibit other cellular functions, results indicated that chloroplast ribosomal RNA was transcribed from chloroplast DNA and that transcription could still be carried out after chloroplast protein synthesis had been inhibited for 4 division cycles. This suggested that chloroplast RNA polymerase was synthesized on cytoplasmic ribosomes (Surzycki et al., 1970). The same authors showed that in the absence of chloroplast RNA synthesis, membrane components of the chloroplast were still present and the organelle was still recognisable, albeit disorganised.

Similar studies have been carried out using higher plants (reviewed by Boulter, 1970; Tewari, 1970; Woodcock and Bogorad, 1971; Ellis, 1976). However, the validity of such experiments, as discussed by Woodcock and Bogorad (1971) and Ellis (1976), depends totally on the specificity of inhibitor action on intact cells.

Interpreting results obtained in inhibitor studies with caution, Ellis (1976) concludes that most of the proteins of the chloroplast are synthesized on the ribosomes of the cytoplasm including those soluble proteins of the carbon dioxide reduction cycle, ferredoxin and RNA polymerase. Those proteins that are synthesized in the chloroplast include RuBPCase, some of the chloroplast ribosomal proteins and lamellar proteins including photosynthetic cytochromes (Ellis, 1976).

(d) Cell-Free Translation of Chloroplast Proteins

Due to the limitations of inhibitor studies, cell-free translation systems have been used extensively to identify the site of translation of chloroplast proteins. Results from inhibitor studies are generally consistent in that they indicate that LSu RuBPCase is synthesized on the 70S ribosomes in the chloroplast whereas SSu is synthesized on the 80S ribosomes in the cytoplasm (for example, see Criddle et al., 1970). Roy et al. (1973) and Gooding et al. (1973) circumvented the limitations of the aforementioned inhibitor studies by using [^{3}H] puromycin to both label and release nascent polypeptides from 80S and 70S ribosomes of greening Wheat leaves, and using antisera to identify the labelled polypeptides. Their

experiments demonstrated *in vivo* association of LSu with 70S ribosomes and SSu with 80S ribosomes.

These studies were extended by Gray and Kekwick (1973, 1974) who showed that cytoplasmic polysomes, extracted from greening Bean leaves, synthesized immunoprecipitable SSu under conditions where initiation of translation did not take place (i.e. a 'polysome runoff') in a cell-free system derived from rat liver. These results were confirmed using polysomes from greening Wheat seedlings in a 'polysome run off' experiment with a Wheat germ cell-free system (Roy *et al.*, 1976). These authors also provided the first evidence that SSu RuBPCase was synthesized as a precursor polypeptide (see later).

Similar experiments were carried out to locate the site of synthesis of LSu RuBPCase. Following the demonstration of the synthesis of LSu in an *E. coli* system directed by chloroplast RNA (Hartley *et al.*, 1975) it was shown that 70S ribosomes from greening Barley leaves directed the synthesis of LSu in a 'polysome run off' experiment in an *E. coli* system (Alscher *et al.*, 1976).

The cell-free systems that have been used to translate chloroplast proteins will be discussed in detail later. Here I will describe the experiments carried out to indicate in which cellular compartment chloroplast proteins are translated.

Bottomley *et al.* (1976) observed that when Spinach total leaf RNA was used to programme the *E. coli* and Wheat germ systems, the translation products obtained from each system were qualitatively different. Although these authors did not identify any of the translation products positively, they concluded that the *E. coli* ribosomes translate selectively the mRNAs of the chloroplast,

whereas the Wheat germ system translated the mRNAs of the cytoplasm. This observation has been confirmed by several workers, however, there are exceptions, e.g. Euglena RNA which only appears to direct the synthesis of LSu RuBPCase in a Wheat germ system and not in an E. coli system (Sagher et al., 1976).

Recently DNA sequencing data has been presented which may explain the apparent selection for translation of higher plant chloroplast RNA by the E. coli ribosomes and Euglena chloroplast RNA by ribosomes of the Wheat germ system. Schwartz and Kössel (1979) have sequenced the DNA coding for the 3' terminal region of Maize 16S chloroplast ribosomal RNA. Comparing this with the sequence of DNA coding for the same region of E. coli 16S ribosomal RNA (Brosius et al., 1978; Carbon et al., 1978), there was found to be extensive homology. It is this region which is thought to interact with the initiation sequences of procaryotic mRNA by means of base pairing (Shine and Dalgarno, 1974; Steitz and Jakes, 1975). In contrast the 3' terminus of Euglena 16S chloroplast ribosomal RNA does not appear to be complementary to the initiation sequence of procaryotic mRNA (Schwarz, Kössel Graf and Stutz, unpublished results). However, it is worth pointing out that Maize chloroplast RNA has defied numerous attempts at translation in an E. coli translation system (D. Coen, personal communication).

The Wheat germ system has been shown to synthesize a number of chloroplast polypeptides thought to be translated in the cytoplasm. These include the SSu RuBPCase (Dobberstein et al., 1977; Highfield and Ellis, 1978), chlorophyll a/b binding protein (Apel and Kloppstech, 1978a Schmidt et al., 1979), ferredoxin

(Huisman et al., 1978), and preliminary results would indicate that the δ and γ subunits of chloroplast ATPase (or coupling factor CF_1) are also synthesized in the system (Price, Watanabe and Zeilinski, unpublished results). However, probably the most important contribution of the studies using the Wheat germ system has been to show that these polypeptides are made as precursors in vitro, as will be discussed in a later section.

(e) Identification of RNA and Protein Synthesized by Isolated Chloroplasts.

A more direct method of investigating the protein synthesizing capacity of chloroplasts is to identify the RNA and protein synthesized in vitro in isolated chloroplasts. If transcription were coupled to translation it would be possible to state that the RNAs and proteins that are synthesized in vitro are coded for by chloroplast DNA. However, as described by Ellis (1976), transcription and translation in isolated chloroplasts are not coupled. Thus it is not possible to state per se that RNA and protein synthesized in vitro are coded for by chloroplast DNA.

The major product of RNA synthesis in isolated chloroplasts is thought to be the precursor molecule of the chloroplast ribosomal RNA (Hartley and Ellis, 1973). Recently it has been shown that this is indeed the case and that the precursor RNA contains the 23S and 16S ribosomal RNA sequences and a spacer sequence (Hartley and Head, 1979) and also the 4.5S ribosomal RNA (Hartley, 1979). The pathway by which 5S ribosomal RNA is accumulated is not known (Hartley, 1979). As mature 23S and 16S ribosomal RNA is not found to be synthesized in vitro presumably

either the processing activity of the chloroplast is disrupted on isolation or cytoplasmic factors are constantly required for processing to take place.

It has also been shown recently that isolated chloroplasts synthesize RNA containing tracts of Poly (A) of between 10-45 (A) nucleotides (Bartoff and Price, 1979). As will be discussed later, although the major RNAs of the chloroplast do not appear to contain poly (A) sequences (Wheeler et al., 1975), 0.5% of total Maize chloroplast RNA contains Poly (A) sequences of on average 45 nucleotides length (Haff and Bogorad, 1976). Whether the poly (A) containing RNA synthesized in vitro in isolated chloroplasts is an mRNA remains to be seen.

It has been known for some time that isolated chloroplasts could incorporate amino acids into protein in vitro (Eisenstadt and Brawerman, 1964; Spencer and Wildman, 1964). This fact has been used to investigate the effect of inhibitors on chloroplast protein synthesis (Ellis, 1969, 1970). Light-driven protein synthesis in isolated chloroplasts was demonstrated by Ramirez et al. (1968). Subsequently, there appeared reports tentatively suggesting that RuBPCase was synthesized in vitro in isolated chloroplasts (Margulies, 1970; Harris et al., 1973). These studies have their limitations and have been reviewed (Woodcock and Bogorad, 1971; Boulter et al., 1972) and the experimental results were inconclusive.

Ellis and co-workers improved and characterised the conditions required for isolated chloroplasts to synthesize discrete polypeptide molecules (discussed in Ellis, 1976, 1977). The first definitive identification of a chloroplast polypeptide synthesized in vitro

in isolated chloroplasts was provided by Blair and Ellis (1973). These authors convincingly showed, by tryptic peptide mapping, that the major soluble product of isolated Pea chloroplasts was LSu RuBPCase. It was later shown by the same group that at least 5 membrane-bound and 2 envelope polypeptides were synthesized *in vitro* (Eaglesham and Ellis, 1974; Joy and Ellis, 1975).

These observations have been confirmed by other workers using isolated Spinach chloroplasts. They report that amongst the *in vitro* products there are at least 4 soluble and 9 membrane-bound polypeptides (Bottomley *et al.*, 1974), or 2 soluble and 8 membrane-bound polypeptides (Morgenthaler and Mendiola-Morgenthaler, 1976). Both groups reported that the major soluble product of the isolated chloroplast co-migrates on SDS-polyacrylamide-gel electrophoresis with LSu RuBPCase.

A major membrane-bound polypeptide product of *in vitro* synthesis in isolated Pea chloroplasts has a molecular weight of approximately 32,000 and has been called 'Peak D' (Eaglesham and Ellis, 1974). This is generally a prominent product and is synthesized when chloroplasts appear to be mature and have stopped synthesizing LSu RuBPCase (Siddell and Ellis, 1975). The labelled polypeptide does not appear to correspond to a stained polypeptide on SDS-polyacrylamide-gel electrophoresis. These observations suggest that the 32,000 M_r polypeptide does not accumulate in the chloroplast but turns over rapidly (Ellis, 1977). The 32,000 M_r polypeptide was also thought to be a major product of the *E. coli* translation system programmed by chloroplast RNA (Hartley *et al.*, 1975), its mRNA appearing not to contain a poly (A) sequence (Wheeler and Hartley, 1975).

More recently it has been shown that the 32,000 M_r polypeptide is synthesized in isolated Pea chloroplasts as a 34,000 M_r precursor which can be processed *in vitro* (Ellis and Baraclough, 1978). This work has been complemented by studies using isolated Maize chloroplasts (Grebanier *et al.*, 1978). These authors showed, by limited proteolytic digestion, that a 34,500 M_r precursor to the 32,000 M_r polypeptide is synthesized *in vitro*. However, in this case, the isolated chloroplasts were unable to process this precursor. These results would suggest the need for caution in the identification of polypeptides that are synthesized in isolated chloroplasts. The labelled polypeptides may be unprocessed precursors to mature chloroplast polypeptides.

It has been shown that the major thylakoid polypeptide labelled *in vivo* in *Spirodela oligorrhiza* is a 32,000 M_r polypeptide that is made as a 33,500 M_r precursor (Edelman and Reisfeld, 1978; Edelman *et al.*, 1979).

The 32,000 M_r polypeptide has defied formal identification. It appears to be deeply embedded in the thylakoid membranes of the chloroplasts (Ellis and Barraclough, 1978) and is possibly associated with chloroplast ATPase (Ellis *et al.*, 1977). It does not appear to be associated with the stacking of grana or the synthesis of chlorophyll a/b binding protein (Ellis and Barraclough, 1978).

It has been shown that isolated Spinach chloroplasts synthesize 3 of the 5 subunits of the chloroplast ATPase (Mendiola-Morgenthaler *et al.*, 1976) and this has been confirmed using Pea chloroplasts (Ellis, 1977). Identification was provided by co-migration with markers on SDS-polyacrylamide-gel electrophoresis of proteins removed from labelled thylakoid membranes of isolated

chloroplasts by treatment known to elute ATPase. This finding has been confirmed more recently by Grebanier et al. (1978). These workers, using limited proteolytic digestion, identified the α and β subunits of ATPase as being products of protein synthesis in isolated Maize chloroplasts.

A number of the more minor products of protein synthesis in isolated chloroplasts have now been identified. These are the elongation factors of chloroplast protein synthesis, EFG and EFT_u (Tiboni et al., 1978) and cytochrome f (Doherty and Gray, 1979). It has also been reported that isolated chloroplasts synthesize cytochrome b_{559} (Zielinski and Price, 1977), the dicyclohexycarbidiimide binding protein (Doherty and Gray, unpublished results) and the apoprotein of chlorophyll protein complex I (Zielinski and Price, unpublished results).

The majority of proteins synthesized in isolated chloroplasts remain unidentified. Autoradiographs of the labelled soluble translation products of isolated chloroplasts fractionated by 2-dimensional-gel electrophoresis (O'Farrell, 1975) reveal at least 80 radioactive 'spots' (Ellis et al., 1977). Unfortunately, this method has not been modified to allow resolution of the membrane-bound polypeptides. However, the 2-dimensional-gel technique can only be considered to give an indication of the possible translation potential of the chloroplast. The technique will resolve polypeptides coded for by one gene but with different isoelectric points (for example, LSu), and individual 'spots' may arise from the modification of polypeptides during protein preparation. Also the recovery of polypeptides in the second-dimension gel may be as low as 40% due to exclusion of polypeptides

from the first-dimension (isoelectric focussing) gel (Peterson and McConkey, 1976).

(f) Physical Mapping of Chloroplast DNA

When coupled with methods to identify specific gene products the most direct way of determining the coding capacity of chloroplast DNA is to physically map the positions of genes on chloroplast DNA. This has lately become possible using recombinant DNA techniques and the rapid progress in this field has been reviewed recently (Bedbrook and Kolodner, 1979).

As previously described, hybridization studies indicated that chloroplast ribosomal RNA cistrons were present in chloroplast DNA. Physical mapping studies have confirmed this and the ribosomal RNA genes of Maize (Bedbrook and Bogorad, 1976; Bedbrook et al., 1977), Spinach (Hobom et al., 1977; Whitfeld et al., 1976; Whitfeld et al., 1978), Chlamydomonas (Rochaix and Malnoë, 1978b; Rochaix, 1978) and Euglena (Gray and Hallick, 1978, 1979; Jenni and Stutz, 1978). The maps of chloroplast DNA obtained by these studies confirm that 2 copies of the genes for 23S and 16S RNA are present in Maize, Spinach and Chlamydomonas. The ribosomal genes are clustered in 2 sets, each set containing a 16S and 23S gene, as well as the genes for low molecular weight ribosomal RNAs, and the sets are arranged in an inverted repeat. The inverted conformation is probably not strictly required for gene function because in Pea the 2 sets of genes are arranged in a tandem repeat (Chu and Tewari, submitted for publication, Kolodner and Tewari, 1979), as are the 3 sets of ribosomal genes in Euglena (Gray and Hallick, 1978; Jenni and Stutz, 1978).

The physical maps of the ribosomal RNA show that the order of the genes is 16S-23S-5S, the same as that found for E. coli ribosomal RNA genes (Lund et al., 1976).

The chloroplast tRNA genes have been mapped on chloroplast DNA from Spinach (Steinmetz et al., 1978; Burkard et al., 1979), Euglena (Hallick et al., 1978) and Chlamydomonas (Malnoë and Rochaix, 1978). Direct evidence has been provided suggesting that chloroplast DNA contains the structural gene for LSu RuBPCase in Chlamydomonas (Gelvin et al., 1977; Rochaix and Malnoë, 1978a), Maize (Coen et al., 1977) and Spinach (Bottomley et al., 1979). These experiments have involved in vitro transcription and translation of total chloroplast DNA (Bottomley et al., 1979) or specific fragments of chloroplast DNA which have been cloned. The most convincing evidence has been provided by Bottomley et al. (1979), who found that LSu RuBPCase was a major product of an E. coli transcription and translation system programmed with Spinach chloroplast DNA. The in vitro synthesized LSu has a limited proteolytic digest profile which is identical with authentic LSu. However, these authors were unable to show that the in vitro synthesized LSu was precipitable with antisera raised against RuBPCase.

Coen et al. (1977) showed that a 4000 base pair fragment of Maize chloroplast DNA programmed the synthesis of a polypeptide, which was immunoprecipitated by antisera raised against RuBPCase, in a transcription and translation system consisting of E. coli RNA polymerase and a rabbit reticulocyte lysate. Further identification was provided by proteolytic digestion of the labelled polypeptide product although this data was not entirely unambiguous.

The data derived from work with Chlamydomonas chloroplast DNA is not so persuasive. The original data from Howell's group (Gelvin et al., 1977) has been called into doubt (Howell, 1978). Also, as will be discussed later, the E. coli translation system used to identify the LSu mRNA did not appear to translate mRNA efficiently and did not provide convincing evidence of correct translation of LSu mRNA. More recently, Rochaix and Malnoë (1978a) have shown that a cloned chloroplast DNA fragment programmed the synthesis in the E. coli system of polypeptides smaller than LSu which could be immunoprecipitated using antisera raised against RuBPCase. Tryptic peptide mapping indicated that one of the immunoprecipitated polypeptides shared partial identity with authentic LSu.

Using techniques similar to those of Coen et al. (1977) described above, it has been shown that chloroplast DNA contains the structural gene of the 32,000 M_r chloroplast membrane-bound polypeptide (Bedbrook et al., 1979a), which is synthesized in vitro as a 34,500 M_r precursor.

Preliminary evidence which has been presented could indicate that the elongation factors of chloroplast protein synthesis are coded for by chloroplast DNA (Ciferri et al., 1979).

Summary

The literature reviewed above indicates that although the chloroplast contains all the components theoretically required for autonomy, it is only a semi-autonomous organelle.

Many chloroplast proteins are coded for by the nucleus and synthesized in the cytoplasm. These include: SSu RuBPCase,

chlorophyll a/b binding protein, the δ and γ subunits of chloroplast ATPase, ferredoxin, malic and lactate dehydrogenases, aldolase, chloroplast RNA polymerase and some chloroplast ribosomal proteins.

The majority of genes present in chloroplast DNA remain to be identified. To date chloroplast DNA has been shown to contain the genes for LSu RuBPCase, the 32,000 M_r chloroplast membrane polypeptide, 4S transfer RNAs and the 23S, 16S, 5S and 4.5 ribosomal RNAs. Amongst the proteins synthesized in isolated chloroplasts are the α, β and ϵ subunits of chloroplast ATPase, the protein synthesis elongation factors G and T_u, cytochromes f and b_{559} and the dicyclohexycarbidiimide binding protein and the apoprotein of chlorophyll protein complex I.

(II) The Transport of Genetically Coded Molecules Across Chloroplast Membranes

(a) Transport into the Chloroplast

The evidence outlined above shows that many chloroplast proteins are synthesized in the cytoplasm, which raises the question of how these proteins enter the chloroplast. This question is also applicable to mitochondrial proteins synthesized in the cytoplasm and the whole topic has been reviewed recently (Chua and Schmidt, 1979; Schatz, 1979).

Interest in the transport of cytoplasmically synthesized proteins into the chloroplast was stimulated by the finding that the Wheat germ translation system synthesized the Wheat 12,000 M_r SSu RuBPCase and *Chlamydomonas* 16,500 M_r SSu as a 20,000 M_r precursor, when

programmed with Wheat polysomes and Chlamydomonas poly $(A)^+$ RNA respectively (Roy et al., 1976; Dobberstein et al., 1977). In both cases the 20,000 precursor (PSSu) was immunoprecipitated by antisera raised against SSu RuBPCase. The latter authors found that SSu was the product of the Wheat germ system programmed by free polysomes, the PSSu being 'processed' by an endoproteolytic activity present in the polysome preparation. It was shown that the endoproteolytic activity present in ribosomal supernatants 'processed' PSSu producing SSu and a small fragment more recently called the 'transit peptide'. These results have since been confirmed (Roy et al., 1977; Cashmore et al., 1978).

This work was followed by the demonstration of a precursor to SSu synthesized in vitro under the direction of poly (A) containing RNA from Pea (Highfield and Ellis, 1978; Cashmore et al., 1978; Chua and Schmidt, 1978) Spinach (Chua and Schmidt, 1978) and Lemna (Tobin, 1978). Cashmore et al. (1978) demonstrated that Pea poly $(A)^+$ RNA directed the synthesis of PSSu which contained tryptic peptides in common with SSu.

Highfield and Ellis (1978) demonstrated that PSSu could be 'processed' by isolated chloroplasts and that the processing apparently coincided with the transport of SSu into the chloroplast. These authors showed that processing and transport took place in the absence of protein synthesis. This has been confirmed by Chua and Schmidt (1978) who showed that, whereas PSSu synthesized in a Wheat germ system programmed by Pea and Spinach poly $(A)^+$ RNA could be processed by chloroplasts isolated from either Pea or Spinach, PSSu programmed by Chlamydomonas poly $(A)^+$ RNA could not. This suggests evolutionary divergence in the processing and

transport of PSSu between lower and higher plants. These authors also showed that the processed SSu entered the assembled RuBPCase holoenzyme inside the chloroplast. This has been confirmed by Smith and Ellis (1979) who also demonstrated that the processing activity was located in the stromal phase of the chloroplast.

These studies have been complemented by the finding that cytoplasmically synthesized chlorophyll a/b binding protein (Kung et al., 1972; Machold and Aurich, 1972) was synthesized in a Wheat germ system, programmed by poly $(A)^+$ RNA from greening Barley, as a precursor 4000 daltons larger than the mature protein (Apel and Kloppstech, 1978a). Recent results by Schmidt et al. (1979) have shown that the chlorophyll a/b binding protein from Pea is composed of two immunologically and structurally related polypeptides (proteins 15 and 16). These polypeptides are also synthesized as precursors (P15, P16) in vitro and can be processed by isolated chloroplasts. In the same series of experiments these authors also noted that a number of other in vitro products were also processed and transported into isolated chloroplasts and integrated into chloroplast membranes. It is possible that one of these polypeptides was ferredoxin which is known to be synthesized as a precursor in a Wheat germ system directed by RNAs from several plants (Huisman et al., 1978).

To date the mechanism by which these precursor polypeptides enter the chloroplast is unknown. About 20 polypeptides destined for passage through cell membranes have been found to be synthesized as precursors. A model has been proposed to explain the passage of proteins across membranes for secretion. This has been termed the 'Signal Hypothesis' (Blobel and Dobberstein, 1975), and it has been

substantiated by experimental results. Many proteins that pass through or are bound to cell membranes are synthesized as precursors with a lipophilic 'signal peptide' (Devillers-Thiery et al., 1975; Habener et al., 1978). The polysomes that are synthesizing these polypeptides become membrane-bound after the synthesis of the N-terminal 'signal peptide' (Blobel and Dobberstein, 1975a, b; Shore and Harris, 1977; Shields and Blobel, 1978; Warner and Dobberstein, 1978). The 'signal peptide' is thought to penetrate the membrane, allowing co-translational passage of the protein through it, and is then cleaved by a specific, membrane-bound peptidase (Jackson and Blobel, 1977; Chang et al., 1978). The remaining portion of the polypeptide is co-translationally passed through the membrane after which it may undergo post-translational modifications, e.g. glycosylation (Rothman and Lodish, 1977; Toneguzzo and Ghosh, 1978; Bielinska and Baime, 1978; Lingappa et al., 1978). Ovalbumin, lacking an N-terminal signal peptide yet transported through a membrane was thought to be an exception to the 'Signal Hypothesis' (Palmiter et al., 1978). However, recently it has been shown that ovalbumin contains an internal 'signal peptide' sequence (Lingappa et al., 1979).

However, the data described here concerning the transport of cytoplasmically synthesized polypeptides into the chloroplast cannot be explained by the 'Signal Hypothesis' largely because PSSu appears to be synthesized on soluble polysomes and transport and processing appears to take place in the absence of protein synthesis (Chua and Schmidt, 1979; Ellis, 1979). One model to explain the passage of proteins into the chloroplast is the 'Envelope Carrier Hypothesis' (Blair and Ellis, 1973; Highfield

and Ellis, 1978; Smith and Ellis, 1979; Ellis, 1979). This proposes that there is a specific carrier protein orientated within the chloroplast envelope in such a way that a segment of the bound PSSu is exposed to the stromal protease. It is suggested that removal of a basic segment of the PSSu triggers a conformational change in the primary structure of the polypeptide resulting in the release of the SSu into the stromal phase of the chloroplast. This model remains to be experimentally tested.

It is thought that the receptor for the PSSu is likely to be a polypeptide or a group of proteins because the processing and transport activity of chloroplasts can be abolished by pre-treatment with protease (Chua and Schmidt, 1978). Processing and transport of PSSu has no requirement for chloroplast ribosomes because mature SSu is found in heat bleached Rye leaves which do not contain chloroplast ribosomes (Feierabend and Wildner, 1978). This would indicate that the receptor and processing activities are coded for in the nucleus and translated on cytoplasmic ribosomes. Inhibitor studies suggest that the proteolytic cleavage involves the breakage of a disulphide-bridge (Dobberstein et al., 1977). These authors draw an analogy between the passage of PSSu into the chloroplast and the passage of diptheria toxin into cells which may involve the cleavage of a disulphide-bridge (Collier, 1975).

Recently it has been suggested that energy is required to process and transport polypeptides of the mitochondrion which are synthesized in the cytoplasm (Schatz, 1979). The results reviewed

here show that all the precursors identified to date are approximately 4000-6000 daltons larger than the mature polypeptides. When considering the cytoplasmic precursors of mitochondrial proteins, the size of the 'transit peptide' is variable (Schatz, 1979). For example, the size of the 'transit peptides' of the subunits of F1 ATPase synthesized in the cytoplasm are 6000 daltons for α and γ subunits and 2000 daltons for the β subunit (Schatz, 1979). Considering that the mature proteins occupy similar locations within the mitochondrion (Sebald, 1977), the difference in 'transit peptide' size is not apparently related to the organellar location of mature polypeptide.

DNA sequencing data has shown that the gene in yeast for Iso-1-cytochrome c, a polypeptide of the inner mitochondrial membrane, synthesized in the cytoplasm, does not contain an expected sequence coding for an N-terminal 'transit peptide' (Smith et al., 1979). If one assumed that the DNA sequence is complementary to the RNA translated in the cytoplasms, it would appear that this organellar protein is not synthesized as a precursor, assuming that an internal 'transit peptide' is not present. Pools of the free apoprotein have been detected in the cytoplasm and haem is added post-translationally (González-Cadavid, 1974). Thus it is tempting to speculate that if a conformational change is required to transport proteins across organellar membranes, this change may be induced by the association of the apoprotein with haem at the outer mitochondrial membrane.

Passage of other gene products (e.g. mRNA, tRNA) from the cytoplasm into the chloroplast, although possible, has yet to be demonstrated. One report, based on inhibitor studies using rifampicin, suggests that the observed lack of inhibition of

chloroplast protein synthesis may be due to the import of mRNA from the cytoplasm (Jennings and Ohad, 1972). However, these authors do not exclude the possibility that although initiation of transcription may be inhibited, chain elongation is not.

(b) Transport from the Chloroplast

The passage of genetically coded information from the chloroplast to the cytoplasm is disputed and remains to be demonstrated convincingly. Data suggesting that this takes place has been obtained using mutants that lack or have defective chloroplast DNA, and measuring the activities of cytoplasmically synthesized polypeptides (Bradbeer et al., 1979).

Using temperature sensitive mutants of Chlamydomonas which are deficient in chloroplast ribosomes it has been shown that these are unable to grow in the presence of reduced carbon (i.e. heterotrophically), indicating that the chloroplast protein synthesis is required for growth even when photosynthesis is not essential (Hanson and Bogorad, 1978). These findings are in contrast to previous results (for example, Harris et al., 1974) and highlights the problems involved in using mutants where the primary effect of the mutation is not known. For example, in bleached Rye leaves lacking chloroplast ribosomes, levels of cytoplasmically synthesized enzymes, including SSu RuBPCase, appear unaffected (Feierabend, 1978), whereas this is apparently not the case with Barley chloroplast DNA mutants lacking chloroplast ribosomes (Bradbeer et al., 1979).

It has been postulated that photoinduced development of dark-grown Euglena is triggered by a blue light receptor located in the nucleus and a blue-red light receptor located in the plastid

(Schiff, 1978). Red light alone can bring about normal light induced development of plastids in Euglena. It would appear necessary to postulate that, in induction by red light, the chloroplast photoreceptor causes a 'signal' to be sent from the chloroplast which overrides the non-plastid blue light receptor and induces the cytoplasmic genetic system to develop (Schiff, 1978). The 'signal' however, need not be of genetic origin. Ion influx or efflux may be a sufficient stimulus.

It has been suggested that Euglena tRNAs coded for by the chloroplast DNA are selectively exported to the cytoplasm (McCrea and Hershberger, 1978). However, more recently this has been disputed (Schwartzbach et al., 1979).

Summary

There is now convincing evidence that chloroplast proteins which are translated in the cytoplasm are synthesized as precursors 4000-6000 daltons larger than the mature protein. These appear to be processed and transported into the chloroplast by a post-translational mechanism. The transport of other genetically coded molecules across chloroplast membranes remains to be demonstrated convincingly.

(III) Chloroplast Development

From the preceding discussion it is clear that any study of the molecular basis of chloroplast development involves the elucidation not only of the control of synthesis of individual chloroplast components but also the interaction of the two genetic systems involved with organelle development.

Chloroplast development has been intensively studied and this interest has been reflected in a recent International Meeting (see Akoyunoglou and Argyroudi-Akoyunoglou, 1978) and review articles (for example, see Kirk, 1970; Rosinki and Rosen, 1972; Bradbeer and Montes, 1976; Leech, 1976). In considering chloroplast development we need to bear in mind the great diversity of photosynthetic organisms and how they adapt to a wide range of environmental conditions which might involve adaptation of the morphological and biochemical characteristics of the chloroplast. The work described in this thesis has been concerned with the synthesis of chloroplast proteins in cotyledons during germination and early development of Cucumber seedlings. Because of this the discussion concerning chloroplast development will be limited to higher plants except where morphological and physiochemical comparisons with lower plants are made.

(a) The Development of Chloroplasts from Etioplasts

Many experimental systems used to study chloroplast development involve the greening of dark-grown, etiolated tissue. Thus these studies concern themselves with the light induced transformation of etioplasts into chloroplasts. However, under natural conditions etioplasts are rarely encountered, and under normal diurnal light

regimes chloroplasts develop directly from proplastids (Leech, 1976). It has been argued that chloroplast development from etioplasts is simply an environmental variation of the developmental pathway of proplastid to chloroplast (Whatley, 1977). However, the etioplast itself is at a more sophisticated level of plastid development than the proplastid (Leech, 1976). This author points out that etioplasts from differing plant sources, or even within a single plant, may not be at the equivalent stage of development. Indeed it has been suggested that in using etiolated higher plant tissues of increasing age, what was considered to be a characteristic of plastid development may be more concerned with the pathology of etiolation (Schiff, 1978).

Etioplasts contain many of the components of the chloroplast; DNA (Jacobsen, 1968), RNA and ribosomes (Jacobsen et al., 1963; Dyer et al., 1971), tRNA and amino acid activating enzymes (Burkard et al., 1972), the enzymes of the Calvin cycle (Bradbeer et al., 1974), many membrane polypeptides (Grebanier et al., 1979; Locksin et al., 1971) including ATPase and components of the electron transport chain including cytochromes f, b_{563}, b_{559LP} and plastocyanin (Plesničar and Bendall, 1973). Etioplasts appear to lack chlorophyll, chlorophyll a/b binding protein and P700 chlorophyll a- protein (for review, see Thornber, 1975).

Thus it would appear that chloroplast development from etioplasts involves the re-organisation as well as the synthesis of components already present in the etioplast and the de novo synthesis of chlorophyll and associated proteins.

(b) Light Induction of Chloroplast Development

The role of light on plant morphogenesis and chloroplast development has been studied extensively and has been the subject of a recent book, Smith (1976a) and several reviews (Kirk, 1971; Zucker, 1972; Mohr and Schopfer, 1977; Schopfer, 1977).

In angiosperms, chloroplast development involves both phytochrome and protochlorophyllide holochrome as photoreceptors (Zucker, 1972). Irradiance of these initiates a complex chain of events leading to change in chloroplast ultrastructure (Kirk, 1971) as well as enzyme induction or repression (Mohr and Schopfer, 1977). However, although phytochrome action has been implicated in effecting the activity of about 50 plant enzymes, the primary action of phytochrome remains in doubt (Schopfer, 1977).

Of interest to us is the control phytochrome might exert over protein synthesis. Phytochrome has been implicated in regulating the synthesis of nucleic acids (Okoloko et al., 1970) and RNA polymerase (Bottomley, 1970) as well as inducing the synthesis of RNA (Glydenholm, 1968; Ingle, 1968b). The latter author found that there appeared to be a differential effect of light on cytoplasmic and chloroplast RNA accumulation. In Radish cotyledons RNA synthesis takes place in the light and the dark. However, in dark-grown tissue, light stimulates the incorporation of [^{32}P] phosphate into cytoplasmic ribosomal RNA by 19%, whereas the incorporation into chloroplast ribosomal RNA is 75% (Ingle, 1968b). These findings have been confirmed with greening Bean leaves (Dyer et al., 1971). Light enhances the synthetic activity of ribosomes (Williams and Novelli, 1968) and this enhancement is

thought to be mediated by phytochrome (Travis et al., 1974), which is also probably involved with polysomal formation (Smith, 1976b; Klein and Pine, 1977). Light also induces the synthesis of at least one tRNA synthetase and several tRNAs (Reger et al., 1970; Burkard et al., 1972).

It is now becoming clear that light effects the accumulation of specific mRNAs (Tobin and Klein, 1975; Rosner et al., 1975). Recently hybridization data has shown that light initiates a complex pattern of chloroplast RNA synthesis in Euglena (Verdier, 1979a and b; Chelm et al., 1979). However, until the identities of these RNAs are established the significance of such data is limited.

The use of cell-free translation systems has enabled the identification of several mRNAs which accumulate in the light. These include the mRNAs coding for the cytoplasmic precursors to SSu RuBPCase (Highfeld and Ellis, 1978; Tobin, 1978) and chlorophyll a/b binding protein (Apel and Kloppstech, 1978a; Apel, 1979). It has been known for some time that plastids isolated from greening Pea leaves synthesize the 32,000 M_r chloroplast membrane polypeptide to an increasing extent relative to LSu RuBPCase as greening proceeds (Siddell and Ellis, 1975). More recently it has been shown that the mRNA coding for the protein precursor of the 32,000 M_r chloroplast membrane polypeptide of Spirodela accumulates in greening tissue (Reisfeld et al., 1978; Edelman et al., 1979). This work has been complemented by the demonstration that in greening Maize tissue there are increases in RNAs which (i) hybridize to specific fragments of chloroplast DNA, and (ii) direct the synthesis of a 34,500 M_r polypeptide in vitro which is thought to be the precursor to

the 32,000 M_r chloroplast membrane polypeptide (Bedbrook et al., 1978).

(c) Co-ordination of Synthesis of Chloroplast Components

The synthesis of different chloroplast components during light induced development appears to be temporally organised (Plesnicar and Bendall, 1973). Considering that the genetic information coding for chloroplast components is located in different cellular compartments, there must be elements of control effecting both compartments. For example, the synthesis of the apoprotein of the chlorophyll a/b binding protein which takes place in the cytoplasm is tightly correlated with chlorophyll synthesis which takes place in the chloroplast (Hiller et al., 1973). It has been proposed that there are two separate light regulation sites involved in the assembly of the chlorophyll a/b binding protein (Apel and Kloppstech, 1978b; Apel, 1979). These authors suggest that light via phytochrome induces the accumulation of the mRNA coding for the apoprotein in the cytoplasm, whereas phototransformation of protochlorophyll(ide) to chlorophyll(ide) takes place in the chloroplast. Another example is RuBPCase, the large and small subunits of which are coded for and translated in separate cellular compartments. There is evidence suggesting that during the cell cycle of Chlamydomonas the synthesis of the two subunits is closely synchronised (Iwanij et al., 1975). However, the previously mentioned results of Feierabend and Wildner (1978) show that the synthesis of SSu RuBPCase can take place in the absence of chloroplast protein synthesis. These authors also found evidence of a pool of free SSu subunits in young tissue. Using protoplasts it has also been shown that the synthesis of the two subunits of RuBPCase can be uncoupled and that the synthesis of complete RuBPCase can

continue after the inhibition of synthesis on cytoplasmic ribosomes (Hirai and Wildman, 1975; Barraclough and Ellis, 1979). These authors suggest that their data indicates that there is a pool of free SSu subunits existing in the cell which sustains the synthesis of the complete RuBPCase after cytoplasmic synthesis has been inhibited. Evidence that there is a pool of free SSu subunits in the cell has been presented by other workers (Roy et al., 1978). It has been suggested that the rate limiting step in the assembly of complete RuBPCase is the synthesis of LSu (Callow, 1974). SSu or another cytoplasmically synthesized protein has been implicated as being a positive initiation factor for the transcription or translation of the LSu gene (Ellis, 1975). Although it has yet to be experimentally tested, evidence suggests that this control may be exerted at the level of transcription (Ellis and Barraclough, 1978).

Summary

Although the study of greening etioplasts provides a useful experimental system to investigate chloroplast development, etioplasts themselves are highly developed organelles. Light is an inducer of chloroplast development, not only activating enzymes but also stimulating de novo protein synthesis. Photoreceptors may be located in the cytoplasm and chloroplast. However, how these two cellular compartments interact during chloroplast development remains unclear.

(IV) Molecular Aspects of Differentiation and Development in Animal Systems

(a) Levels of Control

Following the elucidation of gene expression in procaryotes (for example, see Lewin (1974)) most of our knowledge of gene expression during differentiation and early development has come from investigations carried out using animal systems (see Davidson, 1976). A large amount of data has been accumulated which suggests that, in general, differentiated cells contain the same quantities of DNA and the same complements of DNA sequence and that the genes expressed selectively in differentiated cells are not present in extra copies (Davidson, 1976). On the whole it appears that, when mRNAs are present on cytoplasmic ribosomes, the structural genes that code for that mRNA are transcriptionally active, and that different cell types may contain, at a particular stage of development, different transcriptionally active genes (Davidson, 1976). This would suggest that the initial control of differentiation and development is at the level of transcription. However, as discussed in Palmiter (1975), many other levels of control are possible and it is probable that all are utilised to some extent (for example, see Guyette et al., 1979).

(b) Methods of Investigation

It has been shown in a variety of experimental systems that during induction of gene expression (i.e. during differentiation and development and/or hormonal induction) increased synthesis of cell specific proteins is correlated with an increase in the concentrations

of specific mRNAs. For example, this appears to be the case in the control of synthesis of ovalbumin (McKnight et al., 1975; Harris et al., 1975), haemoglobin (Ross et al., 1974), fibroin (Suzuki and Suzuki, 1974), and vitellogenin (Shapiro and Baker, 1977; Baker and Shapiro, 1977). This data was obtained by hybridization studies carried out to quantitate the amount of mRNA complementary to a specific DNA probe. Hybridization studies have limitations not least because the mRNAs that are quantitated may not be rigorously identified as coding for a specific polypeptide. This difficulty may be overcome by the use of cell-free protein-synthesizing systems to translate the specific mRNA into a polypeptide that can be identified.

Caution is required in the interpretation of experiments that are carried out using cell-free protein-synthesizing systems to assay the accumulation of a specific mRNA. All mRNA species in a particular tissue may not have the same assay requirements, minor changes in conditions may have dramatic effects on translation efficiencies (Tse and Taylor, 1977). It is important to evaluate the translation requirements of individual mRNAs with respect to Mg^{2+} and K^{+} ion levels (Tse and Taylor, 1977; Benveniste, et al., 1976). Suboptimal ion concentrations may result in premature termination of translation of a particular mRNA (Tse and Taylor, 1977). Cell-free systems may exert a high degree of selectivity in their interaction with mRNA and the translation characteristics of isolated mRNAs may not provide an accurate reflection of their behaviour in intact cells (Cereghini et al., 1979). Indeed, as will be discussed later, various mRNAs may exhibit concentration-dependent differences in their peptide

initiation rates, Lodish (1974). Nevertheless, cell-free systems have been used extensively to assay changes, induced by different stimuli, in the levels of translatable mRNAs and identify the proteins that we coded for by these mRNAs, (for example, Paterson et al., 1974; Kurtz et al., 1976; Tata, 1976; Weeks and Collis, 1976; Shapiro et al., 1976; Chang and Littlefield, 1976; Spencer et al., 1976; Reeves, 1977; Alton and Lodish, 1977a and b; Sekenis and Scheller, 1977; Gelinas and Kafatos, 1977; Adams et al., 1977; Thomson et al., 1978; Goodridge et al., 1979).

In experiments which have been carried out using both hybridization and cell-free systems to quantitate levels of a specific mRNA the data obtained by both techniques is essentially similar. For example, compare the data concerning ovalbumin accumulation from Palmiter (1973) with Harris et al. (1975), vitellogenin accumulation from Shapiro and Baker (1977); Baker and Shapiro (1977) with Shapiro et al. (1976), and see Toole et al. (1979) and Hastie et al. (1979). It is not clear whether the accumulation of mRNA is due purely to increased transcription, precursor RNA processing or a decrease in the degradation of RNA.

Summary

Studies using a variety of animal systems have shown that the induction of differentiation and development of certain cell types involves the accumulation of mRNA coding for specific proteins. Studies involving DNA-RNA hybridization and cell-free protein-synthesizing systems to measure the accumulation of mRNA produce similar results. The processes that result in the accumulation of mRNA remain unclear.

(V) Cell-Free Protein-Synthesizing Systems

This thesis concerns itself with the use of *in vitro* protein-synthesizing systems to assay levels of translatable mRNAs present during the development of cotyledons of Cucumber seedlings. The use of these systems has already been discussed briefly. However, in this section I propose to discuss how these systems have been used in detail. Many cell-free protein-synthesizing systems have been developed (for examples, see Shafritz, 1977), but those which have been used most widely to translate mRNAs from plants have been the *E. coli* and Wheat germ sytems.

(a) The *E. coli* System

The concept that genetic formation encoded in DNA is transcribed into an RNA which serves as a template or 'message' in protein synthesis was first introduced by Jacob and Monod (1961). It was subsequently proposed that the amino acid sequence of proteins was determined by the sequence of bases along a particular part of a nucleic acid (Crick *et al.*, 1961). The first direct evidence that this was the case appeared almost simultaneously with the work of Nirenberg's group who used an RNA dependent *E. coli* translation system under the direction of specific synthetic polynucleotide templates. For example, they found that polyuridylic acid (poly U) stimulated the uptake of phenylalanine, suggesting that the codon for phenylalanine is UUU (Nirenberg and Matthaei, 1961). Subsequent experiments showed that polyadenylic acid encourages the uptake of lysine and polycytidylic acid stimulates incorporation of proline (for discussion, see Speyer *et al.*, 1963). This work has been extended to assign nucleotide sequences to all amino acids

(for example, see Nirenberg et al., 1963; Speyer et al., 1963; Nirenberg et al., 1966; Khorana et al., 1966).

Definitive evidence for the presence of a specific mRNA in an RNA extract requires the translation of that mRNA by a cell-free system into a specific polypeptide product (see discussion Singer and Leder, 1966). This was accomplished with the translation of f2 and R17 bacteriophage RNA into viral proteins by the E. coli system (Nathans et al., 1962; Cappechi, 1966). A wide selection of viral RNAs have been translated in vitro using the E. coli system, for example: MS2 (Nathans et al., 1966; Viñuela, 1967), T_4 (Salser et al., 1967), M12 (Konings et al., 1970), Qβ (Jockusch, 1970; Horuichi et al., 1971), T_3 and T_7 (Hercules et al., 1974), PP7 (Davies and Benike, 1974). A coupled transcription and translation system derived from E. coli has been used to transcribe ØX 174 DNA efficiently (Bryan et al., 1969), and translate the resulting RNA into phage protein (Gelfand and Hayashi, 1969; Hayashi and Hayashi, 1970).

Because bacteriophage RNA is readily translated by the E. coli system this RNA has been used to investigate many aspects of protein synthesis. For example, the gene order of viral genomes (Konings et al., 1970), the control of translation by product feedback repression (Konings et al., 1970; Lodish, 1968), the mechanism of the initiation of translation (Clark and Marker, 1966a, 1966b; Schwartz, 1967; Lodish and Robertson, 1970) the secondary structure of mRNA (Lodish, 1970; Lodish and Robertson, 1970), the processing of pre-mRNA (Hercules et al., 1967), the enzymology of protein synthesis (Nirenberg, 1962) and translation termination (Atkins and Gesteland, 1975).

Attempts to translate eucaryotic mRNAs in the E. coli system have met with varying degrees of success. Of the mRNAs used in the E. coli system the translation of eucaryotic viral products have been demonstrated with Avian Myceloblastosis RNA (Siegert et al., 1972), Poliovirus RNA (Warner et al., 1963; Rekosh et al., 1970), Alfalfa Mosaic Virus (Van Ravenswaay et al., 1967), Satellite Tobacco Necrosis Virus (Clark et al., 1965), Rauscher Leukemia Virus and Mouse Mammary Tumour Virus (Gielkens et al., 1972). Two viral polypeptides have been recovered from a transcription and translation system programmed with SV40 DNA (Greenenblatt et al., 1976). On the other hand there are eucaryotic viral RNAs that are not translated into viral proteins in the E. coli system for example, TMV RNA. Used to programmed the E. coli system TMV RNA greatly stimulates the incorporation of amino acids into protein (Nirenberg and Matthael, 1961). Indeed, what was perhaps the first example of immunoprecipitation of in vitro translation products indicated that proteins carrying TMV protein antigenic determinants were synthesized in vitro (Tsugita et al., 1967). Later work however found this not to be the case (Aach et al., 1964), and this has since been confirmed by other workers (Happe and Jockusch, 1973). This then would suggest that the proteins that are synthesized upon the addition of TMV RNA are spurious. This may be caused by the mis-reading of certain base triplets in the TMV RNA as initiators by the E. coli initiation complex. If initiation resulted from mis-reading of TMV RNA, one would expect the polypeptides synthesized in vitro to be relatively short if one assumed termination signals to follow closely the site of false initiation in a randomised base sequence. The

polypeptides synthesized *in vitro* are relatively large (Schwartz, 1967), suggesting that the *E. coli* translation complex does not recognise TMV termination signals.

The correct translation of Rabbit globin mRNA in the *E. coli* translation system has been reported (Laycock and Hunt, 1969) and to my knowledge this is the only non-viral, non-organellar eucaryotic mRNA whose product has been identified amongst *E. coli* system translation products.

The use of the *E. coli* system to translate eucaryotic organellar RNAs is a relatively recent innovation. Mitochondrial DNA from Rat liver has been used to direct a coupled transcription and translation system to yield 4 polypeptides that had a similar molecular weight to polypeptides that are synthesized in the isolated organelle (Chuang and Weissback, 1973). This work extended the findings of previous workers who had used a 'hybrid' system in which Neurospora DNA was transcribed *in vitro* with *E. coli* RNA polymerase. The RNA was extracted and used to programme a sub-mitochondrial system to yield 2 polypeptides that co-migrated with polypeptides that are synthesized *in vitro* in isolated mitochondria (Blossey and Küntzel, 1972; Küntzel and Blossey, 1975). Scragg and Thomas (1975) showed that a system in which Yeast mitochondrial DNA was transcribed by *E. coli* RNA polymerase, and resultant RNA extracted and used to programmed an *E. coli* translation system, produced 6 polypeptides. 3 of these polypeptides were shown by immunoprecipitation to be the components of cytochrome oxidase (Scragg and Thomas, 1977). This was also found to be the case when mitochondrial polyadenylated RNA from Yeast was used to programme an *E. coli* system (Padmanaban, 1975). However, the use

of the E. coli system to translate mitochondrial RNA has been limited. An example of the problem of using the E. coli system to translate mitochondrial RNA has been provided by the work of Moorman and co-workers. They showed that Yeast mitochondrial RNA directed the synthesis of polypeptides that carried the antigenic determinants to cytochrome oxidase as well as ATPase (Moorman et al., 1976). However, electrophoretic analysis of the immunoprecipitated polypeptides revealed that complete proteins were absent from the translation products and that the translation products of the E. coli system bore no resemblance to those polypeptides synthesized in the isolated mitochondria (Moorman et al., 1977, 1978). Thus doubts still remain concerning the identity of the translation products directed by mitochondrial RNA in the E. coli system.

More recently it has been shown that the addition of Ca^{2+} ions to the translation mixture leads to a specific enhancement of translation of mitochondrial RNA (Halbreich, 1979). It was postulated that Ca^{2+} ions effect the secondary structure of the mitochondrial RNA improving the efficiency of the translation.

The first unsuccessful attempts to use the E. coli system to translate higher plant chloroplast RNA were made using a linked transcription and translation system programmed with Spinach chloroplast DNA (Whitfeld et al., 1973). Later Hartley et al. (1975) as previously described, were successful in using an E. coli translation system to translate Spinach RNA. These workers found that the major products of the system were proteins of 52,000 and 35,000 M_r which appeared to be analogous to the major proteins synthesized by isolated Pea chloroplasts, LSu RuBPCase and 'Peak D' (Eaglesham

and Ellis, 1974). Peptide mapping showed that the 52,000 M_r protein synthesized *in vitro* was similar to LSu, but it was 1500 M_r smaller than the native protein. The authors suggested that this may have been the result of spurious initiation or termination of translation in the system, or that the system only translated one of the isoelectric variants of LSu. On the other hand it may be explained by RNAase activity in the *E. coli* extract. When MS2 RNA was used to programme the system, only coat protein was detected amongst the translation products. This may have been due to the sensitivity of the technique used to analyse the translation products but one would expect to see a viral replicase protein amongst the translation products (Nathans *et al.*, 1966; Viñuela *et al.*, 1967; Kozak and Nathans, 1972). The absence of replicase amongst the translation products is indicative of RNAase activity in the *E. coli* extract (J. Atkins, personal communication).

Subsequent work, described elsewhere in this Chapter, using the *E. coli* system has shown that LSu mRNA lacks tracts of poly (A) (Wheeler and Hartley, 1975) and that a light inducible RNA, also lacking poly (A) tracts, isolated from *Spirodela* programmes the synthesis of proteins *in vitro* (Rosner *et al.*, 1975).

The *E. coli* system has been used to identify LSu mRNA amongst fractionated RNA in attempts to isolate this mRNA from *Chlamydomonas*. However, the results that have been obtained have not been totally convincing. Howell *et al.* (1977a, 1977b) showed that although the *E. coli* system programmed with *Chlamydomonas* RNA synthesizes polypeptides which could be immunoprecipitated with antisera raised against RuBPCase, the immunoprecipitated polypeptides had molecular weights of between 14,000 and 18,000. Once more it is probable

that RNAase activity was present in the *E. coli* extract. More recently Sano *et al.* (1979) have reported the isolation of LSu mRNA identified by translation in the *E. coli* system.

(b) The Wheat Germ System

The cell-free protein-synthesizing system derived from untoasted Wheat germ, developed by Marcus and co-workers (Marcus, 1979; Marcus *et al.*, 1974) and modified by Roberts and Paterson (1973), has been used to translate a variety of mRNAs. Originally the system was used in a study of the initiation of protein synthesis in plants (Marcus *et al.*, 1970; Seal *et al.*, 1972; Weeks *et al.*, 1972; Klein *et al.*, 1974).

The Wheat germ system has been used to translate RNAs from a wide range of sources, for example, mRNAs coding for globin (Efron and Marcus, 1973), collagen (Benveniste *et al.*, 1976), viral proteins (Roberts *et al.*, 1973; Higgins *et al.*, 1976) and peptide hormones (Kemper *et al.*, 1976; Cox *et al.*, 1976; Chatterjee *et al.*, 1976). The extensive use of the Wheat germ system has been reviewed by Shafritz (1977).

The system has been used to translate RNA from a variety of plant sources, what proved to be the first report of the isolation and *in vitro* translation of a plant mRNA used the Wheat embryo system to translate leghaemoglobin mRNA (Verma *et al.*, 1974). Subsequently the same group demonstrated the synthesis of cellulase in the same system programmed by RNA extracted from auxin-treated Pea epicotyls (Verma *et al.*, 1975).

The Wheat germ system has been used successfully to investigate the existence of 'long-lived' RNA in seeds (for review see Payne, 1976).

Higgins et al. (1976) have used the system to demonstrate the hormonal control of the level of translatable mRNA for α amylase in Barley Aleurone layers. Using the Wheat germ system, the in vitro synthesis of several seed proteins has been demonstrated: Zein in Maize (Larkins and Dalby, 1975; Larkins et al., 1976; Jones et al., 1977), G1 globulin in Bean (Sun et al., 1975, 1978; Hall et al., 1978), Hordein in Rye (Fox et al., 1977) and the storage proteins of Soybean (Beachy et al., 1978), Pea (Higgins et al., 1977; Higgins and Spencer, 1979) and Oat (Luthe and Paterson, 1977).

In addition, as previously discussed, the Wheat germ system has been used extensively to translate mRNAs which code for proteins which are normally synthesized in the cytoplasm but have a functional role in the chloroplast. Also, as previously discussed, chloroplast mRNAs from Euglena and Spirodela have been translated in the Wheat germ system (Sagher et al., 1976; Edelman et al., 1979).

Summary

The cell-free protein-synthesizing systems described here have been shown to translate a wide variety of mRNAs. Of interest to us is that the E. coli system can be used to translate chloroplast mRNAs. On the other hand, the Wheat germ system can be used to translate cytoplasmic mRNAs, including those that code for proteins with a functional role within the chloroplast.

(VI) The Aims and Approach of the Project

(i) Aims

The aims of this project were to study the control of synthesis of chloroplast proteins during chloroplast development. I have been particularly interested in the control of the synthesis and accumulation of mRNAs that code for chloroplast proteins, especially the two subunits of RuBPCase, and how this is related to the appearance of the native protein *in vivo*.

(ii) Approach

The developmental system chosen for study was the cotyledon of Cucumber (*Cucumis sativus*) seedlings grown either under a 12 hour photoperiod or in the dark. Thus with this system I was studying the development of the proplastid into chloroplasts or etioplasts.

Cotyledons exhibiting epigeal germination provided us with a useful system to study the development of plastids. The acquisition of photosynthetic competence has been studied in the cotyledons of Squash (Lott, 1970), and Mustard (Mohr, 1977). Using the Cucumber cotyledon system the developmental stages were reproducible under defined growth conditions. The cotyledons contain a supply of storage material (primarily lipid) providing energy prior to the development of photosynthetic competence. I also assumed that the development of the photosynthetic apparatus in the cotyledons of Cucumber, as in Mustard (Mohr, 1977), reflected the corresponding events in young 'true' leaves in every respect.

In the absence of specific DNA probes for the mRNAs coding for LSu and SSu RuBPCase I used cell-free translation systems to assay for changes in levels of translatable mRNAs for these proteins.

To carry this out cell-free translation systems derived from *E. coli* and Wheat germ were prepared and optimised to quantitatively translate mRNAs present in total RNA extracted from Cucumber cotyledons of different stages of light- and dark-grown development.

