STUDIES ON THE SYNTHESIS OF THE SMALL SUBUNIT OF FRACTION I PROTEIN

A thesis submitted for the degree of Doctor of Philosophy of the University of Warwick

by

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February 1978

SUMMARY

The small subunit of Fraction I protein from pea leaves has been synthesised *in vitro* by translation of poly(A)-containing RNA , obtained from pea shoot polysomes, in a cell-free protein-synthesising system prepared from wheat-germ. The products of this translation were characterised by gel electrophoretic and immunological techniques. It was found that the small subunit of Fraction I protein was synthesised as a precursor of 20,000 molecular weight; this precursor has been called P20.

In the presence of isolated, intact chloroplasts, P20 is processed to the small subunit. The small subunit so produced is resistant to trypsin-digestion and so is assumed to have entered the chloroplasts. The processing activity is apparently associated with the chloroplast envelope. A mechanism for this processing and transport is proposed and is discussed in the light of other models currently available for the transport of proteins across membranes.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Professor R.J. Ellis for all his advice and encouragement during the course of this work. I wish to extend my appreciation to all members of the Department of Biological Sciences, both past and present, for providing a stimulating atmosphere in which to do research. I must also thank Ruth Ashforth for typing parts of this thesis and for her support. Finally I would like to thank wheat-germ 5 without whose help and ceaseless activity this thesis would not have been possible.

ABBREVIATIONS

A	adenosine base
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BSA	bovine serum albumin
C	cytosine base
chlDNA	chloroplast DNA
Ci	Curie (3.7×10^{10} disintegrations per second)
CM	carboxy methyl
cpm	counts per minute
DEAE	diethylaminoethyl
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
E_x	extinction at wavelength x nm
EDTA	ethylene diamine tetraacetic acid
EMC	encephalomyocarditis
G	guanosine base
g_{ave}	average gravitational field
GTP	guanosine triphosphate
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid
HnRNA	heterogeneous nuclear RNA
IgG	immunoglobulin G
mA	milliampere
mRNA	messenger RNA
NP40	Nonidet-P40
NURB	Neville's upper reservoir buffer
oligo-dT	oligo deoxythymidylic acid
PBS	phosphate buffered saline

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PCMB	para-chloro mercuri benzoate
pH	$\log_{10}$ hydrogen ion concentration
pI	isoelectric point
PMSF	phenyl methyl sulphonyl fluoride
poly(A),(U)	poly adenylic acid; poly uridylic acid
POPOP	1,4-bis-(5-phenyloxazol-2-yl) benzene
PPO	2,5-phenyl oxazole
RNA	ribonucleic acid
RNAase	ribonuclease
rRNA	ribosomal RNA
tRNA	transfer RNA
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SDS	sodium dodecyl sulphate
SFV	Semliki Forest virus
SIM	sucrose isolation medium
SSC	standard saline-citrate
TCA	trichloroacetic acid
TEMED	N,N,N,N'-tetramethylene diamine
TMV	tobacco mosaic virus
TNS	tri-iso-propylnaphthalene sulphonic acid sodium salt
TPCK	L-1-p-tosylamino-2-phenylethyl chloro methyl ketone

CONTENTS

SECTION II - MATERIALS AND METHODS

SECTION I - LITERATURE REVIEW

1. INTRODUCTION

Eukaryotic cells are distinguished from prokaryotic cells by the possession of internal, membrane-bound organelles. Plant cells contain a class of organelles, termed plastids, which are not found in animal cells. The most commonly occuring form of plastid is the chloroplast.

Chloroplasts are the sites of photosynthetic activity in plant cells. All the components required for the conversion of light into chemical energy, and the subsequent utilization of that chemical energy to fix carbon dioxide into carbohydrate, can be found in the chloroplasts (Gibbs, 1971). The major soluble protein of the chloroplast is ribulose bisphosphate carboxylase (RBPCase), often referred to as Fraction I protein. This protein consists of two subunits, as will be described in more detail later (section I3). The enzyme is responsible for the fixation of the carbon dioxide.

Chloroplasts have been found to contain all the components theoretically necessary for self-replication (Boulter et al, 1972). However, attempts to culture chloroplasts outside the plant cell have failed (Ridley and Leech, 1970; Giles and Serafis, 1972). It is now known that many chloroplast functions are encoded in the nucleus, and that the proper functioning of both chloroplast and cytoplasmic protein-synthesising systems is required for the development of functional chloroplasts (Börner, 1973; Ellis et al, 1973). Thus it is not suprizing that chloroplasts will not replicate outside the cell.

In this laboratory, over the last few years, a great deal of work has been done towards elucidating the functions of chloroplast ribosomes (Blair and Ellis, 1973; Eaglesham and Ellis, 1974; Joy and Ellis, 1975; Siddell and Ellis, 1975;

Highfield and Ellis, 1976). However, to gain a fuller understanding of the functioning of the chloroplast's genetic and protein-synthetic machinery, it is necessary to study how the chloroplast interacts with the cytoplasm.

This thesis presents data relating to this topic from the study of a chloroplast protein which is synthesised in the cytoplasm and subsequently enters the chloroplast.

The literature review is intended to describe the background to the work presented in the thesis. In particular, it is intended to outline the concepts prevalent, and the techniques available, when this project was started. At the end of this section, the aims and approach adopted to study cytoplasmically synthesised chloroplast proteins will be described. Only material published up to the end of 1975 will be included in the literature review, except in certain cases where it is felt the the inclusion of more recent material clarifies a particular point. All the relevant findings which have been published between the beginning of 1976 and the time of writing are included in the discussion sections of the thesis. In the discussion sections it is intended to compare the results obtained here with those reported for related systems.

2. CHLOROPLAST-CYTOPLASMIC INTERACTIONS.

A. Structure of the chloroplast.

Chloroplasts are lens-shaped bodies, about 5μm in diameter, present in the cytoplasm of green plant cells. They are one member of the family of organelles known as plastids, but are the most commonly occuring form of plastid. In higher plants, chloroplasts develop through a series of ill-defined intermediate structures, the earliest of which are termed proplastids.

Chloroplasts are separated from the cytoplasm by a double membrane known as the chloroplast envelope. This envelope can be isolated and highly purified preparations can be obtained (Mackender and Leech, 1970; Poincelot, 1973; Douce et al. 1973; Joy and Ellis, 1975; Wellburn and Cobb, 1974). The chloroplast envelope can be distinguished from the nuclear envelope because it does not contain pores.

The lipid and protein compositions of envelope preparations from a variety of species have been investigated (Mackender and Leech, 1974; Pineau and Douce, 1974; Joy and Ellis, 1975). The lipid composition of the envelope is similar to that of the internal thylakoid membrane from the same species, but there are sufficient differences for the two membrane types to be distinguished. In Vicia faba, each membrane has a similar amount of phosphatidyl glycerol but neither contains any phosphatidyl ethanolamine; however, the envelope fraction contains significantly more phosphatidyl choline than does the thylakoid fraction (Mackender and Leech, 1974). When analysed by electrophoresis on polyacrylamide gels, the pattern of proteins from the two membrane preparations differs. Joy and Ellis (1975) have shown that at least two of the polypeptides found in the envelopes of pea chloroplasts are synthesised on chloroplast ribosomes.

The envelope acts as a barrier to the free diffusion of metabolites between the chloroplast and the cytoplasm. This aspect of the envelope has been well reviewed recently (Heber, 1974; Heldt, 1976). The basic results of research in this area are that the outer membrane of the envelope is freely permeable to small molecules, and that the inner membrane is responsible for the control of transport into and out of the chloroplast.

Specific translocation systems have been described for some metabolites, e.g. oxaloacetate and malate.

The internal space of the chloroplast is divided into a granular, electron-dense, soluble phase, known as the stroma and an array of membranes, known as the thylakoid lamellae.

The stroma contains DNA (Ris and Plaut, 1962), which may be membrane-associated (Woodcock and Fernandez-Moran, 1968); ribosomes, tRNAs and amino acid charging enzymes (Francki et al 1965) and intermediary metabolites (Givan and Leech, 1971). The enzymes of the Calvin cycle are also found in the stroma (Trebst et al, 1958). One of these enzymes is ribulose bis-phosphate carboxylase, which brings carbon dioxide into organic combination; the other enzymes carry out a series of reactions such that the carbon dioxide acceptor, ribulose-1,5-bisphosphate is regenerated and the fixed carbon is exported to the rest of the cell as triose phosphates (Gibbs, 1971).

The thylakoid membrane extends throughout the chloroplast and consists of stacks of membrane disks, called grana, connected by single thylakoid lamellae. The whole system appears to be one continuous membrane (Muhlethaler, 1971). The thylakoid membrane contains the pigments, enzymes, cytochromes and other factors necessary for electron transport and photophosphorylation. The organization of the thylakoid membrane has been reviewed recently (Anderson, 1975b) as have electron transport chains (Trebst, 1974) and the pigment-protein complexes (Thornber, 1975). Basically, the thylakoid membrane can be described by a fluid-mosaic type of model with a highly asymmetric distribution of protein. The stromal face of the membrane has photosynthetic coupling factor (CF_1), ferredoxin and ferredoxin-NADP reductase attached. The electron acceptor

sites for the two photosystems are on the stromal side of the membrane, whereas the donor sites are on the inner face of the membrane.

The membrane also serves to divide the chloroplast into stromal and thylakoid spaces; there is evidence that movement of ions between these compartments can change the ionic enviroment of the stroma and so affect the activities of stromal enzymes (Walker, 1976). Heldt *et al* (1973) have shown that light can cause a movement of protons from the stroma into the thylakoid space; this may be connected with the known, light-dependent efflux of Mg^{2+} from the thylakoid space into the stroma (Lin and Nobel, 1971).

B. The sites of coding and synthesis of chloroplast proteins.

(i) Sites of coding.

Chloroplast DNA (chlDNA) consists of a covalently-closed circle about 40μm in circumference; *Pisum* chlDNA has a contour length of 39μm (Kolodner and Tewari, 1972), *Antirrhinum* chlDNA one of 46μm (Hermann *et al*, 1975). Assuming the base sequence is unique, this size is sufficient to code for around 125 proteins, each of 50,000 molecular weight; however, the known chloroplast genes cannot account for more than 10% of the potential coding capacity (Ellis and Hartley, 1974).

Chloroplast ribosomal RNA (rRNA) will hybridise with chlDNA and, in higher plants at least, there are two copies of each rRNA species per genome (Thomas and Tewari, 1974a). Chloroplast rRNA from pea will hybridise with chlDNA from a variety of plants, which would suggest that this part of the genome is conserved (Thomas and Tewari, 1974b).

In both tobacco (Tewari and Wildman, 1970) and *Euglena*

(Schwartzbach et al. 1975) total chloroplast tRNA will hybridise to about 0.4 to 0.7% of the chlDNA. This amount of DNA is sufficient to code for about twenty tRNA molecules i.e. probably one per amino acid.

By the end of 1975 no chloroplast mRNA had been isolated in a sufficiently pure form to allow hybridization studies with chlDNA. Hartley et al (1975) had described the synthesis of the large subunit of Fraction I protein in vitro using RNA isolated from spinach chloroplasts in a cell-free protein-synthesising system from E. coli. This report pointed the way to the subsequent isolation of the mRNA for this protein.

Nicotiana is one of the few genera which affords the opportunity to obtain a wide range of interspecific hybrids. Wildman and his colleagues have used this property of Nicotiana to study the site of coding of some chloroplast proteins. It is possible to study genes present on chlDNA because this DNA is transmitted only by the maternal line (Wildman et al, 1975). The approach adopted by Wildman is to determine some characteristic of a protein which differs in two species of Nicotiana, e.g. isoelectric point or peptide map; the two species are then crossed and the mode of inheritance of the character in the F1 progeny determined. If the character is maternally-inherited then it is assumed that the protein is encoded on chlDNA; if it is inherited in a Mendelian fashion then it is assumed to be due to a nuclear gene.

The two subunits of Fraction I protein have been studied extensively; characteristics such as the tryptic peptides or isoelectric focussing properties of the subunits and the solubility and enzymic activity of Fraction I protein have been used as markers for the subunits (Chan and Wildman, 1972;

Kawashima and Wildman, 1972; Wildman *et al*, 1974; Sakano *et al* 1974; Singh and Wildman, 1973). All the results show that the large subunit of Fraction I protein is encoded in chlDNA, whereas the small subunit is encoded in nuclear DNA.

Ferredoxin is another chloroplast protein which has been investigated in this way. The isoelectric point of ferredoxin varies between different *Nicotiana* species and, using this marker, this protein was found to be inherited as expected for a nuclear gene (Wildman *et al*, 1975).

Similarly Kung *et al* (1972) have shown that the photosystem II-chlorophyll protein (chlorophyll *a*/*b*-binding protein) is inherited in a Mendelian manner. Using tryptic peptide maps as markers, they were able to show that, in crosses between *N. tabacum* and *N. glauca*, the protein behaved as if it were coded in the nucleus.

The green alga *Chlamydomonas* has been used as another system for studying chloroplast genetics. Some genes affecting chloroplast ribosomes have been shown to be inherited uniparentally (Mets and Bogorad, 1972; Boynton *et al*, 1972). The uniparental inheritance is unexpected because sexual reproduction involves fusion of two gametes, each of which contains one chloroplast. When the gametes fuse, the chloroplasts have been observed to also fuse (Cavalier-Smith, 1970). This observation would suggest that both parental copies of chlDNA ought to be present in the daughter cell. Sager and Ramanis (1974) have proposed a restriction-modification system which would explain the loss of paternal chlDNA after fusion.

Some mutants of *Chlamydomonas*, resistant to or dependant upon antibiotics, were found to have chloroplast ribosomes with altered sedimentation coefficients (Gillham *et al*, 1970).

It was found that these mutations were inherited in a Mendelian fashion in some cases yet in a maternal fashion in others (Mets and Bogorad, 1971; Burton, 1972; Boynton *et al*, 1973; Schlanger *et al*, 1972). These mutants have been identified with changes in ribosomal proteins (Mets and Bogorad, 1972; Davidson *et al*, 1974) or defects in ribosomal assembly (Harris *et al*, 1974) which may be due to changes in ribosomal proteins (Nomura, 1970). So far seven Mendelian loci and at least six maternal loci have been identified, which have a role in ribosomal functioning in *Chlamydomonas* (Harris *et al*, 1974).

(ii) Sites of synthesis.

A much-used approach to study the sites of synthesis of chloroplast proteins is to monitor the effect of various inhibitors of protein synthesis when administered either to whole plants or excised leaves or shoots. This method relies on the differential sensitivity of the cytoplasmic and chloroplast ribosomes to these inhibitors. Isolated chloroplast ribosomes have many similarities with bacterial ribosomes in terms of size (Stutz and Noll, 1967), RNA composition (Loening and Ingle 1967; Payne and Dyer, 1971) and number of ribosomal proteins (Hanson *et al*, 1974), and differ in all these properties from the cytoplasmic ribosomes. Functionally, chloroplast ribosomes are similar to bacterial ribosomes; they require f-met-$tRNA_F$ for initiation (Schwartz *et al*, 1967) and functional hybrid ribosomes can be formed with *E. coli* subunits (Lee and Evans, 1971). On the other hand, the ribosomes present in the plant cell cytoplasm have been found to be similar to the 80S ribosomes of animal cells. Protein synthesis by chloroplast ribosomes is also sensitive to antibiotics which inhibit

bacterial protein synthesis, e.g. chloramphenicol, but not to some inhibitors of protein synthesis on 80S ribosomes, e.g. cycloheximide (Ellis, 1969,1970; Boulter *et al*, 1972).

The use of inhibitors to determine the site of synthesis of chloroplast proteins is based on the assumption that the inhibitors have the same effects *in vivo* and *in vitro*, i.e. if an inhibitor of protein synthesis on 70S ribosomes caused an inhibition of the synthesis of a particular protein, then that protein was assumed to be synthesised on chloroplast ribosomes. To draw such a conclusion, it must be shown that the inhibitor is behaving specifically *in vivo*. Both chloramphenicol and cycloheximide, which are the most commomly used inhibitors, have been shown to affect processes other than protein synthesis in some plant cells (Ellis and McDonald, 1970). All stereoisomers of chloramphenicol affect ion uptake and oxidative phosphorylation in higher plants but only the D-*threo* isomer is specific for protein synthesis on chloroplast ribosomes (Ellis, 1969); this stereospecificity provides a useful means of determining whether chloramphenicol is acting solely on chloroplast ribosomes in a given tissue.

Even if the inhibitors *are* specific for a particular protein-synthesising system, it may not be possible to reach an unequivocal conclusion from an inhibitor experiment. If synthesis of a particular protein is inhibited by treatment with an inhibitor of cytoplasmic protein synthesis then either the protein is synthesised on cytoplasmic ribosomes, or it may require a cytoplasmically-synthesised factor for its synthesis elsewhere. An additional problem occurs with studies on multi-component enzymes; if enzymic activity is measured, then inhibition of the synthesis of just one component may affect

the activity of the whole complex.

The results obtained by this type of approach have been sumarised by Ellis *et al* (1973). Most enzymes of the Calvin cycle appear to be synthesised in the cytoplasm. Fraction I protein, some ribosome and some membrane proteins, as well as the photosynthetic cytochromes appear to be synthesised in the chloroplast. Fraction I protein can be affected by both types of inhibitor which is consistent with the two subunits of the protein being encoded in different genomes, one in the nucleus and one in the chloroplast. Criddle *et al* (1970) have shown that the synthesis of the large subunit is preferentially inhibited by chloramphenicol, whereas that of the small subunit is inhibited by cycloheximide.

The use of inhibitors of RNA synthesis to study the sites of coding of chloroplast proteins has also been attempted (Armstrong *et al*, 1971). However, the specificity of the inhibitor used, rifampicin, is in doubt. Rifampicin is specific for baterial as apposed to mammalian RNA polymerases (Wehli and Staehelin, 1971). However, Bottomley *et al* (1971) could not find any inhibitor, rifampicin included, which was specific for chloroplast RNA synthesis, as judged by both *in vitro* assays and *in vivo* measurements of RNA synthesis.

A much more convincing method of determining which proteins are synthesised inside the chloroplasts, is to remove the chloroplasts from the cells and allow them to carry out protein synthesis. Many attempts were made to achieve this (App and Jagendorf, 1963; Spencer and Wildman, 1964; Goffeau and Brachet 1965; Spencer, 1965; Hall and Cocking, 1966; Davies and Cocking, 1967; Gnanam *et al*, 1969; Margulies, 1970). However, bacterial contamination was a problem in the earlier studies

(App and Jagendorf, 1964; Gnanam et al, 1969). A system in which chloroplasts were capable of the synthesis of discrete polypeptides was developed by Blair and Ellis (1973). It was shown that this synthesis was not due to contaminating bacteria and had the characteristics expected for protein synthesis in intact chloroplasts, e.g. RNAase resistance and light-dependance. This method succeeded where the others failed by using rapid isolation techniques, and the inclusion of K^+ as osmoticum; K^+ is required for protein synthesis in isolated chloroplasts and cannot be replaced by Na^+ (Ellis, 1977).

Using this system, it was possible to investigate the products of chloroplast protein synthesis. Blair and Ellis (1973) showed that the major soluble product of chloroplast protein synthesis was the large subunit of Fraction I protein; the small subunit was not synthesised. Eaglesham and Ellis (1974) investigated the membrane proteins synthesised by the isolated chloroplasts and found five discrete peaks of radioactivity when the products were analysed on SDS-polyacrylamide gels. None of these peaks could be identified. Two proteins present in the chloroplast envelope have also been shown to be synthesised on chloroplast ribosomes; however, their identity is also unknown (Joy and Ellis, 1975).

These findings have been confirmed, using chloroplasts isolated from spinach (Bottomley et al, 1974; Morgenthaler and Mendiola-Morgenthaler, 1976) and Euglena (Vasconcelos, 1976). In particular, Morgenthaler and Mendiola-Morgenthaler (1976) used chloroplasts prepared by banding on gradients of silica sol, which results in pure preparations of intact chloroplasts.

Bottomley et al (1974) used autoradiography of dried gels to detect their in vitro products. They claimed that there were

more polypeptides synthesised than had been detected by Blair and Ellis (1973), who had determined the radioactivity profile along their gels by slicing and scintillation counting. Further improvements in the techniques used to analyse chloroplast proteins have led to the detection of many more polypeptides amongst the products of protein synthesis by isolated chloroplasts. Using slab polyacrylamide gels containing a gradient of acrylamide concentration (Chua and Bennoun, 1975), it has been possible to detect at least 25 discrete bands of radioactivity amongst the soluble proteins, and at least 12 in the thylakoids (Ellis, 1977). The soluble protein can be analysed on two-dimensional gels, which separate on the basis of isoelectric point and molecular weight (O'Farrell, 1975); by this method about 80 discrete products can be seen (Ellis et al, 1978). It has not yet proved possible to analyse the chloroplast membrane fractions on such gels (Ellis and Ellis, personal communication).

In spite of the improvements in techniques, the identification of the majority of these in vitro products has not been achieved. Mendiola-Morgenthaler et al (1976) have shown that three of the five subunits of the chloroplast ATPase, coupling factor CF_1, are synthesised by isolated chloroplasts from spinach. This has been confirmed by Ellis (1977) using chloroplasts from pea. It was possible to identify these polypeptides because a procedure which selectively removes CF_1 from the thylakoid membrane was available. These subunits represent only about 1% of the labelled membrane protein, and so were not detectable before this method was used (Ellis,1977).

Protein synthesis in the intact chloroplasts is not sensitive to actinomycin D, and so does not require concomitant

RNA synthesis (Blair and Ellis, 1973). Therefore, all the protein synthesis taking place in isolated chloroplasts must be due to mRNA which was synthesised before the chloroplasts were isolated. It might be thought that such a system does not represent a good model for protein synthesis in chloroplasts *in vivo*. However, it has been shown that the isolated chloroplasts are capable of initiating further rounds of protein synthesis after they have been isolated (Highfield and Ellis, 1976). The *in vitro* products of isolated chloroplasts which have been identified, correspond closely to their *in vivo*-synthesised counterparts. These observations would suggest that the isolated chloroplasts are capable of faithful translation of chloroplast mRNA.

The site of synthesis of the subunits of Fraction I protein has been investigated by another method. Gooding *et al* (1973) isolated polysomes from the cytoplasm and chloroplasts of wheat leaves. They discharged the nascent polypeptide chains of these polysomes by adding [^{3}H]-puromycin; the nascent chains were thus both discharged and labelled. Using antibodies against either the large or small subunits of Fraction I protein to identify the nascent chains, they were able to show that the small subunit was synthesised on cytoplasmic ribosomes and the large subunit on chloroplast ribosomes. Gray and Kekwick (1974) performed similar experiments on polysomes isolated from *Phaseolus vulgaris*. They allowed the polysomes to "run-off" in a cell-free system from rat liver, and so labelled the released chains. Again using antibodies, they were able to show that the small subunit was synthesised on cytoplasmic ribosomes.

(iii) Summary.

The results of all these investigations can be summarised as follows:-

1) chlDNA codes for the rRNAs and at least some of the tRNAs found in the chloroplast;

2) the large subunit of Fraction I protein is coded for by chlDNA, and is synthesised by chloroplast ribosomes;

3) the small subunit of Fraction I protein is coded for by nuclear DNA and is synthesised by cytoplasmic ribosomes;

4) at least 3 of the subunits of CF_1 are synthesised on chloroplast ribosomes; the other 2 subunits might be synthesised on cytoplasmic ribosomes but this is not positively established;

5) in algae, at least, some chloroplasts ribosomal proteins are coded for by chlDNA and may be synthesised on chloroplast ribosomes;

6) ferredoxin and the Calvin cycle enzymes are coded for by nuclear DNA and are synthesised on cytoplasmic ribosomes;

7) chloroplast ribosomes synthesise a large number of proteins which have not been identified.

There is no evidence for the transport of mRNA either to or from the chloroplast; however, there is a formal possibility that the mRNA for a polypeptide might be encoded in the nucleus and yet translated in the chloroplast, or vice versa. For this reason, the fact that a polypeptide is synthesised in one part of the cell can not be taken to indicate anything about its site of coding. Thus, while it is likely that all the polypeptides synthesised by isolated chloroplasts are coded for by chlDNA, this cannot be regarded as established.

C. Changes in plastid proteins during greening

In section A it was stated that proplastids were the developmental progenitors of chloroplasts. Proplastids have been recognised only by their appearance in the electron microscope. They appear to be oval sacs, about 1μm in diameter, and have very little internal structure apart from a few pieces of membrane (Bradbeer et al, 1974a). It has not proved possible to isolate proplastids because of the low amounts present in tissue and the lack of a suitable marker, either biochemical or structural. It has only been possible to describe the path of development of the chloroplast from the proplastid in terms of the structural changes detectable by electron microscopic examination (Whatley, 1974).

When plants are grown in continuous darkness, another form of plastid, termed the etioplast, develops from the proplastid. If such dark-grown plants are transferred into a light-dark regime, the etioplasts will develop into chloroplasts via a series of transient forms; this process is called greening or de-etiolation.

Greening plants have been used as a system for studying the development of chloroplasts because etioplasts and all the intervening stages (often referred to as etio-chloroplasts) to fully-differentiated chloroplasts can be isolated and characterised. In the rest of this section, it is intended to briefly describe the characteristics of etioplasts, and then to describe what is known about the etioplast-to-chloroplast transition.

Etioplasts are ellipsoidal in shape with a diameter of 2 to 3μm (Bradbeer et al, 1974a). The organelle is bounded by a double membrane and the internal space is split into a

stromal phase and a membranous phase, as in chloroplasts. This membrane is organised into a structure known as the prolamellar body and each etioplast may contain up to four of these bodies (Kirk and Tilney-Bassett, 1967). These prolamellar bodies have a highly organised structure of interconnecting tubules (Gunning and Steer, 1976).

It has been found that etioplasts contain many components of the chloroplast but are not able to carry out photosynthesis. The stroma of the etioplast contains DNA (Jacobson, 1968), ribosomes (Dyer et al, 1971), tRNA and amino acid activating enzymes (Burkard et al, 1972) and all the enzymes of the Calvin cycle (Bradbeer et al, 1974a). Analysis of the polypeptides of the prolamellar body by electrophoresis on polyacrylamide gels has shown that some polypeptides appear to be common with those of the chloroplast thylakoid, whereas each membrane has some polypeptides which are peculiar to itself (Bogorad, 1975). It has been shown that etioplasts contain an ATPase activity similar to that of the chloroplasts (Gregory and Bradbeer, 1975; Bogorad, 1975). The etioplasts also contain many of the components of the photosynthestic electron transport chain i.e. cytochromes f, b_{563} and b_{559LP}, and plastocyanin (Plesnicar and Bendall, 1972, Gregory and Bradbeer, 1973).

The major components which etioplasts lack are chlorophyll and the chlorophyll-binding proteins, P700-chlorophyll a-protein and chlorophyll a/b-binding protein (see Thornber, 1975 for a review). Etioplasts contain a complex of protochlorophyllide and protein, called the protochlorophyllide-holochrome (Schopfer and Siegelman, 1968). The protochlorophyllide is reduced, by light, chlorophyllide, which is then transformed into chlorophyll a by addition of the phytyl side-

chain. In light-grown bean leaves there is no detectable protochlorophyllide (Bradbeer, 1975).

Etioplasts are not able to carry out the reactions of photosynthesis because they lack chlorophyll. Although etioplasts have been shown to contain an ATPase, similar to that found in chloroplasts, they cannot form ATP from ADP and inorganic phosphate when transferred from acidic to alkaline solution. (Bogorad, 1975). This process is known as acid/base (A/B) phosphorylation and can be carried out by chloroplasts (Jagendorf and Uribe, 1966).

This evidence suggests that the development of chloroplasts from etioplasts involves both the synthesis of a small number of new components and a re-organisation of those already present. This is borne out by studies of the greening process.

Bradbeer (1975) summarizes much of the work done on the greening of Phaseolus vulgaris. The amounts of chloroplast proteins increase dramatically in the whole leaf during greening; however, when expressed on a plastid basis, the changes are less dramatic in most cases.

During the greening process, the aquisition of individual components of the photosynthetic apparatus occurs at different times after the onset of greening. Plesnicar and Bendall (1973), working with greening barley leaves, were able to correlate the development of grana with the appearance of detectable Photosystem II activity. In particular, the concentration of cytochrome b_{559HP} closely followed the activity of Photosystem II.

The appearance of the chlorophyll a/b-binding protein has been correlated with the synthesis of chlorophyll b (Hiller et al. 1973). When plants are grown under intermittent light

regimes (2 minutes light, 98 minutes dark), only chlorophyll a is synthesised and only the P700-chlorophyll a-protein appears. It has been postulated that the synthesis of chlorophyll is controlled, at least partially, by the availability of acceptor proteins in the thylakoid membranes (Kirk, 1974). This would therefore, represent a system in which the continued synthesis of one component is dependant upon the continued synthesis of the other.

The acquisition of A/B phosphorylation during greening has been correlated with the ability of membrane vesicles to respond to changes in osmotic pressure (Bogorad, 1975). It was suggested that the appearance a 46,000 molecular weight protein in the thylakoid membrane might be connected with the change in membrane properties required to develop A/B phosphorylation.

Thus it is apparent that during the greening process, the assembly of a functioning thylakoid membrane system occurs in a highly organised way. This is not surprising. When a large number of components have to be assembled into a functional whole, it is clearly advantageous to have mechanisms which ensure that this process occurs correctly.

D. Conclusions.

The intention of this section was to show the extent of interaction between the chloroplast and the cytoplasm.

In section I2A it was suggested that many chloroplast proteins are encoded on nuclear DNA and synthesised in the cytoplasm. This class of proteins includes many of those catalysing known chloroplast functions. Of the large number of proteins which can be synthesised by isolated, intact

chloroplasts very few have been identified, e.g. the large subunit of Fraction I protein and some of the subunits of the CF_1. In all cases where both the site of synthesis and coding are known, it appears to be a general rule that chlDNA-coded proteins are synthesised in the chloroplast and nuclear-coded proteins in the cytoplasm. This implies that there is no transport of mRNA into or out of the chloroplast. From this argument, all the proteins synthesised in the chloroplast might well be encoded on the chlDNA.

Some proteins, such as Fraction I protein, have subunits which are coded and synthesised in different parts of the cell, which suggests that there may be some control mechanism to co-ordinate the synthesis of the subunits. Criddle *et al*(1970) were able to show that the synthesis of the large subunit of Fraction I protein was affected by cycloheximide; this would suggest that a cytoplasmically-synthesised protein was required for the continued synthesis of the large subunit. Ellis (1975) has confirmed this using 2-(4-methyl-2,6-dinitroanilino)-N-methyl propionamide (MDMP) to inhibit cytoplasmic protein synthesis. It was postulated that the small subunit was required for the continued synthesis of the large subunit.

During the greening of tissue there must be extensive co-ordination between the chloroplast and the cytoplasm; especially for the assembly of a functioning thylakoid membrane. The available evidence suggests that the genetic and protein-synthetic systems of both the nucleus/cytoplasm and the chloroplast are responsible for the components of this membrane.

The greening process has two points of relevance to this thesis:-

1) particular components are synthesised preferentially

during greening,

2) in the leaf as a whole there is a dramatic increase in chloroplast proteins generally.

The aim of this thesis was to isolate the messenger RNA for the small subunit of Fraction I protein. The general increase in the synthesis of chloroplast proteins during greening would seem to recommend greening tissue as a system for the isolation of mRNAs for chloroplast proteins.

3. FRACTION I PROTEIN.

A. Introduction.

Fraction I protein has been studied extensively for many years and many reviews have appeared recently covering all or some aspects of this protein (Kawashima and Wildman, 1970; Wildman *et al*, 1975; Chollet and Ogren, 1975; Ellis, 1976b; Jensen and Bahr, 1977). It is intended that this section give a short review of Fraction I protein. It is hoped to establish the importance of this protein to the functioning of the chloroplast, and to describe the salient features of the composition and structure of the protein.

Fraction I protein was the name given by Wildman and Bonner (1947) to a protein fraction isolated from the leaves of *Spinacia oleracea* which appeared homogeneous on Tiselius moving-boundary electrophoresis. This fraction accounted for 70-80% of the total soluble protein of the leaf. Analytical ultracentrifugation resolved the fraction further, but the material which sedimented with a coefficient of 18S still accounted for 50% of the total soluble protein. This fraction was later shown to have the ribulose bisphosphate carboxylase (RBPCase) activity of the leaf (Weissbach *et al*, 1956). By

using further fractionation techniques it proved possible to isolate this enzymic activity free from other activities and proteins, e.g. phosphoriboisomerase, phosphoribulokinase and protochlorophyllide-holochrome (Trown, 1965; Paulsen and Lane, 1966; Akoyunoglou et al, 1970).

It would seem reasonable to use the name RBPCase rather than Fraction I protein when referring to the protein as well as to its enzymic activity. However, Andrews et al (1973) found an oxygenase activity associated with RBPCase. This oxygenase activity is present on the same polypeptide as the carboxylase activity, and might even share the same active site (Ryan and Tolbert, 1975a). It has been argued that the name Fraction I protein should be used to refer to the highly-abundant protein present in oxygen-evolving, photosynthetic cells (Ellis, 1976b). This suggestion is followed in the present work.

Fraction I protein (3-phospho-D-glycerate carboxylase (dimerising), E.C. 4.1.1.39) catalyses the following reactions:-

1. CO_2 + D-ribulose-1,5-bisphosphate + $H_2O \rightarrow$ 2 3-phospho-D-glycerate

2. O_2 + D-ribulose-1,5-bisphosphate $\rightarrow$ 3-phospho-D-glycerate + phosphoglycolate

The first reaction is the actual carbon-fixing step in the Calvin cycle of photosynthetic dark reactions (Gibbs, 1971). The oxygenase activity has been implicated in photorespiration (Lorimer et al, 1973). Photorespiration is the process whereby carbon fixed in photosynthesis can be lost again to the atmosphere as carbon dioxide (Chollet and Ogren, 1975). Thus, Fraction I protein has a pivotal role in both photosynthesis and photorespiration. Upto 50% of the carbon fixed in photosynthesis may be lost through the photorespiratory pathway,

and so is an important factor in determining crop yields (Zelitch, 1973).

This oxygenase activity has been found in a large number of species, including the obligate anaerobic bacteria Chromatium (Takabe and Akazawa, 1973b) and Rhodospirillum rubrum (McFadden, 1974). It may seem surprising that anaerobic organisms should possess this activity, but this observation might be taken as indirect support for the view that the oxygenase activity is an "inevitable consequence" of the reaction mechanism of the carboxylase (Lorimer and Andrews, 1973). The transition state of the carboxylase reaction involves the formation of a carbanion; this state is analogous to that found in Grignard-type reagents which can react with both oxygen and carbon dioxide. This type of reaction pathway also explains why this oxygenase lacks the co-factors normally associated with oxygenases (Chollet et al, 1975).

B. Protein chemistry.

Fraction I protein has a molecular weight about 5.1 to 5.5 x 10^5 (Kawashima and Wildman, 1971; Akazawa et al, 1972; McFadden et al, 1975). The protein can be split into subunits by urea (Moon and Thompson, 1969; Sugiyama and Akazawa, 1970), sodium dodecyl sulphate (SDS) (Rutner and Lane, 1967) or high pH (Kawashima et al, 1971). Fraction I protein from almost all organisms studied is composed of two types of subunit; a large subunit of molecular weight 52-55,000 and a small subunit of molecular weight 11-16,000 (Gray and Kekwick, 1974; Iwanij et al, 1974; Ellis, 1976). Fraction I protein from Rhodospirillum rubrum and Chlorobium thiosulphatophilum has been reported to consist of large subunit only (Tabita and McFadden, 1974;

Tabita et al, 1974).

Native Fraction I protein probably contains eight large and eight small subunits, arranged with the large subunits forming a cube and the small subunits attached to the outside of this cube (Baker et al, 1975, 1977). This structure was determined on the basis of data from optical diffraction and electron microscopic studies; the errors in the molecular weight determinations are such that it is not possible to accurately estimate the number of small subunits present.

The large subunit carries both the carboxylase and oxygenase activities; the small subunit may have a role in regulating these activities (Nishimura and Akazawa, 1973, 1974a). The small subunit appears to affect the catalytic activity of the large subunit such that the pH optimum of the reactions is both altered and affected by the concentration of Mg^{2+} ions (Nishimura and Akazawa, 1973). Changes in Mg^{2+} concentration and pH in intact isolated chloroplasts have been detected, and so may have roles in activation of the enzyme during photosynthesis (Walker, 1976).

On the basis of amino acid composition, peptide mapping and immunological cross-reactivity, the large subunits of Fraction I protein are very similar no matter from which species they are isolated, e.g. antiserum against spinach large subunit will cross-react with Fraction I protein from the blue-green algae Anabaena and Plectonema (Takabe et al, 1976). The small subunit, however, varies greatly even between closely related species, e.g. different species of Nicotiana have small subunits which give significantly different peptide maps (Kawashima et al, 1971). The large subunits have not been sequenced from any organism, and so comparison at this level

is not possible. The first twenty amino acids at the NH_2-terminus of the small subunits from Pisum sativum, Vicia faba and Nicotiana tabacum have been determined (Haslett et al, 1976). These twenty amino acids are very similar in sequence in these three species, whereas the small subunits as a whole are not. Therefore, the rest of the sequence must vary greatly between the different species. It is not surprising that some part of the sequence might be common to small subunits from different organisms. The large subunit appears to be highly conserved between various species, and there must be some part of the small subunit in contact with the large subunit in native Fraction I protein. Therefore, it would be likely that this part of the small subunit would also be conserved in sequence.

When the subunits of Fraction I protein are analysed on isoelectric focussing gels, the large subunit appears to have 3 variants, while the small subunit gives any number from 1 to 4 (Kung et al, 1974). The inheritance of these isoelectric variants has been used to study the site of coding of the proteins (Sakano et al, 1974), and the evolutionary origin of some modern species of Nicotiana (Gray et al, 1974).

The large subunit is always resolved into 3 bands no matter from what organism it is isolated. The bands are evenly spaced and cover a very small range of pH values (about 0.3 of a unit). The isoelectric points of the large subunit variants differ from species to species, but the separation between them does not. It is not known what gives rises to these three isoelectric variants; there are no detectable differences between the peptide maps of these three variants (Wildman et al 1975).

The small subunits focus around pH 6 compared with pH 7 for the large subnits. The small subunits from Nicotiana have been extensively studied. In particular, the two isoelectric variants of the small subunit of N. tabacum are thought to be products of separate genes (Gray et al, 1978). The large subunit variants are thought to arise by modification of a single gene product.

Thus Fraction I protein is a key chloroplast protein, which has the following characteristics:-

1)it carries two enzymic activities which have crucial roles in the important processes of photosynthesis and photorespiration;

2) it consists of two types of subunit; one synthesised and encoded in the chloroplast, the other in the cytoplasm and nucleus; it therefore represents a potentially interesting protein for studying the chloroplast-cytoplasm interaction;

3) it is a highly abundant protein and can be easily identified as a protein as well as by its enzymic activity.

4. TRANSPORT OF PROTEINS ACROSS MEMBRANES.

A. Introduction.

Many cells within a multicellular organism secrete protein from the cytoplasm into the extracellular space, e.g. lymphocytes cells of the Islets of Langerhans and pancreatic cells. These secreted proteins are diverse in structure and function, ranging from small polypeptide hormones, through enzymes such as pepsin, to non-enzymic proteins which have important physiological roles, e.g. albumin, ovalbumin and the immuno-globulins. Cells have glycoproteins on their surface which

must also cross the plasma membrane. Conversely, some extracellular proteins must enter cells by re-crossing the cellular membrane, e.g. bacterial toxins. From the evidence presented in section I2, it is clear that proteins must pass from the cytoplasm into the chloroplast during chloroplast development. Mitochondria also have their genetic information split between the nuclear and mitochondrial genomes, and the same situation must occur during the development of a mitochondion, i.e. the transport into the organelle of cytoplasmically-synthesised proteins. Very little work has been done on the transport of proteins across membranes within a cell. Therefore, this section will consider what is known about the transport of proteins across the plasma membrane, i.e. the synthesis of proteins for subsequent export. In particular, the signal hypothesis of Blobel and Sabatini (1972) will be described in detail. At the time the work of this thesis was being performed, this hypothesis seemed capable of providing a model for the transport of proteins into organelles.

B. The Signal Hypothesis.

Palade (1975), in his Nobel lecture, has reviewed the historical background to the study of membrane-bound ribosomes and their role in protein secretion. Initially, it was suspected that membrane-bound polysomes synthesised proteins which were secreted from the cell because cells known to secrete large amounts of protein had extensive arrays of rough endoplasmic reticulum. It was for his work on the elucidation of the pathway whereby secreted proteins were synthesised on rough endoplasmic reticulum, passed onto the Golgi bodies where modifications such as glycosylation were made, and finally

exported from the cell by reverse pinocytosis, that Palade received his Nobel prize.

This work on the synthesis and transport of secreted proteins prompted several questions:-

1) were all the proteins synthesised on membrane-bound polysomes destined for export?

2) were all exported proteins synthesised on membrane-bound polysomes?

3) did free polysomes synthesise only soluble proteins?

A great deal of work has been performed over many years in trying to answer these questions. This field has been well reviewed recently (Tata, 1971; Rolleston, 1974; Palade, 1975; McIntosh and O'Toole, 1976; Shore and Tata, 1977c).

McIntosh and O'Toole (1976) have pointed out that the results of many studies on the proteins synthesised by free and membrane-bound polysomes are open to question. The reason for this rests with the methods used to prepare the free and membrane-bound polysome fractions themselves. Even the simple procedure of checking for cross-contamination of one polysome type by the other is not carried out by all workers. The methods used to prepare the polysomes can also result in artefacts. It has been claimed that the loosely-bound class of membrane-bound polysomes,which has been observed by some workers (Harrison et al. 1974), can arise by association of free polysomes with the membranes during isolation, probably due to the low-salt conditions used (Zauderer et al, 1973). In other situations, loosely-bound polysomes may not be artefacts (Mechler and Vassalli, 1975c).

Another potential point of confusion is illustrated by Shafritz (1974a&b). When RNA was isolated from preparations

of free and membrane-bound polysomes, prepared from rabbit liver, and then translated in a cell-free system, albumin was found to be synthesised by both RNA fractions. However, if the polysomes themselves were added to the cell-free system, and allowed to run-off, then albumin was found to be a product of only the membrane-bound polysomes. Ferritin behaved similarly except that it was synthesised only by free polysomes in the run-off experiment. These observations show that care must be taken when interpreting the results of a particular type of experiment. The run-off type of experiment identifies those proteins that are being synthesised by a given type of polysome. The translation of RNA from a given polysome fraction shows what mRNAs are present in that fraction; however, these mRNAs need not be in polysomes.

Bearing all these problems in mind, Shore and Tata (1977) conclude that, for membrane-bound polysomes, there is a case for believing that a discrete class of proteins may be synthesised; for free polysomes the situation is less clear. For any particular protein, it is always possible to detect some protein synthesis which occurs on free polysomes (5-25% of the total), but this can be explained in terms of the signal hypothesis (see later).

For free polysomes, it is difficult to show that any protein is synthesised exclusively on these polysomes. There is always a substantial level of synthesis on membrane-bound polysomes as well, e.g. tubulin in brain tissue (Floor *et al*, 1976). In fact it has been argued that all protein synthesis starts on the membrane, and that polysomes subsequently either become free or stay membrane-bound (Faiferman *et al*, 1973; Branes and Pogo, 1975). McLauglin and Pitot (1976a&b) have

conducted a careful study of free and membrane-bound polysomes from rat liver. They investigated the location of the polysomes synthesising serum albumin and serine dehydratase, and found that both were predominantly membrane-bound. However, the proportion could be altered by changing the physiological state of the rat before isolating the liver. They also found evidence that newly-synthesised polysomes appeared first on the membranes, and then moved to the soluble phase of the cytoplasm. One point which might confuse interpretation of these results is that the tissue used contains large amounts of membrane, and about 75% of all polysomes are membrane-bound.

Before describing the signal hypothesis it is necessary to describe some of the physical and biochemical properties of membrane-bound polysomes which must be explained by any model.

Examination of rough endoplasmic reticulum in the electron microscope shows that the membrane-bound polysomes appear to be in contact with the membranes via the 60S ribosomal subunits. Treatment of these polysomes with both puromycin and high-salt (500mM) is required to remove them from the membrane. RNAase-digestion removes only a very small proportion of the polysomes (Adelman et al, 1973; Blobel and Potter, 1968). Neither puromycin nor high-salt by itself will remove the polysomes from the membrane. These polysomes are therefore attached by both the nascent polypeptide chain and the 60S subunit.

The signal hypothesis was proposed to explain how the polysomes synthesising a protein destined for export from the cell came to be associated with the endoplasmic reticulum and maintained this association, and how the protein crossed the

membrane (Blobel and Sabatini, 1972). The most advanced version of the hypothesis, and the strongest evidence in support of it, is given by Blobel and Dobberstein (1975a&b).

The signal hypothesis states the following:-

1) that all protein synthesis is initiated in the cytoplasm;

2) that there is a sequence (the signal) of amino acids at the NH_2-terminus of all secreted proteins which is not found on the exported protein itself;

3) that when this signal is present on the nascent polypeptide chain, it causes the polysome to bind to the membrane;

4) that this binding is due to the hydrophobic nature of the signal peptide;

5) that membrane proteins then move laterally through the membrane to interact with the 60S ribosomal subunit to form a hydrophilic pore;

6) that the peptide being synthesised moves through this pore as it is being extended;

7) that the signal sequence is removed from the growing polypeptide as it appears in the cisternal space;

8) that the ribosome dissociates from the membrane when synthesis is complete.

Blobel and Dobberstein (1975a&b) were able to show that the synthesis of immunoglobulin light chain *in vitro*, in a reconstituted system using rabbit reticulocyte ribosomal subunits, rough microsomes "stripped" of polysomes, and light chain mRNA, could be described in terms of the signal hypothesis. In the absence of added membranes, the light chain was synthesised as polypeptide of higher molecular weight; the

signal had been retained. When membranes were present from the beginning of translation then the normal light chain was synthesised; the signal had been removed. This polypeptide was trypsin-resistant unless a non-ionic detergent was added with the trypsin; this was interpreted to mean that the polypeptide had moved across the membrane of the added endoplasmic reticulum. Globin mRNA was used as a control, and the globin synthesised was found to be released into the soluble phase where it was trypsin-sensitive. The synthesis, processing and transport through a membrane of the immunoglobulin light chain could take place in a completely reconstituted system; therefore it was assumed that all the information required for these steps was present in the nascent polypeptide chain. On this hypothesis, interaction between the mRNA and the membrane is not necessary.

Thus the signal hypothesis states that the ultimate location of a protein depends upon its amino acid sequence, and not upon the location of the mRNA, or upon synthesis by a distinct sub-population of ribosomes. The suggestion that membrane-bound ribosomes have a different protein composition from the free ribosomes has been refuted by Lewis and Sabatini (1977). They found that there was a protease which bound to the membrane-bound polysomes during isolation; this protease cleaved two of the ribosomal proteins and so gave membrane-bound ribosomes an apparently different protein composition. If the protease was removed from the polysomes, or inhibited, then the protein compositions of the free and membrane-bound polysomes were identical.

The question as to whether or not binding of the mRNA to the membrane occurs is not clearly answered. Mechler and Vassilli (1975c) present convincing evidence that, in myeloma

cells, the mRNA for the immunoglobulin light chain is bound to the membrane by its 3'-end. Adesnik et al (1976), using human diploid cells, were able to show that the mRNA associated with membrane-bound polysomes remained on the membrane in the absence of any bound ribosomes. Yet the completely reconstituted system of Blobel and Dobberstein (1975b) was able to direct the synthesis of the immunoglobulin light chain and globin into their respective compartments, and in this case the mRNA could not have been membrane-bound. However, Kruppa and Sabatini (1977) have shown that on rat liver rough microsomes, the 3'-end of the mRNA fraction is not associated with the membrane, and so cannot play any role in the binding of the polysomes to the membrane. They did find that use of the same techniques, which removed all the mRNA from the microsomes of rat liver, still left some mRNAs associated with the membranes from tissue culture cells. They concluded that these differences may reflect differences in the extents to which particular mRNAs associate with the membrane; or that the mRNA which remains on the membrane is not polysomal in origin. The observation that all newly-synthesised mRNA may initially be associated with the membrane is relevant to this point (Branes and Pogo, 1975; McLaughlin and Pitot; 1976b).