I sought to identify LSu and the precursor of SSu (PSSu) amongst the translation products of the *E. coli* and Wheat germ systems respectively. Once this had been carried out the amounts of LSu and PSSu synthesized *in vitro* by the optimised translation systems programmed with Cucumber cotyledon RNA could be used as an assay of the amounts of translatable mRNA for those proteins present in the RNA sample. The amounts of radioactively labelled protein synthesized *in vitro* was quantitated by the estimation of radioactivity present either in an immunoprecipitate using antisera raised against the LSu and SSu RuBPCase or in the polypeptide excised from the translation products fractionated by SDS-polyacrylamide-gel electrophoresis.

Once the changing levels of translatable mRNAs for certain polypeptides were known these were related to the appearance of both LSu and SSu and native RuBPCase *in vivo*. Finally as a measure of the protein synthesizing capacity of the chloroplasts themselves a cell-free protein synthesizing system was optimised using isolated chloroplasts from Cucumber cotyledons. The capacity of developing chloroplasts to synthesize proteins was investigated using plastids extracted from cotyledons from different days of Cucumber seedling development.

Chapter 2. Materials and Methods

Section (A) Materials

(I) Plant Material

Cucumber *Cucumis sativus* (var. Long Green Ridge)

Pea *Pisum sativus* (var. Feltham First)

Spinach *Spinacia oleracea* (var. Miragreen) Lawson Donaldson Ltd.

Wheat *Triticum vulgaris* (var. Maris Ranger) a gift from the Plant Breeding Institute, Cambridge, U.K.

Spirodela polyrrhiza cultured in Hoagland's media, a kind gift from Ms. C. Smart, University of Edinburgh U.K.

(II) Bacterial Strains

Escherichia coli strain: PR7, RNAase 1^-, polynucleotide phosphorylase$^-$ and sus$^+$ (Reiner, 1969).

Escherichia coli strain: CSH 73, Lac$^-$, Ara$^-$, Leu$^-$, B1$^-$ and sus$^+$ (Miller, 1972). Both strains were a gift from the Cold Spring Harbor Laboratory, N.Y., U.S.A.

Staphylococcus aureus: Cowan Serotype 1 NCTC No. 8530. A gift from Dr. R. Meagher, University of Georgia, Ga., U.S.A.

(III) Radiochemicals

L-[^{35}S] methionine specific activity 1005 - 1400 Ci/mmol, obtained as an aqueous solution and stored in 1 mM DTT at -80° C.

[^{14}C] Protein Hydrolysate, specific activity 57 mCi/m Atom. Obtained as solution, stored at 4°C.

Both were obtained from The Radiochemical Center, Amersham, U.K.

(IV) Enzymes

Pyruvate Kinase, RNAase (A) obtained from Sigma Chemical Co.

Creatine Phosphokinase obtained from Boehringer Mannheim GmbH.

Spinach Chloroplast ATPase a gift from Professor G. Schatz University of Basel, Switzerland.

(V) Chemicals

Common reagents were obtained, unless otherwise indicated, from BDH Chemicals Ltd. "Analar" grade was used except Ammonium Sulphate which was "Aristar" grade.

Acrylamide, used for protein gels, and Tris, Eastman Kodak.

SDS (especially pure), bis and Acrylamide (especially pure for electrophoresis, used for nucleic acid gels) BDH Chemicals Ltd.

PMSF, ATP (Sodium Salt Grade 1), TEMED, Sucrose, Agarose Spermidine, BSA and Cycloheximide, Sigma Chemical Co.

GTP (Trisodium Salt), PEP (Potassium Salt) and Chloramphenicol, Boehringer Mannheim GmbH.

Calcium Leucovorin, Lederle Division of Cyanamide of G.B. Ltd.

PPO, International Enzymes Ltd.

Coomassie Brilliant Blue R, Raymond A. Lamb Ltd.

Triton X-100, Hopkins and Williams Ltd.

Butyl - PBD, Intertechnique Ltd.

Glass distilled water was used throughout unless otherwise indicated.

(VI) Viral RNAs

MS2 a gift from Dr. J. Atkins, Cold Spring Harbor Laboratory, N.Y., U.S.A.

Qβ
R17
PP7
Cowpea TMV
Wild type TMV

} a gift from Dr. J. Davies, John Innes Institute, Norwich, U.K.

BMV a gift from Dr. T. Hall, University of Wisconsin, Wi., U.S.A.

(VII) Miscellaneous

L-Amino acids and proteins used for molecular weight markers. Sigma Chemical Co.

Sephadex G-200, Medium and G25, Course. Pharmacia.

Antisera raised against:

(a) Spinach SSu RuBPCase.

(b) Spinach RuBPCase.

(c) Wheat SSu RuBPCase.

(d) Spinach RuBPCase linked to Sepharose 4B.

(a) and (b) were gifts from Dr. W. Bottomley, C.S.I.R.O., Canberra City, Australia, (c) and (d) were gifts from Dr. A. Gatenby, University of Edinburgh, U.K.

Wheat Basal and Apical Region RNA, a gift from Dr. C.J. Leaver, University of Edinburgh, U.K.

Cucumber cotyledon poly $(A)^+$ and poly $(A)^-$ RNA, prepared by the method of Aviv and Leder (1972), a kind gift from Ms. E. Weir, University of Edinburgh, U.K.

Section (B) Methods

(I) Cucumber Seedling Growth Conditions and Harvesting

Seeds of Cucumber were imbibed in the dark at 4°C for 16 hours in distilled water. They were planted in trays of Vermiculite (Vermi Peat Ltd.) at a depth of about 1 cm, underlaid with John Innes Potting Compost No. 1. Trays were either kept in continuous darkness or illuminated with a 12 hour photoperiod with a mixture of fluorescent and incandescent lamps at an approximate intensity of 6500 lux. In both light- and dark-grown conditions the temperature was maintained at 26-28°C for 12 hours followed by a night depression to 22°C for 12 hours. Trays were watered daily with tap water, in the dark, watering was carried out under a green safe light. Cotyledons were harvested at daily intervals from Day 0 (cold-imbibed only) to Day 7 (light-grown seedlings) and from Day 1 to Day 8 (dark-grown seedlings). Harvesting was initiated about 1 hour after the onset of illumination for the light-grown seedlings. Cotyledons were harvested onto dry ice and stored at -80°C.

(II) Pea, Spinach and Wheat Growth Conditions and Harvesting

These plants were grown under normal greenhouse conditions in John Innes Potting Compost No. 1. For Pea and Spinach expanding first green leaves were harvested, in Wheat, leaves from 7-day old seedlings were used. The tissue was used either immediately or stored at -80°C.

(III) Cucumber Cotyledon Protein Preparation

All operations were carried out at 4°C. 20 cotyledons from each day of light- and dark-grown development were ground in 8 ml of Grinding Buffer: 50 mM Tris-acetate pH 8.5; 50 mMK acetate; 5 mM Mg acetate; 2 mM DTT using a Willem's Polytron, (Northern Media Supply Ltd.). The homogenate was poured into preweighed centrifuge tubes and the volume estimated by weight. 1 ml samples are taken and are here referred to as Homogenate Proteins. The rest of the homogenate was centrifuged at 10,000 x g for 15 minutes. The supernatants were removed and are here referred to as Supernatants or Soluble proteins. Both Homogenate and Supernatant proteins were stored at -20°C.

Enzyme, protein and chlorophyll assays were carried out using the Homogenate or Soluble fractions.

(IV) Protein Estimation

The method was based on that of Lowry et al. (1951), whereby production of a blue colour relies on the reduction of Folin reagent by the amino acids tyrosine, tryptophan, and cysteine.

Stock Solutions

(1) 2.5% (w/v) $CuSO_4 5H_2O$.

(2) 6% (w/v) Na-K Tartrate.

(3) 6% (w/v) Na_2CO_3 in 0.5 N NaOH.

(4) 0.01 N NaOH.

(5) 0.4 N NaOH.

(6) A standard solution of Bovine Serum Albumin, (BSA), 1 mg/ml in 0.4 N NaOH.

(7) Commercial Folin and Ciocalteu's Phenol reagent diluted 1:7 with water.

Solutions (1, 2 and 6) were stored at 4°C; solutions (3, 4 and 5) were stored at room temperature and solution (7) was made fresh.

An appropriate amount of sample to be estimated for protein (approximately 20-100 ug) was precipitated in Eppendorf centrifuge tubes with an equal volume of 8% (w/v) TCA or if a pellet, was resuspended in 4% (w/v) TCA and left on ice for 20 minutes. If the sample contained chlorophyll this was removed prior to TCA precipitation by the addition of acetone to a final concentration of 80% (v/v). Tubes were centrifuged in Micro-Haematocrit (Gelman Hawksley Ltd.) or Eppendorf bench centrifuge at 12,000 x g for 3 minutes. The pellet was washed twice with 4% TCA. The resulting pellet was hydrolysed in a known amount of 0.4 N NaOH at 45°C for an hour or overnight at room temperature. 100 ul of the resulting solution was assayed. This was made to 1 ml by the addition of 0.01 N NaOH. and 1 ml of reagent mixture was added. Reagent mixture was made fresh by the addition of solutions (1), (2) and (3) in a 1:1:50 ratio. After 30 minutes at room temperature 1 ml of solution (7) was added while vortex-mixing. Samples were allowed to stand at room temperature for 30 minutes after which the colouration was measured with a Corning Colorimeter 252 using a red (600 nm) filter. Protein content was determined relative to a calibration curve constructed by testing dilutions of solution (6) (0-100 ug).

(V) Chlorophyll Estimation

Chlorophyll was extracted by solubilisation in 80% acetone and was quantitated by determining the absorbance at the absorption maxima for chlorophylls a and b and applying an equation which

related absorbance to the amount of chlorophyll in the acetone extract.

Routinely, 1 ml of Supernatant was added to 20 ml acetone and filtered. 1 ml of 0.05 M phosphate, pH 7.5 was also added to 20 ml acetone and filtered, this serves as a blank. The absorbance of the extract, read against the blank, was obtained at A_{645} and A_{663} using a Pye Unicam SP8-100 Spectrophotometer.

Quantitation of the concentration of chlorophyll in the acetone extract (in mg/ml) was obtained using the equation

$$20.2\ (A_{645}) + 8.02\ (A_{663})$$

Chlorophyll estimations can be carried out on smaller amounts of starting material if required. When a large number of chlorophyll estimations were being carried out it was more convenient to centrifuge the extract at 1,500 x g for 10 minutes in a Gallenkamp Junior Bench Centrifuge, than carry out the filtration step. The absorbance of the supernatant being determined.

(VI) SDS-Polyacrylamide-Gel Electrophoresis of Proteins

Polyacrylamide-gel electrophoresis was carried out using modifications of the method of Laemmli (1970). Slab gels were used essentially as described by Laemmli and Favre (1973), using two glass plates, 18 x 22 x 0.4 cm and 18 x 20 x 0.2 cm separated by Perspex spacers lightly coated with vacuum grease. The dimensions of the side spacers were 18 x 1 x 0.1 cm and the bottom spacers 18.5 x 1 x 0.1 cm. The assembled glass plates were held in position by bulldog clips.

Stock Solutions

(1) Acrylamide: 30% (w/v) Acrylamide, 0.2% (w/v) bis-acrylamide.

(2) Stock Buffers: (a) x 5 Separating-gel Buffer: 1.875 M Tris-HCl pH 8.8.

(b) x 10 Stacking-gel Buffer: 0.6 M Tris-HCl pH 6.8.

(3) Electrophoresis Buffer: 0.05 M Tris, 0.384 M Glycine, 0.1% (w/v) SDS.

(4) 10% (w/v) SDS.

(5) 10% (w/v) AMPS.

(6) TEMED.

(7) Protein Sample Buffer: 60 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (w/v) Sucrose, 0.001% Bromo-phenol blue.

Solution (1) was filtered, stored in the dark at 4°C and used within 2 weeks of preparation, solutions (2a) and (2b) were made fresh each week and stored at 4°C, solutions (3) and (5) were made fresh, solutions (4) and (6) were stored at room temperature at 4°C respectively and solution (7) was made a x4 strength and stored at -20°C.

Slab gels of 50 ml total volume consisted of 40 ml of separating gel: 0.375 M Tris-HCl pH 8.8, 0.1% SDS, 20 µl TEMED 200 µl 10% AMPS with the appropriate amount of Stock Acrylamide to give a final concentration of either 15% or 20%. The Acrylamide, Tris and distilled water were mixed, degassed using a vacuum pump before the addition of the SDS, TEMED and AMPS. This was then cast into the gel apparatus. Once cast the separating gel was gently

over-laid with water. When set, the water was removed from the top of the gel and 10 ml of the stacking gel were cast on top of the separating gel. The stacking gel consisted of: 0.06 M Tris-HCl, pH 6.8, 0.01% SDS, 15 µl TEMED, 100 µl 10% AMPS and Acrylamide to produce a final concentration of 5%. A Perspex slot former, producing either 10 or 20 slots, was then carefully inserted into the stacking gel and the gel left to polymerise.

Once polymerised, the slot former and the bottom spacer were removed and the gel placed in the bottom electrophoresis tank containing Electrophoresis Buffer. Care was taken to remove trapped bubbles of air at the bottom of the gel. The slots were filled with Electrophoresis Buffer and in each slot was underlaid the protein sample in Sample Buffer. Electrical contact was made between the top of the gel and the top electrophoresis tank by four layers of Whatman 3 MM chromatography paper, saturated with Electrophoresis Buffer. The electrodes were connected, (-)-ive at the top tank, (+)-ive at the bottom tank. Gels were electrophoresed at a constant current 8-10 mA overnight or until the Bromophenol blue reached the bottom of the gel. In the case of 20% gels to produce a better separation of the proteins, if required, gels were electrophoresed for up to 90 minutes after the dye front had reached the bottom of the gel.

(VII) Staining, Drying Down, Autoradiography, Fluorography and Photography of SDS-Polyacrylamide-Gels

(a) Staining

Gels were fixed and stained for two hours by shaking in 0.2% (w/v) Coomassie Brilliant Blue R in 40% (v/v) Methanol, 7% (v/v) Acetic acid and destained in changes of 40% Methanol, 7% Acetic acid at

room temperature.

(b) Drying Down Gels

The gels were washed in tap water for 3 minutes and sandwiched between a sheet of Whatman 3 MM chromatography paper and a sheet of polythene sheeting. This was then placed, polythene side down, on an aluminium sheet over a water bath, set at 70°C. Two sheets of porous polythene (Vyon: Porvair Ltd.) were placed on top of the Whatman paper and the whole was covered by a rubber mask clamped at the edges and attached by a tube to a vacuum pump. The gels were dried under vacuum for about 2 hours. The whole apparatus was then disassembled and the polythene sheet peeled away from the gel, now dried onto the Whatman paper.

(c) Autoradiography

Dried gels were exposed to Kodak Regulix BB5 at room temperature for between one and two weeks. The film was developed in Ilford Phen-X developer and fixed in Ilford IF-23 Standard fixer. Autoradiographs were scanned using a Kipp and Zonen Densitometer with an Integrator to estimate the percentage total radioactivity incorporated into a particular protein band.

(d) Fluorography

The first step of the technique was to impregnate the gel with PPO as described by Bonner and Lasky (1974). This was done directly after destaining the gel or on a dried gel that had been rehydrated. Rehydration was carried out by soaking the dried gel in warm water, approximately 37°C, for 5 to 10 minutes and carefully removing the Whatman paper.

Gels were rinsed in water and then soaked for 90 minutes in three changes of 5 gel volumes of DMSO. The gels were then soaked in 3 gel volumes of DMSO containing 20% (w/v) PPO for 3 hours. The PPO was precipitated in the gel by washing in 4 changes of 20 gel volumes of water in 30 minutes. The gel was then dried onto Whatman 3MM paper as previously described.

Gels were exposed to Kodak X-Omat XH1 film which had been prefogged as described by Laskey and Mills (1975). This was achieved by exposing the film to a single 1/1000 second flash from a Sun Pak GT32 flash gun covered with Ilford S902 filter and this in turn covered by Whatman Grade 1 filter paper acting as a diffuser at a distance of approximately 22 cm from the film. The flashed side of the film was placed next to the dried gel and exposed at -80°C for between one and two weeks. Developing was carried out in complete darkness in Ilford Phen-X developer and fixed in Ilford IF-23 standard fixer.

(e) Photography

Photography of gels, autoradiographs or fluorographs was carried out by illuminating them on a light box and using Ilfodata HS23 film rated at 20 ASA, developed for 10 minutes in Ilford Microphen and printed on Ilfospeed Grade 5 paper.

(VIII) Electrophoretic Assay of RuBPC Protein

(a) Estimation of Complete RuBPCase

Amounts of complete RuBPC protein can be determined by electrophoresis under non-denaturing conditions (Bennett and Scott, 1971). Homogenate proteins were fractionated on non-denaturing 5% (w/v) polyacrylamide tube-gels and the amount of RuBPC quantitated by

scanning the stained protein in the ultraviolet. This was carried out essentially as described in Becker et al. (1978).

Stock Solutions

(1) Acrylamide: 20% (w/v) Acrylamide, 0.5% bis-acrylamide.

(2) Stock Buffer: 0.35 M Tris adjusted to pH 9.5 with 20% Glycine.

(3) 0.15% (w/v) AMPS.

(4) TEMED.

(5) Protein Sample Buffer: 50 mM Tris-Glycine pH 9.5, 10% (w/v) Sucrose, 0.001% Bromophenol blue.

Solution (1) was filtered, stored in the dark at 4°C and used within 2 weeks of preparation, solutions (2), (3) and (5) were made fresh and solution (4) was stored at 4°C.

Gels of 7.5 cm length were cast in Perspex tubes (1 x 9 cm inside diamter 7 mm) at the final concentration of 5% Acrylamide; 0.125% bis-acrylamide; 0.0875 M Tris-Glycine pH 9.5, degassed and polymerised with 0.75% AMPS and TEMED (20 µl per 60 ml gel solution) and over-laid with water. Electrophoresis buffer was a 1:5 dilution of the Stock Buffer with distilled water. Gels were pre-run at 1-1.5 mA/gel for 30 minutes. Samples of Homogenate protein (100 µg-250 µg) were loaded in approximately 100 µl of Protein Sample Buffer. Gels were run at 1-1.5 mA/gel for about 3 hours, twice the time it takes the Bromophenol blue to reach the bottom of the gel. Gels were removed by air pressure and stained overnight in: 0.1% (w/v) Napthalene Black 12 B; 20% (v/v) Ethanol; 7% (v/v) Acetic acid and destained with frequent changes of 20% Ethanol; 7% Acetic acid.

The tube gels were scanned using a Joyce Loebl Polyfrac UV Scanner with a 265 nm Interference filter. The area of the print out of the RuBPCase peak, identified by co-electrophoresis of a marker RuBPCase, was estimated. Thus values obtained are purely a relative estimation.

(b) Estimation of RuBPCase Subunits

Homogenate proteins were fractionated on 15% polyacrylamide-gels containing SDS as previously described. The gels were stained with Coomassie Brilliant Blue R as described previously. The polypeptide bands of LSu and SSu RuBPCase were identified by co-electrophoresis of marker RuBPCase. The wet gel was scanned on a Vitatron "Flying Spot" TLD 100 Densitometer with a 585 nm Interference filter. Areas under the print out corresponding to LSu and SSu were estimated. Thus, again, values obtained are purely a relative estimation.

(IX) Nucleic Acid Extraction

(a) Total Nucleic Acid

The method used was adapted from Leaver and Ingle (1971).

Stock Solutions

(1) Grinding Buffer: 6% (w/v) Para-aminosalicylic acid, (PAS), 1% (w/v) Tri-propylnaphthalenesulphonic acid, (TNS), 100 mM Tris-HCl pH 8.5.

(2) Phenol/Cresol Solution: Phenol containing: 10% (v/v) re-distilled m-cresol 0.1% (w/v) 8-hydroxyquinoline and saturated with 0.1 M Tris-HCl pH 8.5.

Solution (1) was made fresh, solution (2) was stored at $4^{\circ}C$ in the dark. All operations were carried out at $4^{\circ}C$.

The tissue was homogenised with a pestle and mortar or with a Willem's Polytron homogeniser at full speed for 5-10 seconds in 2-5 volumes of Grinding Buffer. An approximately equal volume of Phenol/Cresol Solution was added and the solutions mixed thoroughly and then poured carefully into a centrifuge tube. After centrifugation at 2,000 x g for 15 minutes the aqueous layer was carefully removed and re-extracted twice with Phenol/Cresol or until no more white precipitate was seen at the aqueous/organic interphase. Nucleic acid was precipitated from the aqueous phase by the addition of 2 volumes of Ethanol and storage at -20°C. The precipitate was collected by centrifugation at 2,000 x g for 15 minutes and washed twice in 80% Ethanol and dried in a vacuum desiccator at 4°C.

The dry pellet was resuspended in cold sterile water and the concentration estimated by the removal of duplicate 5 µl aliquots and measuring the A_{260} of a 1:200 dilution with water. It was assumed that 1 O.D. unit at 260 nm was equivalent to 40 µg/ml of nucleic acid. An indication of purity of the nucleic acid was obtained by measuring the A_{260}/A_{280} ratio which was generally approximately 1.97. The nucleic acids were stored at -20°C. Quantitation of total RNA was carried out by the Perchloric acid method as described by Becker et al. (1978), which was modified from Schmidt and Thannhauser (1945).

(b) Chloroplast RNA

Chloroplast extraction was carried out by a modification of the method of Cockburn et al. (1968); the RNA extraction was a modification of that of Leaver and Ingle (1971).

Stock Solutions

(1) Chloroplast Extraction Buffer: 0.33 M Sorbitol, 50 mM Tris-HCl pH 7.0, 5 mM $MgCl_2$, 1 mM NaCl.

(2) Nucleic Acid Extraction Buffer: 50 mM Tris-acetate pH 8.5, 50 mM KCl, 2% TNS, 12% PAS.

Both solutions were made fresh.

Before harvesting tissue was destarched (by leaving it in the dark) for 24 hours. All operations were carried out at 4°C (Leaver 1973). Chilled tissue was homogenised in 4 volumes of semi-frozen Chloroplast Extraction Buffer with a Willem's Polytron homogeniser with two, 4 second bursts at half speed. The macerate was squeezed through 2 layers of muslin and filtered through 8 layers of muslin into 50 ml plastic centrifuge tubes and centrifuged from rest to 4,000 x g to rest, in approximately 90 seconds. The supernatant was removed and the pellet was gently resuspended in Chloroplast Extraction Buffer using a paint brush. The chloroplasts were washed three times using this procedure. At this point the chloroplasts were either frozen at -80°C or the RNA extracted directly.

The chloroplasts were resuspended in Chloroplast Extraction Buffer lacking Sorbitol, which lyses the chloroplasts. To the resulting solution was added an equal volume of Nucleic Acid Extraction Buffer and the whole was vortex-mixed thoroughly. To this was added an equal volume of Phenol/Cresol Solution and mixed thoroughly (as previously described). The aqueous layer was extracted twice with Phenol/Cresol. The RNA was precipitated, washed, dried and resuspended in sterile water as described in the preceding section and stored at -20°C.

(c) Polysome Extraction

Polysome extraction was carried out essentially as described by Leaver and Dyer (1974).

Stock Solutions

(1) Grinding Buffer: 100 mM Tris-acetate pH 7.5, 250 mM Sucrose, 20 mM Mg acetate, 200 mM K acetate, 1 mM DTT.

(2) Sucrose Cushion: 100 mM Tris-acetate pH 8.5, 20 mM Mg acetate, 200 mM K acetate, 1 mM DTT, 1 M Sucrose.

(3) Resuspension Buffer: 50 mM HEPES pH 7.5, 5 mM Mg acetate, 50 mM K acetate, 1 mM DTT.

All solutions were made fresh.

All operations were carried out at 4°C, 4 g of fresh tissue were homogenised with two, 5 second bursts at full speed with a Willem's Polytron homogeniser in 7.5 ml of Grinding Buffer. Either 20% (v/v) Nonidet P40 or 20% (w/v) Triton X-100 both in 100 mM Tris-acetate pH 7.5, 0.25 mM Mg acetate, 200 mM K acetate was added to give a final concentration of 3% detergent and vortex-mixed. The solution was filtered through Miracloth (Calbiochem. Ltd.) and centrifuged at 20,000 x g for 10 minutes. The supernatant was carefully layered over a 3 ml Sucrose Cushion and centrifuged at 105,000 x g (in an angle rotor) for 5 hours. The pellet was resuspended in 500 µl - 600 µl Resuspension Buffer and 10 µl aliquots were removed for cell-free incubations, each incubation containing approximately 20 µg RNA.

If required the RNA was extracted by the addition of 6% PAS, 1% TNS in 100 mM Tris-HCl pH 8.5, as previously described.

(X) Polyacrylamide-Gel Electrophoresis of Nucleic Acids.

The method used was that of Loening (1967) as modified by Leaver and Ingle (1971) and Leaver (1973).

Stock Solutions

(1) Acrylamide: 15% (w/v) Recrystalised Acrylamide , 0.75% (w/v) bis-acrylamide.

(2) x 5 Stock Buffer: 150 mM NaH_2PO_4, 180 mM Tris pH 7.6-7.8, 5 mM EDTA.

(3) 10% (w/v) AMPS.

(4) TEMED.

Solution (1) was made monthly and stored in the dark at 4°C, solutions (2) and (4) were stored at 4°C and solution (3) was made fresh.

Gels were made up with the final concentration of Acrylamide of 2.4% in a 1:5 dilution of x 5 Stock Buffer. The mixture is degassed and for each 5 ml of the gel mixture was added 25 µl of TEMED and 0.25 ml 10% AMPS. 7.5 cm gels were cast in Perspex tubes (1 x 9 cm, inside diameter 7 mm) and gently overlaid with water. The electrophoresis buffer used was a 1:5 dilution of x 5 Stock Buffer with water and SDS added to a final concentration of 0.05% (w/v). Electrophoresis was carried out at 4°C. The gels were pre-run at 5 mA/gel for 30 minutes. The RNA was loaded onto the gels in electrophoresis buffer made 5% (w/v) Sucrose. Between 10-100 ugm of RNA were loaded onto each gel. Gels were electrophoresed at 6 mA/gel for 3½ hours. Gels are removed from the tubes by air pressure, washed in distilled water for 30 minutes and scanned in a Joyce Loebl Polyfrac UV scanner at 265 nm using a liquid filter of 100 ug /ml of p-dimethylaminobenzaldehyde in

Methanol. To quantitate chloroplast and cytoplasmic RNAs, individual peaks were cut from the scans, weighed and expressed as a fraction of the total area under the trace for a particular scan. Each such fraction was then multiplied by the total RNA content (in µg/cotyledon) determined as described previously. RNA fractionation by this method also allowed me to assess the purity of chloroplast RNA preparations which generally contained less than 5% cytoplasmic ribosomal RNA.

(XI) Preparation of Cucumber Etioplasts

The method used for etioplast preparation and purification was modified from that of Leese et al. (1971).

Stock Solutions

(1) Stock Buffer: 67 mM KH_2/Na_2HPO_4 pH 8.0, 1 mM $MgCl_2$ 0.2% BSA.

This was made fresh.

All operations were carried out at 4°C using prechilled materials. 1 g of Day 5 dark-grown Cucumber cotyledons were harvested and homogenised in 4 ml of Stock Buffer containing 0.5 M Sucrose using a Willem's Polytron. The homogenate was squeezed through 2 layers of muslin and then filtered through 8 layers of muslin into centrifuge tubes and centrifuged from rest to 4,000 x g to rest, in 90 seconds. The pellet was gently resuspended in 5 ml of the grinding buffer using a paint brush. The suspension was layered onto a 20 ml cushion of Stock Buffer containing 0.6 M Sucrose and centrifuged at 440 x g (in a swinging-bucket rotor) for 15 minutes. The pellet was carefully resuspended in 5 ml of Stock Buffer containing 0.6 M Sucrose. The etioplast

suspension was further purified by layering onto a discontinuous gradient made up of a 10 ml step of Stock Buffer containing 1.3 M Sucrose over a 10 ml step of Stock Buffer containing 2 M Sucrose and centrifuged at 2,000 x g (in a swinging-bucket rotor) for 15 minutes. The etioplasts appear 'hazily white' at the interphase and were collected and dialysed for 30 minutes against Stock Buffer containing 0.3 M Sucrose to reduce the Sucrose concentration. Samples from the resulting solution were heated in protein gel sample buffer at 90°C for 3 minutes and analysed by SDS-polyacrylamide-gel electrophoresis as previously described.

(XII) Partial Purification of Cucumber Chloroplast ATPase

Partial purification of Chloroplast ATPase was carried out using the preliminary steps of the method of Younis et al. (1977). Chloroplasts were isolated from Day 6 light-grown Cucumber cotyledons using the Sorbitol Extraction Buffer as previously described and washed once in this medium. The chloroplast pellet was resuspended in: 10 mM NaCl, 2 mM Tricine-NaOH pH 8.0 and allowed to stand on ice for 15 minutes. Thylakoids were collected by centrifugation at 20,000 x g for 10 minutes and washed twice. All subsequent operations were carried out at room temperature. The washed thylakoid membranes were resuspended at a chlorophyll concentration of 2.5-30 mg/ml in 0.35 M Sucrose, 10 mM Tris-SO_4 pH 7.6; 1 mM EDTA and 5 mM DTT. To this was added a 0.5 volume of chloroform and shaken for 15 seconds. The aqueous layer was separated by centrifugation at 1,000 x g for 5 minutes, collected and recentrifuged at 50,000 x g for 30 minutes. Protein in the supernatant was precipitated by the

addition of 1.5 volumes of saturated $(NH_4)_2 SO_4$. The precipitate was resuspended in 20 mM Tricine- Na OH pH 8.0, 2 mM EDTA, 10 mM NaCl, 1 mM DTT and analysed by SDS-polyacrylamide-gel electrophoresis as previously described.

(XIII) Preparation of Cucumber RuBPCase

The method used was a modification of that of Chen et al. (1976).

All operations were carried out at 4°C. 30 g of Day 6 light-grown cotyledons were homogenised with a Willem's Polytron in 3 volumes of 50 mM Tris-HCl pH 7.8, 0.2 M NaCl, 10 mM Sodium metabisulphite, 1 mM KCN, 0.1% (w/v) BSA, 20% (w/v) Dowex AG2-X8, 50-100 mesh (Bio-Rad Laboratories Ltd.), 50 µg/ml PMSF. The homogenate was squeezed through 2 layers of muslin and centrifuged at 100,000 x g for 1 hour. The supernatant was removed and protein precipitated between 20%-50% (w/v) $(NH_4)_2SO_4$. The precipitate was taken up in 50 mM Tris-HCl pH 7.8, 0.1 M NaCl and loaded onto a Sephadex G200 Medium column, (80 x 2.5 cm, Pharmacia) equilibrated with 50 mM Tris-HCl pH 7.8, 0.2 M NaCl.

Fractions were collected and to identify those containing RuBPCase 2 µl aliquots were assayed by Single Dimension Immunodiffusion (described in detail later) using antisera raised against Spinach RuBPCase. Those fractions containing RuBPCase were collected and their purity assayed by fractionation on SDS-polyacrylamide-gels as previously described.

To purify LSu and SSu RuBPCase, protein prepared as described above, was fractionated by SDS-polyacrylamide-gel electrphoresis. The subunits were located by staining with Coomassie Brilliant Blue

R, as previously described, for no longer than 15 minutes. The gel was destained for no longer than 15 minutes. The regions of the gel containing the subunits were excised and the protein recovered by electroelution. Gel pieces were placed in Perspex tubes, used normally, as previously described, for polyacrylamide-tube gels. One end was covered by a bag of dialysis tubing. Electro-elution was carried out using SDS-polyacrylamide-gel Electrophoresis Buffer, (previously described) at 5 mA per tube for 3 hours, or, until the Coomassie stain had migrated completely into the dialysis bag. When the electroelution was complete, the polarity of the electric current was reversed for 30 seconds. The contents of the dialysis bag were carefully collected using a syringe. Aliquots were analysed by SDS-polyacrylamide-gel electrophoresis as described previously and antisera was raised against the protein present in each sample as described later.

(XIV) Preparation of the E. coli Extract

The extract was prepared essentially as described by Modollel (1971). E. coli cells (either PR7 or CSH 73 strain) were grown at 37°C in L broth in a 50 litre fermentor under vigorous aeration. Cells were grown to mid-log phase (A_{675} 0.6 units) and harvested using an Alpha Leval continuous flow centrifuge. Cell growth was terminated by placing the collected cells on ice. Harvesting the cells at later stages (A_{675} 1.6 units) resulted in extracts with reduced activities which is probably due to increased RNAase activity in the extract (J. Atkins, personal communication).

Cells were washed three times in Buffer A; 10 mM Tris-acetate pH 8.2, 10 mM Mg acetate, 60 mM NH_4 acetate, 6 mM β-mercaptoethanol, centrifuging at 12,000 x g for 5 minutes at 4°C. After each wash the cells were resuspended with a glass rod. Unless the cells were to be used immediately they were frozen and stored at -80°C.

Cells were broken using a pestle and mortar, as described by Nirenberg and Matthaei (1961). All operations were carried out at 4°C using pre-chilled materials. Fresh, or thawed stored cells, approximately 10 g, were ground with twice the cell weight of Alumina (Sigma Chemical Co.) until the mixture was homogenous and emitted 'popping sounds', in an unglazed pestle and mortar. Two volumes of Buffer A per g of ground cells was added and ground for a further minute. The paste was carefully poured into centrifuge tubes and alumina, whole cells and debris sedimented by centrifugation at 22,000 x g for 10 minutes. The top three quarters of the supernatant was removed and clarified by centrifugation at 30,000 x g for 30 minutes. The supernatant was removed and placed in a pre-cooled graduated test tube. Then for each ml of supernatant was added: 100 µl 1 M Tris-acetate pH 8.2, 20 µl 0.14 M Mg acetate, 6.6 µl 120 mM ATP, 18 µl 500 mM PEP, 10 µl 10 mM GTP, 10 µl 100 mM DTT, 20 µl 0.5 mM amino acids lacking methionine, 20 µl 0.5 mM methionine, 0.5 µl 10 µg/ml (0.45 units/µg) Pyruvate Kinase (PK), 88 µl sterile distilled water and the whole incubated at 37°C in the dark for 80 minutes.

The preincubated extract was dialysed for 18 hours against at least four changes of Buffer A. The extract was clarified

by centrifugation at 30,000 x g for 30 minutes and 200 µl aliquots of the supernatant were stored under liquid nitrogen or at -80°C. The A_{260} was determined by a 1 to 200 dilution in water and the concentration in the order of 200 O.D. units/ml. Assuming that 1 O.D. unit at 260 nm is equivalent to 40 µg/ml of nucleic acid gives an approximate estimation of ribosomal content. Hereafter the extract will be referred to as the E. coli extract or the S-30 extract.

(XV) Conditions of Incorporation of [^{35}S] met by the E. coli Extract

What will be referred to hereafter as the E. coli translation system (or E. coli system) was made up of 10 µl E. coli extract (A_{260} 2 units), incubated with 10 µl of E. coli Reaction mixture and 30 µl of sterile distilled water containing an appropriate amount of RNA. Each 50 µl incubation was carried out at 37°C for 20 minutes.

Generally a cell-free protein-synthesizing experiment was carried out using 20 separate 50 µl incubations. First the appropriate amount of RNA solution was pipetted into 500 µl Eppendorf centrifuge tubes, the volume made up to 30 µl with sterile distilled water and the tubes placed on ice. Each incubation could be carried out using up to 200 µg RNA, however, as described in the text, 15-30 µg RNA were generally used.

The Reaction mixture was made up of the following constituents:

x 20 Salts: 960 mM Tris-acetate pH 8.0

160 mM Mg acetate

200 mM K acetate

1.26 M NH_4 acetate

2 mg/ml Calcium Leucovorin

x 20 Energy: 120 mM ATP pH 7.0

20 mM GTP

200 mM PEP

x 20 Amino acids: 0.5 mM of each of 19 amino acids (-met)

Pyruvate Kinase (PK) 4.2 mg/ml (450 units/mg)

500 mM DTT

$[^{35}S]$ met.

The x 20 Salts, x 20 Energy, x 20 Amino acids and DTT were made up separately and stored at -20°C. The Pyruvate Kinase was stored at 4°C and the $[^{35}S]$ met was stored at -80°C. The concentrations of the constituents of the Reaction mixture were such that 10 µl in 50 µl incubations gave the final concentrations: 50 mM Tris-acetate pH 8.0, 75 mM NH_4 acetate, 10 mM Mg acetate, 10 mM K acetate, 10 mM PEP, 6 mM ATP, 1 mM GTP, 0.48 units PK, 2 mM DTT, 160 mM Calcium Leucovorin, 0.025 mM 19 amino acids (-met), 5 µCi $[^{35}S]$ met. This also takes into account the ions contributed by the E. coli extract.

10 µl aliquots of the Reaction mixture were added to the centrifuge tubes containing RNA and water and then 10 µl of the E. coli extract were added. The final mixture was briefly vortex-mixed and the incubation started by placing the centrifuge tubes in a water bath at 37°C. The time course of incorporation of radioactivity into protein was followed by the removal of 5 µl aliquots at 5 minute intervals and spotting onto Whatman 3 MM chromatography paper and analysing as described later. If immunoprecipitation were not being carried out the incubation was

terminated after 20 minutes by the addition of acetone to produce a final concentration of 80% (v/v) and the tubes stored at 4°C. The precipitates were prepared for protein gel electrophoresis as described later.

(XVI) Preparation of the Wheat Germ Extract

The method of preparation was adapted from that of Marcu and Dudock (1974). All operations were carried out at 4°C using pre-chilled materials.

5 g of Wheat germ (General Mills, stored at 4°C) were ground in a pestle and mortar with 5 g of powdered glass to produce a powder. To this was added 13 ml of Buffer B: 20 mM HEPES pH 7.6, 120 mM KCl, 5 mM Mg acetate and ground further to produce a paste. The homogenate was centrifuged at 23,000 x g for 15 minutes. The supernatant was removed with care to avoid collecting lipid and loaded onto a Sephadex G25 Course column (26 x 15 cm, Pharmacia) equilibrated with Buffer B. The column was then eluted with Buffer B at a flow rate of 3 ml/min. Fractions containing more than 0.5 O.D. unit at 260 nm per 5 µl were collected, bulked and centrifuged at 23,000 x g for 15 minutes. The supernatant was dialysed for 18 hours against at least 3 changes of Buffer B. The extract was finally centrifuged at 23,000 x g for 15 minutes and 200 µl aliquots of the supernatant were stored under liquid nitrogen or at -80°C. A_{260} was determined by a 1 to 100 dilution in water and the concentration in the order of 140 O.D. units/ml.

(XVII) Conditions of Incorporation of [^{35}S] met by the Wheat Germ Extract

What will be referred to hereafter as the Wheat germ translation system was made up of 10 µl of Wheat germ extract (A_{260} 1.4 units) incubated with 10 µl of Wheat germ Reaction mixture and 30 µl of sterile distilled water containing the appropriate amount of RNA. Each 50 µl incubation was carried out at 25°C for 90 minutes.

Generally a cell-free protein-synthesizing experiment was carried out using 20 separate 50 µl incubations. First the appropriate amount of RNA solution was pipetted into 500 µl Eppendorf centrifuge tubes, the volume made up to 30 µl with sterile distilled water and the tubes placed on ice. Each incubation could be carried out using up to 100 µg RNA, however, as described in the text, 5-30 µg RNA were generally used.

The Wheat germ Reaction mixture was made up of the following constituents:

x 20 HKS	480 mM HEPES-NaOH pH 7.6
	1.6 M K acetate
	25 mM Mg acetate
	5 mM Spermidine
x 20 Energy	20 mM ATP pH 7.0
	1 mM GTP
	160 mM Creatine Phosphate
x 50 Creatine Phosphokinase	5 mg/ml in 50% glycerol
x 20 Amino acids	0.5 mM each of 19 amino acids (-met)
100 mM DTT	
[^{35}S] methionine	

The [^{35}S]met was stored at -80°C, all other solutions were made up separately and stored at -20°C.

The concentrations of the constituents were such that 10 µl of Reaction mixture in 50 µl incubations gave the final concentrations: 28 mM HEPES-NaOH pH 7.6, 104 mM K acetate, 2.25 mM Mg acetate, 0.25 mM Spermidine, 1 mM ATP, 0.05 mM GTP, 8 mM Creatine Phosphate, 5 µg Creatine Phosphokinase, 2 mM DTT, 0.025 mM 19 amino acids (-met), 5 µCi [^{35}S]met. This also takes into account the ions contributed by the Wheat germ extract. The Reaction mixture was added to the Wheat germ extract and 20 µl. aliquots of this were pipetted into each centrifuge tube containing the RNA and water. The incubation was started by vortex-mixing the incubation mixture briefly and placing the centrifuge tubes in a water bath at 25°C. The time course of incorporation of radioactivity into protein was followed by the removal of 5 µl aliquots at 10, 20, 60 and 90 minutes and spotting onto Whatman 3 MM chromatography paper and analysing as described later. If immunoprecipitations were not being carried out the incubation was terminated by the addition of acetone to a final concentration of 80% (v/v) and the tubes were stored at 4°C. The precipitate was prepared for protein gel electrophoresis as described later.

(XVIII) Isolation of Plastids for In Vitro Protein Synthesis

Plastid isolation and incubation was essentially as described by Siddell and Ellis (1975). All materials used were sterilized by autoclaving or by washing in methanol and rinsing in sterile water. Isolation and Resuspension Buffers were sterilized by membrane filtration using sterile Sartorius Membrane Filter

apparatus (membrane pore size 0.2 µm).

The following buffers were used:

Isolation Buffer:	0.35 Sucrose
	25 mM HEPES-NaOH pH 7.6
	2 mm EDTA
	2 mM Sodium Isoascorbate.
Resuspension Buffer:	0.2 M KCl
	66 mM Tricine-KOH pH 8.3
	6.6 mM $MgCl_2$

In experiments carried out to optimise the system, 20 g of Day 5 or 6 light-grown cotyledons were homogenised in 100 ml of semi-frozen Isolation Buffer. Homogenisation was carried out with two, 4 second bursts at half speed in a Willem's Polytron. The homogenate was strained through 2 layers of muslin then filtered through 8 layers of muslin into centrifuge tubes. These were then centrifuged from rest to 4,000 x g to rest in approximately 90 seconds. The pellet was carefully resuspended in 5 ml of Resuspension Buffer, using a paint brush. This suspension was kept on ice in the dark and samples removed for chlorophyll and protein estimations and <u>in vitro</u> incubations.

(XIX) <u>Conditions of Incorporation of [^{35}S]met by Isolated Plastids</u>

What will be referred to hereafter as the isolated chloroplast or plastid system was made up of isolated plastids, prepared as described in the previous section, and Resuspension Buffer containing [^{35}S]met. Sterile media and materials were used throughout. Each 300 µl incubation contained between 100-350 µg protein and 10 µCi [^{35}S]met.

Generally a cell-free protein-synthesizing experiment was carried out using 12 separate 300 µl incubations. First the appropriate amount of Resuspension Buffer was pipetted into 7.5 ml glass test tubes with any chemical addition required (see details in text) and 10 µCi [^{35}S] met. Plastids, isolated and resuspended as described in the previous section, were added and the tubes were capped with aluminium tops and reactions were started by placing the tubes in a 20°C water bath under a filtered red light of 3000 lux. Those tubes incubated in the dark were wrapped in aluminium foil. 20 µl aliquots were periodically removed and spotted onto Whatman 3 MM chromatography paper to estimate the time course of the incorporation of radioactivity into protein, and analysed as described later. After 90 minutes the incubation was terminated by the addition of acetone to a final concentration of 80% (v/v). The precipitate was prepared for SDS-polyacrylamide-gel electrophoresis as described later.

Incubations using plastids extracted from differing days of development were carried out in one experiment. In sequence, 5 g of tissue from each Day of development were harvested and homogenised in 40 ml of Isolation Buffer. The crude plastid pellets were resuspended in 1 ml of Resuspension Buffer and incubations were carried out using equal volumes of resuspended plastids with light or 2 mM ATP as a source of energy. Protein concentration of each incubation was between 1.6 - 2.2 mg.

(XX) Estimation of the Incorporation of [^{35}S] met into Protein

Estimation of the incorporation of radioactivity into protein was carried out essentially as described by Mans and Novelli, (1961). Aliquots were removed from the *in vitro* systems periodically and spotted onto 1.5 cm squares of Whatman 3 MM chromatography paper, dried, placed in ice-cold 10% (w/v) TCA for 30 minutes. They were then transferred to 5% (w/v) TCA at 90°C for 15 minutes and then through 4 changes of 5% TCA at room temperature allowing at least 5 minutes in each. The filters were then transferred to a 1:1 (v/v) Ether-Ethanol mixture for at least 15 minutes and then transferred to Ether for at least 10 minutes at room temperature. The filters were then dried and placed in 6 ml scintillation fluid made up of 0.4% (w/v) butyl-PBD in Toluene. Radioactivity was estimated by scintillation counting using an Intertechnique SL-30 liquid scintillation counter. By this method we are in effect estimating the amount of [^{35}S] met incorporation into hot 5% TCA insoluble material. In the text this will be referred to as either, incorporation of [^{35}S] met (or radioactivity) into protein, or incorporation.

(XXI) Preparation of Proteins from the *In Vitro* Systems for SDS-Polyacrylamide-Gel Electrophoresis.

In each case incubations were terminated by the addition of acetone to a final concentration of 80% (v/v). The precipitate was collected by centrifugation in a Micro-Haemotocrit centrifuge at 12,000 x g for 30 seconds. The pellet was resuspended in SDS-polyacrylamide-gel Sample Buffer (described in Section VI). Before loading onto SDS-polyacrylamide-gels, the samples were

heated at 100°C for 2 minutes or at 37°C for 3 hours. The latter treatment was used in the case of the proteins from the isolated plastid system, with frequent vortex-mixing to ensure solubilisation of the protein precipitate. It was found that proteins from a complete E. coli or Wheat germ incubation could be loaded into each slot of a 10 slot gel without overloading. With plastid incubations approximately 250 µg protein were loaded into each slot.

It was found that the polyacrylamide gel profiles of labelled translation products of the E. coli and Wheat germ systems, prepared as described above, were similar to the profiles obtained if the Sample Buffer was added directly to the in vitro translation mixture. The method of acetone precipitation was adopted for convenience in handling.

(XXII) Estimation of Radioactivity Present in Polyacrylamide Gel Slices

The method used was an adaption of that of Tishler and Epstein (1974). The relevant labelled polypeptide was located by autoradiography and excised from the SDS-polyacrylamide-gel. Each gel slice was dissolved in 0.6 ml of 100 volume H_2O_2 in a loosely capped scintillation vial. The top was tightened before the vial was placed in a drying oven at 80°C for 3 hours. The vial was cooled and 5 ml of Triton/Toluene Scintillation fluid (0.4% (w/v) butyl-BPD in a 1:2 mixture of Triton x-100 - Toluene) was added. The vial was briefly shaken and radioactivity estimated by using an Intertechnique SL-30 liquid scintillation counter.

(XXIII) Fractionation of Chloroplast Membranes

The method of fractionation was adapted from that of Douce et al. (1973). All operations were carried out at 4°C. Chloroplasts were extracted from Day 6 light-grown cotyledons in a Sorbitol extraction buffer as described previously (Section IX b). To the chloroplast suspension was added 24 volumes of 10 mM Tris-HCl pH 7.6, 4 mM $MgCl_2$ and the suspensions were allowed to stand for 4 minutes at 4°C. Each 3 ml of the suspension was loaded onto an 8 ml step gradient made up of 2 ml steps of 0.6 M, 0.93 M, 1.2 M and 1.5 M Sucrose in 5 mM Tris-HCl pH 7.6, 5 mM $MgCl_2$. The gradients were centrifuged at 70,000 x g for 60 minutes in an MSE 6 x 14 ml swinging-bucket rotor. The supernatant and the material between each step were collected. It was inferred from the results of Douce et al. (1973) that the supernatant fraction contained stromal proteins and that between the steps in order down the gradient were: envelope membranes, stroma lamellae and finally a mixture of grana and stroma lamellae. The latter fraction was deep green in colour. Proteins were precipitated from each fraction by the addition of an equal volume of 8% (w/v) TCA. The precipitates were analysed by SDS-polyacrylamide-gel electrophoresis as previously described. In the cases where chloroplasts were incubated in vitro with [^{35}S] met and components fractionated, a x5 volume incubation was carried out and the plastid suspension was used directly. Labelled polypeptides were visualized by fluorography following SDS-polyacrylamide-gel electrophoresis as previously described.

(XXIV) Preparation of Staphylococcus aureus Bound Protein A

The preparation was carried out essentially as described by Kessler (1975).

Stock Solutions

(1) PBS Buffer: 150 mM NaCl, 40 mM KH_2PO_4 - NaOH pH 7.2, 0.05% (w/v) Na azide.

(2) NET Buffer: 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 7.4, 0.02% (w/v) Na azide.

Both solutions were stored at -20°C.

The S. aureus cells were grown in supplemented Penassay broth. Before the cells were heat inactivated, all manipulations were carried out in a fume cabinet, all glassware used was autoclaved prior to washing and gloves and face masks were used. The cells were collected by centrifugation at 8,000 x g for 15 minutes and washed twice in PBS Buffer. Between 25-35 g net weight of cells were used and resuspended at a final concentration of 10% (w/v) in PBS. The cells were fixed by the addition of formamide to a final concentration of 1.5% (v/v) and incubated at 22°C for 90 minutes. The cells were recovered by centrifugation at 8,000 x g for 15 minutes, washed in PBS and finally resuspended at a concentration of 10% (w/v) in PBS. The cells were heat inactivated by addition to a 1 litre conical flask and swirling for 5 minutes in a water bath at 80°C, followed by rapid cooling in ice. The cells were then washed three times and finally resuspended at a final concentration of 10% (w/v) in PBS and stored at 4°C.

Just prior to use an appropriate amount of cells were pelleted and resuspended in NET buffer containing 0.5% (v/v) Nonidet P40

and incubated at room temperature for 15 minutes. The cells were washed once in NET buffer containing 0.5% (v/v) Nonidet P40 and finally resuspended in NET buffer at 10% (w/v) concentration. This treatment removed any free or lightly bound Protein A.

(XXV) Preparation of Antisera

Antisera was raised against Cucumber LSu and SSu RuBPCase by an adaptation of the method of Brandt et al. (1967). The samples of protein were prepared by electroelution as described in Section XIII and the final concentration of protein was approximately 30 μg/ml. To this solution was added an equal volume of Freunds Complete Adjuvant and 0.2 ml of emulsion were injected per mouse intraperitoneally with a 25-gauge needle. The injections were repeated every 7 days until 5 injections had been made. Four days after the last injection 0.1-0.2 ml of Sarcoma 180 cells were injected peritoneally to initiate ascites. The Sarcoma cells were obtained from freshly drawn ascitic fluid from a mouse with Sarcoma using a 22-gauge needle. Ascitic fluid was withdrawn from the mouse 4-12 days post-tumor injection using an 18-gauge needle. Between 10-15 ml could be withdrawn from each mouse. Ascitic fluid was usually obtained 2-3 times allowing 5-10 days between fluid collections. The ascitic fluid was clarified before use by centrifugation at 10,000 x g for 30 minutes at 4°C.

(XXVI) Immunodiffusion Tests

The reactions of antisera used against Cucumber proteins were examined by Single or Double Immunodiffusion tests. These were carried out on a micro-scale essentially as described by

Crowle (1961).

Stock Solution

NAT Buffer: 0.14 M NaCl

0.05% Na Azide

20 mM Tris-HCl pH 7.4

(a) Single Immunodiffusion Tests

Gel solutions containing 2% (w/v) Agarose and between 0.1% - 1.0% (v/v) antisera in NAT Buffer were prepared at 60°C. Gel volumes used were either 400 µl, cast on coverslips or 800 µl, cast on microscope slides. If the gel was cast on a coverslip this in turn was attached to a microscope slide by 4 layers of Parafilm (American Can Co.). Once set, holes were punched in the gel in an asymmetric array using a Pasteur pipette attached to a water vacuum line. Approximately 1.5 µl of antigen solution were pipetted into the well, with care to avoid air bubbles. The gels were incubated in sealed Petri dishes or plastic lunch boxes on tissue paper soaked in NAT Buffer at 37°C for 24-48 hours.

Immunoprecipitates were visualised by staining in Coomassie Brilliant Blue R as follows: Gels were dried under pressure with Mediwipes (Kimberley Clark Ltd.) and washed twice for an hour in NAT Buffer. The gel was finally washed for 10 minutes in distilled water, dried with Mediwipes under pressure and finally fixed onto the microscope slide in a warm oven. The gel, dried onto the microscope slide was placed into 0.4% (w/v) Coomassie Blue R, 45% (v/v) Methanol, 7% (v/v) Acetic Acid, (filtered prior to use) for 10 minutes. Destaining for 2 minutes was carried out in 45% (v/v) Methanol 10% Acetic acid. Excess destain was carefully removed from

the microscope slide with Mediwipes and the slide was allowed to stand on a bench to dry at room temperature.

(b) Double Immunodiffusion Tests

This was carried out in the same manner as described for Single Immunodiffusion Tests, except that the antisera was not added to the gel. Once the gel was set a pentagonal array of holes were punched around a central hole at a distance of 7 mm. Antisera was pipetted into the central hole and into the surrounding array of holes were pipetted the antigen solutions. Diffusion was carried out as described in the previous section. The sensitivity was increased by the addition of 0.8% Ficol (Pharmacia) to each well 24 hours after the start of the immunodiffusion. Precipitates were stained and fixed as previously described.

(XXVII) Immunoprecipitation of Labelled Proteins from Cell-free Translation Products

(a) Immunoabsorption

This was carried out using an immunoabsorbant column, (3 x 1.5 cm) of anti-Spinach RuBPC covalently linked to Sepharose 4B, prepared by the method of Gray and Wildman (1976) (a kind gift from A. Gatenby). Labelled E. coli translation products (approximately 10^6 cpm), were added to 120 µl of buffer to produce a final concentration of 50 mM Tris-HCl pH 7.8, 200 mM NaCl and this was loaded onto the column and the binding, washing and elution carried out exactly as described by Gatenby and Cocking (1977). 40 µg of BSA was added to the elutant as carrier and the protein precipitated by the additon of acetone to a final concentration of 80% (v/v). The precipitate was resuspended in

SDS-protein-gel Sample Buffer and fractionated by electrophoresis as previously described. Labelled polypeptides visualised by fluorography of the dried gels.

(b) Direct Immunoprecipitation

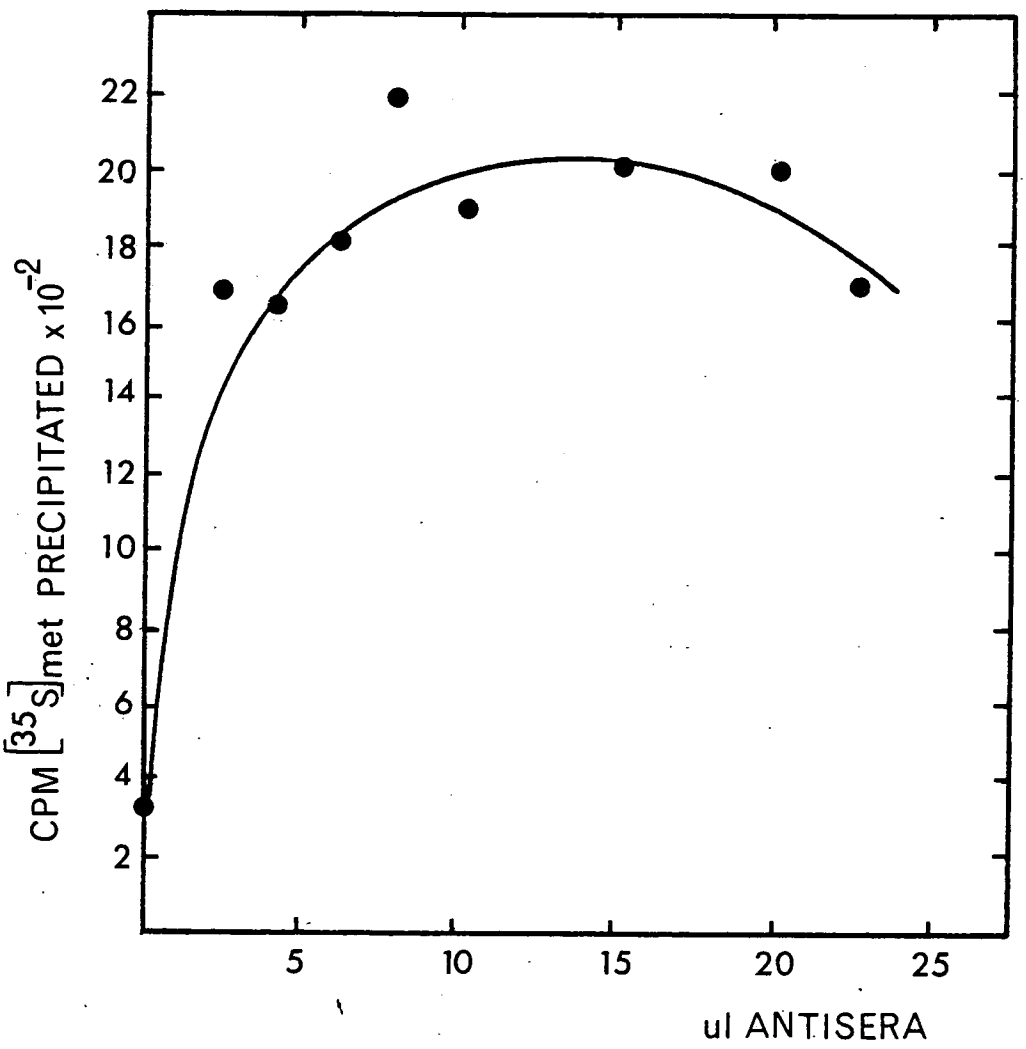
The antisera used to precipitate cell-free products were raised against either Spinach RuBPC or Cucumber LSu and SSu RuBPC as previously described. Precipitation of the antigen-antibody complex was carried out with Staphylococcus aureus bound Protein A (Kessler, 1975). This technique relies on the fact that Protein A binds specifically and strongly to the Fc regions of most mammalian IgG subclasses (Kornvall et al., 1970).

Immunoprecipitations were carried out using the complete incubation mix of the E. coli system or the ribosomal supernatant from Wheat germ incubations. In the latter case this was prepared by centrifugation of the incubation mixture at 105,000 x g for 1 hour and aliquots of the supernatant were used for immunoprecipitation. Generally to 25 µl or 50 µl of the incubation mix or the ribosomal supernatant was added a suitable amount of antisera and the volume was made up to 150 µl or 300 µl by the addition of Saline Buffer: 10 mM Tris - HCl pH 7.5, 5 mM EDTA, 1% (w/v) Triton X-100 and 500 mM NaCl. The amount of antisera to be used in each immunoprecipitation was obtained by titration experiments, where the amount of radio-activity immunoprecipitated by increasing amounts of a specific antisera was estimated. A typical titration curve is shown in Figure 2.1. Immunoprecipitations were carried out at 27°C for an hour and then at 4°C overnight. S. aureus suspension, after treatment with NET Buffer (as previously described) was then added and the

Figure 2.1 Titration of Antisera raised against Spinach RuBPCase with the Translation Products of the *E. coli* System

Increasing amounts of antisera raised against Spinach RuBPCase were added to 50 µl of the *E. coli* translation products programmed by RNA extracted from Day 5 light-grown Cucumber cotyledons. Immunoprecipitates were carried out in a final volume of 150 µl as described in Materials and Methods. Immunoprecipitates were collected by centrifugation after the addition of *S. aureus*-bound protein A. The immunoprecipitates were removed from the *S. aureus* cells by SDS treatment as described in Materials and Methods and the amounts of radioactivity were estimated by the scintillation counting of duplicate 5 µl aliquots.

Fig.2.1


CPM [35S]met PRECIPITATED x10-2
22
20
18
16
14
12
10
8
6
4
2
5
10
15
20
25
ul ANTISERA

incubation continued at 27°C for an hour. Titration experiments showed that a maximum precipitation of antigen-antibody complexes was obtained by addition of x5 antisera volume of 10% (w/v) S. aureus suspension.

The S. aureus cells were collected by centrifugation at 12,000 x g for 3 minutes in a Micro-Haematocrit Centrifuge, washed once in Saline Buffer and then washed 3 times in Saline Buffer containing 0.1% (w/v) SDS. The antigen-antibody complexes were removed from the S. aureus cells by resuspension in SDS-protein-gel Sample Buffer and the cells were pelleted by centrifugation at 12,000 x g for 5 minutes in a Micro-Haematocrit centrifuge. The supernatant was carefully removed and fractionated on 15% or 20% polyacrylamide gels containing SDS. The immunoprecipitated proteins were visualised by autoradiography or fluorography of the dried gels. To estimate the amounts of radioactivity present in immunoprecipitates, duplicate 5 µl samples were removed from supernatant prior to gel electrophoresis, spotted onto discs of Whatman 3 MM Chromatography paper and analysed as previously described.

(XXVIII) In Vivo Labelling of Cucumber Cotyledons

Developmentally related changes in protein synthesis were investigated by in vivo pulse labelling of the cotyledons with [^{35}S] met. In order to minimise bacterial contamination all glass-ware and media were sterilised by autoclaving before use.

Cucumber seeds were surface sterilised with a 1:30 dilution of Sodium Hypochlorite (BDH Stock Solution) for 5 minutes, neutralised in 0.01 N HCl for 10 minutes and washed 8 times in

20 volumes of sterile water. The seeds were then imbibed overnight at 4°C in the dark and planted in sterile vermiculute as normal and watered daily with sterile water.

In vivo labelling was carried out 24 hours before harvesting. Four cotyledons were excised from separate seedlings of each stage of development and placed between Whatman 3 MM chromatography paper and the side of clear plastic containers (6 cm x 4.5 cm diameter). The 3 MM paper, which was 1.5 cm wide allowed at least half of the cotyledon to be exposed above the top of the 'wick' thus facilitating gaseous exchange. 2.3 ml of sterile water containing 25-28 μCi/ml $[^{35}S]$met was placed in the bottom of the containers and this was enough to allow saturation of the 3 MM paper over the period of the labelling. For Day 0 cotyledons, four excised cotyledons were imbibed in 2 ml of labelling media at 4°C in the dark for 24 hours. After the labelling period the cotyledons were rinsed several times in sterile water and dried on tissue paper. Total homogenate protein was prepared by homogenisation of the cotyledons in 2.5 ml of 50 mM Tris-acetate pH 8.5, 50 mM K acetate, 5 mM Mg acetate and 2 mM DTT in a glass on glass Potter homogeniser at 4°C.

Homogenate volume was estimated by weight and a 1.5 ml aliquot was removed and centrifuged at 12,000 x g for 10 minutes in a Micro-Haematocrit centrifuge. The supernatant was removed and is referred to here as the supernatant fraction. Uptake and incorporation of radioactivity was determined by: i) Duplicate 20 μl aliquots were removed from the homogenate, spotted onto discs of Whatman 3 MM chromatography paper and the radioactivity estimated by scintillation counting directly, this is a measure of uptake of $[^{35}S]$met by the cotyledons. ii) Duplicate 20 μl aliquots of the

homogenate and supernatant fractions were processed by the method of Mans and Novelli (1961) as previously described, to give an estimation of incorporation of [^{35}S]met into protein. iii) Duplicate 20 µl aliquots of the labelling media were also taken to determine the amount of radioactivity supplied to the cotyledons.

The labelled proteins were analysed by fractionation on 15% polyacrylamide-gels containing SDS and visualised by fluorography of the dried gel, as described previously. Approximately 20,000 cpm of radioactivity from a particular day of development were loaded onto each slot of the gel.

Chapter 3. Characterisation of the *E. coli* Translation System

(I) Introduction

The aim of this thesis is to examine the regulation of synthesis of chloroplast proteins within the cotyledons during germination and early development of Cucumber seedlings. Thus I have been interested not only in the synthesis of proteins within the chloroplast, but also those proteins synthesized in the cytoplasm but destined to have a functional role within the chloroplast. To this end I have used *E. coli* and Wheat germ translation systems programmed with RNAs extracted from the tissue at different stages of development, as well as a translation system using isolated chloroplasts extracted from different stages of light-grown development and incubated with [^{35}S] met *in vitro*. In consequence, an important part of this project has involved the preparation and optimisation of these systems, as well as characterisation of the labelled translation products. This Chapter, and Chapters 4 and 5 will describe these aspects of the work.

(II) The *E. coli* Translation System

As we have seen in Chapter 1, the *E. coli* translation system has been used successfully to translate chloroplast mRNAs (Hartley *et al.*, 1975; Bottomley *et al.*, 1976, 1977). Thus it appeared to be a useful system to assay changes in translatable chloroplast mRNAs extracted from cotyledons of different stages of Cucumber seedling development.

(III) Characterisation of the Conditions for Incorporation of [^{35}S]met using the E. coli Translation System

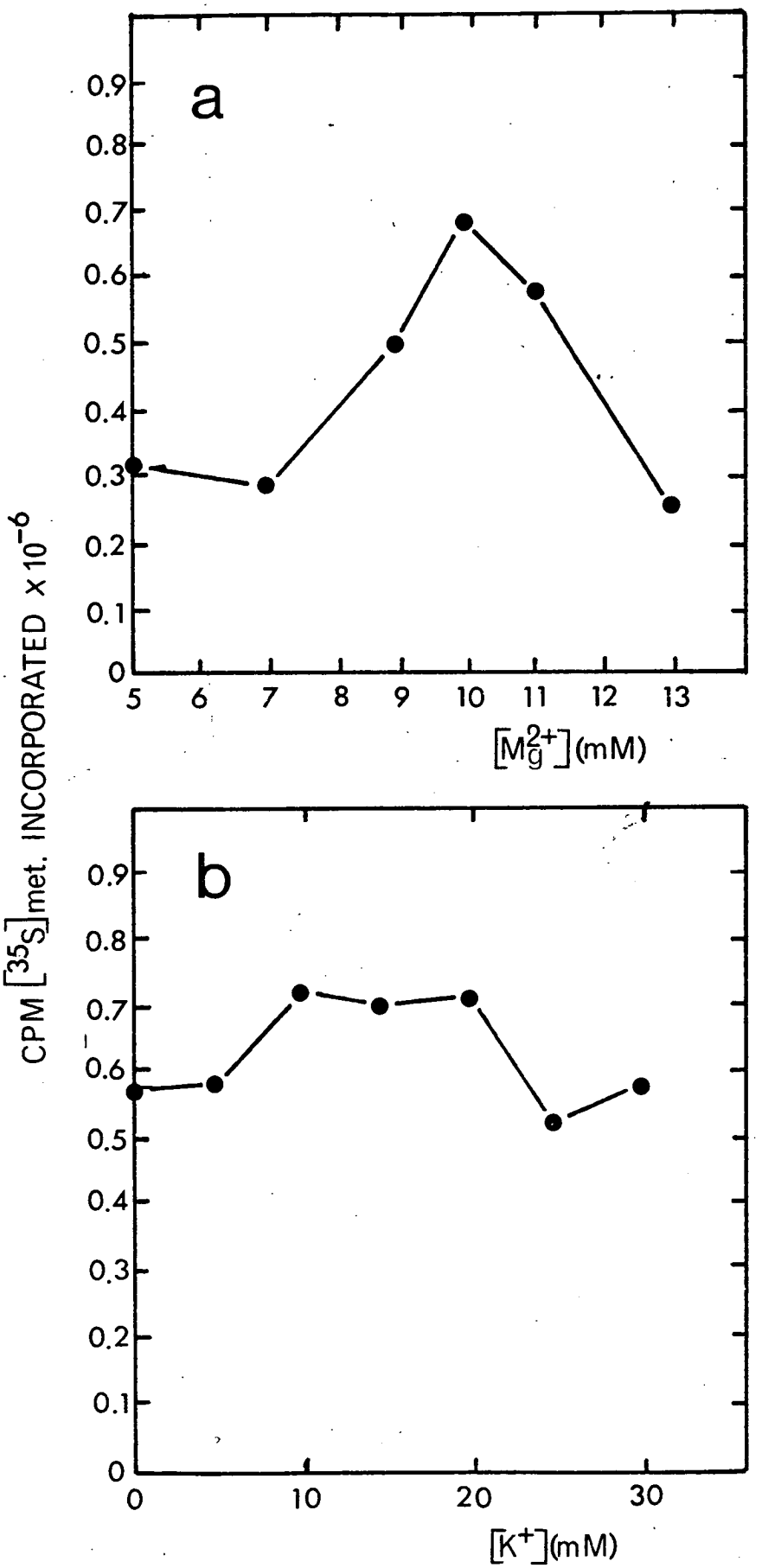
Routinely each preparation of S-30 extract, prepared as described in Materials and Methods (p. 67), was optimised to give the maximum incorporation of radioactivity into TCA-precipitable protein with respect to different magnesium and potassium ion concentrations. Spinach or Pea leaf total RNA or Cucumber Cotyledon total RNA was used to programme the E. coli translation system in optimisation experiments. The results obtained with each RNA were similar and were also found to be similar to results obtained when the system was programmed with chloroplast RNA purified from either Spinach and Pea leaves or Cucumber cotyledons. In Figure 3.1 the effect of different ion concentrations on incorporation of radioactivity by the system is shown. It was found that whereas there was an optimal Mg^{2+} ion concentration to produce maximum incorporation of [^{35}S]met, this was not the case for K^{+} ion concentration. This observation has been reported by other workers (Schwartz, 1967; Modollel, 1971). The concentration of Tris-HCl and NH_4Cl can be varied within reasonable limits (30-100 mM) without affecting the activity of the system (Modollel, 1971).

Autoradiography of the [^{35}S]met labelled translation products following SDS-polyacrylamide-gel electrophoresis shows that although the overall levels of radioactivity incorporated into protein may change, the spectrum of the translation products remains similar, and there is no enhancement of translation of a particular polypeptide at different ion concentrations.

Figure 3.1 Effect of Mg^{2+} and K^{+} Concentration on [^{35}S] methionine Incorporation into Protein by the *E. coli* Translation System

An *E. coli* translation system was prepared and incubated as described in Materials and Methods (p. 67) with 15 μg total Spinach RNA. The ion concentration was adjusted by addition of the appropriate amount of either (a) 50 mM Mg acetate, the K^{+} ion concentration being held constant at 10 mM, or (b) 100 mM K acetate, the Mg^{2+} ion concentration held constant at 10 mM

Fig.3.1


a
b
CPM [35S]met. INCORPORATED x 10-6
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
5 6 7 8 9 10 11 12 13
[Mg2+](mM)
0 10 20 30
[K+](mM)

This observation is in contrast to the observation of other workers who found that fidelity of translation, using viral RNAs, was maximal at Mg^{2+} concentration lower than that required for maximal incorporation (Capecchi, 1967; Salser et al., 1967).

Routinely, 10 µl (A_{260} 2 units) of the E. coli extract, prepared as described in Materials and Methods, were incubated at 37°C with additions of Stock solutions, described in Materials and Methods, to produce in a volume of 50 µl the final concentrations of:

Tris-acetate pH 8.0	50 mM
NH_4 acetate	75 mM
Mg acetate	10 mM
K acetate	10 mM
PEP	10 mM
ATP	6 mM
GTP	1 mM
Pyruvate Kinase	0.48 units
DTT	2 mM
Calcium Leucovorin	160 mM
19 ^{12}C amino acids (- met)	0.025 mM each
$[^{35}S]$met	5µCi
RNA	1-100µg

Initial work with this translation system showed that the addition of a formyl donor was unnecessary (Whitfeld et al., 1973; Bottomley et al., 1976). However, in my hands, the translation system programmed with total and chloroplast RNA extracted from Spinach leaves and Cucumber cotyledons showed a stimulation of

incorporation of up to 10-fold by the addition of Calcium Leucovorin at a concentration of 160 mM (5 μg per incubation). It was found that the optimum Mg^{2+} ion concentration varied with the presence or absence of the formyl donor. Similar results have been reported for protein synthesis directed by MS2 RNA (Kolakofsky and Nakamoto, 1966) and TMV RNA (Schwartz, 1967). It has also been shown that there is an increased requirement for a formyl donor for maximum incorporation following storage of the S-30 extract (Kolakofsky and Nakamoto, 1966). These authors suggested that the increase in Mg^{2+} ion concentration required for maximum incorporation in the absence of a formyl donor may make non-formylated aminoacyl-tRNA more effective as an initiator.

The time course of the incorporation of [^{35}S]met into TCA-precipitable protein by the translation system programmed by various RNAs is shown in Figure 3.2. It can be seen that incorporation is approximately linear for the first 10 minutes and continues to increase for another 5 minutes after which there is an apparent decline. This time course of incorporation has been observed by other workers (Schwartz, 1967; Modollel, 1971), and it has been suggested that the decline in the overall radioactivity incorporated may be caused by proteolysis (Schwartz, 1967). Thus incubations were terminated after 20 minutes and in the following data, where radioactivity incorporated by the E. coli system is quoted, the 15 minute time point is used.

Viral RNAs are good templates in the system and their well-defined translation products allowed me to verify the fidelity of translation of the system. As discussed in Chapter 1,

Figure 3.2. Time course of [^{35}S] methionine Incorporation into Protein by the E. coli Translation System

An E. coli translation system was prepared and incubated as described in Materials and Methods (p. 67) with various RNAs in separate experiments.

(a) Viral RNA

Minus RNA (■-■), Cowpea strain of TMV, 4 μg (▽-▽), Wild type TMV, 5.2 μg (●-●), BMV, 19.2 μg (□-□), PP7, 6 μg (▼-▼), R17, 4 μg (○-○), Qβ, 16 μg (▲-▲).

(b) Chloroplast RNA

Minus RNA (■-■), Spinach, 29.2 μg (○-○), Pea, 28.8 μg (▲-▲), Cucumber cotyledon Day 5 light-grown, 28.0 μg (△-△).

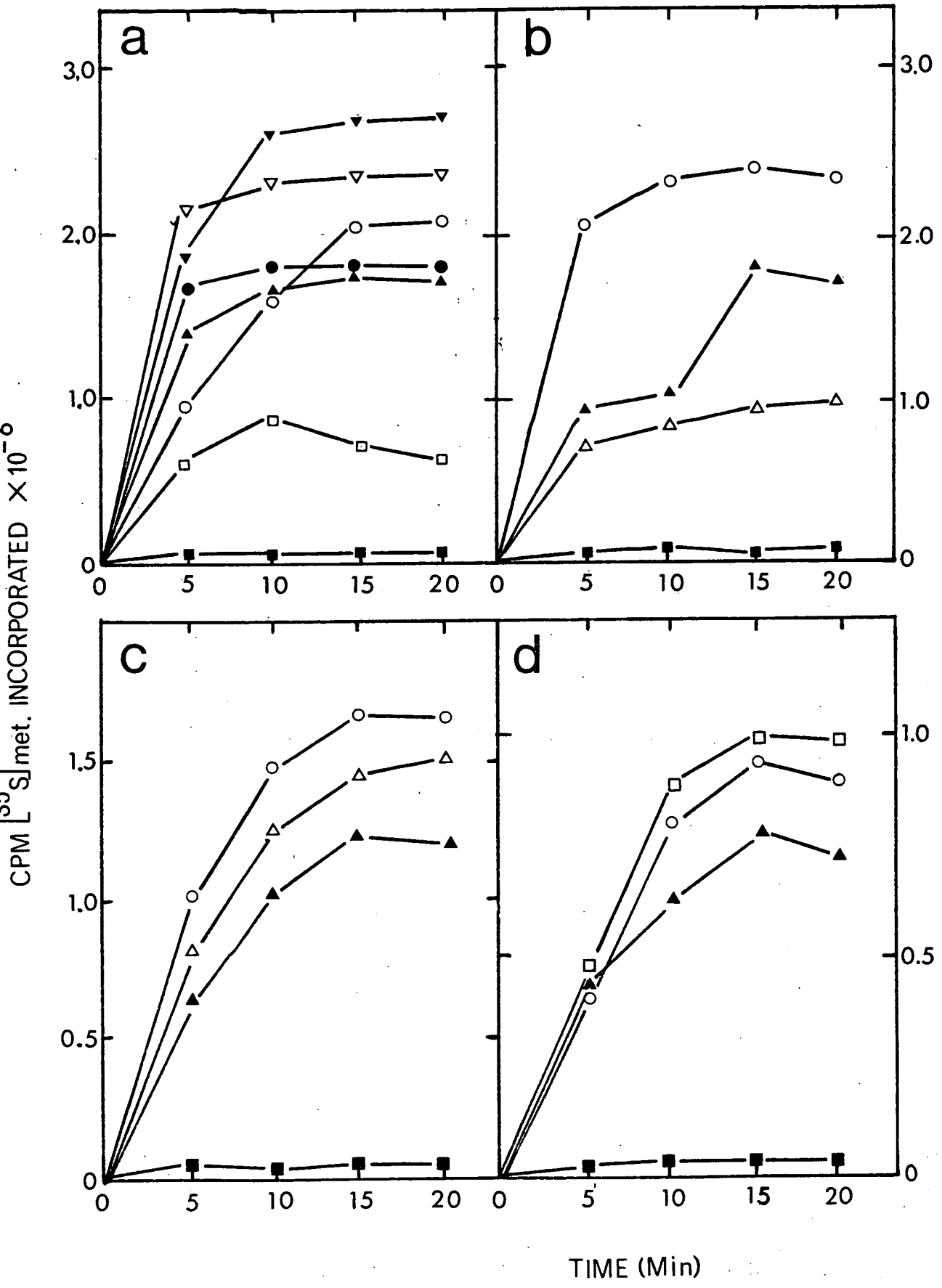
(c) Total RNA

Minus RNA (■-■), Spinach leaf, 29.2 μg (○-○), Pea leaf, 28.8 μg (▲-▲), Cucumber cotyledon Day 5 light-grown, 28.8 μg (△-△).

(d) Fractionated Cucumber Day 5 Cotyledon RNA

Minus RNA (■-■), Total 28.8 μg (○-○), Poly(A)$^{+}$, 2.2 μg (▲-▲), Poly(A)$^{-}$, 29.0 μg (□-□).

Fig.3.2


a
b
c
d
3.0
2.0
1.0
0
1.5
0.5
0 5 10 15 20
CPM [35S]met. INCORPORATED X10-6
TIME (Min)

eucaryotic viral RNAs are very active in the system. Indeed, in the example shown in Figure 3.2, the RNAs from the two strains of TMV appear to be as effective as bacteriophage RNA: Qβ, R17, PP7, in the levels of stimulation produced (see Fig. 3.2a). In general, total and chloroplast RNA extracted from the leaves of Pea and Spinach and the cotyledons of Cucumber stimulate the system to a lesser extent on a per μg basis (see Fig. 3.2 b-c). When similar amounts of total leaf and chloroplast RNA are used to direct the system, chloroplast RNA gives higher levels of incorporation on a per μg basis as previously observed (Bottomley et al., 1976). However, in contrast, this is not generally the case when comparing the incorporation programmed by Cucumber total cotyledon RNA and chloroplast RNA from the same tissue (see Fig. 3.2 b-c).

Total cotyledon RNA was fractionated using an oligo (dT)-cellulose column into poly (A) containing RNA: (poly(A)$^+$ RNA) and RNA excluded from the oligo (dT) column: (poly(A)$^-$ RNA). On a per μg basis the incorporation produced using total cotyledon RNA and poly(A)$^-$ RNA from the same tissue is similar (see Fig. 3.2 d). It was found that the poly(A)$^+$ RNA fraction on a per μg basis was a very good template in the system and this will be discussed further in Chapter 3, Section VII.

(IV) Estimation of Molecular Weights of Polypeptides Synthesized In Vitro

In general, the translation products from each translation system were analysed by fractionation on 15% polyacrylamide-gels containing SDS and the [^{35}S]met labelled polypeptides were

visualised by autoradiography of the dried gel as described in Materials and Methods (p. 55). The apparent molecular weights of the labelled polypeptides were estimated by comparison with standard proteins of known molecular weights according to the method of Weber and Osborn (1969).

Figure 3.3 shows a plot of the electrophoretic mobility of standard polypeptides against the $\log_{10}$ of their molecular weights. Using such calibration curves, the molecular weights of the labelled polypeptides were estimated.

(V) Analysis of the Translation Products of the *E. coli* System Directed by Viral RNAs

Analysis of the translational products of the *E. coli* translation system directed by viral RNAs not only allowed me to investigate which viral polypeptides can be translated on *E. coli* ribosomes *in vitro*, but also, in the case of bacteriophage RNA, the products of which are well characterised, to check the fidelity of the translation system

Schwartz (1967) previously reported that eucaryotic viral RNAs programme the synthesis of a variety of polypeptides (see Fig. 3.4, tracks a-b-c) by the *E. coli* translation system.

TMV RNA has been reported to direct the synthesis of three polypeptides in eucaryotic translation systems. These polypeptides have molecular weights of 165,000, 140,000 and 17,500, the latter being the viral coat protein (Hunter *et al.*, 1976; Whitfeld and Higgins, 1977). No high molecular weight polypeptides are seen amongst the translation products of the *E. coli* system directed by RNA from two strains of TMV.

Figure 3.3 Calibration Curve for Determination of Molecular Weight of Polypeptides Fractionated by SDS-Polyacrylamide-Gel Electrophoresis

Electrophoresis was carried out using 15% (w/v) polyacrylamide slab gels containing SDS, as described in Materials and Methods (p. 53). The electrophoretic mobilities of the standard marker proteins were plotted against the log of their known molecular weights as described in Weber and Osborn (1969).

Standard Marker Proteins: Bovine Serum Albumin (68,000 M_r); Catalase (60,000 M_r); Aldolase (40,000 M_r); Carbonic Anhydrase (29,000 M_r); Trypsin Inhibitor (Soybean) (21,000 M_r); Myoglobin (17,000 M_r); Lysozyme (14,300 M_r); Cytochrome c (11,700 M_r).

Fig.3.3

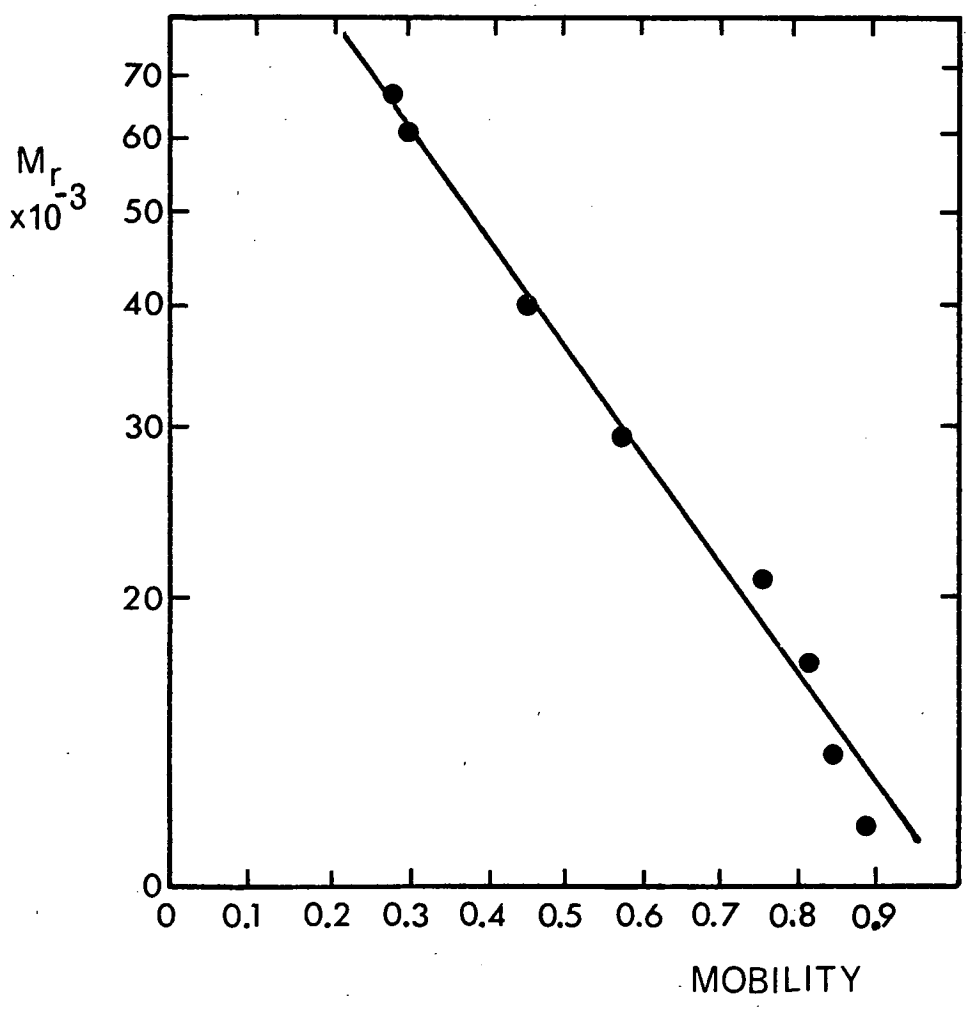
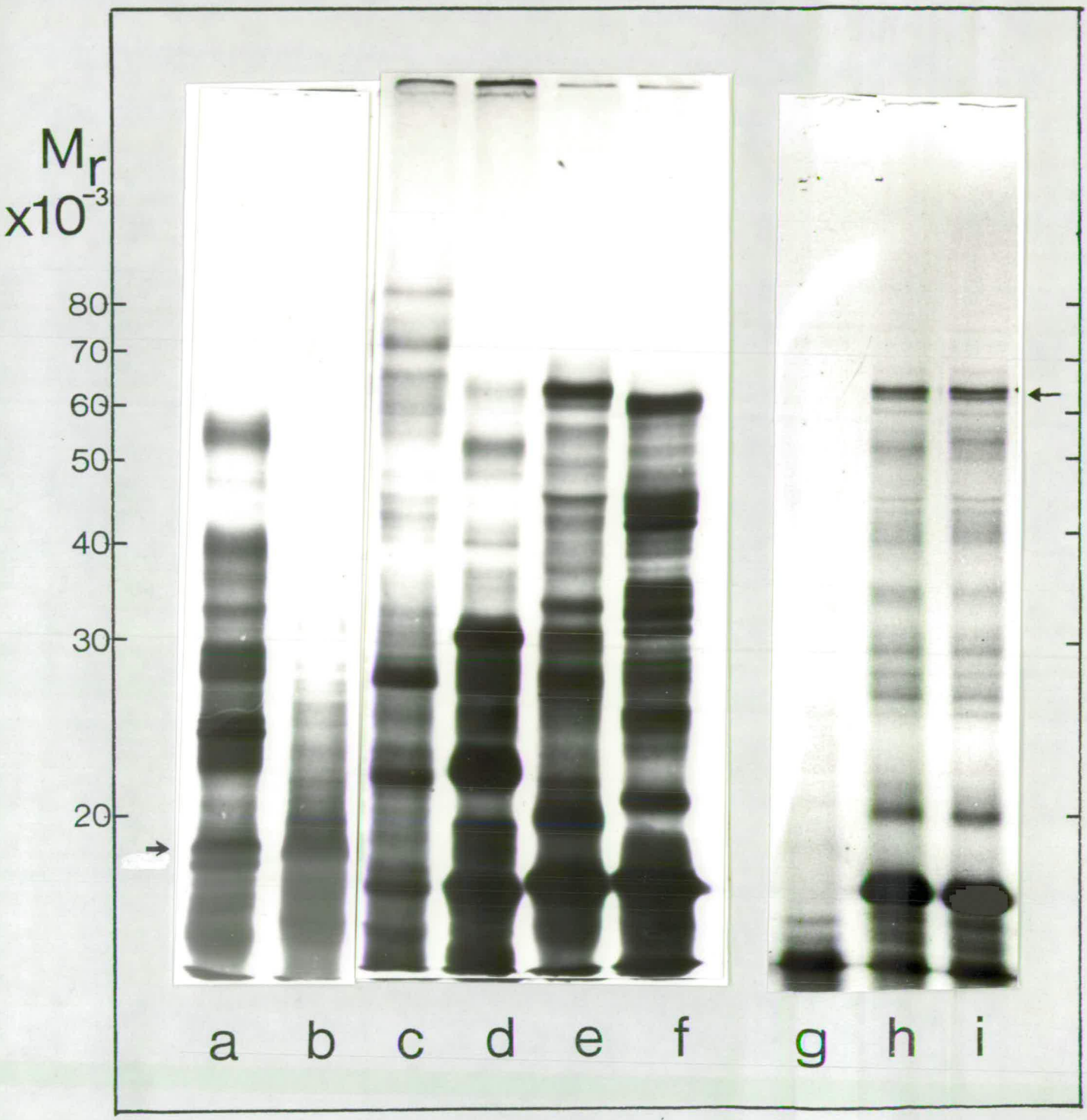


Figure 3.4 Analysis of the Translation Products of the *E. coli* Translation System Directed by Viral RNAs

The *E. coli* translation system was prepared and incubated, as described in Materials and Methods, with viral RNAs. Total translation products were fractionated on 15% polyacrylamide-gels containing SDS in parallel with marker proteins as described for Figure 3.3. Radioactive polypeptides were visualised by autoradiography of the dried gel as described in Materials and Methods. The time courses of the incorporation correspond with those in Figure 3.2 (except for tracks i-h). Arrows indicate the expected position of TMV coat protein (track a) and bacteriophage coat protein and replicase (track i). Tracks (a-f) and (g-i) were on separate gels.

Track (a) Cowpea strain TMV RNA; (b) Wild type TMV RNA; (c) Total BMV RNA; (d) Qβ RNA; (e) R17 RNA; (f) PP7 RNA; (g) minus RNA; (h) MS2 RNA, (3 μg); (i) MS2 RNA, (6 μg).

Fig.3.4



However, a polypeptide of approximately 17,000 M_r is seen amongst the translation products directed by the RNA of Cowpea strain of TMV (Fig. 3.4 arrowed). This RNA has previously been shown to code for TMV coat protein in the Wheat germ translation system (Higgins et al., 1976). In the absence of immunoprecipitation data it can only be tentatively suggested that this polypeptide is the coat protein which previously was thought not to be translated correctly in the E. coli translation system (Schwartz, 1967; Hunter et al., 1976).

In eucaryotic translation systems BMV RNA directs the synthesis of two major polypeptides of 20,000 and 35,000 M_r (Shih and Kaesburg, 1973), the former being the viral coat protein. Amongst the E. coli translation products (Fig. 3.4 c) two prominent labelled polypeptides are seen. However, neither appears to correspond in molecular weight to the viral polypeptides. It has been reported that the E. coli system programmed by BMV RNA yields a polypeptide, which on tryptic peptide mapping "resembles in some aspects coat protein" (Stubbs and Kaesburg, 1967).

My results confirm previous reports that although eucaryotic viral RNAs stimulate the E. coli system, they do not appear to act as effective templates for correct translation of viral proteins.

The bacteriophages, the RNA of which is used here, are all closely related, are well characterised and have proved to be model sources of polycistronic mRNAs (Kozak and Nathans, 1972; Atkins and Gesteland, 1975). In each case three polypeptides are coded for. However, differences in the efficiency of polypeptide chain initiation result in only two viral polypeptides

being seen among the products of the E. coli translation system (Lodish and Robertson, 1970). These are the coat protein and replicase of approximately 15,000 and 66,000 M_r respectively. In the examples shown here (Fig. 3.4, tracks d-e-f-h-i) a range of polypeptides is seen. The majority of these are thought to be the result of premature termination of replicase mRNA translation (Atkins and Gesteland, 1975). This may be the reason for the relatively small amounts of replicase and increased amounts of intermediate sized polypeptides seen amongst the translation products directed by Qβ RNA compared with R17 RNA. It has also been found that intermediate sized polypeptides arise from the read through of the termination signal of the coat protein mRNA (Horiuchi et al., 1971; Weiner and Weber, 1971, 1973). The coat protein of Qβ does not contain methionine (Jockusch et al., 1970) hence it would not be expected to appear among the translation products labelled with [^{35}S]met. However, it does appear to be present, as previously reported (Atkins et al., 1975; Atkins and Gesteland, 1975), suggesting that the E. coli system does not remove the N-formyl methionine from the completed polypeptides (Davies and Kaesburg, 1973).

The translation products directed by R17 and MS 2 RNAs are similar, reflecting their similar genomes (Atkins and Gesteland, 1975). Both code for two polypeptides with a molecular weight similar to replicase (Fig. 3.4 arrowed). It is thought that both are products of translation of the replicase mRNA with the larger polypeptide arising from the read through of a naturally occurring amber codon (Atkins and Gesteland, 1975). Both strains of E. coli used to prepare the S-30 extract are 'suppressor' strains

which 'suppress' the effect of amber codons and thus in this case would cause a read through.

Bacteriophage PP7 infects Pseudomonas strains of bacteria. However, two major polypeptides with molecular weights corresponding to a replicase and coat protein are among the translation products in the E. coli system (Fig.3.4f) as has been reported by others (Davies and Benike, 1974).

It appears then that the E. coli system translates bacteriophage RNAs with fidelity and is thus apparently free of RNAase activity (J. Atkins, personal communication), but possibly fails to remove the N-formyl methionine from completed polypeptides. These results also illustrate the need for caution in analysis of translation products of cell-free translation systems in general, showing that distinct polypeptides may not be specific gene products, but the result of premature termination or termination read through.

(VI) Analysis of the Translation Products of the E. coli System Directed by Plant RNAs

The optimised E. coli translation system when programmed with plant RNA gives translation products which are similar to those previously reported (Hartley et al., 1975; Bottomley et al., 1976, 1977, 1979), (see Fig. 3.5). The spectrum of translation products programmed by RNA extracted from Pea and Spinach leaves and Cucumber cotyledons are similar, and those directed by total and chloroplast RNA are essentially the same. These findings confirm earlier work (Bottomley et al., 1977) which suggested that the E. coli translation system preferentially translated

Figure 3.5 Analysis of the Translation Products of the *E. coli* Translation System Directed by Plant RNAs

The *E. coli* translation system was prepared and incubated as described in Materials and Methods with Plant RNAs. Total translation products were fractionated on SDS-polyacrylamide-gels and visualised as described in Materials and Methods. The time courses of incorporation correspond to those in Figure 3.2.

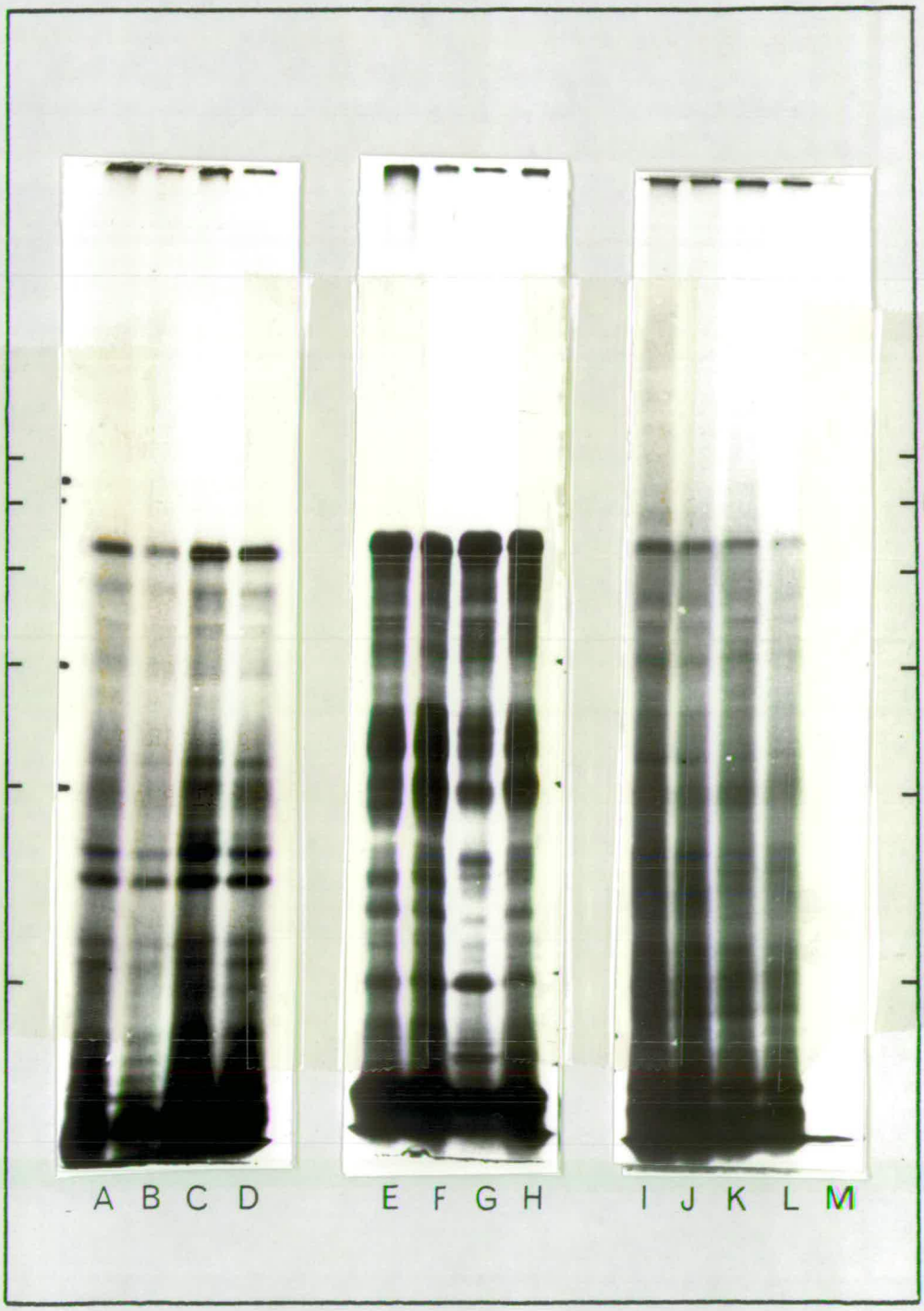
Tracks (A-D) RNA extracted from Cucumber Day 5 light-grown cotyledons: (A) Chloroplast RNA, 28 µg; (B) Chloroplast RNA, 14 µg; (C) Total RNA, 28,8 µg; (D) Total RNA, 14,4 µg.

Tracks (E-H) RNA extracted from Spinach Leaves: (E) Chloroplast RNA, 29,2 µg; (F) Chloroplast RNA, 14.6 µg; (G) Total RNA, 29.2 µgm; (H) Total RNA, 14.6 µg.

Tracks (I-L) RNA extracted from Pea Leaves: (I) Chloroplast RNA, 28,8 µg; (J) Chloroplast RNA, 14.4 µg; (K) Total RNA, 28.2 µg; (L) Total RNA, 14.1 µg.

Track (M) minus RNA.

Fig.3.5



chloroplast mRNAs present in total RNA.

Close inspection reveals that total and chloroplast Cucumber cotyledon RNA directs the synthesis of at least 14 distinct polypeptides, and total leaf and chloroplast RNA, extracted from Spinach and Peas, 16 each. It is apparent that several of the polypeptides appear common to the products directed by RNAs from all three sources. Analysis of the translation products showed that they did not change qualitatively with time of incubation once the full spectra of products had become resolvable by autoradiography. The product profile was not affected by the addition of unlabelled methionine after 15 minutes of incubation indicating that no proteolysis or precursor processing takes place in the system. Nor was it affected by the treatment of the RNA with formaldehyde to destroy the secondary structure of the RNA (Lodish and Robertson, 1969).

Incubation of the E. coli translation system, programmed with Cucumber total RNA, with a $[^{14}C]$ protein hydrolysate yielded similar translation product profiles to those labelled with $[^{35}S]$-methionine. This indicates that all the translation products of the E. coli system programmed by Cucumber total RNA contain methionine.

Obviously caution is required in the identification of the translation products, but the appearance of polypeptides of similar molecular weight directed by RNA extracted from different plant tissue, if not fortuitous, would indicate that these are translation products of chloroplast mRNAs common to each tissue.

To date only one polypeptide amongst the E. coli translation products has been positively identified. This is the major

product of the system programmed with Pea and Spinach chloroplast RNA with an apparent molecular weight of 55,000 which co-migrates with the LSu RuBPCase, produces similar enzymatic digests with tryptic peptide mapping (Hartley et al., 1975) and limited proteolytic digestion (Bottomley et al., 1979).

A major product of the system directed by Cucumber RNA is a 54,000 M_r polypeptide which does not co-migrate with a stained E. coli S-30 protein on SDS-polyacrylamide-gel electrophoresis, which may be tentatively identified as LSu RuBPCase.

Positive identification of LSu RuBPCase among the translation products of Cucumber, total and chloroplast RNA, has been shown by (i) co-migration with authentic LSu RuBPCase on SDS-polyacrylamide-gel electrophoresis and (ii) immunoprecipitation and subsequent analysis of the precipitated polypeptides on SDS-polyacrylamide-gels. Preliminary results using limited proteolytic digestion (Cleveland et al., 1978) would indicate that the 54,000 M_r polypeptide is indeed LSu RuBPCase (not shown).

Immunoprecipitation of the LSu RuBPCase from the in vitro translation products has been carried out in two ways:

(i) the translation products have been passed through an immunoabsorbant column of anti-Spinach RuBPCase covalently linked to Sepharose 4B. The bound material was eluted and analysed by SDS-polyacrylamide-gel electrophoresis,

(ii) direct immunoprecipitation was carried out on the complete translation products or the supernatant obtained after centrifugation of the translation products at 105,000 x g for an hour, (referred to

hereafter as the ribosomal supernatant). Antisera used was raised against Spinach RuBPCase or Cucumber LSu RuBPCase. Precipitation of the antigen-antibody complex was carried out by the addition of *S. aureus* bound Protein A as described in Materials and Methods (p. 83).

In each case the 54,000 M_r polypeptide was immunoprecipitated (see, for example, Fig. 3.6). Thus LSu RuBPCase appears to be a product coded for by Cucumber RNA as assayed in the *E. coli* translation system. It also appears to be a major product *in vitro* of Pea and Spinach RNAs, in the latter case amounting to approximately 17% of the total radioactivity incorporated. Close inspection of the 55,000 M_r region of the SDS-polyacrylamide-gel profile of the translation products of the *E. coli* system directed by total and chloroplast RNA extracted from Spinach leaves reveals that the major polypeptide is a doublet. Bottomley *et al.* (1979) have observed that an *E. coli* transcription and translation system programmed with Spinach Chloroplast DNA produces a polypeptide slightly larger than LSu RuBPCase.

(VII) Analysis of the Translation Products of the *E. coli* System Directed by Poly $(A)^+$ and Poly $(A)^-$ Fractions of Total Cucumber Cotyledon RNA

Total cotyledon RNA was extracted from Day 5 light-grown Cucumber seedlings and fractionated into poly $(A)^+$ and poly $(A)^-$ RNA on a column of oligo (dT)-cellulose. The translation products of the *E. coli* system programmed with poly $(A)^+$ and

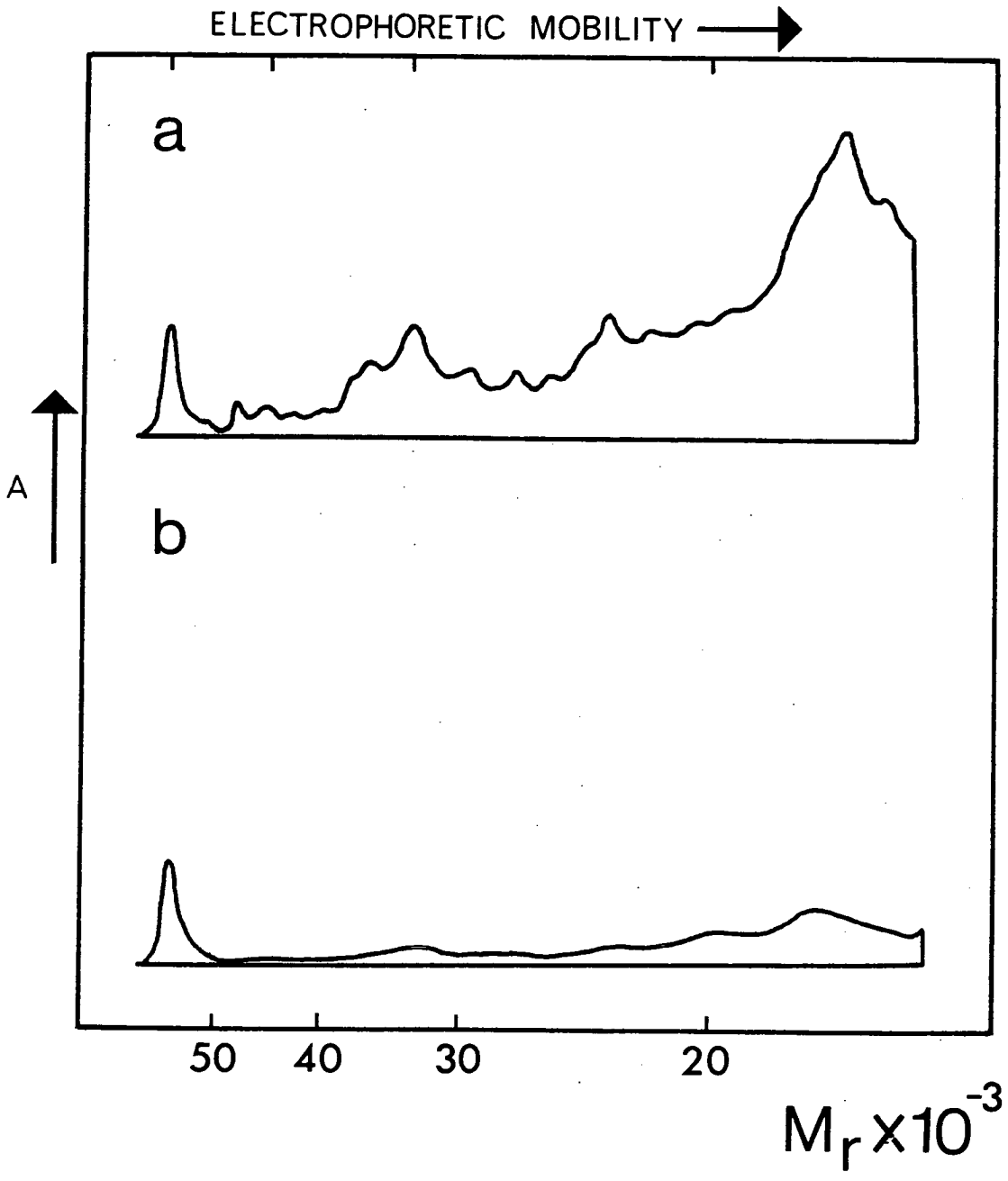
Figure 3.6 Immunoprecipitation of LSu RuBPCase from the Translation Products of the *E. coli* System Directed by Cucumber Total RNA

The *E. coli* system was incubated with Day 5 light-grown Cucumber cotyledon total RNA and immunoprecipitated directly as described in Materials and Methods (p. 83) using antisera raised against Spinach RuBPCase. The immunoprecipitated proteins were analysed by SDS-polyacrylamide-gel electrophoresis with autoradiography of the dried gel as described in Materials and Methods. The autoradiograph was scanned using a Kipp and Zonen Densitometer. Analysis of the precipitate obtained by the addition of pre-immune sera yielded only low molecular weight polypeptides over the same period of autoradiograph exposure.