The binding of the mRNA to the membrane, or lack of it as the case may be, does not fundamentally affect the signal hypothesis. The presence or absence of a signal peptide will still determine the ultimate location of the polypeptide being synthesised. Strong support for this idea comes from work with E. coli. Silhavy et al (1976) were able to show, by inserting the gene for β-galactosidase into the maltose operon, that changing the NH_2-terminal sequence of β-galactosidase could

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result in the enzyme becoming membrane-bound. They were able to fuse together parts of the mal F and lac z genes such that the promotor and NH_2-terminal part of the mal F gene product must have been joined to part of the lac z gene, which was lacking part of the NH_2-terminal region of β-galactosidase. The mal F gene product is a membrane protein which has a role in the transport of maltose and, in the four separate isolates where such protein fusion had occurred, the β-galactosidase activity was found to be membrane-bound. In normal cells, this enzyme is soluble.

The signal hypothesis could be adapted to account for the entry of proteins into organelles by assuming that the signal present on such proteins was different from that present on the proteins to be exported from the cell. This might mean that the polysomes would be found on the envelope of the organelle, as has been claimed for mitochondria from yeast (Butow et al, 1975), because the signal was specific for the organelle envelope. Alternatively, the signal might direct the synthesis of the protein into a class of vesicle which would fuse with the organelle membranes, and so discharge their contents in to the organelle. In this case this class of vesicles must be able to specifically recognise the correct organelle. These two alternatives only differ as to the location of the polysomes synthesising the organelle proteins; they both assume that the signal has a role in determining the final location of the polypeptide. This assumption implies that there are different signals for different classes of proteins, but that they all operate in a similar fashion. Apart from the report of ribosomes bound directly to the outside of yeast mitochondria, very little is known about the site of synthesis, within the

cytoplasm, of organelle proteins. Some mitochondrial proteins are known to be synthesised on the rough microsomal fraction from rat liver (Bingham and Campbell, 1972; Gonzalez-Cadavid *et al*, 1968; Gonzalez-Cadavid and Saez De Cordova, 1974). The small subunit of Fraction I protein may be synthesised on membrane-bound polysomes (Gooding *et al*, 1973).

The signal hypothesis makes certain predictions about the characteristics of the synthesis and processing of proteins which cross membranes:-

1) the *in vitro* translation of the mRNA for such a protein in the absence of membranes should result in the synthesis of a polypeptide which is larger than that found *in vivo*;

2) in the presence of membranes the product should be cleaved to a polypeptide of lower molecular weight;

3) as a result of this cleavage the polypeptide should become trypsin-resistant as it crosses the membrane; it should be rendered trypsin-sensitive by addition of non-ionic detergents, which dissolve membranes;

4) such processing and transport can only occur if the membrane is present at the start of translation, soluble released chains cannot either be processed or rendered trypsin-insensitive;

5) *in vivo*, the polysomes synthesising such a protein will be membrane-bound.

The signal-hypothesis therefore represents a model for the synthesis and transport of cytoplasmically-synthesised proteins which makes several testable predictions.

5. DETECTION AND ISOLATION OF mRNA.

A. Introduction.

The concept of messenger RNA (mRNA) was introduced by Jacob and Monod (1961). However, it was not until 1969 that it proved possible to isolate an RNA which could be translated *in vitro* into an identified protein. Proir to this, "messenger RNA" fractions had been identified by other criteria. The rapidly-labelled, non-ribosomal RNA found in the polysome fraction was regarded as messenger RNA but this RNA could not be translated into protein by any cell-free protein-synthesising system then available (Chantrenne *et al*. 1968; Darnell, 1968).

Lockard and Lingrel (1969) were able to show that the 9S RNA isolated from mouse reticulocytes would direct the synthesis of mouse haemoglobin β-chain in a cell-free system from rabbit reticulocytes. They detected and identified the product by chromatography of the reaction mixtures on CM-cellulose; the mouse β-chain can be distinguished from the endogenous rabbit globin β-chain in this way. The α-chains of rabbit and mouse haemoglobin co-chromatograph with each other on this system, and so it was not possible to detect synthesis of the mouse α-chain.

Since this time, the mRNAs for a very large number of proteins have been isolated, or detected by translation in cell free systems. The whole area of mRNA isolation, structure and function has been reviewed regularly in recent years (e.g. Lingrel, 1972; Brawerman, 1974b; Kaesberg, 1976; Darnell, 1976). In this section it is intended to describe the structure of mRNAs in so far as they relate to experiments described in the thesis, i.e. with regard to poly(A)-tails (section I5B) and "caps" (section I5C). The procedures which have been used to

detect and isolate specific mRNAs will be described (sections I5D&E).

B. Poly(A)-tails.

Tracts of poly(A) have been detected at the 3'-ends of most eukaryotic mRNAs; the size of this tract is heterogeneous, and varies from about 50 to 200 adenylate residues (for a review of early work see Darnell et al, 1973). Poly(A)-tails have been detected at the 3'-ends of polysomal RNA from plants (Van de Walle, 1973; Sagher et al, 1974). However, chloroplast RNA does not appear to have any poly(A) sequences present (Wheeler and Hartley, 1975). On the other hand, mitochondrial RNA has poly(A)-tails about 60-80 bases long; very short pieces of poly(A) (15-50 residues) have been detected on RNA isolated from bacteria (Srinivassan et al, 1975; Ohta et al, 1975). The variation in size of the poly(A) is found even when the mRNA for a single protein is studied, e.g. mouse globin mRNA (Gorski et al, 1974). There is evidence for the shortening of the poly(A)-tail in the cytoplasm, and this could explain the heterogeneous size observed (Sheiness et al, 1975).

Only histone mRNAs (Greenberg and Perry, 1972) and reovirus mRNAs (Stoltzfus et al, 1973) have so far been established as lacking poly(A)-tails at their 3'-ends. The occurrence of poly(A)-tails raises the question as to what function they serve, and why histone and reovirus mRNAs do not contain them. The major site of addition of the poly(A) is in the nucleus (Darnell et al, 1973). Heterogeneous nuclear RNA (HnRNA) was also reported to have poly(A) at its 3'-end (Darnell et al, 1973). Many studies have been made of the metabolism of the poly(A) attached to HnRNA as a way of investigating the relation-

ship between HnRNA and cytoplasmic mRNA (Darnell *et al*. 1973; Perry and Kelley, 1973; Puckett *et al*. 1975; Brandhorst and McConkey, 1975). The results of these experiments are difficult to interpret because of the complexity of the systems studied. As with all *in vivo* labelling experiments, the effect of endogenous pools of unlabelled material on the kinetics of labelling must be taken into account. Moreover, the relationship between the poly(A) found in the nucleus and the cytoplasm is complicated. Poly(A) addition can occur in the cytoplasm as well as the nucleus (Brawerman and Diez, 1975), and so poly(A)-tails in the cytoplasm are undergoing the competing processes of elongation and shortening. The general conclusion from these studies is that the poly(A)-tails found in the cytoplasm are the same as those found in the nucleus (Greenberg 1975).

It has been suggested that the poly(A) sequences found on mRNA in the cytoplasm might have a function in stabilizing the mRNA (Darnell *et al*. 1973). However, Perry and Kelley (1973) found that poly(A)-containing RNA decayed with 1^{st}-order kinetics, i.e. both "young" and "old" mRNA was equally likely to be degraded. Since it was assumed that older mRNA had a shorter poly(A)-tail than newly-synthesised mRNA, these workers concluded that the size of the attached poly(A) had no influence on the stability of the mRNA. Sheiness *et al* (1975) found that the shortening which occurred in the cytoplasm led to an accumulation of poly(A)-tails around 50 residues in length. They argued that this size distribution of poly(A)-tails could be generated by assuming that shortening of the poly(A) occurred by a random endonucleolytic cleavage and that, when the poly(A) was below a certain size, the mRNA was degraded.

The randomness of the cleavage would explain why both old and young mRNAs could be degraded.

More direct evidence that the poly(A)-tail has an effect on the stability of mRNA has been provided by *in vitro* studies. Initial attempts to demonstrate any effect of removal of the poly(A) on the translational properties of mRNA were unsuccessful (Williamson *et al*, 1974; Bard *et al*, 1974). However, it became apparent that when cell-free, protein-synthesising systems which were capable of a large number of rounds of translation on each mRNA molecule were used, then an effect of the poly(A) could be demonstrated (Huez *et al*, 1974). The poly(A)-tail can be removed from RNA by means of polynucleotide phosphorylase, and so the properties of a particular mRNA can be compared with and without the poly(A)-tail being present (Soreq *et al*, 1974). If globin mRNA, either with or without poly(A), is injected into *Xenopus* oocytes, then it is found that the non-poly(A)-containing globin mRNA is degraded much faster than the poly(A)-containing RNA (Marbaix *et al*, 1975). By preparing globin mRNA which had progressively shorter poly(A)-tails, Nudel *et al* (1976) were able to show that when the length of poly(A) fell below 32 residues the stability of the mRNA decreased until, below 16 residues, the mRNA was as unstable as non-poly(A)-containing RNA. This finding was confirmed by Hieter *et al* (1976) who investigated the effect of adding poly(A) onto 5S rRNA, which does not naturally have poly(A) attached. They found that 5S rRNA was more resistant to digestion by spleen RNAase when poly(A) was attached. They also found that the poly(A) had to be more than 20 residues in length to confer maximum protection. Poly(G) would also give protection; Hieter *et al* (1976) postulate that histone

mRNAs do not need a poly(A)-tail for protection against nuclease-digestion because they are rich in A and G due to the amino acid sequence they encode.

Thus poly(A) sequences are found at the 3'-end of most cytoplasmic mRNAs, and this sequence probably has a role in determining the stability of the mRNA *in vivo*.

C. Caps.

The term cap was applied to the unusual structures detected at the 5'-end of eukaryotic mRNAs (Rottman *et al*. 1974). The cap consists of 7-methyl-guanosine linked to the 5'-end of the messenger RNA by a triphosphate bridge from the 5'-carbon of the 7-methyl-guanosine. This type of structure has been detected at the 5' terminus of a large number of RNAs, e.g. Rous sarcoma virus RNA (Keith and Fraenkel-Conrat, 1975); reovirus mRNA (Furuichi *et al*, 1975) and mouse myeloma mRNA (Cory and Adams, 1975). As a result of the large number of caps identified, it has been possible to divide them into two classes (Perry and Kelley, 1976). Cap I has the structure $m^7G(5')ppp(5')X_mpYpZp.....$ where X,Y, and Z are any of the four bases, but X is usually either A or G. Cap II has the structure $m^7G(5')ppp(5')X_mpY_mpZp.....$

Cap structures have been detected on HnRNA (Schibler and Perry, 1976), and kinetic studies have shown that these caps are conserved in the cytoplasm (Perry and Kelley, 1976). The cap II structures are derived from cap I structures by methylation of the ribose moiety of the Y base (Perry and Kelley 1976). The cap II structures are derived from a specific subset of cap I structures,and are found on older mRNA; it is not clear whether the cap II structure is responsible for

the increased lifetime of these RNAs, or whether the structure is formed because mRNAs are present in the cytoplasm long enough for the methylation of the Y base to take place.

Initially it was postulated that the cap structure was essential for the translation of mRNA (Both et al, 1975a&b). The early work which indicated that caps were required for translation of mRNA used chemical methods to remove the m^7G group from the 5'-end (Muthukrishnan et al. 1976; Shih et al 1976). When RNA treated in this way was added to the wheat-germ system, its translation was inhibited by about 90%. However, in the case of the RNAs from Brome Mosaic virus, the inhibition could be overcome by increasing the RNA concentration (Shih et al. 1976). Viruses such as Vaccinia (Wei and Moss, 1974), reo (Furuichi et al, 1976), vesticular stomatitis (Rhoads et al, 1974) and cytoplasmic polyhedrosis (Shimotohno and Muira, 1976) contain enzymes which can form caps on the viral RNA. It has proved possible to generate viral mRNAs, which have been capped to different extents, by inhibiting these enzyme activities (Both et al,1976b). Using such mRNA as a template in the wheat-germ system, it was demonstrated that the wheat-germ extract contained an endogenous capping mechanism which was dependant upon protein synthesis (Both et al. 1976b; Muthukrishnan et al, 1975a). It was found from these studies that the cap is essential for the translation of these mRNAs.

Subsequently, it was reported that compounds such as m^7G, m^7Gp and $m^7GpppXm$ inhibit the translation of capped mRNAs in the wheat-germ cell-free system (Canaani et al, 1976; Hickey et al, 1976). These compounds prevent the formation of an initiation complex on capped mRNA (Roman et al, 1976).

Inhibition of translation of a particular mRNA by such compounds has been taken as evidence that the mRNA is capped (Groner *et al*, 1976; Sharma *et al*, 1976). Proteins have been detected in cell-free extracts which will bind to caps, and it has been postulated that this binding represents the site of action of the cap analogues. However, in *Artemia salina* extracts, the binding protein is different from any previously identified initiation factor (Filipowicz *et al*, 1976) while the binding protein found in rabbit reticulocytes is identical to IF-M3 (Shafritz *et al*, 1976).

These results present a consistent picture in which the presence of a cap is essential for the binding of ribosomes to mRNA, and the subsequent translation of that mRNA. However, the situation has been shown recently to be not so straightforward. Objections have been raised against the use of chemicals to remove caps from mRNAs (Rose and Lodish, 1976). These workers found that it was the rate, rather than extent, of binding to ribosomes which was affected by cap removal, this might explain why increasing the concentration of uncapped mRNA can reduce the apparent inhibition of translation (Shih *et al*, 1976). It has become apparent that the wheat-germ system has a greater requirement for the mRNA to be capped than does the reticulocyte cell-free system (Lodish and Rose, 1977). The importance of the cap is also affected by the K^+ concentration in the *in vitro* translation system; at high K^+ concentrations the cap becomes more important (Weber *et al*, 1977).

Abraham and Pihl (1977) enzymatically removed the cap from the mRNAs for globin and immunoglobulin light chain, and found that this removal had no effect on the translation of

these mRNAs in a wheat-germ system. They concluded that there was no absolute requirement for a cap to obtain translation of these mRNAs. This report must be contrasted with other reports which also enzymically remove caps from mRNA. When Shimotohno et al (1977) removed the caps from cytoplasmic polyhedrosis viral, tobacco mosaic viral and globin mRNA, the messengers had greatly reduced activity in cell-free translation assays. Zan-Kowalczewska et al (1977) obtained the same result when they removed the caps from reovirus and TMV RNAs.

A possible explanation of these conflicting results is provided by the observation that uncapped reovirus mRNA is much less stable in wheat-germ extracts and Xenopus oocytes than in the reticulocyte lysate cell-free system (Furuichi et al, 1977). In particular, it was found that capping caused a large increase in the stability of the mRNA in the wheat-germ and oocyte systems but had no effect on stability in the reticulocyte system. Both Shimotohno et al (1977) and Zan - Kowalczewska et al (1977) observed that the enzymically de-capped mRNA was very unstable in the wheat-germ system, most of the RNA being degraded within a few minutes. It was postulated that the wheat-germ extracts contained a 5'-exonuclease, and that a cap at the 5'-terminus of the mRNA blocked its action.

The presence of such a nuclease would explain most of the observed requirement for a cap for translation of mRNA in the wheat-germ system. The reason for the differential sensitivity of the wheat-germ and reticulocyte cell-free systems to the presence of a cap then becomes apparent. The result of Abraham and Pihl (1977) is, at present, the only report which has found no effect of capping on translation in the wheat-germ

system. It may be that the particular wheat-germ extract used in this work did not contain such a 5'-exonuclease, but this remains to be demonstrated.

Thus it may be that the function of the cap, like that of the poly(A)-tail at the 3'-terminus, is to prevent degradation of the mRNA by nucleases present in the cytoplasm. An enzyme which can hydrolyse m^7G from the 5'-end of oligonucleotide fragments has been detected in HeLa cells (Nuss et al, 1975). Clearly mRNA degradation could be an important point of control and perhaps removal and addition of caps represents a means of exerting this control.

However, not all the effects of caps can be explained in this way. The inhibition of binding of mRNA to ribosomes by cap analogues may indicate that the cap also has some function in the binding of ribosomes to the mRNA. It has been suggested that the presence of Aand U bases next to the cap improves the binding of ribosomes (Muthukrishnan et al, 1976). The ribosome binding site of brome mosaic virus RNA4 has both the cap and the initiator AUG codon within it, and the sequence between the cap and the AUG codon consists of A and U residues (Dasgupta et al, 1975). However, this is not the case for the rabbit globin mRNAs (Baralle, 1977).

Some viral RNAs have been found which do not have caps at their 5'-ends, e.g. RNA from polio virus (Hewlett et al, 1976; Lee et al, 1976; Flanegan et al, 1977), encephalomyocarditis virus (Frisby et al, 1976) and cowpea mosaic virus (Klootwijk et al, 1977). The translation of these RNAs is not inhibited by cap analogues (Weber et al, 1976). However, Brooker and Marcus (1977) were able to cap the RNA from satellite tobacco necrosis virus (STNV), even though it is not

capped *in vivo*, using an extract from vaccinia virus. The capped STNV RNA then behaved like a normal, capped mRNA. The RNA bound to 80S ribosomes 2.5 times faster than the uncapped RNA, and its translation, in a wheat-germ system, was inhibited by m^7Gp. These results suggest that the lack of capping of this RNA *in vivo* is not due to some special feature of the RNA which prevents it being used in a capping reaction.

Thus it may be that the cap on an mRNA does play some role in the initiation of protein synthesis on that mRNA; the inhibition of initiation by cap analogues and the presence of proteins which bind to caps would suggest this. However, most of the evidence which suggests a major role for caps is questionable because the wheat-germ system has been used to assay the effects of alterations to the cap. Caps, therefore, may be just one of the many features of an mRNA which influences the efficiency with which the mRNA is translated. Its importance will vary depending upon other parameters, e.g. the sequence of the mRNA next to the cap, the type of ribosome present and the ionic enviroment.

D. Isolation of mRNAs.

Theoretically the messenger RNA for any protein can be isolated by coupling techniques for fractionation of RNA with an *in vitro* assay for messenger activity. A very large number of different messengers have been detected but only a few of these have been isolated in pure form. The reason for this is that whilst it is easy to detect the presence of an mRNA by a translation assay, it is more difficult to separate one particular mRNA from all the others. In this section it is intended to discuss the various ways in which mRNAs have been

isolated.

(i) Detection of mRNA.

The ability to direct the synthesis of a particular protein *in vitro* is the only criterion which can be used to assess whether or not an RNA preparation contains the messenger for that protein. Thus it is necessary to have a suitable *in vitro* protein-synthesising system and to be able to detect the protein of interest among the total products of translation.

The systems most commonly in use, when this work was started, were based on the rabbit reticulocyte lysate and the wheat-germ extract. These two systems fulfil most of the requirements for a good cell-free protein-synthesising assay, which are:-

1) high activity and high sensitivity, i.e. they incorporate high amounts of radioactivity in response to low concentrations of RNA;

2) non-selectivity, i.e. no requirement for special factors for certain mRNAs;

3) low endogenous levels of protein synthesis;

4) reproducibility;

5) ease of preparation and storage.

The reticulocyte lysate has a very high activity, up to 50% of the rate found in reticulocytes *in vivo* (Lodish and Jacobson, 1972). However, the lysate also has a high level of endogenous globin mRNA, which means that the system has a high endogenous level of incorporation. This endogenous incorporation is almost entirely into globin and so is detrimental only in some circumstances. The wheat-germ system is probably not as active as the reticulocyte lysate, but it has a much lower endogenous rate of synthesis, and so has

become popular for assaying messenger activity.

The methods used to detect a particular protein amongst the total translation products synthesised in a cell-free system in response to added RNA depend upon the protein in question and upon what proportion of the total products it represents.

Co-electrophoresis with standard proteins isolated from cells on SDS-polyacrylamide gels can be used as an initial method for detecting and identifying proteins amongst the translation products. However, this method is of limited applicability. Co-electrophoresis by itself cannot be taken as sufficient to identify a particular protein, but must be coupled with some of the other analytical methods. Some proteins, as isolated from cells, contain post-translational modifications such as glycosylations, phosphorylations and acetylations, which may affect their electrophoretic mobility such that it differs from the unmodified product of *in vitro* translation. If the protein of interest is only a minor component of the total products then it may not be possible to detect it at all when the unfractionated products are analysed on polyacrylamide gels.

A commonly used method for identification and detection of particular polypeptides is immune precipitation of the translation products. This method has been used to identify the products of translation of a large number of mRNAs, e.g. those for placental lactogen (Hubert and Cedard, 1975), ovalbumin (Rhoads *et al*, 1971) and 3,4-dihydroxyphenylalanine decarboxylase (Fragonlis and Sekeris, 1975). This method of identification is often coupled with electrophoresis on polyacrylamide gels and is especially useful if the protein

is only a minor component of the total protein synthesised.

Comparison of the tryptic, or chymotryptic, peptides of an *in vitro*-synthesised polypeptide with those of the standard protein can establish whether or not the two are related. The peptides can be separated by either two dimensional electrophoretic and chromatographic methods (Ingram, 1963) or by chromatography on columns of ion-exchange resin (Rhoads *et al* 1971). Usually it is necessary to isolate the required polypeptide from the *in vitro* products by immune precipitation and/or electrophoresis on polyacrylamide gels. Maizels (1971) describes a method for isolating polypeptides from polyacrylamide gels and preparing them for tryptic peptide analysis. Peptide mapping of *in vitro* products has been widely used as an identification procedure (e.g. Laycock and Hunt, 1969; Jacobs-Lorena *et al*, 1972; Taylor and Schimke, 1973).

In certain circumstances methods other than these can be used to identify a protein. In the case of interferon, for example, there is no monospecific antibody available, nor has the protein been purified. Thus the only way to identify this protein as a product of *in vitro* protein synthesis is to assay its biological activity (Reynolds *et al*, 1975; Pestka *et al*, 1975). Biological assays have also been used for other polypeptides, e.g. *Petunia hybrida* proteins (van der Donk, 1975), TMV coat protein (Roberts *et al*, 1974) and rat brain tubulin and actin (Gozes *et al*, 1975). The unusual solubility properties of zeins, the storage proteins of maize, have been used to identify them as the products of cell-free translation (Larkins and Dalby, 1975). However, all these are exceptional cases, and most polypeptides are identified and characterised by the other methods described.

Thus the positive identification of a particular protein amongst the total products of an *in vitro* protein-synthesis assay requires the combination of more than one diagnostic test. Once it has been established that a polypeptide is related to the protein of interest, then a single parameter, such as its mobility on polyacrylamide gels, can be used for routine identification.

(ii) Isolation of mRNA.

Once a method for detecting the polypeptide of interest has been devised, it is possible to use *in vitro* protein-synthesising systems to look for the presence of the mRNA for this polypeptide. Thus, isolation of mRNA can theoretically be accomplished by applying various RNA-fractionation techniques.

All the mRNAs which have so far been purified have been isolated from tissue in which they constituted the most abundant mRNA species. In this situation it is possible to isolate pure mRNA by simple physical sizing of the RNA, plus chromatography on oligo-dT cellulose to remove any contaminating rRNA. Several examples are described.

Globin mRNA was isolated from mouse reticulocytes by fractionation of the RNA on a sucrose gradient in a zonal rotor; the RNA which had a sedimentation coefficient of 9S was the globin mRNA (Williamson *et al*, 1971). The RNA which sedimented between 10 and 14S on a sucrose gradient fractionation of mouse myeloma RNA was chromatographed on oligo-dT cellulose. The material which bound to the column was further analysed by digestion with T_1-nuclease. It was concluded that about 50% of the RNA so obtained was the mRNA for the immunoglobulin light chain (Brownlee *et al*. 1973). This shows the purification

which can be achieved, using only relatively crude fractionation procedures, when dealing with an abundant mRNA.

Lens crystallins are very abundant proteins in calf-lens, and the mRNAs coding for them are also abundant; about 70% of protein synthesis in calf-lens is due to translation of δ-crystallin mRNA (Zelenka and Piatigorsky, 1974). Berns et al (1972) isolated the mRNA for αA_2-crystallin by fractionating calf-lens polysomal RNA on a sucrose gradient and removing the 14S RNA. Zelenka and Piatigorsky (1974) isolated δ-crystallin mRNA by oligo-dT cellulose chromatography of polysomal RNA. This simple procedure results in mRNA which is about 80% pure, as judged by protein synthesis assays.

Ovalbumin accounts for about 60% of the protein synthesised by chick oviduct cells. The ovalbumin mRNA has been purified by a combination of sucrose gradients, chromatography on Sepharose-6B and binding to Millipore filters (Rosen et al, 1975).

These procedures described in the above examples are only applicable to the special circumstances found on the starting material, i.e. a high proportion of the mRNA being specific for just one protein. To isolate an mRNA for which this is not the case requires a different approach.

Immune precipitation of polysomes is a technique whereby the polysomes which are synthesising a particular protein can be isolated by means of antibodies specific for that protein. This technique has been extensively described for polysomes synthesising ovalbumin (Palmiter et al, 1972; Shapiro et al, 1974) and immunoglobulin light chain (Schechter, 1974). This technique has also been applied to the isolation of vitellogenin mRNA (Wetekam et al, 1975). There are some situations

where this technique is the only way of separating two mRNAs; the α_s and β-caseins of ewe mammary gland are very similar in size, and the mRNAs are probably also very similar (Gaye and Houdebine, 1975).

The technique of immune precipitation has only recently been applied successfully to the isolation of specific polysomes. Holmes et al (1971) concluded that it was impossible to use the technique, because of non-specific interaction between the Fc portion of the immunoglobulin molecule and the ribosomes. Removal of the Fc portion gave an improvement (Delovitch et al, 1972). However, the use of antigen-affinity matrices has allowed the preparation of immunoglobulin fractions which are absolutely specific for a particular antigen (Palmiter et al, 1972; Schimke et al, 1974; Shapiro et al, 1974). With such highly specific preparations it has not proved necessary to remove the Fc part of the immunoglobulin molecule.

6. AIMS AND APPROACH.

A. Aims.

The principal aim of this project is to isolate the mRNA coding for the small subunit of Fraction I protein from pea leaves. The isolation of this mRNA is a necessary first step for the study of the ways in which the synthesis of nuclear-coded chloroplast proteins is regulated. The small subunit of Fraction I protein is an obvious protein to study in this respect. A great deal is known about Fraction I protein itself, and about its subunits, and about its role in the functioning of the chloroplast (section I3). The large subunit is synthesised in the chloroplast, and there appears to be some co-ordination between the chloroplast and the cytoplasm in the

synthesis of these subunits. Moreover, other workers in this laboratory are attempting to isolate the mRNA for the large subunit (Hartley et al, 1975), and so it might be ultimately possible to study the co-ordinate synthesis of these two polypeptides.

Radioactive complementary DNA (cDNA) to particular mRNAs has been synthesised by use of reverse transcriptase from avian myeloblastosis virus (AMV) and oligo-dT as a primer (Ross et al 1972; Verma et al, 1972; Diggelman et al, 1973). By studying the hybridization of this DNA with RNA isolated from whole cells or polysomes it is possible to measure changes in the amount of one particular mRNA (Orkin et al, 1975).

Thus the isolation of the mRNA for the small subunit of Fraction I protein would enable a cDNA probe to be synthesised. With such a probe, it would be possible to follow changes in the level of this mRNA during chloroplast development. It would also be possible to measure the number of genes for this protein present in the nuclear DNA.

B. Approach.

The first decision to be made when attempting to isolate a specific mRNA is which cell-free system is to be used to assay for messenger activity. The small subunit has a molecular weight of about 14,000 (Haslett et al, 1975) which is very close to that of globin from rabbit reticulocytes. Thus the rabbit reticulocyte system would be inconvenient to use because of the high endogenous synthesis of globin which would mask any synthesis of the small subunit. Immunological methods might detect the small subunit but any minor non-specific interaction with the globin could still present problems. For

this reason it was decided to use the wheat-germ system for *in vitro* protein synthesis, as described by Roberts and Paterson (1973). The cell-free system will be extensively characterised to ensure that it is faithfully translating the added mRNA.

The products of *in vitro* translation will be characterised by electrophoresis on SDS-polyacrylamide gels, initially, and then the presence of the small subunit in these products will be shown by peptide mapping and immune precipitation.

As described in section I5Dii, it may be necessary to use immune precipitation of polysomes as a preliminary step in isolating the mRNA for the small subunit. To do this will require the preparation of antibodies specific for the small subunit of Fraction Iprotein from pea leaves. Whether or not the immune precipitation technique is successful, it will also be necessary to characterise the mRNA with respect to poly(A)-content and to size. Thus it is envisaged that the mRNA for the small subunit of Fraction I protein can be isolated by the following procedures:-

1) the small subunit-synthesising polysomes will be isolated by immune precipitation using antibodies specific for the small subunit;

2) the RNA will be isolated from these polysomes and then chromatographed on oligo-dT cellulose to isolate the poly(A)-containing RNA which will be assumed to be equivalent to mRNA;

3) the poly(A)-containing RNA will then be further fractionated by sedimentation through sucrose gradients or by electrophoresis on polyacrylamide gels; this should result in pure mRNA.

SECTION II - MATERIALS AND METHODS

1. MATERIALS

A. Plant material.

Pea seeds (*Pisum sativum* var. Feltham First) were obtained from S.Dobie, 11, Grosvenor St. Chester.

The unimbibed seeds were sown into a tray half-full of damp compost (J. Arthur Bowers compost from Lindsay and Kestevens Ltd., Saxilby, Lincoln). They were then covered with more compost. The trays kept in a well-ventilated room at 20°C under a 12 hour photoperiod of 3,000 lux white light, provided by Philips "Warmwhite" fluorescent tubes. The trays were watered with tap water. The apices obtained from such plants were fully green and were used at the ages given in the text; day zero was the day of sowing.

For the growth of etiolated tissue a different procedure was used. The seeds were sown over a thick layer of vermiculite (Micafil, Dupre Vermiculite, Hertford, Herts., U.K.) which was saturated with tap water. A thin layer of water-saturated vermiculite was placed over the seeds. The trays were then placed in light-tight cupboards or boxes for as long as required. Again day zero was the day of sowing.

In greening experiments the etiolated seedlings were taken out of the dark, watered and then placed under continuous illumination of approximately 10,000 lux white light.

B. Chemicals, biochemicals and radiochemicals.

All materials used were of Analar grade, the source of specific chemicals and biochemicals is given below.

Sigma(London) Chemical Co. Ltd., London. U.K.

Proteins:- aldolase, bovine serum albumin (BSA) fraction V, carbonic anhydrase, chymotrypsinogen, cytochrome c, fumarase,

β-galactosidase, lactate dehydrogenase, β-lactoglobulin, myoglobin, ovalbumin, phosphorylase.

Chemicals:- adenosine triphosphate (tris salt) (ATP), S-adenosyl homocysteine (SAH), S-adenosyl methionine (SAM), blue dextran, chloramphenicol, Coomassie Brilliant Blue R, creatine phosphate, cycloheximide, diethyl pyrocarbonate, Dowex 1x8-100, heparin (sodium salt), N-2-(hydroxyethylpiperazine-N'-yl) ethane sulphonic acid (HEPES), sodium isoascorbate, phenyl methyl sulphonyl fluoride (PMSF), spermidine hydrochloride, spermine hydrochloride, N-tris(hydroxymethyl)methyl glycine (TRICINE), 2-amino-2-hydroxymethyl propane-1,3-diol (tris, TRIZMA base).

BDH Chemicals Ltd., Dorset, U.K.

acrylamide, bromophenol blue, ethylene diamine tetraacetic acid (EDTA), Nonidet-P40, polyethylene glycol, average molecular weight 6,000 (PEG6000), 2,5-diphenyl oxazole (PPO), 1,4-bis-(5-phenyloxazole-2-yl)benzene (POPOP), sodium dodecyl sulphate (SDS).

Koch-Light Ltd., Bucks., U.K.

N,N,N,N'-tetramethylene diamine (TEMED)

Hopkins and Williams Ltd, Essex, U.K.

cyanogen bromide

Eastman Kodak, Rochester, New York, U.S.A.

N,N'-methylene bisacrylamide

Pharmacia (Great Britain) Ltd, Paramount House, London, U.K.

All Sephadex and Sepharose gel chromatography media; also poly(U)-sepharose and Ficol.

L.K.B., Bromma, Sweden.

ampholines (ranges pH 3-10 and pH 5-8)

Collaborative Research, Waltham, Massachusetts, U.S.A.

Oligo-dT cellulose, type T2.

Difco Laboratories, Detroit, Michigan, U.S.A.

Nobel agar, Freunds adjuvant (complete and incomplete)

Whatman Ltd., Kent, U.K.

DEAE-cellulose, DE52.

Biorad, Kent, U.K.

CM-cellulose

Calbiochem, Lucerne, Switzerland.

TPCK-trypsin

Radiochemical Centre, Amersham, Bucks., U.K.

L-[^{35}S]-methionine (400-1000 Ci/mmole)

L-[^{3}H]-lysine (25 Ci/mmole)

[^{125}I]-iodine (carrier free in NaOH solution)

New England Nuclear, Dreieich, West Germany.

L-[^{3}H]-arginine (23 Ci/mmole)

2. METHODS

A. Isolation of plastids.

(i) Differential centrifugation.

This was the method used by Blair and Ellis (1973) to isolate chloroplasts active in protein synthesis. All solutions and equipment were sterile, where possible, to reduce contamination by micro-organisms.

Apices (15g.,fresh weight) from pea seedlings (10-12 day old) were homogenized for 6 seconds in a Polytron homogenizer (Northern Media Supplies, Hull, U.K.) at setting 7 in 100ml. of semi-frozen, sterile sucrose isolated medium (SIM) (0.35M sucrose, 25mM HEPES-NaOH, pH 7.6, 2mM EDTA, 2mM sodium isoascorbate). The homogenate was strained through eight layers of muslin and centrifuged at 2,500g for one minute at 0°C. The supernatant solution was poured off and the inside of the tube was quickly wiped with a tissue to remove most of the remaining liquid. The subsequent treatment of the chloroplast pellet varied depending on requirements. (see sections II2Aii and II2K).

(ii) Isopycnic centrifugation.

The chloroplast preparations obtained by the previous method (II2Ai)are typically 40-50% intact as judged by the criterion of refractility in the phase-contrast microscope (Kahn and von Wettstein, 1961). The density-gradient method developed in Price's laboratory, gives preparations which are about 80% intact, and, moreover, are active in photosynthesis (Morgenthaler et al, 1974) and protein synthesis (Morgenthaler and Mendiola-Morgenthaler, 1976). This method employs gradients of silica sol, which do not cause any osmotic damage

to the chloroplasts as do high concentrations of sucrose. However, it is crucial to remove any impurities from the silica sol (Ludox AM from E. I. du Pont de Nemours and Co.); the purification is described by Morgenthaler et al (1974). The purified Ludox used in these experiments was a generous gift from Dr. Carl Price. To improve the separation of intact from broken chloroplasts, the Ludox was "fortified" by the addition of polyethylene glycol (PEG 6000) to 10% (w/v); this is the Ludox/PEG stock (Morgenthaler et al, 1975). Linear gradients (10-80% v/v) of Ludox/PEG in SIM were poured, using an MSE gradient maker, to a final volume of 14mls and were kept cool (10°C) but not cold. These gradients were made no more than 2 hours before they were required.

Pea apices (8g., fresh weight) were homogenised and a chloroplast pellet obtained as described in section II2Aii. This pellet was gently resuspended in 4mls of SIM by using a cotton wool bud to disperse the chloroplasts. Aliquots (1ml) of this suspension were then layered on top of each gradient. To obtain a preparation of lysed chloroplasts, 1ml of distilled water, followed by 3mls of SIM, was used to resuspend the chloroplasts; this lysed preparation was then treated as for the intact chloroplast suspension. It was found that centrifugation at 7,000 rpm for 15 minutes in the MSE 6x15 ml swing-out head ($8,600g_{ave}$) was sufficient to allow equilibrium to be reached.

The intact plastids could be seen as a green band approximately a third of the way down the tube; the lysed plastids stayed almost at the top of the gradient. The band of intact plastids occasionally appeared double, but this was not always observed.

The lysed and intact chloroplast bands were removed separately with a pasteur pipette, mixed with an equal volume of SIM and pelleted again by a 4000g centrifugation for 5 minutes. The plastid pellets so obtained were used in processing studies as described in section II2K.

B. Preparation of anti-serum specific for pea Fraction I protein.

(i) Isolation of pure Fraction I protein.

The protein was purified by the method routinely used in this laboratory (Blair and Ellis, 1973) which is a modification of a procedure devised by Kawashima and Wildman (1971). All operations were carried out at $4^{o}C$. The 2-mercaptoethanol present in various buffers was added just before use, to minimise losses due to evaporation and oxidation.

The top-most leaves (200g) of 14-day old pea seedlings were homogenised in 300mls of ice-cold buffer A (25mM tris-HCl, 50mM NaCl, 1mM $MgCl_2$, 0.5mM EDTA, 40mM 2-mercaptoethanol, pH 7.4) with PMSF added to a final concentration of 2mM, in an atomix blender at top speed for 1 minute. The PMSF is a potent inhibitor of serine proteases and has been shown to prevent proteolytic attack on Fraction I protein in Phaseolus extracts (Gray and Kekwick, 1974a). The homogenate was strained through eight layers of sterile muslin. A high speed supernatant was obtained by centrifugation at $10,000g_{ave}$ for 10 minutes followed by a further centrifugation at $30,000g_{ave}$ for 60 minutes. The yellowish liquid was then passed through a column (6.6x50cm) of coarse grade Sephadex G-25 to remove low molecular weight contaminants. The column was washed with buffer B (25mM tris-HCl, 50mM NaCl, 0.5mM EDTA and 10mM

2-mercaptoethanol, pH 7.4). The protein-containing material could be seen as a fast-moving brown band. The brown material was collected in the void volume of the column and a 35-45% ammonium sulphate cut taken as follows. Neutral, saturated ammonium sulphate was added, with stirring for 30 minutes and the precipitate collected by centrifugation at 10,000g_{ave} for 10 minutes. The material precipitated by increasing the ammonium sulphate concentration from 35-45% saturation was resuspended in as small a volume as possible of buffer C (50mM tris-HCl, 0.5mM EDTA and 10mM 2-mercaptoethanol, pH 8.0). This suspension was dialysed overnight against 1 litre of buffer C.

The protein was absorbed onto a column (1.5x15cm) of DEAE-52 cellulose, previously equilibrated with buffer C. The column was washed with this buffer until no more 280nm absorbing material could be eluted. Buffer C was then made 100mM in sodium chloride, by addition of the solid salt, and the solution used to wash the column. The protein-containing portion of the eluate, as determined by E_{280} measurment, was collected; the column retained a large amount of yellow material. The protein was precipitated by addition of ammonium sulphate to 50% saturation and pelleted at 10,000g_{ave} for 10 minutes. This pellet was resuspended in 5ml buffer B and applied to a Sephadex G-200 column (2.5x90cm). Elution was by buffer B at a rate of 2.5ml/h and 2ml fractions were collected. Those fractions which had a E_{280}/E_{260} ratio greater than 1.8 were pooled. The Fraction I protein was precipitated by 50% saturated ammonium sulphate and the precipitate collected as before.

For storage as native Fraction I protein the pellet was resuspended in 5ml of PBS (phosphate buffered saline) and

dialysed against 1 litre of the same. The dialysed solution was then stored as a freeze-dried powder at 4°C.

For storage in its dissociated form or as a prelude to separation of its subunits (section II2Bii), the Fraction I protein was resuspended in the appropriate SDS-containing buffer and heated in a boiling water-bath for 2 minutes. This boiled preparation was then stored at -20°C.

(ii) Separation of Fraction I protein into its subunits.

The native Fraction I protein obtained in section II2Bi was resuspended in SDS-buffer (50mM tris-HCl, 0.1mM EDTA, 0.5% w/v SDS, 10mM 2-mercaptoethanol, pH 8.6) and heated to 90°C for 2 minutes.

The subunits were isolated by chromatography of this heated material on a column (1.5cm x 90cm) of Sephadex G-100 equilibrated with SDS-buffer. The column was pumped at 25ml/h and fractions (1ml) were collected for E_{280} measurements. If no more than 30mg. of protein were loaded onto the column, the large and small subunits were well separated.

Before the isolated subunits were used to raise anti-serum in rabbits, the SDS was removed (Gray and Kekwick, 1974a). This was done by ion-exchange chromatography as described by Weber and Kuter (1971); the SDS is replaced by urea which can subsequently be dialysed away.

Dowex 1 x 8-100 anionic-exchange resin was washed with urea buffer (50mM tris-HCl, 6M urea, 0.1M EDTA, 10mM 2-mercaptoethanol,pH 8.6), and the fines removed. The resin was then poured into the column (1.5x15cm). The column was washed with 100mls of distilled water, 100mls of 1N HCl and then distilled water until the eluate was no longer acidic; 100mls

of 1N NaOH was poured through the column. This procedure was repeated, and the column then washed with distilled water followed by 100mls of 1N HCl; the column was finally equilibrated with the urea-containing buffer.

The samples of isolated subunits were made 6M urea by addition of the solid. Aliquots (10mg protein) of the subunits were passed through the column and 1ml. fractions collected for E_{280} measurement. The column was regenerated as described above, after each use. The protein-containing fractions from the column were pooled and dialysed against two changes of 1 litre of 50mM tris-HCl, 0.1mM EDTA pH 8.6. The protein concentration was determined as described in section II2P.

(iii) Isolation of rabbit immunoglobulin specific for Fraction I protein and its subunits.

a) Injection of protein into rabbits.

The preparations of Fraction I subunits obtained in II2Bii were concentrated by dialysis against solid PEG 6000 until they reached a protein concentration of 2 mg/ml. The lyophilised native Fraction I protein was dissolved in sterile distilled water at a concentration of 2 mg/ml. These protein solutions were prepared for injection by emulsification with Freunds complete adjuvant; 1ml. adjuvant was mixed with 1ml of protein solution.

The rabbits used were male New Zealand Whites obtained from Buxted Olac rabbits, Great Totease Farm, Buxted, Sussex. The rabbits were maintained in a temperature-controlled room on a diet of pellets and water ad libidum. Prior to the injection of antigen in complete adjuvant, the rabbits were

of 1N NaOH was poured through the column. This procedure was repeated, and the column then washed with distilled water followed by 100mls of 1N HCl; the column was finally equilibrated with the urea-containing buffer.

The samples of isolated subunits were made 6M urea by addition of the solid. Aliquots (10mg protein) of the subunits were passed through the column and 1ml. fractions collected for E_{280} measurement. The column was regenerated as described above, after each use. The protein-containing fractions from the column were pooled and dialysed against two changes of 1 litre of 50mM tris-HCl, 0.1mM EDTA pH 8.6. The protein concentration was determined as described in section II2P.

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bled twice to obtain the pre-immune serum. All bleeding was done from the ear by cutting one of the veins; each bleed gave, on average, 10-12mls of serum.

After the pre-immune serum had been obtained, each rabbit was given 1mg of one of the antigens as a series of subcutaneous injections between the shoulders; each site of injection received no more than 0.2mls of the antigen/adjuvant emulsion. Each antigen was injected into two rabbits to allow for any variation in response between individuals. Three weeks after the primary immunisation, each rabbit was given a further 1mg of antigen as before, except that the antigen had been emulsified with incomplete adjuvant. Seven days later the rabbits were bled to obtain the first immune serum. Each rabbit was bled regularly over the next two months, sometimes as often as every other day, to obtain large amounts of serum.

The blood samples obtained by bleeding the rabbits were collected in sterile tubes, and were left to clot at room-temperature for 1 hour, followed by overnight storage at 4°C. The serum was removed using a pasteur pipette.

The immunological reactivity of the sera was determined by Ouchterlony double-diffusion assay (Ouchterlony, 1968). Gels of Difco Agar Noble (1% w/v) in 0.9% NaCl were set in 5cm petri dishes, and wells 0.5cm in diameter cut out with a template.There was one central well with six more wells equally spaced around it. Usually the serum was placed in the central well and various antigens in the surrounding wells but occasionally the converse arrangement was used. Each well could take a maximum of 80µl. Once the plates had been loaded they were left in the dark at room-temperature in a damp atmosphere. Usually the precipitin lines could be observed

after 24-48 hours.

b) Isolation of IgG fraction from serum.

It was intended to use the antibody preparations in an attempt to isolate polysomes synthesising the small subunit of Fraction I protein. It was thus necessary to isolate a pure immunoglobulin G (IgG) fraction from the sera. The goat anti-rabbit serum from Miles-Yeda was purified in the same way. The procedure followed was essentially that of Schimke et al (1974).

The serum was diluted with an equal volume of distilled water, to reduce the protein concentration. Saturated ammonium sulphate (adusted to pH7) was added to 50% saturation at 4°C and left to stir for 30 minutes. The precipitated protein was collected by centrifugation at 10,000g for 30 minutes, and the pellet washed once in 50% saturated ammonium sulphate. The protein was resuspended in PBS and the procedure repeated. This procedure removes most of the serum albumin.

At this stage in the purification the protein is predominantly IgG but still contains enzymic activities, particularly RNAase. Never the less this relatively crude preparation was used for some purposes e.g. synthesis of IgG-Sepharose 4B affinity columns (section II2Ci).

The IgG preparation from the immune sera was dialysed against two changes of one litre of 10mM phosphate, 15mM NaCl pH 7.2. The preparation was then fractionated on a Fraction I Sepharose 4B column as described in section II2Ciia. The fractions which eluted at pH 4.8 were precipitated by 50% saturated ammonium sulphate, and the precipitates resuspended in 10mM phosphate, 15mM NaCl pH 7.2.

The ammonium sulphate was removed by dialysis against the resuspension buffer.

c) Removal of RNAase.

Before any of the IgG preparations (anti-Fraction I, pre-immune or goat anti-rabbit) could be used in attempts to specifically precipitate the small subunit-synthesising polysomes (section II2Fiii) the RNAase activity had to be removed by a column procedure (Shapiro et al, 1974).