(a) Complete translation products

(b) Immunoprecipitate

Fig.3.6



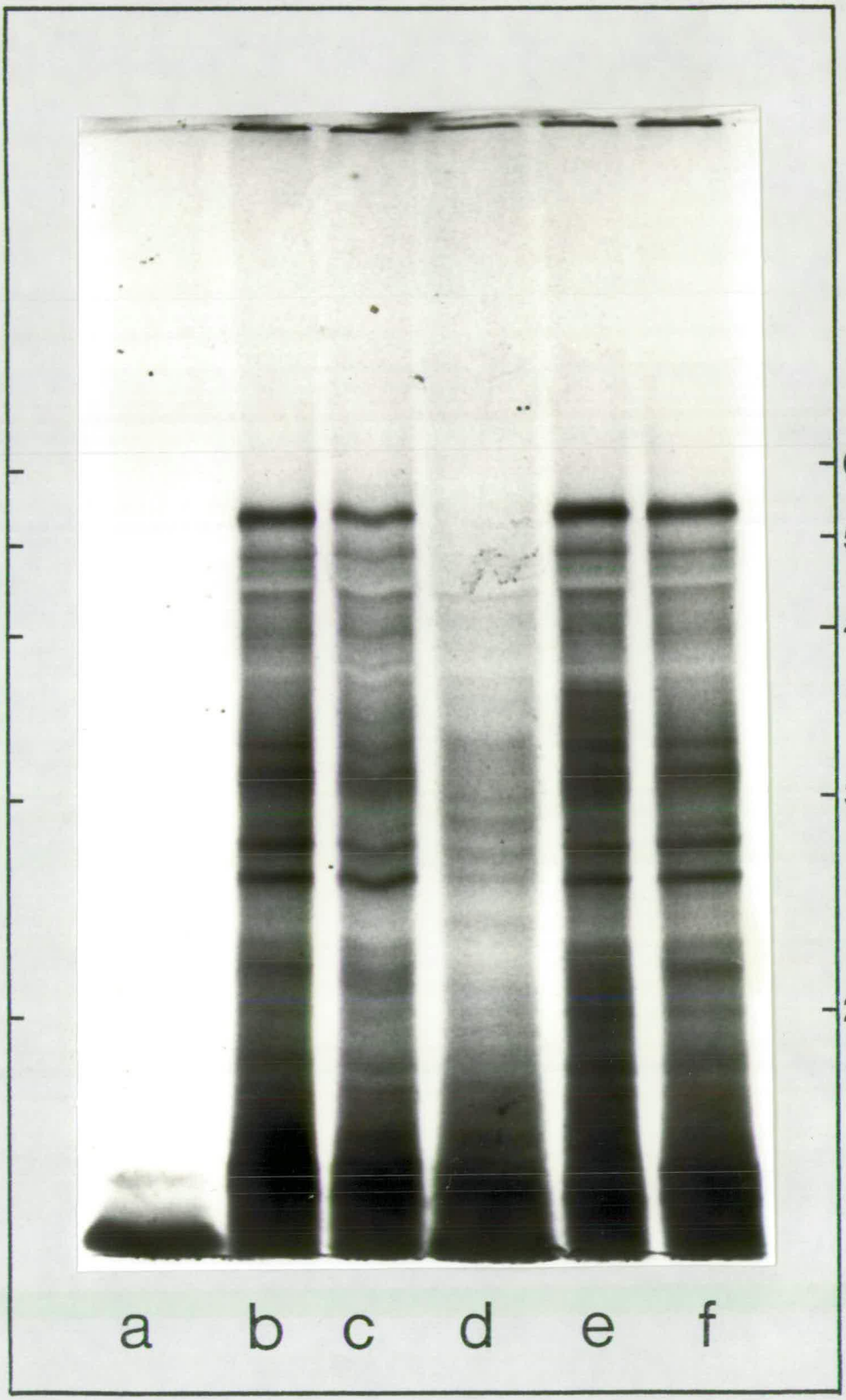
poly $(A)^-$ RNA and total and chloroplast RNA extracted from tissue at the same stage of development are shown in Figure 3.7. My results confirm the previous findings that the LSu RuBPCase is not present among the translation products directed by poly $(A)^+$ RNA (Wheeler and Hartley, 1975; Sagher et al., 1976). The products that are programmed by poly $(A)^-$ RNA resemble those directed by total and chloroplast RNAs. As previously shown in Figure 3.2 d, poly $(A)^+$ RNA on a per µg basis provides a very active template for the E. coli translation system. Analysis of the translation products of the E. coli system directed by poly $(A)^+$ RNA reveals a number of polypeptides, some of which co-migrate with polypeptides programmed by total and chloroplast RNA. It is possible that these polypeptides are coded for by the chloroplast RNA containing short sequences of poly (A) similar to those reported in Maize chloroplast RNA (Haff and Bogorad, 1976). The presence of these polypeptides among the translation products directed by total and chloroplast RNAs would indicate that they are not produced as a result of premature termination of translation of the LSu mRNA. Although it has not been experimentally proven, it is thought that fractionation of poly $(A)^+$ on oligo (dT)-cellulose does not allow the recovery of RNA containing less than 20 adenosine nucleotides (Cabada et al., 1977). The apparent presence of some polypeptides common to the translation products programmed by poly $(A)^+$ and poly $(A)^-$ RNA suggests that these polypeptides are coded for by RNA containing tracts of poly (A) of more or less than 20 adenosine nucleotides respectively.

Figure 3.7 Analysis of the Translation Products of the *E. coli* System Directed by Poly(A)$^+$ and Poly(A)$^-$ Fractions of Cucumber Cotyledon Total RNA

The *E. coli* translation system was prepared and incubated as described in Materials and Methods with various RNA fractions prepared from Day 5 light-grown cotyledons. Equal amounts of radioactivity were loaded onto each slot of the SDS-polyacrylamide-gel and analysed as described in Materials and Methods.

Track (a) minus RNA; (b) Cotyledon total RNA, 28.8 μg; (c) Chloroplast RNA, 28.0 μg; (d) Poly(A)$^+$ RNA, 1.1 μg; (e) Poly(A)$^-$ RNA, 29.0 μg; (f) Cotyledon total RNA, 28.8 μg.

Fig.3.7



(VIII) Analysis of the Translation Products of the *E. coli* System Directed by Isolated Polysomes

It was thought that, if successful, analysis of the polypeptides produced in the translation system under the direction of polysomes isolated from Cucumber cotyledons of different stages of development would provide an indication of when the RNA in question is being translated *in vivo*. Such 'polysome run-off' experiments have been described using an *E. coli* system (Alscher *et al.*, 1976), and have also been successful using a Wheat germ translation system (Sun *et al.*, 1975; Beachy *et al.*, 1978; Larkins and Hurkman, 1978).

After several unsuccessful attempts using polysomes from Cucumber cotyledons, it was decided to compare the translation products directed by polysomes from this tissue with those isolated from the Spinach leaves in a similar manner. Although it has been proposed that LSu RuBPCase is translated in the stroma of the chloroplast (Ellis, 1977), there is some evidence that chloroplast polysomes are membrane bound (Falk, 1969; Philipovich *et al.*, 1973; Chua *et al.*, 1973). Thus parallel extractions were carried out using 1% (w/v) Triton X-100 and 3% (v/v) Nonidet P40 in the extraction buffer, the latter being reported to remove chloroplast polysomes from the membranes (Margulies and Weisstrop, 1976). Figure 3.8 shows the translation products directed by polysomes extracted from the two types of tissue with each extraction procedure. Whereas active polysomes were not recovered from Cucumber cotyledons, it appears that the use of the two detergents in the extraction media does not result in the isolation of different populations of polysomes. It was thought that RNAase

Figure 3.8 Analysis of the Translation Products of the *E. coli* System Incubated with Polysomes

Polysomes were isolated from Day 5 light-grown Cucumber cotyledons or the expanding first leaves of Spinach, in media containing 1% (w/v) Triton X-100 or 3% (v/v) Nonidet P 40 and A_{260} 0.5 units were incubated in an *E. coli* translation system as described in Materials and Methods (p. 62). Total translation products were loaded onto each slot of 15% polyacrylamide-gels containing SDS and analysed as described in Materials and Methods. The minus RNA control contained no prominent bands.

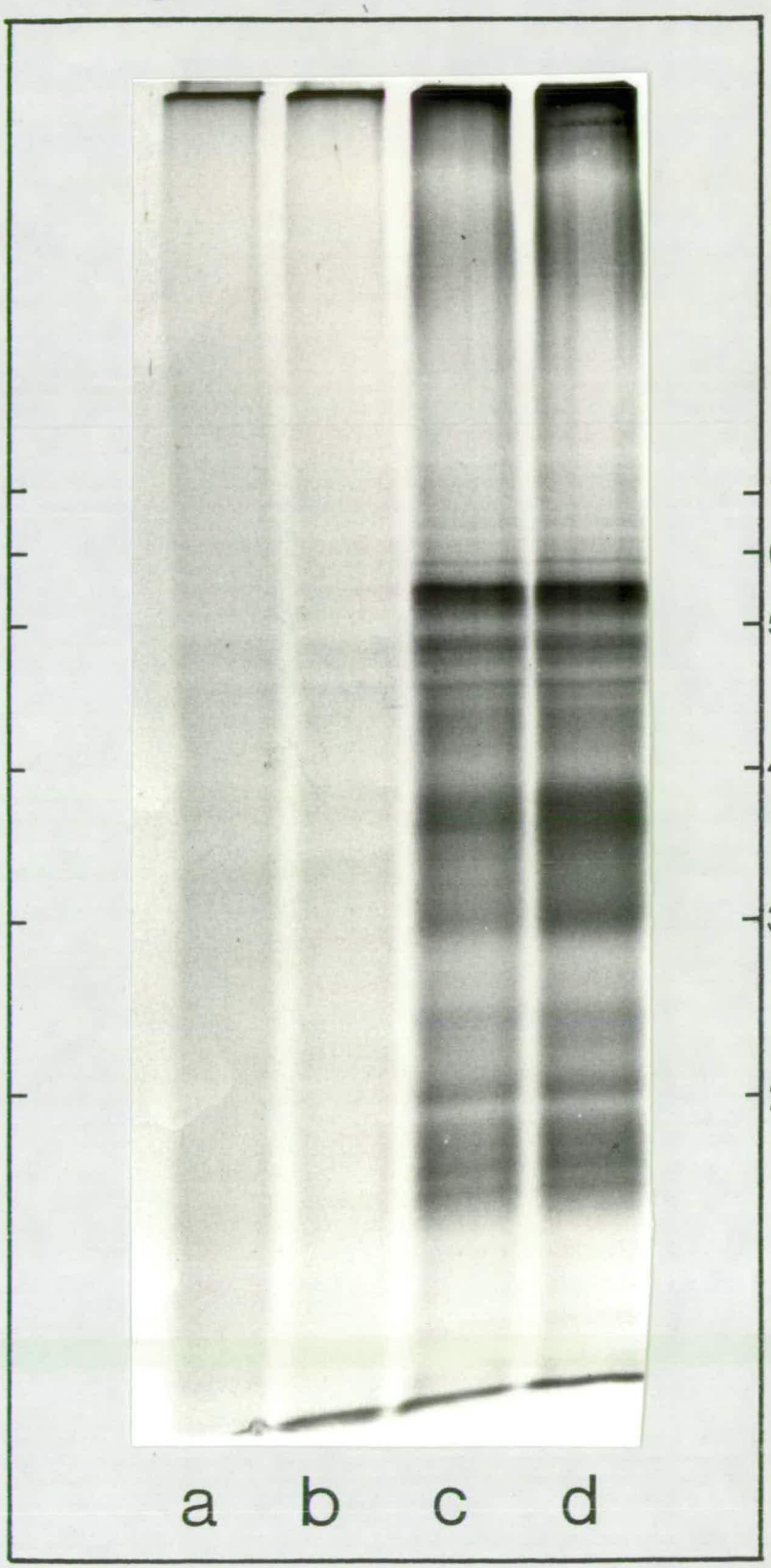
Tracks (a) Cucumber polysomes, Triton X-100 (2.59×10^5 cpm)

(b) Cucumber polysomes, Nonidet P 40 (3.2×10^5 cpm)

(c) Spinach polysomes, Triton X-100 (9.34×10^5 cpm)

(d) Spinach polysomes, Nonidet P 40 (1.36×10^6 cpm)

Fig.3.8



activity in the cotyledons may be the cause of my inability to extract active polysomes from Cucumber and more recently it has been found that indeed this is the case (J-M. Grienenberger, personal communication).

Spinach polysomes appear to be quite active in the system and it is useful to compare these 'polysome run-off' products with those directed by Spinach RNA (Fig. 3.5, tracks E-F-G-H). The polypeptide products synthesized by 'polysomal run-off' appear to be more clearly defined and there is an absence of labelled small molecular weight material.

(IX) The Effect of RNA Concentration on Incorporation by the *E. coli* Translation System

Incubation of increasing amounts of RNA from plant tissue with *E. coli* system, generally leads to an increase in overall incorporation of [^{35}S]met (Fig. 3.9). At low levels (up to 20 µg per incubation, 400 µg/ml) of chloroplast and total RNA, [^{35}S]met incorporation is proportional to the amount of RNA added.

As previously shown (Fig. 3.2 b-c), Spinach chloroplast RNA is more active than Spinach total RNA on a per µg basis (Fig. 3.9 a). Increasing the amounts of total RNA further results in a reduction in overall radioactivity incorporated at approximately 90 µg per incubation, (1.8 mg/ml) whereas this is not seen when chloroplast RNA is used at concentrations of up to 200 µg per incubation (4 mg/ml). Although these results are similar to those previously reported (Bottomley *et al.*, 1976), these workers did not observe a reduction in overall

Figure 3.9 Effect of Increasing RNA Concentrations on [^{35}S] Methionine Incorporation into Protein by the E. coli System

The E. coli translation system was prepared and incubated as described in Materials and Methods in separate experiments with increasing amounts of plant RNAs.

(a) Spinach leaf RNA

Chloroplast (■ - ■), Total leaf (□ - □).

(b) Cucumber Day 5 light-grown Cotyledon RNA.

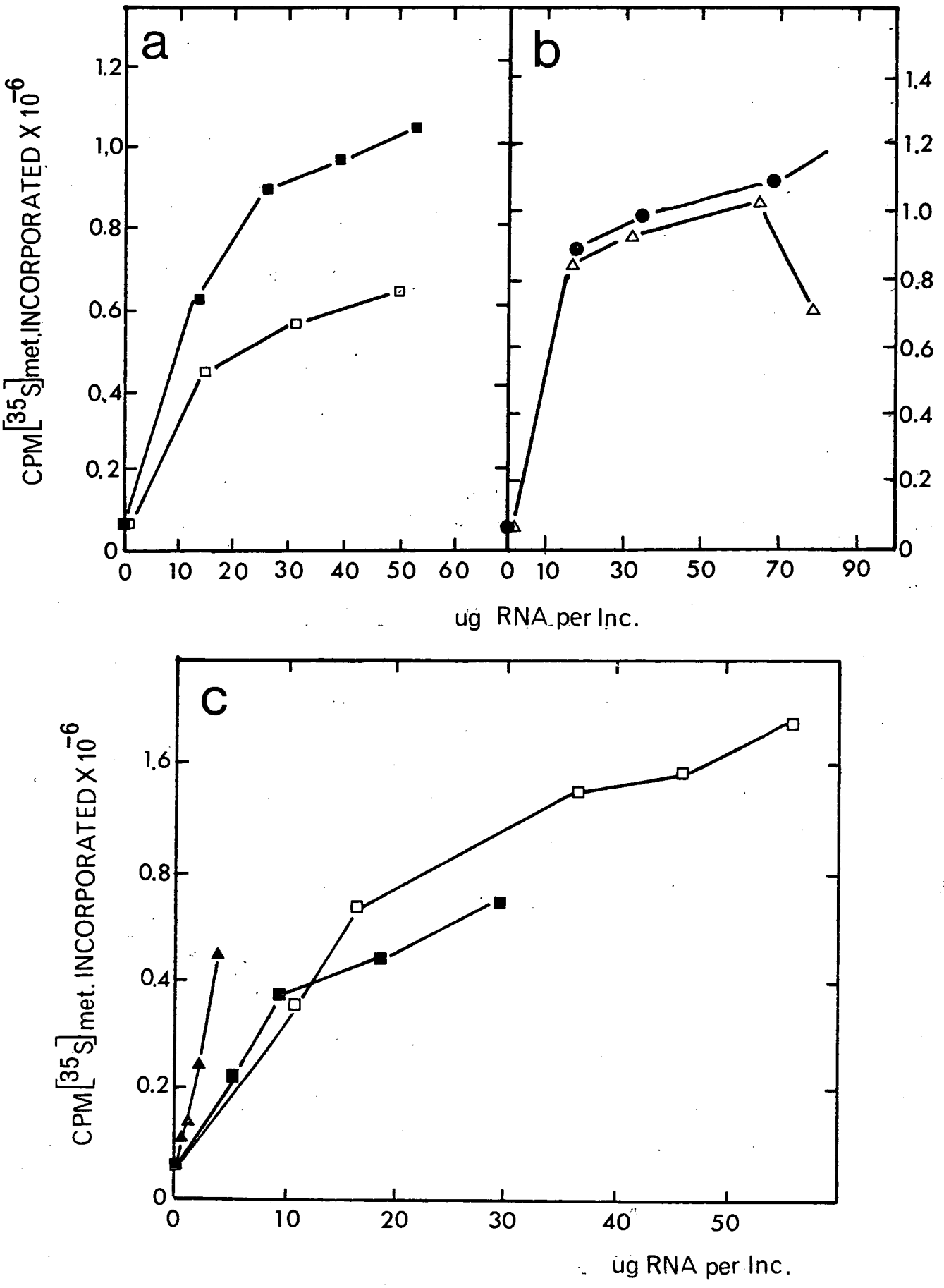
Chloroplast (● - ●), Total cotyledon (△ - △).

(c) Cucumber Day 5 light-grown cotyledon RNA

Total cotyledons (□ - □), Poly(A)$^+$ (▲ - ▲),

Poly(A)$^-$ (■ - ■).

Fig.3.9


a
b
c
CPM[35S]met.INCORPORATED X 10-6
ug RNA per Inc.
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
10
20
30
40
50
60
70
90

incorporation when using even larger amounts of total RNA.

Total Cucumber cotyledon RNA appears as active as chloroplast RNA on a per μg basis (Fig. 3.9 b). Once more, at higher levels of total RNA there is a reduction in incorporation which is not seen when chloroplast RNA is used. As we have observed, Cucumber poly $(A)^+$ RNA appears to be a very active template in the E. coli system (Fig. 3.9 c). It is apparent that total and Poly $(A)^-$ RNA from the same tissue stimulate incorporation of $[^{35}S]$ met in the system in a similar manner.

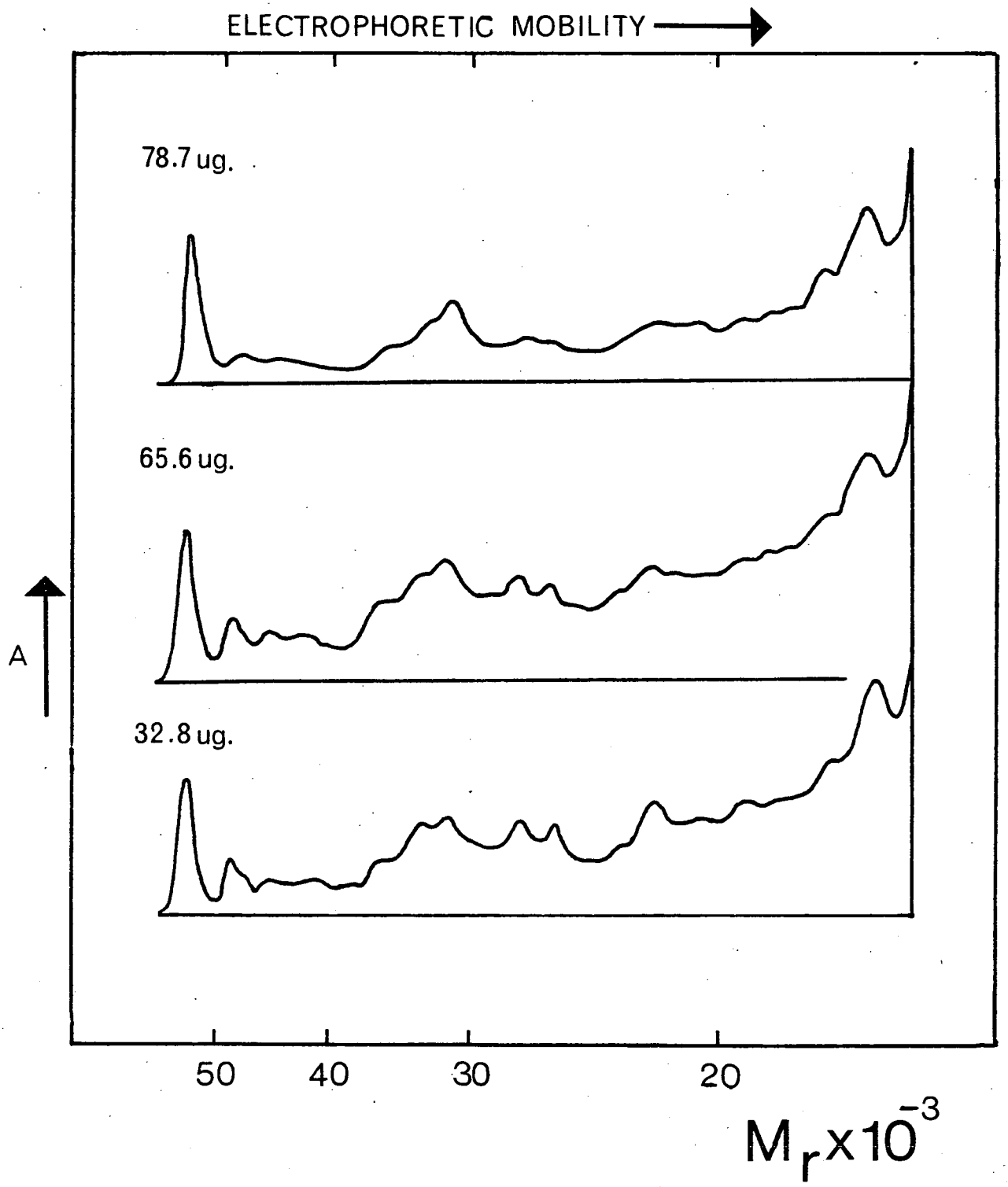
When increasing amounts of RNA are used in the system, most of the labelled polypeptide products appear to become more distinct among the total translation product profile. However, at the high input levels of total RNA where the overall incorporation declines, the relative proportions of the translation products change.

Densitometric scans of the autoradiographs of the translation products directed by increasing amounts of total Cucumber cotyledon RNA are shown in Figure 3.10, and these correspond to the total amounts of radioactivity incorporated as shown in Figure 3.9 b. Although the reduction in the overall incorporation with increasing RNA concentration is reflected in a reduction in the total amount of $[^{35}S]$ met labelled translation products that are seen, the amounts of LSu RuBPCase synthesized relative to the other products increase. Similar differential translation has been seen in other systems (for example, Stewart et al., 1973; Hall and Arnstein, 1973; Sonenshein and Brawerman, 1976, 1977). This has been shown to be due to differences in the

Figure 3.10 Effect of Increasing RNA Concentrations on the Translation Products of the E. coli System

The E. coli translation system was prepared and incubated as described in Materials and Methods with increasing amounts of Cucumber Day 5 light-grown cotyledon RNA. The corresponding total radioactivity incorporated is shown in Fig. 3.9b. The total translation products were fractionated on 15% polyacrylamide-gels containing SDS as described in Materials and Methods and the autoradiographs of the translation products were scanned using a Kipp and Zonen Densitometer.

Fig.3.10


ELECTROPHORETIC MOBILITY
78.7 ug.
65.6 ug.
32.8 ug.
A
50
40
30
20
$M_r \times 10^{-3}$

rate of initiation of translation of specific mRNAs (Lodish and Jacobsen, 1972; McKeehan, 1974; Lomedico and Saunders, 1977). As a result of this a model has been proposed that such differential translation is dependent on the relative affinities of specific mRNAs to rate limiting initiation factors (Lodish, 1974).

The results reported here could be interpreted similarly. At low concentrations of RNAs, competition for 'rate controlling elements' is not severe and all RNAs are translated with equal efficiency. With increasing quantities of RNAs, the translation apparatus becomes saturated and the mRNAs (in our case the mRNA for LSu RuBPCase) with higher affinity for the relevant 'rate controlling element' are translated preferentially. This would be in agreement with the proposal that efficient initiation is an important factor in the regulation of synthesis of those proteins that are particularly abundant in nature (Sonenshein and Brawerman, 1976, 1977). Such results would lead us to question the indiscriminate use of cell free systems with the assumption that the resultant translation products reflect the true complexity and abundance of mRNAs within the total RNA used to direct the system. Hence, in the following experiments conducted to investigate the levels of LSu mRNA in developing Cucumber cotyledons, care was taken to use concentrations of RNA that would not result in the preferential synthesis of LSu.

Chapter 4. Characterisation of the Wheat Germ Translation System

(I) Introduction

The observation that the Wheat germ system preferentially translated those mRNAs normally translated in the cytoplasm when programmed with total leaf RNA (Bottomley et al., 1976, 1977) suggested that the system programmed with total cotyledon RNA would translate cytoplasmic mRNAs including those that code for chloroplast polypeptides. Thus it appeared to be a useful system, when programmed with RNA extracted from cotyledons of different stages of Cucumber seedling development, to assay changes in translatable mRNA coding for chloroplast proteins which are synthesized in the cytoplasm. These experiments were carried out using a Wheat germ translation system optimised for use with Cucumber RNA by Ms. E. M. Weir.

(II) Conditions for Incorporation of [^{35}S] met using the Wheat Germ Translation System

Routinely, 10 µl (A_{260} 1.4 units) of the Wheat germ extract prepared as described in Materials and Methods (p. 71), were incubated at 25°C with additions of Stock solutions described in Materials and Methods (p. 72), to produce in a volume of 50 µl a final concentration of:

HEPES pH 7.6	28 mM
K acetate	104 mM
Mg acetate	2.25 mM
Spermidine	0.25 mM
ATP	1 mM

GTP	0.05 mM
Creatine Phosphate	8 mM
Creatine Phosphokinase	5 µg
DTT	2 mM
19 [^{12}C] amino acids (-met)	0.025 mM each
[^{35}S]met	5 µCi
RNA	1-60 µg

The time course of incorporation of [^{35}S]met into TCA-precipitable protein by the system programmed with various RNAs is shown in Figure 4.1. After an initial lag, incorporation is approximately linear for 60 minutes and thereafter the rate declines. The incubations were terminated at 90 minutes and in the following data, where radioactivity incorporated by the Wheat germ system is quoted, the 90 minute time point is used.

Like the E. coli system, eucaryotic viral RNAs prove to be very good templates in the Wheat germ translation system. This is not the case when the system is programmed by bacteriophage RNA (see Fig. 4.1 a).

On a per µg basis, RNA extracted from the leaves of Spinach and Wheat, Cucumber cotyledons and Spirodela polyrrhiza stimulates incorporation of radioactivity by the system to a lesser extent than that found for eucaryotic viral RNAs (see Fig. 4.1 b). The time courses of incorporation of radioactivity by the Wheat germ system, programmed by the poly $(A)^+$ and poly $(A)^-$ fractions of Cucumber cotyledon total RNA and Cucumber cotyledon chloroplast RNA are similar to that shown for total Cucumber cotyledon RNA. However, as will be discussed in Chapter 4, Section VI, on a per µg basis poly $(A)^+$ RNA serves as a very efficient template in the Wheat

Figure 4.1 Time Course of [^{35}S] methionine Incorporation into Protein by the Wheat Germ Translation System.

The Wheat germ translation system was prepared and incubated as described in Materials and Methods (p. 71) with various RNAs.

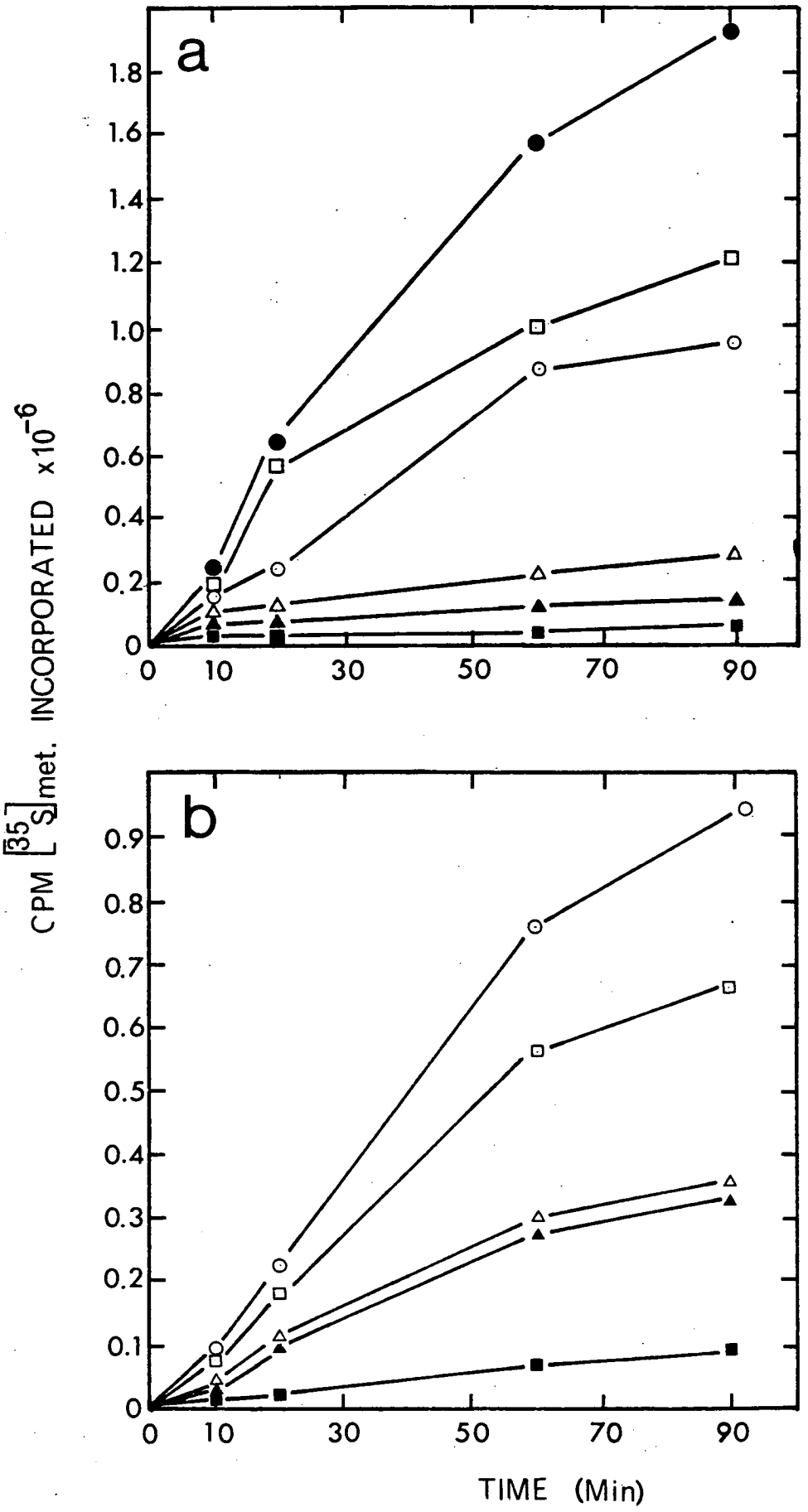
(a) Viral RNA

Minus RNA (■-■), Cowpea strain TMV 4 µg (●-●), Wildtype TMV 5 µg (□-□), Total BMV 9.5 µg (○-○), PP7 5.6 µg (△-△), R17 8 µg (▲-▲).

(b) Plant total RNA

Minus RNA (■-■), Spinach leaf 20 µg (□-□), Cucumber cotyledon 19.5 µg (○-○), Spirodela polyrrhiza 14.4 µg (△-△), Wheat leaf 15 µg (▲-▲).

Fig.4.1


a
b
CPM [35S]met. INCORPORATED x10-6
TIME (Min)
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
0.1
0.3
0.5
0.7
0.9
10
30
50
70
90

germ system whereas poly $(A)^-$ and chloroplast RNA do not.

(III) Analysis of the Translation Products of the Wheat Germ System Directed by Viral RNAs

The labelled translation products obtained from the Wheat germ incubations directed by viral RNA, the time courses of which are shown in Figure 4.1, were fractionated by SDS-polyacrylamide-gel electrophoresis, visualised by autoradiography and are shown in Figure 4.2. In all cases with the Wheat germ translation system, the water control contained one radioactive band of approximately 10,000 M_r (not shown).

Bacteriophage RNA does not appear to be an effective template in this system. However, some polypeptides appear to be programmed by PP7 and R17 RNA. The polypeptide programmed by PP7 does not appear to correspond to the molecular weight of any known viral proteins. However, the 14,000 M_r polypeptide that is programmed by R17 RNA corresponds in size to that of the viral coat protein. It has been reported that Qβ RNA, which is closely related to R17, is translated with fidelity in the Wheat embryo system (Davies and Kaesburg, 1973).

The lack of translation products in the Wheat germ system directed by the bacteriophage RNA would also indicate that the addition of heterologous RNA does not stimulate the translation of a large amount of endogenous RNA as has been reported with this system (Senger and Gross, 1976).

TMV RNA directs the synthesis of a number of polypeptides in the Wheat germ system as found by previous workers (Higgins et al., 1976; Bruening et al., 1976). Although there are a

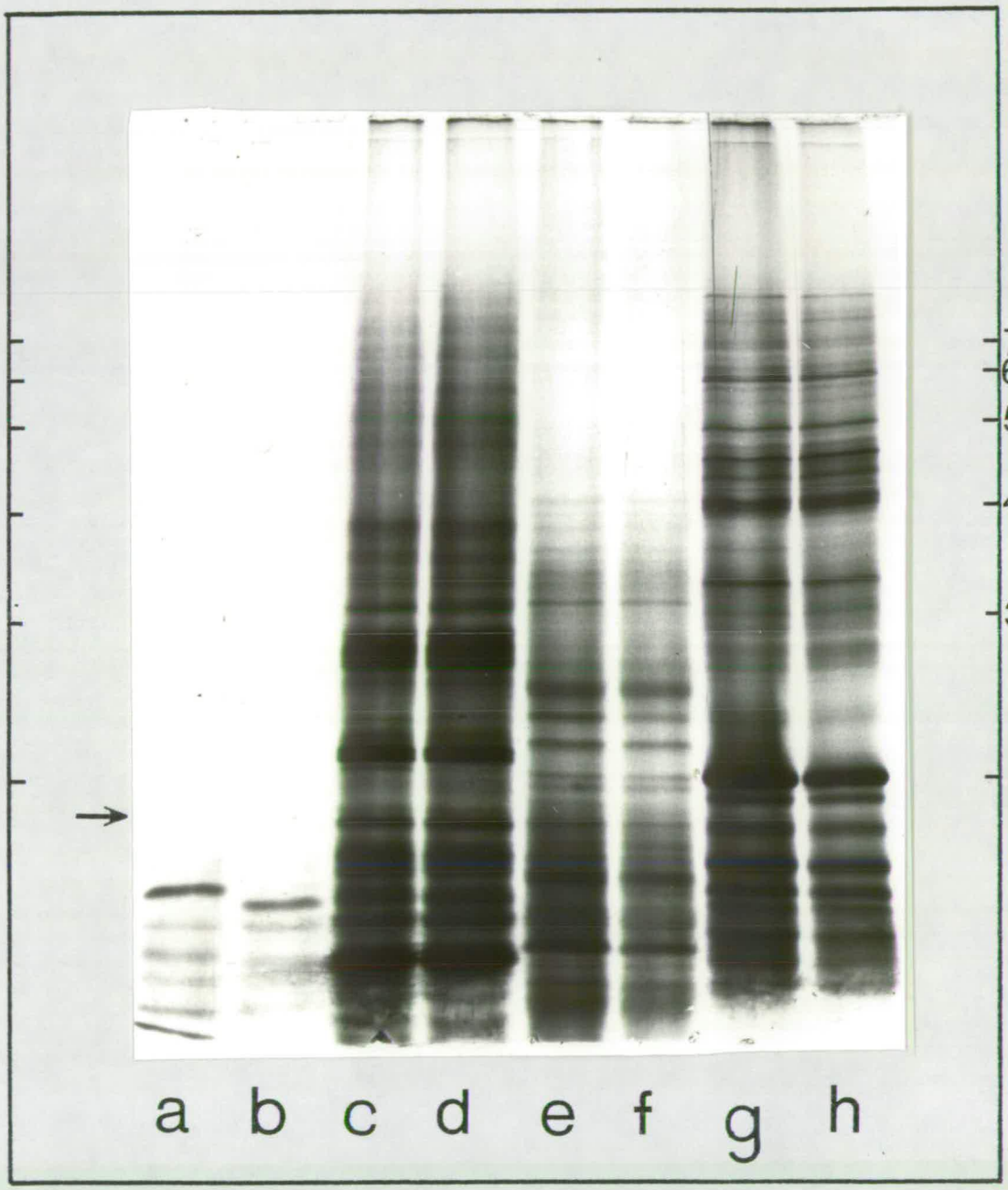
Figure 4.2 Analysis of Translation Products of the Wheat Germ Translation System Directed by Viral RNAs

The Wheat germ translation system was prepared and incubated as described in Materials and Methods with viral RNAs and the total translation products fractionated on 15% polyacrylamide-gels containing SDS in parallel and analysed as described in Materials and Methods. Time courses of the incorporation shown in Figure 4.1a correspond to tracks (a), (b), (c), (e) and (g). Minus RNA control contained a smear of radioactivity of approximately 10,000 M_r.

Track (a) R17 (b) PP7 (c) Cowpea strain TMV (d) Cowpea strain TMV 2.0 μg (7.6×10^5 cpm) (e) Wildtype TMV, (f) Wildtype TMV, 2.5 μg (7.0×10^5 cpm) (g) Total BMV (h) Total BMV 4.8 μg (8.6×10^5 cpm).

Arrow indicates the expected position of TMV coat protein in tracks (c), (d), (e) and (f).

Fig.4.2



large number of high molecular weight polypeptides present, none appear to correspond to those of 140,000 and 160,000 M_r seen by Hunter et al. (1976), in both their Wheat germ and Reticulocyte lysate system translation products. Previous authors have shown that, whereas TMV coat protein (17,500 M_r) is not a major product of cell-free translation of Wild type TMV RNA (Roberts et al., 1973; Efron and Marcus, 1973), it is a major product when the translation is carried out with RNA from the Cowpea strain of TMV (Whitfeld and Higgins, 1977). Among the translation products shown here, coat protein does not appear to be a major product. However, a polypeptide of corresponding molecular weight is present among the products directed by Cowpea strain TMV RNA and to a lesser extent by Wild type TMV RNA (arrowed).

Among the translation products directed by BMV RNA there are two polypeptides of approximately 20,000 and 35,000 M_r which may correspond to the products of the same size reported by other workers to be synthesized in a Wheat embryo system directed by BMV RNA (Shih and Kaesburg, 1973). Indeed, BMV can be considered as a source of homologous RNA because this virus can infect wheat (Davies and Kaesburg, 1974).

These results would indicate that the Wheat germ system translates viral RNAs from eucaryotic and possibly procaryotic sources, although, in agreement with previous observations (Davies and Kaesburg, 1974), the system in general only translates with fidelity small RNAs thought to contain the coat protein cistron. The presence of high molecular weight polypeptides among the translation products might indicate that there is little or no RNAase activity in the extract.

(IV) Analysis of Translation Products of the Wheat Germ System Directed by Plant RNAs

The labelled translation products of the Wheat germ system directed by total RNA from various plant sources, the time courses of which are presented in Figure 4.1b, were fractionated by SDS-polyacrylamide-gel electrophoresis, visualised by autoradiography and are shown in Figure 4.3. It is clear that the translation products of the Wheat germ system directed by Spinach leaf and Cucumber cotyledon total RNA are apparently qualitatively different from those that are seen when the E. coli system is programmed with the same RNA (Fig. 3.5). This is also the case with polypeptides programmed with Wheat and Spirodela total RNA, the E. coli products of which are not shown. This is in agreement with previous findings and would lend support to the proposition that the Wheat germ and E. coli systems selectively translate cytoplasmic and organellar RNAs respectively (Bottomley et al., 1976, 1977).

As with the E. coli system translation products directed by RNA extracted from tissue from different plants, the similarity in translation product profile, if not fortuitous, would suggest that the Wheat germ system translates mRNAs common to each source of RNA. The profile of translation products is not affected by the addition of unlabelled methionine after 45 minutes of incubation suggesting that no proteolysis or precursor processing takes place in the system.

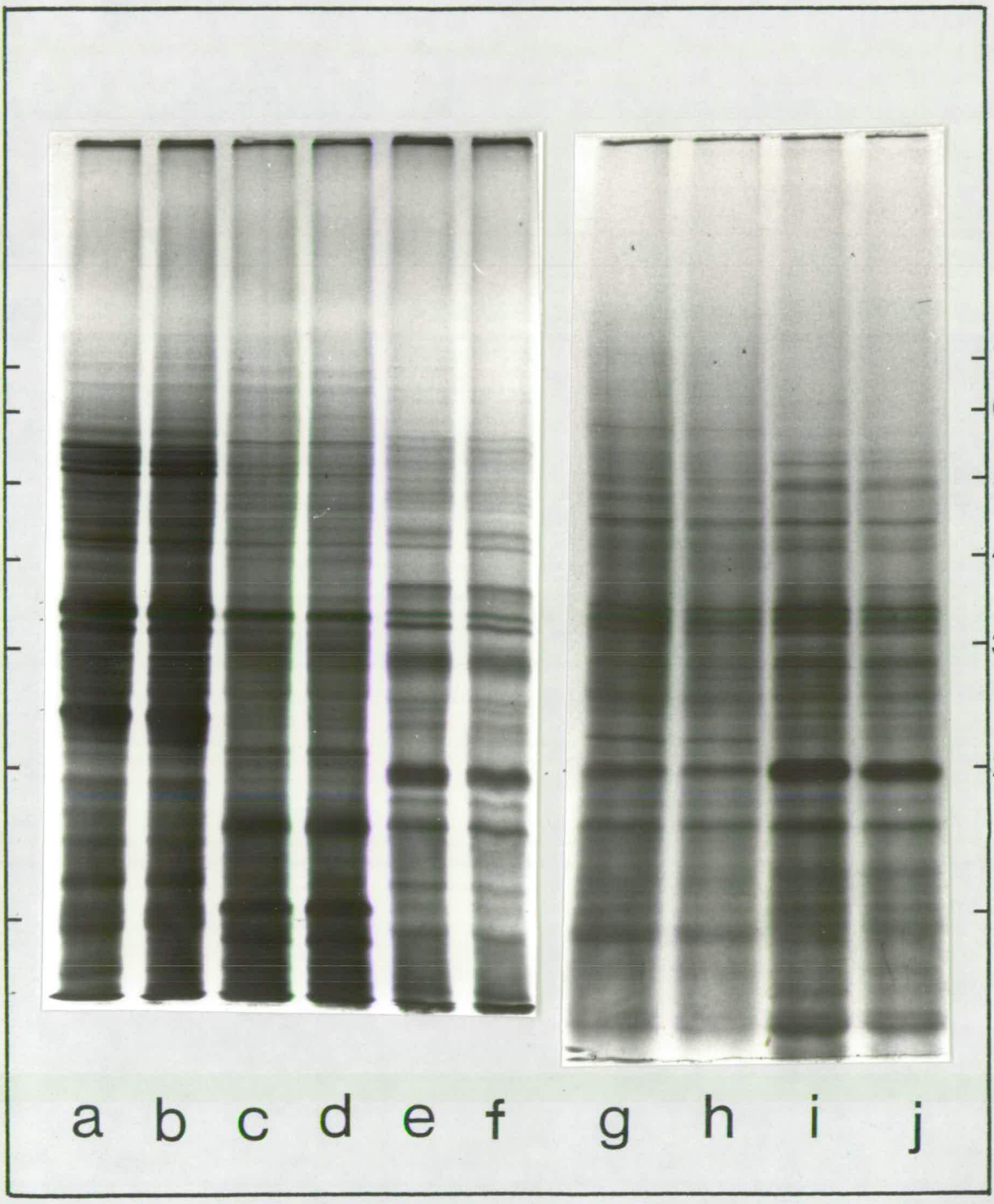
A number of workers have used immunoprecipitation to identify specific polypeptides among the translation products of the Wheat germ system programmed by plant RNA.

Figure 4.3 Analysis of the Translation Products of the Wheat Germ System Directed by Plant Total RNAs

The Wheat germ translation system was prepared and incubated as described in Materials and Methods with various plant RNAs. Total translation products were fractionated on 15% polyacrylamide-gels containing SDS and visualised by autoradiography as described in Materials and Methods. The corresponding time courses of the incorporation are shown in Fig. 4.1 (b) for tracks (b), (d) and (f). Tracks (a)-(f) and (g)-(j) were fractionated on separate gels, correct matching of the two was achieved by co-electrophoresis on a separate gel of the total translation products of the Wheat germ system directed by total RNA extracted from Spirodela and Wheat. The minus RNA control contained a smear of radioactivity of 10,000 M_r.

Tracks (a) Cucumber cotyledon Day 5, 14 µg (7.2×10^5 cpm), (b) Cucumber cotyledon Day 5, 19.5 µg (c) Spinach, 10 µg (5.86×10^5 cpm) (d) Spinach, 20 µg (e) Spirodela 7.2 µg, (3.54×10^5 cpm) (f) Spirodela, 14.4 µg (g) Wheat basal region 8.6 µg (3.31×10^5 cpm) (h) Wheat basal region, 12.9 µg (4.23×10^5 cpm) (i) Wheat apical region, 8.6 µg (6.77×10^5 cpm) (j) Wheat apical region, 12.9 µg (5.51×10^5 cpm).

Fig.4.3



These are the precursors to:

SSu RuBPCase	18,000-20,000 M_r (Dobberstein et al., 1977; Highfield and Ellis, 1978; Cashmore et al., 1978; Tobin, 1978; Chua and Schmidt, 1978).	
Chlorophyll a/b binding protein	32,000 M_r (Apel and Kloppstech, 1978a).	
	33,000/35,000 M_r (Schmidt et al., 1979).	
Ferredoxin	20,500 M_r (Huisman et al., 1978)	
Catalase	55,500 M_r)	
Isocitrate lyase	61,500 M_r) 59,000 M_r)	(Reizman, Weir, Titus, Leaver and Becker, unpublished results)
Malate synthase	57,000 M_r)	
Malate dehydrogenase	38,000 M_r)	

The 33,500 M_r precursor to the Spirodela 32,000 M_r chloroplast membrane polypeptide is also thought to be translated in the system and has been identified by partial proteolytic digestion and by the lack of lysine in both the native protein and the precursor (Edelman et al., 1979). It has also been reported that the cytoplasmically synthesized δ and γ subunits of chloroplast ATPase are synthesized as precursors in a Wheat germ system programmed with Spinach poly $(A)^+$ RNA (Price, Watanabe, and Zielinski, unpublished results).

I was primarily interested in the translation of the mRNA extracted from Cucumber cotyledons that codes for SSu RuBPCase.

It is now well documented that SSu mRNA is translated to produce a precursor *in vitro*. By immunoprecipitation I have identified this precursor. Direct immunoprecipitations were carried out using the ribosomal supernatants of the Wheat germ system directed by total Cucumber cotyledon RNA, prepared as described in Materials and Methods (p.83). The antisera used were raised against Spinach RuBPCase and Cucumber SSu RuBPCase. With each antisera a 25,000 M_r polypeptide was immunoprecipitated (for example, see Fig. 4.4). This was of interest not least because it appears to be a precursor 5,000 daltons larger than those previously reported and almost twice the size of the SSu RuBPCase of Cucumber, (14,000 M_r).

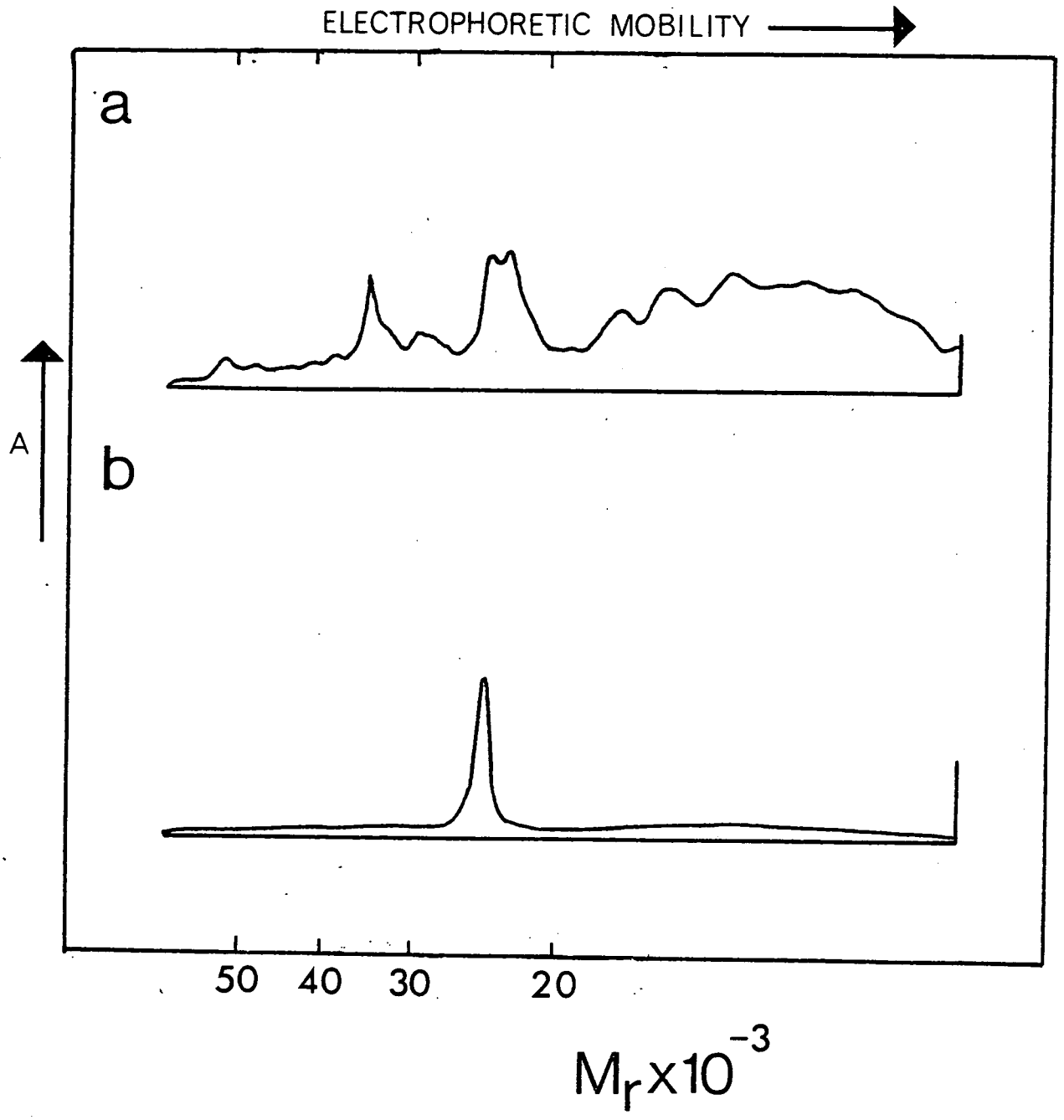
Two possible artifacts of *in vitro* translation were considered that might result in the appearance of a 25,000 M_r polypeptide that could contain the RuBPCase antigenic determinants - firstly, incorrect translation of the SSu mRNA by the Wheat germ system, or secondly, premature termination of translation of LSu mRNA. To test the first possibility, I attempted to identify PSSu amongst the translation products directed by other sources of RNA, e.g. Wheat and Spinach. Wheat and Spinach RNA have been reported to direct the synthesis *in vitro* of a PSSu polypeptide of 20,000 M_r (Roy *et al.*, 1976) and 18,000 M_r (Chua and Schmidt, 1978) respectively. Incorporations were carried out using total RNA extracted from two regions of the first leaf of Wheat, the basal and apical regions. In linear monocotyledonous leaves proplastids are found in the youngest cells at the basal region of the leaf. Successively larger and more developed plastids are present in cells towards the apical regions of the leaf (Leech, 1976). Thus it might be

Figure 4.4 Immunoprecipitation of PSSu RuBPCase from the Translation Products of the Wheat Germ System Directed by Total Cucumber Cotyledon RNA

The Wheat germ system was prepared and incubated with Cucumber Day 5 light-grown cotyledon RNA and the translation products were immunoprecipitated directly using anti-Spinach RuBPCase sera, as described in Materials and Methods (p. 83). Immunoprecipitates were analysed on 20% (w/v) polyacrylamide-gels containing SDS with subsequent fluorography. The fluorographs were scanned using a Kipp and Zonen Densitometer. Preimmune antisera precipitated products of only low molecular weight polypeptides over the same exposure period.

(a) Complete translation products (b) Immunoprecipitate

Fig.4.4



expected that synthesis of chloroplast proteins will be taking place towards the apical region of the leaf. Comparing the translation products directed by the two RNA samples (Fig. 4.3 g,h and i,j). We see an enhancement of the 20,000 M_r polypeptide amongst the translation products directed by the RNA extracted from the apical region of the leaf. This polypeptide has also been immunoprecipitated using antisera raised against Wheat SSu. A polypeptide of approximately 20,000 M_r has also been immunoprecipitated using antisera raised against Spinach SSu from the translation products of the Wheat germ system directed by Spinach RNA. The 20,000 M_r polypeptide does not generally appear to be a major translation product of the Wheat germ system programmed by Spinach RNA (see Fig. 4.3, tracks c and d). Why this should be the case has not been investigated, however, it may be due to the age of the tissue from which the RNA is extracted. The difference in the apparent molecular weight of the Spinach PSSu reported here, approximately 20,000 M_r and that of other workers - 18,000 M_r (Chua and Schmidt, 1978), may be due to differences in the gel electrophoretic technique (for example, compare the estimation of the molecular weight of Pea PSSu, 18,000 M_r (Chua and Schmidt, 1978) and 20,000 M_r (Highfield and Ellis, 1978).

By comparison of the report of the immunoprecipitation of a 20,000 M_r PSSu from Lemna RNA directed Wheat germ products (Tobin, 1978), I assume that the major polypeptide of the same molecular weight among the translation products directed by RNA extracted from Spirodela shown here (Fig. 4.3, tracks e and f) is also PSSu.

I have previously shown that translatable LSu mRNA is absent from poly $(A)^+$ RNA, (Fig. 3.7). When poly $(A)^+$ and poly $(A)^-$ RNA is used to programme the Wheat germ translation system, the 25,000 M_r polypeptide is present among the translation products directed by both of the RNAs. This would argue against the possibility that the 25,000 M_r polypeptide is an artifact from the premature termination of translation of LSu mRNA.

Thus it would appear that the Wheat germ system translates SSu mRNA in a similar manner to those reported by other workers and that the 25,000 M_r polypeptide is the PSSu coded for by Cucumber cotyledon RNA. However, I have not excluded the possibility that some specific factor of Cucumber cotyledon RNA causes the mis-translation of SSu mRNA <u>in vitro</u>.

Other polypeptides among the translation products have been tentatively identified by co-electrophoresis with marker proteins and other methods, the details of which are given in later sections. These are the α and β subunits of chloroplast ATPase (59,000 and 62,000 M_r), LSu RuBPCase (54,000 M_r), the precursor to chlorophyll a/b binding protein (32,000 M_r) and possibly the 35,000 M_r precursor to the 34,000 M_r chloroplast membrane protein synthesized in isolated Cucumber chloroplasts (see Chapter 5, Section II).