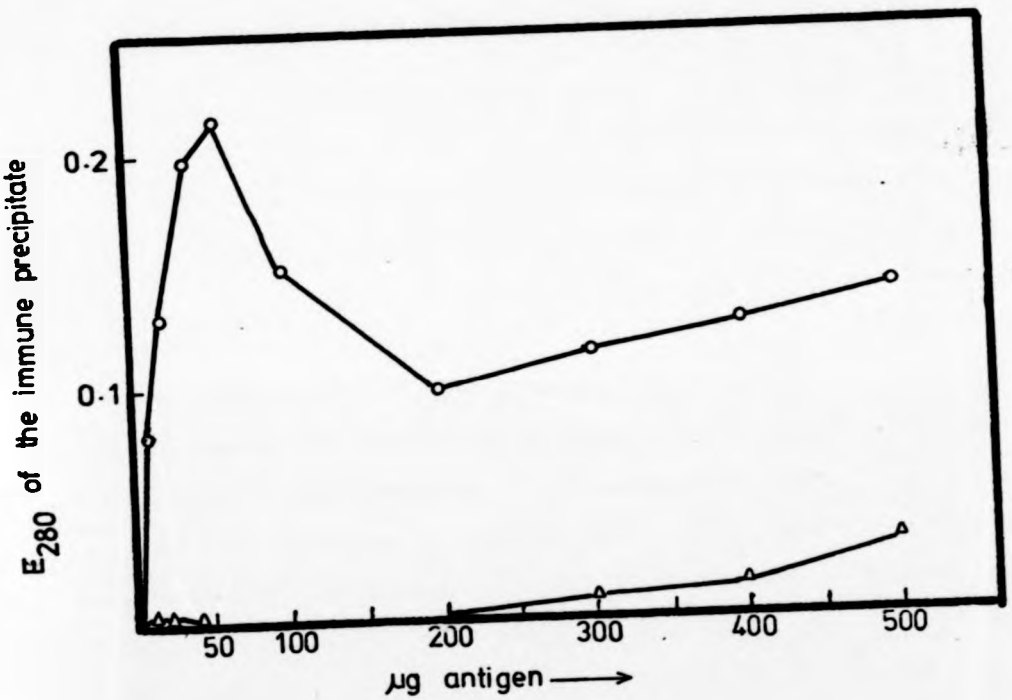
The column (1.5cm i.d.) consisted of a 10cm bed of CM cellulose with a 10cm bed of DEAE-cellulose on top. Both celluloses had been equilibrated with 10mM phosphate, 15mM NaCl pH 7.2. No more than 300mg of protein were loaded onto the column at a time. The column was washed with the buffer and 7ml fractions collected for E_{280} measurements. The fractions containing the protein which eluted in the void volume were pooled, and the protein precipitated by 50% ammonium sulphate. The ammonium sulphate was removed by dialysis against 50mM tris-HCl, pH 7.4, 150mM NaCl, 10mM magnesium acetate. The IgG preparations were titrated and tested for RNAase activity as described in section II2Bivb. They were then stored as 100µl aliquots at -20°C.

(iv) Characterisation of immunoglobulin fractions.

a) Titration against antigen.

The procedure used was based on that of Hudson and Hay (1976) in that the ability of a constant amount of IgG to form a precipitate with various amounts of antigen was measured.

The reactions were carried out in a final volume of 600µl of PBS with NP40 (1%, w/v). These reaction mixtures contained around 1mg of protein if the IgG fraction was crude, or about


E_{280} of the immune precipitate
0·2
0·1
50
100
200
300
400
500
µg antigen ⟶

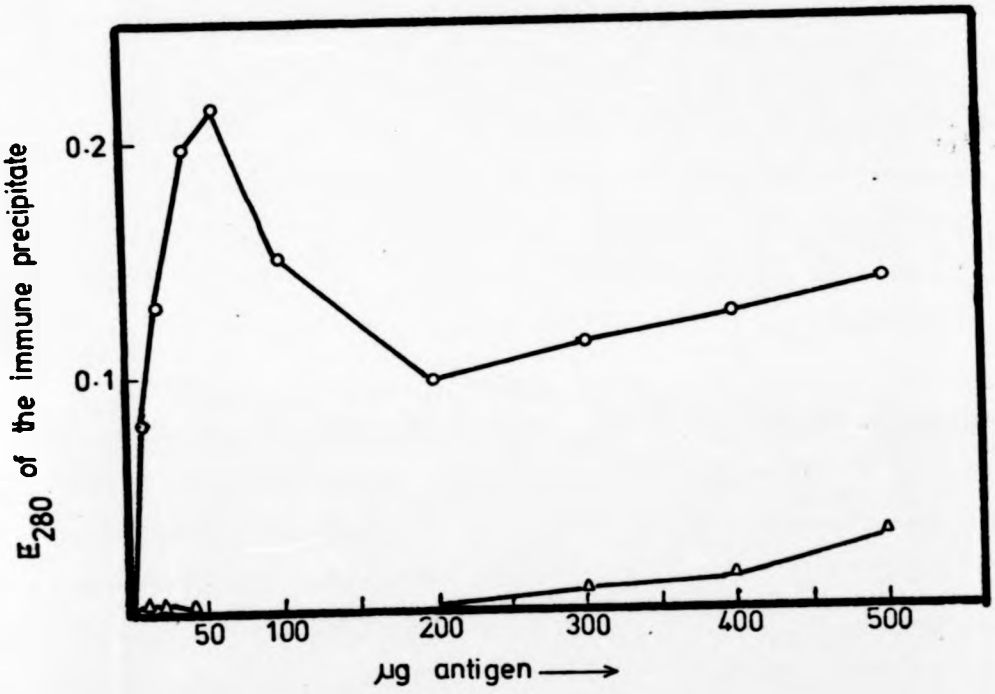

E_{280} of the immune precipitate
0·2
0·1
50
100
200
300
400
500
µg antigen ⟶

Figure 1. Titration of rabbit anti-Fraction I protein immunoglobulin against pure pea Fraction I protein.

Increasing amounts of pure pea Fraction I protein were incubated with either anti-Fraction I or pre-immune immunoglobulin (660µg) as described in section II2Biva. The immune precipitate was dissolved in 350µl of 0.1N NaOH and the E_{280} measured.

o——o anti-Fraction I immunoglobulin

Δ——Δ pre-immune immunoglobulin

100µg protein with more purified preparations. Pure Fraction I protein was used in the range 10-500µg per assay. Whenever the anti-Fraction I protein IgG preparations were being titrated, a parallel series of assays using IgG from pre-immune serum was used to control for non-specific effects. The reaction mixtures containing both antigen and antibody were incubated for 1 hour at 37°C and then left for at least 16 hours at 4°C for the precipitate to form. Sometimes the reactions were left for up to 36 hours to ensure complete precipitation.

The antibody-antigen complexes were pelleted by a short spin in a bench centrifuge, washed twice with 500µl of PBS+NP40 and finally dissolved in 350µl of 0.1 N NaOH. The E_{280} of the solution was measured and the results obtained are shown in Fig1. From this Figure, it is possible to obtain a value for the amount of antigen maximally precipitated by a given amount of IgG protein. The ratio of IgG protein to antigen in such a precipitate was used as a measure of the "strength" of the IgG preparation.

From the E_{280} of the immune precipitate, it is possible to calculate the amount of IgG present by assuming that all the added antigen has been precipitated, and by using known extinction coefficients for Fraction I protein and rabbit IgG in 0.1 N NaOH. It was determined that 1mg/ml solutions, in 0.1 N NaOH, of Fraction I protein and rabbit IgG give 1.52 and 0.92 E_{280} units respectively. These determinations allowed quantitation of the amount of anti-Fraction I antibody.

The goat anti-rabbit antibody was titrated in a similar way except that [^{125}I]-labelled rabbit IgG (section II2M) was used as antigen. The amount of antigen in the pellet and

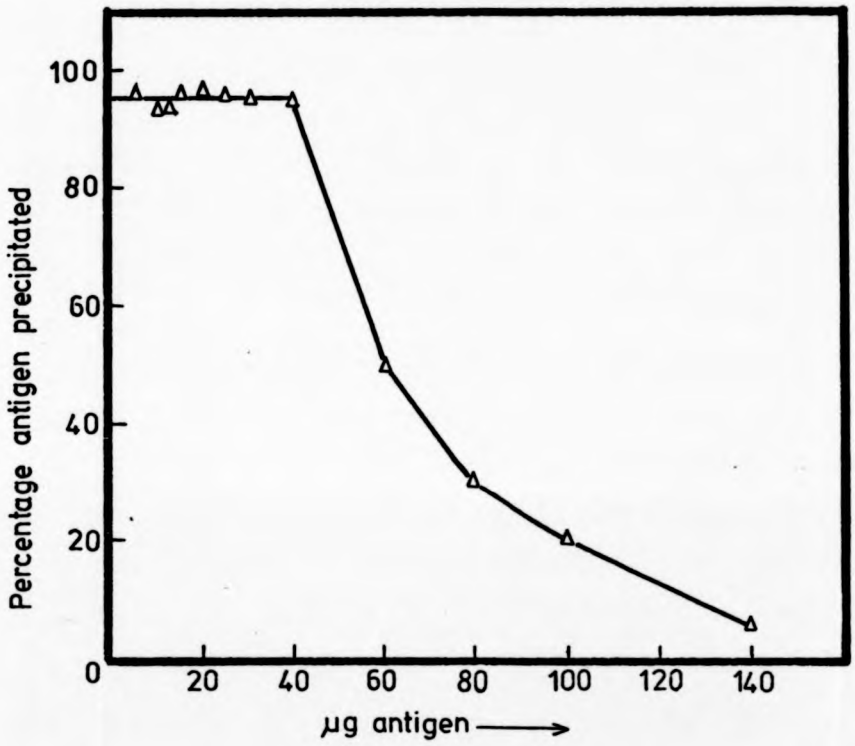

Percentage antigen precipitated
100
80
60
40
20
0
20
40
60
80
100
120
140
µg antigen

Figure 2. Titration of goat anti-rabbit immunoglobulin against [^{125}I]-labelled rabbit immunoglobulin.

Goat anti-rabbit immunoglobulin (1mg) was incubated with increasing amounts of [^{125}I]-labelled rabbit immunoglobulin (2550 cpm/µg) in a final volume of 500µl as described in section II2Biva. The radioactivity present in both the pellet and supernatant was measured in an LKB Ultrogamma counter and the percentage of added rabbit immunoglobulin precipitated was calculated.

supernatant solution could easily be determined by counting the two fractions in an LKB Ultrogamma γ-radiation counter. The results are shown in Figure 2. The equivalence point can be determined as the largest amount of antigen added which will all be completely precipitated by a given amount of antibody.

b) Assay of RNAase activity in IgG fractions.

In order to attempt the specific immune precipitation of polysome preparations as a preliminary step in a messenger RNA isolation procedure, it is essential that the antibodies used contain no active nuclease, which could destroy the messenger activity.

Pea polysomes were isolated as described in section II2Fi and resuspended in 20mM tris-HCl pH 8.5, 20mM KCl, 10mM $MgCl_2$ at a concentration of 10 E_{260} units/ml. Aliquots (100µl) of the polysome suspension were mixed with an equal volume of buffer containing 50µg of various IgG preparations. The mixtures were incubated at 37°C for 90 minutes. The entire sample was then loaded onto a gradient of sucrose and analysed as described in section II2Fii.

In control tubes the polysomes showed a particular size distribution. It was taken as diagnostic of RNAase activity if this size distribution shifted towards smaller polysomes. It was not possible to accurately quantitate the nuclease activity by this method. However, this assay was useful to detect the presence or absence of nuclease activity.

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C. Preparation and use of affinity columns.

(i) Preparation

The coupling of specific substrates to an inert support has been used increasingly in recent years in many fields of biochemistry. In particular, procedures for coupling proteins to Sepharose 4B beads have been worked out (Cuatracasas and Anfinsen, 1971).

The Sepharose must first be activated by reaction with cyanogen bromide. To introduce the maximum number of reactive sites onto the Sepharose beads, a high ratio of cyanogen bromide to Sepharose (300 mg/ml of gel) must be used. It is important that, during the activation reaction, the temperature does not rise above 20°C and the pH of the reaction mixture is maintained around 11. Therefore, a thermometer and pH electrode must be accommodated in the reaction vessel. It is advisable to carry out the reaction in a high-speed fume-hood because of the noxious nature of cyanogen bromide. It was not possible to store the activated Sepharose once made and so only sufficient was prepared for immediate coupling with protein; 1ml of gel was used for every 8mg of protein to be coupled.

The required volume of Sepharose 4B was diluted with an equal volume of water and transferred to a beaker on ice. For large-scale reactions (>10ml Sepharose) 6N NaOH was used to keep the pH at 11; for smaller reactions 2N NaOH was used. Two drops of the NaOH solution were added to raise the pH and the solid cyanogen bromide added at once. The reaction took about 20 minutes for completion, and during this time NaOH and ice were added to maintain the optimum conditions. The reaction was judged complete when the pH did not change and all the cyanogen bromide had dissolved. The reaction mixture

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was then quickly poured onto a Whatman No.1 paper disc in a Buchner funnel and washed with 20 volumes of 0.2M sodium citrate pH5.5. The Sepharose was allowed to drain slightly and was then transferred to a suitable reaction vessel. The protein was added at a concentration of 8 mg/ml in citrate buffer and left to react at 4°C overnight. Vigorous agitation must be avoided, and so the mixture was placed on a slow roller.

The reacted Sepharose was poured into a suitable column and washed with citrate buffer. For small volumes, Pasteur pipettes plugged with glass wool were used. The amount of protein present in the wash from the column was measured as described in section II2O; usually >80% of the protein had bound. Any activated sites which had not reacted with protein were blocked by reaction with 0.5M glycine in citrate buffer overnight at room-temperature.

(ii) Uses

a) To isolate IgG specific for Fraction I protein

The IgG-containing fraction obtained by ammonium sulphate precipitation of serum from immune rabbits (section II2Biiib) was chromatographed on a column of Fraction I protein-Sepharose 4B. The column consisted of 200mg of native Fraction I protein bound to Sepharose 4B and the IgG fraction was titrated against native Fraction I protein (section II2Biva) to ensure that the column was not over-loaded. The chromatography was performed as described by Shapiro *et al* (1974).

All the IgG fraction (2 g) was loaded onto the column in 10mM phosphate, 15mM NaCl pH7.2 and the column washed with the same buffer. Fractions (7ml) were collected and monitored

for E_{280}. When the column eluate had dropped below 0.1 E_{280} units/ml, the column was washed with 100mM sodium acetate, 1M NaCl pH4.8. When the E_{280} of the eluate again dropped below 0.1, the column was washed with 100mM Na_2CO_3, 1M NaCl pH7.6. This cycle was repeated once. Finally the column was washed with 0.1M glycine-HCl, pH 2.8.

The fractions representing the column wash, pH4.8-eluted and pH2.8-eluted protein species were pooled and made 50% in ammonium sulphate. The precipitates were taken up in PBS and the ammonium sulphate removed by dialysis against PBS.

b) To assay products of <u>in vitro</u> protein synthesis.

The columns, which had been made by binding the ammonium sulphate cut of serum to Sepharose 4B (section II2Biiib) were used to determine whether or not any of the products of <u>in vitro</u> protein synthesis (section II2Jii) contained any peptides immunologically related to Fraction I protein.

The <u>in vitro</u>-synthesised products were diluted 10-fold by the addition of 200µl of 50mM tris-HCl, pH 7.8, 0.2M NaCl and the unincorporated radioactivity removed by a small desalting column of Sephadex G-25 (coarse). A series of G-25 columns were set up in small Pasteur pipettes plugged with glass wool. The void volume of a representative column was determined using blue dextran (M.W. $2x10^6$). The samples were loaded onto the columns and eluted with 50mM tris-HCl, pH 7.8 0.2M NaCl; the void volume eluted from each column was collected. Each sample was split into two equal parts, one of which was chromatographed on the anti-Fraction I protein column, the other on the pre-immune column. Alternatively the entire sample was loaded onto the pre-immune column, and the material which did not bind was re-chromatographed on the

immune column. After loading onto the relevant column, the sample was washed through with 10 column volumes of 50mM tris-HCl, pH 7.8, 0.2M NaCl, followed by 10 volumes of 50mM tris-HCl, pH 7.8, 1M NaCl; finally any bound material was eluted by 50mM tris-HCl, pH 7.8, 0.2M NaCl and 8M urea. Fractions (0.1ml) were collected and 10µl aliquots assayed for radio-activity in 4ml of Triton-toluene scintillant (TTS).

To prepare the various bound or non-bound fractions for analysis on polyacrylamide gels (section II2Eic) it was necessary to first precipitate out the labelled proteins. The procedure of Clewley and Kennedy (1976) was followed . Ammonium bicarbonate was added to a final concentration of 100mM fcllowed by 9 volumes of acetone. This mixture was stored overnight at -20°C . The precipitate was pelleted at 4000g for 10 minutes and dried at 80°C in a vacuum oven. This heat-treatment caused the ammonium bicarbonate to sublime away. The dry sample was taken up in a suitable volume (500µl) of $1/10^{th}$ strength gel running buffer containing 1% SDS and heated at 95°C for 2 minutes. These samples were stored at -20°C before analysis on gels.

c) To isolate polysomes synthesising specific proteins.

The IgG preparations, which had been purified as described in section II2Biiib and c (except that they had not been chromatographed on the Fraction I protein-Sepharose column) were used to make affinity columns as described (section II2Ci).

Polysomes, isolated as described in section II2Fi, were resuspended in 50mM tris-HCl, pH 7.8, 0.2M NaCl, 5mM $MgCl_2$, 1% NP40 to a final concentration of 10 E_{260} units/ml. Aliquots of this suspension were chromatographed on both anti-Fraction I protein and pre-immune columns. The samples

were run into the columns, left at room-temperature for 30 minutes and then washed through with buffer. Any bound material was eluted with 50mM tris-HCl, pH7.8, 0.2M NaCl, 8M urea. Fractions (1.5ml) were collected and E_{260} monitored. RNA was obtained from any chosen fraction by phenol extraction as described in section II2Gi. All glassware was chromic acid washed, and solutions were either autoclaved or diethylpyrocarbonate (1% v/v)-treated to remove RNAase activity.

D. Preparation of heat-inactivated Staphylococcus aureus.

Staphylococcus aureus cells, which contain A-protein on their outer surfaces, have been shown to bind to the Fc region of IgG molecules (Jonsson and Kronvall, 1974). To be useful as an immune absorbent, the bacteria must be processed to inactivate them without denaturing the A-protein. This was done as described by Jonsson and Kronvall (1974); the preparation was carried out by Mrs C. J. Ellis. The bacterial preparation was stored at 4°C as a 10% (w/v) suspension in PBS plus 1% NP40.

E. Polyacrylamide gel electrophoresis systems.

(i) Protein gels.

a) Non-denaturing cylindrical gels.

These gels were cast in perspex tubes (10cm x 0.6cm) according to the method of Blair and Ellis (1973). The acrylamide:bisacrylamide ratio was 50:1 by weight and the gels contained a final concentration of 375mM tris-HCl, pH8.5. Stock solutions of 40% (w/v) acrylamide, 0.8% (w/v) bisacrylamide and 3.0M tris-HCl, pH8.5 were used. In a final volume of

40ml, the volume of acrylamide stock was adjusted to give the required gel concentration, and TEMED was added to a final concentration of 0.06% (v/v). The reaction was initiated by addition of ammonium persulphate (0.07% (w/v), final concentration). The gel mixture was poured into the tubes and overlaid with water to give a smooth top surface.

When polymerised, the gels were pre-electrophresed for 1 hour at 10mA/gel to remove any unreacted persulphate. The running buffer was 50mM tris-380mM glycine, 10mM 2-mercapto-ethanol, pH8.5. The top electrophoresis tank contained the cathode. The samples were loaded in 5mM tris-38mM glycine pH8.5 containing 5% (w/v) sucrose and 0.01% bromophenol blue. Electrophoresis was either at 5mA/gel until the dye had just reached the bottom of the gels in the case of 5% gels, or overnight at 3mA/gel for higher percentage gels.

The gels were removed from the tubes by means of air-pressure. The protein bands were visualised by staining overnight in 0.1% Coomassie Brilliant Blue R, 50% methanol, 7% acetic acid, followed by destaining in sucessive changes of 50% methanol, 7% acetic acid over 48 hours. The gels were scanned at 620nm in a Joyce-Loebl Chromoscan.

b) Denaturing cylindrical gels.

These gels were made exactly as described above (section II2Eia) except that the gel contained a final concentration of 0.1% (w/v) SDS and 3% (w/v) urea. The gels were pre-electro-phoresed at 10mA/gel for 1 hour; the running buffer was as above but containing 0.1% (w/v) SDS. The samples were made up in 10-fold diluted running buffer containing 1% (w/v) SDS and 1% (w/v) 2-mercaptoethanol, and were heated for 2 minutes in a

boiling water bath. Sucrose was added for ease of loading (10µl of 50% sucrose per 100µl of sample). Bromophenol Blue (0.01%,w/v) was used as a tracking dye.

The gels were electrophoresed at 5mA/gel for about 2 hours, when usually the bromophenol blue was just running off the bottom. The staining and scanning of the gels was as described as above.

c) SDS-containing polyacrylamide slab gels; discontinuous buffers.

This method used a discontinuous buffer system described by Laemmli (1970), and the polyacrylamide slab arrangement described by Studier (1973). The glass plates used were 20 x 20cm square and 0.3cm thick. In each pair of plates, one had a notch 15.8cm x 1.5cm cut out of one end and the other had a strip of perspex 20cm x 1.0cm x 0.15cm glued to each of two parallel edges. These perspex strips acted as formers for the vertical edges of the gel and were placed 0.4cm in from the extreme edges of the plate; they were also 0.4cm in from the bottom of the plate. The arrangement was made leak-proof by placing a length (60cm) of Esco rubber tubing (0.25cm external diameter) along the sides and bottom of the plates. The plates were clamped together with bulldog clips. When the gel had been cast, the rubber tubing could easily be removed by releasing the clamping pressure. This method of forming the sides of the gel was found more convenient than using neoprene rubber strips and agar as described by Studier (1973).

The resolving gel, 16cm long, contained either a single concentration or a linear concentration gradient of acrylamide. In the first case the monomer:crosslinker ratio

was 37.5:1 regardless of the final concentration; in gradient gels the low and high concentration stocks had monomer:cross-linker ratios of 37.5:1 and 160:1 respectively. The final volume of reaction mixture required to form each type of gel was 40mls. For high percentage gels the volume of acrylamide/bisacrylamide stock was adjusted to give the required final concentration (percentage,w/v); the final concentrations of the other gel constituents were 375mM tris-HCl pH8.8, 0.1% (w/v)SDS, 0.1% (v/v) TEMED and 0.1% (w/v) ammonium persulphate. The ammonium persulphate was added last, thoroughly mixed in, and the mixture poured into the plates. To get a smooth horizontal surface to the gel, 0.5mls of butan-2-ol (AR) were layered on top and left to polymerise.

In the case of gradient gels the low and high concentration reaction mixture contained the same final concentrations of tris-buffer and SDS; however the TEMED and ammonium persulphate concentrations were reduced so that the polymerisation did not occur during the time taken to form the gradient; the low concentration stock contained 0.04% of each, the high 0.03% of each. Both reaction mixtures also contained 0.2%(w/v) linear polyacrylamide; this helped to stabilise the gradient once formed. The gradient was formed by taking 20mls of each reaction mixture and pumping 1 volume from chamber containing the low concentration acrylamide into the chamber containing the high concentration acrylamide whilst at the same time, pumping 2 volumes from this chamber into the gel plates. The chamber where the high and low concentration solutions mixed was stirred vigorously. The gel normally required 10 minutes to pour and the top surface was overlaid as above with butan-2-ol.

was 37.5:1 regardless of the final concentration; in gradient gels the low and high concentration stocks had monomer:cross-linker ratios of 37.5:1 and 160:1 respectively. The final volume of reaction mixture required to form each type of gel was 40mls. For high percentage gels the volume of acrylamide/bisacrylamide stock was adjusted to give the required final concentration (percentage,w/v); the final concentrations of the other gel constituents were 375mM tris-HCl pH8.8, 0.1% (w/v)SDS, 0.1% (v/v) TEMED and 0.1% (w/v) ammonium persulphate. The ammonium persulphate was added last, thoroughly mixed in, and the mixture poured into the plates. To get a smooth horizontal surface to the gel, 0.5mls of butan-2-ol (AR) were layered on top and left to polymerise.

In the case of gradient gels the low and high concentration reaction mixture contained the same final concentrations of tris-buffer and SDS; however the TEMED and ammonium persulphate concentrations were reduced so that the polymerisation did not occur during the time taken to form the gradient; the low concentration stock contained 0.04% of each, the high 0.03% of each. Both reaction mixtures also contained 0.2%(w/v) linear polyacrylamide; this helped to stabilise the gradient once formed. The gradient was formed by taking 20mls of each reaction mixture and pumping 1 volume from chamber containing the low concentration acrylamide into the chamber containing the high concentration acrylamide whilst at the same time, pumping 2 volumes from this chamber into the gel plates. The chamber where the high and low concentration solutions mixed was stirred vigorously. The gel normally required 10 minutes to pour and the top surface was overlaid as above with butan-2-ol.

Each type of gel used the same stacking gel system, which contained 3% (w/v) acrylamide, 0.16% (w/v) bisacrylamide (or 4.5% acrylamide, 0.24% bisacrylamide) 125mM tris-HCl pH 7.5, 0.1% (w/v) SDS, 0.1% (v/v) TEMED and 0.1% (w/v) ammonium persulphate.

Immediately after pouring the stacking gel mixture into the plates a 12-toothed Teflon slot-former was inserted and left till polymerisation was complete. The slot-former consisted of a piece of Teflon (14cm x 3.8cm x 0.15cm) with teeth (1.8cm x 1cm) cut out of it; the teeth were 0.2cm apart, and were attached to a block of perspex (2cm x 14cm).

When the stacking gel had set, the tubing was removed from the edges of the plate and the apparatus transferred to the eletrophoresis tank. This tank was exactly as described by Studier (1973); however the seal between the gel plates and the upper buffer reservoir was made leak-proof by paraffin wax rather than by agar. The running buffer was 25mM tris-190mM glycine pH 8.5, 0.1% (w/v) SDS, 50mM 2-mercaptoethanol; this was used in both the anode and cathode compartments. Any air bubbles at the bottom of the gel were removed with a syringe to give good contact between the gel and the buffer. Samples (10-100µl, 10-200µg protein) were loaded on using a syringe, and contained 5% (w/v) sucrose and 0.005% bromophenol blue. Initially the samples were dialysed against 10-fold diluted running buffer, and then placed in a boiling water-bath for 2 minutes in the presence of 2% SDS, 100mM 2-mercaptoethanol. Subsequently, it was found that resolution was improved if the samples contained NURB (Neville's Upper Reservoir Buffer, 41mM tris-40mM borate pH 8.64,0.1% SDS; Neville, 1971). This improvement was probably because the

samples stacked to a certain extent before entering the stacking gel.

Electrophoresis was continued until the bromophenol blue had just run off the gel. This required various combinations of current and time depending upon the type of gel; typically a gel containing a 7.5-25% linear gradient of acrylamide required 16 hours at a constant 15 mA current. When electrophoresis was finished the plates were separated, and the stacking gel cut away from the resolving gel. The resolving gel was then left for at least 4 hours in staining solution (II2Eia). Gels were destained by sucessive changes of 1l. of 40% methanol, 10% acetic acid.

At this stage the gels could be dried down onto a sheet of filter paper. The method used was a modification of that due to Maizels (1971). The wet gel was placed onto a damp sheet of Whatmann 3MM (30cm x 20cm) and covered with "cling-film". This sandwich was placed, filter-paper side downwards, onto the gel drier itself, which consisted of a sheet of porous polyethylene located in a metal frame. The gel was held flat by 8 layers of heavy-duty cartridge paper , and the whole arrangement placed inside a plastic bag. This was heated to 80°C in an oven and a vacuum line attached to a nozzle on the back of the metal frame. Gels were normally dry after 90 minutes. They were removed from the drier and left under a heavy weight to keep them flat whilst they cooled. The gels could be stored in the dark in this state as a permanent record of the pattern of Coomassie Blue-stained proteins.

d) SDS-containing polyacrylamide slab gels; continuous buffers.

To measure the molecular weights of proteins on gels

the use of a continuous buffer system as opposed to a discontinuous one is claimed to give more accurate results (Clegg et al, 1976). The system of Fairbanks et al (1971) was used.

The slab gel apparatus described in the previous section was used. The gels contained 12% (w/v) acrylamide, 0.45% (w/v) bisacrylamide, 1% (w/v)SDS, 0.4M tris-acetate pH7.4, 0.2M sodium acetate, 0.02M EDTA, 0.1% (w/v) ammonium persulphate. The polymerisation mixture was poured between the plates and allowed to set with the slot-former in place. All other procedures were as described in section II2Eic except that the electrophoresis buffer was 40mM tris- acetate pH7.4, 20mM sodium acetate, 2mM EDTA and 1% SDS.

e) Two-dimensional analysis of proteins.

A two-dimensional gel system described by O'Farrell (1975) was used to analyse proteins on the basis of both pI and molecular weight. The first dimension was isoelectric focussing in a polyacrylamide gel; the second dimension was a slab gel containing acrylamide as described (section II2Eic).

Samples were prepared for analysis by lyophilisation and subsequent resuspension in 9.5M urea (ultrapure), 2% (w/v) NP40, 2% ampholines (5-7 and 3.5-10 pH ranges mixed 2:1 v/v) and 5% (w/v) 2-mercaptoethanol; they were then stored at -20°C. The isoelectric focussing gels were prepared and,exactly as described by O'Farrell (1975), were used to fractionate the samples. Two types of pH gradient were used; one from pH 4.0-6.5 (pH5-7 and pH3.5-10 ampholines 2:1 by volume, 2% final concentration) the other pH4.0-8.0 (2% final concentration of pH3.5-10 ampholines).

When the gels had come to equilibrium which usually

required 20 hours at 300V, they were removed from the tubes. Those gels used to analyse samples were equilibrated for 30 minutes, with vigorous shaking, in 15mls of 70mM tris pH6.8, 2.2% (w/v) SDS, and 7% (v/v) 2-mercaptoethanol; the gels were then stored at -70°C. Two blank gels were always used to determine the characteristics of the pH gradient which had been generated. These gels were cut into 1cm sections and each section shaken for 30 minutes with 2mls of distilled water. The pH of the liquid was measured with pH electrode.

The slab gels used for the second-dimension analysis were exactly as described in section II2Eic, except that the stacking gel was poured right to the top of the plates and had a flat surface. The first dimension gel was placed on top of the slab gel and fixed in place with molten agarose-bromophenol blue, 18mM tris-HCl, pH6.8). Marker proteins were loaded into slots formed in the agarose. Electrophoresis was for 20 hours at 14mA. The gels were stained and destained as described (section II2Eic).

All the two-dimensional gels were impregnated with PPO and fluorographed as described by Bonner and Laskey (1974). The sensitivity of the film was increased by pre-flashing procedure (Laskey and Mills, 1975). The X-ray film was fogged to an optical density of 0.15 before placing next to the dried gel. A two-second flash of white-light from a dark-room enlarger with one layer of Whatman 3MM over the lens (set at f22) at a distance of 94cm from the film was found to be sufficient for the film used in this work (Cronex 4, Du Pont Nemours). The gels were subsequently stored at -70°C.

ii) RNA gels.

All RNA samples were analysed on 2.4% (w/v) acrylamide cylindrical gels as described by Loening (1967). The acrylamide was recrystallised from chloroform; the bisacrylamide was recrystallised from acetone.

The gels contain 2.4% (w/v) acrylamide, 0.12% (w/v) bisacrylamide, 40mM tris-acetate pH7.8, 20mM sodium acetate, and 2mM EDTA. This mixture was degassed and then TEMED (0.1%, v/v) added; the polymerisation was initiated by addition of ammonium persulphate (0.2% w/v, final concentration). The gels were pre-electrophoresed at 50V for 1 hour.

RNA samples were dissolved in 15% sucrose, 5mM tris-HCl, pH 8.1, 15mM NaCl, and 0.1% SDS before loading onto the gels. To ensure denaturation, RNA samples were resuspended in 8M urea, 5mM tris-acetate, pH 7.8, 2.5mM sodium acetate, 0.5mM EDTA, and 15% (w/v) sucrose, and heated to 60°C for 2 minutes before loading. Electrophoresis was at 50 V for 4 hours. The gels were removed from the tubes and soaked in water for 30 minutes before scanning at 260nm in a Gilford gel scanner.

F. Isolation and analysis of polysomes.

(i) Isolation.

Polysomes were isolated from pea apices by a method modified from that described by Davies *et al* (1972). This method was developed specifically for pea apices and has been shown to reduce degradation due to RNAase. All buffers and equipment were autoclaved or treated with diethyl pyrocarbonate (Ehrenberg *et al*, 1976) before use.

Pea apices were pinched off by hand, and dropped into liquid nitrogen. The frozen tissue (3-10g.) was ground, in

the absence of buffer, in a cooled mortar and pestle until a frozen fine powder was obtained. At this point the grinding buffer (5mls / g. tissue) was added and the grinding continued for a further minute. The grinding buffer consisted of 200mM tris-HCl pH 8.5, 200mM sucrose, 60mM KCl, and 30mM $MgCl_2$ plus heparin, cycloheximide and the non-ionic detergents NP40 and sodium deoxycholate to concentrations detailed in the results section. The homogenate was poured rapidly through 2 layers of muslin and then centrifuged at 30,000 g_{ave} for 20 minutes. Aliquots of the clear supernatant were layered on top of 7ml sucrose pads (50% w/v sucrose in 40mM tris-HCl pH 8.5, 20mM KCl, and 10mM $MgCl_2$) in 25ml tubes. The polysomes were centrifuged through the pad by 60 minutes at 200,000 g_{ave}.

At this stage the pellets could be dried with a stream of nitrogen and the tubes stored inverted at -20°C. Alternatively the pellets were resuspended in the buffer appropriate for the next stage of the experiment; see sections II2Fii and II2G.

ii) Analysis of polysome size.

Velocity sedimentation through gradients of sucrose concentration has been used extensively to obtain size distribution profiles of polysomes. With gradient systems of good resolution it is possible to see each polysome size class as a discrete peak. The system used was based on that described by Davies et al (1972).

The gradients were formed by placing 1ml of 50% (w/v) sucrose, in 20mM tris-HCl pH 8.5, 20mM KCl, and 10mM $MgCl_2$, in the bottom of a 6.5ml polycarbonate centrifuge tube and overlaying with 2mls. 37.5% sucrose, 2mls. 25% sucrose and

1ml 12.5% sucrose in the same buffer. The linear gradient forms as the steps diffuse into each other. The gradients were left 24-48 hours before use at 4°C to allow diffusion to produce the gradient.

The polysome pellets were resuspended gently in the gradient buffer to a concentration of approximately 10 E_{260} units / ml, and 0.5-1.0 E_{260} units loaded onto each gradient. The tubes were spun at 205,000 g_{ave} for 75 minutes; this period did not include the run-up and run-down times and the brake was on during run-down.

The E_{260} profile down the gradient was obtained using an ISCO gradient analyser. A saturated solution of sucrose, containing bromophenol blue as marker, was pumped to the bottom of the centrifuge tubes and the gradient displaced upwards. The E_{260} was monitored continuously.

iii) Indirect immune-precipitation of polysomes.

a) Analytical.

Polysomes were isolated from eight pea apices which had greened in the presence of 1mCi of [^{35}S] -methionine (1000 Ci/mmole) as described in section II2N. The polysome pellet obtained was resuspended in 50mM tris-HCl pH 7.6, 150mM KCl, 10mM $MgCl_2$ and 1% NP40. The E_{260} and hot TCA-precipitable radioactivity were determined.

The polysome suspension was split into aliquots containing 4 E_{260} units each and incubated, in a final volume of 400µl, with the concentrations of either anti-Fraction I IgG or pre-immune IgG given in the text. Incubation was for 1 hour at 37°C; then goat anti-rabbit IgG was added, to the final concentrations indicated. After a further hour at 37°C

the precipitates were left to form at 4°C overnight.

The precipitates were pelleted by a 5 minute spin in a bench microfuge (11,000g) and washed twice with resuspension buffer plus NP40. Finally the pellets were resuspended in 200µl of 50mM tris-HCl, 150mM KCl, 0.5% SDS, 50mM 2-mercaptoethanol and the protein precipitated by TCA at 4°C for 30 minutes. The samples were heated at 90°C for 15 minutes to hydrolyse RNA and then filtered onto Whatmann GFC discs. The discs were washed extensively with 5% TCA followed by 20mls ethanol, and finally dried. The dry filters were placed in 4mls toluene scintillant and counted in a Packard TriCarb Scintillation Spectrophotometer at 12% gain, open window.

b) Preparative.

Polysomes were isolated from pea apices as described (section II2Fi) and suspended in 50mM tris-HCl, 150mM KCl, 10mM $MgCl_2$, 1% NP40, 500 µg/ml heparin and 10µg/ml cyclohexamide to a final concentration of 10 E_{260} units/ml. Aliquots (10ml) of this suspension were incubated at 37°C with 500µg/ml of either anti-Fraction I IgG or pre-immune IgG. After 1 hour, a 90:1 excess, by weight, of goat anti-rabbit IgG was added to each incubation mixture which was then incubated for a further hour and left overnight at 4°C.

The immune precipitate was collected by pelleting through 4ml of 0.5M sucrose over a 7ml pad of 1M sucrose; both sucrose solutions were in the buffer used for the immune reactions. The precipitates were pelleted after 30 minutes at 10,000g_{ave}; some material was visible at the interface between the two sucrose pads. The pellets were resuspended in 1ml of buffer and the procedure repeated. The RNA was obtained from such polysome preparations as described in section II2G.

G. Isolation of RNA.

(i) Phenol extraction.

The methods used were based on those of Brawerman (1974) and Aviv and Leder (1972). The phenol reagent consisted of the following:- phenol/8-hydroxyquinoline/redistilled *m*-cresol (500g:0.5g:70ml) mixed 50:50:1 (by volume) with chloroform and isoamyl alcohol.

Polysomes were isolated as described in section II2Fi and the pellets resuspended in 100mM tris-HCl, pH9.0, 100mM NaCl, 2mM EDTA and 1%(w/v) SDS to a final concentration of 10 E_{260} units/ml. An equal volume of phenol reagent was added and the mixture shaken vigorously. After 30 minutes standing at room-temperature, the mixture was separated into two solvent phases by a 10 minute centrifugation at low-speed. The aqueous layer was removed and the phenol layer re-extracted with a half-volume of the resuspension buffer. The aqueous phases were pooled and extracted a further two times with phenol, to ensure that all the protein had been extracted from the aqueous phase. Phenol present in the aqueous phase was removed by two extractions with an equal volume of diethyl ether. The last traces of ether were removed by bubbling nitrogen through the aqueous phase. Potassium acetate, pH6.5, was added to a final concentration of 2%(w/v) and the RNA precipitated by addition of two volumes of redistilled ethanol at -20°C.

(ii) Lithium chloride precipitation.

This was based on the method described by Schimke *et al* (1974).

Polysome preparations (section II2Fi) were resuspended in 100mM tris-HCl, pH9.0, 100mM NaCl, 1%(w/v) SDS and 10mM

EDTA to a final concentration of 10 to 15 E_{260} units/ml and then precipitated by addition of 2 volumes of redistilled ethanol at -20°C. After 3 hours, the precipitates were collected by centrifugation at 4,000g for 10 minutes, dried with a stream of nitrogen and resuspended in 10mM EDTA (adjusted to pH7.0 by addition of tris base).

Any insoluble material was removed by centrifugation at 4,000g for 10 minutes. The supernatant solution was removed and an equal volume of 4M LiCl was added. The RNA precipitated out of solution at 4°C.

(iii) Isolation of tRNA from wheat-germ.

Wheat-germ, which had been enriched for embryo by floatation (section II2Ji), was used to obtain a tRNA preparation as described by Roberts et al (1973).

Wheat-germ (25g) was ground in a pestle and mortar with 25g of glass beads (80 mesh). When a powder had been obtained, 100ml of grinding buffer (50mM KCl, 50mM tris-HCl, pH7.6, 10mM $MgCl_2$ and 5mM 2-mercaptoethanol) were added and homogenisation continued for two minutes. The slurry was spun at 30,000g_{ave} for 20 minutes. The fatty layer on the surface was removed, and discarded; the brown, opaque supernatant solution was removed and centrifuged at 260,000g_{ave} for 60 minutes. The supernatant solution was decanted and centrifuged for a further 120 minutes at 260,000g_{ave}. The final, clear, yellowish supernatant solution was adjusted to 100mM potassium acetate, pH5.0 and 2%(w/v) SDS and extracted twice with an equal volume of phenol reagent (section II2Gi). The aqueous layer was made up to 1M NaCl by addition of the solid salt, and re-extracted with an equal volume of phenol. Finally RNA was precipitated

by addition of 2 volumes of redistilled ethanol, at -20°C, to the aqueous layer.

The precipitate was collected by centrifugation at 2,000g for 10 minutes. The pellet was dried under nitrogen and then incubated at 37°C in 0.1M tris-HCl, pH8.5 for 1 hour; this should result in the de-acylation of the tRNA. The sample was then extracted twice with an equal volume of diethyl ether. Finally a quarter volume of 10% potassium acetate, pH6.5 was added and the RNA precipitated at -20°C by addition of 2 volumes of redistilled ethanol.

H. Analysis of poly (A)-containing RNA.

(i) Oligo-dt cellulose chromatography.

Chromatography of RNA on columns of oligo-dT cellulose has been used for both the detection and isolation of poly(A)-containing RNA. The method used was based on that described by Aviv and Leder (1972), except that LiCl was used in place of KCl since it was found that potassium dodecyl sulphate invariably came out of solution at 4°C.

The RNA sample had normally been stored as an ethanol precipitate at -20°C and so was pelleted and dried in a stream of nitrogen. The dry RNA pellet was dissolved in loading buffer (10mM tris-HCl pH 7.6, 0.4M lithium chloride, 0.4% SDS) to a final concentration of not more than 10 E_{260} units/ml. The column of oligo-dT cellulose (type T_2, Collaborative Research) consisted of 1mg of the matrix in a large Pasteur pipette plugged with glass wool. The column was placed inside a water-jacket so that its temperture could be controlled.

The column was equilibrated with loading buffer, and the sample passed through at 4°C. The eluate from the column

by addition of 2 volumes of redistilled ethanol,at -20°C, to the aqueous layer.

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The column was equilibrated with loading buffer, and the sample passed through at 4°C. The eluate from the column

was continuously monitored at 260nm by an LKB Uvicord, and material which passed through the column in the void volume was collected. The column was washed until no more 260nm-absorbing material was eluted. The salt concentration was then decreased from 0.4M to 0.1M LiCl and elution continued. A small amount of E_{260}-absorbing material was eluted; this was usually too little to be worth collecting. The temperature of the column was raised to 37°C and no-salt buffer (10mM tris-HCl pH 8.5, 0.4% SDS) passed through. The RNA which was bound to the column was eluted by this treatment. This bound fraction was collected, made 150mM in LiCl and precipitated by addition of 2 volumes redistilled ethanol at -20°C.

If necessary, the material which did not bind after one passage through the column could be passed through again, in case the column had been saturated on the first occasion A column of 1g. oligo-dT cellulose can bind about 150-200µg of poly(A)-containing RNA. Alternatively the non-bound RNA was chromatographed on polyU-Sepharose (section IIHii) or precipitated by ethanol for storage.

Material which binds to oligo-dT cellulose may still contain rRNA due to aggregation between rRNA and poly(A)-containing RNA. Thus a second passage through the column was required after any aggregates had been disrupted. The method of Bantle *et al* (1976) was followed.

The poly(A)-containing RNA was pelleted from ethanol, dried, and dissolved in 50µl 10mM tris-HCl pH 7.6 plus 450µl DMSO (dimethyl sulphoxide) and 50µl 1M LiCl, 2% (w/v) SDS, 10mM tris-HCl pH 6.5, 50mM EDTA. This mixture was heated to 55°C for 5 minutes before 4.5mls of ice-cold loading buffer

were added. The RNA was then chromatographed on the oligo-dT cellulose as before.

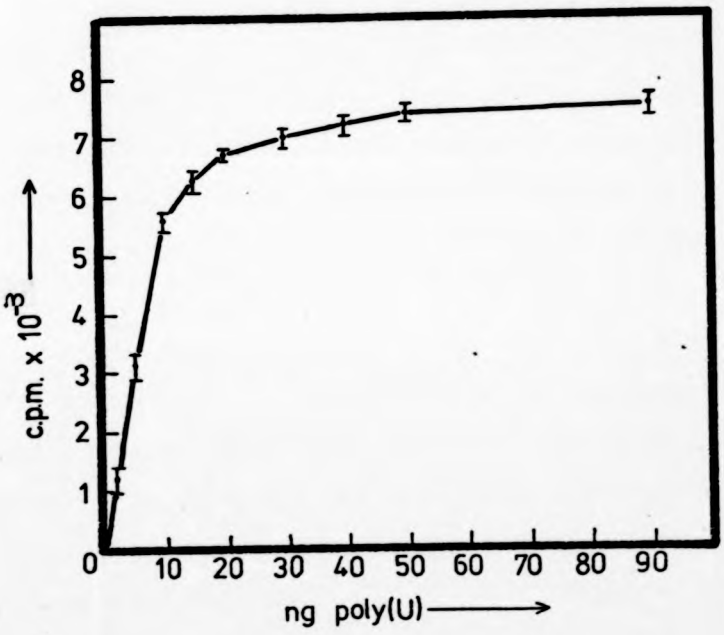
(ii) Poly (U)-Sepharose chromatography.

The poly(U)-Sepharose (Pharmacia) was used in exactly the same way as the oligo-dT cellulose, except that the intermediate 0.1M salt wash was omitted. The poly(U)-Sepharose method was used only with RNA samples which would not bind to oligo-dT cellulose.

(iii) Estimation of poly(A)-content by hybridisation with [^{3}H]-poly(U).

The method was based on that described by Covey and Grierson (1976). The [^{3}H]-labeled poly(U) was obtained from the Radiochemical Center, Amersham, as a freeze-dried solid and was dissolved in sterile 2 x SSC (Standard Saline Citrate, 0.15M sodium chloride, 0.015M sodium citrate).

The [^{3}H]-poly(U) was calibrated against poly(A) (Sigma). Increasing amounts (0 to 90ng of [^{3}H]-poly(U) were incubated at 20°C with 10ng of poly(A) in a final volume of 100µl of 2 x SSC. After 2.5 hours RNAase A was added to a final concentration of 20µg/ml, and incubated for 30 minutes. This treatment served to hydrolyse any single-stranded RNA. Then 100µg of 2' and 3' UMP were added to lower the specific activity of the [^{3}H]-nucleotides formed by the RNAase A digestion. Yeast RNA (400µg) was added and the volume made up to 1ml with water before addition of 1ml of 10% (w/v) TCA. The RNA was left to precipitate at 4°C for 1 hour. The precipitates were collected on Gelman nitrocellulose membranes, 0.22µm pore size. Each filter was washed with 50mls


c.p.m. x 10^{-3}
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50
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70
80
90
ng poly(U)

Figure 3. Calibration curve for the reaction of [^{3}H]-poly(U) with poly(A).

In a final volume of 100µl, 10ng of poly(A) were incubated with various amounts of [^{3}H]-poly(U) as described in section II2Hiii. Triplicate assays were performed for each point on the curve and the bars represent the range of the triplicates. 1ng of poly(A) is equivalent to 740 cpm [^{3}H]-poly(U)

5% (w/v) TCA and 25ml ethanol before drying at 60°C for 30 minutes. Each filter was counted in 4mls toluene scintillant at 70% gain, open window, in a Packard TriCarb scintillation counter.

Figure 3 shows the plot of RNAase A-resistant counts against amount of [^{3}H] poly(U) per incubation. The plateau shows that all the poly(A) has hybridised and that, in the presence of excess [^{3}H]-poly(U), 740 RNAase A-resistant counts per minute are equivalent to 1ng of poly(A).

The hybridisation assays on RNAs of unknown poly(A) content were performed exactly as above, except that a constant 50ng of [^{3}H]-poly(U) was used per assay and two different amounts of the unknown were used to ensure that the poly(U) was in excess.

I. Analysis of RNA on sucrose gradients.

(i) Fixed-angle rotor method.

Usually RNA is fractionated on sucrose gradients at relatively low-speed centrifugations in swing-out heads. This method requires overnight or longer periods of centrifugation to get reasonable resolution of the RNA (see Parish,1972). The use of such lengthy spinning times means that the diffusion of sample bands becomes an important problem affecting the resolution achieved. To overcome this problem, a method using short spins in an angled rotor has been developed in this laboratory by Dr. S. Covey(unpublished), and this was the method used.

Exponential gradients were formed by pumping 15mls of 30% (w/v) sucrose through a constant volume of 10mls 7% (w/v) sucrose. This procedure gave gradients of 7% to about 25%

5% (w/v) TCA and 25ml ethanol before drying at 60°C for 30 minutes. Each filter was counted in 4mls toluene scintillant at 70% gain, open window, in a Packard TriCarb scintillation counter.

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sucrose. The gradient buffer contained 50mM tris-HCl pH 8.0, 150mM LiCl, 0.7% SDS and 2mM EDTA. The gradients were pumped into the bottom of 25ml centrifuge tubes; the light sucrose was displaced upwards, and the loading tube carefully removed when the gradient was complete. The RNA samples were resuspended in 3% (w/v) sucrose in the gradient buffer at a final concentration of about 400µg/ml, and 0.5ml aliquots loaded onto the top of each gradient. The tubes were then filled with liquid paraffin, the caps fitted, and the tubes balanced. An MSE 8 x 25 Ti rotor was used in an MSE highspeed 65 centrifuge. The rotor was run up to 55,000 rpm ($230,000g_{ave}$), left at this speed for exactly 1 hour at 10°C and allowed to decelerate with the brake on.

The gradients were displaced from the bottom by a saturated solution of sucrose, and the E_{260} profile obtained by means of an LKB Uvicord monitor. Particular regions of the gradients were collected, and the RNA precipitated with ethanol as before.

A major point to note in this method is the very short time required for centrifugation. However the shortness of this time means that the characteristics of the run-up and run-down periods of the centrifuge have a profound effect on the resolution. Thus any particular centrifuge must be calibrated to give the required resolution on the gradient. Attempts to use the same conditions on a number of different centrifuges resulted in either pelleting of the 28S rRNA, or insufficient resolution (Covey, personal communication).

(ii) Zonal centrifugation.

The titanium zonal rotor, XVB, was used to fractionate large quantities of RNA. The rotor was run up to loading speed (about 2,000rpm) and the gradient pumped in. The gradient was formed by pumping 650mls of 30% (w/v) sucrose in gradient buffer (50mM tris-HCl, 250mM LiCl, 1mM EDTA and 0.2% SDS, pH 7.6) through a constant volume of 300mls initially containing 7% (w/v) sucrose. The gradient was pumped to the edge of the rotor. The sample (25mls of RNA, 1mg/ml, in 3.5% (w/v) sucrose) was loaded into the centre of the rotor and 50mls of gradient buffer pumped in as an overlay. The function of the overlay is to displace the sample zone out from the centre of the rotor into an annulus. In such an annulus the width of the band is lower than it is closer to the rotor spindle. This reduces the difference in centrifugal force experienced across the sample zone and so improves resolution.

The rotor was run at 30,000 rpm for 13 hours before running down to 2,000rpm for unloading. The gradient was displaced out through the centre of the rotor by 40% (w/v) sucrose. The E_{260} profile was monitored and 10ml fractions collected. The required fractions were pooled and the RNA precipitated by addition of 2 volumes of ethanol at -20°C.

J. Preparation and use of a cell-free protein synthesising system from wheat-germ.

(i) Preparation.

a) Method of Roberts and Paterson (1973).

Wheat-germ was obtained from Harrods of London (milled by Marriage's, Chelm Mill, Chelmsford, Essex) and

stored in a dessicator at 4°C. Such commercial preparations contain large amounts of endosperm. There are reports that cereal endosperm contains RNAase activity (Bog-Hansen *et al* 1974) and so it must be removed. Floatation of wheat-germ on carbon-tetrachloride/cyclohexane mixtures can separate the germ from the endosperm (Marcus *et al*, 1974). The germ and endosperm can be easily distinguished under a low-power microscope and reduction in the amount of endosperm was the criterion used to assess the success of floatation. The exact proportions of the two solvents required for optimum separation varies between different batches of wheat-germ. It was found that a carbon tetrachloride/cyclohexane ratio of about 3:1 gave the best separation of germ from endosperm with the sample used. A 500ml beaker containing about 450mls of the solvent mixture was used; 20g. of wheat-germ was scattered onto the surface and quickly, but gently, stirred in; after 20 seconds the material which floated was scooped off with a filter-paper disc. The residual solvent was removed by blowing cold air over the germ. In order to reduce to a minimum, any detrimental effects, the time the germ was in contact with the solvent was kept as short as possible. This meant that a second cycle of floatation was required for the best results. The wheat-germ was stored at 4°C in glass bottles.

In preparing the wheat-germ extract great care was taken to reduce contamination by nucleases. All solutions and apparatus were autoclaved where feasible; this included the Sephadex G-25, where the column itself was washed with a 1% solution of diethylpyrocarbonate. The glass beads (80 mesh) were washed with concentrated hydrochloric acid, rinsed

extensively with distilled water and dried at 120°C.

The wheat-germ (6g.) was ground, in a cooled mortar and pestle, with an equal weight of acid-washed glass beads until a fine powder was obtained; this usually took about 30 seconds. At this point 5mls of grinding buffer (50mM HEPES, 100mM potassium acetate, 1mM magnesium acetate, 2mM calcium acetate, and 6mM dithiothreitol, pH 7.6) were added and the mixture worked into a thick paste with the pestle. A further 10mls of buffer was then added and the grind continued for 30 seconds. The homogenate was transferred to 15ml chromic acid-washed corex tubes and spun at $30,000g_{ave}$ for 10 minutes. The supernatent solution was removed; there was always a layer of lipid floating on the surface and care was taken not disturb it.

The wheat-germ extract was then placed on ice and the following additions made:-

$1/400^{th}$	volume 1M magnesium acetate
$1/20^{th}$	volume ATP/GTP (20mM/2mM respectively)
$1/50^{th}$	volume 100mM dithiothreitol.
$1/20^{th}$	volume 200mM creatine phosphate.
$1/100^{th}$	volume 5mg/ml creatine phosphokinase.

This mixture was then incubated at 30°C for 10 minutes. The mixture was then passed down a column (30cm x 1.5cm) of Sephadex G-25 (coarse) and eluted with 50mM HEPES, 120mM potassium acetate, 5mM magnesium acetate and 6mM dithiothreitol, pH 7.6. The material which eluted in the void volume was collected and allowed to pass through a 21 gauge needle under gravity, into liquid nitrogen. The small frozen spheres of

extract were stored in liquid nitrogen; only sufficient extract for a particular experiment was thawed at any one time. After any extract had been thawed out, it was not re-frozen. Each sphere had an approximate volume of 10µl.

b) Method of Marcu and Dudock (1974).

This procedure is similar to that of Roberts and Paterson (1973) in that the same buffers are used; however there is no pre-incubation step.

Wheat-germ (3g.) was ground with acid-washed glass beads (3g.) in 10mls of grinding buffer. The supernatent from the $30,000g_{ave}$ centrifugation was put straight onto the Sephadex G-25 column and the material eluted in the void volume collected. This material was spun for 20 minutes at $30,000g_{ave}$ and the clear supernatent stored in liquid nitrogen as described above.

(ii) Use as assay system for mRNA.

a) Preparation of incubations and analysis of TCA precipitable counts.

Preparations of RNA were assayed for messenger activity by incubation with wheat-germ extract in the presence of labelled amino acids. The incubations were carried out in conical, plastic vials (0.5 mls total capacity, Sarsted (U. K.) Ltd., Leicester.) in a final volume of at least 20µl.

Before any RNA can be tested for messenger activity in the wheat-germ system it must have all traces of SDS removed from it. This was achieved by twice reprecipitating the RNA from 0.2M HEPES-KOH, pH 7.6 by addition of two volumes of redistilled ethanol at-20°C. The final RNA precipitate was

dried with a stream of nitrogen and redissolved in sterile distilled water to a suitable final concentration, usually about 1mg/ml. RNA concentrations were calculated on the basis of 1mg/ml = 20 E_{260} units.

The wheat-germ incubations contained the following components at the indicated final concentrations; wheat-germ extract, one quarter volume; tris-ATP, GTP (type III), 0.1mM; creatine phosphate, 10mM; all amino acids, except those used as label, 0.05mM each; HEPES-KOH, 25mM; dithiothreitol, 2mM. The incubations also contained potassium acetate, magnesium acetate, spermidine and spermine at concentrations indicated in the text. The radioactive amino acid used as label was dried down, and then resuspended in sterile distilled water before addition to the incubations at the indicated concentrations. The incubations were made up to volume with sterile distilled water. The incubations were kept on ice whilst all the additions were made and the wheat-germ extract added last.

Incubation was usually for 60 minutes at 27°C, although in characterisation experiments both these parameters were varied. At the end of this time duplicate 2µl aliquots were taken and spotted onto strips (2cm x 1cm) of Whatmann N°. 1 paper. The strips were heated to 95°C for 20 minutes in 10% TCA containing (w/v) 0.5% D,L-methionine (>10mls/strip), washed with fresh 10% TCA containing, 0.5% D,L-methionine (>10mls/strip), washed twice with absolute ethanol (>5mls/strip) and finally washed with diethylether (5mls/strip). The strips were dried at 70°C and counted in toluene-scintillant (0.5% PPO, 0.03% POPOP) in a Packard Tricarb scintillation counter.

(b) Analysis of products on polyacrylamide gels.

The reaction products remaining after the determination of the TCA-precipitable counts were analysed on SDS-containing polyacrylamide gels. At the end of the incubation, SDS, 2-mercaptoethanol, sucrose and bromophenol blue were added to final concentrations of 2%, 100mM, 5% and 0.05% respectively. Then 20 times concentrated NURB (Neville's Upper Reservoir Buffer; Neville, 1971) was added to give a final concentration of 1 x NURB. The samples were heated in a boiling water-bath for 2 minutes to fully denature the protein. After this the samples were stored at -20°C. Before running on a gel the samples were always placed in a boiling-water-bath for 2 minutes. The samples were analysed on gels as described in sections II2Eic and d.

For two-dimensional gels the incubations were lyophilised before resuspension in the urea-ampholine mixture (section II2Eie).

c) Immunological analysis of in vitro products.

Two methods were used. The first was chromatography on columns of immunoglobulin bound to Sepharose 4B (section II2Ciib). The second was indirect immunoprecipitation (Herrlich and Schweiger, 1974). Both goat anti-rabbit IgG and heat-inactivated Staphylococcus aureus were used as second precipitants.

The incubation products were diluted 10-fold with cold PBS, and then NP40 and BSA added to 1% (v/v) and 200µg/ml respectively. In some experiments the ribosomes were removed by spinning for 1 hour at 250,000g_{ave} at 4°C; the supernatents were used for the immune reactions. Highly

purified anti-Fraction I or pre-immune IgG was added to each sample, usually in the range 50-1000 µg/ml, and the mixtures incubated at 37°C for 1 hour.

When used as the second precipitant, a 30-fold excess by weight of goat anti-rabbit IgG was added and incubation continued for a further hour at 37°C. Complete precipitation was obtained after at least 18 hours at 4°C. The precipitate was pelleted in a bench microfuge ($10,000g_{max}$) and washed twice with 500µl of PBS containing 1% NP40. The pellet was finally dissolved in a suitable volume of NURB containing 2% SDS and 100mM 2-mercaptoethanol. Sucrose and bromophenol blue were added and the sample prepared for running on an SDS acrylamide slab gel (II2Jiib).

When used as the second precipitant, a 100-fold excess by weight of heat-inactivated Staphylococcus aureus was added at the end of the hour, and the incubation continued for 30 minutes at 4°C (Kessler, 1975). The bacteria were pelleted by a 3 minute spin in a bench microfuge, washed twice with 500µl of PBS containing 1% NP40 and finally resuspended in a suitable volume (100-500µl) of NURB containing 2% SDS and 100mM 2- mercaptoethanol. The antigen-antibody complex was removed from the surface of the bacteria by warming to 50°C for 15 minutes. The bacteria were spun down as before and the supernatent removed for analysis on gels (see above).

K. Assay of processing activity.

Poly(A)-containing RNA from pea apices was translated in large wheat-germ incubations (100µl-400µl) as described in section II2J(ii)a. The incubations were then split into aliquots (10-20µl, see text for details) in small, conical

plastic vials. These aliquots were kept on ice whilst chloroplasts were isolated as described in section II2Ai or ii. The chloroplasts were resuspended in plastid resuspension buffer (25mM HEPES-KOH, 110mM KCl, 3mM magnesium chloride, 10mM dithiothreitol, pH 7.6).

Aliquots were added to the wheat-germ products, in plastic vials, and incubated at 30°C for 60 minutes. The final incubation volume and the chlorophyll concentration varied from experiment to experiment but were in the ranges 50-100µl and 50-200µg/ml respectively. Any additions such as inhibitors were added dissolved in plastid resuspension buffer.

Sub-fractions of the chloroplasts were also used. These were prepared by hypotonic lysis of the chloroplasts in 10mM HEPES-KOH, 10mM dithiothreitol, pH 7.6 followed by addition of an equal volume of double strength plastid resuspension buffer. The chloroplast lamellae were pelleted at 4,000g for 5 minutes and were resuspended in plastid resuspension buffer before use in the assay. The supernatant solution from this lamellar preparation is referred to as low speed stroma. High speed stroma was obtained by centrifuging the low-speed stroma at $200,000g_{ave}$ for 30 minutes.

The incubations were stopped by adding SDS to 2% (w/v) final concentration and were prepared for analysis on gels as before (section II2Jiib). If the incubations were to be immunologically analysed, no SDS was added, but the reaction was terminated by addition of an equal volume of cold PBS containing NP40 (2%, v/v). This mixture was spun at 10,000g for 5 minutes to remove any insoluble material, and the supernatant solution used for immunological analysis as described (II2JiiC).

L. Peptide mapping.

The procedure described by Clegg *et al* (1976) was used, with some modifications.

The peptides to be mapped were recovered from preparative polyacrylamide slab gels, these were normal Laemmli-type gels (section II2Eic) except that the stacker had a flat surface instead of slots. The proteins were labelled with [^{3}H]-arginine and lysine (section II2Jii and II2N) and so could not be detected by autoradiography. The *in vivo* standard proteins were detected by staining with Coomassie Blue.

The *in vitro* synthesised, unknown proteins could only be detected by a combination of protein staining and autoradiography. The gels containing the [^{3}H]-labelled *in vitro* products also had small slots at the edge which contained [^{35}S]-methionine-labelled *in vitro* products. The gels were stained with Coomassie Blue which located the wheat-germ proteins. Autoradiography of the dry gel would locate the [^{35}S]-labelled polypeptides. Using the stained wheat-germ proteins as a guide, it was then possible to locate the regions of the gel which contained the [^{3}H]-labelled polypeptides corresponding to any particular [^{35}S]-labelled polypeptide. These bands of gel were cut out using a razor blade and treated exactly as described by Clegg *et al* (1976).

The silica thin-layer plates were impregnated with 20% (w/v) PPO in acetone and dried before being left at -70°C to fluorograph as described by Laskey and Mills (1975).

Removal of the correct bands from the gels was checked by taking a vertical strip (1cm) down the length of the gel and cutting it into 1mm fractions. Each gel fraction was dissolved by 0.2 mls hydrogen peroxide at 80°C and counted

in 4mls TTS (triton-toluene scintillant, 0.5% PPO, 0.03% POPOP, 30% (w/v) triton X -100 in toluene) at 70% gain, open window in a Packard Tri-Carb scintillation counter. The profile of radioactivity down the gel could be matched with a scan of an autoradiograph of similar products.

M. Iodination of proteins.

The method of Greenwood et al (1963) was used.

The protein to be iodinated (1mg) was mixed with 1mCi of [^{125}I-] sodium iodide (carrier-free, Radiochemical Centre, Amersham) and 100µl of buffer added (250mM tris-HCl, 0.5M NaCl, 5mM EDTA, pH 7.5). The volume was made up to 400µl with sterile distilled water. The reaction was started by addition of 100µl of chloramine T (5mg/ml) and left to proceed for 30 seconds at room-temperature. The reaction was terminated by addition of 500µl sodium metabisulphite (1mg/ml) and 100µl of potassium iodide (5mg/ml). The entire reaction mixture was loaded onto a Sephadex G-50 column (25ml bed volume) in a 50ml disposable plastic syringe. The column was washed with 50mM tris-HCl, 0.1M NaCl, 1mM EDTA, pH 7.5, and 2ml fractions taken by hand. The material which eluted in the void volume was collected and dialysed overnight against 1 litre of 10mM phosphate, 15mM sodium chloride pH 7.2. The specific activity was determined from the results of protein assay by the Lowry method (section II20) and from the TCA-precipitate radioactivity.

N. Isolation of labelled proteins from pea leaves.

(i) Labelling of pea leaves.

Excised pea shoots can take up radioactive amino acids

through the cut ends of their stems. Etiolated pea seedlings, seven days old, were removed from the vermiculite (section II1A) and the stems cut with a razor blade, about 5cm below the apex. As soon as it was cut, the stem was placed in a vial containing the radioactive label in 1ml of water. Usually six to eight seedlings were used and either 1mCi [^{35}S]-methionine (845Ci/mmole) or 4mCi [^{3}H]-lysine (25Ci/mmole) and [^{3}H]-arginine (23Ci/mmole). The shoots were kept under white light (10,000 lux), and a fan used to blow air over them to increase transpiration. Labelling was for 24 hours and when the initial liquid had all been taken up, further distilled water was added to the vial.

(ii) Isolation of soluble protein..

The apices were cut from the labelled shoots and ground at 4°C in a pestle and mortar with 10mls of hypotonic buffer (25mM tricine-KOH, 1mM EDTA, pH 7.8 with 2mM PMSF added). After 5 minutes of vigorous grinding, the homogenate was passed through two layers of muslin and spun at 30,000g_{ave} for 20 minutes. The supernatent solution was decanted, and the protein precipitated from it by addition of an equal volume of neutral, saturated ammonium sulphate at 4°C.

The ammonium sulphate precipitate was collected by centrifugation at 10,000g_{ave} for 10 minutes and half was resuspended in PBS. The remainder was resuspended in 1 x NURB, 2% SDS, 100mM 2-mercaptoethanol. The PBS solution was used for immunological analysis by either antibody columns (II2Ciib) or indirect immunoprecipitation (section II2JiiC). The other preparation was used for polyacrylamide gel analysis (section II2Eic).

(iii) Isolation of chlorophyll a/b-binding protein.

The pellet obtained in the high speed spin described in section II2Nii contains thylakoid fragments and nuclei; it was resuspended in 5mls of 30% (w/v) sucrose, 0.1M tricine-KOH, 10mM potassium chloride, 1mM magnesium chloride, 1% (w/v) Ficol, 0.1% (w/v) BSA, pH 7.5. The fragments of thylakoid membranes were isolated from this preparation by using the gradient method described by Miflin and Beevers (1974).

The gradients were made up in 0.1M tricine-KOH,pH 7.5 and were prepared about 2 hours before they were used. Each gradient consisted of a pad of 2.7mls of 60% (w/v) sucrose, overlaid with a 4ml linear gradient of 60-42% (w/v) sucrose, another 3.3mls of 42% (w/v) sucrose, a 6.7mls linear gradient of 42-30% (w/v) sucrose and finally 2mls of 30% (w/v) sucrose. Aliquots (2.5mls) of the membrane preparation were loaded on to the gradients at 10°C. The gradients were then spun in a 3 x 25 centrifuge head (MSE rotor) according to the following schedule:-

a) 5 minutes at 5,000 rpm,

b) 10 minutes at 10,000 rpm,

c) deceleration to 5000 rpm without the brake,

d) bringing to rest with the brake.

The gradients had a green band about a third of the way down and a green"smear" extending from the top to about halfway down. The smear probably reflects the great size heterogeneity of the membrane fragments. The top half of the gradients were removed, pooled and diluted by addition of 0.1M tricine-KOH pH 7.5. The membranes were pelleted at 30,000g_{ave} for 10 minutes, and finally resuspended in 1.25mls 0.1M

sodium carbonate, 0.1M dithiothreitol.

The chlorophyll a/b-binding protein can be selectively extracted from chloroplast membranes by chloroform/methanol (2:1,v/v) according to Chua et al (1975). The membrane suspension was shaken with 25mls of chloroform/methanol and left overnight at 4°C. The insoluble material was removed by spinning at 4,000g for 10 minutes. The green supernatant solution was removed and the solvent blown off with a stream of nitrogen. The green residue still contained lipid and chlorophyll which were removed by acetone extraction. The residue was resuspended in 3mls 0.1M Na_2CO_3, 0.1M dithiothreitol; it was found that addition of acetone to about 5% (v/v) helped solubilisation. The suspension was then made 80% (v/v) in acetone and left on ice for 30 minutes. The precipitate was collected by centrifugation at 4000g for 10 minutes and the last traces of acetone removed by a stream of nitrogen gas. The precipitate was dissolved in 1ml of 0.1M sodium carbonate, 0.1M dithiothreitol. For analysis of this material on polyacrylamide gels, NURB and SDS were added as described in section II2Jiib.

O. Protein determinations.

Protein was determined by the method of Lowry et al (1951).

Solution A. 0.5% (w/v) $CuSO_4 .5H_2O$ in 1% (w/v) sodium potassium tartrate.

Solution B. 50mls of 2% (w/v) Na_2CO_3 plus 1ml of solution A.

Solution C. 1M Folin-Ciocalteau reagent.

A standard curve was prepared by taking aliquots (5-500µl)

of a standard (1mg/ml) solution of BSA in 1N NaOH. Each aliquot was made up to 0.7mls by addition of 1N NaOH. Solution B (7.0mls) was added, mixed well and left for 10 minutes at room temperature. Solution C (0.7mls) was added and the reaction left for 10 minutes at 50°C. The samples were cooled under running tap-water and then the E_{750} measured.

The unknown samples were precipitated by an equal volume of 10% (w/v) TCA at 4°C for at least 30 minutes. The precipitate was collected by centrifugation at 2,500g for 10 minutes and the pellet resuspended in 0.7mls of 1N NaOH. This solution was treated as above, and the protein concentration determined from the standard curve.

P. Estimation of chlorophyll.

The chlorophyll concentration in chloroplast preparations was determined by the method of Arnon (1949). Aliquots (0.1ml) of the chloroplast suspension were made up to 5mls with 80% (v/v) acetone. These mixtures were left in the dark for 10 minutes. The precipitated protein was removed by filtration through Whatmann N°1 paper. The extinctions at 640nm and 655nm were determined in a Unicam SP500 spectrophotometer, 80% acetone was used as a blank. Chlorophyll concentrations were calculated from the formula.

$$[\text{chlorophyll}]_{mg/l} = (20.2 \times E_{645}) + (8.02 \times E_{663})$$

SECTIN III - RESULTS AND DISCUSSION

1. ISOLATION AND ASSAY OF mRNA FROM PEA LEAVES.

A. Characteristics of the wheat-germ, cell-free, protein-synthesising system.

(i) Method of preparation.

In the literature, wheat-germ extracts have been made from wheat-germ obtained commercially (Roberts and Paterson, 1973; Shih and Kaesberg, 1973) or isolated by hand from wheat seeds (Marcus *et al*, 1974; Shih and Kaesberg, 1973). The wheat-germ used throughout this work was obtained from Harrods of Knightsbridge, London and was milled by Marriage's, Chelmer Mill, Chelmsford, Essex. This wheat-germ was recommended to us by Dr. Alan Smith of I.C.R.F., London.

Any system to be used to assay messenger RNA activity should have only a low level of RNAase activity. There is evidence that barley endosperm contains RNAase activity (Bog-Hansen *et al*, 1974) and so it would seem advisable to remove the endosperm from wheat-germ preparations. However, very few published methods include steps to achieve this removal. Most workers follow the method of Roberts and Paterson (1973), which does not include the removal of endosperm. Marcus *et al* (1974) used floatation on a carbon tetrachloride/cyclohexane mixture to remove endosperm. Therefore it was decided to prepare wheat-germ extracts by a variety of different methods and to compare their protein-synthesising abilities under identical assay conditions. The variables investigated included removal of endosperm, pre-incubation to lower the endogenous activity and passage of the extract through a Sephadex G25 column.

Table I shows the results of such a comparison. The procedure of Roberts and Paterson (section II2J(i)a) was

Extract number	incorporation cpm/hr/2.5µl		incorporation per E_{260} unit	stimulation over background
	-TMV	+TMV		
WG3	350	5,000	32,350	14.3
WG4	1,150	31,300	83,150	27.2
WG5	1,200	49,000	146,850	40.8
WG6	2,400	43,000	118,100	18.1
WG7	1,050	32,000	101,000	30.5
WG8	160	2,600	3,670	16.2
WG9	1,100	27,300	74,000	24.8
WG10	950	49,000	121,500	51.6
WG11	800	41,400	110,000	51.7
WG12	530	26,000	60,000	49.0
WG13	600	20,000	50,000	33.3

Table I. Comparison of the protein-synthesising abilities of wheat-germ extracts prepared in different ways

The protein-synthesising ability of various wheat-germ extracts was tested by determining the incorporation of [^{35}S]-methionine into hot TCA-precipitable material as described in section II2Jiia. Each reaction mixture (20µl) contained 8µg of TMV RNA, 3µCi [^{35}S]-methionine, 100mM K^+, 3mM Mg^{2+} and incubation was at 29°C for 60 minutes. Duplicate aliquots (2.5µl) were taken for determination of TCA precipitable radioactivity. In control tubes the TMV RNA was omitted. The zero-time amount of radioactivity was 500 cpm and was subtracted from all the incorporations.

WG 3 to 9 were prepared as described by Roberts and Paterson (1973); WG3 was prepared with unfloated wheat-germ, WG4 was the control; WG7 was prepared without the pre-incubation step, WG6 was the control; WG8 was prepared without the Sephadex-G25 step, WG9 was the control; WG 10-13 were prepared as described by Marcu and Dudock (1974).

followed for preparations 3-9, that of Marcu and Dudock (section II2Jib) for preparations 10-13. Whenever an alteration was made to the procedure, a parallel preparation was made using the unaltered procedure. Thus comparison of WG4 (wheat-germ preparation 4) with WG3 shows that removal of the endosperm by a floatation step was useful, giving a more active preparation. It is interesting to note that the non-pre-incubated WG6 has a higher total incorporation than the comparable pre-incubated WG7. However, the stimulation by added RNA is much higher for WG7 compared with WG6. Therefore, the preincubation step does cause some reduction in the efficiency of the translation system but has a much greater effect on the endogenous activity. When assaying RNA fractions for messenger activity it is necessary to have not only a high incorporation of radioactivity for ease of detection but also a high stimulation over endogenous levels so that any new products are not obscured by a high background. Thus the pre-incubation step is necessary. If chromatography on Sephadex G25 was omitted the resulting WG8 had a much lower rate of incorporation compared with the control WG9. This difference can be explained if WG8 contains a pool of non-radioactive methionine which dilutes the specific activity of the added [^{35}S] -methionine.

It is concluded from these experiments that when using the Roberts and Paterson procedure, it is desirable to enrich the wheat-germ by floatation, to pre-incubate the extract and to remove endogenous amino acids on a Sephadex G25 column.

A second method of preparation was that of Marcu and Dudock (1974), which differs from the previous method in that there is no pre-incubation step but there is a second

centrifugation after the G-25 column step (section II2Jib).

WG5 was the most active preparation made by the Roberts and Paterson procedure. WG10 was made, using the same batch of floated wheat-germ, by the Marcu and Dudock procedure. Each extract showed a comparable level of incorporation but WG10 showed a greater stimulation by added mRNA. It seems odd that the pre-incubation step is necessary for low endogenous incorporation using one method but not by the other. However, every wheat-germ extract made by the Marcu and Dudock procedure (WG10-13) showed endogenous levels of incorporation as low as any of the others.

It is clearly necessary to know how reproducible are the results obtained with different batches of wheat-germ, and to establish how long the wheat-germ extract can be stored without loss of activity. WG11 was made from the same batch of floated wheat-germ as WG3-10. The germ had been stored at $4^{o}C$ in a glass bottle (Marcus *et al*, 1974) for six weeks after WG10 had been made. The stimulation by added mRNA was almost identical to that shown by WG10 but the level of incorporation was lower by about 8%, on an E_{260} basis. This reduction is slight and is probably within the variation inherent in the methods.

WG12 and WG13 were each made from different batches of wheat-germ which had been enriched by floatation at the same time. Each preparation was less active than either WG10 or 11. The wheat-germ for WG12 was purchased before that for WG13 and both were purchased about 10 weeks after the wheat-germ used for WG3-11. These results show that the wheat-germ bought commercially can vary in its activity. This variation might well be just due to the effect of storage in shop

conditions. An alternative explanation is that the time since the wheat was harvested is important. The batch of wheat-germ used for WG3-11 was obtained around October/ November whereas the two other batches were obtained in December and January. Subsequent experiences of other members of this department, who have purchased wheat-germ, from both Harrods and directly from the mills, during the Spring and early Summer, would seem to support this idea (E. Sturgess, J. Silverthorne and J. Morser, personal communications).

The wheat-germ extracts are stored in liquid nitrogen and there has been no evidence of any loss of activity during storage. Some extracts have been stored for over two years without showing any detectable deterioration.

(ii) Conditions of assay.

Having established the procedures required to make wheat-germ extracts which incorporate amino acids into protein when mRNA is added,it was necessary to optimise the conditions of the assays.

For the comparison in table I the assay conditions were as described by Roberts and Paterson (1973), except that deacylated tRNA was not added. During the early experiments on optimisation of the assay conditions, it was found that the results varied markedly from day to day. Supposedly identical incubations set up on consecutive days could give incorporations differing by as much as four-fold. This situation was intolerable and serves to emphasise that to obtain consistent, meaningful results with cell-free systems requires great care.

Much of the variation was traced to the components used

in making a complete incubation e.g. ATP, GTP and amino acids. It was found that only sterile, glass-distilled, de-ionised water (this is referred to as SDW, sterile distilled water) should be used to make up each component. The individual components must be stored at -20°C as small aliquots (<100µl); this reduces the cycles of freezing and thawing to a minimum. Another major source of trouble appeared to be the creatine phosphokinase used in the incubations. Initially this component was stored at -20°C in solution but it did not retain its activity. Subsequently the enzyme was added as a small crystal to the energy-mix and then split into aliquots for the individual incubations. However, neither method gave consistent results; eventually it was found that leaving the enzyme out altogether gave more consistent results without reducing the incorporation.

The reproducibility was further improved by the addition of polyamines to the incubations. Polyamines lower the requirement for magnesium ions and increase the level of incorporation by stimulating the elongation rate (Hunter et al, 1977). How they might improve the reproducibility of the assay is not clear. According to Glanville et al (1976), 250µm spermidine and 50µm spermine are the optimal concentrations, and these concentrations were used in every subsequent assay.

Figure 4 shows the results of varying some of the incubation parameters. The time-course of incorporation (Fig.4A) shows that at 29°C there was an initial lag-period of about 5 minutes followed by a period of linear incorporation, which then levelled off by 60 minutes. The drop in incorporated counts seen with prolonged incubation was reproducible, and so may well reflect degradation of the newly-synthesised

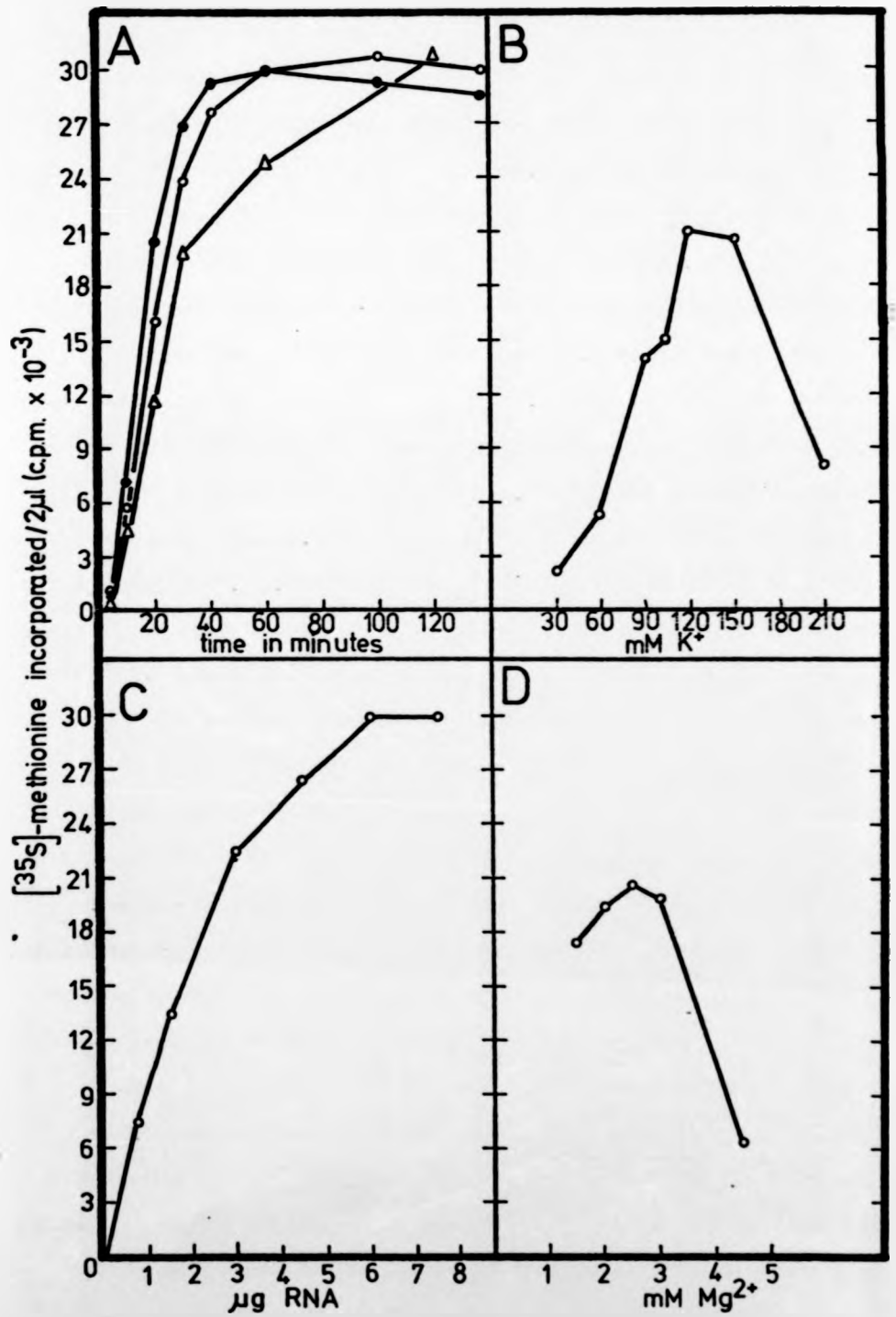
A
B
C
D
[35S]-methionine incorporated/2µl (c.p.m. x 10-3)
time in minutes
mM K+
µg RNA
mM Mg2+

Figure 4. Characteristics of the wheat-germ assay system.

A wheat-germ extract, prepared as described by Roberts and Paterson (1973), was assayed under various conditions. All reaction mixtures contained 1mM tris-ATP, 0.1mM GTP, 10mM creatine phosphate, 0.05mM of each unlabelled amino acid, 25mM HEPES-KOH, pH 7.6, 2mM dithiothreitol and one quarter volume of wheat-germ extract. The concentrations of potassium acetate, magnesium acetate, [^{35}S]-methionine and RNA are given below. Duplicate aliquots (2µl) were taken for determination of TCA-precipitable radioactivity (section II2Jii).

A. Time-course of incorporation. Each incubation was as described above, plus 110mM K^+, 2.5mM Mg^{2+}, 3uCi [^{35}S]-methionine and 10µg TMV RNA in a final volume of 30µl. Incubation was at either 25°C or 29°C and aliquots (2µl) were removed at the indicated times.

●——● ○——○ duplicate incubations at 29°C

△——△ incubation at 25°C

B. Effect of K^+-concentration on incorporation. Incubations contained the components described above, plus 2.5mM Mg^{2+}, 1µCi [^{35}S]-methionine, 6µg TMV RNA and the indicated K^+ concentration in a final volume of 20µl. The reaction mixtures were incubated at 27°C for 60 minutes before aliquots were removed.

C. Dependance upon added RNA. Incubations were as described in A except that the reaction volume was only 20µl and contained 1µCi[^{35}S]-methionine; spermine (50µM) and spermidine (250µM) were also added. The

amount of TMV RNA added to the reaction mixtures was as indicated.

D. Effect of Mg^{2+} concentration on incorporation. The reaction mixtures contained all the components as described in C, 6μg of TMV RNA was added to a final volume of 20μl; the Mg^{2+} concentration was as indicated.

	incorporation
Complete	100
-RNA	1-2
+cycloheximide (100μg/ml)	0.5
+chloramphenicol (100μg/ml)	98
-ATP/GTP	0
-CP	40

Table II Characteristics of the incorporation by the wheat germ system.

Wheat-germ reaction mixtures, as described in Figure 4A, were incubated for 60 minutes at 27°C. The incorporation of such a reaction mixture was defined as 100%. The incorporation in the presence of inhibitors or in the absence of various components was related to this value.

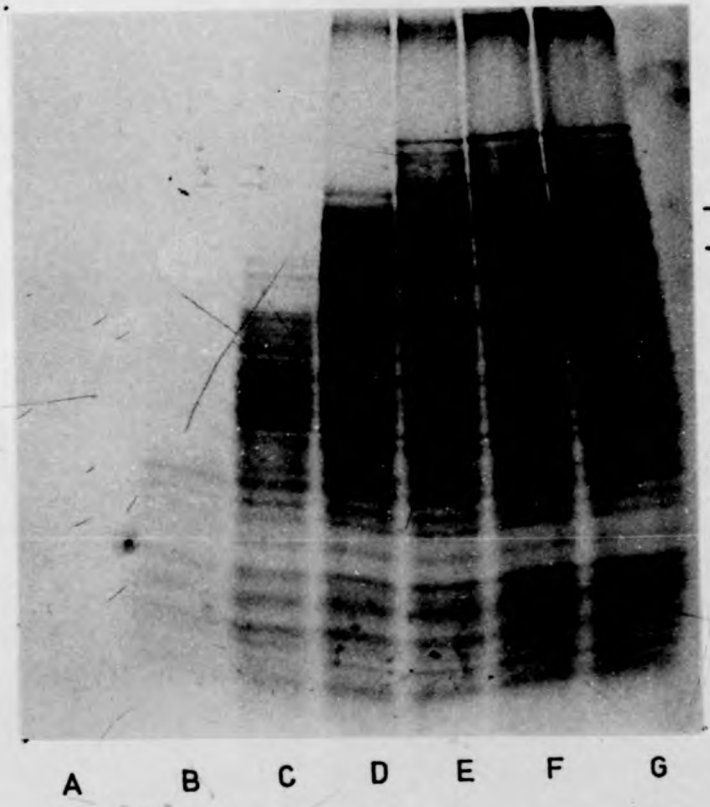
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Figure 5 Time-course of protein synthesis in a wheat-germ cell-free system.

A wheat-germ cell-free protein-synthesising system was set up as described in section II2Jiia. In a final volume of 20µl, each incubation mixture contained all the components as described plus; 110mM K^+, 2.5mM Mg^{2+}, 250µM spermidine, 50µM spermine, 5µCi [^{35}S]-methionine and 6µg of TMV RNA. Each reaction mixture was incubated at 27°C for the times indicated before the reaction was stopped by addition of SDS to a final concentration of 1%. Each reaction mixture was prepared for electrophoresis as described in section II2Jiib. Aliquots of each sample, equivalent to 4µl of the original protein synthesis reaction mixture, were analysed by electrophoresis on a slab polyacrylamide gel (section II2Eic). Electrophoresis was at a constant current of 15mA for 16 hours, the resolving gel contained a gradient of acrylamide concentration from 7.5% to 25%. The dried gel was left at room temperature to expose a sheet of X-ray film (Cronex 4, Du Pont) for seven days. Times of incubation:-

A. zero time; B. 5 minutes; C. 10 minutes;
D. 20 minutes; E. 30 minutes; F. 40 minutes;
G. 60 minutes.

Molecular weight markers:- 1. cytochrome c (12,500); 3. β-lactoglobulin (18400); 2. myoglobin (17,200); 4. chymotrypsinogen (25,000); 5. large subunit of pea Fraction I protein (55,000); 6. BSA (68,000).

polypeptides. At 25°C the initial rate was lower than at 29°C but incorporation was linear for longer, up to 120 minutes. However, because the drop in incorporation after prolonged incubation at 29°C suggested that proteolysis might occur, all subsequent incubations were for 60 minutes at 27-28°C.

The effect on incorporation of varying the K^+ and Mg^+ concentration is shown in Fig. 4 (B and D). The maxima appeared at 110mM K^+ and 2.5mM Mg^{2+}. These values agree well with the optimum conditions obtained by Roberts and Paterson (1973). These concentrations are the nominal concentrations present in the incubations, no allowance having been made for the changes caused by some components of the incubation e.g. ATP which binds metal ions.

Incorporation by the wheat-germ system reaches saturation on addition of increasing amounts of TMV RNA (Fig. 4C). The response to the RNA concentration was linear up to about 150µg/ml and was saturated at 300µg/ml. In all the experiments characterizing the wheat-germ system, saturating levels of TMV RNA were used, so that any small variations in the amount of RNA added did not cause any changes in the incorporation.

Table II shows the effect of leaving out particular components and of adding either cycloheximide or chloramphenicol to the incubation mixtures. The system requires added ATP and GTP to function and is sensitive to cycloheximide but not to chloramphenicol. These results are expected if the incorporation is due to isolated 80S ribosomes rather than to contaminating micro-organisms.

Figure 5 shows the time-course of appearance of the TMV RNA directed polypeptides; this time-course is very similar to that obtained by Hunter *et al* (1977). It is obvious that,

for the smaller polypeptides, the bands appear after a certain time, rapidly reach a plateau and then stay at the same intensity for the rest of the incubation. This would imply that the time during which initiation was possible was quite short (about 10 minutes). The wheat-germ extract was able to synthesise high molecular weight polypeptides; the slowest migrating band in the pattern is thought to be part of the TMV-polymerase and has a molecular weight about 140,000 (Paterson et al, 1975).

This pattern of synthesis, i.e. a short period of initiation followed by run-off of the polysomes, has also been observed in cell-free systems prepared from L-cells (Kerr et al, 1972) and Krebs II ascites cells (Boime and Leder, 1972). The time taken for the first appearance of a particular peptide can be used to calculate the rate of elongation of the system. The 140,000 peptide has appeared by 30 minutes of incubation yet is not present at all at 20 minutes. If it is assumed that an average amino acid has a molecular weight of 115, then the elongation rate can be calculated to be at least 40 amino acids per minute, and be as high as 50 amino acids per minute. The variation is due to the uncertainty about the exact time of first appearance of the 140,000 polypeptide. A value of 100 amino acids per minute was obtained by Hunter et al (1977) for a wheat-germ system but a Krebs II ascites system was found to have a rate of only 25 amino acids per minute (Mathews and Osborne, 1974).

(iii) Fidelity.

To be of use as a method for detecting mRNA, any cell-free system must faithfully translate the messengers into

discrete polypeptides. Doubts have been raised about the ability of wheat-germ systems to translate the mRNAs for high molecular weight proteins (Anderson et al. 1974). However, it has subsequently been shown that, by altering the conditions of the assay, the wheat-germ system is perfectly able to synthesise high molecular weight polypeptides. The addition of polyamines (Roewekamp et al, 1976; Benveniste et al. 1976) or raising the K^+ concentration to 100mM or higher (Shapiro et al, 1976; Rosen, 1976) will increase the relative synthesis of polypeptides larger than about 30,000 molecular weight. The system used throughout this work was shown to synthesise the high-molecular weight products directed by TMV RNA in easily detectable yield (Fig.5).

In the literature, wheat-germ extracts have been shown to faithfully translate a wide range of mRNAs; for example, globin mRNA (Roberts and Paterson, 1973); rat liver preproalbumin mRNA (Taylor and Tse, 1976); brome mosaic virus RNAs (Shih and Kaesberg, 1973); dihydrofolate reductase mRNA (Chang and Littlefield, 1976) and cellulase mRNA (Verma et al, 1975). However, it has also been shown that many of the numerous polypeptides synthesised in response to added TMV RNA arise by premature termination (Hunter et al.1977). This means that, on large mRNAs, the ribosomes may terminate at specific, yet non-physiological, points along it; the reason for this is not known. It is thus possible that any large mRNA molecule will programme the synthesis of a range of polypeptides, all with definite molecular weights, as well as the full-sized product. It must be pointed out that the only situation where this has definitely been shown to occur is during the translation of large, viral RNA, e.g. TMV and EMC. For both these RNAs, this

premature termination occurs in other cell-free systems as well as in the wheat-germ (Boime and Leder, 1972).

It has been shown that wheat-germ extracts can cap added mRNA which is uncapped (Muthukrishnan et al, 1975; see section IC). The cap may also be required for the translation of the mRNA (Both et al, 1975; Canaani et al, 1976; Shatkin, 1976; although see Abraham and Pihl, 1977). The situation is further complicated by the observation that variations in ionic concentrations in vivo can affect the synthesis of some peptides but not others (Nuss and Koch,1976). In fact, the maximum synthesis of preproalbumin in a wheat-germ system occurs at K^+ and Mg^{2+} concentrations much higher than those which give maximum TCA-precipitable counts (Tse and Taylor, 1977).

It is apparent, from these reports, that cell-free, protein synthesising systems may give rise to artifacts if it is not fully characterised. This characterisation essentially involves showing that the system will faithfully translate the added mRNAs into defined products. The effect of changes in the ionic conditions on the spectrum of proteins synthesised in response to a particular mRNA population should also be investigated.

The fidelity of translation by the wheat-germ preparations used during this project was tested using known mRNAs. RNA isolated from embryos of Xenopus laevis was translated into a range of products, amongst which were polypeptides which co-electrophoreses in two dimensions with authentic Xenopus laevis histones(Ballentine and Woodland, personal communications). The 26S RNA of Semliki Forest Virus (Clegg et al, 1976) was translated by the wheat-germ system into its authentic gene products, the viral structural polypeptides (Kennedy, personal communication). Finally, RNA isolated from

chick embryo kidney cells directed the synthesis of a wide range of products, amongst which was actin (Morser and Lomniczi, personal communication; see also Highfield et al, 1978). As mentioned above (section III1Aiii) TMV virion RNA gives a pattern of products similar to that obtained in other wheat-germ systems (Roberts et al, 1973; Hunter et al 1977). The system would thus appear to be capable of faithful translation.

The effects of ionic conditions and capping upon translation must be investigated using the particular RNA preparations under study. So, whilst the characteristics of RNA isolated from pea leaf polysomes are discussed in section III1B, it is felt that certain aspects of the translation of this RNA should be discussed here because they relate to the use of the wheat-germ system.