(V) <u>Analysis of Translation Products of the Wheat Germ System Directed by Poly $(A)^+$ and Poly $(A)^-$ RNA</u>

The translation products of the system directed by poly $(A)^+$ and poly $(A)^-$ RNA fractions of total Cucumber cotyledon RNA are

essentially similar, as has been found previously with Pea poly $(A)^+$ and poly $(A)^-$ RNA (Gray and Cashmore, 1976). However, small amounts of LSu RuBPCase appear to be present among the translation products directed by poly $(A)^-$ and total RNA, but not poly $(A)^+$ RNA. Figure 4.5 shows a detail of the 40,000 to 60,000 M_r region of a SDS-polyacrylamide-gel containing the Wheat germ translation products directed by Cucumber total, poly $(A)^-$, poly $(A)^+$ and chloroplast RNA. A labelled polypeptide that co-migrates with LSu synthesized in the E. coli system directed by total RNA is present among the translation products of the Wheat germ system directed by total, poly $(A)^-$ and chloroplast RNA, but not poly $(A)^+$ RNA. If immunoprecipitation of the translation products of the Wheat germ system directed by total RNA is carried out using antisera raised against RuBPCase and the autoradiograph of the immunoprecipitated polypeptides is overexposed a faint band of 54,000 M_r is seen (not shown).

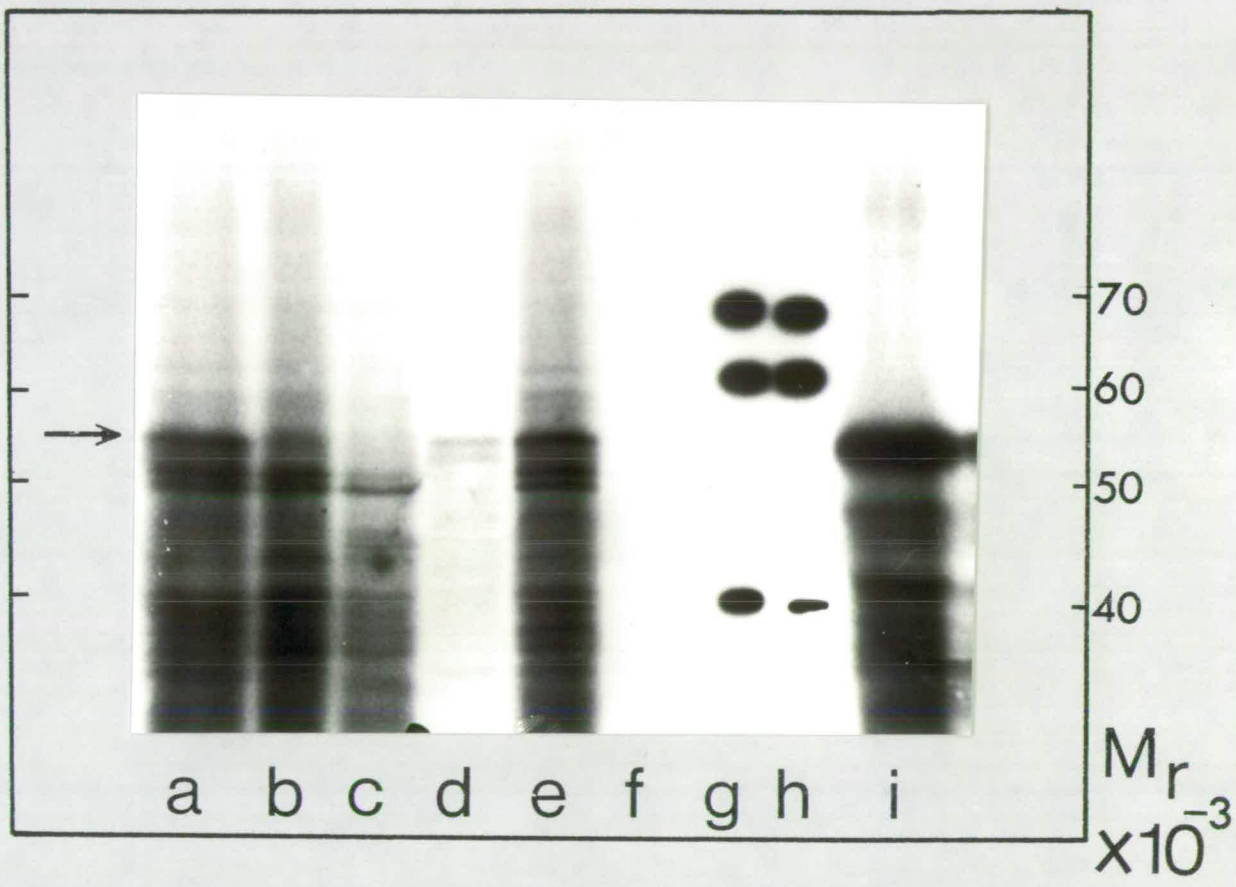
Thus it would appear that the Wheat germ system translates Cucumber LSu mRNA, albeit with a low efficiency. As discussed in Chapter 1 (Section I d) it has been previously shown using RNAs extracted from lower plants LSu mRNA is efficiently translated in the Wheat germ system (Sagher et al., 1976; Howell, 1978). The relative amounts of LSu among the translation products of the Wheat germ system compared with the E. coli system directed by the same RNA would indicate that the 80S ribosomes of the Wheat germ system translate cytoplasmic mRNAs preferentially. However, this does not totally exclude the translation of organellar mRNAs. When chloroplast RNA is used to programme the Wheat germ system there is a small amount of incorporation

Figure 4.5 Analysis of the Translation Products of the Wheat Germ System Directed by Poly(A)$^+$ and Poly(A)$^-$ Fractions of Cucumber Total RNA

The Wheat germ and, for comparison, the E. coli translation systems were prepared and incubated as described in Materials and Methods with various fractions of Day 5 light-grown Cucumber cotyledon RNA. The products of the translation systems were fractionated on polyacrylamide gels and visualised as described in Materials and Methods. The 40,000 -70,000 M_r region of the resulting autoradiograph is shown here. Tracks (a) - (f) Wheat germ translation products, track (i) E. coli translation products. Arrow indicates the expected position of LSu RuBPCase.

Track (a) Cucumber total cotyledon RNA, 28.8 µg (6.17 x 10^5 cpm), (b) Cucumber cotyledon poly(A)$^-$ RNA, 29.5 µg (4.50 x 10^5 cpm), (c) Cucumber cotyledon poly(A)$^+$ RNA, 0.5 µg (4.85 x 10^5 cpm), (d) Cucumber chloroplast RNA, 28.0 µg (1.70 x 10^5 cpm), (e) as slot (a), (f) minus RNA (g) - (h) molecular weight markers (i) Cucumber total cotyledon RNA 28.8 µg (9.19 x 10^5 cpm).

Fig.4.5



(see the following section), with the only apparent products being faint labelled bands of 54,000, 35,000 and 33,000 M_r.

(VI) The Effect of RNA Concentration on [^{35}S]met Incorporation by the Wheat Germ System

Incubating the Wheat germ system with increasing amounts of RNA generally leads to an increase in the overall incorporation of [^{35}S]met into protein (Fig. 4.6). After a peak with increasing concentrations of RNA the overall incorporation of [^{35}S]met declines. Analysis of the translation products showed that over the range of RNA concentrations used there was apparently no preferential translation of a specific mRNA.

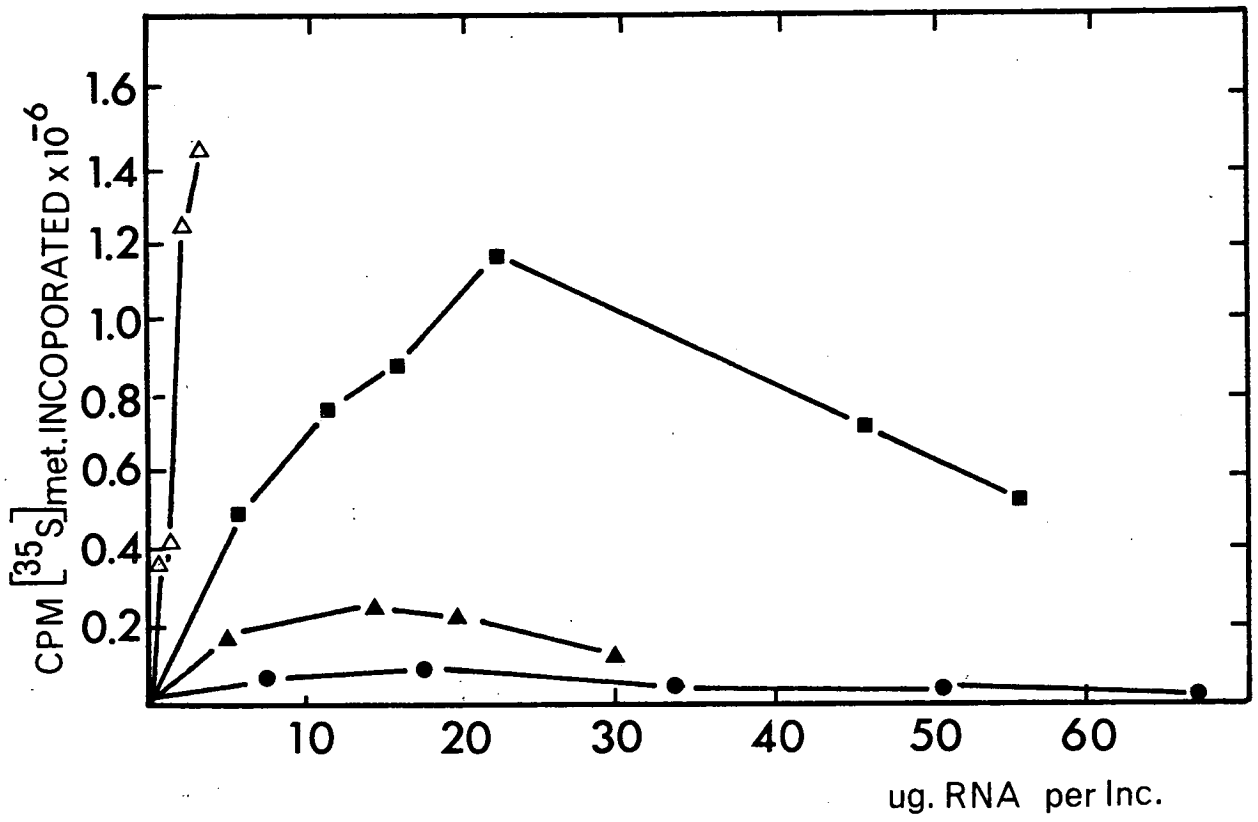
It is apparent that poly $(A)^+$ RNA is a very efficient template in the Wheat germ system. Over the range of RNA concentrations used there was no apparent saturation of the system. Poly $(A)^-$ RNA is not as an efficient template as total RNA in the Wheat germ system. Chloroplast RNA, as indicated in the previous section, does not stimulate a great amount of incorporation of [^{35}S]met into protein by the Wheat germ system at the RNA concentrations used. This is in agreement with previous observations (Bottomley et al., 1976).

Figure 4.6 Effect of Increasing RNA Concentrations on [^{35}S] Methionine Incorporation into Protein by the Wheat Germ System

The Wheat germ system was prepared and incubated as described in Materials and Methods with increasing amounts of different fractions of Day 5 Cucumber cotyledon RNA.

Cucumber cotyledon poly(A)$^{+}$ RNA ($\triangle - \triangle$), Cucumber total cotyledon RNA ($\blacksquare - \blacksquare$), Cucumber cotyledon poly(A)$^{-}$ RNA ($\blacktriangle - \blacktriangle$), Cucumber chloroplast RNA ($\bullet - \bullet$).

Fig.4.6


CPM [35S]met. INCOPORATED x10-6
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
10
20
30
40
50
60
ug. RNA per Inc.

Chapter 5. Characterisation of Protein Synthesis in Chloroplasts Isolated from Cucumber Cotyledons

(I) Introduction

As we have seen in the preceding sections, two heterologous cell-free translation systems directed by the same RNA yield polypeptide products that are qualitatively different. Thus, in order to gain a more accurate picture of protein synthesis within the chloroplast, I sought to optimise an *in vitro* translation system using isolated Cucumber cotyledon chloroplasts.

(II) Optimisation of the Isolated Chloroplast System

For optimising the system chloroplasts were extracted from Day 5 or Day 6 light-grown cotyledons and incubated *in vitro*, as described in Materials and Methods (pp.73,74). Incubations were carried out at 20°C in a final volume of 300 µl containing 100-350 µg of chloroplast protein suspended in the following medium:

Tricine-KOH pH 8.3	66 mM
KCl	200 mM
$MgCl_2$	6.6 mM
^{35}S met.	10 uCi

The system used was essentially that of Siddell and Ellis (1975). However, to optimise the incorporation of [^{35}S]met the effects of varying the Mg^{2+} ion concentration and the addition of increasing levels of ATP were investigated. Chloroplasts were prepared as described in Materials and Methods and were resuspended in the above media lacking $MgCl_2$. Magnesium ion concentration was adjusted by the addition of the appropriate amount of 100 mM $MgCl_2$ with ATP concentration maintained at 2 mM. ATP concentration was

adjusted by the addition of 240 mM ATP with the Mg^{2+} ion concentration maintained at 6 mM. Incubations were carried out as described in Materials and Methods (p. 74). Under the conditions used the Mg^{2+} ion concentration did not appear to be critical to produce maximal incorporation, indeed Mg^{2+} ions are not required in the resuspension buffer for incorporation to take place (see Fig. 5.1 a). The results obtained by varying the ATP concentration show that maximal incorporation took place at 2 mM ATP (see Fig. 5.1 b). This latter result is similar to that found by Siddell and Ellis (1975) who used plastids isolated from greening Pea tissue. However, in contrast to the findings of these authors, incorporation was not dependent on the addition of ATP. Under the conditions of the incubation the provision of an Energy generating system (4 mM ATP, 1 mM GTP, 10 mM PEP and 0.48 units of Pyruvate Kinase) appeared to inhibit incorporation by the isolated chloroplasts.

The time course of incorporation of [^{35}S]met into protein is shown in Figure 5.1 c. In agreement with other workers (Blair and Ellis, 1973), we see that after approximately 20 minutes the rate of light-driven incorporation declines. In the following data, where radioactivity incorporated by isolated plastids is quoted, the 60 minute time point is used.

Some of the characteristics of protein synthesis in isolated chloroplasts are outlined in Table 1. Protein synthesis in the isolated chloroplast takes place in the light, and as an energy source light can be supplemented or replaced by ATP. The ability of ATP to replace light energy has been observed by Bottomley *et al*. (1974) with isolated Spinach chloroplasts. These authors suggested that this may be due to the incorporation of

Figure 5.1 Characteristics of $[^{35}S]$ Methionine Incorporation by Chloroplasts Isolated from Day 6 Cucumber Cotyledons

Chloroplasts were isolated and incubated with $[^{35}S]$ met as described in Materials and Methods (p. 73).

(a) The effect of Mg^{2+} ion concentration (ATP maintained at 2 mM)

(b) The effect of ATP concentration (Mg^{2+} maintained at 10 mM)

(c) Time course of $[^{35}S]$ met incorporation.

Light dirven, 500 µg protein per incubation (■ - ■)

Light driven, 250 µg protein per incubation (▲ - ▲)

Light driven + 2 mM ATP, 250 µg protein per incubation (● - ●)

Dark + 2 mM ATP, 250 µg protein per incubation (o - o)

Dark, 250 µg protein per incubation (▼ - ▼)

Dark + 20 mM NaAc, 250 µg protein per incubation (Δ - Δ)

Fig.5.1

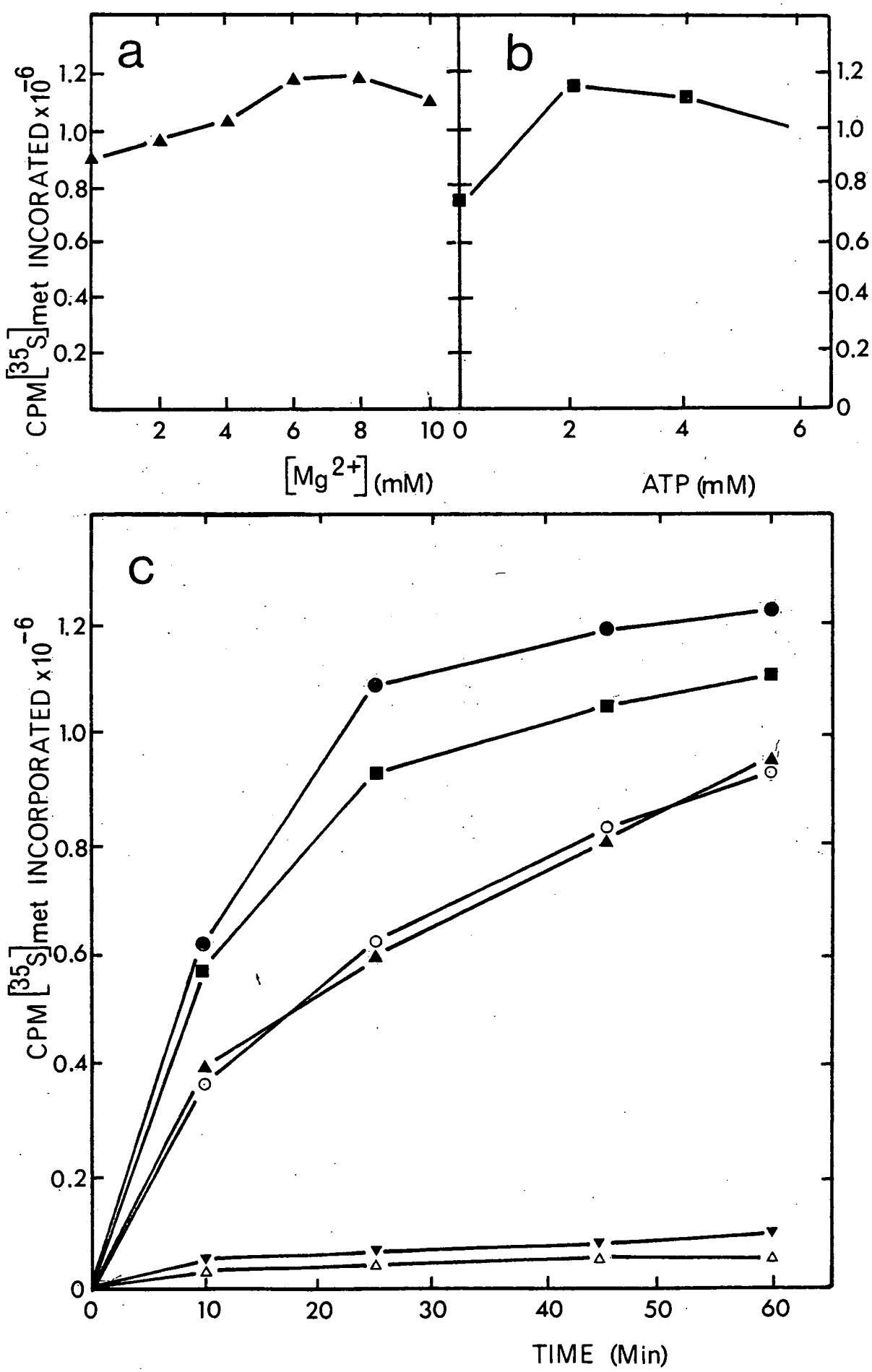

a
b
c
CPM [35S]met INCORPORATED x10-6
1.2
1.0
0.8
0.6
0.4
0.2
0
2
4
6
8
10
[Mg2+] (mM)
ATP (mM)
TIME (Min)
0
10
20
30
40
50
60

Table 1. The Effect of the Energy Source and Inhibitors on the Incorporation of [^{35}S]met into Protein by Isolated Day 6 Cucumber Cotyledon Chloroplasts

Chloroplasts were isolated from Cucumber cotyledons from Day 6 of light-grown seedling development and incubated as described in Materials and Methods. Incorporation of [^{35}S]met into protein was assayed as described in Materials and Methods. In each case where ATP was used the final concentration was 2 mM. Incorporation by the light-driven system is called 100%

Table 1

Energy Source	Treatment	Incorporation (%)
Light	Complete	100
None	Complete	8
Light + ATP	Complete	125
ATP	Complete	100
Light	+ D-threo-chloramphenicol (50 µg/ml)	27
None	+ D-threo-chloramphenicol (50 µg/ml)	6
Light	+ Cycloheximide (12.5 µg/ml)	106
None	+ Cycloheximide (12.5 µg/ml)	6
Light	+ Ribonuclease (33 µg/ml)	80
Light	+ CCCP (5 µM)	50
Light + ATP	+ CCCP (5 µM)	70
None	+ Na acetate (20 mM)	7

radioactivity by lysed chloroplasts. This suggestion can be ruled out by the observation that the incorporation by the isolated chloroplasts is largely insensitive to ribonuclease. It has been suggested that sensitivity to ribonuclease is directly related to chloroplast intactness (Blair and Ellis, 1973; Ellis et al., 1973). Incorporation by isolated chloroplasts is inhibited by D-threo-chloramphenicol but not cycloheximide. Thus it appears that, in common with previous findings (for example, Blair and Ellis, 1973; Siddell and Ellis, 1975), protein synthesis in this system takes place in intact chloroplasts.

Carbonyl cyanide m-chlorophenyl-hydrazone (CCCP), a commonly used inhibitor of photophosphorylation, only inhibited incorporation by 50% (Table 1). This is in contrast to the findings of Blair and Ellis (1973) who, using isolated Pea chloroplasts, found complete inhibition. Siddell and Ellis (1975) using etioplasts isolated from etiolated Pea tissue, with ATP as an energy source, found that inhibition was only 22%. The intermediate effect of CCCP with chloroplasts isolated from Day 6 Cucumber cotyledons may be a reflection of the intermediate age of the plastid used. Etioplasts unable to use light energy would presumably be unaffected by an inhibitor of photophosphorylation, whereas mature chloroplasts able to use light as an energy source (which can only be partially replaced by ATP) would be inhibited by CCCP.

The amount of incorporation in the dark with the addition of Na acetate allowed me to assess the amount of bacterial contamination in the chloroplast preparation and indicates that it was very low.

The translation products of the isolated chloroplasts were fractionated by SDS-polyacrylamide-gel electrophoresis and

visualised by autoradiography or fluorography. The translation products from each of the above treatments, if they were resolvable, were essentially similar.

(III) Translation Products of the Isolated Chloroplast System

The translation products of the isolated chloroplasts were fractionated by SDS-polyacrylamide-gel electrophoresis and visualised by autoradiography or fluorography (see Fig. 5.2, tracks c and d).

It is clear that a number of labelled polypeptides correspond to stained chloroplast proteins. The translation products have been fractionated by the method of Douce et al. (1973), as described in Materials and Methods (p. 78) into those which are membrane-bound or soluble. The majority of the polypeptides were membrane-bound and those that were soluble are arrowed between tracks b and c in Figure 5.2. These include 3 polypeptides between 70,000 and 80,000 M_r which appear on the stained gel and are labelled to a low level and are only seen when autoradiographs are over-exposed. Those polypeptides that are membrane-bound are associated with the fraction containing grana and stroma lamellae. No polypeptides, other than contaminating LSu RuBPCase, appear to be specifically associated with the chloroplast envelopes.

Those polypeptides previously found to be synthesized in isolated chloroplasts and positively identified by other workers are: LSu RuBPCase of Pea, 55,000 M_r (Blair and Ellis, 1973), the α, β and ϵ subunits of Spinach and Pea chloroplast ATPase, 59,000, 56,000 and 13,000 M_r (Mendiola-Morgenthaler et al., 1976; Ellis, 1977), the protein synthesis elongation factors G and T_u of

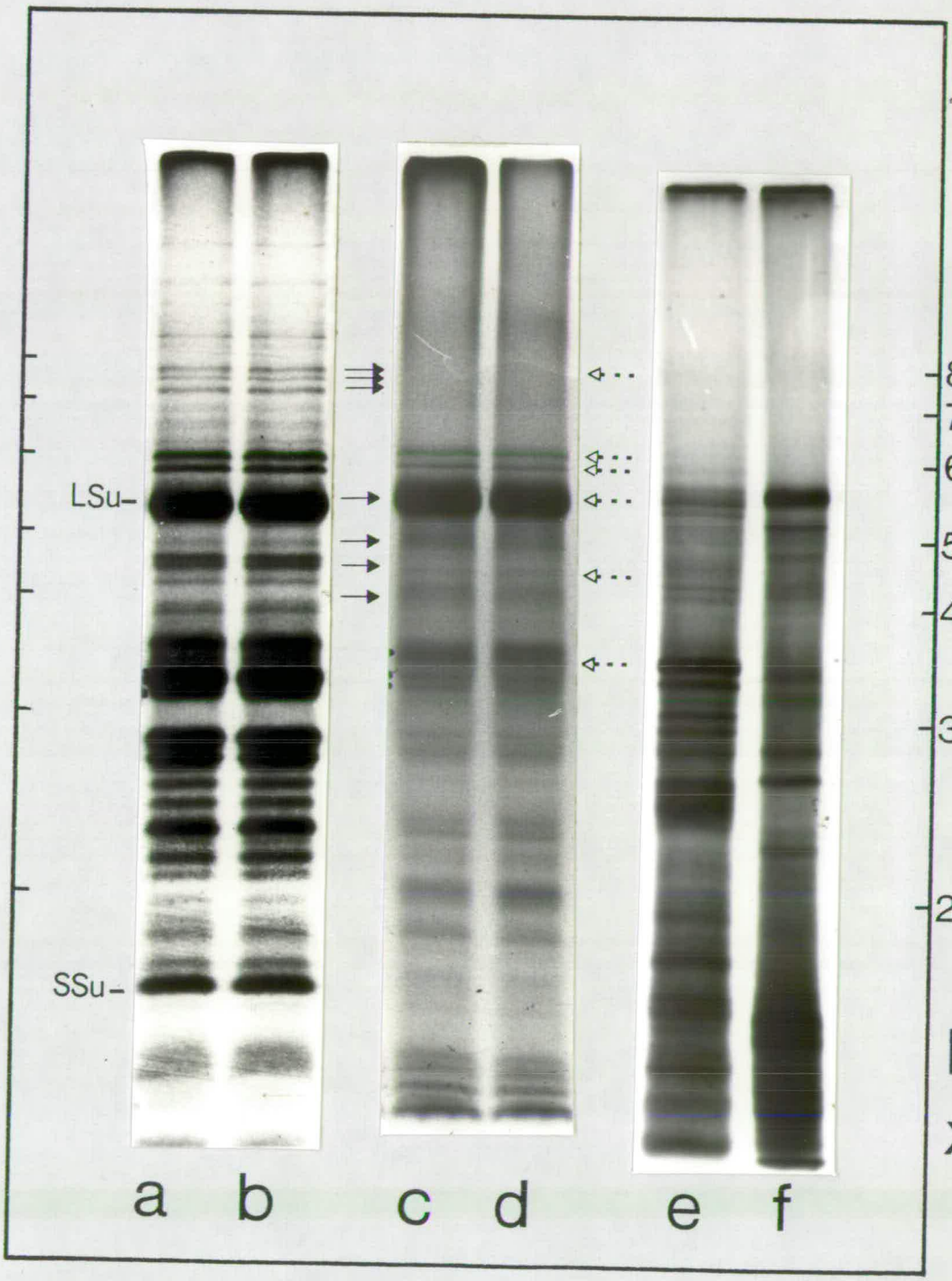
Figure 5.2 Analysis of the Translation Products of Isolated Chloroplasts and Comparison with the Translation Products of the Wheat Germ and *E. coli* Systems

Chloroplasts were isolated from Day 6 Cucumber cotyledons and incubated with [^{35}S]met as described in Materials and Methods. Wheat Germ and *E. coli* systems were programmed with 28.8 µg of RNA extracted from Day 6 light-grown cotyledons. Translation products were fractionated on polyacrylamide gels and visualised as described in Materials and Methods.

Track (a) and (b) stained gel of proteins of isolated chloroplasts (approx. 250 µg protein per track), (c) and (d) autoradiograph of tracks (a) and (b) (1.5×10^5 cpm per track), (e) Wheat germ translation products (2.5×10^5 cpm), (f) *E. coli* translation products (2.5×10^5 cpm).

Arrows between tracks (b) and (c) indicate polypeptides which are synthesized in vitro and are soluble; broken arrows between tracks (d) and (e) indicate polypeptides synthesized *in vitro* which have been tentatively identified.

Fig.5.2



Spinach, 77,000 and 45,000 M_r (Tiboni *et al.*, 1978), Spinach cytochrome f, 32,000 M_r (Doherty and Gray, 1979) and a membrane-bound polypeptide of unknown function of Pea, 32,000 M_r (Eaglesham and Ellis, 1974), which is made as a precursor of slightly higher molecular weight, which is completely processed by the chloroplast *in vitro* (Ellis and Barraclough, 1978). Grebanier *et al.* (1978) have also shown that a major product of protein synthesis in isolated Maize chloroplasts is a polypeptide of 34,500 M_r which is an unprocessed precursor of the 32,000 M_r Maize chloroplast membrane polypeptide. Preliminary reports also suggest that cytochrome b_{559} (Zieblinski *et al.*, 1977) dicyclohexycarbidiimide (DCCD) binding protein (Doherty and Gray, unpublished results) and the apoprotein of chlorophyll protein complex I (Zieblinski and Price, unpublished results) are also synthesized in isolated chloroplasts.

By comparison with purified marker proteins on SDS-polyacrylamide-gel electrophoresis it has been possible to tentatively identify the proteins synthesized by isolated Cucumber cotyledon chloroplasts. These are the LSu RuBPCase, the α and β subunits of ATPase, and possibly the elongation factors G and T_u of protein synthesis (see Fig. 5.2, broken arrows). The polypeptide of approximately 34,000 M_r reported to be a major product of chloroplasts isolated from Pea and Maize (Ellis and Barraclough, 1978; Grebanier *et al.*, 1978) is not a major product synthesized by isolated Cucumber cotyledon chloroplasts. I will show later that this is not due to the stage of development of the tissue from which the chloroplasts were extracted, nor is it due to the incubation conditions because, with Pea and Spinach chloroplasts

incubated under the same conditions, the 34,000 M_r polypeptide is a major translation product.

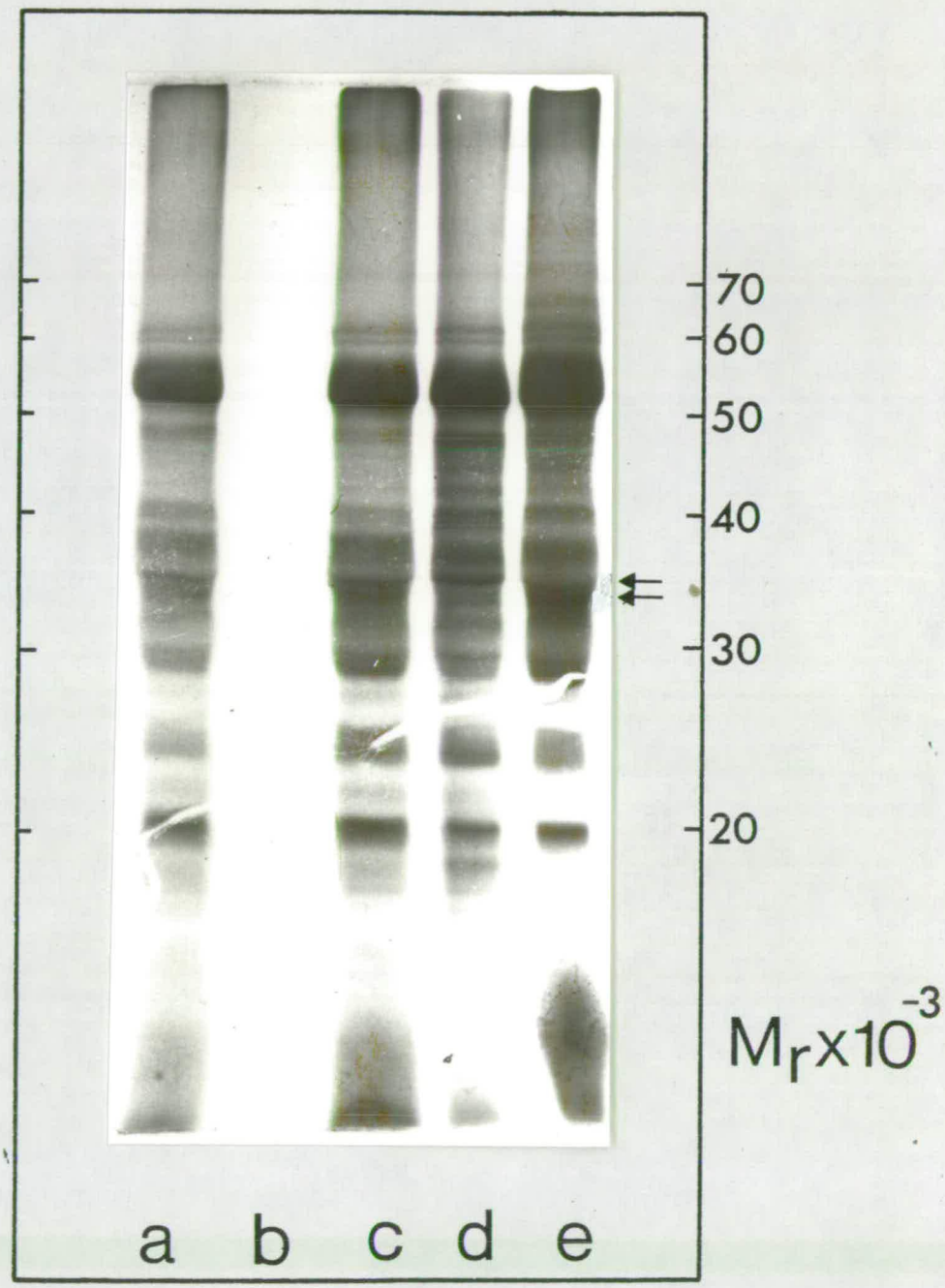
Comparing the isolated chloroplast translation products with those of the Wheat germ and E. coli systems directed by RNA extracted from tissue of the same age, a number of labelled polypeptides from the in vitro systems co-migrate on SDS-polyacrylamide-gel electrophoresis. Only one polypeptide synthesized by all the in vitro systems can be identified with any confidence, namely LSu RuBPCase. Co-migration of other polypeptides may be fortuitous and thus only tentative comparisons of the in vitro translation products can be made. The products of the isolated chloroplast system co-migrating with the products of the Wheat germ system include, amongst some other minor polypeptides, the α and β subunits of ATPase and a polypeptide of approximately 35,000 M_r. It has been shown by other workers that a 33,500 M_r polypeptide precursor to the 32,000 M_r chloroplast membrane protein of Spirodela is synthesized in the Wheat germ system (Reisfeld et al., 1978). A preliminary investigation as to whether any polypeptides synthesized in the isolated chloroplasts were synthesized as precursors and processed by the chloroplasts in vitro was carried out. Isolated chloroplasts were incubated in the light as normal. After 20 minutes, excess unlabelled methionine was added as a 'chase' and the incubation allowed to continue for a further 40 minutes. The translation products were analysed by SDS-polyacrylamide-gel electrophoresis in parallel with polypeptides synthesized in isolated chloroplasts incubated in the light and the dark, with and without ATP, for 60 minutes (see Fig. 5.3). The translation products of isolated chloroplasts incubated in the light are similar

Figure 5.3 Analysis of the Translation Products of Chloroplasts Isolated from Cucumber Cotyledons

Chloroplasts were isolated from Day 6 Cucumber cotyledons and incubated with [^{35}S]met as described in Materials and Methods In a 'pulse-chase' experiment chloroplasts were incubated in the light with ATP and [^{35}S]met for 20 minutes and then excess unlabelled methionine was added and the incubation continued for a further 40 minutes. Translation products were fractionated on SDS-polyacrylamide-gels and visualised as described in Materials and Methods.

Track (a) chloroplasts incubated in light, (b) chloroplasts incubated in dark, (c) chloroplasts incubated in light + 2 mM ATP, (d) chloroplasts incubated in dark + 2 mM ATP, (e) 'Pulse-chase' chloroplasts incubated in light + 2 mM ATP.

Fig.5.3



whether they were incubated in the presence or absence of ATP, or with a 40 minute 'chase' (see Fig. 5.3, tracks a, c and e). Close comparison of these with the translation products of isolated chloroplasts incubated in the dark with ATP shows that, in the latter case, a 35,000 M_r polypeptide appears more prominent (see Fig. 5.3, track d). Amongst the translation products of isolated chloroplasts incubated in the light, the relative amount of the 35,000 M_r polypeptide appears reduced and there is an apparent increase in amount of a polypeptide of approximately 34,000 M_r (see Fig. 5.3 arrowed).

Although this has not been studied in detail it is tempting to speculate that the 34,000 M_r is analogous to the 32,000 M_r polypeptide synthesized as a precursor in isolated Pea chloroplasts (Ellis and Barraclough, 1978). In this case the 34,000 M_r polypeptide is synthesized as a precursor of 35,000 M_r. In the light, isolated chloroplasts can process the precursor, whereas in the dark, the processing activity is reduced.

Recently it has been shown that in intact Spirodela a 33,500 M_r precursor of the 32,000 M_r chloroplast membrane polypeptide is processed rapidly in the light. However, in the dark this activity decays with time (Edelman et al., 1979).

Chapter 6. The Cucumber Developmental System

(I) Introduction

The cotyledons of germinating Cucumber seedlings, used successfully for the investigation of lipid metabolism during germination (Trelease et al., 1973; Becker et al., 1978), provide a useful system to study chloroplast development. Grown under controlled conditions the cellular and morphological events surrounding the development of photosynthetic competence are temporally reproducible. This section will describe this developmental system in detail with special reference to the synthesis of chloroplast proteins and those parameters that are indicative of the acquisition of photosynthetic competence.

(II) Morphology

Cucumber seedlings grown under controlled conditions of a 12 hour photoperiod or in constant dark are shown in Figure 6.1. In both cases development is similar until Day 3. During this initial period the energy for seedling development is provided by the metabolism of stored lipid within the cotyledons, which is accompanied by an increase in glyoxysomal enzyme activities (Trelease et al., 1971; Becker et al., 1978). At Day 4 the cotyledons emerge above the soil. In the light-grown seedlings there is a marked increase in cotyledon size and an accumulation of photosynthetic pigment. This does not occur in dark-grown seedlings where the only obvious morphological change is a dramatic elongation of the hypocotyl. In the light-grown seedling the fresh weight of the cotyledon increases 10-fold between Day 0 and Day 8, whereas in the dark-grown tissue the increase is only 2-fold

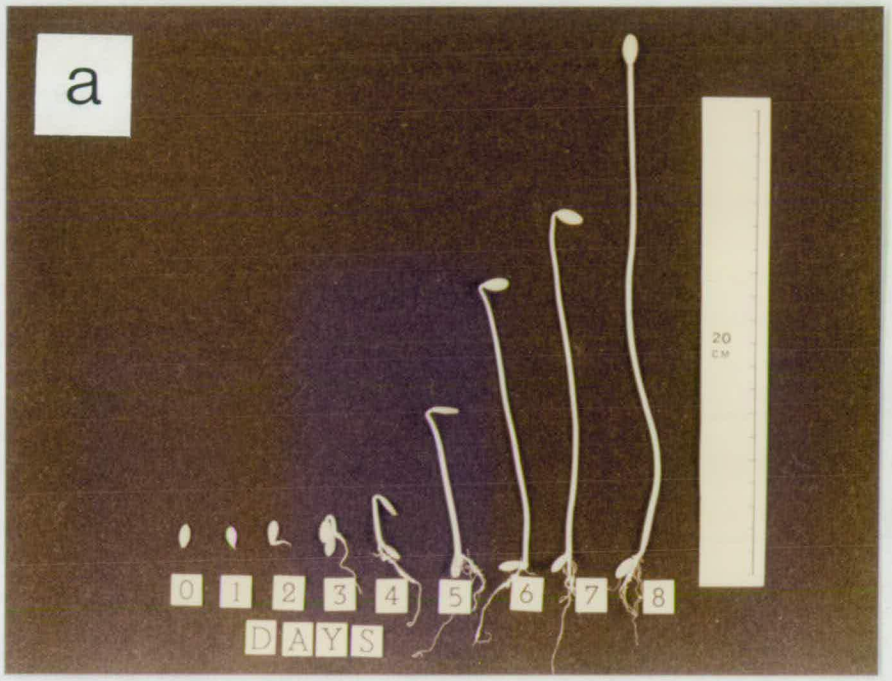
Figure 6.1 Growth and Development of Light- and Dark-grown Cucumber Seedlings

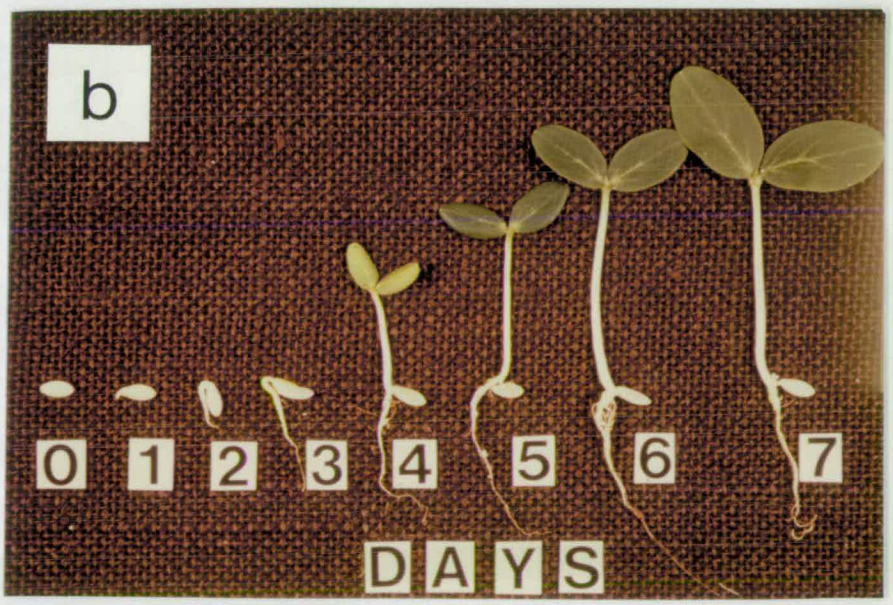
Seedlings were grown as described in Materials and Methods (p. 50).

(a) Dark-grown seedlings.

(b) Light-grown seedlings.

Fig.6.1





(Becker et al., 1978). During the first 8 days of development the cell number of the cotyledons remains constant at approximately $6.78 \pm 0.08 \times 10^5$ in the light and in the dark $6.84 \pm 0.15 \times 10^5$ cells per cotyledon (Becker et al., 1978). Thus all data presented here on a per cotyledon basis can in effect be extrapolated to a per cell basis.

(III) Cotyledonary Protein

The protein content of the cotyledonary homogenates and supernatants prepared as described in Materials and Methods (p. 51), presented as a function of time of germination and early development are shown in Figure 6.2. It is apparent that the amount of homogenate protein declines after Day 3 in both light- and dark-grown cotyledons. It is thought that this decline is associated with the mobilisation of protein bodies (Becker et al., 1978). Accompanying this decline is an increase in the supernatant proteins in light-grown and to a lesser extent in dark-grown cotyledons. There is also an apparent increase in the homogenate proteins in light-grown cotyledons after Day 5.

Coomassie Blue stained SDS-polyacrylamide-gel profiles of the homogenate and soluble proteins are shown in Figure 6.3. To facilitate direct comparison of polypeptide band intensities the actual amount of protein applied to each gel slot corresponded to 1.0% (homogenate) and 2.5% (supernatant) of the protein content of a single cotyledon at a given stage. The most prominent feature of the homogenate polypeptide profiles is the progressive disappearance of a cluster of polypeptides of molecular weights between 20,000 and 30,000 which are probably storage proteins. At Day 3 there appears

Figure 6.2 Developmental Changes in Protein Content of Cucumber Cotyledons

The protein content of light- and dark-grown Cucumber cotyledons homogenate and supernatant fractions was estimated as described in Materials and Methods (p. 51). The data is plotted on a per cotyledon basis.

Homogenate (light-grown) (□ - □), Homogenate (dark-grown) (■ - ■), Supernatant (light-grown) (△ - △), Supernatant (dark-grown) (▲ - ▲).

Fig.6.2

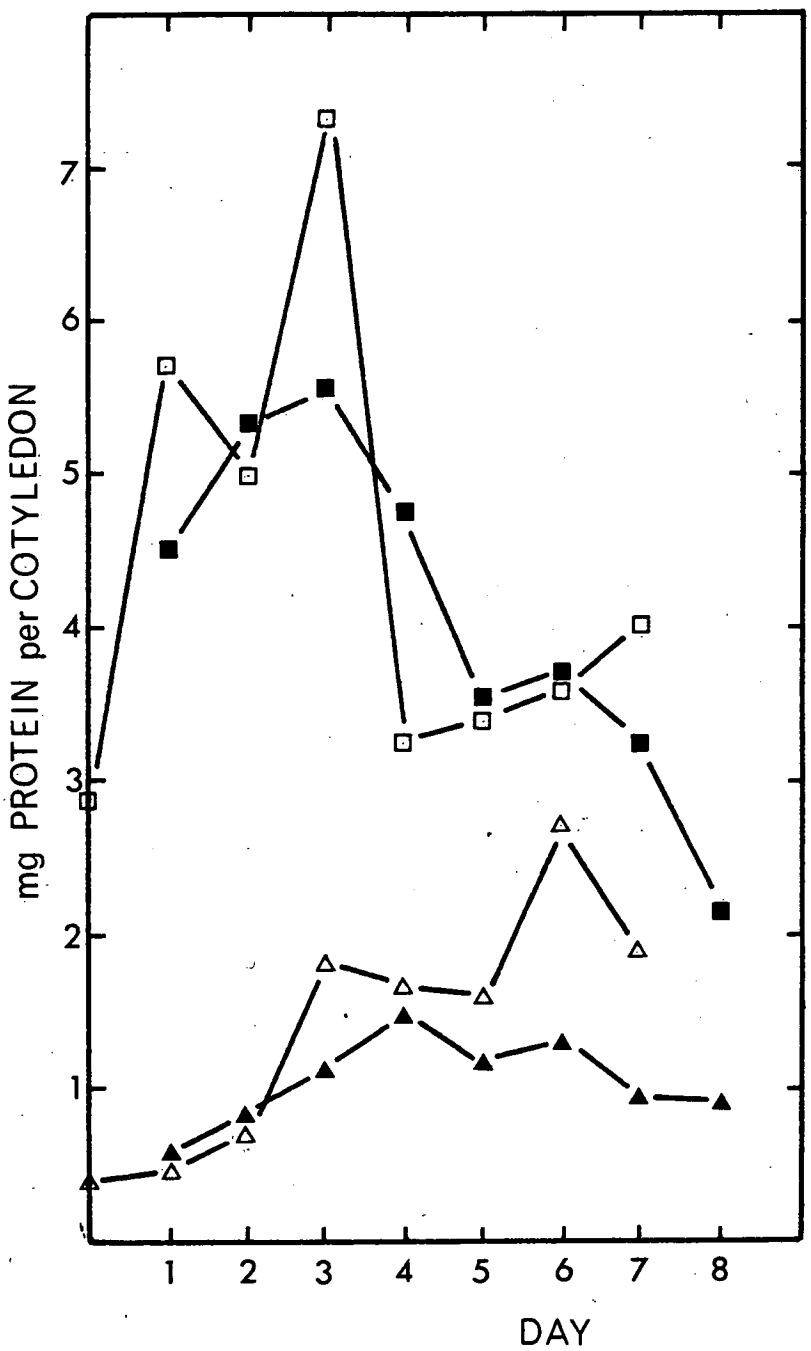


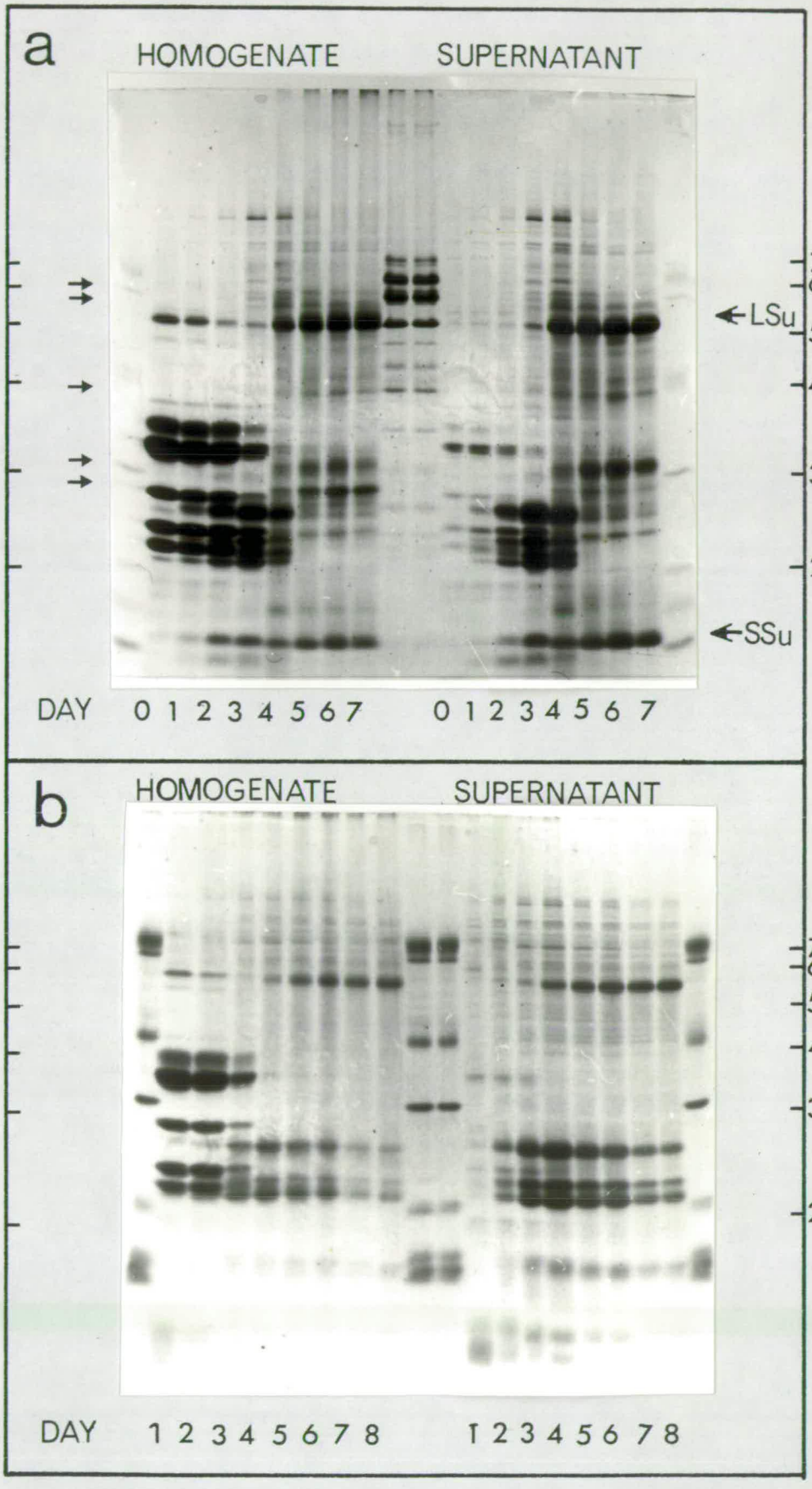
Figure 6.3 SDS-Polyacrylamide-Gels of Homogenate and Supernatant Protein from Cotyledons of Light- and Dark-Grown Cucumber Cotyledons

Proteins were prepared, loaded on a per cotyledon basis (described in text), fractionated on 15% polyacrylamide-gels containing SDS and stained with Coomassie Blue as described in Materials and Methods.

(a) Proteins extracted from light-grown tissue.

(b) Proteins extracted from dark-growh tissue.

Fig.6.3


a
HOMOGENATE
SUPERNATANT
LSu
SSu
70
60
50
40
30
20
DAY 0 1 2 3 4 5 6 7 0 1 2 3 4 5 6 7
b
HOMOGENATE
SUPERNATANT
70
60
50
40
30
20
DAY 1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8
Mr x10-3

a series of lower molecular weight polypeptides which possibly represent the partial solublisation of the storage polypeptides. This apparent solubilisation occurs at the same time in the light- and dark-grown tissue, however, the lower molecular weight polypeptides appear to persist longer in the dark-grown tissue.

It is apparent that some polypeptides appear in the polyacrylamide-gel profile at Day 3 and subsequently increase in staining intensity. Two of the most prominent of these are polypeptides of 54,000 and 14,000 M_r (arrowed). These 2 polypeptides co-migrate with the subunits of purified RuBPCase. Several polypeptides appear to be light dependent in their appearance at Day 4, the most prominent of these being a membrane-bound polypeptide of 29,000 M_r, supernatant polypeptides of 31,000 and 39,000 M_r, and less clear in this photograph membrane-bound polypeptides of 62,000 and 59,000 M_r (arrowed).

Tentative identification of some of these polypeptides, the synthesis of which is light dependent, can be provided by co-migration with marker proteins on SDS-polyacrylamide-gel electrophoresis. For example, Figure 6.4 shows the stained SDS-polyacrylamide-gel profiles of the proteins of Day 5 Cucumber cotyledon etioplasts (the major polypeptide at 68,000 M_r is BSA present in the extraction media), Day 5 Cucumber cotyledon chloroplasts, light-grown Day 5 Cucumber cotyledon homogenate proteins, partially purified Cucumber chloroplast ATPase and Spinach Chloroplast ATPase. The polypeptides of 59,000 and 62,000 M_r co-migrate with the α and β subunits of partially purified ATPase. The membrane-bound 29,000 M_r polypeptide co-migrates with a chloroplast membrane-bound

Figure 6.4 SDS-Polyacrylamide-Gels of Plastid Proteins

Various Cucumber cotyledon plastid proteins were prepared, loaded onto 15% polyacrylamide-gels containing SDS, electrophoresed and stained with Coomassie Blue, as described in Materials and Methods.