The wheat-germ system can cap added mRNA (Both et al, 1975). Only those mRNAs which have capped 5'-termini in vivo are capped by wheat-germ extracts (Muthukrishnan et al, 1975). Satellite tobacco necrosis virus RNA is neither capped in vivo, nor when added to a wheat-germ extract (Leung, 1976). The methyl donor is S-adenosyl methionine (SAM) and S-adenosyl-homocysteine (SAH) acts as a competative inhibitor of the reaction (Muthukrishnan et al, 1975). The wheat-germ extract seems to contain endogenous SAM, but exogenous SAM will cause an increase in the capping activity (Both et al, 1975). If an mRNA, which required a fully capped 5'-terminus for proper functioning, is isolated in an uncapped state, then the addition of SAM to the wheat-germ system will stimulate its translation but the addition of SAH will inhibit. It is thus possible to test whether or not an RNA preparation contains such an mRNA by looking at the effect of both SAM and SAM plus excess SAH,

RNA	incorporation (cpm/2µl)			stimulation over endogenous		
	control	+SAM	+SAM +SAH	control	+SAM	+SAM +SAH
TMV	117,500	97,000	92,500	56.2	78.3	79.0
EMC	9,180	7,150	6,500	4.4	5.8	5.5
poly(A)-RNA	47,900	41,000	34,600	22.9	33.1	29.6
-RNA	2,090	1,240	1,170			

RNA	incorporation (cpm/2µl)			stimulation over endogenous		
	control	+SAM	+SAM +SAH	control	+SAM	+SAM +SAH
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EMC	9,180	7,150	6,500	4.4	5.8	5.5
poly(A)-RNA	47,900	41,000	34,600	22.9	33.1	29.6
-RNA	2,090	1,240	1,170			

dogenous

+SAM

+SAH

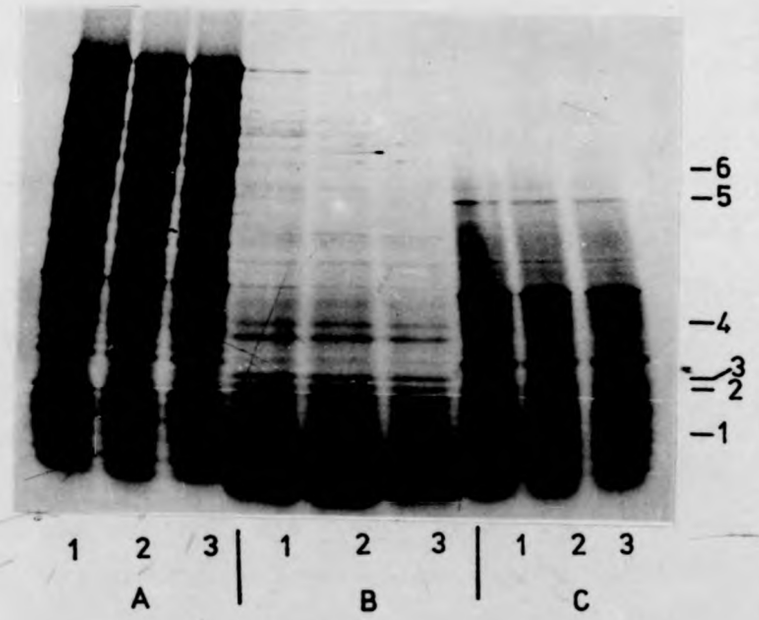
79.0

5.5

29.6

Table III The effect of SAM and SAH on the translation of different mRNAs.

Wheat-germ reaction mixtures (20µl) as described in section II2Jiia, contained 110mM K^+, 2.5mM Mg^{2+}, 50µM spermine, 250µM spermidine, 4µCi [^{35}S]-methionine plus TMV RNA (6µg), poly(A)-RNA (3µg) or EMC RNA (1µg) as indicated. Some incubations contained SAM (4µM) or both SAM (4µM) and SAH (1.6mM). The incorporation was measured by determining the TCA-precipitable radioactivity in 2µl aliquots. The stimulation is expressed relative to the appropiate minus-RNA control. Zero-time values of 900 cpm were subtracted from all the incorporations.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1 2 3 1 2 3 1 2 3
A
B
C

Figure 6 Effect of SAM and SAH upon the products of translation of various RNAs.

A wheat-germ cell-free system was set-up as described in section II2Jiia. The final concentrations of K^+, Mg^{2+}, spermidine and spermine were as in Fig.5. Each incubation contained, in a final volume of 20µl, 4µCi [^{35}S]-methionine and the indicated amount of each RNA. Some incubations contained SAM (4µM) or SAM plus SAH (1.6mM) as indicated.

A. TMV RNA (6µg) 1. control
2. added SAM
3. added SAM plus SAH

B. EMC RNA (1µg) 1,2 and 3 as above.

C. Poly(A)-containing RNA from etiolated pea shoots after 24 hours in continuous light (3µg), 1,2 and 3 as above.

Each set of three tracks contained the equivalent of either 2µl of reaction mixture (A&C) or 10µl (B). The molecular weight markers are as in Fig. 5.

on the products of translation.

Table III shows the results of such an experiment on poly(A)-containing RNA isolated from pea leaf polysomes. TMV RNA is capped (Keith and Fraenkel-Conrat, 1975) and EMC is uncapped (Frisby et al. 1976) yet both are translated in the wheat-germ system. These RNAs should be unaffected by any changes in the capping activity of the system and so act as controls. The presence of either SAM or both SAM and SAH resulted in a slight drop in the total counts incorporated by all the RNAs; each was affected to the same extent when expressed as a percentage. The endogenous incorporation was also inhibited and so the stimulation by added RNA was actually increased. Since the translation of all the RNAs was equally affected, neither effect can be due to changes in capping activity.

The poly(A)-containing RNA was isolated from polysomes and so there must be a large number of different mRNAs present. It might be that only some of them require capping and methylation for translation and so there would not be any detectable change in total incorporation. The products of the wheat-germ incubations were analysed on polyacrylamide gels (section II2EiC). Figure 6 shows an autofluorograph of the products from each RNA. Each set of tracks for a particular RNA contained equal TCA-precipitable counts so that if the synthesis of some polypeptides were stimulated or inhibited by the addition of SAM, or SAM plus SAH, there would be a change in the intensity of some bands. Even with a much longer exposure than that shown, it was not possible to detect any qualitative or quantitative changes in the pattern of products given by any RNA under the different conditions of

on the products of translation.

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incubation.

These results do not permit any conclusions to be drawn about the 5' termini of the polysomal, poly(A)-containing RNAs. They may be either capped or uncapped. However, it is clear that the translation of these RNAs is not affected by any changes in the ability of the wheat-germ system to carry out capping reactions. Since the RNA was isolated from polysomes, it seems unlikely that it is necessary to modify it before it can be translated in a cell-free system.

According to the paper of Lodish (1974), which analyses the effect of partial inhibitions of the various steps in protein synthesis on the relative rates of translation of different mRNAs, some mRNAs are translated more often than would be expected on the basis of their concentration. This situation arises when there is competition between messengers for factors required for initiation; some mRNAs are initiated more efficiently than others. A consequence of this effect is that the pattern of products obtained in such a situation does not reflect the actual composition of the mRNA population. Lodish used the α and β- globin mRNAs as examples in his model, and other systems have been found which behave as he predicted (Nuss and Koch, 1976). Under conditions of saturating RNA in a cell-free system there may well be competition for initiation factors, and so the pattern of polypeptides obtained would over-emphasise the efficiently initiated mRNAs, and not represent the mRNA population as a whole. To test for this effect, TMV RNA and polysomal poly(A)-containing RNA were translated in the wheat-germ system at about 1µg/ml concentrations. This is at least 100-fold lower than the saturating concentration and so might be expected to give a representative spectrum of products.

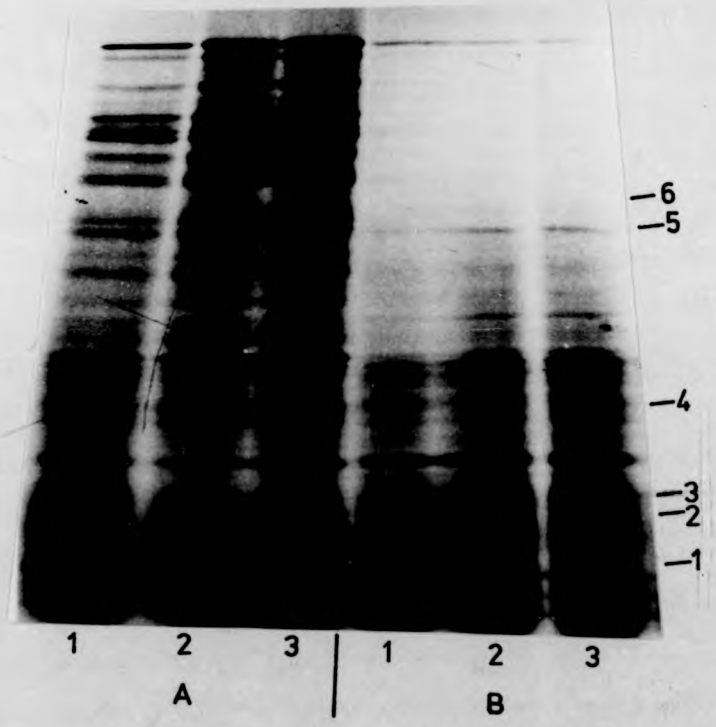
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Figure 7 Products of translation of TMV RNA and pea polysomal poly(A)-containing RNA at low RNA levels.

TMVRNA and pea polysomal poly(A)-containing RNA from etiolated tissue greened for 24 hours in continuous light, were translated in a wheat-germ protein synthesis system (section II2Jiia). Each incubation mixture was as described in Fig. 5, the final volume was 20µl.

A. TMV RNA 1. 0.04µg added RNA
2. 0.08µg added RNA
3. 0.12µg added RNA

B. Poly(A)-containing RNA 1. 0.02µg added RNA
2. 0.04µg added RNA
3. 0.06µg added RNA

Each track was loaded with either 50,000cpm (A) or 25,000cpm(B) and the autofluorograph exposed for seven days. The molecular weight markers were the same as in Fig. 5.

Figure 7 shows that the pattern of products obtained with the low concentration of each RNA was the same as at saturating RNA concentration (compare with Fig. 7 with Fig. 6). This RNA concentration was as low as it was feasible to go; at 1μg/ml each RNA stimulated incorporation by only 2-fold. Thus, within a 100-fold range of RNA concentrations, the products of poly(A)-RNA in the wheat-germ system were the same. This result implies that, at saturating concentrations of RNA, the wheat-germ does not selectively translate some mRNAs.

Finally the effect of varying the K^+ concentration on the products of translation was investigated. Poly(A)-containing RNA was translated in the wheat-germ system at K^+ concentrations ranging from 30mM to 180mM. The total TCA-precipitable radioactivity incorporated gave a curve practically identical to that shown in Fig. 4B. The products were analysed on gels and Figure 8 shows the result of this analysis. Equal counts were loaded onto each track and so, if any mRNA had optimum translation at a K^+ concentration different from the other mRNAs, this would be reflected in a change in the relative intensities of the bands. This does not occur and so it can be concluded that all the mRNAs in the RNA preparation have optimal translation at the same K^+-concentration i.e. 110mM.

The results described in this section establish that the wheat-germ system used in this work is capable of translating poly(A)-containing RNA from pea leaf polysomes into a range of discrete polypeptides. These polypeptides differ, in terms of electrophoretic mobility on SDS-polyacrylamide gels, from those obtained by translation of TMV RNA, EMC RNA and 26S SFV RNA; therefore they are a feature of the added mRNA and not

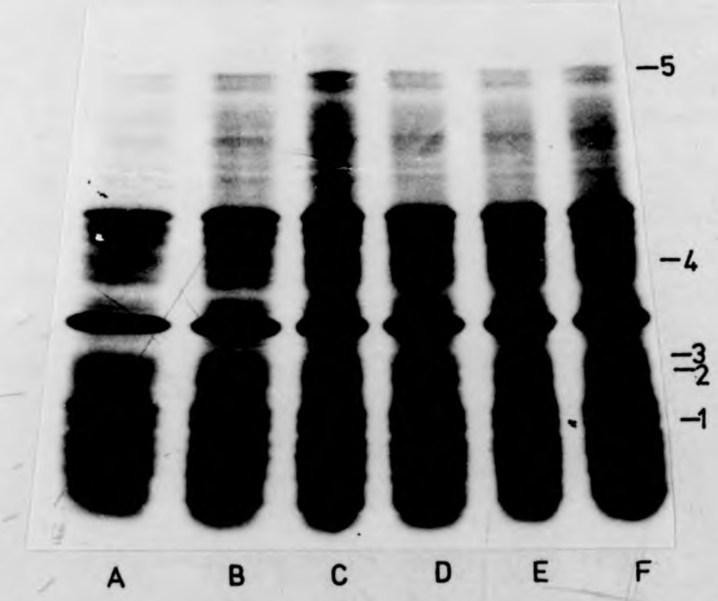
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Figure 8 Effect of K^+-concentration on the products of translation of pea shoot poly(A)-containing RNA.

Polysomal poly(A)-containing RNA from etiolated tissue which had greened for 36 hours in continuous light was translated in a wheat-germ cell-free system as described in section II2Jiia. The final volume of each incubation mixture was 20ul and contained the following:-
all the components as indicated in Fig. 5 except that there were 3µCi [^{35}S]-methionine and 3µg RNA per incubation; the K^+-concentration was as
A. 30mM B. 60mM C. 90mM D.120mM E. 150mM
F. 210mM

Each track contained 50,000 cpm and the autofluorograph was exposed at -70°C for six days. The molecular weight markers are as in Fig. 5.

of endogenous incorporation by the system. The pattern of products has been shown to be independent of the conditions of assay, which makes the system very useful for the study of pea polysomal mRNAs.

B. Characteristics of RNA isolated from pea leaf polysomes.

In the previous section it was shown that polysomal RNA from pea leaves could be translated into a series of polypeptides by the wheat-germ system. In this section it is intended to describe the procedures used to isolate these cytoplasmic leaf polysomes and to prepare from them functional mRNA. The RNA was characterised with respect to some physical properties, as well as by its ability to stimulate incorporation in the wheat-germ system.

(i) Isolation of polysomes.

There are many published methods describing the isolation of polysomes from a variety of tissues e.g. Palmiter and Schimke (1973) and Gielkens et al (1971). All methods must aim to reduce any potential RNAase activity to a minimum and to keep the polysomes structually intact. This is especially important when the RNA from the polysomes is to be assayed for messenger activity; any nicks, however few, introduced into the RNA could completely destroy its ability to programme the synthesis of a defined polypeptide.

A method has been published which was developed especially for the isolation of polysomes from pea shoots (Davies et al, 1972). The potential RNAase activity is reduced by using a high buffer concentration and pH i.e. 200mM tris-HCl, pH 8.5. This method was modified slightly, and used throughout this

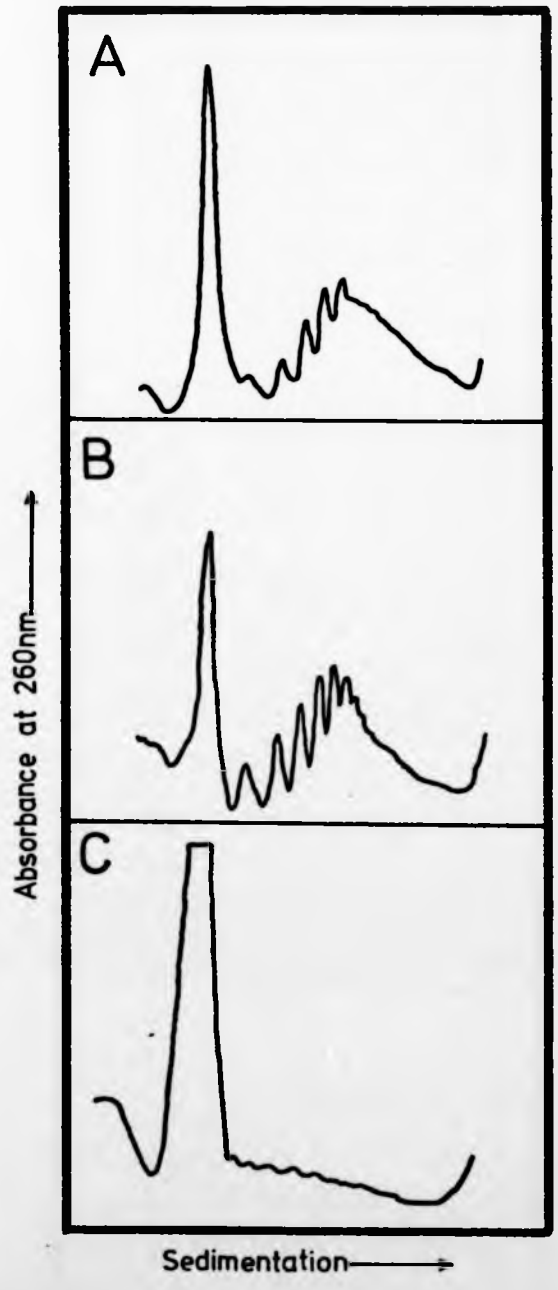
A
B
C
Absorbance at 260nm
Sedimentation

Figure 9. Analysis of pea leaf polysomes by sedimentation through sucrose gradients.

Polysomes were isolated from 10 day old pea leaves either as described by Davies *et al* (1972) or using the modified method described in section II2Fi. Aliquots (5 E_{260} units) of these polysomes were analysed on 12.5% to 50% linear gradients of sucrose concentration as described in section II2Fii, centrifugation was for 75 minutes at 205,000g_{ave}.

A. Polysomes isolated as described by Davies *et al* (1972).

B. Polysomes isolated by the modified method.

C. Polysomes incubated for 10 minutes at 37°C with 50µg/ml of RNAase A.

work. The modifications are described in section II2Fi. The basic criterion which was used to compare extraction methods was the size of polysomes obtained. The polysome profiles were analysed by velocity centrifugation in sucrose density gradients. The larger the size of polysomes seen on the gradient the less they are assumed to be degraded.

Figure 9 shows the absorbance profiles obtained for polysomes isolated from 10 day old pea shoots. Comparing Fig.9A with 9B we can see that the modifications introduced into the extraction procedure result in increased numbers and sizes of polysomes. In Fig. 9A the highest polysome size distinguishable as a discrete peak contains hexamers, whereas in Fig. 9B octamers can be seen. In addition, a higher proportion of the 260nm-absorbing material appears to be in the polysome, as opposed to monosome, part of the gradient when the modified method is used. Incubation of a preparation of polysomes with RNAase A (50µg/ml) at 37°C for 30 minutes reduces all the polysomes to monosomes (Fig. 9C); this confirms that the profiles obtained were due to polysomes.

(ii) Properties of the RNA isolated from polysomes.

a) Total polysomal RNA.

Polysomes were used as the starting material for all RNA isolations during this work. Attempts to isolate active RNA from whole tissue were unsuccessful. Total nucleic acids were isolated from pea shoots by the Kirby-phenol method as described by Hartley and Ellis (1973). The DNA was removed by digestion with RNAase-free DNAase (Worthington). The RNA obtained in this way proved to be contaminated with starch, and some preparations contained dark-coloured residues which


-6
-5
-4
-3
-2
-1
A B

Figure 10 Comparison of different RNA extraction procedures by translation in the wheat-germ system.

Polysomes were prepared from 10 day old pea shoots (section II2Fi) and one half of the preparation was extracted with phenol (section II2Gi), the other half was treated as described in section II2Gii. The polysomal RNA preparations so obtained were translated in the wheat-germ cell-free system (section II2Jiia). Each incubation mixture (20µl) contained 10µCi [^{35}S]-methionine and 4µg of one of the RNA preparations. Each track contained 100,000cpm and the autofluorograph was developed for 2 days at -70°C.

A. Phenol-extracted RNA

B. RNA prepared by the LiCl-precipitation procedure

Molecular weight markers as in Fig. 5

could be traced to the Kirby-phenol reagent. All such RNA preparations were inactive in the wheat-germ system.

Polysomes offer two advantages for the isolation of RNA with messenger activity. The first is that the RNA is associated with protein during homogenisation and so has a certain amount of protection from nucleases. This means that harsh nuclease inhibitors such as TNS do not have to be used; there is also less protein to be removed in the subsequent phenol extractions, when polysomes are used instead of whole cells. The second advantage is that any RNA found in polysomes can reasonably be assumed to have been engaged in protein synthesis *in vivo*. Therefore, it ought to be active in a suitable *in vitro* protein synthesising system.

RNA was extracted from the polysomes using phenol/SDS as described in section IIG(i). The extraction was performed at pH 9.0 to improve the recovery of poly(A)-containing RNA (Brawerman, 1974). It was always found necessary to extract the RNA with diethyl ether to remove all traces of phenol.

All SDS must be removed from the RNA before assaying for messenger activity in the wheat-germ system. The RNA was taken through two cycles of ethanol precipitation in the absence of SDS. The final ethanol precipitation was from 0.25M HEPES-KOH pH 7.6. Finally the RNA was dissolved in sterile water and was stored at -70°C.

The products of the translation of total polysomal RNA in the wheat-germ system are shown in Figure 10 (track A). The RNA usually stimulated incorporation by 15-20 fold at saturation (about 500µg/ml). The products range in molecular weight from 8,000 to 60,000; two bands at around 20,000 and 30,000 are prominent. The endogenous products could never be

seen when an equal volume of all incubation mixtures were loaded onto the gel. The characteristics of the products given by the RNA are described in section III2. For the purposes of the rest of this section the pattern of products, as shown in Fig. 10, will be used as a standard by which to judge the template properties of RNA preparations.

The pattern of <u>in vitro</u>-synthesised polypeptides shows a preponderance of bands between 10,000 and 20,000 in molecular weight; there is relatively little incorporation into polypeptides larger than 30,000. Therefore it was necessary to investigate the integrity of the RNA used. The wheat-germ system has been shown capable of synthesising high-molecular weight polypeptides in response to TMV-RNA (section III1Aiii) so it might be that the RNA was degraded. This possibility can be checked by electrophoresis on polyacrylamide gels (section II2Eii). Figure 11A show a scan, at 260nm, of a cylindrical gel containing total polysomal RNA; the sample had been heat-denatured before loading to show up any nicks in the RNA. The rRNAs, derived from both cytoplasmic and chloroplast ribosomes, are the most prominent peaks of absorbance. It must be remembered that it is not possible to check the integrity of the messenger RNA molecules themselves. The mRNA species represent only 1-2% of the polysomal RNA and are heterogeneous in size; thus they cannot be expected to be seen in Fig. 11A. Therefore for practical purposes, it is assumed that if any degradation of the RNA had taken place it would affect both rRNA and mRNA equally. This assumption means that any activity which nicked the mRNA molecules will also nick the rRNA molecules, and so change the absorbance profile of the gel.

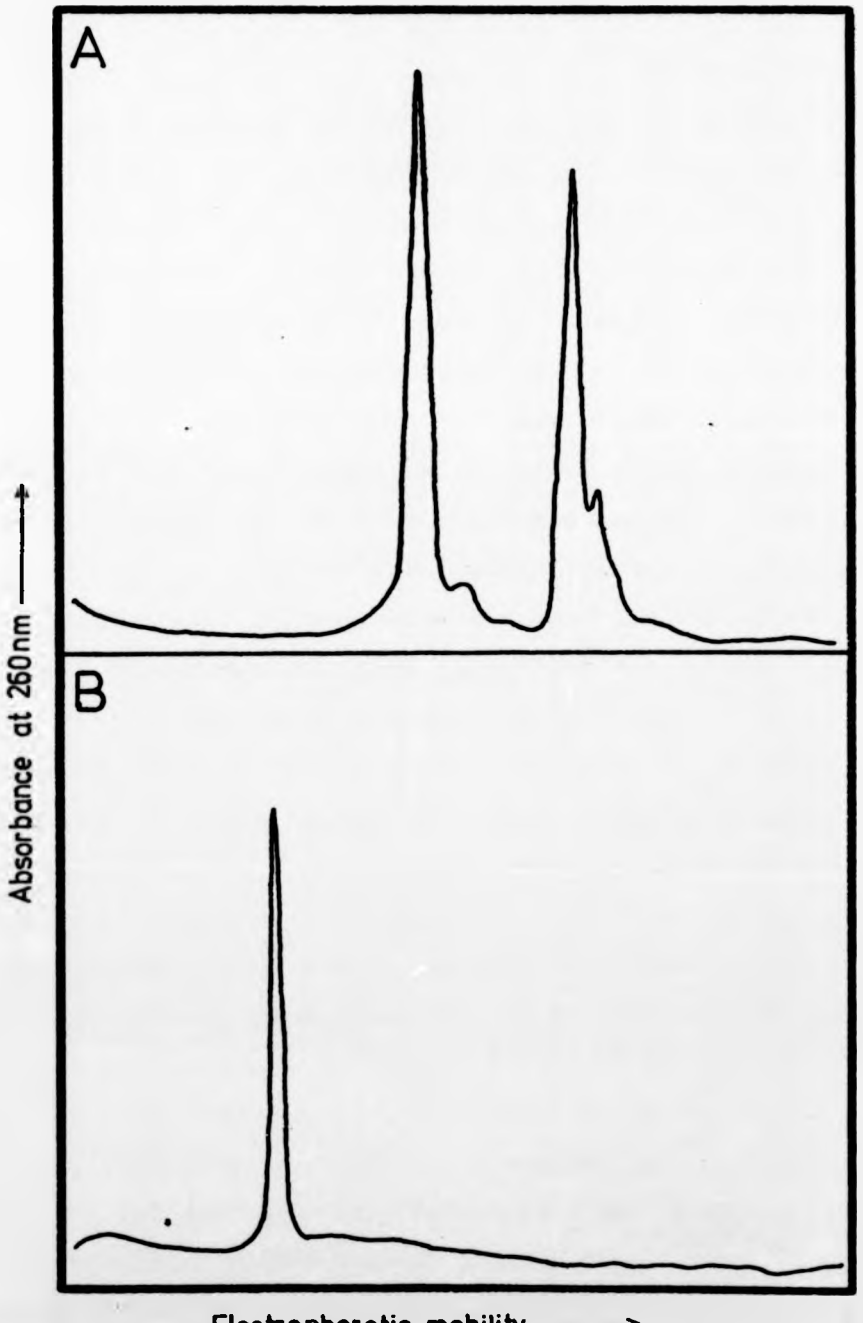
A
B
Absorbance at 260nm
Electrophoretic mobility

Figure 11. Electrophoretic analysis of RNA.

Total polysomal RNA or TMV RNA was analysed by electrophoresis for 4 hours at 50V (constant) on 2.4% polyacrylamide gels (section II2Eii). The samples were denatured by heating to 60°C for 2 minutes in 8M urea, 5mM tris-acetate, pH 7.8, 2.5mM sodium acetate, 0.5mM EDTA and 15% (w/v) sucrose before loading onto the gels. The gels were scanned at 260nm in a Gilford gel scanner.

A. Total polysomal RNA (50μg)

B. TMV RNA (20μg)

rRNA	units of rRNA[1] initial	expected[2]	corrected	ratio
large cytoplasmic	17.0		17.0	
small cytoplasmic	12.0		8.7	1.95
large chloroplast	0.6	6.0	6.0	
small chloroplast	3.0		3.0	2.0

1. based on the area of the peaks of the individual rRNAs

2. assuming that the ratio of large to small chloroplast rRNAs is 2.0

atio

1.95

2.0

As is 2.0

Table IV Ratios of rRNAs in total polysomal RNA.

Polysomal RNA from pea shoots was analysed by electrophoresis on 2.4% polyacrylamide gels (section II2Eii) and the gels were scanned at 260nm as described in Figure 11. The areas under the various peaks seen in the scan of the gel (Fig 11A) were measured by cutting out and weighing the paper and the units are milligrams. Each value is the average of six determinations. From the value for the small chloroplast rRNA, it is possible to estimate the amount of chloroplast large rRNA which should be present based on the relative molecular weights (0.56×10^6 and 1.1×10^6). The discrepancy (5.4) between the expected and actual values for this RNA is assumed to be due to breakdown of the RNA into fragments of 0.7 and 0.4×10^6 molecular weight. This extra RNA present in the peak of the small chloroplast rRNA must be subtracted. This extra RNA is calculated as follows:-

5.4 ("lost" 1.1×10^6 rRNA) x 0.7/1.1 (relative molecular weights) = 3.3 units

This is subtracted from the cytoplasmic small rRNA value to give the corrected value.

The profile shown in Figure 11A does not support this assumption. Four RNA species can be seen but it would be expected that any nuclease activity would give rise to additional absorbance peaks or, at least, produce shoulders on the existing peaks. The relative proportions of the various rRNAs are as expected. The large rRNA from chloroplast ribosomes is known to be nicked *in vivo* and gives rise to two fragments of 0.4×10^6 and 0.7×10^6 molecular weight (Ingle *et al*, 1971). The larger fragment co-migrates with the cytoplasmic small rRNA and so reduces the ratio of large to small cytoplasmic rRNAs. It is possible to apply a correction to this ratio because the small rRNA of the chloroplast is not degraded, and so the breakdown of the larger chloroplast rRNA can be quantified. Table IV shows that when this correction is applied, the ratio of large to small cytoplasmic rRNA is as expected. Thus, it is highly unlikely that any extensive degradation has occured with the rRNAs; it is assumed that this conclusion also applies to the mRNAs.

The TMV RNA used in all this work was isolated from TMV virions and prepared for addition to the wheat-germ system by procedures similar to those used to prepare the polysomal RNA from pea shoots. This RNA, when analysed on polyacrylamide gels, gave a single, symmetrical absorbance peak (Fig. 11B), indicating that it was not degraded. This RNA gave a pattern of products in the wheat-germ system similar to those obtained by other workers (e.g. compare Fig. 6 with Fig. 2 in Hunter *et al*, 1977).

b) Translation of poly(A)-containing RNA from polysomes

As discussed in section I5B it is often assumed that all cytoplasmic mRNAs, apart from those coding for histones,

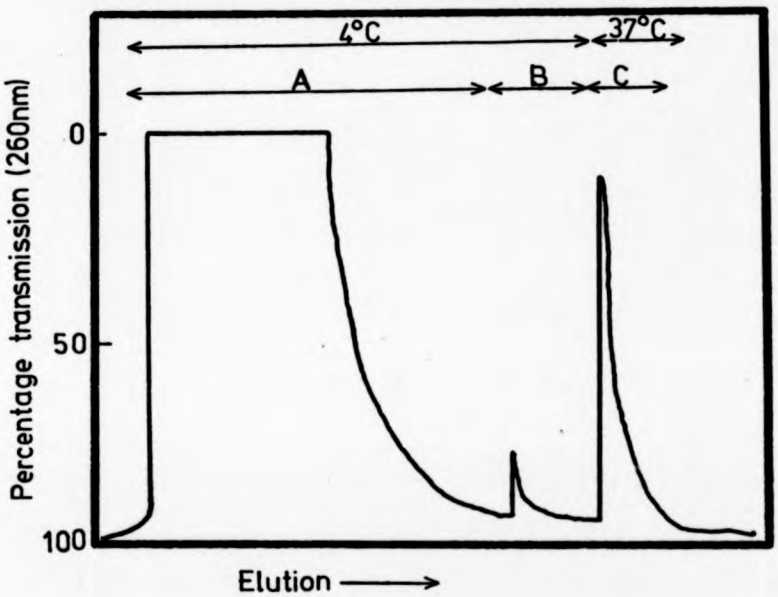
4°C
37°C
A
B
C
Percentage transmission (260nm)
0
50
100
Elution

Figure 12. Chromatography of polysomal RNA on oligo-dT cellulose.

Total polysomal RNA, isolated from 10 day old etiolated tissue which had greened for 16 hours, was resuspended, at a concentration of 10 E_{260} units/ml, in buffer A (10mM tris-HCl, pH 7.6, 0.4% SDS, 0.4M LiCl). The sample was loaded onto a column containing 1g of oligo-dT cellulose and washed through, at 4°C, with buffer A. The column was subsequently washed with buffer B (10mM tris-HCl, pH 7.6, 0.1M LiCl, 0.4% SDS) at 4°C and then the temperature raised to 37°C before elution of the bound RNA with buffer C ("no-salt" buffer, 10mM tris-HCl, pH 7.6, 0.4% SDS). The transmission at 260nm was monitored continuosly using an LKB Uvicord. The total loading was 185 E_{260} units.

contain a tract of polyadenylate at their 3'-ends. This poly(A)-tail is used, in all procedures for isolating specific mRNAs, as a convenient handle to separate mRNA molecules from the total RNA (section 15D). Such poly(A)-tails have been found on RNA from plants (Higgins et al, 1973; Sagher et al, 1974) and, in most of the work presented in this thesis, the poly(A)-containing RNA fraction was used.

The polysomal RNA was chromatographed on oligo-dT cellulose as described in section II2Hi. Figure 12 shows a typical elution profile. The material which was eluted with the "no-salt" buffer was regarded as poly(A)-containing RNA, while that which did not bind to the column was regarded as non-poly(A)-containing RNA. The question of the actual poly(A) content of the various fractions is discussed in section III1Biv. The material which did not bind to the column was re-chromatographed to ensure that its failure to bind was not just due to saturation of the oligo-dT sites. It was found that the material eluted by 0.1M LiCl was a specific sub-population of RNA molecules. When the non-bound material was re-chromatographed, there was no material which subsequently eluted at 0.1M salt. Now it might be that the 0.1M-salt-wash material arose because of binding of rRNA to poly(A)-containing RNA which was bound to the column via its poly(A)-tail. Such binding might be disrupted by lowering of the salt concentration from 0.4M to 0.1M. On re-chromatography there would not be any poly(A)-containing RNA present and so there would be nothing for the rRNA to associate with. However, there is evidence that the 0.1M salt-wash-RNA contains some poly(A)-containing RNA. This evidence is discussed in section III1Biv.

Both the poly(A)-containing and non-poly(A)-containing RNA

fractions were translated in the wheat-germ system, and the products of synthesis analysed by electrophoresis on SDS-polyacrylamide slab gels and autoradiography. It was found that the patterns of products obtained were indistinguishable by one-dimensional electrophoresis, but that the poly(A)-containing RNA was much more active as a template. Activity is defined as the total incorporation in counts per minute per microgram of added RNA at sub-saturating levels of RNA. By this criterion, the poly(A)-containing RNA was never less than 40 times more active than the non-poly(A)-containing RNA. Although poly(A)-tails are regarded as diagnostic of an mRNA the converse does not seem to be true. In fact, other workers have noticed that the two types of RNA, when isolated from pea shoots, give practically identical patterns of products, both qualitatively and quantitatively, when translated in the wheat-germ system (Gray and Cashmore, 1976). This has also been found for other plants (Ragg et al, 1977) and some animal systems (Lodish et al, 1974). However, with some tissue culture cells, major differences can be found in the products of the two types of RNA (Sonenshein et al, 1976), and hybridization data would suggest that non-poly(A)-containing RNA does contain sequences different from those found in poly(A)-containing RNA (Nemer et al 1974, Milcarek et al, 1974). It must be pointed out that the definition of non-poly(A)-containing RNA is lack of binding, at high LiCl concentrations, to oligo-dT cellulose. Evidence is presented in section III1B(iv) which suggests that lack of binding to oligo-dT cellulose does not indicate the complete absence of poly(A)-tails, but rather that the poly(A)-tails are of insuffient length to bind to the oligo-dT. On this basis it is easier to understand the similarity of products;

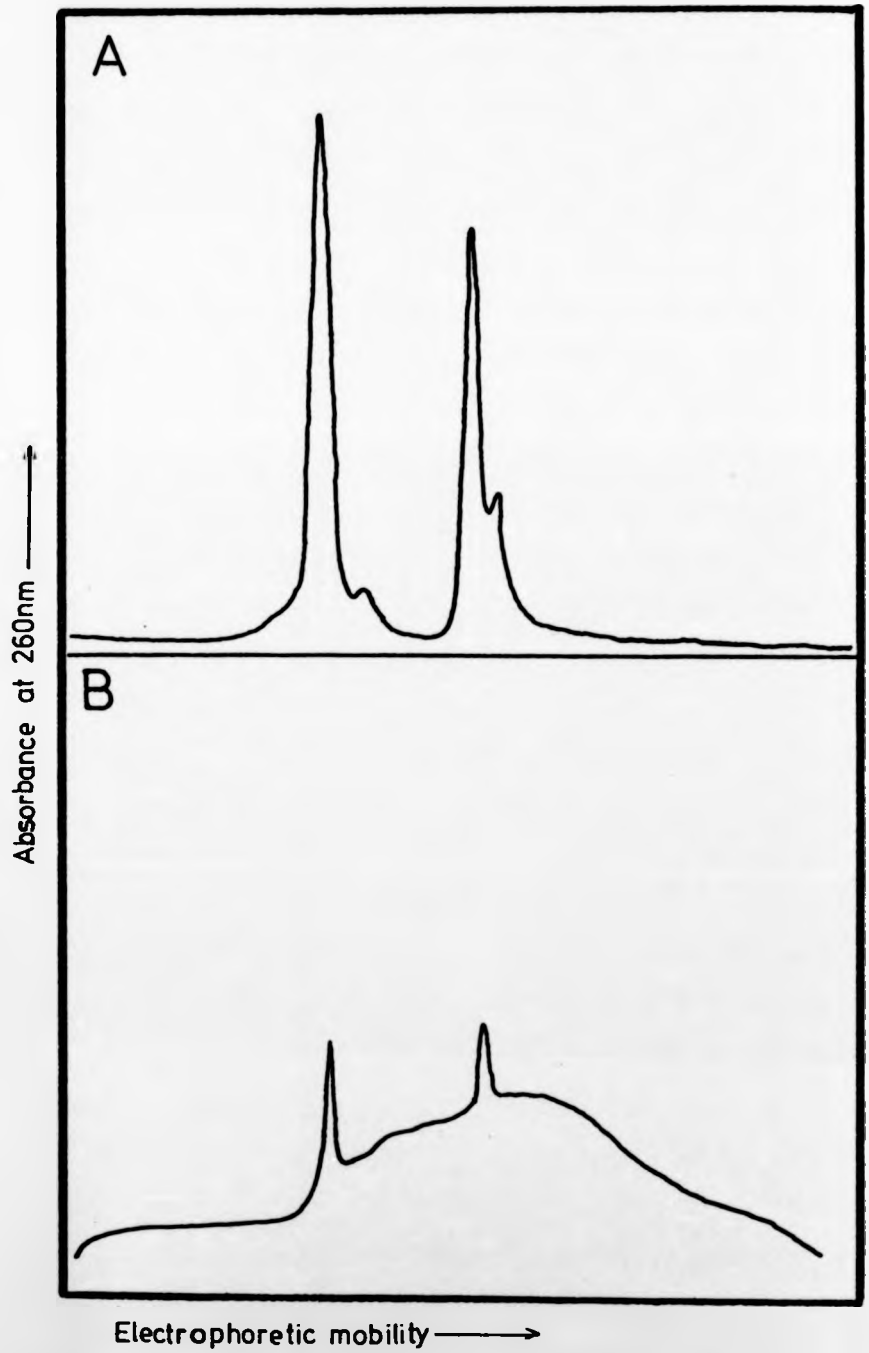
A
B
Absorbance at 260nm
Electrophoretic mobility

Figure 13. Electrophoretic analysis of RNA after chromatography on oligo-dT cellulose.

The poly(A)-containing or non-poly(A)-containing RNA samples obtained from Fig. 12, were analysed on polyacrylamide gels as described in Fig. 11.

A. Non-poly(A)-containing RNA (50μg)

B. Poly(A)-containing RNA (40μg), the scale was expanded two-fold relative to A.

the non-poly(A)-containing RNA fraction probably contains all the mRNAs present in the poly(A)-containing RNA, but they have shorter poly(A)-tails and are present in lower amounts. Such mRNAs could arise by the shortening of the poly(A)-tails, a process known to occur *in vivo* (Sheiness and Darnell,1972). The difference in activities of the two RNA fractions probably *does* reflect the lower concentration of mRNA in the non-poly(A)-containing RNA. The shortening or removal of poly(A) from ovalbumin mRNA has been shown to have no effect on its translation in a wheat-germ system (Doel and Carey, 1976).

The poly(A)-containing and non-poly(A)-containing RNAs were analysised by electrophoresis on polyacrylamide gels (section II5b), and Figure 13 shows the results. The poly(A)-containing RNA still contains some rRNA which arises because of aggregation between rRNA and mRNA (Bantle *et al*, 1976; Kabat, 1975). The non-poly(A) containing RNA contains only rRNA, as far as can be determined. The poly(A)-containing RNA has the rRNA superimposed on a heterogeneous background of absorbing material. This is the observation expected of a mRNA population which consists of a large number of different sequences present in varying amounts.

It was thought valid to regard the poly(A)-containing RNA as largely messenger RNA for a variety of reasons:-

1) the RNA exhibited a heterogeneous molecular weight distribution as expected for a population of mRNAs,

2) the products of translation, as analysed by one-dimensional SDS, gel-electrophoresis, were the same as those of total polysomal RNA.

3) the RNA had a much higher specific activity than total polysomal RNA,

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2) the products of translation, as analysed by one-dimensional SDS, gel-electrophoresis, were the same as those of total polysomal RNA.

3) the RNA had a much higher specific activity than total polysomal RNA,

4) the residual messenger activity present in the non-poly(A)-containing RNA could easily be due to a limitation of the technique used to separate the two RNA fractions. This interpretation would explain the extreme similarity of the products of the two RNA fractions.

The products of translation of the non-poly(A)-containing RNA were routinely examined throughout this work but were found to be absolutely identical to the poly(A)-containing RNA-directed products.

It was decided to test an alternative method of extracting RNA from polysomes, in case the phenol extraction procedure was being selective in its effect. As part of the experiments described in section III4A, it also proved necessary to extract RNA from solutions containing heparin; phenol extraction does not remove heparin, which acts as a potent inhibitor of protein synthesis (Schimke et al, 1974). Heparin is not soluble in 2M LiCl and so the procedure outlined in section II2Gii was tested.

Polysomes were prepared, as usual, from 10 day old pea shoots and one half of the preparation was phenol-extracted while the other half was extracted with LiCl as described in section II2Gii. The yield of RNA was better using the LiCl-procedure compared with the phenol-procedure (89% yield compared with 75%). The main reason for this could be that with phenol extractions there are losses at the phenol/buffer interface, whereas the LiCl method consists of a series of precipitations which should be almost quantitative. The two RNA samples were translated in the wheat-germ system; that prepared by the LiCl-procedure had a higher specific activity than the phenol-extracted RNA (120,000 cpm/μg compared with

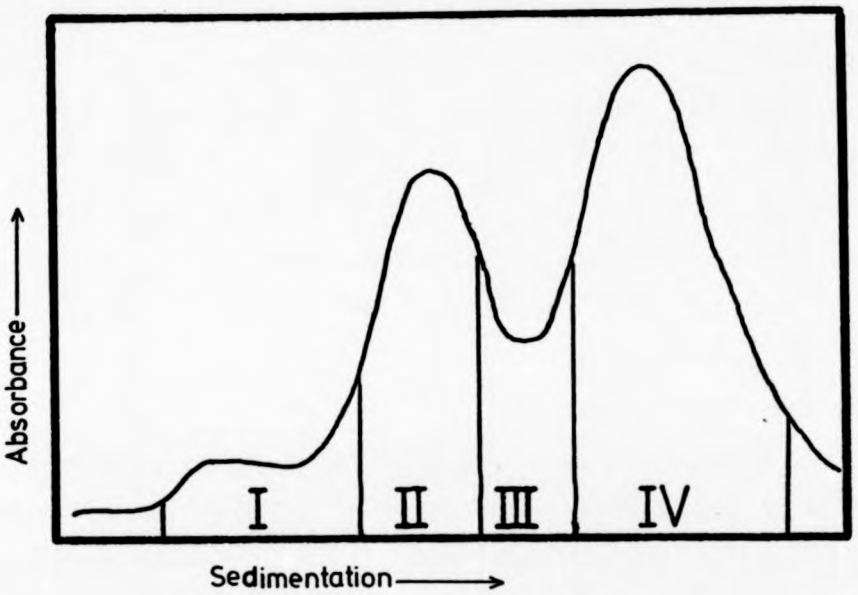
Absorbance
I
II
III
IV
Sedimentation

Figure 14. Fractionation of total polysomal RNA by sedimentation through a sucrose gradient in a zonal rotor.

Total polysomal RNA (500 E_{260} units) from etiolated pea leaves, which had greened for 48 hours continuously, was centrifuged through a 7% to 25% sucrose gradient on the zonal rotor XVB, as described in section II2Iii. The gradient was unloaded and monitored continuously at 260nm using an LKB Uvicord. The RNA was divided into four fractions, I to IV, as shown.

75,000 cpm/μg).

Figure 10 shows an analysis of the products directed by the two RNA samples; equal counts have been loaded onto each track for ease of comparison. It can be seen that the products of each RNA are identical. Thus the increased activity of the LiCl-prepared RNA was due to the wheat-germ system translating it more efficiently than the phenol-extracted RNA, not because it contained additional different messenger sequences. Since the products synthesised in response to the two RNA preparations were the same, this would imply that the pattern obtained was not just a result of the particular method of preparation used.

The LiCl-procedure gave high yields of RNA which was very active in the wheat-germ system; it was used on many occasions to prepare RNA. However, it became apparent that the RNA prepared this way was highly aggregated. Whenever RNA prepared by the LiCl-procedure was chromatographed on oligo-dT cellulose, the bound material accounted for about 5-6% of the total 260nm-absorbing material loaded onto the column. This is to be compared with the 2-3% binding obtained with the phenol-extracted RNA. When the poly(A)-containing RNA from LiCl-prepared RNA was analysed on gels, it would seem that a higher proportion of rRNA was present compared with that seen in phenol-extracted RNA.

When a large amount of LiCl-prepared polysomal RNA was fractionated on a density gradient in a zonal rotor (section II2Iii), it was found that all the messenger activity was associated with the ribosomal RNA peaks. Figure 14 shows the separation of the RNA on the gradient. This result was obtained in a preliminary experiment with a view to isolating preparative

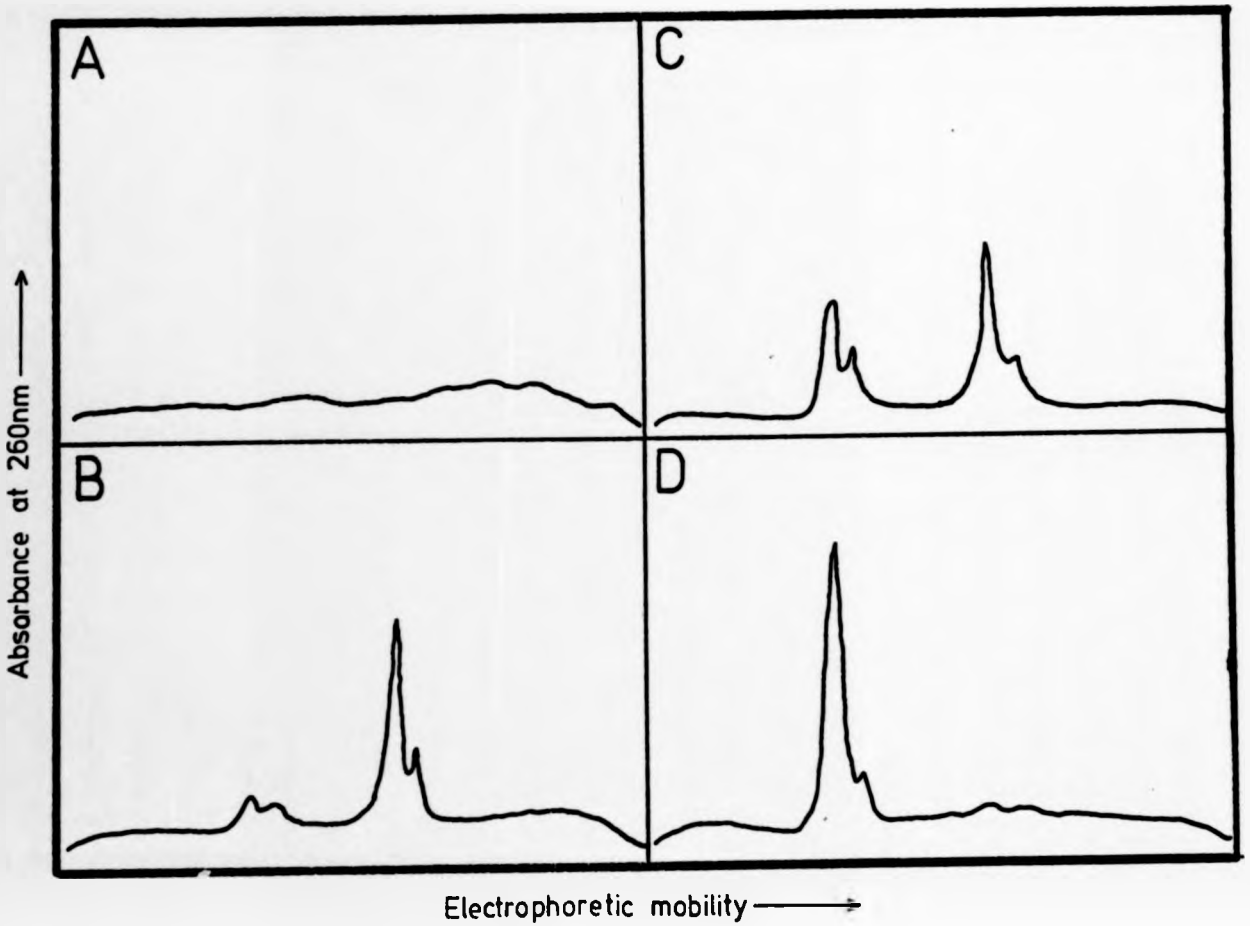
A
C
B
D
Absorbance at 260nm
Electrophoretic mobility

Figure 15. Electrophoretic analysis of RNA after fractionation on a zonal sucrose gradient.

The fractions obtained by sedimentation of total polysomal RNA on a sucrose gradient in the zonal rotor (XVB) as described in Fig. 14, were electrophoresed on 2.4% acrylamide gels as described in Fig. 11.

A. Fraction I

B. Fraction II

C. Fraction III

D. Fraction IV

amounts of specific mRNAs; the aggregation of the RNA was not suspected at this stage. The centrifugation was not long enough to achieve good separation of the two rRNAs; the large rRNA had moved only half-way down the gradient. Neverless the gradient was split into four fractions as shown in Fig.14. and the RNA recovered by ethanol precipitation. Fraction I should have contained all RNAs with sedimentation coefficients up to about 16S. Now globin mRNA sediments at 9S and ovalbumin mRNA at about 16S, so it might be expected that the RNA in fraction I should have directed the synthesis of polypeptides up to about 40,000 molecular weight; the other fractions should contain mRNAs for larger polypeptides but consist mainly of rRNA.

When the RNAs from each fraction were translated in the wheat-germ system it was found that fraction A had no mRNA activity at all. The other fractions had specific activities of 60,000 cpm/µg, 16,000 cpm/µg and 55,000 cpm/µg (respectively II, III and IV). Thus the messenger activity was associated with the rRNA fractions. The products of translation were analysed by electrophoresis on SDS-slab gels. All the fractions with activity direct the synthesis of the same products. These results indicate that the mRNAs were associated with the rRNAs, and this, in turn, explains the unusually high amount of binding to oligo dT-cellulose.

When the RNA, from the four gradient fractions, was analysed on polyacrylamide gels (Fig.15)it was obvious that the rRNAs were not aggregating with one another. The zonal technique was successful in separating the rRNAs, yet the messenger activity was associated with the rRNAs. The most likely explanation is that there are mRNA:rRNA aggregates.

There has been a report (Kabat, 1975) that aggregates between mRNA and rRNA can occur and that aggregates of 18S rRNA and globin 9S mRNA are more efficiently translated than the unaggregated mRNA. The improved translation of the LiCl-prepared RNA may be due to a phenomenon such as this, although the results shown in Fig.15 suggest that the aggregation occurs with both rRNAs. Thus, while the LiCl-method of RNA isolation gives good yields of active mRNA, it also causes aggregation. So before the mRNA can be fractionated on the basis of size or poly(A)-content, it must be denatured.

(iii) Variations in the mRNA population during greening of etiolated pea shoots.

Whenever studying the properties of mRNA populations; it is obviously of interest to know how different growth conditions affect the activity of the isolated mRNA and to establish whether or not there are changes in the spectrum of mRNAs. The original aim of this project was to investigate the possibility of isolating specific mRNAs, in a pure form, as a prelude to studying the expression of plant genes during development. As discussed in section I5D, most of the mRNAs which have been isolated in a pure state have come from tissue in which the mRNA has accounted for at least 40% of the total mRNA. It is obviously desirable to know when during development the mRNA for a particular protein is present in maximum amount and to use tissue in such a developmental state as the starting material.

In the laboratory where this work was carried out the area of interest is the interaction between the nuclear and chloroplast genomes in the development of the fully functioning

chloroplast (section I2). In particular, the changes which take place in the transition from etioplast to chloroplast are of interest. As discussed in section I3 and I6, the changes which take place in going from etiolated to green tissue are relatively few, and probably the main effect is to increase the rate of synthesis of the chloroplast proteins. This rate increase may be achieved via increased amounts of mRNA for the cytoplasmically-synthesised proteins, and so the greening of etiolated pea seedlings may offer a system suitable for the isolation of pure mRNAs. Peas can be grown completely in the dark for at least 14 days because they have large seeds; no special precautions and equipment are needed and large amounts of material can easily be obtained.

It must be emphasised that the experiments which are to be described cannot be used to say anything meaningful about the course of development of the chloroplast from the etioplast. In section I6 it was pointed out that the greening pea shoot was not a good developmental system from some points of view. The shoots consist of a heterogeneous population of cells and the greening process starts in these cells at different times as the leaves open out. Cell division also occurs during greening, and both the number of plastids per cell and the number of chloroplast DNA molecules per chloroplast change (Bennett and Radcliffe, 1976). However, it is only necessary, for the present purposes, to know how the mRNA population varies within the whole shoot, since this is the starting tissue for any RNA isolation. In particular, it is most important to know when a particular mRNA is at its maximum concentration relative to the other mRNAs. It is assumed that the pattern of products of translation in the wheat-germ system reflect the

tissue	time (h)	weight (g)	RNA (mg)	poly (A) (%)	mRNA specific activity	total mRNA activity	activity per g. tissue
etiolated	0	4.2	1.9	1.5	9.35×10^5	26.6×10^6	6.3×10^6
etiolated	12	5.0	2.8	2.4	7.15×10^5	48.0×10^6	9.6×10^6
etiolated	24	7.0	6.4	2.25	6.80×10^5	97.9×10^6	14.0×10^6
etiolated	36	7.5	3.8	3.7	4.90×10^5	68.9×10^6	19.7×10^6
etiolated	48	4.0	4.5	3.4	5.75×10^5	88.0×10^6	22.0×10^6
green	0	4.0	3.0	2.0	7.40×10^5	44.4×10^6	11.1×10^6
green	24	4.5	3.3	2.2	7.35×10^5	53.3×10^6	11.8×10^6

Table V Changes in parameters of poly(A)-containing RNA from pea shoot polysomes during greening.

Polysomes were prepared from pea seedlings which had either been grown in complete darkness for 12 days and the transferred to continuous light or had been grown in a light/dark regime before transfer to continuous light. The poly(A)-containing RNA was isolated from these polysomes and translated in a wheat-germ cell-free system. Each 20µl incubation contained 1µg of RNA. The specific activity of the RNA was determined as the radioactivity incorporated per ug of added RNA. The total mRNA activity in the tissue was obtained by multiplying the specific activity of the poly(A)-containing RNA by the total amount of this RNA present in the tissue (Assuming 20 E_{260} units are equivalent to 1mg of RNA)

relative concentrations of different mRNAs, and that changes in the amount of a particular polypeptide synthesised are due to changes in the amount of the mRNA coding for it. By using the wheat-germ assay with poly(A)-containing RNA isolated at different times of greening, it should be possible to establish when a particular mRNA forms the highest proportion of the poly(A)-containing RNA. This proportion is a more important parameter, within limits, than the absolute amount of mRNA. This is because the most difficult part of any mRNA isolation from pea shoots is not in obtaining sufficient material or separating the mRNA from rRNA, but in separating one particular mRNA from the other mRNAs.

A number of trays of pea seeds were sown on the same day and either kept in the dark for 12 days or grown under a 12 hour light (3,000 lux)-12 hour dark regimen. After 12 days all the trays, both dark and light-treated, were placed under continuous illumination of 10,000 lux of white light (section II1A). The trays of green seedlings were used as controls for the continuous-light treatment. At intervals, polysomes were isolated from the shoots on a particular tray of peas as described in section II2Fi. Part of the polysome preparation was analysed on sucrose gradients as described in section II2Fii; the rest of the preparation was extracted with phenol (section II2Gi), and the RNA split into poly(A)-containing and non-poly(A)-containing fractions by oligo dT-cellulose chromatography (section II2Hi). The poly(A)-containing RNA was translated in the wheat-germ system, and TCA-precipitable counts determined.

Table V shows the results obtained from this experiment. The following points should be noted:-

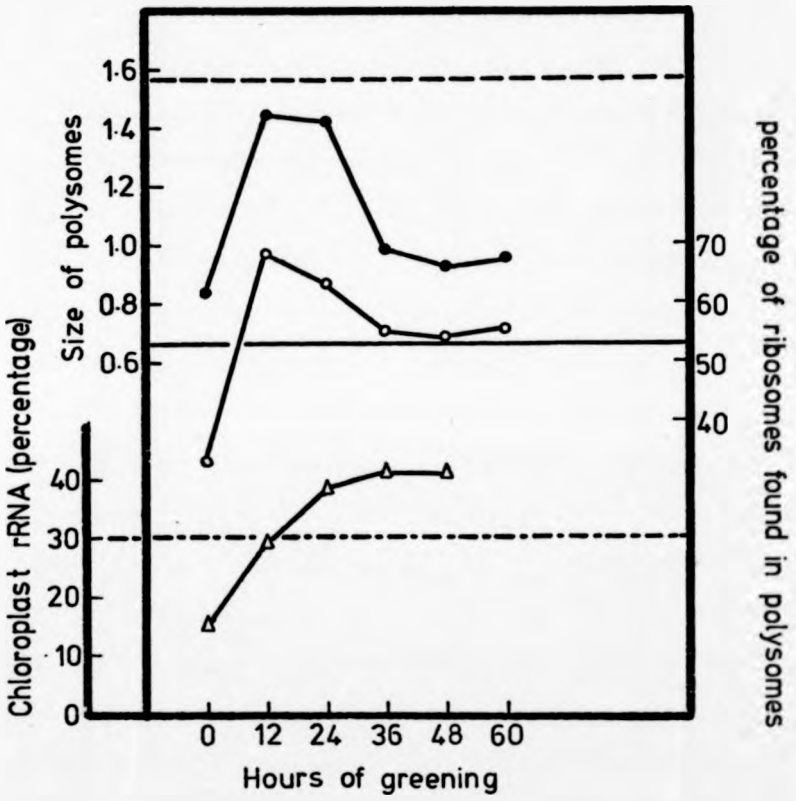
1·6
1·4
1·2
1·0
0·8
0·6
Size of polysomes
70
60
50
40
percentage of ribosomes found in polysomes
40
30
20
10
0
Chloroplast rRNA (percentage)
0
12
24
36
48
60
Hours of greening

Figure 16. Changes in polysome parameters during the greening of etiolated tissue.

During the greening of etiolated tissue, polysomes were isolated at 12 hour intervals (section II2Fi). The polysomes were analysed on sucrose gradients (section II2Fii) and RNA extracted from the polysomes was analysed by electrophoresis on polyacrylamide gels (section II2Eii). From measurements on the areas under the E_{260}-profiles of the polysomes, it was possible to determine the percentage of ribosomes which were present in polysomes, as apposed to monosomes. In the same way it was possible to measure the ratio of polysomes larger than pentamers to those smaller than pentamers, this is a measurement of the size of polysomes. From the profiles of polysomal RNA on polyacrylamide gels, it was possible to estimate the percentage of chloroplast rRNA in the total RNA. Each of these parameters was also measured on controls which had been grown under a 12 hour dark/12 hour light regimen and then transferred to the same continuous light conditions as the etiolated tissue.

o——o percentage of ribosomes present in polysomes

——— green control

•——• size of polysomes (ratio of polysomes larger than pentamers to those smaller)

– – – – – – – green control

Δ——Δ percentage of chloroplast rRNA

—·—·—·— green control

1) there is no significance in the variations of the amount of shoot tissue obtained at each time point; this just reflects variations in the number and size of the germinated seedlings on any particular tray at the start of the greening phase;

2) the percentage of polysomal RNA which binds to oligo dT-cellulose increases during greening, yet the specific activity of the RNA, as a messenger population in the wheat-germ system, goes down. This could be because the later samples contain more RNA, and so there may be an increase in non-specific binding at the oligo dT-fractionation step,

3) the total messenger activity obtained per gram of tissue can be seen to increase during the greening; this might be expected since the tissue is now growing more rapidly, than when in the etiolated state,

4) the green control shows more activity after 24 hours under continuous light, implying that the increased exposure to light can cause a stimulation of protein synthesis.

From the polysome profiles, it is possible to obtain data concerning the amount of ribosomes in polysomes compared to monosomes. The polysome region of the gradients was divided arbitrarily at the position of the pentamers; by comparing the amount of material larger than pentamers with that less than pentamers, a measure of the size of polysomes could be gained. All measurements on the polysomes were made by cutting out and weighing areas under the traces; at least six measurements were made on each profile and averaged. Figure 16 presents the results.

It must be pointed out that the figures presented for the percentage of ribosomes in polysomes, and also for the

proportion presented as polysomes larger than pentamers are both over-estimates. The polysomes were prepared by pelleting through a cushion of sucrose; this method has been shown to enrich for polysomes over monosomes, and for heavy polysomes over light polysomes (Leaver and Dyer, 1974). However, the conditions used were such that at least 80% of all ribosomes were pelleted (see Fig. 1 of Leaver and Dyer, 1974) and each sample was prepared in exactly the same way. It is valid, therefore, to make comparisons between preparations made at different times.

The percentage of polysomal RNA which is derived from chloroplast ribosomes can be estimated by electrophoresis of the RNA on polyacrylamide gels. As shown in Figure 11A, the chloroplast rRNAs are visible as discrete peaks and, by applying a correction factor for the breakdown of the large chloroplast rRNA as shown in table IV, the proportion of chloroplast RNA can be estimated.

Figure 16 presents all these data. The points to note are as follows:-

1) in the sample from etiolated tissue the amount of polysomes, their size and the amount of chloroplast rRNA present are all less than those from the green control;

2) after 12 hours of continuous illumination the polysomes from etiolated shoots areabout the same size as those from the green shoots, but there are now more ribosomes in polysomes compared with the green controls.

3) with longer periods of illumination the percentage of ribosomes in polysomes from etiolated tissue approaches that of the green control, whereas the size of the polysomes decreases.

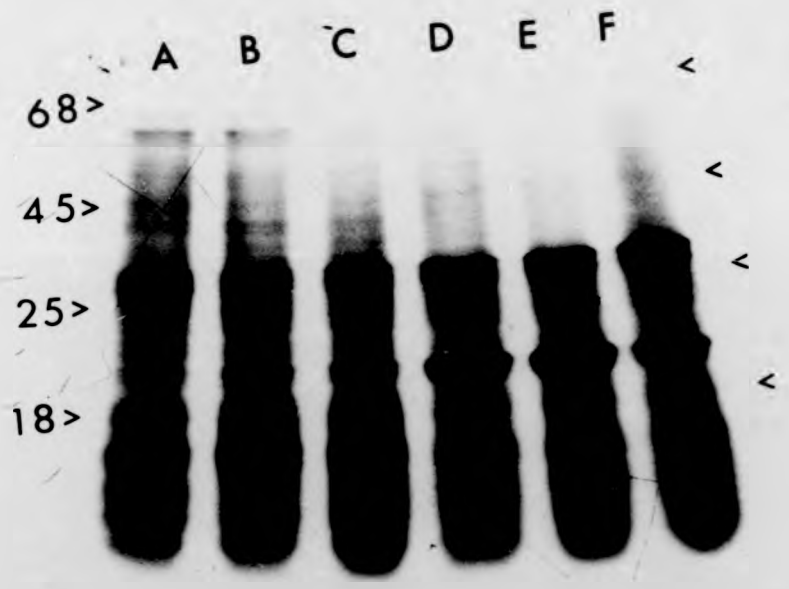

Figure 17 Changes in the translation products of poly(A)-containing polysomal RNA during the greening of etiolated tissue.

Poly(A)-containing RNA was isolated from polysomes prepared from etiolated pea shoots at 12 hourly intervals during growth under continuous illumination. The RNA was translated in a wheat-germ system as described (see section II2Jiia and Fig. 5). The products of translation were analysed on a slab polyacrylamide gel (7.5% to 25% acrylamide concentration gradient) as described in Fig. 5 and the autofluorograph exposed for two days at -70°C, each track contained 150,000 cpm.

The RNA samples were from:-

A. Etiolated tissue

B. etiolated tissue which had been in continuous light for 12 hours

C. as B but after 24 hours D. as B but after 36h

E. as B but after 48 hours

F. green tissue which had been in continuous kight for 24 hours.

4) the proportion of chloroplast rRNA steadily increases with greening; this is expected since the amount of chloroplast rRNA in the cells increases (Smith *et al*, 1970), and plastid protein synthesis increases (Siddell and Ellis, 1975).

The increase in the amount of polysomes during greening is consistent with the increase in total messenger activity of RNA isolated during greening. As more polysomes are formed, more mRNA appears in the polysome pellet. It must be remembered that, because only pelletable mRNA is measured, it is impossible to distinguish between increased utilization of pre-existing mRNA within the tissue or *de novo* mRNA synthesis. The time scale is far too long (12 hours) to distinguish between these two possibilities.

The products of translation of poly(A)-containing and non-poly(A)-containing RNA in the wheat-germ system were analysed by electrophoresis on SDS-polyacrylamide gels. The poly(A)-containing RNA-directed products are shown in Figure 17; the non-poly(A)-containing RNA-directed products were similar to these at each time-point. Equal TCA-precipitable counts were loaded onto each track so that if any polypeptide changed relative to the others it would be seen as a change in band intensity.

The major change during greening is seen at around 20,000 molecular weight; a band grows in intensity throughout greening until it is the single, most highly labelled band. Comparing the products after 48 hours of greening with the green control, the band at 20,000 is more intense in the former, implying that the mRNA for this polypeptide accounts for a higher proportion of the total mRNA in the 48 hour-

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greened tissue than in normally grown tissue. As will be explained in section III2A, this band has been called P20 and has been identified in the present work as a precursor to the small subunit of Fraction I protein.

Other changes in the pattern of mRNA activity take place at around 30,000 molecular weight. There are three bands visible in this region as products of the translation of the RNA from etiolated tissue. During the course of greening, the relative labelling of these bands changes until the 48 hours-greened products contain just one band in this region.

It should be noted that, the fact that some bands decrease in intensity, e.g. at around 50,000 molecular weight, does not mean that the amount of mRNA for these polypeptides has decreased. Equal amounts of radioactivity were loaded onto each track shown in Figure 17 so that the pattern and intensity of products should reflect the constitution of the mRNA population. A decrease in intensity of a band would imply that the relevant mRNA formed a decreased part of the total mRNA population. However, the total mRNA activity increased markedly during greening and so, in certain circumstances, a given mRNA could increase in amount yet, if it did not increase to the same extent as the total mRNA, its product could form a lower proportion of the total products.

It is not easy to quantitate the changes taking place in the amounts of particular polypeptide products. Microdensitometer traces of the tracks cannot resolve some bands which are close together; the high background in some parts of the gel also makes it difficult to quantitate the darkening of the autoradiograph. Attempts at cutting out the labelled bands and determining their radioactivity by

scintillation counting were unsucessful. This was probably because the regions of interest, e.g. around 20 and 30,000 molecular weight, contain many highly labelled bands close together on a fairly high background. Thus any error in cutting out bands from the gel would result in large variation in the radioactivity apparently located in the bands. This is just the result found; some bands obviously differing in their intensity apparently had similar amounts of radioactivity, while similar bands differed in their radioactivity. Therefore, it was not found possible to quantitate any changes in the pattern of products.

An important point to note, when considering the pattern of products, is that variations in the conditions of the wheat-germ assay did not cause any changes in the pattern of products obtained with any particular poly(A)-containing RNA preparation. Yet addition of the same amount of RNA, isolated from different times of greening, to identical wheat-germ incubation mixtures did give different patterns. These observations confirm that the wheat-germ system does respond to differences in the population of RNA molecules added by synthesising different polypeptides.

These results establish that tissue which has greened for 48 hours under continuous illumination contains a higher level of mRNA activity than normal green tissue and that one mRNA in particular has increased to become a major mRNA species. Such tissue provides a good starting material for attempts to isolate the mRNA for the polypeptide known as P20 (see section III4B).

(iv) Poly(A)-containing RNA from polysomes.

a) Poly(A)-content of polysomal RNA.

In section I5B, the possible function of poly(A) in mRNA was discussed. When this work was started, the general opinion was that poly(A)-containing RNA was synonymous with mRNA, histone mRNA being the only exception. Although there was evidence that in sea-urchins and Hela cells there exists a non-poly(A)-containing, non-histone mRNA fraction which codes for polypeptides different from those coded by poly(A)-containing mRNA; this conclusion was based on labelling kinetics and hybridisation studies rather than on direct translation of this RNA (Nemer et al, 1974; Milcarek et al, 1974). Lodish et al (1974) found that the non-poly(A)-containing RNA from slime mould cells was translated into a similar range of products as the poly(A)-containing RNA; Rosen et al (1975b)found that rat casein mRNA could be found in equal amounts in both fractions. A criticism of these studies is that the non-poly(A)-containing RNA is defined by its failure to bind to either nitrocellulose filters, oligo dT-cellulose or poly(U)-Sepharose; the fraction is rarely checked for the actual presence of poly(A) by any more direct method. Thus it might be that the similarity between the translation products of the two RNA fractions is due to the presence in the non-poly(A)-containing RNA fraction of poly(A)-containing RNA with a poly(A) tail too short to bind to an affinity column. It is known that, for oligo dT-cellulose, poly(A)-tails less than about 30 residues will not bind (Nudel et al, 1976).

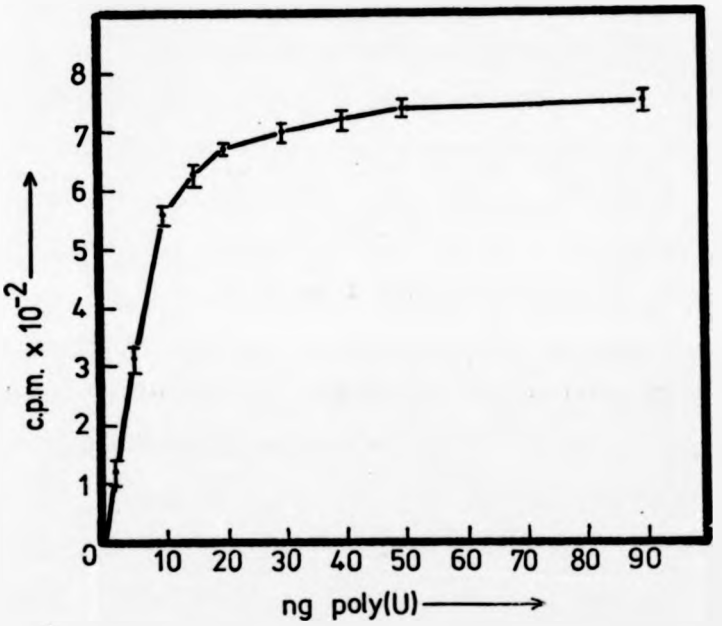
In section III1Bii it was noted that the pattern of translation products directed by non-poly(A)-containing RNA from pea leaf polysomes was similar, if not identical, to

that directed by poly(A)-containing RNA. The poly(A)-containing RNA was, however, much more active at directing protein synthesis. Gray and Cashmore (1976) have also found that the two RNA fractions from pea polysomes give this result. They concluded that the material which did not bind to poly(U)-Sepharose was completely free from any poly(A)-sequences. They determined this by measuring the nuclease-resistant radioactivity of RNA labelled *in vivo* with [^{32}P]-orthophosphate. About 27% of the poly(A)-containing RNA was nuclease-resistant, whereas only 0.27% of the non-poly(A)-containing RNA was nuclease-resistant. When the two RNA fractions were translated in a wheat-germ system, they each gave the same products and the non-poly(A)-containing RNA accounted for 50% of the total activity. The non-poly(A)-containing RNA contained about 60-65% of the total radioactivity in RNA, after correction for the rRNA labelled during the 2-hour labelling period. They concluded that there was a population of mRNA molecules which did not contain poly(A) but which did contain the same sequences as the poly(A)-containing RNA. A shortening of poly(A)-tails has been reported as occuring in the cytoplasm of tissue-culture cells (Sheiness *et al*, 1975) and so Gray and Cashmore considered that this non-poly(A)-containing RNA arose by such a mechanism. They comment that this would mean about 50% of the poly(A)-containing RNA would have to lose its poly(A) during the *in vivo* labelling period, about 2 hours. However, such a conclusion is not necessary from their data.

The basic problem with their work is that they are correlating the rapidly labelled non-poly(A)-containing RNA with the RNA which is active in the wheat-germ system; this

is not a valid correlation. The labelling period was short relative to the functional lifetimes of mRNAs (Perry and Kelley 1973; Bastos and Aviv, 1977) and so the labelling experiment may only be dealing with a rapidly turning-over sub-population of mRNAs which might be predominantly non-poly(A)-containing. On the other hand, the translation experiment deals with steady-state populations of mRNAs, in which the mRNAs detected in the in vivo-labelling experiment might only comprise a small percentage; so the non-poly(A)-containing RNA fraction might contain poly(A)-containing RNA which has a poly(A)-tail too short to bind to poly(U)-Sepharose. The fact that the non-poly(A)-containing RNA contained only a very small amount of nuclease-resistant labelled material does not rule out this possibility. It is known that the older an mRNA is, the shorter the poly(A)-tail is likely to be (Nokin et al, 1976; Merkel et al, 1976), so the mRNA which does not bind to the poly(U)-Sepharose may well have been synthesised before the labelling started and would, therefore, be undetected.

It is important to check the conclusions of Gray and Cashmore by the use of a method which will measure the steady-state levels of poly(A). Their conclusion must be checked because it implies that there is a very rapid loss of poly(A) from mRNA in the cytoplasm of pea cells, and that mRNA, which has lost all its poly(A), is still quite stable; it accounts for about 50% of the translational activity. It has been shown for globin mRNA, that once the poly(A)-tail has been reduced below about 20 residues, the mRNA is very much less stable (Nudel et al, 1976); so it would not be expected that such mRNA would form such a large proportion of the total mRNA. However, other workers with other systems have also found a large


c.p.m. x 10^{-2}
0
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30
40
50
60
70
80
90
ng poly(U)

Figure 18. Calibration curve for the reaction of [^{3}H]-poly(U) with poly(A).

In a final volume of 100µl, 10ng of poly(A) were incubated with various amounts of [^{3}H]-poly(U) as described in section 112Hiii. Triplicate assays were performed for each point on the curve and the bars represent the range of the triplicates. 1ng of poly(A) equivalent to 740 cpm of [^{3}H]-poly(U)

proportion of total activity in the non-poly(A)-containing RNA fraction (Rosen et al, 1975; Lodish et al, 1974).

Hybridization of RNA with [^{3}H]-labelled poly(U) has been used to quantitated the amount of poly(A) in steady-state populations of plant RNA, and is capable of great sensitivity (Covey and Grierson, 1976). This technique was used in an experiment designed to investigate the properties of polysomal RNA fractionated on both oligo dT-cellulose and poly(U)-Sepharose. RNA was isolated from polysomes; the LiCl-method (section II2Gii) was used because the problem of aggregation had not yet been detected (section III1Bii). The RNA was chromatographed upon oligo dT-cellulose as described in section II2Hi. The fraction which eluted with 0.1M LiCl and that which eluted in no-salt buffer were precipitated with ethanol. The material which did not bind to the oligo dT-cellulose (referred to as dT_{NB}) was rechromatographed to confirm that it did not contain any material which would bind. The dT_{NB} RNA was then chromatographed on poly(U)-Sepharose (section II2Hii) and the bound (U_B) and non-bound (U_{NB}) fractions precipitated by addition of 2 volumes of ethanol.

Each RNA fraction obtained was assayed by translation in the wheat-germ system (section II2Jii) and by hybridization to [^{3}H]-poly(U) (section II2Hiii).

The poly(U)-hybridization assay was calibrated by determining how much radioactive poly(U) would hybridise to 10ng of poly(A) with saturating poly(U). Figure 18 shows such a calibration curve. Increasing amounts of poly(U) were reacted with 10ng of poly(A) and it was found that a ratio of poly(U)/poly(A) of at least 5 was necessary to obtain saturation in the 2.5 hours of the hybridization incubation.

RNA	amount	cpm poly(U) hybridised	ng poly(A)	poly(A) (%)
TOTAL	2µg	1510	2.04	0.102
	5µg	3960	5.36	0.107
dT_B	200ng	1990	2.70	1.35
	500ng	4488	6.06	1.21
$dT_{0.1}$	1µg	756	1.02	0.102
	2µg	1520	2.05	0.103
dT_{NB}	2µg	306	0.41	0.02
	5µg	733	0.99	0.02
U_B	1µg	1410	1.91	0.191
	2µg	3010	4.06	0.203
U_{NB}	5µg	157	0.21	0.0042
	10µg	345	0.47	0.0047
MS2	10µg	280	0.38	0.0038

Table VI Hybridization of RNA with [^{3}H]-poly(U).

In a final v. ume of 100µl of 2xSSC, the indicated amounts of the various RNA fractions were incubated with 50ng of [^{3}H]-poly(U) at 20°C. The extent of hybridization between the [^{3}H]-poly (U) and the RNA was determined as described in section II2Hiii. The nuclease-resistant radioactivity was measured in triplicate assays, each filter was counted for 20 minutes and a background of 50 cpm was subtracted from each filter.

The RNA fractions were as follows:- dT_B, the RNA which binds to oligo-dT cellulose and is only eluted at 37°C in no-salt buffer; $dT_{O.1}$, the RNA which is eluted from the oligo-dT column at O.1M salt; dT_{NB}, the RNA which does not bind to the oligo-dT; U_B, the RNA which does not bind to oligo-dT but does bind to poly(U); U_{NB}, the RNA which binds to neither.

	amount of RNA		mRNA activity			poly(A)-content		
	(µg)	(%)	sp. act. ($x10^{-3}$)	total act $x10^{-6}$	% of total	% of RNA	total (µg)	% of total
TOTAL	5200	100	26.3	136.76	100	0.101	5.25	100
dT_B	305	5.8	226.25	69.00	50.4	1.276	3.89	74.1
$dT_{O.1}$	265	5.2	41.0	10.86	7.9	0.098	0.26	5.0
dT_{NB}	4620	88.8	10.5	48.51	35.4	0.016	0.73	13.9
dT_{NB}	4330	100	10.5	45.46	100	0.016	0.69	100
U_B	280	6.5	50.0	14.00	30.7	0.196	0.54	78.3
U_{NB}	4050	93.5	4.5	18.22	40.0	-	-	-

Table VII Fractionation of messenger activity by chromatography on oligo-dT cellulose and poly(U)-Sepharose.

Total polysomal RNA was chromatographed on oligo-dT cellulose as described in section II2Hi. The RNA which did not bind to the oligo-dT cellulose was then chromatographed on poly(U)-Sepharose (section II2Hii). All the fractions of RNA thus obtained were translated in a wheat-germ extract (section II2Jiia) and the specific activity of the RNA, in terms of cpm incorporated per ug of RNA was determined. The total activity in a fraction was obtained from the specific activity and the total RNA in the fraction. The percentage of total activity is expressed relative to TOTAL for dT_B, $dT_{O.1}$ and dT_{NB} and relative to dT_{NB} for U_B and U_{NB}. The data from Table VI is used to calculate the amount of poly(A) in each RNA fraction and hence the percentage recovery of poly(A) by these procedures.

The calibration curve shows that 740 c.p.m. = 1ng of poly A. Each point on the curve is the average of 3 determinations and the bars show the spread of individual results.

Table VI shows the results obtained when the fractionated polysomal RNA was assayed for poly(A)-content. The dT_B-fraction contained the highest proportion of poly(A); the content of 1.28% poly(A) is lower than might be expected since poly(A)-containing mRNA from other systems usually contains 5-10% poly(A) (Grierson et al, 1976). The reason for this rather low poly(A)-content is found in Table VII, which shows that 5.8% of the total polysomal RNA binds to the oligo dT. With hindsight, it is known that LiCl-prepared RNA is aggregated and so the dT_B fraction must contain a high proportion of rRNA; this rRNA would reduce the apparent percentage of RNA which is poly(A). This interpretation was confirmed by analysis of the dT_B-RNA on polyacrylamide gels. It was not possible to estimate how much rRNA was present but it obviously formed a higher percentage of the dT_B RNA than was usually the case. The 0.1% of the total polysomal RNA which was poly(A) is what would be expected for an RNA population in which 1-2% of the molecules were mRNA with poly(A)-tails about 150 residues long (Grierson et al, 1976); this calibration assumes an "average mRNA" to be about 2000 bases long (Grierson et al, 1975). The dT_B fraction contains over 70% of the total poly(A) sequences (Table VII) and so it is likely that the low percentage of dT_B which is poly(A) is due to the presence of rRNA, rather than that the poly(A)-containing RNA has short poly(A)-tails.

It is interesting to note that there is poly(A)-containing RNA which does not bind to oligo dT-cellulose yet

which does bind to poly(U)-Sepharose (Table VII). This (U_B) must also contain rRNA because, if all the molecules contained only 0.2% poly(A), then each poly(A) tail would be only 3-4 residues long and such short tails would not bind. Examination of U_B RNA on polyacrylamide gels shows mainly rRNA. It was not possible to estimate accurately the amount of non-rRNA; thus the size of those poly(A) tails which did not bind to oligo dT but which did bind to poly(U)-Sepharose is not known.

It is important to compare the amount of poly(A) present in the U_{NB} RNA, which does not bind to oligo dT-cellulose or poly(U)-Sepharose, with the value for poly(A) in MS2 RNA; this bacteriophage RNA is known not to contain any poly(A) from sequence analyses. The U_{NB} RNA gives a value of 0.0044% poly(A) while the MS2 RNA gives a value of 0.0038% poly(A). These values are not significantly different and so it would appear likely that the RNA which does not bind to either oligo dT-cellulose or poly(U)-Sepharose does not contain any poly(A).

Table VII brings together the results of the poly(A) determinations and the translational activity assays. Equal amounts of each RNA fraction were translated in the wheat-germ system and total TCA-precipitable counts determined; the counts incorporated into protein per µg added RNA are used to define the specific activity of the RNA.

The main points to emerge from Table VII are as follows:-

1) the material which binds to the oligo dT-cellulose has a higher specific activity than any other fraction even though it was shown earlier that there is a high contamination by rRNA which will reduce this specific activity;

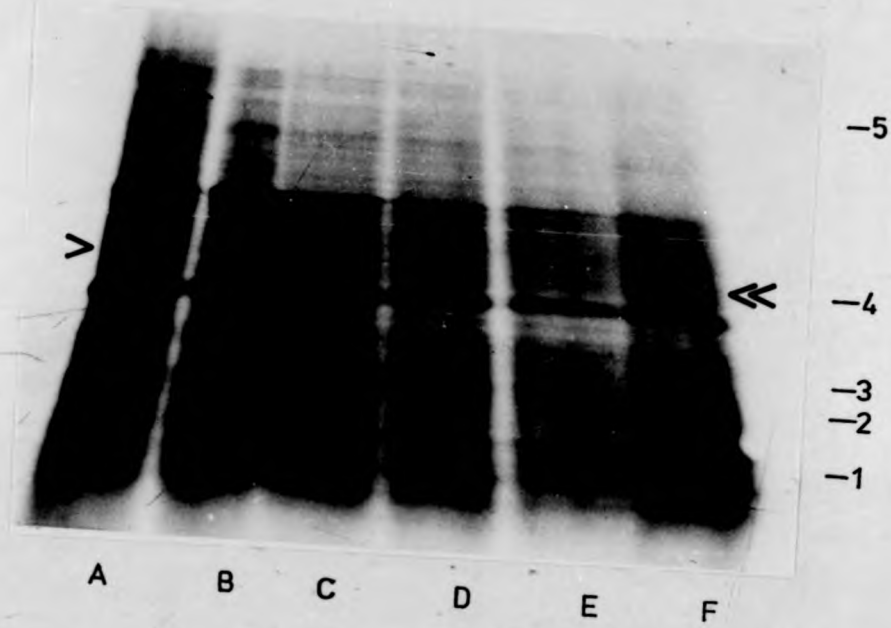

Figure 19 Comparison of the translation products of RNA fractionated on the basis of poly(A)-content.

The various RNA fractions obtained by chromatography of polysomal RNA on both oligo-dT cellulose and poly(U)-Sepharose were translated in a wheat-germ system (section II2Jiia). Each reaction mixture (20µl) contained 5µCi [^{35}S]-methionine and 3µg of each RNA. Incubation was for 60 minutes at 27°C and the products analysed on a slab polyacrylamide gel as described in Fig. 5. each track contains 60,000 cpm and the autofluorograph was exposed for 8 days at -70°C. The molecular weight markers were as in Fig. 5

A. Products of total polysomal RNA

B. Products of dT_B RNA

C. Products of $dT_{0.1}$ RNA

D. Products of dT_{NB} RNA

E. Products of U_{NB} RNA

F. Products of U_B RNA

2) only about 50% of the activity is bound to the oligo dT-cellulose but about 30% of the remaining activity binds to poly(U) Sepharose.

3) Only about 15-20% of the total activity is associated with the U_{NB} fraction i.e. non-poly(A)-containing RNA.

4) Almost all (>90%) of the poly(A) present in the total RNA can be accounted for in the various fractions, the dT_B RNA containing 74% of the total.

Thus it can be concluded that at least 80% of the mRNA activity is associated with RNA which contains at least a short poly(A)-tail.

b) Translation of poly(A)-containing RNA.

The next point to consider is whether the various RNA fractions are translated into different polypeptides. The products were analysed by electrophoresis on SDS polyacrylamide gels (Fig. 19) Equal amounts of radioactivity were loaded onto each track.

Although each track seems to contain the same pattern of polypeptides there are changes in the intensity of the bands. In particular the intensity of P20 and P32, the two major products of dT_B RNA, drops in the dT_{NB}, U_{NB} and U_B tracks. This is what is expected if the mRNAs for these polypeptides were to become a lower proportion of the total mRNA population. For U_B especially, the fact that equal amounts of radioactivity are present in each track yet the intensity of blackening for particular bands decreases must mean that other polypeptides are being synthesised in increasing amounts. This observation would fit with a situation where the mRNA derived from the poly(A)-containing RNA was being removed, and the non-poly(A) containing mRNA was forming a larger proportion of the total

mRNA. This conclusion would mean that U_{NB} RNA does not consist entirely of de-adenylated-poly(A)-containing RNA even though it produces a pattern of products qualitatively similar to that of poly(A)-containing RNA and does not have any measurable poly(A) content. The corollary of this conclusion is that not all the 20% activity associated with U_{NB} RNA can be due to mRNA derived from poly(A)-containing RNA, so that perhaps as little as 10% of the total activity of polysomal RNA is due to de-adenylated poly(A)-containing RNA.

In comparing these results with those of Gray and Cashmore (1976), the main point of difference is the amount of messenger activity associated with the non-poly(A)-containing RNA. They found 50% of the total activity did not bind to poly(U)-Sepharose whereas the figure was only 20% in this study. The most obvious difference in the protocols of the two experiments is at the poly(U)-Sepharose chromatography step. Gray and Cashmore put the RNA straight onto poly(U)-Sepharose and washed with 25% formamide before eluting the bound material; whereas, in the present work, the fraction which had not bound to oligo dT-cellulose was used and there was no intermediate wash. Therefore, the U_B fraction in this experiment may well contain molecules which would not have bound under the conditions of Gray and Cashmore. They also concluded that the non-poly(A)-containing fraction was translated into the same products as the poly(A)-containing RNA and so associated all the activity of the non-poly(A)-containing RNA with the RNA derived from the poly(A)-containing fraction. However, Figure 19 shows that the U_{NB} RNA products are qualitatively similar to yet are quantitatively different from the products of dT_{NB} RNA. This observation suggests that only part of the

activity of the non-poly(A)-containing RNA can be due to RNA derived from the poly(A)-containing fraction. This interpretation also fits with data that suggest that there is a non-poly-containing mRNA population which is distinct from the poly(A)-containing RNA (Nemer et al, 1974, 1975; Milcarek et al, 1974).

These results confirm the finding of Gray and Cashmore that the RNA which does not bind to poly(U)-Sepharose does not contain any measurable poly(A). A different method was used which measured the poly(A)-content of the steady-state population not just that of the rapidly labelled RNA.

However, since these experiments were done, it has become apparent that the method used to assay the poly(A)-content has a major drawback. Both the poly(U)-hybridization and the nuclease-resistance methods rely, finally, on the TCA-precipitation of polynucleotide fragments. This precipitation is not efficient with fragments smaller than about 20 residues; precipitation by cetyl triethylammonium bromide (CTAB) is much better in this situation (Ford P.J., personal communication). So it may be that short poly(A)-tails, which would not bind to oligo dT-cellulose (Hruby and Roberts, 1976; Nudel et al,1976) or probably even poly(U)-Sepharose, would not have been detected in either of these assays. Therefore, the poly(A)-content of RNA from pea leaf polysomes which does not bind to poly(U)-Sepharose, is still not known with certainty.

From Figure 19 it can be seen that some polypeptides do seem to be predominantly translated from either poly(A)-containing or non-poly(A)-containing RNA. The band indicated

isoelectric focussing

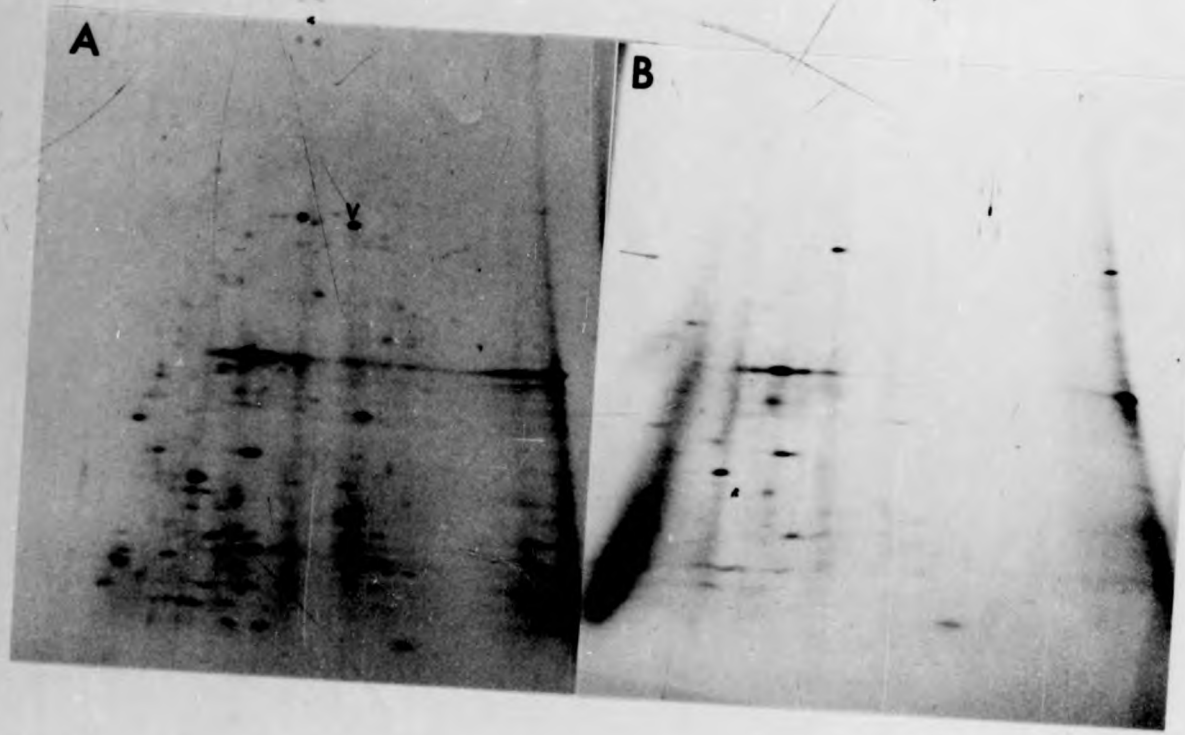

Figure 20 Comparison of *in vitro* translation products on two dimensional gels.

The dT_B and dT_{NB} RNAs obtained in section III1Biv were translated in a wheat-germ system (section II2Jiia) and analysed by two-dimensional electrophoresis (section II2Eie). The pH gradient was from 4.0 to 8.0 (from left to right as the gel is viewed). The autofluorographs were exposed for 21 days at -70°C.

A. Products of dT_B RNA (100,000 cpm)

B. Products of dT_{NB} RNA (80,000 cpm)

by the single arrow is present predominantly in the products translated from the dT_B and $dT_{0.1}$ fractions; it does not appear to be present in the U_B fraction. This difference must mean that most of the mRNA molecules for this polypeptide have long poly(A)-tails. The band indicated by the double arrow is present in every track apart from that of the U_{NB}-directed products. This would imply that the mRNA molecules for this polypeptide are only rarely found with very short poly(A)-tails. There does not seem to be any band present in the U_{NB} RNA-directed products which is not found elsewhere. These statements carry the qualification that bands, present in different tracks, which co-electrophorese are assumed to be the same.

Figure 20 shows a comparison of dT_B and dT_{NB} RNA-directed products analysed on a two-dimensional electrophoretic system (section II2C). The two spots arrowed in Fig.20A are not present, or present in very much lower amounts, in Fig.20B most of the other spots seem to be common to each. The identities of these two polypeptides are unknown. This result again shows that the non-poly(A)-containing RNA directs the synthesis of products very similar to those directed by poly(A)-containing RNA. There are no spots visible on Fig.20B which are not present on Fig.20A. However, for technical reasons, the non-poly(A)-containing RNA used was dT_{NB}not U_{NB} and it has already been shown that almost 50% of its activity is due to poly(A)-containing RNA (Table VII). There is less radioactivity in Fig.20B compared with Fig.20A and minor spots, which might be different, cannot be seen. Longer exposure of the autoradiograph did not give better results because the background fogging of the film increases and the

[^{35}S]-methionine radioactivity decays. The histone mRNAs do not have poly(A)-tails in most tissue looked at, but histones are too basic to focus on the first dimension used in this system.

C. Discussion.

The results presented in this section establish:-

1) that the wheat-germ cell-free system used in this work was capable of the faithful translation of added mRNA and that it could be used as a system for following changes in a population of mRNA;

2) that the RNA preparations used in this work were not degraded, and could be translated by the wheat-germ system into discrete products;

3) that the methods for RNA isolation and its cell-free translation could be used to gain information about changes which occur during the greening of etiolated pea seedlings;

4) that the messenger activity associated with pea shoot polysomes was found predominantly in poly(A)-containing RNA.

Each point will be now discussed in more detail.

1) Translation by the cell-free system from wheat-germ.

The results described in section III1A show that the wheat-germ system translates known mRNAs (SFV 26S RNA, chick embryo kidney RNA and Xenopus embryo RNA) into authentic products. The pattern of polypeptides produced in response to added TMV RNA was very similar to published patterns, especially in that the high-molecular weight polypeptides (greater than 100,000) account for a large proportion of the products. Many published studies have shown that the wheat-germ system can faithfully translate RNA isolated from a

wide variety of sources, including plant cells, into many different types of proteins (enzymes, structural proteins and hormones). In particular, some products have been shown to have biological activity e.g. T4 lysozyme (Beck and Gassen, 1977) and human interferon (Raj and Pitha, 1977; Pestka et al. 1975).

An important point to establish in the use of an mRNA assay is that the pattern of translation products reflects the pattern of individual mRNAs in the RNA population. If this is not the case, then the in vitro assay will give misleading information about what mRNAs are present and about their relative concentrations. When it is desired to isolate a pure mRNA, one criterion of purity is that the mRNA directs the synthesis of only one product. If the assay system is selective in the translation of some mRNAs then this criterion will not be valid.

It has been suggested by Tse and Taylor (1977) that the wheat-germ system is poor as a quantitative assay for mRNA. They based this conclusion on the thorough study they made of the translation of rat liver poly(A)-containing RNA in a wheat-germ extract. They found that albumin, which they measured by immunological techniques, had its optimum synthesis at K^+ and Mg^{2+} concentrations which were high compared with those which were optimal for total protein synthesis. Under conditions which were not optimal, albumin fragments only were made not the full sized polypeptide. This result means that the proportion of total products which represented albumin varies extensively with changes in incubation conditions. Similar results have been found for the synthesis of pro-collagen (Benveniste et al, 1976), myosin (Patrinou-Georgoulas and

John, 1977) and vitellogenin (Shapiro *et al*, 1977). It is interesting to note that all these proteins have high molecular weights; albumin has a molecular weight of about 64,000 while the other proteins are all larger than 150,000. It may be that the synthesis of large proteins require a high K^+ concentration, to increase elongation rate, for complete synthesis.