Tracks (A), (E) and (H) Marker proteins,

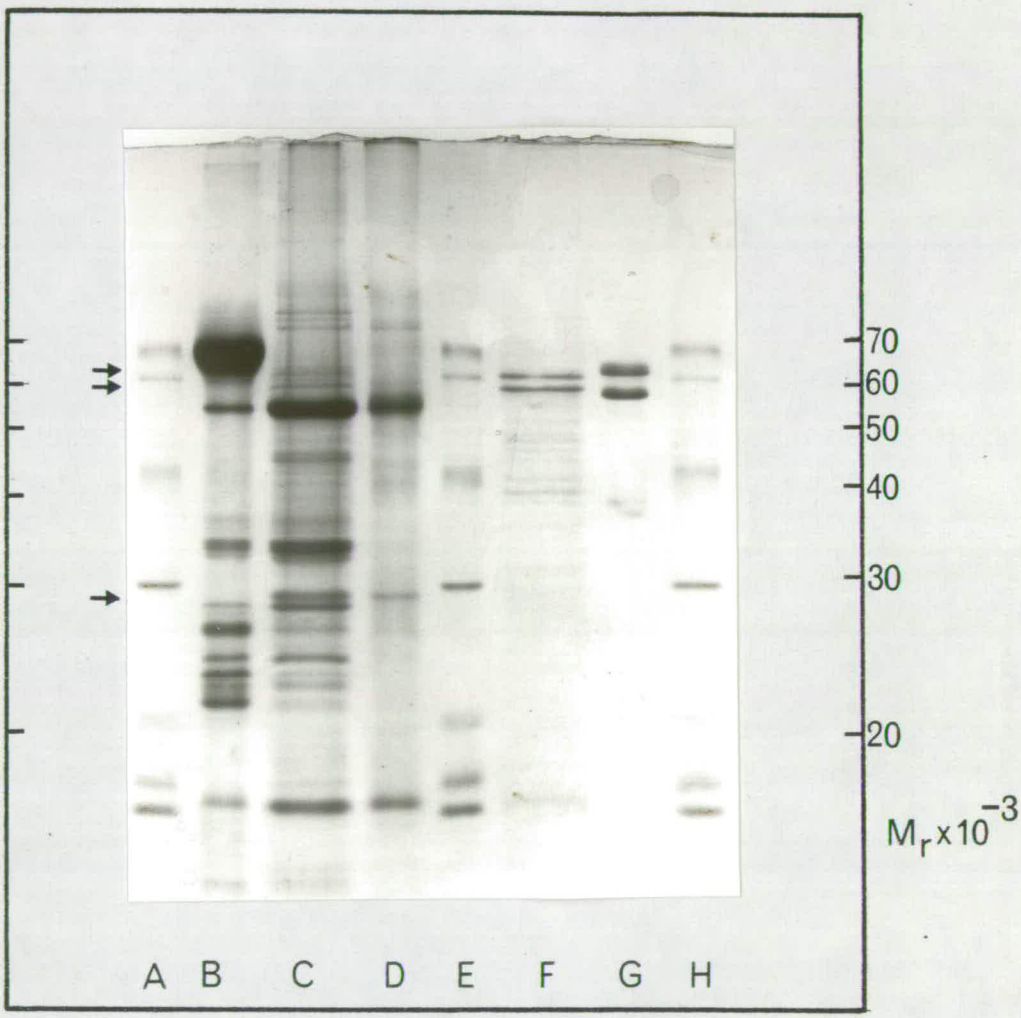
(B) Day 5 etioplast proteins (approx. 150 µg),

(C) Day 5 chloroplast proteins (approx. 250 µg),

(D) Light-grown Day 5 cotyledon homogenate proteins (approx. 70 µg), (F) partially purified Cucumber cotyledon chloroplast ATPase (approx. 20 µg),

(G) purified Spinach chloroplast ATPase (approx. 20 µg).

Fig.6.4



polypeptide which has been found to be associated with chlorophyll on non-denaturing-gel electrophoresis by the method of Markwell et al. (1979) and is thought to be the chlorophyll a/b binding protein (J. Schouten, personal communication).

Comparing the stained protein gel profile of plastids isolated from dark- and light-grown tissue of the same age, it is apparent that some polypeptides are dependent upon light for their synthesis. These include the β subunit of ATPase (the α subunit is obscured by the BSA in the extraction buffer), and the chlorophyll a/b binding protein. However, this observation is based purely on the appearance of stained polypeptides in the polyacrylamide gel. The appearance of chlorophyll a/b binding protein has been shown to be light dependent (for example, see Apel and Kloppstech, 1978a). However, although the synthesis of the ATPase is stimulated by light (Horak and Hill, 1972) it has been found that ATPase is present in etioplasts (Lockshin et al., 1971; Horak and Hill, 1972; Börner et al., 1979). Thus the results presented here are consistent with the findings of others and suggests that the synthesis of some chloroplast proteins are light dependent whereas others are not.

It is clear from Figure 6.3 that although light is not required for the initial synthesis and accumulation of the subunits of RuBPCase it does influence the final levels of the subunits present in the light- and dark-grown tissue.

To estimate the relative amounts of the subunits of RuBPCase synthesized in light- and dark-grown cotyledons, homogenate proteins extracted from cotyledons of each day of development were fractionated

on 15% polyacrylamide-gels containing SDS. As previously described a fixed proportion of the total protein per cotyledon was loaded onto each slot of the gel. After electrophoresis gels were stained with Coomassie Blue as described in Materials and Methods and LSu and SSu identified by their co-migration with purified RuBPCase subunits. The stained polypeptides were scanned using a Densitometer as described in Materials and Methods (p. 59), and the relative amounts of each subunit were estimated by measuring the areas under the peaks of the Densitometric scans (see Fig. 6.5 a-b). The rate of accumulation of the two subunits of RuBPCase appear to be essentially similar, and higher levels of both subunits are present in light-grown tissue. This data depends on the observation that the Coomassie Blue staining is directly related to the amount of protein in the gel (Bennett and Scott, 1971).

Levels of native RuBPCase can be assessed by electrophoresis of total homogenate protein on 5% non-denaturing polyacrylamide-gels, staining the gels with Naphthalene Black 12B and scanning the stained gels with a Densitometer as described in Materials and Methods (p. 57). The accumulation of native RuBPCase protein in the Cucumber cotyledons during seedling development is similar to the accumulation of the individual subunits (see Fig. 6.5 c). RuBPCase protein is first detectable in the light- and dark-grown cotyledons between Days 2 and 3 and increases in amount until Day 4. Thereafter the accumulation of RuBPCase protein continues in the light-grown tissue at the same rate but the rate declines in the dark-grown tissue.

In contrast to these findings the synthesis of chlorophyll, a marker of photosynthetic competence, is totally light dependent

Figure 6.5 Developmental Changes in RuBPCase Protein and Chlorophyll in Cotyledons of Light- and Dark-Grown Cucumber Seedlings

Measurements were carried out using Cucumber cotyledon homogenates and supernatants, as described in Materials and Methods.

(a) Developmental changes in LSu.

Homogenate proteins were fractionated on 15% polyacrylamide-gels containing SDS, stained with Coomassie Blue and amounts of LSu estimated by Densitometric scanning, as described in Materials and Methods. Light-grown cotyledons (□ - □), Dark-grown cotyledons (■ - ■).

(b) Developmental changes in SSu.

Estimated as described in (a). Light-grown cotyledons (□ - □), Dark-grown cotyledons (■ - ■).

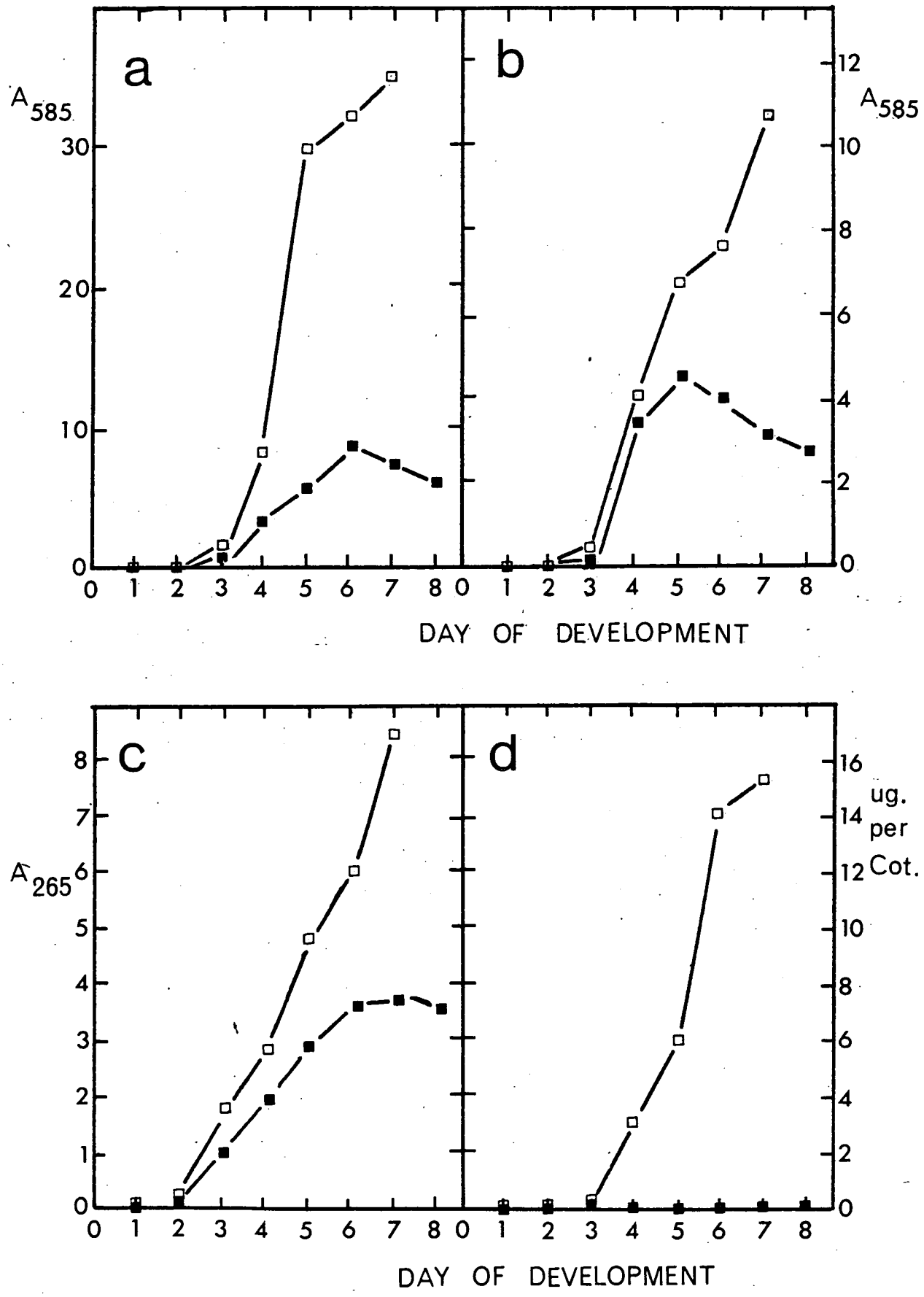
(c) Developmental changes in complete RuBPCase.

Homogenate proteins were fractionated on non-denaturing 5% polyacrylamide-gels, stained with Naphthalene Black and the amount of RuBPCase estimated by Densitometric scanning as described in Materials and Methods. Light-grown cotyledons (□ - □), Dark-grown cotyledons (■ - ■).

(d) Developmental changes in chlorophyll.

Estimations were carried out using cotyledon supernatants as described in Materials and Methods. Light-grown cotyledons (□ - □), Dark-grown cotyledons (■ - ■).

Fig.6.5


a
b
c
d
A585
A265
ug. per Cot.
DAY OF DEVELOPMENT
DAY OF DEVELOPMENT

(Fig. 6.5 d). Comparing Figure 6.5 c and d it is apparent that the initiation of synthesis of RuBPCase protein precedes that of chlorophyll by approximately one day.

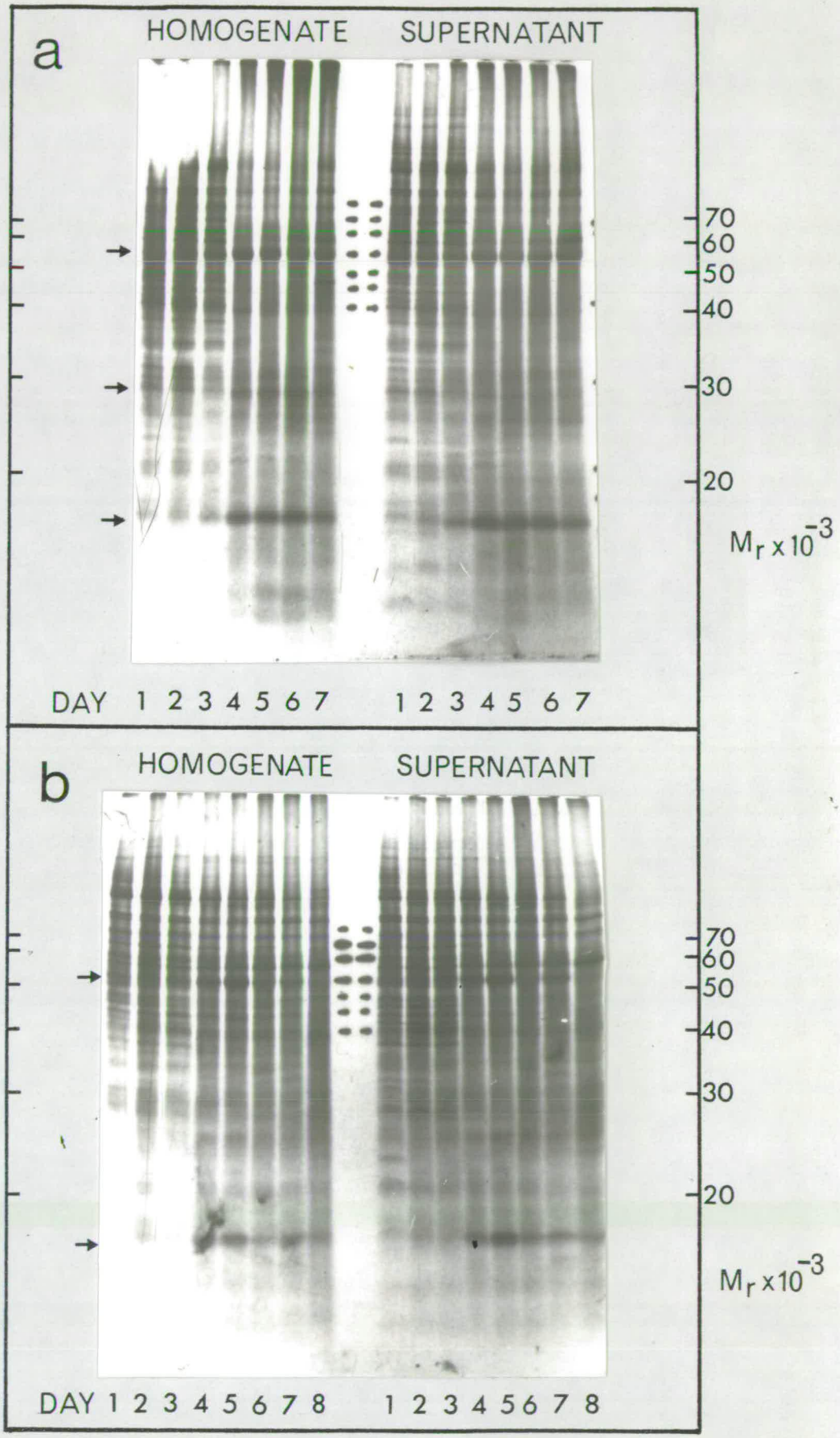
To determine when specific polypeptides are being synthesized in the cotyledons of the developing seedlings *in vivo* labelling experiments were carried out and these have been described in detail in Materials and Methods (p. 84). This involved labelling excised cotyledons with [^{35}S]met at various stages of development for the 24 hour period before the cotyledons were homogenized. Homogenate proteins and proteins of the 10,000 x g supernatants were fractionated on 15% polyacrylamide-gels containing SDS and the labelled polypeptides were visualised by fluorography (see Fig. 6.6). No precise quantitative measurement, however, could be carried out due to possible changes in the endogenous amino acid pool, which was not measured. Comparing the fluorographs with the stained polypeptide profiles (Fig. 6.3) we can see many differences. This may be due to the increased sensitivity of the fluorographs, the possibility that some of the labelled polypeptides are precursors or that some of the polypeptides are rapidly turned over in the cotyledons. It is clear that those polypeptides thought to be storage proteins (molecular weights 20,000 - 30,000) are absent from the *in vivo* labelled protein profiles. Conclusions concerning the time of initiation of synthesis of certain polypeptides can only be assessed accurately by immunoprecipitation. Visual analysis can at best, be only subjective, but considering the previous evidence the data here supports the finding that the synthesis of LSu and SSu RuBPCase (arrowed) is initiated between Day 2 and Day 3, both in the light and in the dark, whereas the appearance of chlorophyll

Figure 6.6 *In vivo* Labelling of Cucumber Cotyledons During Seedling Development

Cotyledons were excised from seedlings of differing stages of development and labelled with [^{35}S] met, as described in Materials and Methods. After a 24 hour labelling period the cotyledons were homogenised and the homogenate and supernatant fractions were fractionated on 15% polyacrylamide-gels containing SDS. Labelled polypeptides were visualised by fluorography as described in Materials and Methods. In each case equal amounts of radioactivity were loaded to each slot of the gel.

(a) Light-grown tissue. (b) Dark-grown tissue.

Fig.6.6


a
HOMOGENATE
SUPERNATANT
70
60
50
40
30
20
$M_r \times 10^{-3}$
DAY 1 2 3 4 5 6 7 1 2 3 4 5 6 7
b
HOMOGENATE
SUPERNATANT
70
60
50
40
30
20
$M_r \times 10^{-3}$
DAY 1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8

a/b binding protein on Day 4 (arrowed) is light dependent.

(IV) Cotyledonary RNA

Measuring the accumulation of RNA in the cotyledons during seedling development in the light and the dark should provide us with an indication of the development of a capcity for protein synthesis within the cotyledons.

Total RNA was extracted from light- and dark-grown cotyledons by the phenol/detergent method and equal amounts of nucleic acid from each stage of development were fractionated on polyacrylamide gels and scanned as described in Materials and Methods (p. 63). Representative gel profiles are shown in Figure 6.7. The scans that were obtained with RNA extracted from dark-grown cotyledons are essentially the same (not shown). Chloroplast ribosomal RNAs are not detectable before Day 3 and thereafter increase in amounts.

To construct the graph shown in Figure 6.8 b, showing the accumulation of ribosomal RNA over the differing days of seedling development the area under each ribosomal peak shown in Figure 6.7 was expressed as a fraction of the total area under the scan curve, and then that fraction was multiplied by the total RNA content (shown in Fig. 6.8 a), obtained as described in Materials and Methods (p. 60).

During cotyledon development there is a striking increase in total RNA. This increase takes place in both the light-and the dark-grown tissue until Day 3. The rate of increase thereafter is maintained in the light-grown tissue until Day 4 but the rate declines in the dark-grown tissue. The decline in the levels of

Figure 6.7 Developmental Changes in Polyacrylamide Gel Profiles of Nucleic Acids Extracted from the Cotyledons of Light-Grown Cucumber Seedlings

Nucleic acids were extracted from light-grown cotyledons by the phenol/detergent method, 25 μg of nucleic acids from each day of development were fractionated on 2.4% (w/v) polyacrylamide gels and these were scanned at 265 nm as described in Materials and Methods.

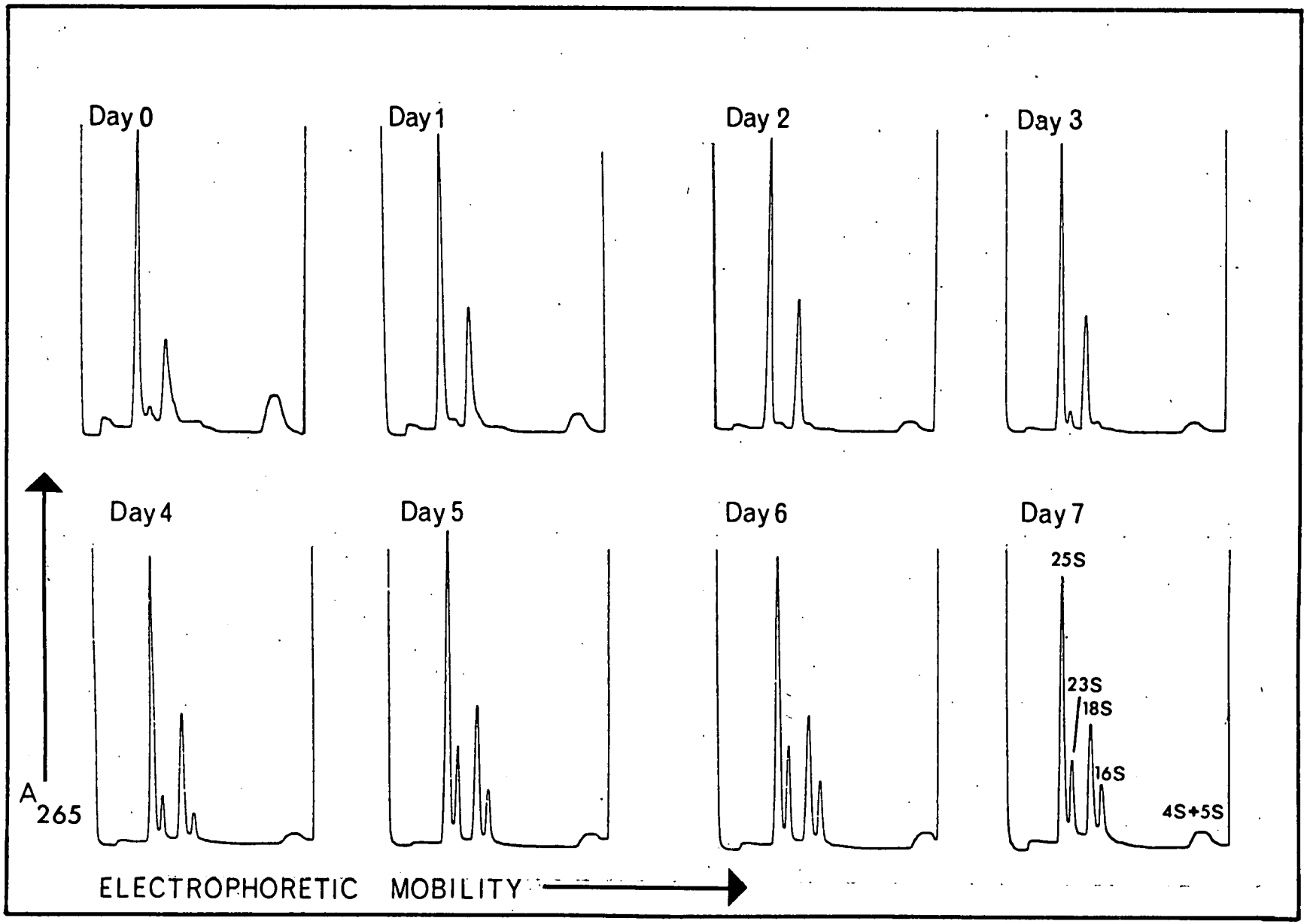

Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
25S
23S
18S
16S
4S+5S
A 265
ELECTROPHORETIC MOBILITY

Fig.6.7

Figure 6.8 Developmental Changes in Ribonucleic Acid Content of Cotyledons of Light- and Dark-Grown Cucumber Seedlings

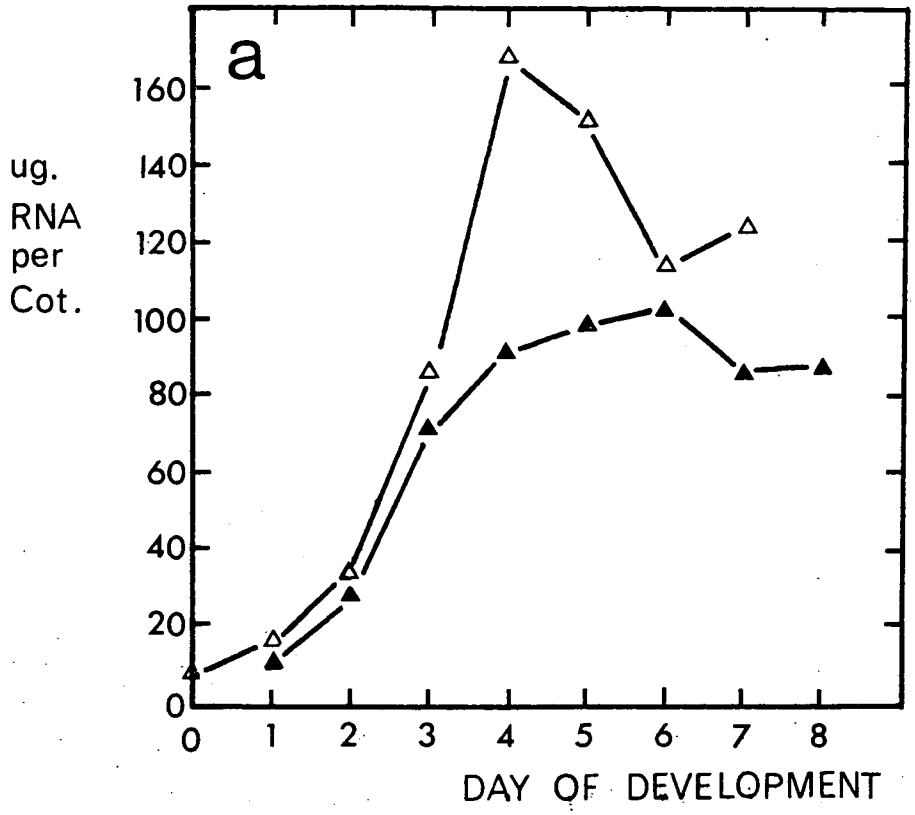
(a) Total Ribonucleic Acid.

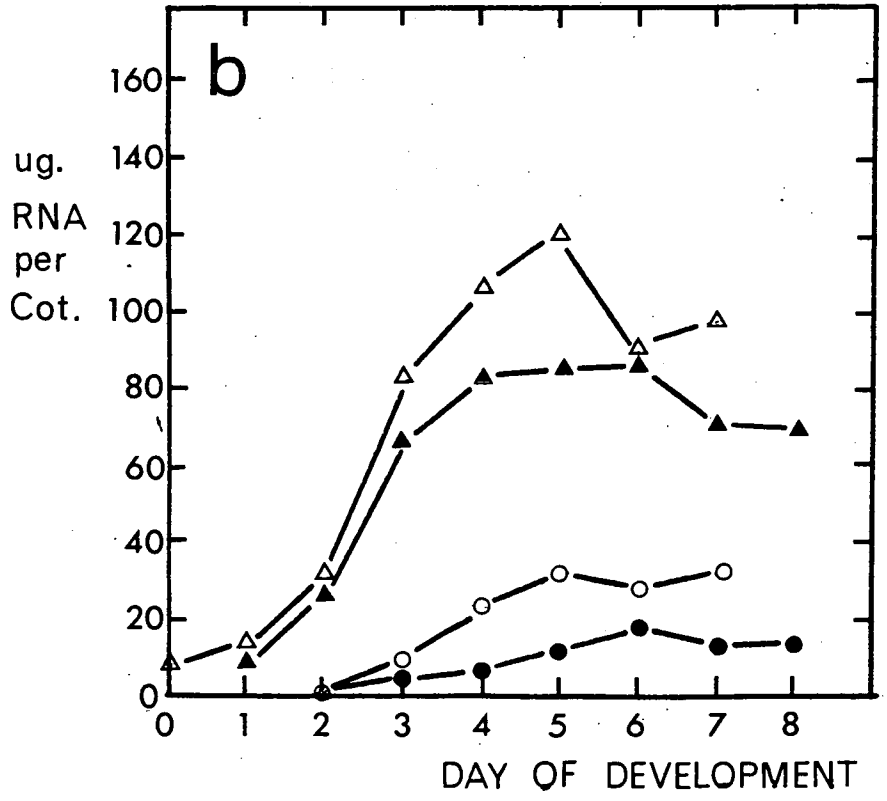
Homogenate samples were assayed for RNA as described in Materials and Methods. Light-grown tissue (△-△), Dark-grown tissue (▲-▲).

(b) Ribosomal RNAs.

The area under each ribosomal RNA peak obtained as described for Fig. 6.7 was expressed as a fraction of the total area of the scan curve and that fraction was multiplied by the total RNA content as shown in (a). Cytoplasmic ribosomal RNA (light-grown) (△-△), cytoplasmic ribosomal RNA (dark-grown) (▲-▲), chloroplast ribosomal RNA (light-grown) (○-○), and chloroplast ribosomal RNA (dark-grown) (●-●).

Fig.6.8


a
ug.
RNA
per
Cot.
160
140
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8
DAY OF DEVELOPMENT


b
ug.
RNA
per
Cot.
160
140
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8
DAY OF DEVELOPMENT

of RNA in the light-grown cotyledons after Day 4 remains unexplained.

Chloroplast ribosomal RNA is undetectable until Day 3 and accumulates to account for 25% (light-grown) and 20% (dark-grown) of the cellular RNA at Day 7. The maximum rate of accumulation of chloroplast ribosomal RNA occurs between Days 3 and 5 in both the light and dark, about 24 hours after the most rapid increase in cytoplasmic ribosomal RNA

Chapter 7. In Vitro Translation of Cucumber Cotyledon RNA from Different Stages of Seedling Development

(I) Developmental Changes in LSu RuBPCase mRNA as Assayed in the E. coli Translation System

As we have seen in Chapter 3, a major translation product of the E. coli system, when programmed with Cucumber cotyledon RNA is, LSu RuBPCase. Thus the optimised E. coli translation system, programmed with RNA extracted from cotyledons of different stages of light- and dark-grown seedling development was used to assay the levels of translatable mRNA for LSu RuBPCase present in the tissue. As the translation products synthesized under the direction of cotyledon total and cotyledon chloroplast RNA were essentially similar, it was decided to use total RNA extracted from the cotyledons of different developmental stages to assay the amounts of translatable mRNA for LSu RuBPCase. In this way I overcame the problem of quantitative extraction of plastids from light- and dark-grown tissue.

Quantitation of the levels of a specific translatable mRNA using an in vitro translation system depends on the quantitative estimation of the translation product programmed by that mRNA. Thus quantitation of mRNA will be affected by inefficient initiation or premature termination of translation of that mRNA in the cell-free system, reducing the levels of complete polypeptide coded for by the mRNA in vitro. Experiments carried out using increasing amounts of total cotyledon RNA to programme the E. coli system show that the translation of LSu mRNA by E. coli ribosomes appears to be the most efficient (discussed in Chapter 3, Section IX). The possibility of premature termination of translation of LSu mRNA by the E. coli system cannot be totally excluded but as discussed in Chapter 3,

(Section VII), this does not appear to be a major difficulty in the system.

In carrying out experiments to assay changes in levels of a specific mRNA in preparations of total RNA it is important to use concentrations of total RNA which will produce quantitative translation of all mRNAs present and not produce preferential translation of a specific mRNA. Thus for each sample of RNA extracted from cotyledons of different days of light- and dark-grown development the effect of increasing the concentration of that RNA on the total incorporation of radioactivity by the system was estimated. The amounts of LSu RuBPCase among the resulting translation products were quantitated by excision of the labelled polypeptide from the gel, and the radioactivity was estimated by scintillation counting as described in Materials and Methods. It was found that, with increasing amounts of RNA up to 20 µg per incubation (400 µg/ml), incorporation of radioactivity into protein was linear and that the synthesis of LSu bore a linear relationship to the total protein synthesis (for example, see Fig. 7.1). Thus in experiments to study the developmental changes in amounts of LSu mRNA the E. coli translation system was programmed with 15 µg per incubation of total RNA extracted from cotyledons of different stages of light- and dark-grown development. The amount of radioactivity incorporated by the E. coli system, programmed with a particular RNA, was calculated on a per µg input basis and is shown in Figure 7.2a. The developmental changes of amounts of radioactivity incorporated by the E. coli system programmed with equal amounts of RNA from light- and dark-grown tissue are essentially similar. The incorporation of radioactivity increases when programmed with RNA extracted from cotyledons up to Day 3 in the light or Day 4 in the dark and thereafter declines.

Figure 7.1 Incorporation of [^{35}S] methionine into Total Protein and LSu RuBPCase by the *E. coli* Translation System Programmed with Total Cucumber Cotyledon RNA

The *E. coli* translation system was incubated, as described in Materials and Methods with increasing amounts of Day 5 light-grown Cucumber cotyledon RNA. The amount of radioactivity incorporated into LSu RuBPCase was estimated by excision of the labelled polypeptide, located by autoradiography and scintillation counting as described in Materials and Methods.

Fig.7.1

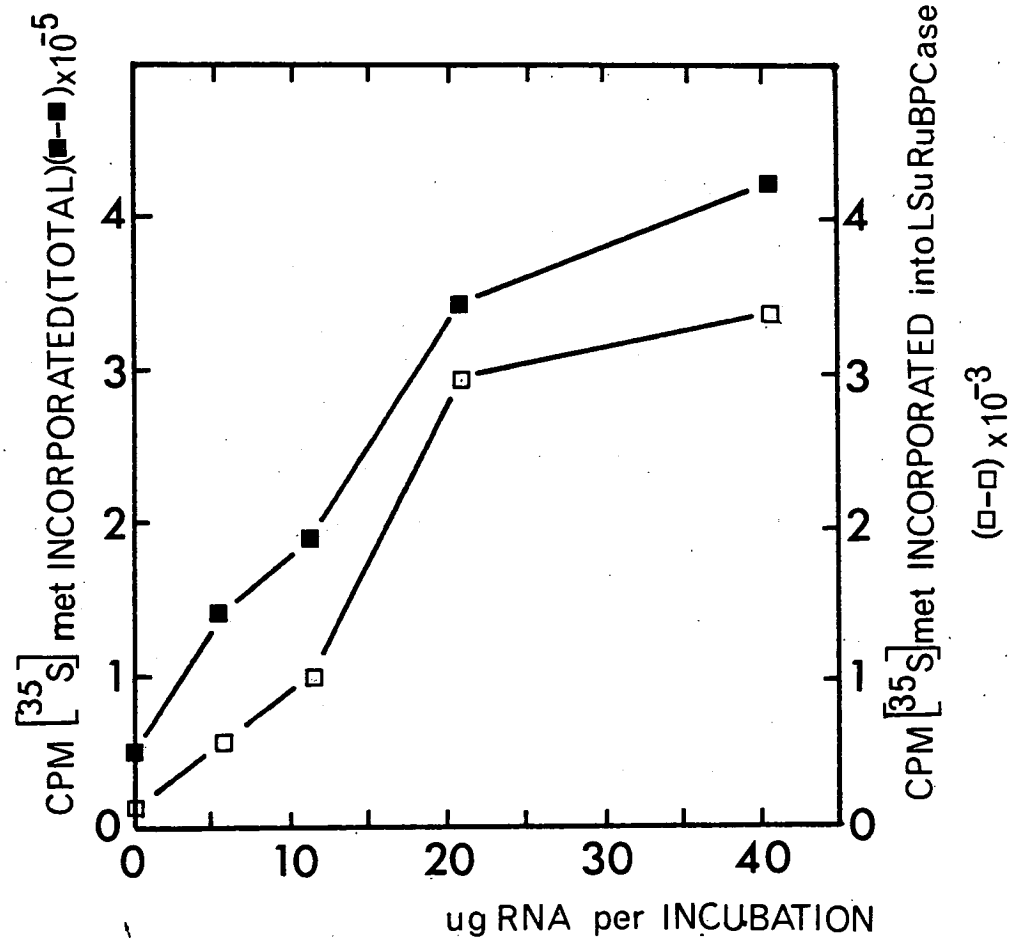

CPM [35S] met INCORPORATED(TOTAL)(■-■)x10-5
CPM [35S]met INCORPORATED intoLSuRuBPCase (□-□) x10-3
0
1
2
3
4
0
10
20
30
40
ug RNA per INCUBATION

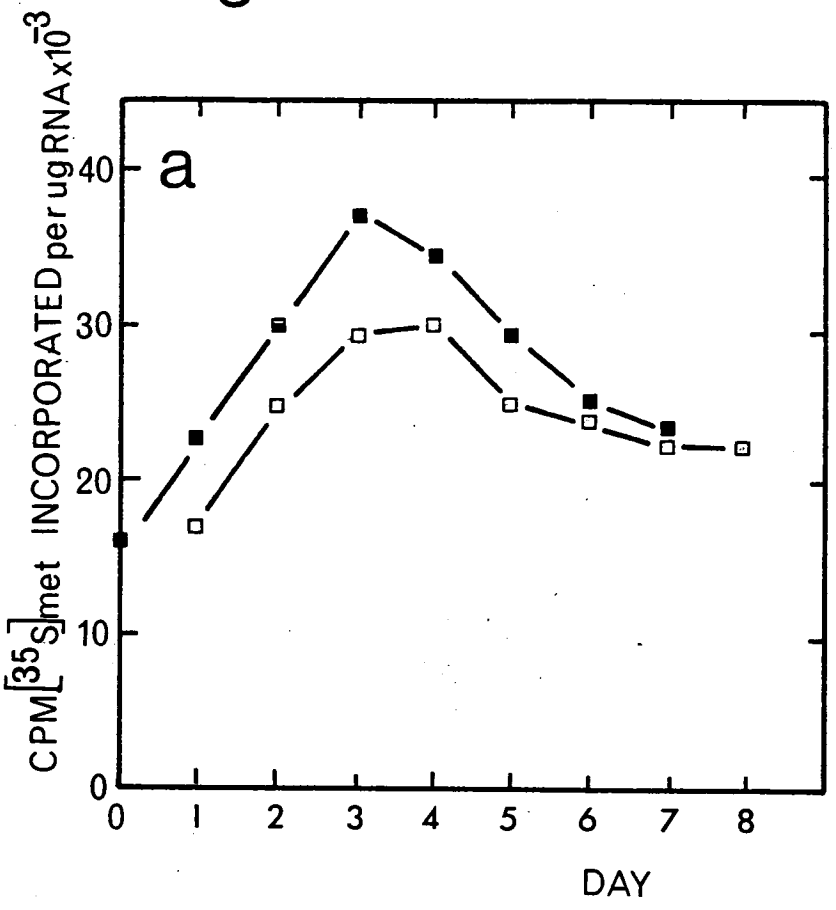
Figure 7.2 Incorporation of [^{35}S] methionine into Protein by the E. coli System Programmed with Equal Amounts of Total RNA Extracted from Cotyledons of Light- and Dark-Grown Cucumber Seedlings.

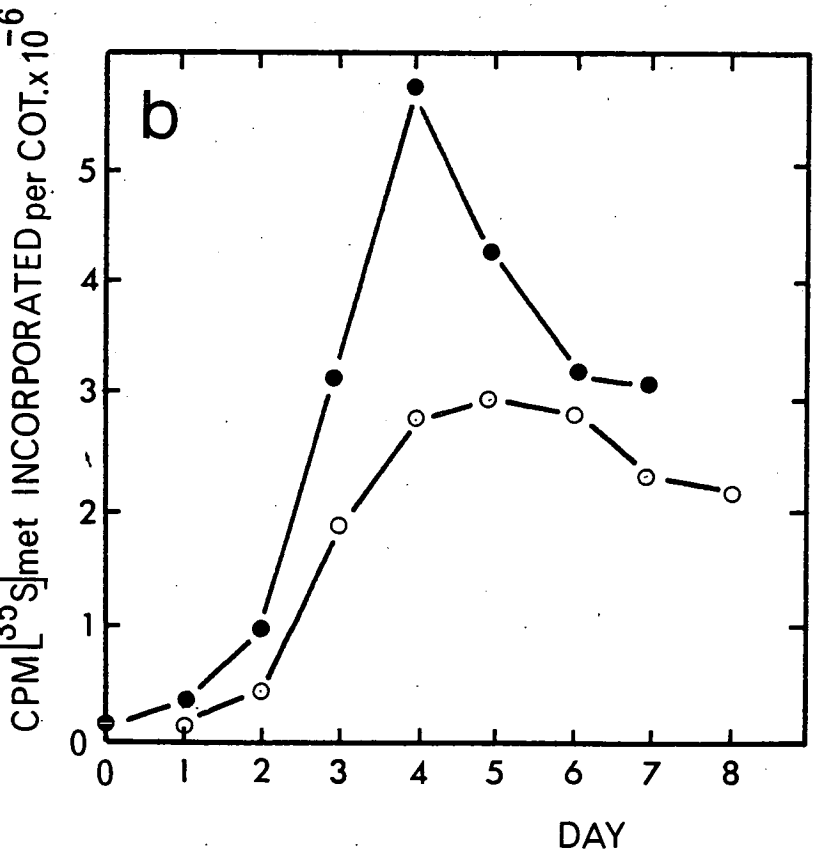
The E. coli translation system was incubated as described in Materials and Methods with 15 µg of total RNA extracted from Cucumber cotyledons from each stage of light- and dark-grown seedling development.

(a) Data plotted on a per µg input basis.
RNA extracted from light-grown cotyledons (■ - ■).
RNA extracted from dark-grown cotyledons (□ - □).

(b) Data plotted on a per cotyledon basis.
RNA extracted from light-grown cotyledons (● - ●).
RNA extracted from dark-grown cotyledons (○ - ○).

Fig.7.2


a
CPM [35S]met INCORPORATED per ug RNA x10-3
0
10
20
30
40
0 1 2 3 4 5 6 7 8
DAY


b
CPM [35S]met INCORPORATED per COT. x 10-6
0
1
2
3
4
5
0 1 2 3 4 5 6 7 8
DAY

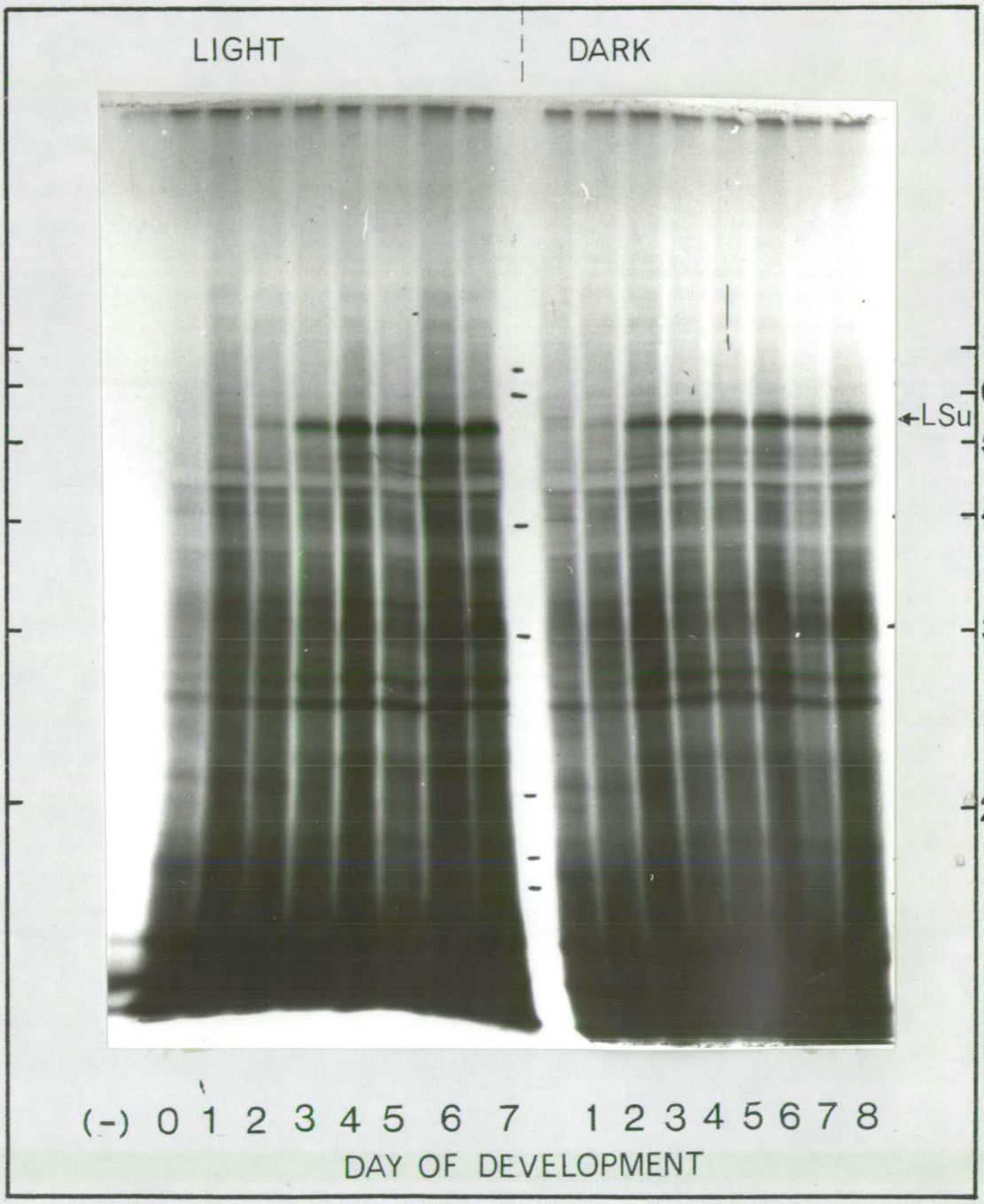
To allow direct comparison with other data presented here, the amount of radioactivity incorporated by the E. coli system programmed by various RNAs is presented on a per cotyledon basis (Fig. 7.2b). To do this the amount of incorporation produced on a per µg input basis (shown in Fig. 7.2a) was multiplied by the estimate of total RNA present in a cotyledon for that particular day (see Fig. 6.8a). Three points emerge from the analysis of the translation products of the E. coli system, programmed with equal amounts of RNA extracted from cotyledons of light- and dark-grown development by SDS-polyacrylamide-gel electrophoresis and autoradiography (see Fig. 7.3). Firstly, there are developmental changes in the profiles of translation products. Secondly, these developmental changes are essentially similar when programmed with RNA extracted from light- and dark-grown tissue. Thirdly, the translatable mRNA coding for a polypeptide, previously identified as LSu RuBPCase, first appears at approximately Day 2 and thereafter its levels appear to increase. A number of other unidentified labelled polypeptides also appear to change in amounts in a developmentally related manner.

Quantitation of LSu RuBPCase amongst the translation products has been carried out, firstly, by excision of the labelled polypeptide from the polyacrylamide gel and by estimating amounts of radioactivity by scintillation counting, secondly, by direct immunoprecipitation of LSu from the translation products. In the second case, the amounts of radioactivity present in aliquots of the immunoprecipitate were estimated by scintillation counting and samples analysed by SDS-polyacrylamide-gel electrophoresis and auto-

Figure 7.3 Analysis of the Translation Products of the *E. coli* System Programmed with Equal Amounts of Total RNA Extracted from Cotyledons of Light- and Dark-Grown Cucumber Seedlings.

The *E. coli* translation system was incubated as described in Materials and Methods with 15 µg of total RNA extracted from Cucumber cotyledons from each stage of light- and dark-grown seedling development. Translation products were analysed on 15% polyacrylamide-gels containing SDS followed by autoradiography as described in Materials and Methods.

Fig.7.3


LIGHT
DARK
70
60
LSu
50
40
30
20
Mr -3
x10
(-) 0 1 2 3 4 5 6 7 1 2 3 4 5 6 7 8
DAY OF DEVELOPMENT

radiography. Both techniques yielded essentially similar results (see Fig. 7.4 a-b), which gave me some confidence in thinking that in excising the labelled band of LSu I was not also excising another radioactive polypeptide that co-migrates with LSu. The slight differences between the data presented remains to be explained although it needs to be borne in mind that these are the results of separate experiments. It is apparent that the amounts of LSu synthesized under the direction of equal amounts of RNA extracted from light- and dark-grown cotyledons are similar. The translatable mRNA for LSu RuBPCase appears between Days 2 and 3 and accumulates at approximately the same rate in the light and dark-grown tissue until Day 4 or Day 5 and thereafter declines. This data presented on a per cotyledon basis (Fig. 7.5 a-b) allows a direct comparison to be made with Figure 6.3 showing the amount of native RuBPCase *in vivo*.

These results would indicate that translatable mRNA for LSu appears in both the light- and dark-grown tissue at about the same time as the *in vivo* appearance of complete RuBPCase between Day 2 and Day 3. Thus the initiation of synthesis of the mRNA appears to be light independent. The amounts of translatable mRNA for LSu increases in both light- and dark-grown cotyledons until Days 4 - 5 and over this period appears to be positively related to the amount of RuBPCase protein synthesized *in vivo*. At later stages of development the amount of translatable mRNA declines quite sharply in the light-grown, but much more slowly in the dark-grown cotyledons. The decline in the amounts of translatable mRNA after Days 4 - 5 remains unexplained as during this period *in vivo* accumulation of complete RuBPCase continues. Mixing experiments were carried out

Figure 7.4 Quantitation of LSu RuBPCase Synthesized by the *E. coli* System Programmed with Equal Amounts of Total RNA Extracted from Cotyledons of Light- and Dark-Grown Cucumber Seedlings

The *E. coli* translation system was incubated as described in Materials and Methods with equal amounts of RNA extracted from Cucumber cotyledons from each stage of light- and dark-grown seedling development. Quantitation of LSu RuBPCase synthesized *in vitro* on a per μg input basis was estimated by

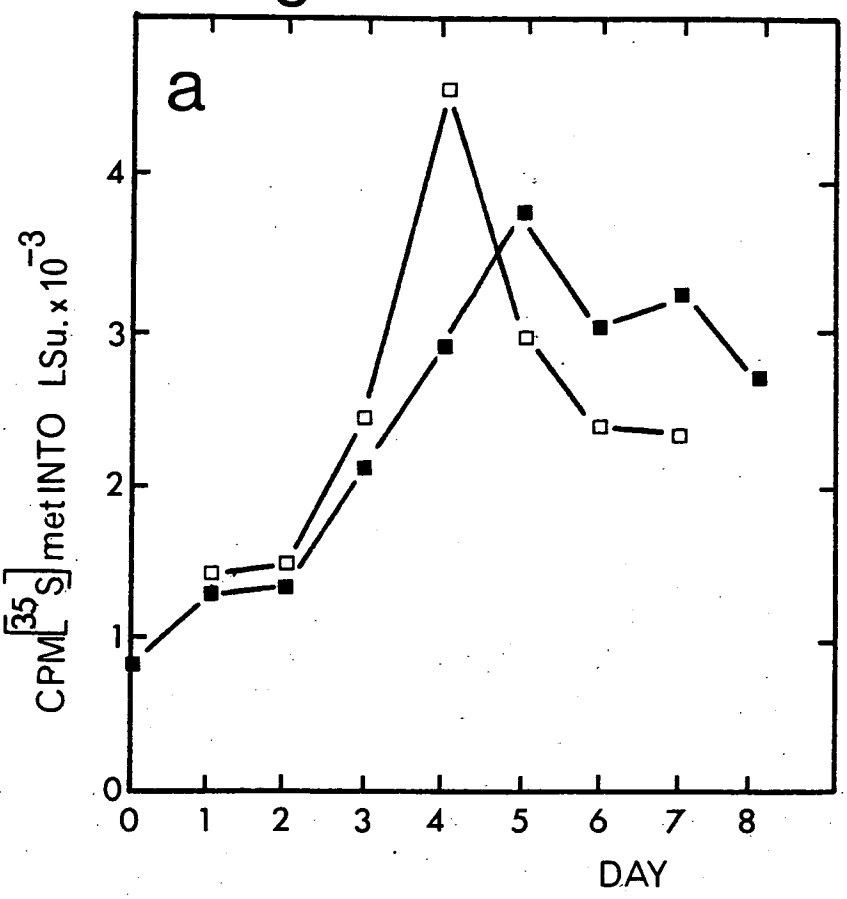
(a) Fractionation of the translation products on 15% polyacrylamide-gels containing SDS, location of polypeptide by autoradiography and estimation of radioactivity by excision of the polypeptide and scintillation counting as described in Materials and Methods.

(b) Direct immunoprecipitation of LSu from the translation products, and radioactivity was estimated by scintillation counting as described in Materials and Methods.

In both cases (■ - ■) RNA from light-grown cotyledons

(□ - □) RNA from dark-grown cotyledons.

Fig.7.4


a
CPM[35S]met INTO LSu. x 10-3
0
1
2
3
4
0 1 2 3 4 5 6 7 8
DAY

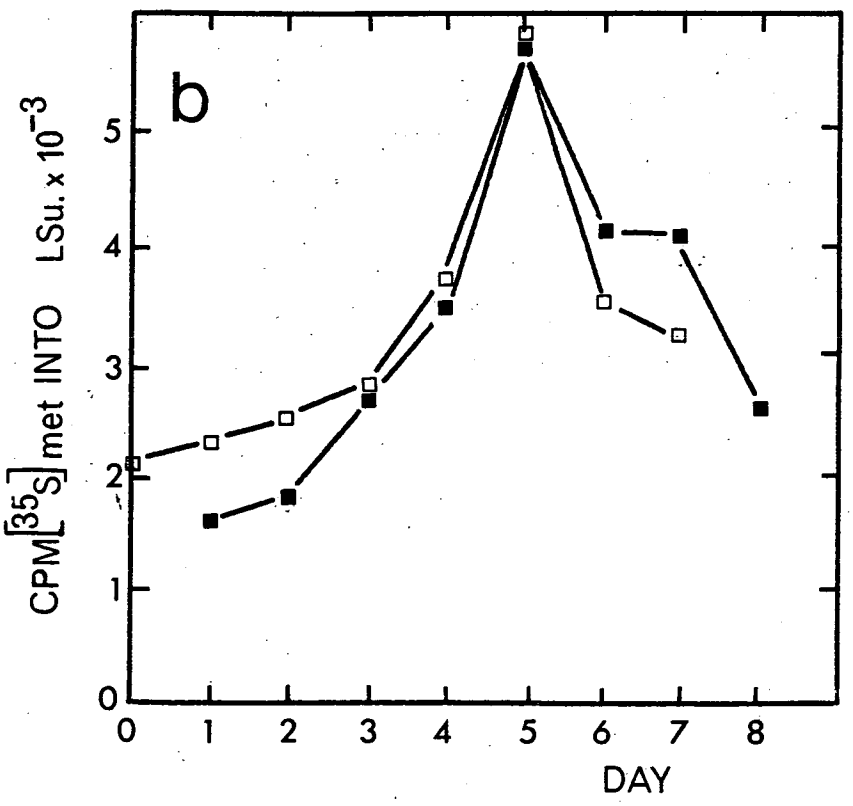

b
CPM[35S]met INTO LSu. x 10-3
0
1
2
3
4
5
0 1 2 3 4 5 6 7 8
DAY

Figure 7.5 Quantitation of LSu Synthesized by the *E. coli* System Programmed with Equal Amounts of Total RNA Extracted from Cotyledons of Light- and Dark-Grown Cucumber Seedlings

Data was calculated on a per μg input basis (shown in Fig. 7.4) and multiplied by the total RNA content of the Cucumber cotyledons (shown in Fig. 6.8a) to give the amounts of LSu RuBPCase synthesized *in vitro* on a per cotyledon basis.