In contrast to these reports, many other studies have shown that translation in the wheat-germ system is capable of reflecting changes in messenger RNA levels. Martial *et al* (1977) followed the changes in rat pituitary cells after treatment with either thyroid or glucocorticoid hormones. They measured the synthesis of growth hormone both by the cells and by a wheat-germ system programmed with RNA extracted from the cells; they also measured the level of mRNA for growth hormone present in the cells by hybridization with a cDNA probe, which was enriched for sequences complementary to the mRNA for growth hormone. They found that all the three assay systems gave consistent results during hormonal induction of growth hormone. Increases in the mRNA level in the cells was correlated with increased *in vitro* synthesis of growth hormone in response to this RNA, which in turn paralled the increased synthesis of growth hormone by the cells i.e. each parameter changed to the same extent relative to the control values. This shows that the wheat-germ system can be used as an assay to follow changes in the mRNA population. Similar studies on the induction of actin in chick embryonic muscle culture (Paterson *et al*, 1974), on thyroid hormones and α_{2u} globulin (Kurtz *et al*,1976), on human placental lactogen and chorionic gonadotrophin during placental development

(Chatterjee et al, 1976), on cAMP induction of phosphoenolpyruvate carboxykinase in rats (Iynedjian and Hanson, 1977), and on dihydrofolate reductase in BHK cells (Chang and Littlefield, 1976), have all shown that the changes in the synthesis of a given protein in the wheat-germ system do parallel those which occur in vivo. Perhaps a clue to the reason for the difference between these results and those of Tse and Taylor (1977) might be found in the report of Adams et al (1977). This paper describes the use of the wheat-germ system to assay for the presence of mRNAs for cell surface proteins in transformed chick embryo fibroblasts. All the proteins investigated have molecular weights in excess of 150,000 yet they were made as full-length products under conditions of optimum synthesis of the bulk cellular mRNAs. Immune precipitation showed that only full-sized polypeptides were synthesised; there were no partial products. This observation is in direct opposition to the findings of Taylor and Tse as well as Benveniste et al (1976) and Patrinou-Georgoulas and John (1977). It must be pointed out that Adams et al do not quantitate the percentage of total incorporation which is due to these particular proteins, but it is obviously quite small (see Fig.1 track 2 of Adams et al, 1977). However, the important difference is that they get full-length, and only full-length, products under conditions where the other cellular mRNAs are translated, whereas the other workers found the full length high molecular weight polypeptides only under conditions where the synthesis of other polypeptides was low or non-existent. In their discussion Adams et al say, "We have examined seven different lots of wheat-germ, and have found four that will translate these

mRNAs into large polypeptides". This would imply that the ability to translate the mRNA for high-molecular weight polypeptides can vary between wheat-germ batches (it is not clear whether these seven batches were all from the same source or not). These authors do not say whether or not the batches, which were incapable of synthesising the high molecular weight polypeptides, could still synthesise the lower molecular weight products. Nevertheless, it would seem that a possible explanation of these different results rests with the particular batch(es) of wheat-germ by different workers for their experiments.

So it would appear that the wheat-germ system can be used to study changes in mRNA populations, and even to quantitate the level of certain mRNAs in favourable circumstances, but the success of such assays depends very much upon the RNA preparations used, upon the protein under investigation and upon the wheat-germ preparation employed.

As mentioned earlier, the wheat-germ S-30 preparations used in the work described in this thesis were capable of the synthesis of high molecular weight polypeptides; in particular the products of translation of chick embryo kidney RNA contained polypeptides with molecular weights up to at least 140,000. With the pea polysomal RNA as template raising the K^+ concentration did not result in the increased synthesis of larger polypeptides. However, variations in the growth conditions of the peas did cause changes in the products of translation in the wheat-germ system.

All these observations suggest that the wheat-germ system used in this work, unlike that used by Tse and Taylor (1977), was capable of responding to added RNA by the faithful

translation of the mRNAs present, and of reflecting changes in that mRNA population.

2) Physical and functional state of pea leaf polysomal RNA

It is difficult to show that a population of mRNA molecules is not at all degraded; complete degradation is easily demonstrated but partial effects are much more difficult to detect. With a single mRNA species the criterion of electrophoretic mobility and the shape of the peak of the absorbance profile can be used to detect any changes in molecular weight due to nuclease action. With a heterogeneous mRNA population, these tests cannot be used because no individual mRNA species can be resolved. In this situation, the only possible course is to obtain evidence which would be inconsistent with RNA degradation taking place.

It was shown in Figure 9 that the modified method of polysome isolation from pea leaves resulted in a preparation which contained larger polysomes than obtained when using the unmodified method. However, this does not necessarily mean that the polysomes, so obtained, were undegraded. In Figure 35 in section III 4A(i) it is shown that polysomes isolated by these methods still contain their nascent chains and, more importantly for this discussion, that there is no radioactivity associated with the monosome peak. These observations imply that there was very little, if any, nicking of the mRNA during the polysome isolation. If the mRNA linking the ribosomes of a polysome together had been nicked then smaller polysomes would result, some of which must be monosomes, and so labelled nascent chains would appear in the monosome region of the gradient. Since this was not observed, it is reasonable to conclude that the mRNA was not nicked. The data

shown in section III1B(ii) indicate that the mRNA was probably not degraded by subsequent handling procedures.

When this polysomal RNA was translated in the wheat-germ system, the pattern of products shown in Figure 10 was obtained. That this pattern does result from the translation of mRNAs present in the pea polysomal RNA and not by some other process is discussed in section III1B(ii). The most striking feature of this pattern of products is the lack of high molecular weight polypeptides. However, the wheat-germ system used has been shown to be capable of translating the mRNAs for large polypeptides if they are present; the RNA is unlikely to be degraded (see above) and altering the conditions of the assay does not change the pattern. So it must be concluded that the polysomal RNA does not contain significant amounts of mRNAs which code for high molecular weight polypeptides. Even very long exposures of autoradiographs failed to detect any radioactivity as discrete bands in the high-molecular weight region; occasionally a band was seen but this was not reproducible, and was barely visible above the background fogging

3) Changes in the mRNA population during the greening of etiolated pea shoots.

The results of an experiment to look at changes in the mRNA population during the greening of etiolated tissue are presented (section III1Biii). It has been argued earlier that the wheat-germ system responds to the added poly(A)-containing RNA by synthesising a range of products in a reproducible and specific manner. Therefore it is assumed that this pattern reflects the mRNA population of the added RNA. On this basis, Figure 17 shows that the mRNAs for two

polypeptides,of molecular weight 20,000 (P20) and 32,000 (P32), form an increasing proportion of the mRNA population during greening; the mRNA for P20, in 48 hour-greened tissue, is a larger proportion of total mRNA than in the green control.

The main aim of the work described in section III1Biii was to determine how the mRNA population varied during greening, because it was possible that greening tissue might be suitable starting material for the isolation of specific mRNAs. This proved to be the case. Table V shows that the mRNA activity isolated from greening etiolated tissue was about 3-fold greater than from etiolated tissue (expressed as cpm/g. tissue), and was twice the value for green tissue. Furthermore the mRNAs for P20 and P32 increased in amount relative to the other mRNAs. So tissue which has greened for about 48 hours under continuous high-intensity illumination would seem to be promising starting material for the isolation of these particular mRNAs.

These results also give some insight into the changes which take place during greening. It was emphasised earlier that the pea shoot does not present a good developmental system in that it is a heterogenous collection of tissues, so all the results effectively represent averages from a population of cells at different developmental stages. However, the whole system is progressing from the etiolated to the green state and can be used to study aspects of chloroplast development at the level of mRNA and protein synthesis.

The development of a fully-functional chloroplast is under the control of a variety of photoreceptors (Smith, 1975a; Bradbeer and Montes, 1976). Phytochrome has been shown to

mediate many of the early events of chloroplast development (Graham et al 1968, 1971) but later events certainly require other photoreceptor systems e.g. the final steps in the synthesis of chlorophyll require a photochemical reaction of protochlorophyllide (Smith, 1975). In particular, the development of granal stacks and co-operativity between the two photosystems of photosynthesis requires continuous illumination of the plants (Armond et al, 1976).

Phytochrome is a proteinaceous photoreceptor which has been implicated in a large number responses of plants to light; these range from seed germination which takes place hours after the light stimulus, to changes in membrane potentials which take place within seconds of the stimulus (for a review of the many responses to phytochrome see Smith, 1975c). It is obvious that responses which take place only hours after the light stimulus are probably secondary, or even tertiary, effects of the initial response of the phytochrome system. There are two main hypothesis as to the mode of action of phytochrome. The first suggests that phytochrome acts directly on the genetic material of the cell, and so switches gene transcription on and off as required. The second says that the phytochrome is associated with membranes, and exerts its effects by changing the properties of these membranes. The evidence for and against these two theories is presented in Smith (1975b) and at the moment the second mechanism seems more likely; this is because there is evidence that the rapid effects of phytochrome do not require changes in the RNA or protein synthetic machinery of the cell. However, many of the long term effects of phytochrome must be mediated at the level of transcription and/or translation. It is of course possible that phytochrome acts directly at more

than one site in the cell.

Smith (1976a) investigated the *in vivo* response of bean cytoplamic polysomes to various light-treatments. He concluded that the increase in the number of ribosomes in polysomes was under phytochrome-mediated control and was due to increased packing on existing polysomes; it did not require the synthesis of any new mRNA. However, he admitted that the evidence was suggestive rather than conclusive, and that it was necessary to investigate the mRNA population of the polysomes to be sure of this conclusion. It might be that the spectrum of proteins synthesised by the polysomes was modulated by a change in the proportions of various mRNAs without the need for the synthesis of new mRNA. The experimental procedure used in section III1Biii could be adopted to determine the mRNAs present in polysomes before and after various light-treatments, as well as the total mRNA activity of the tissue. By these means it would be possible to detect any changes in the mRNA population of polysomes and to determine how these arose. A change in the pattern of proteins synthesised by polysomes, which did not involve *de novo* synthesis of mRNA, could be explained by the action of phytochrome on membranes. There is evidence from animal systems that changes in the cellular ionic enviroment can produce changes in protein synthesis. Carrasco and Smith (1976) have shown that the translation of picorna virus RNA is stimulated by Na^+, whereas the translation of globin mRNA is inhibited. They postulated that in virus-infected cells the coat protein of the virus alters the cellular membrane so that the concentration of Na^+ in the cell rises; this increase switches off host-protein synthesis and stimulates the viral protein synthesis. This

stimulatory effect is thought to be mediated at the initiation step. Nuss and Koch (1976) have shown that "salt-shock" of cultured myelome cells can affect the initiation of protein synthesis within the cells, and thereby change the spectrum of proteins synthesised. Therefore, any theory which involves phytochrome acting upon the plant cell membranes could easily be adapted to explain any changes in protein synthesis observed. The experiments reported in section III1Biii were conducted over much too long a time-scale to have any relevance to this discussion; Smith (1976a) found that the maximum effect of phytochrome on polysome size was reached after 2 hours.

Armond *et al* (1976) have shown that pea plants which are grown under intermittant light conditions (2minutes light, 118 minutes dark) can develop full photosynthetic activity but have reduced levels of chlorophyll *b* and chlorophyll *a*/*b*-binding protein, no granal stacking, smaller photosynthetic units and a reduced quantum efficiency compared with normally grown plants. When transferred to continuous light conditions, the plants acquire all the characteristics of normal plants. A system such as this, where there is only one major protein which changes, the chlorophyll *a*/*b*-binding protein, would seem ideal for studying the control of the mRNA for this protein. Indeed, when the intermittently-grown material has been transferred to continuous-light conditions, it might be expected that the mRNA for this protein would be abundant; thus facilitating its isolation. The methods of polysome isolation and mRNA translation described in the present work could be used to gain a great deal of information about the synthesis of this protein. It would, for instance, be possible to determine whether or not the mRNA for this protein was

stored in an untranslated state in intermittently-illuminated plants or whether de novo synthesis of the RNA occurred only under continuous illumination.

4) Location of messenger activity in the poly(A)-containing RNA.

In section III1Biv it was argued that the bulk (80%) of the mRNA activity found associated with pea polysomes was due to the poly(A)-containing RNA. This percentage was higher than the figure of 50% obtained by Gray and Cashmore (1976), but it was pointed out that the procedures used by them may have resulted in RNA with short poly(A)-tails appearing in the non-poly(A)-containing fraction. It is known that RNA with poly(A)-tails less than about 20-30 residues long will not bind to either oligo-dT cellulose or poly(U)-Sepharose (Hruby and Roberts, 1976; Nudel et al, 1976). It was also argued that the method used by Gray and Cashmore (1976) to detect poly(A) would not have detected poly(A) synthesised before the labelling period began. The poly(U)-hybridization assay used in section III2Biv also showed no detectable poly(A) in the non-poly(A)-containing fraction. However, it was pointed out that the poly(A) which might have been present would have been of such a short length that it would not have been precipitated by the TCA. Thus very short poly(A)-tails may go undetected by methods which rely on TCA-precipitation.

The problem of ensuring that non-poly(A)-containing RNA really does not contain any poly(A) segments at all is very difficult to solve. It has been reported above that poly(A)-tails below a certain length will not bind to the affinity columns used to obtain poly(A)-containing and non-poly(A)-containing RNA fractions. In these cases it was possible to detect the poly(A) in the material which did not bind to the

column because a single mRNA species was used, i.e. EMC RNA (Hruby and Roberts, 1976) and globin mRNA (Nudel et al, 1976). The non-poly(A)-containing RNA fraction is predominantly rRNA, and so it would be necessary to detect the presence of small poly(A)-tails on only a small percentage of the RNA in the fraction. This usually means working at the limits of the assay. Furthermore, the small size of these poly(A)-tails poses difficulties other than those due to the difficulty in precipitating them. In hybridizations with [^{3}H]-poly(U), the thermal stability of any hybrid is related to its length by the equation $b\Delta T = 820$,

where b= the number of bases in the hybrid

and ΔT= the reduction in melting temperature of the hybrid compared to that of an infinitely long hybrid (Thomas and Dancis, 1973).

Using this relationship it can be seen that the melting temperature drops rapidly for short poly(A)-tails, e.g. when b=20, $\Delta T = 41^{\circ}$; when b=15, $\Delta T = 54.5$. So to detect such short tails by hybridization would require less stringent conditions which, in turn, increases non-specific hybridizations.

In section III1Biv it was suggested that there is evidence for a population of mRNAs which does not have a poly(A)-tail, and which contains sequences different from those present in the poly(A)-containing RNA. However, whenever the non-poly(A)-containing RNA fraction from pea polysomes is translated in a cell-free system, the pattern of products very closely resembles that due to poly(A)-containing RNA. This would imply that there is also a non-poly(A)-containing RNA population which has the same sequences as poly(A)-containing RNA. There are three ways in which this might arise:-

1) by the transcription of the same gene, or two genes for the same protein, into two populations of mRNA, one which is polyadenylated and one of which is not;

2) by de-adenylation of pre-existing polyadenylated mRNA in the cytoplasm;

3) by the inability of present techniques to detect the presence of short poly(A)-tails.

The third alternative is clearly a trivial explanation, but is one which cannot be decisively eliminated at the moment. It is particularly important to establish whether or not the second and third alternatives are correct; they only differ in the degree of de-adenylation postulated. The work of Huez et al (1974) and Nudel et al (1976) has shown that the length of the poly(A)-tail of an mRNA may bear a relation ship with its stability in the cell. In particular, they have shown that tails less than 16 residues long confer nothing to the stability of the mRNA but that longer tails increase the stability; tails greater than 32 residues give maximum stability. Now many of the so-called non-poly(A)-containing RNA fractions can contain at least 50% of the total mRNA activity (Gray and Cashmore, 1976; Rosen, 1975). If this were due to poly(A)-containing mRNA which had been completely de-adenylated, then such RNA would have to be quite stable to account for so much of the mRNA activity. This postulate of stability does not fit with the data relating poly(A)-content to stability. If the activity were due to poly(A)-containing RNA which no longer bound to an affinity column, then the residual poly(A) would still stabilize the mRNA to some extent; 32 A residues is around the limit of binding to oligo-dT cellulose. Thus, the presence of activity in the non-poly(A)-containing RNA due to contamination by poly(A)-containing RNA

could be rationalised if the third possibility were correct.

The data presented in section III1Biv do not establish that there is an mRNA population, lacking poly(A), which codes for proteins different from those coded by poly(A)-containing RNA. However, it does suggest that such a population might exist. Although Figure 19 shows that there are no obvious, new polypeptides synthesised from non-poly(A)-containing RNA when compared with poly(A)-containing RNA-directed products, it is apparent that less synthesis of the proteins P20 and P32 occurs from the non-poly(A)-containing RNA. This must mean that other polypeptides form an increasing proportion of the products. One explanation would be that another set of mRNAs (non-poly(A) containing mRNAs ?) form an increasing proportion of the mRNA population. However another explanation is possible. The stimulation over endogenous activity is very low with such RNA preparations due, at least in part, to the high proportion of rRNA present. There is known to be some RNAase activity present in wheat-germ extracts (Hunter et al, 1977) and so it might be that the rRNA added to the system would compete with endogenous RNA for degradation by the nuclease. This competition would result in a stimulation of synthesis of products encoded by endogenous mRNA.

It is clear from this discussion that many problems of interpretation arise when relatively crude RNA preparations are translated in cell-free systems. Nevertheless, the results presented in this section establish that RNA prepared from pea leaf polysomes can be translated with fidelity at a rate sufficient to allow characterization of the products and purification of individual messenger species.

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PAGE

MISSING

2. CHARACTERIZATION OF IN VITRO-SYNTHESISED POLYPEPTIDES.

In section III1 it was shown that the wheat-germ protein synthesising system translates poly(A)-containing polysomal RNA from pea shoots into a range of polypeptides. Evidence was presented which suggested that the RNA was probably being faithfully translated. In this section it will be shown that this is the case from studies of the identities of some of the polypeptides synthesised *in vitro*.

One of the aims of this work was to investigate the possibility of isolating the mRNA for a particular protein in pure form. The results reported in section III1Biii showed that the mRNAs for two polypeptides appeared in increasing amounts during the greening of etiolated tissue. These mRNAs would thus seem to be the best candidates for isolation. It is therefore necessary to characterise the polypeptides coded for by these mRNAs with the hope of identifying them; because these polypeptides form such a large proportion of the products synthesised by RNA from 48 hours-greened or fully-green tissue, they are the easiest to study.

In section I3B, it was argued that the small subunit of Fraction I protein ought to be a major polypeptide synthesised in greening tissue. The two major products synthesised *in vitro* have molecular weights of around 20 and 30,000 respectively whereas the small subunit has a molecular weight of about 14,000. Therefore it is also necessary to see whether or not the small subunit is present in the *in vitro* products. If the small subunit could not be detected in the *in vitro* products, this would cast doubt upon the fidelity of translation *in vitro*.

A. Electrophoretic analysis of the in vitro-synthesised polypeptides.

An important initial step in the characterization of a polypeptide is to determine its molecular weight. The most convenient method to use, when dealing with a range of radioactive polypeptides present in small amounts, is SDS-polyacrylamide gel electrophoresis, followed by autoradiography of the dried gel. The in vitro products were analysed on slab gels of the type described by Laemmli (1970); this type uses a stacking gel system, and the resolving gel contains a linear gradient of acrylamide concentration from 7.5% to 25% (section II2Eic). This gradient gave excellent resolution of a wide range of polypeptides, e.g. Fig.6 shows the resolution of polypeptides from 140,000 to less than 10,000 molecular weight on the same gel. However, it was not possible, using this system, to construct a molecular weight calibration curve. Therefore, another gel system was used for molecular weight measurements only. The system of Fairbanks et al (1971) has been used by other workers to obtain accurate molecular weights for viral proteins (Clegg et al,1976). These workers also found that the gradient gel system could not be used to determine molecular weight (Brzeski and Clegg, personal communication).

The gel system of Fairbanks et al (1971) was used to construct the calibration curve shown in Fig.21. The mobilities are expressed relative to that of cytochrome c. The standards and unknowns were run on the same slab gel for ease of comparison. The calibration curve consists of two straight lines which meet at around 45,000. This type of curve has been obtained by others, but not using this gel system

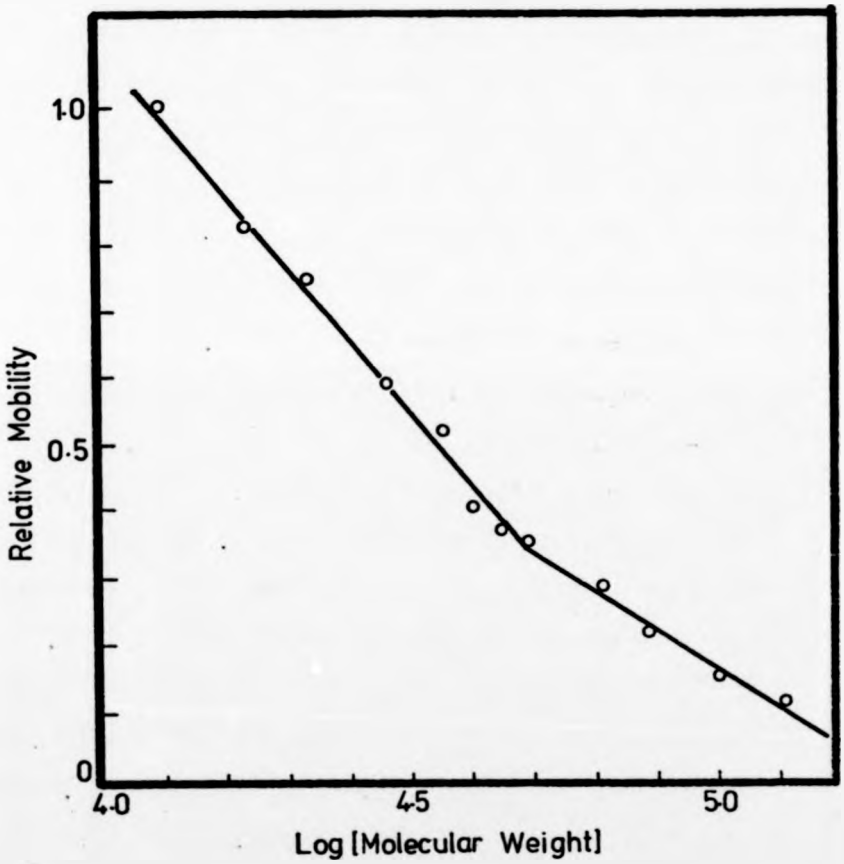

1·0
0·5
0
Relative Mobility
4·0
4·5
5·0
Log [Molecular Weight]

Figure 21 Molecular weight calibration curve.

The mobility of various standard proteins on SDS-containing polyacrylamide slab gels, using a continuous buffer system (section II2Eid), was measured relative to cytochrome c. The $\log_{10}$ of molecular weight is plotted against the mobility of the protein to give the calibration curve. The standard proteins were:- β-galactosidase (130,000); phosphorylase (100,000); bovine serum albumin (68,000); fumarase (49,000); ovalbumin (44,000); aldolase (40,000); lactate dehydrogenase (36,000); carbonic anhydrase (29,000); soyabean trypsin inhibitor (21,500); myoglobin (17,200); cytochrome c (12,600).

(Neville, 1971; Eaglesham and Ellis, 1974). Clegg *et al* (1976) did not observe such a biphasic relationship; however, they constructed their calibration curve only for molecular weights above 40,000.

The electrophoretic mobilities of the *in vitro* products are such that the main polypeptides fall on the same straight line, and so the graph in Fig.21 can be used to estimate their molecular weights. The two main *in vitro* products have molecular weights of 20,600±1,000 and 32,400±1,500. These products will be called P20 and P32 in all further discussion; the P is intended to stand for polypeptide while the number is the approximate molecular weight x 10^{-3}. The small subunit of Fraction I protein, when run in this gel system, had an apparent molecular weight of 14,000±1,000. This figure compares very well with the published value of 13,500 (Haslett *et al*, 1976).

This gel system was not used for routine analysis of the *in vitro*-synthesised products because it does not have the same resolution as the gradient gel system. The lack of a stacking system means that the sample bands do not sharpen, and so tend to be more diffuse. In fact, most of the uncertainty in the molecular weight determination comes from the measurement of relative mobility.

The characterization of P20 and P32 will form the main part of the work described in this section. In tissue which is greening, and so developing chloroplasts, it would be expected that cytoplasmically-synthesised polypeptides destined for the chloroplast would occur in high abundance. Thus P20 and P32 may be such polypeptides, and study of them may lead to some insight into the relationship between the

cytoplasm and the chloroplast.

In initial experiments all the *in vitro* products were labelled with [^{35}S]-methionine. This is not a good precursor to employ when attempting to characterise these polypeptides. Methionine occurs with only a low abundance in most proteins, and some do not contain it at all. This means that in a population of polypeptides the low molecular weight components will be under-represented when labelled with [^{35}S]-methionine. It was also intended to carry out a tryptic peptide analysis of the *in vitro* products for comparison with that of known proteins (section III2C). With low molecular weight polypeptides this procedure might lead to only one or two labelled peptides being produced. The small subunit of Fraction I protein, for example, contains only one or two methionines (Haslett *et al*, 1976). It was therefore decided to use [^{3}H]-arginine and [^{3}H]-lysine together to label polypeptides *in vivo* and *in vitro*, because these two amino acids are much more prevalent and evenly distributed throughout the polypeptides than is methionine. An additional advantage is that trypsin cleaves proteins on the C-terminal side of both lysine and arginine, and so every tryptic peptide should be labelled.

When using the tritiated arginine and lysine mixture in the wheat-germ system, it was found that the endogenous incorporation was higher than when using [^{35}S]-methionine. One reason for this is probably that the small peptides which constitute the endogenous products are relatively poor in methionine. This means that the stimulation over the endogenous incorporation was lower for any given RNA, e.g. poly(A)-containing RNA from 48 hours-greened tissue stimulated


A
B
C
D
E
F
G

Figure 22 Comparison of *in vitro*-synthesised polypeptides with labelled standard proteins.

Total soluble protein and the chlorophyll *a*/*b*-binding protein were isolated from pea shoots grown in the presence of a mixture of [^{3}H]-lysine and arginine (section II2M). RNA was translated in the wheat-germ system as described in section II2Jiia. Each reaction mixture (20µl) contained 10µCi each of [^{3}H]-arginine/lysine. The products were analysed by electrophoresis on slab polyacrylamide gels as described in Fig. 5.

A. Endogenous products of the wheat-germ system

B. Total soluble pea shoot proteins

C. Products of poly(A)-containing polysomal RNA from etiolated pea shoots which have greened for 48 hours.

D. Chlorophyll *a*/*b*-binding protein fraction

E. Products of non-poly(A)-containing polysomal RNA from etiolated pea shoots which had greened for 48 hours.

F. Total soluble pea shoot proteins

G. Products of poly(A)-containing RNA from etiolated pea shoots.

[^{35}S]-methionine incorporation 52-fold (115,600 cpm/2µl compared with 2,200 cpm/2µl) but stimulated [^{3}H]-arginine/lysine incorporation only 30-fold (82,000 cpm/2µl compared with 2750 cpm/2µl).

The preparation of the labelled protein fractions from pea shoots is described in section II2N. Figure 22 shows a comparison of the *in vitro* products with the *in vivo*-labelled polypeptides on an SDS polyacrylamide gel. This gel shows the following points:-

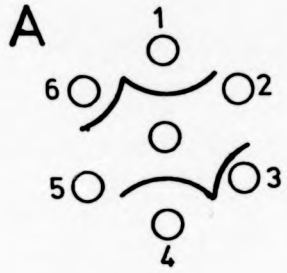
1)that the endogenous products (track A) consist mainly of one polypeptide with a molecular weight of about 14,000; this product has a slightly slower mobility than the small subunit of Fraction I protein. When labelled with [^{35}S]-methionine, the endogenous product was not usually visible so the appearance of this polypeptide correlates with the increased endogenous incorporation. This endogenous polypeptide can be seen in the *in vitro* products (tracks C&E).

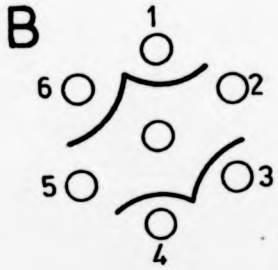
2) Neither P20 nor P32 in track C correspond to any band present in the *in vivo*-labelled soluble chloroplast proteins (track B) or to the chlorophyll *a*/*b*-binding protein (track D).

3) Neither the small subunit nor the chlorophyll *a*/*b*-binding protein appears to be present in the products of either poly(A)-containing or non-poly(A)-containing RNA from 48 hours-greened tissue (track C&E).

4) The products of poly(A)-containing RNA from etiolated tissue (track G) do not contain any polypeptides which exactly co-electrophorese with the *in vivo*-labelled proteins.

5) There is a difference in the relative intensities of P20 and the other *in vitro*-synthesised products when the arginine/lysine is used for labelling compared to methionine.





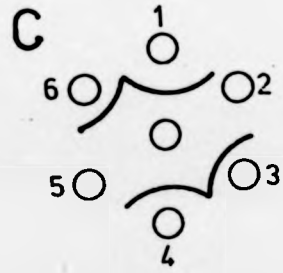


Figure 23 Ouchterlony double diffusion assay of rabbit antiserum.

Agar(1%, Difco Nobel agar in 0.9% NaCl) was poured into petri dishes to a depth of about 3mm and holes cut out using a template. The antigen was placed in the centre well and the antisera in the outer wells. The wells contained 80ul. The plates were left to develop for 24 hours in the dark in a damp box. The antisera were distributed as follows:-

wells 1&4, anti-Fraction I serum diluted 10-fold with PBS; wells 2&5, anti-small subunit serum, undiluted; wells 3&6, anti-large subunit, undiluted

Centre wells:-

A. Pea Fraction I protein (0.5mg/ml)

B. Pea large subunit (1mg/ml)

C. Pea leaf soluble protein (1mg/ml)

This indicates that P20 has a relatively poor methionine content.

Thus it was not possible to even tentatively identify any of the polypeptides synthesised *in vitro*. In particular, neither the small subunit nor the chlorophyll a/b-binding protein could be correlated with any of the *in vitro* products, even though they would be expected to be major products *in vivo*. This lack of correspondence between the *in vitro* products and the *in vivo* standards is unexpected, and might imply that the wheat-germ is translating the mRNAs for two minor polypeptides preferentially.

B. Characterization of the *in vitro* products by immunological methods.

Serum was obtained from the blood of rabbits which had been injected with either Fraction I protein or one of its subunits as described in section II2Bi. Initially the antibody activity of these sera was determined by Ouchterlony double-diffusion assays (Ouchterlony, 1968). By this method it is possible to tell whether or not a particular serum contains antibody to a particular protein.

Figure 23 shows the pattern of precipitin lines obtained with various combinations of anti-sera and antigens. The serum from rabbits injected with Fraction I protein gave a single, sharp precipitin line against both pure Fraction I protein and large subunit, as would be expected. The serum from rabbits injected with the large subunit behaved in the same way; there was less antibody present than in the anti-Fraction I serum. Both these anti-sera gave a single, sharp precipitin line when total leaf soluble protein was used as

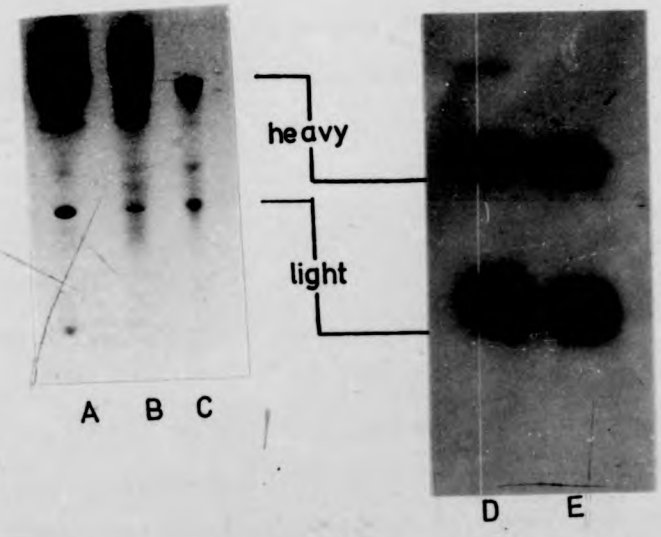


Figure 24 Purification of immunoglobulin G from rabbit serum.

The serum from rabbits was fractionated as described in section II2Biii. Samples of immunoglobulin from the pre-immune serum or from immune serum after chromatography on Fraction I-Sepharose 4B (section II2Ci) were iodinated with [^{125}I] as described in section II2L. The iodinated samples were chromatographed on DEAE/CM cellulose. The samples were analysed by electrophoresis either on a 12.5% polyacrylamide slab gel (A,B and C) or on a gradient polyacrylamide slab gel (D and E). The proteins were visualised either as Coomassie blue-staining bands (A,B and C) or by autoradiography (D and E).

A. Total rabbit serum (150µg)

B. Protein precipitated from rabbit serum by 50% saturated ammonium sulphate.

C. The protein from B which does not bind to DEAE cellulose at pH 7.6 (section II2Biiic).

D. [^{125}I]-labelled immunoglobulin from pre-immune serum after chromatography on DEAE/CM cellulose.

E. [^{125}I]-labelled immunoglobulin from anti-Fraction I serum after chromatography on DEAE/CM cellulose.

antigen. Since the precipitin lines were concurrent, each antibody must be recognising at least some part of the same molecule. The serum obtained from rabbits injected with the small subunit did not give a visible precipitin line with any antigen and varying both the serum and antigen concentrations over a wide range did not alter this situation. One possible explanation for this result is that there was so little antibody against small subunit in the serum that it would never be possible to obtain equivalence in this type of assay. The precipitin line forms at the point in the agar where the antibody and antigen concentrations are in the correct proportions for precipitation; this is the equivalence point. In whole serum the immunoglobulin fraction is only a relatively small part of the total protein and so it is an easy matter to increase the effective antibody concentration by purification procedures. The serum from the small subunit-injected rabbits was therefore taken through part of the immunoglobulin purification procedure, even though it may not contain any anti-small subunit activity.

The procedures which can be used to isolate immunoglobulin preparations of increasing purity are described in section II2Biii. Figure 24 shows the purifications which can be achieved. Track A shows the pattern for unfractionated serum, and the most striking feature is the large amount of serum albumin present. After two cycles of precipitation with 50% saturated ammonium sulphate (track B), most of the albumin has been removed, but there are many other polypeptides present as well as the immunoglobulin chains. However, chromatography on DEAE cellulose at pH7.6 (section II2Biii) results in most proteins, apart from the IgG, remaining

bound to the column. Track C shows the material which does not bind; this material was usually only 15% of the material loaded onto the column, and was predominantly IgG. Track D shows an autoradiograph of [^{125}I]-labelled IgG. A sample of serum, which had been ammonium sulphate-precipitated and so had a composition similar to that of track B, was iodinated with [^{125}I]-iodide (section II2M). This labelled sample was then chromatographed on a DEAE/CM cellulose column as described (section II2Biii); the material which did not bind is shown in Fig. 24D. This Figure shows that the IgG fraction obtained is extremely pure; the only extra labelled band visible accounts for about 1% of the total radioactivity. Track E also shows [^{125}I]-labelled material which does not bind to a DEAE/CM cellulose column. However, before this step, the IgG was further enriched by chromatography on a column of Fraction I-Sepharose (section II2Ciia). This preparation only contains material which co-electrophoreses with the heavy and light chains of IgG.

Aliquots of all the sera were taken through the ammonium sulphate precipitation procedure and DEAE/CM cellulose chromatography steps, and then bound to Sepharose-4B as described in section II2Ci. Such material acts as an affinity matrix and so can be used repeatedly to assay for the presence of particular polypeptides. Such an affinity column has already been described for Fraction I protein by Gray and Wildman (1975). These columns can also be used to compare the antibody activities of the IgG fractions bound to them.

Total leaf soluble protein was chromatographed on all the columns, and the bound and non-bound fractions were analysed on SDS-polyacrylamide gels. Figure 25 shows the result of one such analysis. The chromatography was as described in

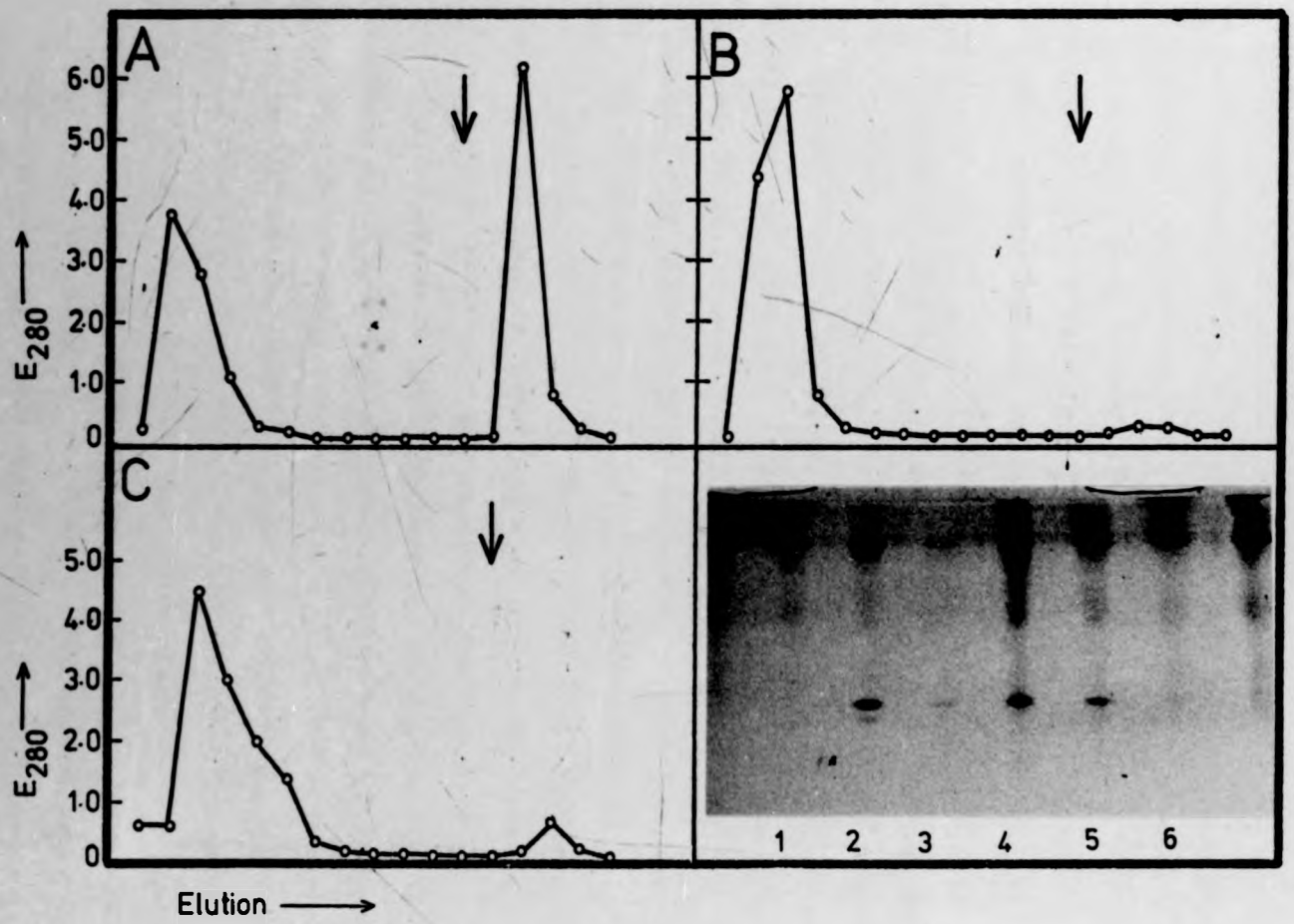


Figure 25 Analysis of pea leaf soluble protein by chromatography on antibody affinity columns.

Pea leaves (20g, 12 days old) were homogenised as described in section II2Bi. The supernatant after the $30,000g_{ave}$ centrifugation was made 50% saturated by ammonium sulphate and the precipitated protein was pelleted by centrifugation at 30,000g This pellet was resuspended in 2ml buffer (50mM tris-HCl, pH 7.8, 0.2M NaCl) and dialysed against this buffer. Aliquots (0.5ml) of this suspension were loaded onto the various columns and washed through with the buffer. The material which had bound to the column was eluted by 50mM tris-HCl, pH 7.8, 0.2M NaCl, 8M urea.

The arrow indicates the change in buffer.

A. Anti-Fraction I-Sepharose

B. Anti-small subunit-Sepharose

C. Anti-large subunit-Sepharose

D. The various fractions obtained by chromatography described above were precipitated by addition of an equal volume of 10% TCA. The precipitates were washed with acetone and then dissolved in electrophoresis buffer (50mM tris-380mM glycine, pH 8.5, 0.1% SDS) with SDS added to a final concentration of 2%. The samples were electrophoresed on a 12.5% slab gel (section II2Eic)

1. the unbound and 2. the bound fraction from A

3. the bound and 4. the unbound fraction from B

5. the bound and 6. the unbound fraction from C

section II2Cii. Panels A, B, C and D show the E_{280} elution profile of the leaf extract on the columns containing anti-Fraction I, anti-large subunit and anti-small subunit antibodies respectively; the arrows mark the start of the elution with 8M urea. Panel D shows a gel analysis of the bound and non-bound fractions from these profiles. Each affinity column was the same size and had the same amount of IgG protein bound to it (3ml bed volume, 24mg protein).

It can be seen that both the anti-Fraction I and anti-large subunit columns bind appreciable amounts of material, 40% and 10% of the input E_{280} respectively whereas the anti-small subunit column binds only 1-2% of the input protein. This level of binding to the anti-small subunit column is of the same order as for columns containing the IgG from pre-immune serum. Comparing the non-bound fractions from the columns (Fig.25D), it is clear that there is hardly any Fraction I protein in the non-bound fractions from the anti-Fraction I and anti-large subunit columns (tracks 1&6). However, the non-bound material from the anti-small subunit column still contains a large amount of Fraction I protein; this would follow from the very low binding of protein. It should be noticed that the prominent band present in the non-bound fractions is probably not large subunit. This band has a slightly slower mobility than the large subunit found in the bound fractions; this similar mobility probably explains the broadness of the large subunit band in the total extracts and in track 4, because this band would overlap with the large subunit. In the non-bound fractions there is no visible small subunit band, which would also suggest that the other band is not the large subunit. The very small amount of protein

which bound to the anti-small subunit column meant that only a very low amount could be loaded onto the gel, and so the presence of both large and small subunits in this fraction (track 4) is not significant. Fraction I accounts for such a high proportion of the soluble protein that, at low loadings, only the large and small subunits might be seen.

The conclusion from this analysis is that the anti-Fraction I and anti-large subunit anti-sera do contain anti-Fraction I activity; it is not possible to say that the anti-large subunit is specific for just the large subunit, and not the whole Fraction I protein, on the basis of these experiments. The anti-small subunit serum does not contain any significant amount of anti-Fraction I protein activity; this would imply that it did not contain any anti-small subunit activity either. So it must be concluded that the attempt to raise antibodies specific for the small subunit of Fraction I protein was not successful. However, the anti-Fraction I antiserum is immunologically active and should contain some antibodies specific for the small subunit. The anti-Fraction I antibody was used, throughout the rest of the work, to identify polypeptides immunologically related to Fraction I protein.

Attempts were made to analyse the <u>in vitro</u> products of various RNA preparations by chromatography on the antibody-containing affinity columns; however, these attempts did not prove successful. The main problem with this approach appeared to be the non-specific binding of the labelled polypeptides to the columns. The wheat-germ assay mixtures contained very small amounts of protein (100 to 200μg), but addition of unlabelled BSA or pea soluble protein did not

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reduce the amount of non-specific binding. The binding obtained was regarded as non-specific because it was the same percentage of input radioactivity (15 to 30%) irrespective both of the column used and of the RNA which had been used in the wheat-germ system. There was a large dilution of the sample by this chromatography step, and hence there were problems in recovering labelled polypeptides for analysis on gels. The columns had a definite life-time. After each column had been used to analyse 20 to 30 samples, it rapidly lost its ability to bind anything at all. Washing the columns with urea, guanidium hydrochloride, low pH buffer or SDS did not restore their binding ability.

After the failure of the affinity chromatography as a method to analyse and identify the in vitro-synthesised products, it was decided to try immune precipitation of the products. This can be attempted in two ways, one direct, the other indirect. The direct precipitation method involves adding excess, unlabelled antigen (in this case, Fraction I protein) to the samples before addition of the antibody. The amounts of antigen and antibody are calculated to be equivalent, and so a precipitate is obtained. The indirect method involves the addition of sufficient antibody to the sample such that every antigen has an antibody molecule bound to it. The precipitation is achieved by addition of a second antibody, usually goat anti-rabbit or sheep anti-rabbit, in an amount equivalent to the first, specific antibody.

In the literature both methods are described, but it was decided that the indirect method would be used in this work. There were two main reasons for this choice. Firstly, the direct precipitation method uses large amounts of the

specific antibody compared with the indirect method. It is difficult to discover exactly what quantities are used in the immune precipitation reactions described in the literature, but Shapiro *et al* (1976) and Daniel *et al* (1977) provide clear data. Shapiro *et al* employed the indirect method, and used 20µg of anti-vitellogenin/75µl incubation mixture followed by sufficient goat anti-rabbit IgG to get precipitation. Daniel *et al* used the direct method to precipitate ligandin from wheat-germ incubation mixtures; they used 300µg of anti-ligandin/300µl of incubation mixture. This difference is only by a factor of 2 to 3 fold, but the ligandin antibody had been purified by affinity chromatography, and so was enriched by a large factor relative to the vitellogenin antibody, which had only been chromatographed on DEAE/CM celluloses. Thus the anti-ligandin antibody should only contain those antibodies specific for ligandin, whereas the anti-vitellogenin antibody would contain all the antibodies present in the serum originally (Shapiro *et al*, 1974). Secondly, it was intended to use the indirect precipitation method to isolate polysomes synthesising a specific polypeptide (section III4A), and so it was decided to use this method to investigate the *in vitro* products as well.

The IgG preparation was enriched for the anti-Fraction I antibodies by chromatography on Fraction I-Sepharose 4B (section II2Ciia). The fractions which did not bind or which were eluted at either pH4.8 or pH2.8 were pooled, and the anti-Fraction I activity in each measured by a quantitative immunoprecipitation assay (section II2Biva). The basis of the assay is that, at around the maximum precipitation (see Fig.1), the antibody and antigen are at equivalence i.e. neither is

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	total protein	antibody (mg)	antibody % of protein	yields total protein	yields antibody
ammonium sulphate precipitated serum	1960	160	8	100	100
affinity column					
- non-bound	1760	60	3.5	90	38
- pH 4.8 eluted	110	35	32.0	5.6	22
- pH 2.8 eluted	42	-	-	2.2	-

Table VIII Recovery of anti-Fraction I antibody after chromatography on Fraction I-Sepharose 4B.

The immunoglobulin fraction obtained by two cycles of precipitation of immune serum by 50% saturated ammonium sulphate, was chromatographed on a column of Fraction I protein bound to Sepharose 4B (section II2Ciia). The anti-Fraction I activity of each fraction was determined by quantitative immunoprecipitation with purified Fraction I protein (section II2Biva). The protein content of each fraction was measured by the Lowry protein determination (section II2O).

in excess. So the precipitate must contain all the added antigen and that amount of antibody present which is specific for that antigen. From the absorbance of the precipitate, and the experimentally determined extinction coefficients of the antibody and antigen, it is possible to estimate the amount of specific antibody present in an antibody preparation.