(a) LSu estimated directly.

(b) LSu estimated by immunoprecipitation.

In both cases: RNA from light-grown cotyledons (△ - △).

RNA from dark-grown cotyledons (▲ - ▲).

Fig.7.5

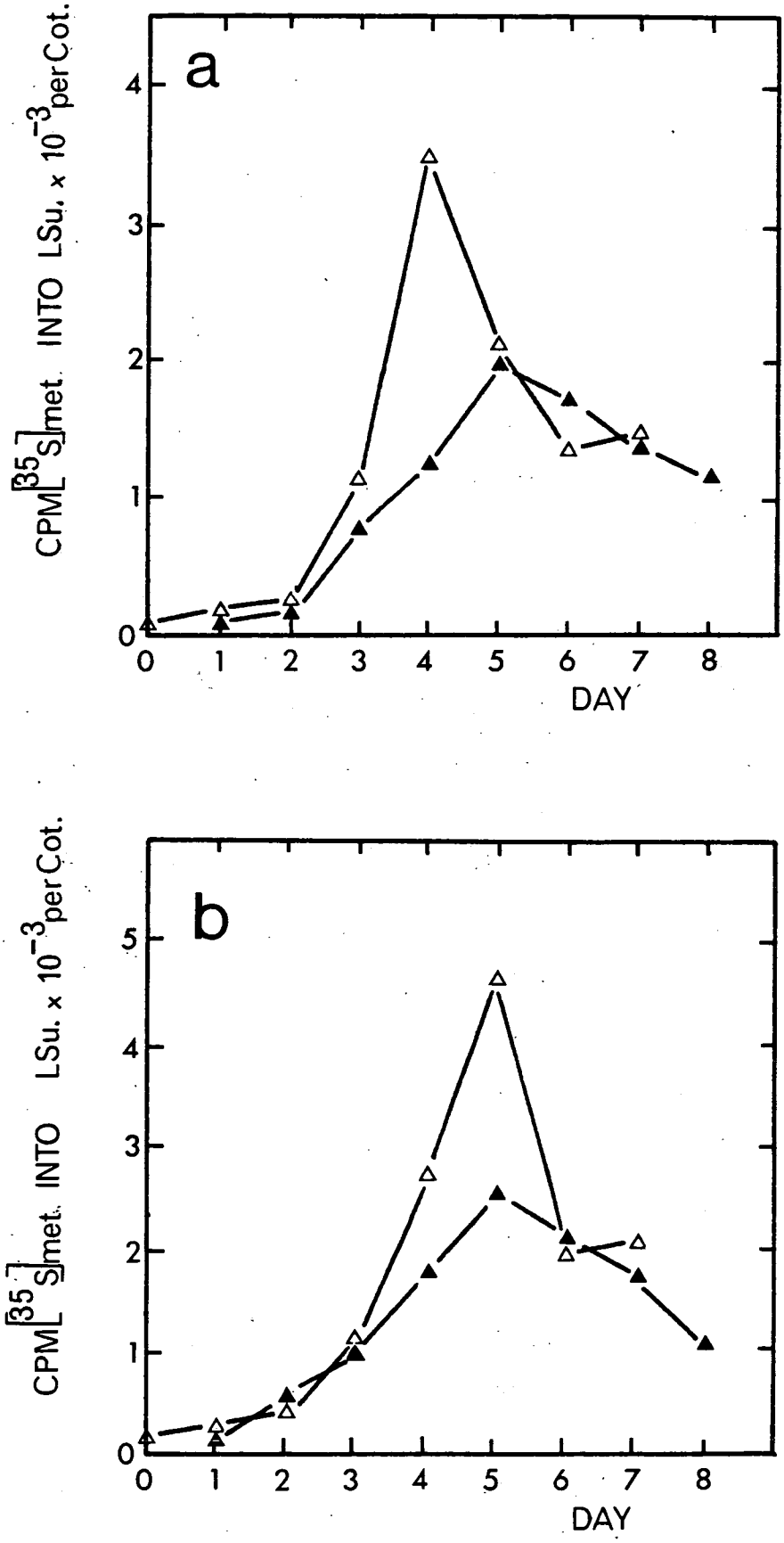
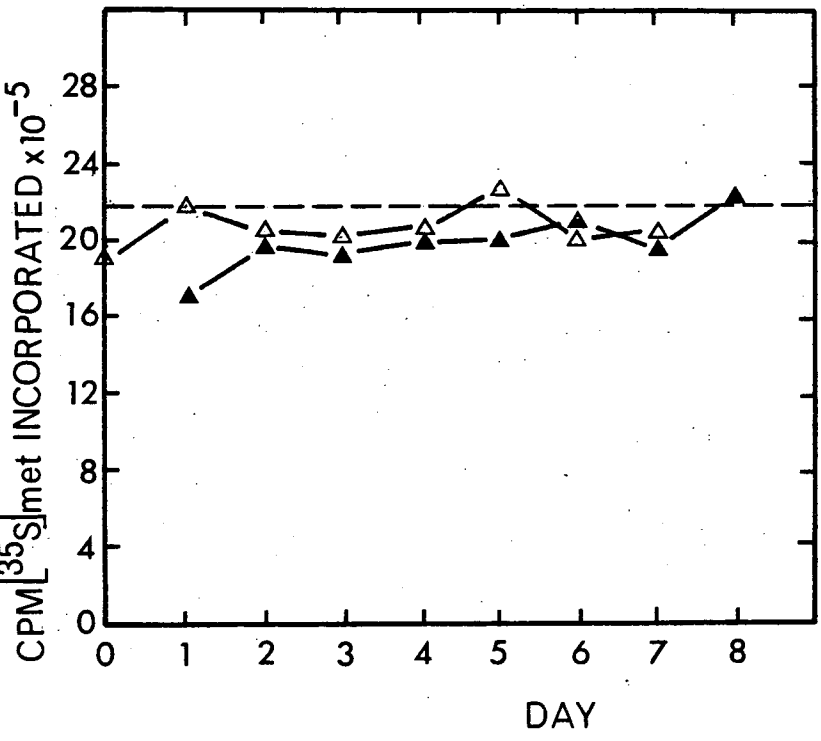


Figure 7.6 Incorporation of [^{35}S] Methionine by the *E. coli* System Programmed with Qβ RNA Mixed with Equal Amounts of Cucumber Cotyledon Total RNA Extracted from Light- and Dark-Grown Seedlings

The *E. coli* translation system was incubated as described in Materials and Methods with 15 µg of total RNA extracted from Cucumber cotyledons from each stage of light- and dark-grown seedling development added to 4 µg Qβ RNA.

Broken line Qβ RNA alone, Qβ RNA added to RNA from light-grown cotyledons (△ - △), Qβ RNA added to RNA from dark-grown cotyledons (▲ - ▲).

Fig.7.6


CPM[35S]met INCORPORATED x10-5
28
24
20
16
12
8
4
0
0
1
2
3
4
5
6
7
8
DAY

in order to show that the decline in incorporation by the E. coli system, when programmed by total cotyledon RNA extracted from the later stages of development, was not due to some inhibitor that co-purifies with the RNA. Equal amounts of RNA, extracted from light- and dark-grown cotyledons, were mixed with 4 µgm of Qβ RNA and used to programme the E. coli system. The amounts of RNA used in these experiments did not result in saturation of the E. coli system. The incorporation of radioactivity by the system programmed with Qβ RNA alone and Qβ RNA mixed with the total cotyledon RNA are shown in Figure 7.6. RNA extracted from light- and dark-grown cotyledons does not appear to have a differential effect on the translation of Qβ RNA in the E. coli system

(II) Developmental Changes in PSSu RuBPCase mRNA as Assayed in a Wheat Germ Translation System

As has been described in Chapter 4, a major translation product of the Wheat germ system when programmed with Cucumber cotyledon total RNA, has been identified as PSSu RuBPCase. Thus the optimised Wheat germ translation system, programmed with RNA extracted from cotyledons of different stages of light- and dark-grown seedling development, was used to assay the levels of translatable mRNA for the PSSu present in the tissue.

To ensure quantitative translation of the total RNA in the Wheat germ system, experiments similar to those described in the previous section for the E. coli system were carried out. For each sample of RNA extracted from the cotyledons of differing days of development the effect of increasing the concentration of that RNA on incorporation in the Wheat germ system was investigated. The results were essentially the same as shown in

Figure 7.1 for the E. coli system.

Thus the Wheat germ system was incubated with 15 µg of total RNA extracted from cotyledons of differing stages of seedling development and the amount of radioactivity incorporated was calculated on a per µg unput basis and shown in Figure 7.7a. The developmental changes of amounts of radioactivity incorporated by the Wheat germ system programmed by RNA extracted from light- and dark-grown cotyledons are similar . A broad peak of incorporation of radioactivity takes place when the system is programmed with RNA extracted from Day 1 - Day 4 cotyledons after which there is a decline.

Once more to allow direct comparison with other data presented here, the amount of radioactivity incorporated by the Wheat germ system programmed with various RNAs are presented on a per cotyledon basis (Fig. 7.7b).

Analysis of the Wheat germ translation products by SDS-polyacrylamide-gel electrophoresis and autoradiography show stage specific changes in the polypeptide profiles encoded by the RNAs extracted from light- and dark-grown cotyledons (Fig. 7.8). Although the polypeptide profiles programmed by RNA from light- and dark-grown tissue are similar, there are some subtle differences between the two.

The translatable mRNA coding for the polypeptide that has been previously identified as PSSu RuBPCase first appears to be present between Day 2 and Day 3 and thereafter accumulates. Essentially similar amounts of PSSu mRNA appear to be present in RNA extracted from light and dark-grown tissue. In addition to PSSu another major labelled polypeptide of 35,000 M_r appears on Day 2. This polypeptide is present amongst the translation products directed

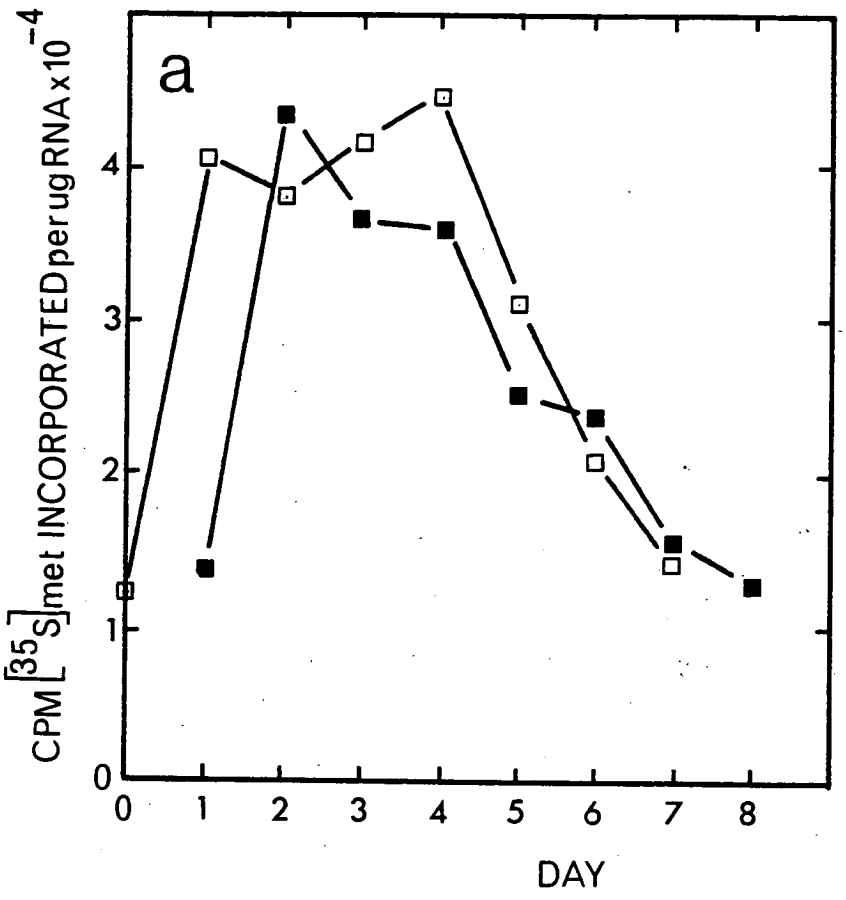
Figure 7.7 Incorporation of [^{35}S] Methionine into Protein by the Wheat Germ System Programmed by Equal Amounts of Total RNA Extracted from Cotyledons of Light- and Dark-Grown Cucumber Seedlings

The Wheat germ translation system was incubated as described in Materials and Methods with 15 µg of total RNA extracted from Cucumber cotyledons from each stage of light- and dark-grown seedling development.

(a) Data plotted on a per µg input basis.
RNA extracted from light-grown cotyledons (□ - □).
RNA extracted from dark-grown cotyledons (■ - ■).

(b) Data plotted on a per cotyledon basis.
RNA extracted from light-grown cotyledons (△ - △).
RNA extracted from dark-grown cotyledons (▲ - ▲).

Fig.7.7


a
CPM [35S]met INCORPORATED per ug RNA x10-4
0
1
2
3
4
0 1 2 3 4 5 6 7 8
DAY

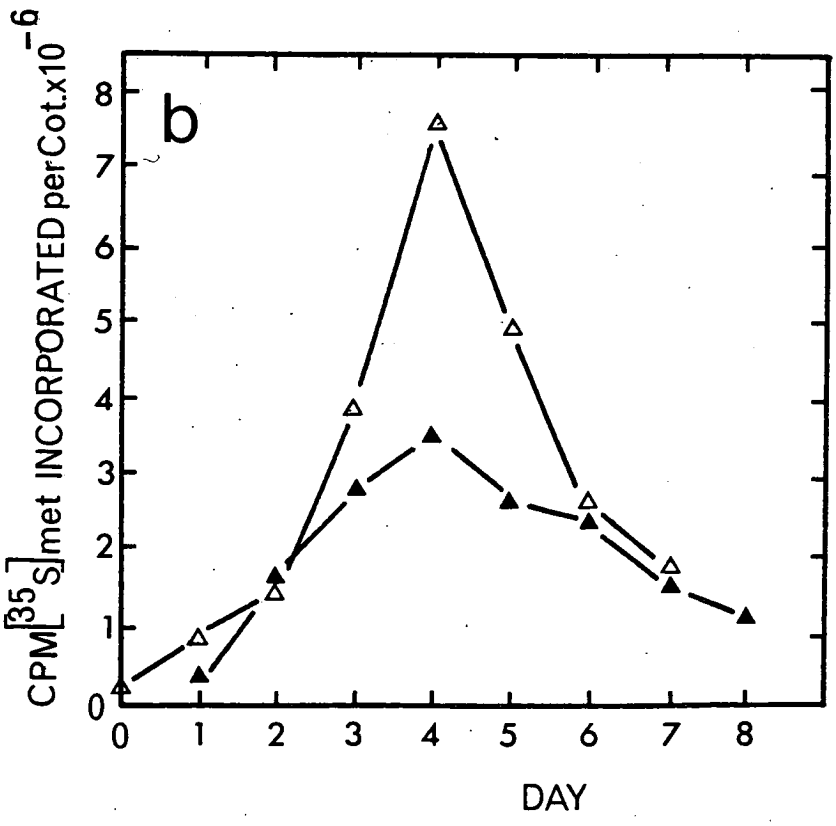
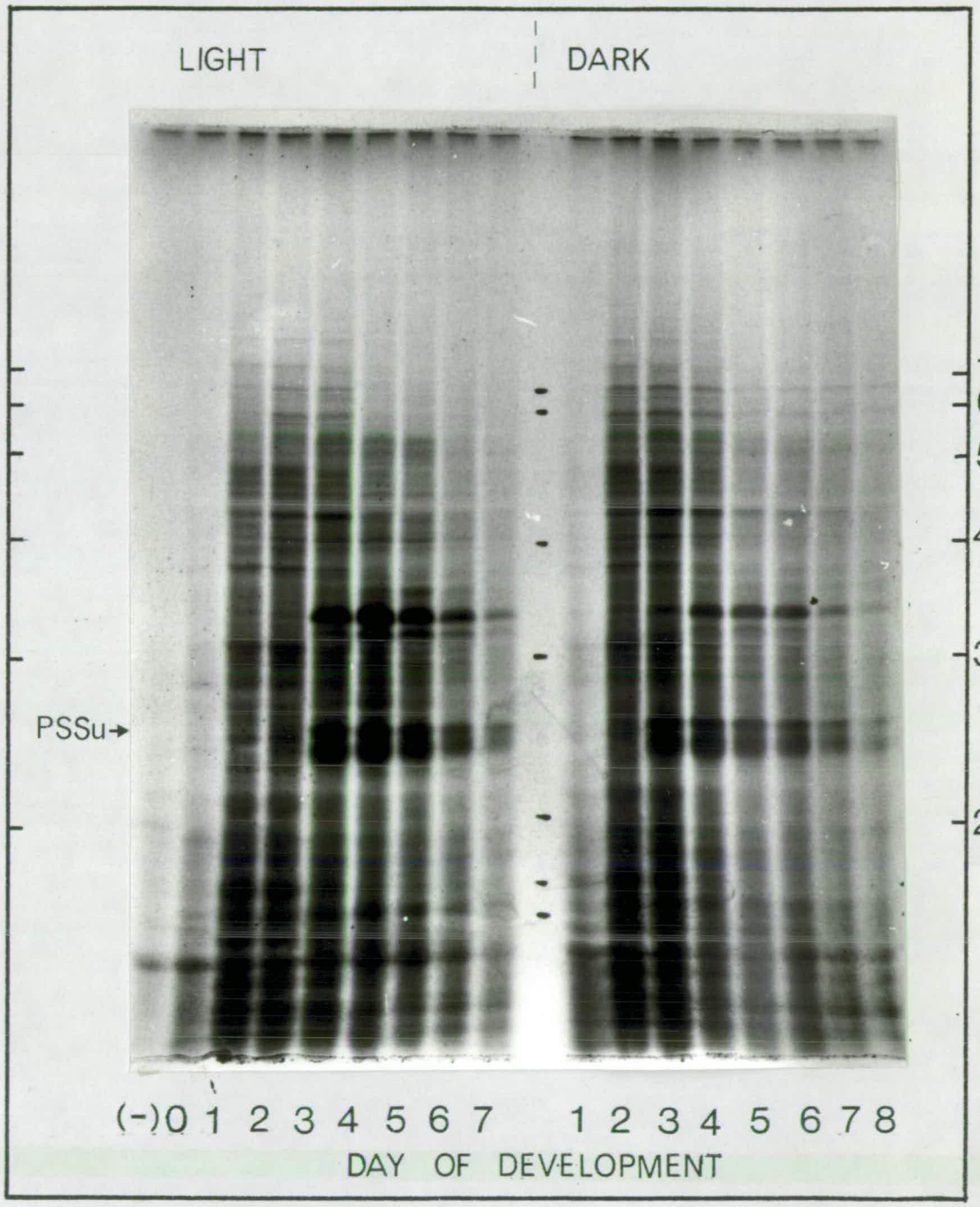

b
CPM [35S]met INCORPORATED per Cot. x10-6
0
1
2
3
4
5
6
7
8
0 1 2 3 4 5 6 7 8
DAY

Figure 7.8 Analysis of the Translation Products of the Wheat Germ System Programmed by Equal Amounts of Total RNA Extracted from Cotyledons of Light- and Dark-Grown Cucumber Seedlings

The Wheat germ translation system was incubated as described in Materials and Methods with 15 µg of total RNA extracted from Cucumber cotyledons from each stage of light- and dark-grown seedling development. Translation products were analysed on 15% polyacrylamide-gels containing SDS followed by autoradiography as described in Materials and Methods.

Fig. 7.8


LIGHT
DARK
PSSu
70
60
50
40
30
20
Mr x10-3
(-)0 1 2 3 4 5 6 7 1 2 3 4 5 6 7 8
DAY OF DEVELOPMENT

by RNA from light-grown tissue and to a lesser extent in the products directed by RNA from dark-grown tissue. As previously discussed this polypeptide co-migrates on gel electrophoresis with the 35,000 M_r membrane polypeptide synthesized in chloroplasts isolated from Cucumber cotyledons. In contrast the mRNA coding for the 32,000 M_r polypeptide first appears at Day 4 and its synthesis appears to be light dependent. Comparing this observation with those of other workers (Apel and Kloppstech, 1978a), would suggest that the 32,000 M_r polypeptide synthesized in vitro is the precursor to the chlorophyll a/b binding protein.

Close comparison of the translation product profiles programmed by RNA from light- and dark-grown tissue reveals a number of differences amongst minor polypeptides, the majority of which have yet to be identified. These include polypeptides that accumulate under the direction of RNA extracted from the early stages of light- and dark-grown development and which subsequently decline and disappear in the light, whereas in the dark, although there is a gradual decline, they persist for longer, and those polypeptides that are only synthesized under the direction of RNA from light-grown tissue.

Quantitation of PSSu RuBPCase amongst the translation products was carried out by excision of the labelled polypeptide, located by autoradiography of an SDS-polyacrylamide-gel and radioactivity estimated by scintillation counting. It had been hoped to use antisera raised against purified Cucumber SSu to quantitate the amounts of PSSu amongst the translation products. However, while the antisera available enabled me to carry out immunoprecipitation to identify PSSu amongst the translation products it was not good enough to yield meaningful results in the quantitation of PSSu

coded for by mRNAs extracted from differing days of development. Because of this, these results do not take into account possible premature termination of translation of PSSu mRNA which may take place in the system. However, we might expect that the amount of premature termination will be proportional to the amount of specific mRNA present at each stage of development. Hence for our purpose any premature termination will only result in the under-estimation of PSSu amongst the translation products and not effect the overall developmental pattern. The possibility that I was excising another polypeptide that co-migrates on gel electrophoresis with PSSu cannot be excluded. When excision of the labelled PSSu was being carried out the period of preparative electrophoresis was extended to ensure the separation of PSSu from a polypeptide of slightly smaller molecular weight.

The amounts of PSSu synthesized by the Wheat germ system directed by equal amounts of RNA extracted from differing stages of development are shown in Figure 7.9, expressed on a per µg input basis. It is apparent that the amounts of PSSu synthesized in the Wheat germ system programmed by RNA extracted from light- and dark-grown tissue are similar. Assuming that the Wheat germ and the E. coli systems are able to translate low levels of RNA with equal efficiency we can compare Figure 7.9 directly with Figure 7.4. The appearance and initial increase of the mRNA for PSSu assayed in the Wheat germ system appears to precede that of LSu mRNA assayed in the E. coli system. The peak in amount of PSSu mRNA assayed in vitro precedes that of LSu mRNA by a day.

The amount of PSSu synthesized in the Wheat germ system directed by equal amounts of RNA extracted from light- and dark-grown cotyledons plotted on a per cotyledon RNA basis is shown

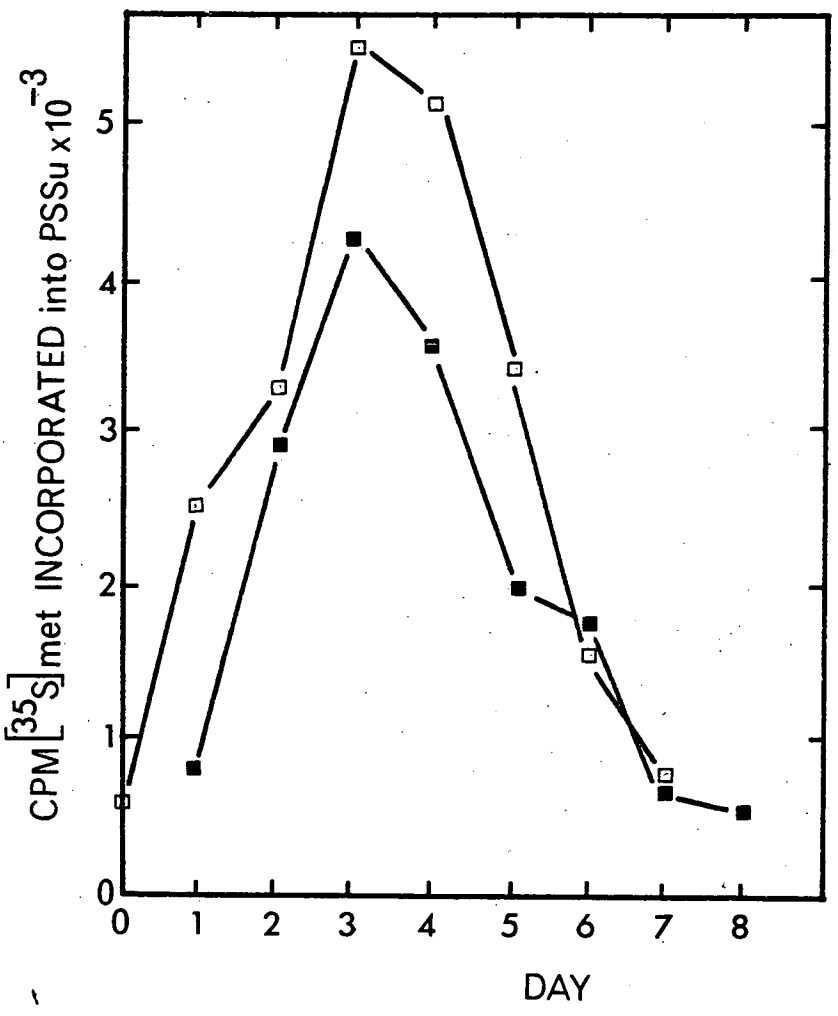
Figure 7.9 Quantitation of PSSu RuBPCase Synthesized by the Wheat Germ System Programmed with Equal Amounts of Total RNA Extracted from Cotyledons of Light- and Dark-Grown Cucumber Seedlings

The Wheat germ translation system was incubated as described in Materials and Methods with 15 µg of total RNA extracted from cotyledons from each stage of light- and dark-grown seedling development. Quantitation of PSSu RuBPCase *in vitro* on a per µg input basis was carried out by fractionation of the translation products on 15% polyacrylamide-gels containing SDS, location of the polypeptide by autoradiography and estimation of radioactivity by excision of the polypeptide and scintillation counting as described in Materials and Methods.

RNA from light-grown cotyledons (□ - □).

RNA from dark-grown cotyledons (■ - ■).

Fig.7.9


CPM[35S]met INCORPORATED into PSSu x10-3
0
1
2
3
4
5
0
1
2
3
4
5
6
7
8
DAY

in Figure 7.10a. The *E. coli* data for the synthesis of LSu, shown in Figure 7.10b is replotted for convenient comparison. The developmental profiles are essentially similar and over the first 4 days of seedling development compare well with the accumulation of RuBPCase protein *in vivo* as shown in Figure 6.5c. However, the decline in translatable mRNA for PSSu *in vitro* is again not reflected *in vivo* where accumulation of both complete RuBPCase and SSu continues (Fig. 6.5 b and c).

The decline in the amount of the PSSu mRNA in the Wheat germ system can be partially attributed to a factor that co-purifies with the total RNA which inhibits the Wheat germ system. Mixing experiments were carried out using equal amounts of total RNA extracted from cotyledons at each stage of light- and dark-grown development mixed with 5 μg total BMV RNA to programme the system. The amounts of RNA used in these experiments did not result in the saturation of the Wheat germ system. The amounts of incorporation of radioactivity by the Wheat germ system, programmed with either BMV RNA alone or BMV added to the RNAs extracted from various stages of cotyledon development are shown in Figure 7.11a. There appears to be a decrease in the incorporation of radioactivity with RNAs extracted from cotyledons of later stages of light-grown development. As a preliminary investigation as to whether the inhibiting factor co-purifies with mRNAs, a similar experiment was carried out using the poly(A)$^+$ fractions of total RNA extracted from cotyledons of light- and dark-grown seedlings, incubated in the Wheat germ system with equal amounts of BMV RNA (see Fig. 7.11b). Once more concentrations of RNA were used which do not saturate the system. The data suggests that whatever factor present in total

Figure 7.10 Quantitation of PSSu RuBPCase Synthesized by the Wheat Germ System Programmed by Equal Amounts of Total RNA Extracted from Cotyledons of Light- and Dark-Grown Cucumber Seedlings

(a) Data as presented in Figure 7.9 except multiplied by the total RNA content of the Cucumber cotyledon (presented in Figure 6.8) to give the amounts of PSSu RuBPCase synthesized *in vitro* on a per cotyledon basis.

(□ - □) RNA from light-grown cotyledons.

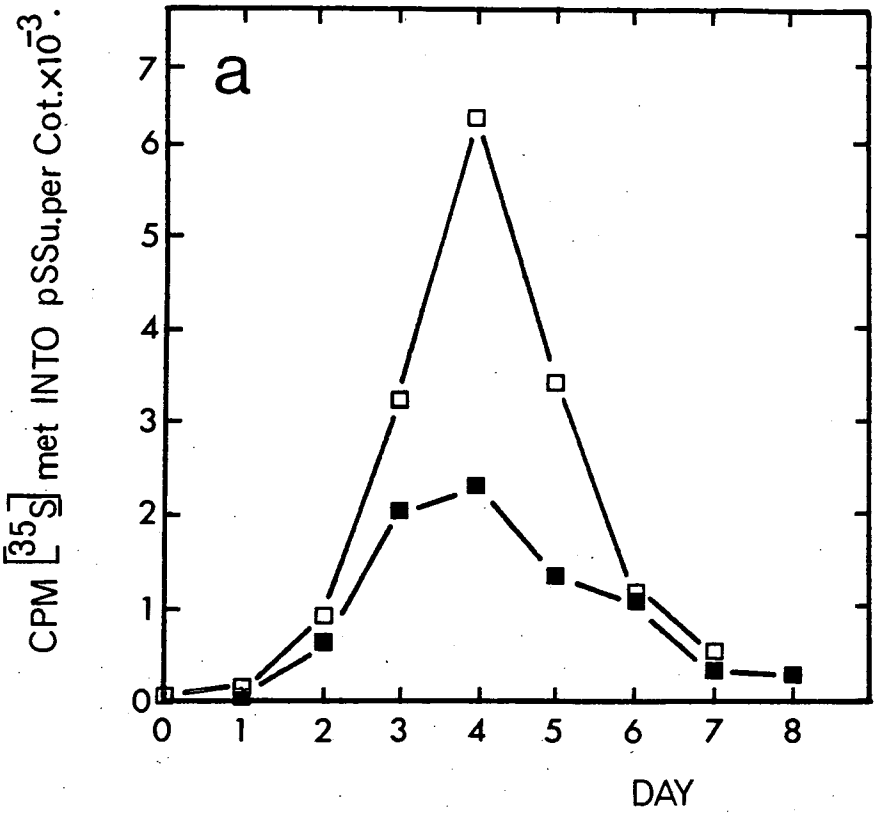
(■ - ■) RNA from dark-grown cotyledons.

(b) For convenient comparison Figure 7.5a showing the amounts of LSu RuBPCase synthesized by the *E. coli* translation system programmed with the same RNA is shown.

(△ - △) RNA from light-grown cotyledons.

(▲ - ▲) RNA from dark-grown cotyledons.

Fig.7.10


a
CPM [35S] met INTO pSSu.per Cot.x10-3.
0
1
2
3
4
5
6
7
0
1
2
3
4
5
6
7
8
DAY

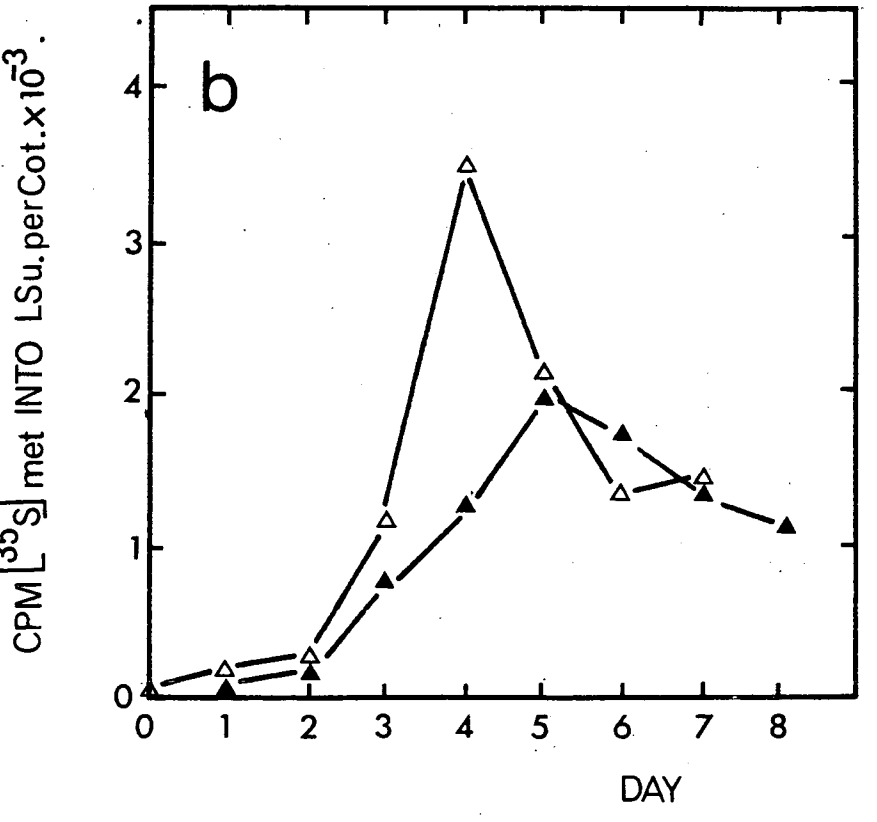

b
CPM [35S] met INTO LSu.perCot.x10-3.
0
1
2
3
4
0
1
2
3
4
5
6
7
8
DAY

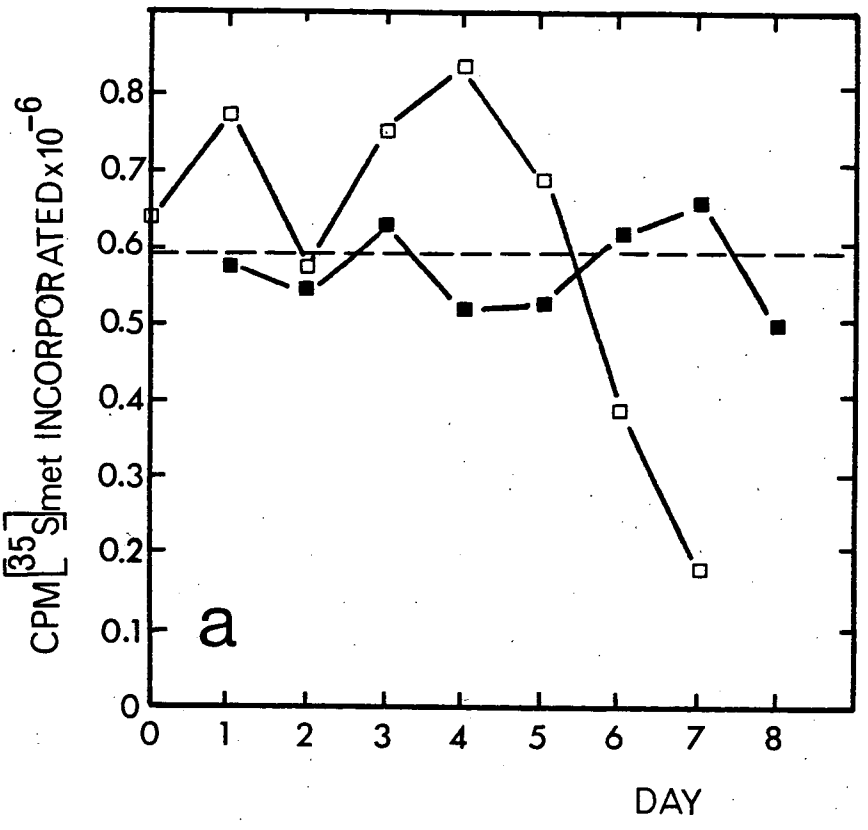
Figure 7.11 Incorporation of [^{35}S] Methionine by the Wheat Germ System Programmed with BMV RNA Mixed with Equal Amounts of Cucumber Cotyledon Total RNA Extracted from Light- and Dark-Grown Seedlings

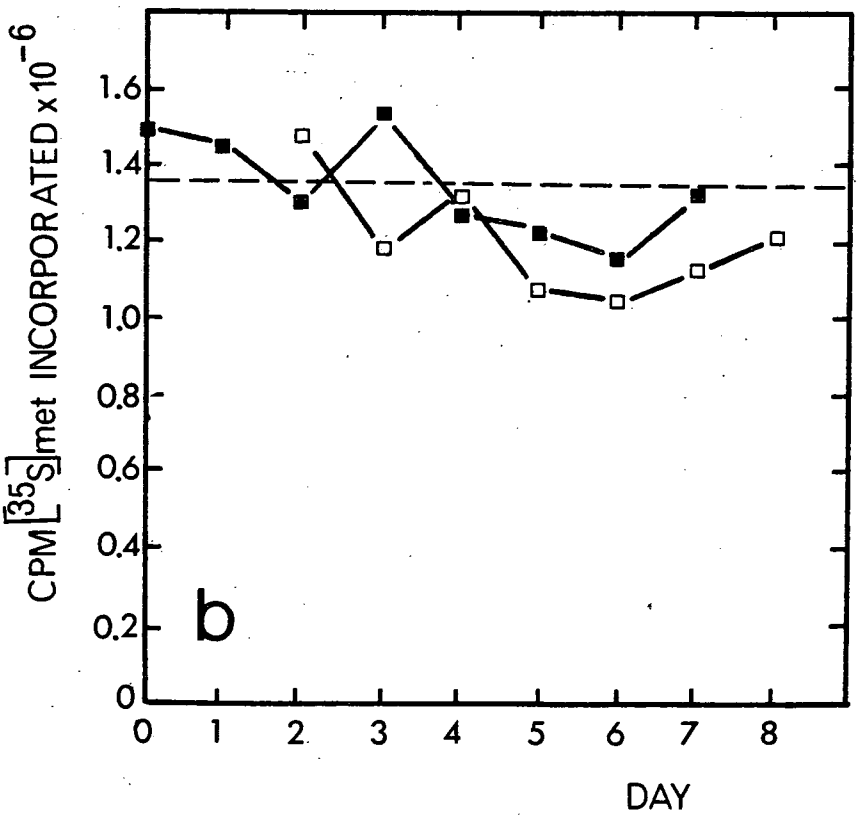
The Wheat germ system was incubated as described in Materials and Methods with RNA extracted from Cucumber cotyledons from each stage of light- and dark-grown seedling development added to 5 µg BMV RNA.

(a) Broken line BMV RNA alone, BMV added to 15 µg total RNA from light-grown cotyledons (□-□), BMV added to 15 µgm total RNA from dark-grown cotyledons (■-■).

(b) Broken line BMV RNA alone, BMV added to 0.5 µg poly(A)$^+$ RNA from light-grown cotyledons (■-■), BMV added to 0.5 µg poly(A)$^+$ RNA from dark-grown cotyledons (□-□).

Fig.7.11


CPM[35S]met INCORPORATED x10-6
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
0 1 2 3 4 5 6 7 8
DAY


CPM[35S]met INCORPORATED x10-6
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
b
0 1 2 3 4 5 6 7 8
DAY

RNA preparations from light-grown cotyledons of the later stages of seedling development does not co-purify with poly(A)$^+$ RNA

(III) Developmental Changes in the Capacity of Isolated Plastids to Synthesize Proteins

In the preceding Sections, I have used heterologous cell-free translation systems to assay changes in the amounts of mRNAs coding for chloroplast specific proteins in Cucumber cotyledons during seedling development. However, although the appearance of specific mRNAs, assayed *in vitro*, appears to be related to the accumulation of the proteins that they code for *in vivo*, it may be unwise to extrapolate *in vitro* data to *in vivo* changes without some caution. Indeed the apparent decline in the amounts of mRNAs extracted from cotyledons of the later stages of Cucumber developmental sequence has only been partially explained in the case of the Wheat germ system. Thus it was hoped that an investigation of the protein synthetic capacity of isolated plastids extracted from light- and dark-grown cotyledons would allow an insight into not only the development of the capacity of the plastids for protein synthesis, but also the range of polypeptides synthesized.

Unfortunately, it was found that plastids extracted from the dark-grown sequence of tissue were not able to incorporate radioactivity into protein under the conditions used, either with light or ATP as an energy source. This is in contrast to the findings of Siddell and Ellis (1975) who, using etioplasts isolated from the apical buds of Pea plants grown in the dark, demonstrated the synthesis of relatively small amounts of LSu RuBPCase using ATP as a source of energy. My lack of success in isolating plastids

able to carry out protein synthesis *in vitro* from dark-grown seedlings may have been due to either the age of the tissue or the tissue itself. Dark-grown cotyledons are morphologically similar to dry seeds and the homogenisation procedure may have disrupted the plastids.

As we have seen in Chapter 5, plastids from light-grown tissue are able to carry out protein synthesis using light or ATP as source of energy. Thus plastids isolated from cotyledons of the light-grown sequence of seedling development were incubated with [^{35}S]met in the light, or in the dark with addition of ATP. The addition of ATP ensured that plastids unable to carry out photophosphorylation had an adequate source of energy.

As described in Materials and Methods (p. 75), plastids were isolated from 5 g. of cotyledons, harvested from Day 2 to Day 7 light-grown seedlings and resuspended in equal volumes of Resuspension Buffer. Equal volumes of the resuspended plastids were used in incubation.

To allow direct comparison with other data here the results are expressed on a per cotyledon basis. In order to do this the amount of radioactivity incorporated by a plastid extract, prepared from 5 g of tissue was divided by the number of cotyledons in 5 g of tissue (the fresh weight of the cotyledons was previously obtained by other workers (Becker *et al.*, 1978) (see Fig. 7.12a). Also the amount of radioactivity incorporated as a function of the amount of incubated protein is shown to indicate the synthetic activities of crude plastid preparations (see Fig. 7.12b).

With light or ATP as a source of energy, the temporal pattern of the development of the protein synthetic capacity of isolated

Figure 7.12 Incorporation of [^{35}S] Methionine into Protein by Isolated Plastids Extracted from Cucumber Cotyledons of Differing Stages of Light-Grown Seedling Development

Plastids were isolated from cotyledons of differing stages of seedling development and incubated with [^{35}S]met as described in Materials and Methods.

(a) Amounts of radioactivity expressed on a per cotyledon basis

(△ - △) Incubation with light as an energy source.

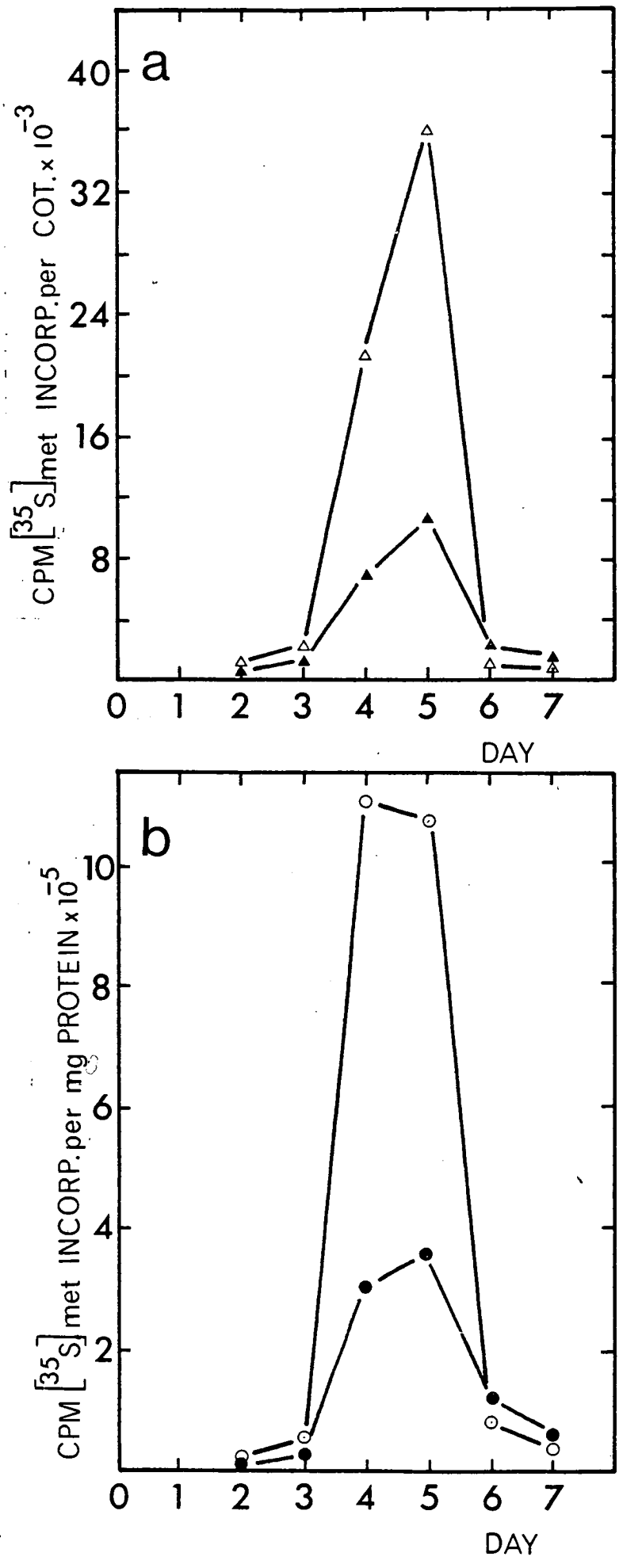
(▲ - ▲) Incubation with 2 mM ATP as an energy source.

(b) Amounts of radioactivity expressed on a per mg protein incubated basis

(○ - ○) Incubation with light as an energy source.

(● - ●) Incubation with 2 mM ATP as an energy source.

Fig 7.12


a
40
32
24
16
8
CPM [35S] met INCORP. per COT. x 10-3
0 1 2 3 4 5 6 7
DAY
b
10
8
6
4
2
CPM [35S] met INCORP. per mg PROTEIN x 10-5
0 1 2 3 4 5 6 7
DAY

plastids is similar. However, ATP alone, up to Day 5, is unable to effectively replace light as a source of energy. After Day 6 light can be replaced by ATP, confirming previous results (see Chapter 5). This observation remains to be explained, but possibly reflects subtle changes either in the permeability of the plastid membranes to ATP or the energy requirements of the isolated plastids to carry out protein synthesis. Siddell and Ellis (1975) found that plastids isolated from Pea shoots at subsequent stages of greening had differential energy requirements. They demonstrated that plastids became less dependent on addition of ATP to carry out protein synthesis. Indeed after 96 h of greening protein synthesis in isolated plastids became totally light dependent.

There appears to be a peak of protein synthetic activity between Day 4 and Day 5 which is then followed by a decline. This pattern has been observed on 3 separate occasions. These results are reflected in the profiles of the labelled translation products analysed by SDS-polyacrylamide-gel electrophoresis and fluorography (Fig. 7.13). In this case, equal amounts of protein from each incubation were loaded on each gel slot so no direct comparison with other data can be made. The labelled proteins synthesized in isolated plastids incubated in the light and in the dark with ATP are similar. It was apparent that plastids from Day 3 synthesize LSu RuBPCase and another polypeptide of approximately 31,000 M_r. On Day 4 other polypeptide bands became apparent including what are thought to be the α and β subunits of chloroplast ATPase. Amongst the polypeptides synthesized in the plastids in the dark with ATP there appears to be a polypeptide of 35,000 M_r (arrowed) from the 'light-driven' polypeptide products. In the

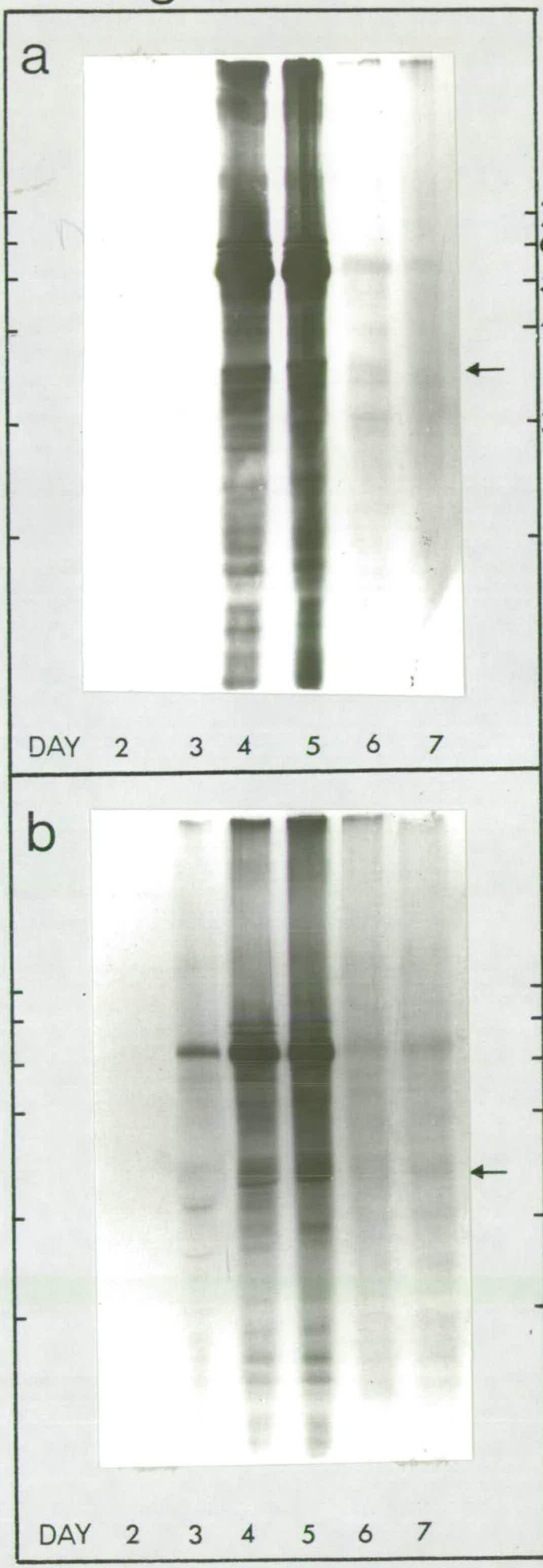
Figure 7.13 Analysis of Translation Products of Isolated Plastids Extracted from Cucumber Cotyledons at Different Stages of Light-Grown Seedling Development and Incubated with $[^{35}S]$ Methionine

Plastids were isolated and incubated with $[^{35}S]$ met as described in Materials and Methods. Equal amounts of protein from each incubation were fractionated on 15% polyacrylamide-gels containing SDS and labelled translation products were visualised by fluorography.

(a) Chloroplasts incubated with ATP as an energy source. Fluorograph exposed 25 days

(b) Chloroplasts incubated with light as an energy source. Fluorograph exposed 7 days

Fig.7.13


a
70
60
50
40
30
20
DAY 2 3 4 5 6 7
b
70
60
50
40
30
20
DAY 2 3 4 5 6 7
$M_r \times 10^{-3}$

latter case a polypeptide band of 34,000 M_r is present (arrowed). As described in Chapter 5, Section III this may be a result of the plastids incubated in the dark being unable to process a 35,000 M_r precursor to the 34,000 M_r polypeptide. Between Day 4 and Day 5 the relative proportions of the polypeptide products do not appear to change.

Although there is a dramatic decline in the incorporation of radioactivity by plastids extracted from Day 6 and Day 7 cotyledons, this reduction in activity is purely relative to Day 4 and Day 5. These plastids are still capable of carrying out protein synthesis and the translation products resemble those that are obtained from Day 5 plastids (for example, see Fig. 5.3).

Chapter 8. Discussion

The aim of this study was to investigate the synthesis of Cucumber cotyledon chloroplast proteins during the development of seedlings grown in the light and in the dark. In carrying this out with the use of cell-free protein-synthesizing systems I studied, in particular, three separate aspects of the topic. Firstly, the accumulation of mRNAs coded by the chloroplast, secondly the accumulation in the cytoplasm of mRNAs coding for chloroplast proteins and thirdly, the development of the capacity of isolated chloroplasts to synthesize proteins. In discussing my results, I intend to (i) describe the use of the cell-free protein synthesizing systems, (ii) inter-relate the observed developmental changes as assayed in the cell-free systems, (iii) correlate these with the developmental changes observed *in vivo* and (iv) outline the advantages and disadvantages of the experimental techniques that have been adopted and discuss possible improvements.

The results shown here indicate that the *E. coli* system can translate the mRNA components of Cucumber total cotyledon RNA and chloroplast RNA to produce a similar profile of polypeptides. The *E. coli* system was extensively characterised and the optimal conditions of incubation were used to ensure that the polypeptide products resulted from the correct translation of mRNAs. The fidelity of RNA translation was verified by the use of viral RNA (Chapter 3, Section V). However, an important point raised by the analysis of the translation products of viral RNAs is that distinct labelled polypeptides may result from either premature termination or termination read through. This should be borne in mind when

considering the translation products of any cell-free protein-synthesizing system.

The data presented confirms previous findings that LSu RuBPCase is a major product of the E. coli system programmed with RNA extracted from photosynthesizing tissue (Hartley et al., 1975; Wheeler and Hartley, 1975; Bottomley et al., 1976, 1977 and 1979; Reisfeld, 1978b; Sano et al., 1979). The 54,000 M_r translation product of the E. coli system when programmed with Cucumber RNA has been identified as LSu RuBPCase by co-migration with marker LSu on SDS-polyacrylamide-gel electrophoresis and immunoprecipitation with antisera raised against Spinach RuBPCase and Cucumber LSu RuBPCase (for example, see Fig. 3.6). Preliminary results of limited proteolytic digestion of the 54,000 M_r polypeptide by the method of Cleveland et al. (1977) indicates that this polypeptide is indeed LSu RuBPCase (data not shown).

It is thought that there are three isoelectric variants of LSu RuBPCase in vivo (Kung et al., 1974; Chen et al., 1976). These appear to be the result of charge differences in the protein (Gray et al., 1978). These authors suggest that the charge differences arise from processing of the polypeptide shortly after translation, possibly the removal of the peptide sequence at the N-terminal. The E. coli system used here apparently does not remove the N-terminal methionine from completed polypeptides (Chapter 3, Section V). Thus it would be interesting to investigate whether LSu synthesized in the system is composed of isoelectric variants. This could be carried out simply by analysing the translation products of the E. coli system by 2-dimensional-gel electrophoresis (O'Farrell, 1975). Other workers have shown that isoelectric variants of LSu are synthesized in isolated chloroplasts (Ellis et al., 1977), a

Wheat germ system programmed with Euglena poly(A)$^+$ RNA (Sagher et al., 1976) and an E. coli system programmed with Spirodela RNA (Reisfeld et al., 1978).

There remains a pressing need to identify some of the other translation products of the E. coli system. Indirectly I have been able to show that the majority of the polypeptides present amongst the translation products of the system are not a result of premature termination of LSu RuBPCase mRNA translation. This has been possible because these translation products are present when the E. coli system is programmed with RNA not expected to contain LSu mRNA, for example, compare the SDS-polyacrylamide-gel profiles of the translation products directed with poly(A)$^+$ RNA extracted from Cucumber cotyledons with those directed by poly(A)$^-$ RNA from the same tissue (Fig. 3.7). It was hoped that some of the E. coli translation products could be tentatively identified by co-migration on SDS-polyacrylamide-gel electrophoresis with either purified chloroplast proteins or the products of protein synthesis in isolated chloroplasts. However, the results were disappointing (see Fig. 5.2), with only the co-migration of LSu being apparent. This could be due to several factors including i) the E. coli system may translate mRNAs that code for minor polypeptides or polypeptides that turn over rapidly and hence these would not be observed as stained chloroplast polypeptides ii) some of the labelled translation products of either the E. coli system or isolated chloroplasts may be unprocessed precursor polypeptides.

Of particular interest is the apparent absence from the translation products of the E. coli system of the chloroplast membrane polypeptide of approximately 34,000 M_r which is synthesized in

isolated chloroplasts. This is in agreement with the observations of Bottomley *et al.* (1976, 1977 and 1979), but in contrast to the findings of Hartley *et al.* (1975) who, using an *E. coli* system programmed by Spinach chloroplast RNA, obtained a major polypeptide product of approximately 35,000 M_r which was thought to be analogous to one of the polypeptides synthesized in isolated Spinach chloroplasts. The apparent absence of this polypeptide from the translation products of the *E. coli* system shown here suggests differential translation of this chloroplast mRNA by the *E. coli* system and isolated chloroplasts.

The results obtained using increasing amounts of Cucumber cotyledon total RNA to programme the *E. coli* system suggests that LSu mRNA is efficiently translated (Chapter 3, Section IX). By analogy with the results of other workers this may be due to the efficiency of initiation of translation (Lodish, 1974; Lomedico and Saunders, 1977). This may serve as a mechanism by which plant tissues accumulate large amounts of RuBPCase. RuBPCase is the most abundant polypeptide in light-grown plant tissue (Kawashima and Wildman, 1970), comprising up to 50% of the soluble protein and more than 25% of the total protein in Spinach or Tobacco leaves (Wildman, 1979). LSu RuBPCase is known to be coded for by a single gene in chloroplast DNA (Bedbrook, 1979a) and although chloroplasts contain as many as 30 copies of circular DNA (Whitfeld *et al.*, 1973) it is not known if all are expressed simultaneously. Several mechanisms may result in high levels of RuBPCase, for example, high rates of transcription or the protein itself being relatively stable. On the other hand the efficiency of translation coupled with chloroplast ribosomes accounting for up to 50% of the total ribosomes of the leaf (Boardman *et al.*, 1966) may account for the

abundance of RuBPCase. It would be useful to investigate whether preferential translation took place in an S-30 protein synthesizing system derived from chloroplasts. The preparation of an active S-30 protein synthesizing system from chloroplasts has been attempted by several workers, but it is yet to be successful (J. Weil and O. Ciferri, personal communication).

Comparison of the SDS-polyacrylamide-gel profiles of the labelled translation products of the E. coli and Wheat germ systems programmed with the same RNA shows that they are qualitatively different (Fig. 5.2). This is in agreement with the observations of Bottomley et al. (1976, 1977) who suggested that the Wheat germ system preferentially translated cytoplasmic mRNAs whereas the E. coli system preferentially translated chloroplast mRNAs. While the majority of translation products of both systems remain to be identified, this suggestion needs to be treated with caution. Support has been provided here by the finding that whereas LSu RuBPCase is a major translation product of the E. coli system it is a minor product of the Wheat germ system (Fig. 4.5). On the other hand, a chloroplast mRNA may be translated in the Wheat germ system if the translation product of approximately 35,000 M_r is a precursor to the 34,000 M_r chloroplast membrane polypeptide synthesized in isolated chloroplasts. The identification of the Wheat germ 35,000 M_r translation product is at best tentative as it has been provided by co-electrophoresis on SDS-polyacrylamide-gel electrophoresis with the translation products of isolated chloroplasts. Although the 35,000 M_r polypeptide is a major translation product (see Fig. 4.3) of the Wheat germ system it does not correspond to a protein amongst the stained protein gel profile of Cucumber cotyledons

(Fig. 6.3) or gel profiles of *in vivo* labelled polypeptides (Fig. 6.6). This suggests that the 35,000 M_r polypeptide may be a precursor polypeptide or is turned over rapidly *in vivo*. Edelman and co-workers have shown that the 33,500 M_r precursor of the 32,000 M_r chloroplast membrane protein is synthesized in a Wheat germ system programmed by *Spirodela* total RNA (Reisfeld *et al.*, 1978a). Both precursor and mature polypeptide turn over rapidly *in vivo* (Edelman *et al.*, 1979). However, even though the 35,000 M_r polypeptide is synthesized when the Wheat germ system is programmed with Cucumber chloroplast RNA, this RNA does not efficiently stimulate incorporation in the system (see Chapter 4, Sections V and VI). This observation remains to be explained, but the possibility that some factor required for the efficient translation of the 35,000 M_r polypeptide mRNA in the Wheat germ is present in the total RNA preparation and not the chloroplast RNA preparation cannot be excluded.

It has been found that when programmed with *Spirodela* RNA the precursor of the 32,000 M_r chloroplast membrane polypeptide is efficiently synthesized in the Wheat germ system, whereas LSu is efficiently synthesized only in the *E. coli* system (Edelman and Reisfeld, 1978; Reisfeld *et al.*, 1978b; Edelman *et al.*, 1979). This suggests a differential affinity of two specific chloroplast mRNAs for the Wheat germ and the *E. coli* translation complexes and could be due to several reasons. The most persuasive of these involves the primary structure of the mRNA. The chloroplast membrane polypeptide mRNA may be 'capped' whereas LSu mRNA may not. 'Cap' structures consisting of a methylated guanosine residue linked by a 5'-5' triphosphate group to the first coded nucleotide have been found at the

5' terminal of most eucaryotic (but not procaryotic) mRNAs (for reviews, see Shatkin, 1979; Filipowitz, 1979 and Clemens, 1979). Although the role of 'cap' structures is not known precisely a great deal of attention has been paid to the influence of 'cap' structures on the translational efficiencies of mRNAs. Recently, elegant experiments have shown that when procaryotic mRNAs, not normally efficiently translated in the Wheat germ system, are 'capped' in vitro their translational efficiencies in the Wheat germ system become comparable with globin mRNA (Paterson and Rosenberg, 1979; Rosenberg and Paterson, 1979). At present it is not known whether mRNAs coded by the chloroplast are 'capped' but this is under investigation (M. Edelman, personal communication).

When comparing the translation products of the Wheat germ system programmed with RNAs from different plant sources similarities are apparent (Fig. 4.3). Obviously care is required in making such a comparison due to possible differences in the developmental stages of tissue from which the RNA is extracted. However, it is apparent that the translation products of Spinach and Cucumber RNA bear a greater similarity to each other than to Spirodela and Wheat and vice versa. The reasons for the relative similarities are not known, the only obvious relationship between the sources of RNA being that Cucumber and Spinach are dicotyledonous whereas Spirodela and Wheat are monocotyledonous.

One of the most significant points resulting from my observations is that the Cucumber cotyledon PSSu is a 25,000 M_r polypeptide (Fig. 4.4). This is 11000 daltons larger than the mature SSu (14,000 M_r). The PSSu has been identified by immunoprecipitation with antisera raised against Spinach RuBPCase and Cucumber SSu

RuBPCase. It would have been useful to confirm this finding by another method such as comparing limited proteolytic digests or tryptic peptides of the 25,000 M_r polypeptide and SSu. However, it was envisaged that difficulties would arise in applying these techniques. The method of limited proteolytic digestion depends on the re-electrophoresis of the polypeptide fragments from protein digestion on SDS-polyacrylamide-gels (Cleveland et al., 1977). Technical difficulties would arise in obtaining adequate resolution of polypeptide fragments smaller than 14,000 M_r on re-electrophoresis. The alternative is tryptic peptide mapping (for example, see Gray et al., 1978). However, due to the relatively large size differences between the precursor and the mature polypeptide any meaningful comparison of the peptide fragments from both polypeptides may be difficult to obtain. Possibly the best form of identification could be provided by investigating whether the 25,000 M_r polypeptide is processed, transported into isolated chloroplasts and integrated into RuBPCase (Chua and Schmidt, 1978; Smith and Ellis, 1978).

The Cucumber cotyledon PSSu is at least 5000 daltons larger than the PSSus previously reported to be synthesized in vitro (Roy et al., 1977; Dobberstein et al., 1977; Highfeld and Ellis, 1978; Cashmore et al., 1978; Tobin, 1978; Chua and Schmidt, 1978). Close examination of the results presented by the latter authors indicate that Chlamydomonas PSSu is larger than those of Pea and Spinach being approximately 21,000 M_r. These authors have shown that whereas Pea and Spinach chloroplasts can process and transport either Pea or Spinach PSSus they do not process and transport Chlamydomonas PSSu. It was suggested that evolutionary divergence

has resulted in this inability of higher plant chloroplasts to process and transport PSSu from a lower plant. This may be related to the larger size of Chlamydomonas PSSu. The relatively large size of Cucumber cotyledon PSSu may also reflect evolutionary variation. Another possibility is that the 25,000 M_r PSSu is unique to the cotyledons and is not found in the leaves of mature plants. However, recent experiments have shown that the Wheat germ translation products programmed by either Cucumber cotyledon or leaf RNA are similar and both contain the 25,000 M_r polypeptide (Dosser and Walden, unpublished results).

Using increasing amounts of total RNA to programme the Wheat germ system did not result in the preferential translation of SSu mRNA, as had been found with LSu mRNA when increasing amounts of total RNA were used to programme the E. coli system. As discussed previously preferential translation of mRNA may be one of the mechanisms by which large amounts of protein are accumulated in vivo. Hence preferential translation of SSu mRNA would be expected considering the previously described abundance of RuBPCase and the recent demonstration that the SSu gene is only present in a few copies at the most in the haploid Pea genome (Cashmore, 1979). Some indirect evidence that SSu mRNA is relatively efficiently translated in vitro was provided by the experiments carried out to investigate the possible inhibitory effects on the translation of BMV RNA in vitro of Cucumber total and poly$(A)^+$ RNA extracted from cotyledons of later stages of seedling development (see Chapter 7, Section II). Due to the efficiency of translation of BMV RNA in the Wheat germ system compared with total RNA (see Fig. 4.1) it was expected that only BMV RNA would be translated in mixing experiments.

However, analysis of the translation products by SDS-polyacrylamide-gel electrophoresis and autoradiography, showed that amongst the translation products of the mixture of BMV and total cotyledon RNA extracted from later stages of seedling development, a 25,000 M_r polypeptide was present. This is not the case when BMV RNA alone is used to programme the Wheat germ system (see Fig. 4.2). This suggests that SSu mRNA competes effectively with BMV RNA for translation in the Wheat germ system.

The Wheat germ translation system directed by Cucumber cotyledon RNA synthesized several enzymes associated with lipid metabolism which have been identified by immunoprecipitation. These are the precursors of isocitrate lyase (61,500 and 59,000 M_r), malate synthase (57,000 M_r), catalase (55,500 M_r) and malate dehydrogenase (38,000 M_r) (Reizmann, Weir, Titus, Leaver and Becker, unpublished results). I have tentatively identified the 32,000 M_r polypeptide precursor of chlorophyll a/b binding protein by analogy with the results of Apel and Kloppstech (1978a). The light dependent synthesis of the mRNA coding for the 32,000 M_r polypeptide is initiated in the cotyledons at Day 4 of the Cucumber seedling developmental sequence (see Fig. 7.8). This coincides with the light dependent appearance of the polypeptide identified as chlorophyll a/b binding protein in the SDS-polyacrylamide-gel profiles of stained proteins (Fig. 6.3) and proteins labelled <u>in vivo</u> (Fig. 6.6). It had been hoped to positively identify the 32,000 M_r polypeptide as the precursor of the chlorophyll a/b binding protein by immunoprecipitation using antisera raised against chlorophyll a/b binding protein (a gift from K. Apel and J. Bennett). However, although the 32,000 M_r polypeptide was immunoprecipitated from the Wheat germ translation

products several other polypeptides appeared as contaminants.

Several other polypeptides have been tentatively identified amongst the Wheat germ translation products by co-electrophoresis on SDS-polyacrylamide-gels with proteins synthesized in isolated chloroplasts. These include the α and β subunits of ATPase and as previously described the 35,000 M_r chloroplast membrane-bound polypeptide. However, as discussed previously chloroplast RNA does not appear to be efficiently translated in the Wheat germ system, thus the suggestion that proteins synthesized in the chloroplasts appear amongst the translation products directed by total RNA needs to be treated with caution. Recently evidence has been presented indicating that a Wheat germ system programmed with Spinach poly(A)$^+$ synthesizes precursors to the δ and γ subunits of chloroplast ATPase (Price, Watanabe, Zielinski, unpublished results). It is thought that these subunits are synthesized in the cytoplasm (for example, see Bouthyette and Jagendorf, 1978).

The characteristics of protein synthesis in chloroplasts isolated from Cucumber cotyledons were similar to those described by other workers (Blair and Ellis, 1973; Siddell and Ellis, 1975; Bottomley et al., 1974; Morgenthaler and Mendiola-Morgenthaler, 1976). The isolated chloroplasts were able to use light or ATP as an energy source to synthesize a number of membrane-bound and soluble polypeptides (see Fig. 5.2). The major products have been tentatively identified by co-migration with purified chloroplast proteins on SDS-polyacrylamide-gel electrophoresis and comparison with previously published results. These include LSu RuBPCase, the α and β subunits of ATPase and a 34,000 M_r polypeptide, the latter thought to be analogous to the major membrane-bound polypeptide of

approximately 32,000 M_r described by other workers (Eaglesham and Ellis, 1974; Bottomley et al., 1974; Morgenthaler and Mendiola-Morgenthaler, 1976; Grebanier et al., 1979). If this is the case, it is of interest that the membrane-bound polypeptide synthesized in isolated Cucumber cotyledon chloroplasts is not a major product as described by the other workers. It was found that this was not due to the conditions of the incubation nor the stage of development of the tissue from which the chloroplast were isolated.

Preliminary evidence would suggest that the 34,000 M_r polypeptide is synthesized in vitro as a 35,000 M_r precursor which is processed when isolated chloroplasts are incubated in the light but not when they are incubated in the dark with ATP as source of energy (Fig. 5.3). This work could be improved and extended by different experiments involving the addition of unlabelled methionine as a 'chase'. In the experiments described in Chapter 5, Section III, translation products of isolated chloroplasts incubated in the light or the dark for 60 minutes with [^{35}S]met were compared with those of a 20 minute incubation in the light with [^{35}S]met followed by a 40 minute 'chase' (Fig. 5.3). It was hoped that this would show that amongst the translation products of the former incubations the 35,000 M_r and 34,000 M_r polypeptides would be labelled whereas those of the latter would lack a labelled polypeptide of 35,000 M_r. However, the translation product profiles of the chloroplasts incubated in the light are similar, this is presumably because the declining rate of incorporation after 20 minutes (see Fig. 5.1) is equivalent to the addition of a 'chase' at this time. An improved experiment would be to compare the translation products of a 20 minute incubation with [^{35}S]met with those of a 20 minute incubation with [^{35}S]met followed

by a 40 minute 'chase' as described by Ellis and Barraclough (1978). This might provide evidence of a direct relationship between the 35,000 M_r and 34,000 M_r polypeptide, which can only be indirectly inferred by the results presented here. Confirmation that the processing activity is light dependent would come from a comparison of the translation products of a 20 minute incubation with $[^{35}S]$met in the dark using ATP as an energy source and a 40 minute 'chase' carried out in the light or the dark.

Although there are apparently dramatic morphological differences between the cotyledons of Cucumber seedlings grown in the light and dark (Fig. 6.1), the protein content and the SDS-polyacrylamide-gel profile of stained proteins of both are similar (see Fig. 6.2 and Fig. 6.3).

In agreement with previous findings approximately 80% of homogenate protein sediments upon centrifugation at 10,000 x g prior to Day 2 (Fig. 6.2), presumably this is because the majority of the protein is contained in protein bodies (Becker et al., 1978). These authors also showed that protein bodies can be recovered by sucrose density centrifugation of organellar preparations from tissue from between Days 0 and 4. Protein bodies are prominent ultrastructural features of the Cucumber cotyledons during the early stages of development (Trelease et al., 1978). There is a decrease in sedimentable protein extracted from light- and dark-grown cotyledons after Day 3. At Day 6 approximately 70% and 50% of the protein respectively from the light- and dark-grown cotyledons no longer sediments at 10,000 x g. The progressive disappearance of a group of polypeptides of between 20,000 and 35,000 M_r from the SDS-polyacrylamide-gel stained protein profile is thought to represent

the degradation of the protein bodies (Fig. 6.3). This takes place during the same period in the light and the dark, but in the latter case what are thought to be partially solublised storage proteins, appear to persist until Day 8.

Between Day 2 and Day 3, several polypeptides appear and subsequently increase in staining intensity, the most prominent of these being the LSu and SSu of RuBPCase. A number of polypeptides appear to be light dependent in their appearance at Day 4. These include soluble polypeptides of approximately 31,000, 39,000 M_r and a 29,000 M_r protein only present in the homogenate protein fraction. The latter protein has been identified as chlorophyll a/b binding protein (J. Schouten, personal communication).

Quantitation of the individual subunits and native RuBPCase has been provided by densitometric scanning of denaturing or non-denaturing polyacrylamide protein gels respectively. These techniques rely on the quantitative staining of proteins by Coomassie Blue and Napthalene Black (Bennett and Scott, 1971 and Becker et al., 1978). The data in Figure 6.5 shows that the initiation of synthesis of the subunits of RuBPCase and the initial appearance of native RuBPCase takes place between Day 2 and Day 3. The initiation of synthesis is light-independent and the accumulation of RuBPCase in light- and dark-grown tissue appears to be similar until Day 4. At Day 4, the cotyledons emerge above the soil and the rate of accumulation of RuBPCase is maintained in the light, whereas in the dark it declines. It is not known whether the decline in the rate of accumulation in the dark is due to a decline in the overall rate of synthesis or increased turn over. These results confirm the data of Becker et al. (1978) and are similar to the findings of Mohr (1977) using Mustard cotyledons.

Due to the difference in assay technique, caution is required in attempting to compare closely the data concerning the accumulation of individual subunits and complete RuBPCase. If this were possible one would be able to investigate whether the synthesis of the individual subunits was tightly coupled with the assembly of the native protein. To my knowledge there have been no reports for free LSu in plant tissue, but results from Ellis' group suggests a mechanism by which a specific protein of cytoplasmic origin serves to aggregate LSu prior to the assembly of RuBPCase (Ellis, 1979). Several workers have presented evidence suggesting that pools of free SSu may be present in protoplasts *in vitro* (Hirai and Wildman, 1975; Barraclough and Ellis, 1979) and in young tissue (Feierabend and Wildner, 1978; Roy *et al.*, 1978). A better technique to assay the levels of individual subunits and native RuBPCase would be Rocket Immunoelectrophoresis. This technique permits the accurate quantitative measurement of the amount of a specific protein present in a mixture of proteins (see Crowle, 1961; Axelsen *et al.*, 1973). The technique has been used successfully to study the accumulation of G1 protein during the development of Bean cotyledons (Sun *et al.*, 1978) and is not only very sensitive, but also would allow direct comparisons of the amounts of individual subunits and the native protein. A variation of Rocket Immunoelectrophoresis, Crossed Immunoelectrophoresis (as described in Axelsen *et al.*, 1973) could be used to investigate the possible presence of pools of free SSu in tissue in which the synthesis of LSu has been abolished (Feierabend and Wildner, 1978). However, such techniques rely on the avidity of the

the antisera and also the specificity of an antisera for complete RuBPCase and not the individual subunits.

The light dependent appearance of chlorophyll takes place at Day 4 and corresponds with the emergence of the cotyledons above the soil (Fig. 6.5d). This follows the initiation of synthesis of RuBPCase by one day (Fig. 6.5c) and coincides with the light-dependent appearance of chlorophyll a/b binding protein, amongst the SDS-polyacrylamide-gel profile of stained proteins (Fig. 6.3) and proteins labelled with [^{35}S]met *in vivo* (Fig. 6.6). It has been known for some time that in greening tissue chlorophyll synthesis is tightly coupled with the acquisition of photosynthetic competence and associated enzymatic activity (Tolbert and Gailey, 1955; Huffaker *et al*., 1966). Indeed it has been suggested that RuBPCase is part of the protein constituent of the protochlorophyll holochrome (Trown, 1965). On the other hand, increased enzymatic activity of photosynthetic enzymes may be related to the structural development of the chloroplast. Both of these possibilities have been excluded by Mohr and co-workers, who, using Mustard cotyledons grown under different light conditions, found that the onset and rate of RuBPCase synthesis was not related to the organisational state of the plastid compartment nor greening (for review, see Mohr, 1977).

Conclusions concerning the results of *in vivo* labelling with [^{35}S]met are limited for at least two reasons, firstly, changes in the uptake of [^{35}S]met into the cotyledons (due to permeability changes) and secondly, changes in the internal methionine pool during cotyledon development. However, with these considerations in mind the results obtained confirm the observation that the initiation of synthesis of LSu and SSu takes place between Day 2 and

Day 3 and is followed in the light by the initiation of synthesis of the chlorophyll a/b binding protein at Day 4 (Fig. 6.3). The method of *in vivo* labelling carried out for 24 hours prior to harvesting did not detect any of the previously described polypeptide precursors observed by other workers using *in vitro* and *in vivo* systems. This is presumably because of the rapid turn over of these precursor polypeptides. Indeed to my knowledge the presence of the precursors to chlorophyll a/b binding protein and SSu have yet to be demonstrated in the intact plant cell. Maccechini *et al.* (1979) have used 'pulse-chase' experiments on spheroplasts of yeast to demonstrate the presence of cytoplasmically synthesized precursors to mitochondrial proteins. Analogous experiments could be carried out using plant protoplasts.

The increases in RNA content in light- and dark-grown tissue (Fig. 6.8a) are similar to the previous findings of Vedel and D'Aoust (1970) using Cucumber cotyledons and Ingle (1968b) using Radish cotyledons. These workers found that there was an increase in the RNA content of the cotyledons during light- and dark-grown seedling development and the amount of RNA in the dark reached 60-70% of the amount found in the light. In the data presented here the cotyledons of dark-grown cotyledons accumulate 50% and 85% of the RNA content of those grown in the light at Day 4 and Day 6 respectively.

Fractionation of the total RNA by polyacrylamide-gel electrophoresis shows that both the light- and dark-grown tissue accumulate chloroplast ribosomal RNA (Fig. 6.7 and Fig. 6.8b). This is in agreement with previous workers (Ingle, 1968b; Grierson and Covey, 1975; Becker *et al.*, 1978). The accumulation of chloroplast ribosomal RNA coincides with the accumulation of complete RuBPCase

confirming the previous findings of Patterson and Smillie (1971) and Becker et al. (1978).

Using [^{32}P] orthophosphate to label ribosomal RNA of Radish cotyledons during seedling development Ingle (1968a) showed that whereas incorporation into cytoplasmic ribosomal RNAs took place throughout the 6 days of seedling development incorporation into chloroplast ribosomal RNA took place only during Days 2, 3 and 4. Similar results were obtained by Patterson and Smillie (1971) who, using [^{3}H] uracil to label RNA, found a sharp decline of incorporation of label into chloroplast ribosomal RNA when the first leaf of Wheat had stopped growing. This data was interpreted as showing that the pattern of net synthesis and degradation was similar and once a certain stage of development was reached, there was little replacement of chloroplast ribosomal RNA, whereas there was turn over of cytoplasmic ribosomal RNA. Although the idea that chloroplast ribosomal RNA is synthesized on a 'once only' basis is persuasive (Ingle et al., 1970) these results may also have been due to changes in the nucleotide pools as discussed in Patterson and Smillie (1971).

The decline in the amounts of total RNA during the latter stages of the developmental sequence (Fig. 6.8) remains to be satisfactorily explained. Using different developmental systems, other workers have observed that an initial increase in the amounts of RNA was followed by a decline (Ingle, 1968a, 1968b, Patterson and Smillie, 1971; Grierson and Covey, 1975). The possibility that the decline in RNA is due to RNAase action during extraction would be excluded by the observation that the polyacrylamide-gel profiles of total nucleic acids shows that the ribosomal RNAs of later

stages of development appear to remain intact (Fig. 6.7). One possibility is that the decline in the total RNA content indicates the initiation of senescence in the tissue (discussed in Butler, 1967). It has been shown that in senescing Cucumber cotyledons the decline in RNA content is associated with an increase in RNAase activity (Lewington et al., 1967).

When equal amounts of total RNA extracted from cotyledons of light- and dark-grown seedling development are used to programme the E. coli system the developmental changes in incorporation are similar (Fig. 7.2a). This would indicate that in both light- and dark-grown tissue there is an accumulation of chloroplast mRNA until Day 4 in the light, Day 5 in the dark, after which there is a decline. Comparison of the translation products directed by equal amounts of total cotyledon RNA from light- and dark-grown Cucumber seedlings show that they are essentially the same (Fig. 7.3). The result indicates that the mRNAs assayed with the E. coli system broadly fall into two categories, the majority are those that are present at every stage of development while the minority appear to be present from Day 2 or Day 3 and thereafter increase in amounts. The most prominent of the latter group has been identified as LSu mRNA.

Until more of the translation products of the E. coli system can be identified as proteins synthesized within the chloroplast any conclusions concerning the overall transcriptional activity of the chloroplast during its development can only be tentative. It may be a feature of the translation system that only those mRNAs that are translated are those that are not quantitatively or qualitatively different in light- and dark-grown tissue.

However, the data presented here would suggest that equal amounts of RNA extracted from light- and dark-grown Cucumber cotyledons contain the same relative proportions of chloroplast mRNAs. The only differences between light- and dark-grown tissue being the amounts of mRNA present in each at any particular stage of development (Fig. 6.8a).

The similarities in the types of mRNA assayed in the E. coli system are reflected in the similarities in the gel profiles of stained proteins present in the light- and dark-grown cotyledons (Fig. 6.3) and labelled in vivo (Fig. 6.6). At first, this result was surprising, considering the dramatic morphological differences between light- and dark-grown seedlings (Fig. 6.1). However, it is perhaps to be expected considering the observations of previous workers (outlined in Chapter 1), that etioplasts and chloroplasts each contain many of the same constituents. The data presented here would extend these observations by showing that etioplasts and chloroplasts contain the same spectra of mRNAs.

The initiation of synthesis of LSu mRNA between Day 2 and Day 3 appears to be light independent and coincides with the appearance of both chloroplast ribosomal RNA (Fig. 6.8b) and LSu in vivo (Fig. 6.5a). The levels of LSu synthesized in vitro with the E. coli system programmed with equal amounts of RNA from light- and dark-grown tissue indicates that the pattern of accumulation of LSu mRNA in the light and dark is similar (Fig. 7.4). Comparing the levels of mRNA assayed in vitro (Fig. 7.5) with the levels of LSu and RuBPCase assayed in vivo (Fig. 6.5b,c) it appears that the synthesis of LSu in cotyledons takes place as soon as the mRNA becomes available and that the accumulation of LSu mRNA is

positively related to the accumulation of LSu and RuBPCase until Day 4 or Day 5. After this time there is a decline in the amounts of LSu mRNA assayed *in vitro* whereas *in vivo* the rate of accumulation of RuBPCase is maintained, this will be discussed later.

Where equal amounts of RNA extracted from light- and dark-grown cotyledons are used to programme the Wheat germ system the peak of mRNA activity extends from Day 1 to Day 4 in the light and from Day 2 to Day 4 in the dark (see Fig. 7.7). This corresponds to the period of rapid accumulation of cytoplasmic ribosomal RNA (Fig. 6.8b) and presumably the most active period of cytoplasmic protein synthesis.

Close comparison of the translation products of the Wheat germ system programmed with equal amounts of RNA extracted from light- and dark-grown cotyledons reveals not only developmental changes but differences between light and dark (Fig. 7.8). The data indicates that translatable mRNAs in the Wheat germ system broadly fall into 4 categories: i) Those that are present early in development in the light and the dark and subsequently decline in both light and dark disappearing in the former but persisting in reduced amounts in the latter case. These include the mRNAs coding for the previously mentioned enzymes that are involved with lipid metabolism (Weir, Leaver, Reizman, Becker and Grienenberger, unpublished results). ii) Those that increase during light- and dark-grown development. These include mRNAs that code for a 24,000 M_r polypeptide and the Cucumber PSSu. iii) Those whose synthesis is light dependent, these include mRNAs coding for a 37,000 M_r and a 32,000 M_r polypeptide, the latter is thought to be the precursor

of the chlorophyll a/b binding protein. iv) Those mRNAs that are present in the light and in lesser amounts in the dark. These include the mRNA coding for the 35,000 M_r polypeptide which co-migrates with a membrane-bound polypeptide synthesized in isolated chloroplasts.

When equal amounts of total cotyledon RNA are used to programme the Wheat germ system we see that the developmental changes in the amount of PSSu mRNA, like LSu mRNA, are similar in seedlings grown in the light and the dark (Fig.7.9). Due to possible differences in translational efficiencies of the two cell-free systems close comparisons cannot be made between the accumulation of PSSu and LSu mRNA assayed in vitro. However, it is apparent that the peak amount of PSSu mRNA precedes that of LSu by one day (See Fig. 7.9 and Fig. 7.4). The accumulation of LSu, like LSu is directly related to the levels of PSSu mRNA, assayed in vitro during the initial period of seedling development (compare Fig. 6.5b and c with Fig. 7.10a).

Comparing the accumulation of LSu and PSSu mRNA assayed in vitro (Fig.7.10) with that of the individual subunits and native proteins assayed in vivo (Fig. 6.5a, b and c) similarities during the initial period of seedling development are apparent. In the dark-grown tissue, after an initial increase, the decline in the rate of accumulation of LSu and SSu and RuBPCase appears to be preceded by a decline in the levels of LSu and PSSu mRNA assayed in vitro. On the other hand the decline in the levels of LSu and PSu mRNA extracted from light-grown cotyledons of the later stages of seedling development takes place while the accumulation of LSu, SSu and RuBPCase is maintained. This remains to be satisfactorily

explained. Indeed this point exposes one of the major limitations of using *in vitro* protein-synthesizing systems to assay changes in levels of mRNAs. Such systems may be used to assay increases in levels of mRNA. However, although a decline in protein synthesis *in vitro* may reflect a real decline in mRNA levels it may also be due to other factors such as the mRNA becoming functionally defective *in vitro* or cell-free protein-synthesis progressively becoming inhibited.

Mixing experiments were carried out to investigate whether a factor co-purifying with RNA isolated from cotyledons from the latter stages of seedling development inhibited either the *E. coli* or Wheat germ translation systems. In each case equal amounts of cotyledon total RNA were added to equal amounts of viral RNAs. Due to the relative efficiency of viral RNA to stimulate protein synthesis *in vitro* in such experiments incorporation of radioactivity takes place largely into viral protein. So, in effect, we are looking at the effect of Cucumber RNA on viral RNA translation. The results of these experiments would suggest that some factor present in the RNA prepared from cotyledons of the later stages of light-grown seedling development inhibits viral RNA translation in the Wheat germ system, but not the *E. coli* system (see Fig. 7.6 and Fig. 7.11a) Preliminary data would suggest that the inhibitory factor is not associated with poly(A)$^+$ RNA (Fig. 7.11b). However, why this factor only affects translation by the Wheat germ system remains unexplained. Unfortunately this preliminary data does not allow an assessment of the contribution of the inhibiting factor to the decline in activity *in vitro* of total RNA extracted from the later stages of light development. This demonstrates the advantage of

using a partially purified mRNA, for example, poly(A)$^+$ RNA, in a study as this, at least when using a Wheat germ system. However, a developmental decline in incorporation after Day 4, similar to the results shown here (Fig. 7.7), is observed when poly(A)$^+$ RNA is used to programme the Wheat germ system (E. Weir, personal communication). Also it has been found that during the later stages of seedling development there is a decline in the proportion of cotyledonary RNA containing sequences of poly(A) (Weir and Leaver, unpublished results). Pillay (1977) has found that in cotyledons of dark-grown Soybean seedlings there is a decline with age in the capacity of ribosomes and supernatant factors to support the incorporation of amino acids in vitro.

As previously described the reasons for studying protein synthesis in plastids isolated from cotyledons at each stage of Cucumber seedling development were two-fold. These were to investigate i) the development of the capacity of isolated plastids to synthesize proteins, ii) the spectrum of polypeptides synthesized by plastids during seedling development. However, it became apparent that there would be difficulties in expressing the results in a meaningful manner. In the results shown here the activity of a plastid preparation from each stage of seedling development was expressed as radioactivity incorporated as a function of the amount of plastid fraction protein incubated (see Fig. 7.12b). To allow direct comparison with other data shown here the amount of radioactivity was also expressed on a per cotyledon basis (see Fig. 7.12a). Each method has its limitations which need to be borne in mind when considering the results obtained. These are that changes in the amount of chloroplast protein are not taken into account and

that it is assumed that the proportion of plastids recovered from the cotyledons at each stage of seedling development is the same. The results expressed in either way are similar. There is an initial increase in protein synthetic activity which reaches a maximum at Day 5 followed by a dramatic decline. The initial increase in activity coincides with the period of the accumulation of chloroplast ribosomal RNA (Fig. 6.8b) and RuBPCase (Fig. 6.5c) *in vivo* and LSu mRNA assayed *in vitro* (Fig. 7.5).

The decline in the relative protein synthetic activity of the isolated plastids after Day 5 remains to be explained. Optimisation experiments were carried out using plastids from Day 6 tissue and these were capable of protein synthesis which was largely insensitive to RNAase (see Table 1). This would argue against the possibility that plastids become increasingly more sensitive to disruption during isolation. Another possibility is that there is an increase in the methionine pool within the plastids at later stages of seedling development leading to a relative decrease in the amount of labelled methionine incorporated. This could be investigated by measuring the levels of free methionine directly or by assaying the incorporation of $[^{35}S]$met by isolated plastids in the presence of increasing levels of unlabelled methionine (for example, see Forde *et al.*, 1979).

The results obtained using isolated plastids resemble those of other workers who examined the ability of plastids isolated from etiolated tissue which has allowed to green for various lengths of time to incorporate radioactivity into protein. Drumm and Marguilies (1970) using plastids isolated from greening Bean leaves observed an increase in the capacity for protein synthesis and showed

that the increase was not due to the decline in the levels of an inhibitor or changes in the amino acid pools in the plastids. Within 48 hours of greening the ability of the plastids to incorporate amino acids *in vitro* on a per leaf basis had declined almost to the level found with plastids which had not been exposed to the light. Siddell and Ellis (1975), using Pea leaves showed that in the first 48 hours of greening there was an increase of approximately 3-fold in the capacity for protein synthesis of isolated plastids. Moreover, these authors showed that after 96 hours of greening ATP was no longer able to stimulate protein synthesis whereas light did. It was suggested that this might have represented a change in the permeability of the plastid envelope to ATP.

The developmental profile of the labelled translation products of the isolated plastids are essentially similar whether they are incubated in the light, or in the dark with ATP as an energy source (Fig. 7.13). Once again among the translation products of plastids incubated in the dark at 35,000 M_r polypeptide is present whereas this is replaced by a 34,000 M_r polypeptide among the translation products of the plastids incubated in the light.

There are only minor developmental changes in the relative amounts of specific polypeptides synthesized *in vitro* in isolated plastids, for example, the amount of the 31,000 M_r polypeptide appears to decline (see Fig. 7.13). This is in contrast to the findings of Siddell and Ellis (1975) and Ellis *et al*. (1977). These workers showed that the ratio of incorporation of [^{35}S]met into LSu RuBPCase and the 32,000 M_r chloroplast membrane polypeptide progressively declined either when these polypeptides were synthesized *in vitro* by plastids isolated from etiolated Pea leaves at different

stages of greening or when they were synthesized in vivo in expanding Spinach leaves. A differential synthesis of the LSu RuBPCase and 34,000 M_r chloroplast membrane polypeptide may take place in Cucumber cotyledons at a developmental stage later than those investigated here.

During the later stages of light-grown seedling development the rate of accumulation of both chlorophyll and chloroplast ribosomal RNA in the cotyledon declines (see Fig. 6.5d and Fig. 6.8a.). Coupled with the results obtained with E. coli, Wheat germ and the isolated plastid system (see Fig. 7.10 and Fig. 7.12), this data could be interpreted as showing that after an initial period of active synthesis of chloroplast components, which is light dependent, there is a decline. This would be in agreement with the findings of Ingle (1968a), Drumm and Margullie (1970) and Patterson and Smillie (1971) which has previously been described. The only evidence obtained here which would argue against this suggestion is that concerning the accumulation of RuBPCase. In the light-grown tissue the rate of accumulation of RuBPCase in vivo is maintained during the period of decline in both the amounts of LSu and SSu mRNA assayed in vitro and the protein synthesizing activity of isolated plastids. This may be interpreted as suggesting that a light-dependent factor increases the efficiency of RuBPCase mRNA translation in vivo. On the other hand, the rate of accumulation of native RuBPCase protein may be sustained by pools of previously synthesized subunits. As previously discussed Rocket Immunoelectrophoresis would be useful to investigate the latter of these possibilities as well as confirm the data concerning the accumulation of RuBPCase presented here.

When the work described in this thesis was commenced it was envisaged that the E. coli and Wheat germ systems could be used to assay the developmental changes in levels of mRNA in the cotyledons of developing Cucumber seedlings. The results presented here have mostly proved this to be the case. However, during the course of the work the previously discussed limitations of the cell-free systems, when used to assay changes in levels of mRNA, became apparent. To overcome these limitations the work described here could be complemented and extended by using specific DNA probes to quantitatively hybridize mRNAs present in a given RNA sample (for example, see Evans et al., 1978).

Advances in recombinant DNA technology make such a study feasible and indeed the cloned LSu gene of Maize (Bedbrook et al., 1979a) and the cDNA synthesized from Pea PSSu mRNA (Bedbrook et al., 1979b) may provide adequate probes for Cucumber LSu and PSSu mRNA. However, here I intend to outline an experimental method using techniques currently available by which DNA could be obtained to use as a probe in carrying out hybridization experiments. As described in Taylor (1979) the experimental approach generally used involves the isolation of a specific mRNA followed by synthesis of a cDNA to that mRNA. However, this approach has several experimental difficulties including the isolation of an mRNA of adequate purity, especially when that mRNA is only present in small amounts. As well as the synthesis of full length cDNA. The method I outline below obviates the need to isolate pure mRNA and synthesize cDNA as well as having the advantages that more than one gene which is differentially expressed during seedling development may be isolated. Once isolated, the genes themselves could be studied further.

The first step of the experimental technique involves the production of libraries of fragments of purified chloroplast and nuclear DNA (for example, see Maniatis et al., 1978). Separate libraries of each DNA should be constructed using different restriction endonucleases so that the presence of a particular gene is not excluded because it is cleaved by one endonuclease. The method adopted to screen the libraries of different DNA fragments is that of Benton and Davies (1977) as adapted by Blattner et al. (1978) and Smithies et al. (1978) to cope with the large number of DNA fragments. Simply, this involves cloning the DNA fragments using a phage vector and using these phages to produce plaques on a bacterial-lawn grown on agar, cast on 'Cafeteria-trays'. Phage DNA is bound to replicate nitrocellulose filters. The principle of plaque screening adopted here relies on the results presented in this thesis using the in vitro translation systems that suggests that several genes are expressed in the cotyledons of developing seedlings at a specific time either in the light or the dark. Plaque screening is carried out by hybridizing separately RNA from Day 1 and Day 5 cotyledons of seedlings grown in the light and dark to the cloned DNA immobilised on the replicate nitrocellulose filters. The RNA is 'end-labelled' to a high specific activity with polynucleotide kinase and $[\gamma\text{-}^{32}P]$ ATP. Thus autoradiography of the hybridized DNA-RNA immobilised on the nitrocellulose filters should reveal those cloned genes that are either expressed between Day 1 and Day 5 or are light dependent for their expression.

It is likely that this technique will also detect fragments of DNA containing ribosomal genes which may not be of interest.

This may be avoided either by hybridization of the cloned DNA, immobilised in the nitrocellulose filters, with unlabelled ribosomal RNA before plaque screening or by using the poly(A)$^+$ fraction of RNA in plaque screening to detect those genes that are transcribed to produce mRNA containing poly(A) sequences. However, there have been reports that the eucaryotic genome contains A-T rich regions (Mol et al., 1967; Blattner et al., 1978), hence in the latter case, prior hybridization with poly(A) (riboadenylate) may be required to inhibit non-specific hybridization (Jeffreys and Flavell, 1977).

The relatively small size of chloroplast DNA producing a relatively low number of DNA fragments upon restriction allows an additional screening step to be carried out. To reduce the number of DNA fragments to be cloned,'Southern Hybridization'can be used to detect DNA fragments containing differentially expressed genes (for example, see Bedbrook et al., 1978).

Once screening has been carried out the next step is to identify the genes contained in DNA fragments. This could be carried out by two techniques: 1) "hybrid-arrest translation" (Paterson et al., 1977) or 2) "hybridization-release translation" (Sobel et al., 1979; Smith et al., 1979).

The technique of "hybrid-arrest translation" involves the comparison of products of a cell-free translation system programmed with total RNA, total RNA previously mixed with cloned DNA under conditions which allow hybridization and as a control total RNA previously mixed with cloned DNA but heated to denature any segments of hybridization. The disappearance of a polypeptide from the translation product profile programmed by total RNA hybridized to cloned DNA indicates that the cloned DNA contains the gene for

that polypeptide.

The technique of "hybridization-release translation" involves the binding of the cloned DNA to cellulose or diazobenzyloxymethyl-paper. Total RNA is added under conditions in which complementary sequences within the RNA and cloned DNA can hybridize. The hybridized RNA is eluted and used to programme a cell-free system and the resulting translation product should be coded for by the cloned DNA which was used for the hybridization.

By the method outlined above, specific genes can be isolated and used as probes to study the accumulation of specific species of mRNAs. However, the sequencing of the promoter regions of these genes may provide further insight into the control of transcription which this thesis indicates as being the initial controlling step in the induction of differentiation and development of the cotyledons of Cucumber seedlings.

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Publications

C.J. Leaver, E.M. Weir, R. Walden and W.M. Becker.

Developmental changes in Cytoplasmic and Organellar mRNAs during Germination of Cucumber (Cucumis sativus) (1977). In Translation of Natural and Synthetic Polynucleotides. Ed. A. Legocki, Academic, London.

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REGULATION OF CHLOROPLAST PROTEIN SYNTHESIS DURING GERMINATION AND EARLY DEVELOPMENT OF CUCUMBER (CUCUMIS SATIVUS)

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ABSTRACT

Accumulation of ribulose bisphosphate carboxylase was measured during germination and early development in both light and dark grown cucumber seedlings. This was related to the appearance and relative amounts of the messenger RNA for the large subunit of ribulose bisphosphate carboxylase as assayed in an in vitro protein-synthesising system prepared from E. coli.

INTRODUCTION

During germination and early development of light grown cucumber seedlings the cotyledons initially serve as the site of lipid storage and utilisation, they later expand and assume a photosynthetic role. The disappearance of glyoxylate cycle enzymes associated with the conversion of stored lipid to carbohydrate usually coincides with the emergence of the seedling above ground and the appearance and progressive increase in enzymes and pigments associated with photosynthetic activity[1].

Chloroplast ribosomal RNA and ribulose bisphosphate carboxylase (RuBPC) are both important markers of chloroplast function which rise in concert beginning at day 3, followed a day later by chlorophyll and a peroxisomal enzyme marker, glyoxylate reductase[1]. Among the photosynthetic indicators assayed only chlorophyll displays an absolute requirement for light. Each of the other markers appears at the same characteristic time during development whether the seedlings are grown in light or dark. In each case however, light clearly affects the levels which are reached at later stages, but does not appear to be required for the initial appearance or increase[1].

We have been particularly interested in both the regulation of the appearance of RuBPC and the controlling effect of light on its accumulation at later stages of cotyledon development. To this

end we have measured the accumulation of RuBPC protein in both light and dark grown cotyledons and related this to the appearance and relative amounts of the messenger RNA (mRNA) for the large subunit (LSU) of RuBPC as assayed in an *in vitro* protein synthesising system derived from *E. coli*.

MATERIALS AND METHODS

Seeds of *Cucumis sativus* var. Long Green Ridge were grown in either continuous darkness or illuminated for 12 hours per day with a mixture of incandescent and fluorescent lamps at an approximate intensity of 6500 lux. The temperature in both cases was maintained at 26-28°C for 12 hours followed by a night depression to 22°C for 12 hours.

All other procedures for the harvesting of cotyledons, protein extraction and the quantitative assay of RuBPC protein by gel electrophoresis were as described in reference 1. Total RNA was extracted as described by Leaver and Ingle[2] and used to programme an *E. coli* S-30 protein synthesising system as described by Bottomley *et al*[3]. The LSU of RuBPC was identified as a major product of the *in vitro* system by coelectrophoresis with authentic LSU on sodium dodecyl sulphate (SDS)-polyacrylamide gels and by immunoprecipitation with monospecific antibodies prepared against purified LSU. Immunoprecipitation was carried out essentially according to the method of Kessler[4].

RESULTS AND DISCUSSION

Figure 1 shows that RuBPC protein appears between days 2 and 3 in both light and dark grown cotyledons and accumulates at the same rate until day 4. Thereafter accumulation continues at a constant rate in the light but essentially ceases in the dark grown cotyledons. Light therefore does not seem to be required for the initial appearance and increase in the RuBPC protein, but does influence the eventual levels which are reached. Amongst other photosynthetic indicators assayed only chlorophyll synthesis displays an absolute requirement for light. It is interesting to note that the cessation of RuBPC protein accumulation in the dark at day 4 coincides with the acquisition of photosynthetic function in the light, as indicated by the initiation of chlorophyll synthesis.

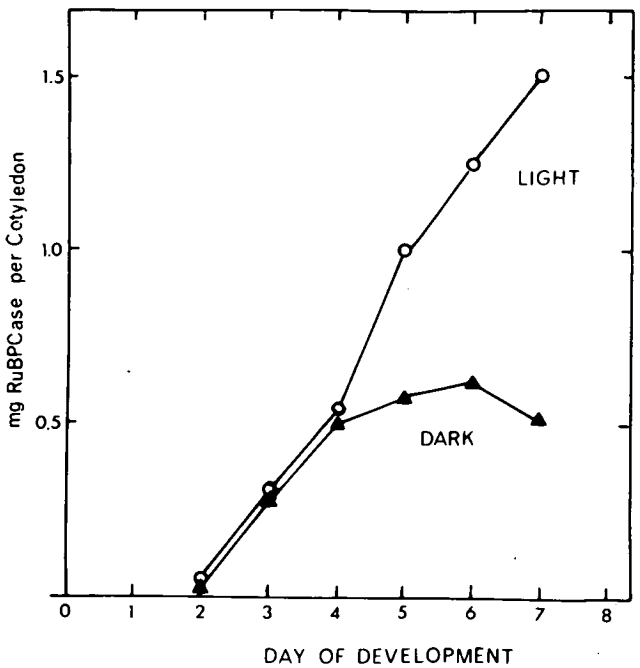

Fig. 1. Developmental changes in amounts of ribulose bisphosphate carboxylase protein in cotyledons of light-grown (○——○) and dark-grown (▲——▲) cucumber seedlings.

As part of a programme investigating the level(s) of regulation of RuBPC synthesis we have sought to develop a quantitative assay for the mRNA's for both the large and small subunits of this organellar protein. In this way we hope to determine (a) when synthesis of these mRNAs is initiated and (b) how the levels of these mRNAs relates to the observed, in vivo developmental changes in RuBPC protein.

We recognise that the most direct method for determining the amounts of a specific mRNA is by hybridisation with a radioactive cDNA probe prepared from that specific message. Here, however, we report our progress in the use of an E. coli cell-free protein synthesising system programmed with total cotyledon RNA, for the quantitative assay of translatable mRNA for the large sununit of RuBPC.

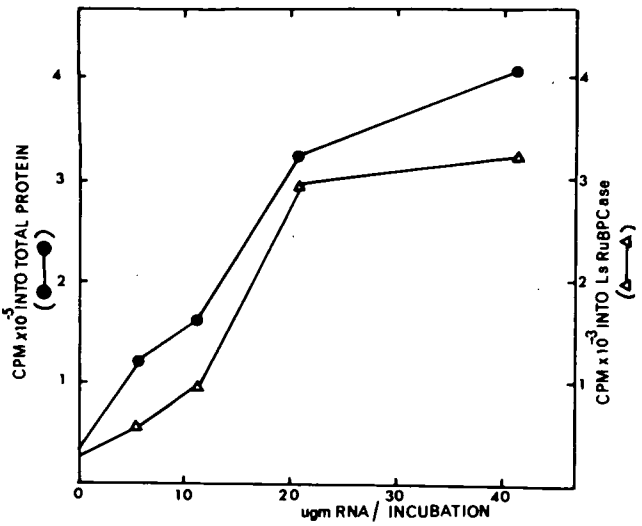

Fig. 2. The effect of varying concentrations of total cucumber cotyledon RNA on [^{35}S]-methionine incorporation into total trichloroacetic acid-precipitable protein (●——●) and into the large subunit of ribulose bisphosphate carboxylase (△——△), in an *E. coli* cell free system.

The *E. coli* cell-free system responded to added total RNA (from cotyledons of any age) in a linear fashion (Fig. 2) and the synthesis of the LSU of RuBPC was found to bear a linear relationship to input RNA over the range 0 to 400 µg/ml (Fig. 2). The amount of LSU synthesised was estimated in two ways (1) [^{35}S]-methionine labelled translation products were analysed by SDS-polyacrylamide gel electrophoresis and the LSU, detected by autoradiography of the dried gel, was excised and counted (2) LSU was immunoprecipitated, with monospecific antibodies, from the labelled cell-free translation products, aliquots were counted directly and analysed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

Total RNA was extracted from light and dark grown cotyledons during the first 7 days of development and equal amounts used to programme the *E. coli* cell-free system. The time courses of the

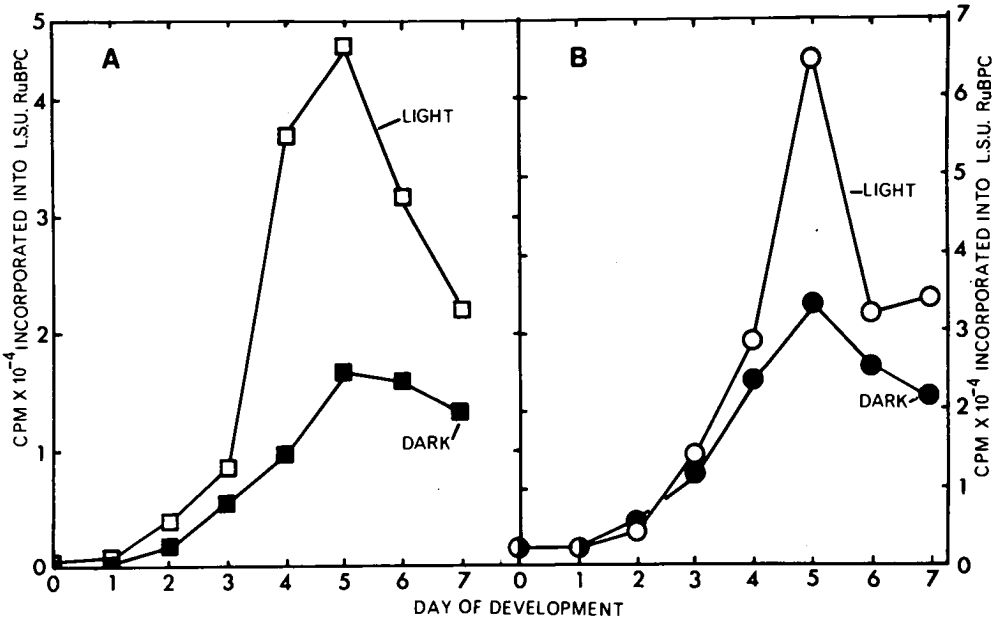

Fig. 3. Developmental changes in the level of translatable mRNA for the large subunit of ribulose bisphosphate carboxylase. Total RNA was extracted from cotyledons of light-grown (□—□, ○—○) and dark-grown (■—■, ●—●) cucumber seedlings and used to programme on E. coli cell-free protein-synthesising system. The amount of large subunit synthesised was estimated (A) by excision and counting of the labelled LSU and (B) by immunoprecipitation, as described in the text.

appearance of translatable mRNA for the LSU of RuBPC are shown in Figure 3, using both the direct counting of labelled LSU excised from the acrylamide gels (Fig. 3A) and by immunoprecipitation (Fig. 3B).

The data produced by both methods are essentially similar and suggest that synthesis of the mRNA for LSU just precedes the in vivo appearance of the complete RuBPC protein.

The amount of translatable mRNA for LSU increases in both light and dark grown cotyledons to day 5 and over this period appears to be positively related to the amount of complete RuBPC protein synthesised in vivo. After day 5, the amount of translatable mRNA drops quite sharply in light grown but much more slowly in dark grown cotyledons.

These preliminary results suggest that the initial rise in the level of translatable mRNA for the LSU of RuBPC is already programmed into the ungerminated seed and requires no environmental stimulus beyond that needed to initiate the imbibition and germination processes. The later involvement of light in the initiation of chlorophyll synthesis would seem to be associated with increased levels of LSU mRNA and the accumulation of higher levels of RuBPC protein in the light grown cotyledons.

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