Table VIII presents the data for the chromatography of anti-Fraction I IgG on Fraction I-Sepharose 4B. The most important point is that the material which stays bound to the column until the pH of the eluting buffer is raised to 2.8 has no activity. This result is unexpected because the reason it stays bound to the column is because it has an affinity for Fraction I protein. The likely explanation is that the IgG has been irreversibly denatured by the eluting buffer. The chromatography was performed at $4^{o}C$, but the pH2.8-eluted fractions stayed in this buffer until their E_{280} had been measured. This explanation was confirmed by pooling all the fractions, precipitating the IgG with ammonium sulphate and repeating the chromatography. This time there was no material which was eluted at pH2.8; this is consistent with the loss of the highly-specific anti-Fraction I activity at the previous pH2.8 elution step. To avoid denaturation at this elution step it might be necessary to use a different buffer, or to collect into un-buffered tris base and so return the proteins to neutrality as quickly as possible. The eluting buffer must disrupt the very strong antibody-antigen interactions and so would, of necessity, be denaturing; the second alternative might thus be a solution.

It is interesting to note that the amount of protein present in this pH2.8-eluted fraction corresponds very well

with the amount of anti-Fraction I antibody which was lost by this chromatographic procedure. This loss of potentially pure, highly-specific anti-Fraction I protein antibody was very unfortunate, and it was not possible to repeat the whole procedure. However, the material which was eluted from the column at pH4.8 was enriched for the anti-Fraction I antibody; 32% of the protein was anti-Fraction I antibody compared with 3.5% for the unbound material. This pH4.8-eluted fraction presumably contains the antibodies which have a lower avidity for Fraction I protein, and so are eluted by less stringent conditions.

The amount of goat anti-rabbit IgG which was necessary to obtain equivalence with the anti-Fraction I antibody was determined by a similar quantitative immunoprecipitation procedure. However, [^{125}I]-labelled rabbit IgG was used as antigen, and so it was possible to quantitate the amount of antigen in both the pellet and supernatant. Thus the results of this assay were expressed as shown in Figure 2; equivalence occurs at the highest amount of added antigen at which 100% of the antigen is precipitated. In this way it was found that a ratio of 30:1 by weight of goat anti-rabbit to rabbit IgG gave equivalence.

Thus the components used for the immune precipitation of the *in vitro* products were anti-Fraction I antibody purified by affinity chromatography, IgG purified from pre-immune serum by ammonium sulphate precipitation and DEAE/CM cellulose chromatography, and goat anti-rabbit IgG purified in the same way. The *in vitro* products were analysed by immune precipitation as described in section II2Jiic.

Numerous attempts were made to obtain clean immune

precipitates from the *in vitro* products i.e. precipitates which contained only a subset of the polypeptides present. However, the pattern of polypeptides in the precipitates always corresponded with the total *in vitro* products. Thus either every polypeptide is immunologically related to Fraction I protein or there is a great deal of non-specific binding to the precipitate. The latter is the most likely explanation. In some experiments, the precipitate obtained using the anti-Fraction I antibody was enriched in P20 relative to the pre-immune control, but this was not a reproducible observation.

Finally another indirect precipitation method was tried. Kessler (1975) has described the use of heat-inactivated *Staphylococcus aureus* to replace the goat anti-rabbit antibody as the second precipitant. The *S. aureus* used was the Cowan I strain (NCTC 8530) which has a protein on its surface known as the A-protein; this A-protein can bind to the Fc regions of IgG molecules and so bind the molecules to the surface of the bacterium. The advantages of this method are reduced non-specific binding combined with great speed and convenience. For example, in transformed cell lysates, the use of the *S. aureus* method makes detection of SV40-specific antigens feasible, where it was not before (Smith, A.E., personal communication). The binding of the IgG to the A-protein is very fast, probably of the order of seconds (Kessler, 1975). This speed means there is no need to incubate overnight, or longer, to get complete precipitation as is necessary in the goat anti-rabbit method. The specificity is probably improved because the shorter incubations allow less time for non-specific effects to take place. Moreover, the goat

Figure 26 Immune precipitation of [^{35}S]-labelled pea leaf soluble protein.

[^{35}S]-methionine-labelled soluble protein was isolated from pea shoots as described (section II2Ni). Aliquots (50µg) of this preparation were incubated for 60 minutes at 37°C, in a final volume of 60µl, with 100µg of either anti-Fraction I or pre-immune IgG (section II2Jiic). Heat-inactivated S. aureus (10mg) was added and incubation continued for 30 minutes on ice. The bacteria were washed and the adsorbed protein removed as described (section II2Jiic). The immune precipitated samples plus an aliquot (25µg) of the original soluble protein preparation were analysed on a 7.5 to 25% gradient slab gel (section II2Eic). The gel was electrophoresed at 15mA for 16 hours then dried down and the dry gel auto-radiographed at room-temperature for 4 days. the autoradiograph was scanned using a Joyce-Loebl Chromoscan.

A. Total soluble protein

B. Anti-Fraction I precipitated protein

C. Pre-immune precipitated protein

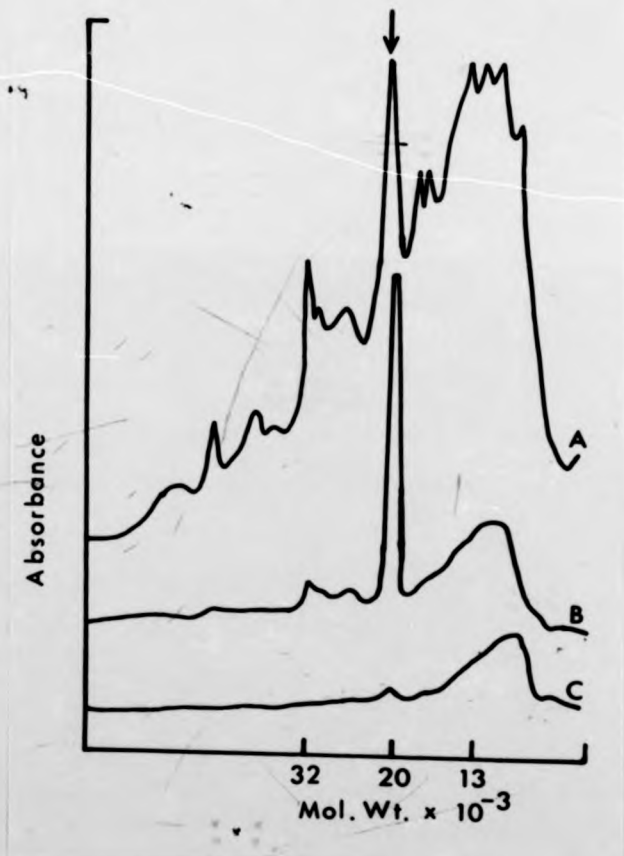

Figure 27 Immune precipitation of poly(A)-containing RNA-directed polypeptides with anti-Fraction I-antibody

Poly(A)-containing RNA was translated in the wheat-germ system and then prepared for immune precipitation (section II2Jiic). In a final volume of 100µl, the *in vitro* products were incubated with 10µg of IgG at 37°C for 60 minutes before addition of 1mg of heat-inactivated *S. aureus*. The polypeptides adsorbed onto the *S. aureus* were prepared for gel electrophoresis as described in section II2Jiib. The products were analysed on slab polyacrylamide gels as described in Fig. 5. The autoradiograph was exposed at room-temperature for 7 days and it was then scanned on a Joyce-Loebl Chromoscan.

A. Trace of total products of poly(A)-containing RNA in the wheat-germ system.

B. Polypeptides precipitated from A by anti-Fraction I antibody and *S. aureus*.

C. As B but using pre-immune IgG and *S. aureus*.

The arrow indicates the position of P20

anti-rabbit antibody preparation contains a range of antibodies, the S. aureus does not, therefore the immunological cross-reactions are reduced.

Figure 26 shows the results of the immune precipitation of in vivo-labelled pea shoot soluble proteins, when S. aureus is used as second precipitant. The result is a very clean precipitation of the subunits of Fraction I protein by the anti-Fraction I antibody, whereas no polypeptide at all is precipitated by the pre-immune IgG. This observation shows that the method is capable of great specificity.

Figure 27 shows the results of the immune precipitation of the in vitro products of poly(A)-containing RNA. The arrow marks the position of P20, and it is striking that this is the only polypeptide precipitated by the antibody. In particular, there is no detectable labelled polypeptide precipitated by the pre-immune IgG.

These observations on their own establish that P20 is immunologically related to Fraction I protein but it is not possible to say to which of the two subunits it is related. However, it is possible to present a case for P20 being related to the small subunit of Fraction I protein and not to the large subunit on the basis of the immune precipitation in the following manner.

It has been shown that the wheat-germ system is capable of faithfully translating added RNA. It was not possible to alter the pattern of products obtained in vitro by changing the incubation conditions. This constancy of pattern makes it unlikely that the large subunit mRNA is translated by the wheat-germ system into a polypeptide much smaller than authentic large subunit (i.e. 20,000 rather than 55,000 M.W.).

In the literature, there are reports both for (Sagher et al, 1976), and against (Bottomley et al, 1976), the ability of the wheat-germ system to translate chloroplast mRNA. It should be noted that Sagher et al found that the large subunit mRNA was faithfully translated by the wheat-germ system, and did not give partial products. When chloroplast RNA (prepared by S. Covey and J. Silverthorne) was assayed under conditions identical to the poly(A)-containing RNA, there was no stimulation above background incorporation. It is also highly unlikely that the poly(A)-containing RNA preparations contained any large subunit mRNA. Chloroplast RNA does not contain any measurable poly(A) and the large subunit mRNA does not bind to oligo dT-cellulose (Wheeler and Hartley, 1975; Sagher et al, 1976). For these reasons it is unlikely that P20 is relatcd to the large subunit and so must be related to the small subunit of Fraction I protein.

C. Tryptic peptide analysis.

To obtain a peptide map of the in vitro-synthesised products requires a procedure capable of handling extremely small amounts of material, which can be detected only by radioactive labelling. The in vitro products are most easily fractionated on polyacrylamide gels, and so the method must also allow for extraction of the polypeptide from the gel before any subsequent analysis. A method which fits these criteria has been described (Clegg et al, 1976). It was designed specifically for the peptide mapping of labelled polypeptides isolated from gels. The procedure used is described in section II2L.

In section III2A it was argued that arginine and lysine

are to be preferred to methionine when labelling a polypeptide for peptide mapping. The tritiated derivatives were used because they have a higher specific activity than the [^{14}C-] labelled compounds, and are cheaper. However, the use of tritium meant that some modifications had to be made to the method. Clegg et al (1976) used [^{35}S]-methionine to label viral polypeptides and located the viral polypeptides on gels by autoradiography. Tritium-labelled compounds cannot be detected directly by autoradiography. If the gel is impregnated with the scintillant PPO then the tritium decay will cause fluorescence, and the labelled material can be detected by autoradiography (Bonner and Laskey, 1974). What effect PPO might have on subsequent analytical procedures in the method is unknown but it may not be beneficial. Therefore, another method of detection had to be found. It was decided to use [^{35}S]-methionine labelled *in vitro* products as markers for the [^{3}H]-labelled *in vitro* products.

Parallel incubations were set up, one containing [^{3}H]-arginine and lysine, the other containing [^{35}S]-methionine. The products were prepared for running on gels as described in section II2Jii. A stacker gel was cast without individual sample slots, and then neoprene rubber inserts were placed on top of the stacker to form one large central slot and two small side slots. The [^{3}H]-labelled *in vitro* products were loaded into the central slot, the [^{35}S]-labelled products into the side slots. The gel was stained with Coomassie Blue after electrophoresis, and the gel then autoradiographed after drying. The autoradiograph serves to locate the [^{35}S]-labelled products Using the stained bands, which are wheat-germ proteins, as guides, it is possible to cut out from the region containing

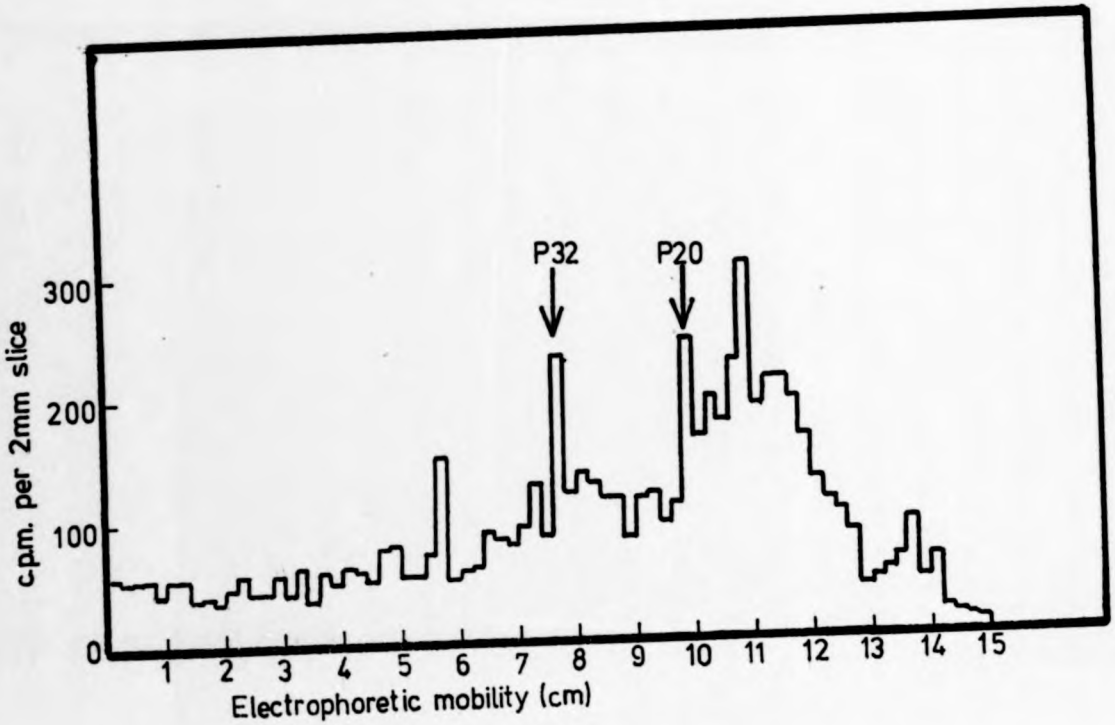

Figure 28 Profile of radioactivity along a gradient polyacrylamide slab gel.

The [^{3}H]-labelled products of poly(A)-containing RNA, from etiolated pea shoots which had greened for 24 hours, were electrophoresed at 15mA for 17 hours on a 7.5 to 25% gradient polyacrylamide gel (section II2Eic). Using [^{35}S]-labelled markers, it was possible to locate the regions of the gel containing [^{3}H]-labelled P20 and P32. These bands were removed and treated as described in section II2L. The radioactivity remaining in the gel and present in the supernatant after trypsin-digestion of these bands was determined by scintillation counting. The profile shown in the figure was obtained by taking 2mm fractions from a strip (1cm wide) of the rest of the gel remaining after removal of the P20 and P32 bands and determining the radioactivity present in the fractions by scintillation counting. The radioactivity present in the regions corresponding to P20 and P32 was calculated back from the known <u>total</u> radioactivity present after trypsin-digestion.

the [^{3}H]-labelled products strips of gel which correspond to the [^{35}S]-labelled bands. In this way it is possible to obtain specific [^{3}H]-labelled polypeptides for peptide analysis.

To test this modified procedure, a trial run was performed. Poly(A)-containing RNA from 24-hours-greened tissue was translated in the wheat-germ system and the [^{3}H]- and [^{35}S]-labelled products were run on a gel as described. The bands corresponding to P20 and P32 were cut out, and digested with trypsin (section II2L). The efficiency of the digestion was tested by measuring both the radioactivity released and that remaining in the gel. For both P20 and P32, between 75 and 80% of the radioactivity was released from the gel.

In order to test that the correct regions of the [^{3}H]-labelled products had been removed, a section along the direction of electrophoresis was taken out of the remaining parts of the gel, and the distribution of radioactivity determined by counting 2mm slices. The radioactivity due to the gel slices which had been removed for the trypsin digestion, was allowed for in the profile; this was easily done because the total radioactivity in the gel slice was known. Figure 28 shows this reconstructed profile. It can be seen that the profile corresponds very well with the autoradiographic profile obtained for the products of 24 hour-greened poly(A)-containing RNA (Fig. 17, track C). The gel slices removed by the modified procedure correspond to the regions expected, i.e. to P20 and P32. The arrows indicate the gel slices removed. These results show that the modified procedure will locate the main *in vitro* products, and that the trypsin digestion will elute most of the radioactivity from the gel slices.

As standards for this procedure, [^{3}H]-labelled large and

Figure 29 Peptide mapping of proteins.

Polypeptides labelled either *in vivo* or *in vitro* with [^{3}H]-arginine and [^{3}H]-lysine were hydrolysed with trypsin and the peptides analysed by chromatography on thin-layer silica plates as described in section II2K. The plates were impregnated with PPO and the autofluorographs left to expose at -70°C for 8 weeks.

A. Small subunit of Fraction I protein (10,000 cpm)

B. P20 synthesised *in vitro* (20,000 cpm)

C. Large subunit of Fraction I protein (30,000 cpm)

D. Region of gel of the products of translation of poly(A)-containing RNA corresponding to the molecular weight of the small subunit of Fraction I protein (10,000 cpm).

1st Dimension

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1st Dimension

small subunits were isolated from total *in vivo*-labelled soluble protein. These polypeptides were also released from gels by trypsin digestion. In this case detection was easy because the labelled bands corresponded to Coomassie Blue-staining bands. Figure 22 shows the poly(A)-containing RNA-directed products and soluble protein preparation used for the isolation of P32, P20 and the large and small subunits. All these samples were analysed as described in section II2L.

Figure 29 shows the patterns of peptide maps obtained. The points to note are:-

1) the large and small subunits give different maps as would be expected for two different polypeptides;

2) the region of the poly(A)-containing products which contained polypeptides with the same molecular weight as the small subunit did not give a map which was similar to that produced by authentic small subunit;

3) P20 did not produce a clear map in this system, all the peptides either streaked or failed to resolve at all in the first dimension;

4) neither P32 nor the chlorophyll *a*/*b*-binding protein gave usable peptide maps, for the same reason, i.e. anomalous behaviour on the first dimension of chromatography.

This anomalous behaviour cannot be due to the procedure of removing the polypeptides from the gels because all samples were treated in the same way. Nor can this behaviour be due to some feature of the *in vitro*-synthesised products. The region of the *in vitro* products corresponding in mobility to the small subunit gave a reasonable map, whereas the *in vivo*-labelled chlorophyll *a*/*b*-binding protein did not. All that can be said is that this method is not suitable for analysis of these

particular polypeptides.

It must be noted that, although arginine and lysine were used as labelled precursors, not all the spots on the maps are equally labelled. This is probably because the peptides must diffuse out of the gel before they can be detected on the two-dimensional chromatograms. A consequence of this is that the larger peptides might be under-represented because they would not diffuse out of the gel as efficiently as smaller peptides.

Although it was not possible to come to any conclusion about P20 or P32, this peptide mapping shows that the *in vitro* products do not contain any polypeptides of the same size as the small subunit which are related to the small subunit. This is consistent with the immune precipitation results, where only P20, not a polypeptide of the same size as the small subunit, was precipitated by anti-Fraction I antibody.

D. Conclusions.

The major point to emerge from this analysis of the *in vitro*-synthesised products is that little can be said about their identities. The most interesting result is the immune precipitation of P20 by anti-Fraction I antibody. This antibody does not precipitate any product which corresponds in size to mature small subunit, and so it is suggested that P20 represents a high-molecular weight form of the small subunit.

This conclusion is supported by results reported recently by Dobberstein *et al* (1977). They found that the products obtained by translation of *Chlamydomonas* poly(A)-containing RNA in a wheat-germ system gave a pattern of polypeptides very similar to that presented in this thesis (compare Fig. 17 with Fig. 1 of Dobberstein *et al*, 1977). In particular, a major

product was a polypeptide with a molecular weight of about 20,000 and this was the only material precipitated by anti-small subunit antibody; there was no material corresponding in size to the mature small subunit. They called this polypeptide pS, and were able to show that pS could be cleaved to give the small subunit by an enzyme present in the soluble protein fraction of Chlamydomonas reinhardtii. This processing activity will be discussed in section III3. Gray and Cashmore (1976) also found this 20,000 molecular weight polypeptide as a major product of translation of poly(A)-containing RNA from pea leaves. They state that it is related to the small subunit, but do not present any data to support this claim.

All the above observations were made on in vitro-synthesised polypeptides, and all the authors used the wheat-germ system to translate the RNA. Dobberstein et al (1977) could not detect any P20 in extracts of C. reinhardtii labelled in vivo. It is thus possible that P20 arises by anomalous translation of the small subunit mRNA. This is an unlikely explanation for the following reasons:-

1) P20 can be processed to authentic small subunit by either cell extracts (Dobberstein et al, 1977) or chloroplasts (section III3); it seems unlikely that there is an activity in vivo for correcting an in vitro "mistake";

2) in section III1C it was argued that the wheat-germ system can faithfully translate added mRNA from a wide range of sources, so its inability to translate the small subunit mRNA is unexpected;

3) the wheat-germ system is known not to be able to carry out any post-translational modification of polypeptides (Kreil et al, 1977), thus if in intact cells small subunit was made

by cleavage of P20, this process would not be expected to occur in the wheat-germ extracts. Such high-molecular weight precursors have been detected for a large number of polypeptides synthesised *in vitro* and these have been shown to represent faithful translation of the mRNA (Campbell and Blobel, 1976). Seeburg *et al* (1977) and Ullrich *et al* (1977) have shown, rat growth hormone and rat insulin respectively, that the "extra" amino acids present *in vitro* are coded for by the mRNA, and lie between the initiator AUG codon and the N-terminal amino acid of the mature protein; thus they must always be present when the mRNA is faithfully translated.

It is important to establish that P20 is a faithful translation product of the small subunit mRNA because it has an obvious bearing on the ability of the wheat-germ system to translate pea mRNA. In particular, if P20 were the result of an aberrant translation of the small subunit mRNA, then the other polypeptides might also be artefacts thus making the system useless for analysing mRNA populations. The three points mentioned above show that this is probably not the case.

It is not obvious why the peptide mapping of P20 and P32 failed to give satisfactory results. It has been shown that the method worked well with the large and small subunits, and so this failure may well be due to some property of the polypeptides themselves; it should be noted that the chlorophyll *a*/*b*-binding protein also gave an unsatisfactory map. It might be that a different mapping procedure would give better results. Recently a method has been described by Cleveland *et al* (1977) for mapping peptides by limited proteolysis on polyacrylamide gels. This method is suitable for mapping *in vitro* products because the samples can be used whilst still in

a segment of gel. The digestion by enzyme takes place in the stacking gel, and a number of different enzymes can be used at once. The peptides which result are a series of partial-digestion products, and are resolved on a one dimensional SDS-polyacrylamide gel. Potentially the resolution in one dimension is not as good as a two-dimensional separation system; however, the use of a range of proteolytic enzymes provides the "second dimension" of the analysis. A significant advantage of this method is that there are no handling steps where losses can occur. The method used in this thesis (section II2L) involves a number of handling steps, and the losses may be in excess of 70%. The method of Cleveland *et al* (1977) has been used to show that polypeptides synthesised *in vitro*, by a coupled transcription-translation system containing influenza virions, are identical to authentic influenza proteins (Minor, P.D., personal communication). So this might well be a suitable method for identifying the *in vitro* products of pea polysomal poly(A)-containing RNA.

It was argued earlier that the precipitation by anti-Fraction I antibodies indicated that P20 was related to the small subunit and not to the large subunit; the key element in this argument is that it is extremely unlikely that there would be any large subunit-related polypeptide present in the *in vitro* products from poly(A)-containing RNA. However, a more convincing argument would be possible if anti-small subunit antibody could have been used. It is not known why the immunological procedure failed to give any anti small subunit antibody; the procedure was based on a published method which gave antibodies to the small subunit from bean leaves (Gray and Kekwick, 1974a). There are no reports of

antibodies to the small subunit from pea leaves, but antibodies against the small subunits of Euglena (Brown et al, 1976), spinach (Nishimura and Akazawa, 1974a), wheat (Gooding et al, 1973) and C. reinhardtii (Dobberstein et al, 1977) have been reported. It might be that the small subunit from pea is less antigenic than the small subunits from these organisms. In section I2B it was mentioned that the small subunits from diffferent organisms are unrelated as determined by peptide mapping, amino acid composition or immunological cross-reactivity. So, although the method used to prepare the small subunit for injection into the rabbits followed the method described for the small subunit from bean leaves, it might not have been suitable.

There is a great variation in the published methods used to prepare Fraction I protein subunits for injection into rabbits. The crucial step is the dissociation of the native protein into the individual subunits. Nishimura and Akazawa (1974) used para-chloromercuribenzoate (PCMB) and Sephadex-G200 chromatography at pH9.0 to obtain isolated small subunit. However, numerous attempts to repeat this observation in this laboratory, using Fraction I protein from both pea and spinach leaves, were unsuccessful (Ballantine, E., personal communication). Furthermore, no other group working in this field has confirmed this observation; so this method probably cannot be used as a basis for the isolation of the small subunit from pea leaves. Gooding et al (1973) used guanidium hydrochloride and urea to dissociate Fraction I protein from wheat leaves; however, the antiserum produced against the small subunit did not appear to be monospecific (Jagendorf, A., personal communication). The two other published methods rely on SDS to dissociate the subunits, and then use either G-200 Sephadex (Brown et al,

1976) or polyacrylamide gels (Dobberstein et al,1977) to separate them; the small subunit is injected into the rabbits without attempting to remove the SDS. Thus it may be beneficial to keep the small subunit denatured with SDS. Any future attempt to raise anti-small subunit antibodies might be successful if the procedure described in section II2B were modified in this way.

Thus the main conclusion of this section is that P20 is related to the small subunit of Fraction I protein, and represents a high-molecular weight precursor of the small subunit. In section III3 it is suggested that this precursor is concerned with the transport of the small subunit across the chloroplast envelope. This suggestion raises the important question as to whether or not other chloroplast proteins are made as precursors. This possibility might explain why there seems to be no obvious correspondence between the in vitro products and the in vivo standards. It must also be emphasised that amongst the in vitro products will be some polypeptides which are not destined to enter the chloroplast. In such a situation, where the in vitro polypeptide might have a higher molecular weight than its in vivo counterpart and/or form only a small fraction of the total product, only the use of specific antibodies can possibly lead to the identification of specific polypeptides. However, this approach also presents problems because very few chloroplast proteins have been purified, and so the basic knowledge required to prepare polypeptides for immunological work is lacking. Even if there had been co-electrophoresis of one of the in vitro products with one of the in vivo-labelled polypeptides, it would not have been possible to identify it, unless it was the small subunit or the

chlorophyll a/b-binding protein; the identity of most of the other *in vivo*-labelled bands is unknown.

The chlorophyll a/b-binding protein is regarded as a likely polypeptide to be found in the *in vitro* products of RNA isolated from greening tissue. This is because it has been shown that this polypeptide is involved in the development of a fully-functional granal membrane system, and its synthesis is closely connected with the synthesis of chlorophyll (Armond *et al*, 1975). During greening these processes occur, and so it would be expected that there would be a large amount of synthesis of this polypeptide; the protein can be one of the major membrane proteins of the chloroplast. P20 is a major product of *in vitro* translation, and this is consistent with the small subunit being a major protein synthesised on cytoplasmic ribosomes during greening. Therefore, it might be expected that, if any other chloroplast protein were to be prominent *in vitro*, it would be related to the chlorophyll a/b-binding protein. The most abundant product, after P20, appears to be P32, which has a molecular weight of 32,000 compared with 26,000 for the chlorophyll a/b-binding protein from pea (Bennett, 1977). Since P20 has a molecular weight about 6,000 larger than that of the small subunit, this molecular weight difference might be expected if the chlorophyll a/b-binding protein is synthesised as a precursor.

There is no direct evidence which supports the idea that P32 and the chlorophyll a/b-binding protein are related. It should be feasible to check this possibility. The attempted peptide mapping of P32 and the chlorophyll a/b-binding protein was not successful, but this is an obvious and straight-forward method by which to compare these two polypeptides. Another

approach would be to raise antibodies to the chlorophyll a/b-binding protein and then to immunoprecipitate the *in vitro* products. It might also be possible to correlate the presence of P32 *in vitro* with situations in which the synthesis of the chlorophyll a/b-binding protein is either repressed or stimulated in intact cells. The intermittent illumination system of Armond *et al* (1976) is the obvious choice in this respect. One complication present in this last sort of approach is that it must always be assumed that a labelled band present in the *in vitro* products under different conditions corresponds to the same polypeptide. In Fig. 17 it can be seen that there are always polypeptides present in the same region of the gel as P32 throughout greening; these polypeptides need not always be the same at each time of greening. Indeed, if P32 were to be related to the chlorophyll a/b-binding protein, it could be argued that it ought not to be present as a product of polysomal RNA from etiolated tissue. It was mentioned in section I2B that most chloroplast proteins can be found in etioplasts; the chlorophyll a/b-binding protein is not one of these, and so it is not expected that its mRNA would be present on polysomes from etiolated tissue. One possibility is that this protein is degraded as soon as it is synthesised in etiolated tissue and so never accumulates.

This section has shown the difficulties involved in attempting to identify the products of *in vitro* translation of pea, polysomal, poly(A)-containing RNA. The only successful approach is to look for a particular protein which must be well-characterised. When dealing with the chloroplast, where the identity of the majority of the observed proteins is unknown, it is difficult to correlate one set of unknowns

(the *in vitro* products) with another set. The identification of P20 with the small subunit was made possible only by the use of immune precipitation methods. It may be that all organelle proteins are made as larger polypeptides *in vitro*, in which case the immunological approach would be the only feasible one. However, there must be techniques available for isolating particular proteins in pure form so that specific anti-serum can be obtained; this requirement in turn implies that something is known about the function and/or behaviour of those proteins. Thus, whilst it is easy to obtain a pattern of bands on a polyacrylamide gel, and also to study variation in this pattern under different conditions, it is much more difficult to interpret this information into particular facts about particular proteins. This is feasible only for proteins like Fraction I protein or the chlorophyll *a*/*b*-binding protein, where there is a basis of background information on which to draw.

PAGE

MISSING

3. PROCESSING OF P20 TO MATURE SMALL SUBUNIT.

In section III2 it was established that P20 was immunologically related to Fraction I protein. It was argued that P20 might be a high-molecular weight precursor of the small subunit. This conclusion was strengthened because it was possible to show that there was no material present which corresponded with the mature small subunit, either immunologically or by peptide mapping.

The signal hypothesis of Blobel and Sabatini (1972) was discussed in section I4B. All the evidence available up to the end of 1975 was presented. The major points which emerge from this hypothesis are that proteins destined for secretion from the cell are made as precursors of higher molecular weight by ribosomes bound to the endoplasmic reticulum; the extra sequence (signal) is at the N-terminus of the protein; the signal is cleaved from the protein as it is transported across the membrane of the endoplasmic reticulum, and this cleavage occurs concomitantly with the synthesis of the polypeptide chain. This mechanism was shown to explain all the observed properties of the synthesis of immunoglobulin light chain by MOPC41 tissue culture cells (Blobel and Dobberstein, 1975 a&b). In particular, it explains why it is necessary to add isolated membranes to the cell-free system at the start of the incubation to obtain cleavage (or processing) *in vitro*.

Subsequently, signal sequences have been detected on a large number of secreted proteins when they are synthesised *in vitro* (Campbell and Blobel, 1976; MacDonald *et al*, 1977; Palmiter *et al*, 1977; Reeves, 1977). According to the signal hypothesis, it would not be expected that such pre-proteins would be found *in vivo*, because the processing occurs before

synthesis is complete. Indeed there is evidence that the processing step occurs very early in synthesis (Rothman and Lodish, 1977; Palmiter *et al*, 1977). However, Schmeckpeper *et al* (1975) were able to detect a putative precursor to MOPC41A immunoglobulin light chain *in vivo* when the cells were treated with the chymotrypsin inhibitor TLCK (100µg/ml). No other protease inhibitor they tested gave this effect, but no other myeloma cell line was sensitive to TLCK. In all instances where it has been investigated, these precursors can be processed when membranes are added to the cell-free system and the processing and translation must be concurrent (Birken *et al*, 1977; Boime *et al*, 1977; Katz *et al*, 1977; Szczesna and Boime, 1976). The processing is linked with transport of the newly-synthesised polypeptide through the membrane (Blobel and Dobberstein, 1975b; Lingappa *et al*, 1977; Shields and Blobel, 1977).

All the proteins which have been shown to have a signal sequence are secreted from the cell, e.g. immunoglobulins. P20 is a precursor to the small subunit, it might be expected that the extra sequence present on P20 has a role in the transport of the small subunit into the chloroplast. At the moment nothing is known about the mechanism by which proteins cross the chloroplast envelope. This section presents some experiments designed to test the possible relationship between P20 and the small subunit, and to determine whether or not this relationship has a role in transport across the chloroplast envelope.

A. Characteristics of processing.

In the absence of any indications from the literature,

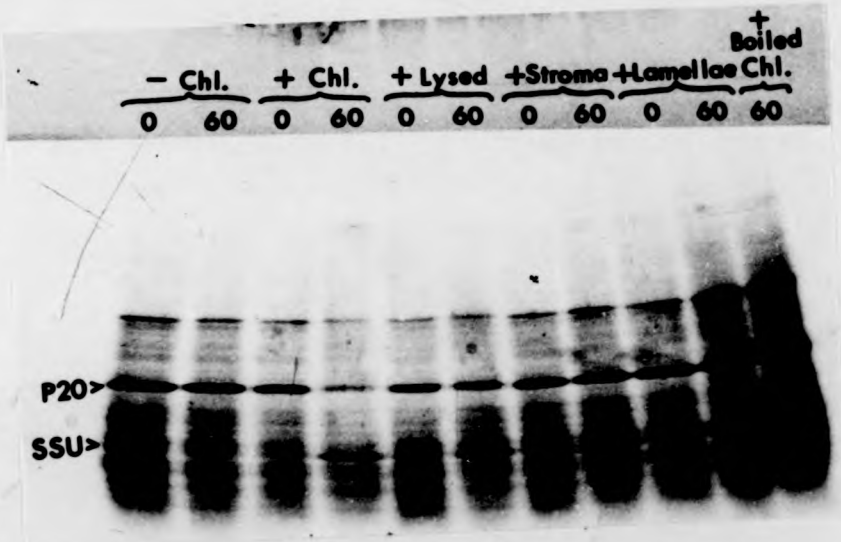

Figure 30 Processing of P20 to the small subunit.

The products of translation of poly(A)-containing RNA from green pea shoots in the wheat-germ system (section II2Jiia and Fig. 5) were incubated with freshly isolated intact chloroplasts or fractions thereof, as described in section II2K. The chloroplasts were isolated from 10 day old green pea shoots (section II2Ai) and one half of the preparation was resuspended in 3ml of intact plastid buffer (25mM HEPES-KOH, pH 7.6, 110mM KCl, 3mM $MgCl_2$, 10mM DTT) the other was resuspended in 1.5ml of 10mM HEPES-KOH, pH 7.6, 10mM DTT, which caused the choroplasts to lyse, followed by 1.5ml of 2x intact plastid buffer. An aliquot (50µl) of the lysed chloroplast preparation was removed before it was centrifuged for 10 minutes at 4,000g; the supernatant was the "stroma" fraction. The pellet was washed once before finally being resuspended in 3ml of intact plastid buffer; this the "lamellae" fraction. An aliquot of the intact plastid preparation was heated in a boiling-water bath for 2 minutes this was the boiled chloroplast preparation. Aliquots (850,000 cpm) of the *in vitro*-synthesised products were incubated, in a final volume of 30µl, with chloroplasts equivalent to 4.7µg of chlorophyll (section II2P), or the fractions from an equivalent amount of chloroplasts, for either 0 or 60 minutes. The reactions were terminated by adding SDS to 1% and the samples then prepared for running on gels as described. The autoradiograph was

Figure 30 exposed for 4 days at room temperature. Each track on the gel contained about 400,000 cpm SSU:- small subunit of Fraction I protein

the most obvious way to start investigating transport of proteins into the chloroplast is to incubate isolated chloroplasts with proteins. The signal hypothesis would suggest that the chloroplasts must be present when the polypeptides are being synthesised, if any processing and transport is to occur. The chloroplasts, as isolated in this laboratory, are active in both protein and RNA synthesis (Blair and Ellis, 1973; Hartley and Ellis, 1973). Thus the presence of chloroplasts in the wheat-germ system at zero-time produces a potentially complex system; the two protein synthesising systems might interact. It might be that, in the presence of chloroplasts the wheat-germ system would make a different range of products and vice-versa. The ionic conditions in the wheat-germ system are also different from those used to obtain high rates of protein synthesis in chloroplasts. There is ATP and GTP present in the wheat-germ assay tubes and this could drive the chloroplast protein synthesis irrespective of whether or not the chloroplasts are illuminated (Blair and Ellis, 1973). Thus a preliminary experiment was to take the wheat-germ assay mixture at the end of the incubation, when all synthesis had finished (see Fig. 4A), and incubate it with isolated chloroplasts and with fractions derived from chloroplasts.

Figure 30 shows the results of such an experiment. Tracks A&B show that incubating the <u>in vitro</u> products for a further 60 minutes does not change the pattern of polypeptides, indicating that there is no significant endogenous proteolytic activity. Track D shows an unexpected result. After 60 minutes incubation with intact chloroplasts there is a reduction in the amount of label associated with P20, and an increase in the label which co-electrophoreses with the small subunit. This

change is not due to some effect of the chloroplast proteins on the electrophoretic pattern, because tracks C&K are the same as the no-chloroplast controls. Thus it must be concluded that there is a processing activity associated with the isolated intact chloroplast preparation which is capable of cleaving P20 to a polypeptide which co-electrophoreses with the small subunit.

The lysed chloroplasts and stroma both have some of this processing activity present (tracks F&H) but at a lower level. The lysed chloroplast preparation is identical to the intact chloroplast preparation, except for the physical state of the chloroplasts, so the reduction in activity must be associated with the act of lysis. This implies that, for maximum activity, the chloroplast components must have a definite organization. The lamellar fraction has no activity at all, and so this cleavage of P20 is not just dependant upon the presence of a membrane but upon the presence of a particular membrane. It is assumed that the activity is membrane-associated because the addition of an amount of stromal protein, equivalent to the amount of intact chloroplasts used, results in much less processing activity (compare tracks D&H). The stroma was prepared by a low-speed centrifugation of lysed chloroplasts, and so will contain some membrane fragments, which could account for the activity associated with this fraction.

This experiment suggests that isolated chloroplasts contain an activity which will specifically cleave P20 to the small subunit, and that this activity may be associated with the outer membranes, or envelope, of the chloroplast. Subsequent experiments were designed to establish the following points:-

1) Is the product of P20 processing authentic small subunit

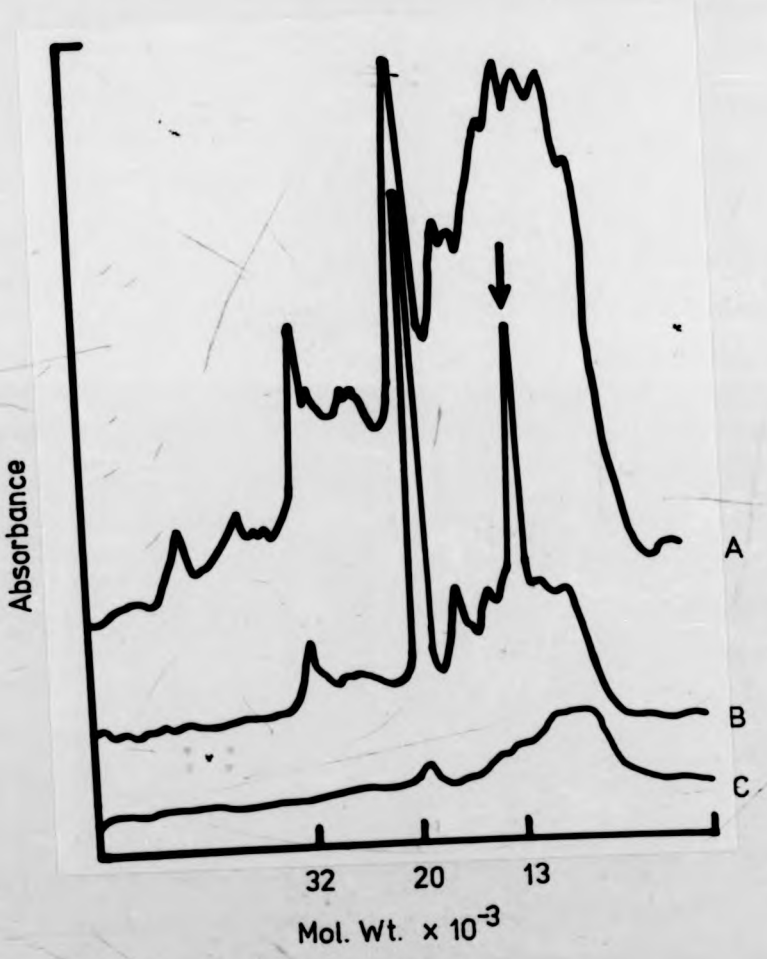

Figure 31 Immune precipitation after processing of P20.

The *in vitro*-synthesised products of poly(A)-containing RNA were incubated with isolated intact chloroplasts as described in Figure 30. At the end of the incubation time, the reaction mixture was made 1% with respect to NP40 and the volume increased from 60µl to 200µl by addition of PBS containing BSA(100µg/ml). Aliquots (100µl) were analysed by immune precipitation. IgG (100µg) was added to the samples and incubated for 60 minutes at 37°C. *S. aureus* (10mg) was added and the samples treated and prepared for gel electrophoresis as described (Figure 27). The autofluorograph was exposed for 4 days at -70°C and was scanned in a Joyce-Loebl Chromoscan

A. Total labelled polypeptides obtained by incubation of *in vitro*-labelled products with chloroplasts.

B. As A but precipitated by anti-Fraction I antibody and *S. aureus*.

C. As B but using preimmune IgG.

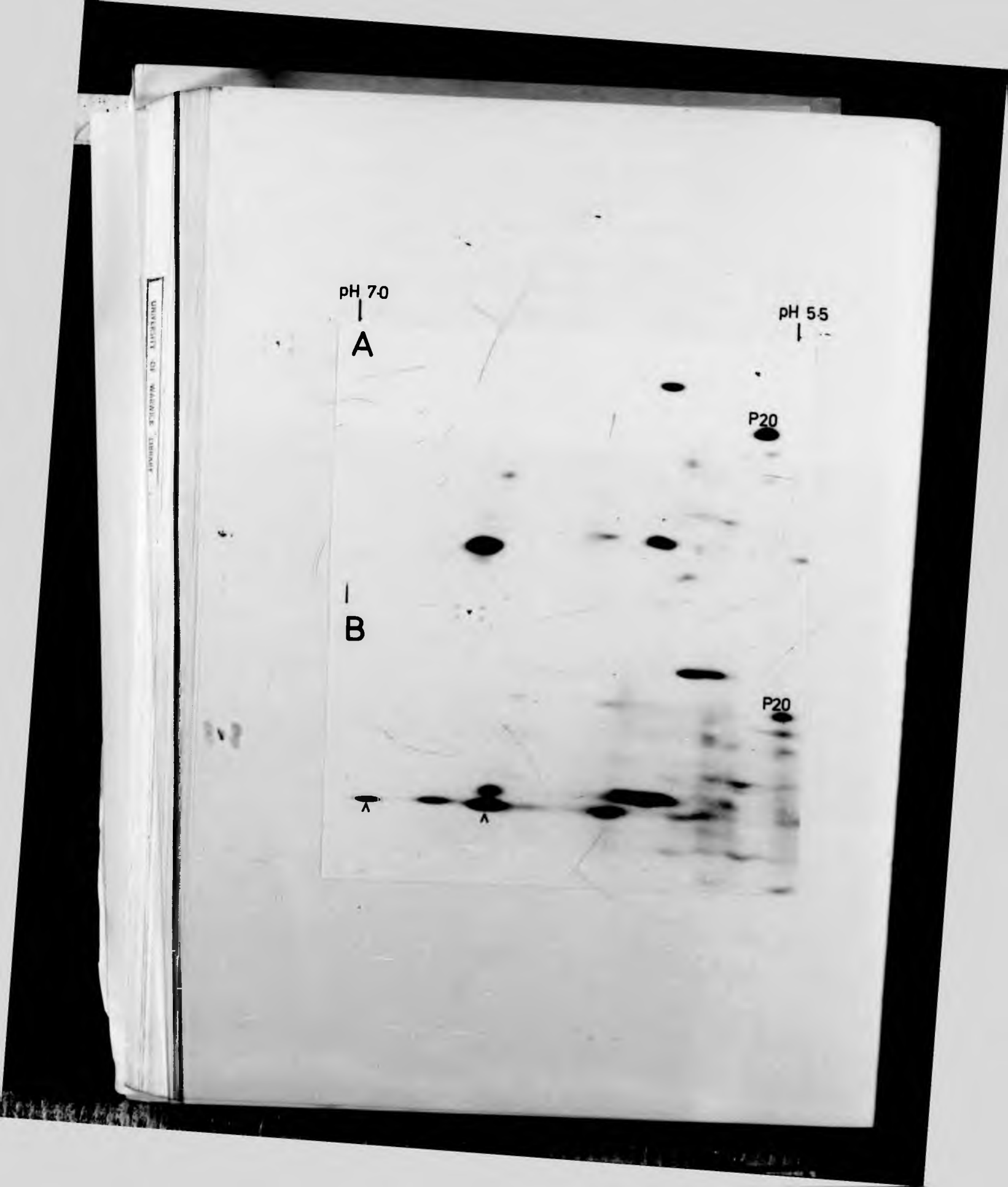
pH 7·0
A
pH 5·5
P20
B
P20

Figure 32 Analysis of the products of processing by two dimensional gel electrophoresis.

[^{35}S]-labelled products of *in vitro* translation of poly(A)-containing RNA were analysed by two-dimensional electrophoresis on polyacrylamide gels (section II2Eie). The autofluorographs were exposed for 10 days at -70°C. Only part of the total pattern on the gels is shown; this covers the area of polypeptides with molecular weights between 10,000 and 25,000 and isoelectric points between pH 5.5 and 7.0

A. Products of translation of poly(A)-containing RNA

B. As A but after incubation with intact chloroplasts.

The arrow-heads indicate the positions of the two isoelectric variants of the subunit of Fraction I protein.

2) Is the processing activity located in the chloroplasts or is it a cytoplasmic contaminant?

3) Which chloroplast fraction contains the processing activity?

4) Does processing occur in the absence of protein synthesis by either cytoplasmic or chloroplast ribosomes? Does processing require cytoplasmic ribosomes to be present?

Chloroplasts can be isolated in a highly purified form by banding on a density gradient of Ludox (section II2Aii). Intact and lysed chloroplasts can be separated from each other, and the preparations are pure as judged by examination in the phase-contrast microscope. Such a preparation of purified intact chloroplasts was incubated with the in vitro-synthesised products, and the mixture then lysed by the addition of the non-ionic detergent NP40. The supernatant was taken for analysis by immune precipitation. Figure 31 shows the results of this experiment; the traces are microdensitometer scans of the autoradiograph. It is clear that the band co-electrophoresing with the small subunit which appears on incubation of intact chloroplasts with the in vitro products is precipitated by the anti-Fraction I antibody. This result confirms that cleavage of P20 gives rise to the small subunit.

Additional confirmation is shown in Figure 32. After incubation for 60 minutes with intact chloroplasts, the in vitro-synthesised products were analysed by 2-dimensional electrophoresis (section II2Eie) and compared with controls incubated in the absence of chloroplasts. Figure 32 shows the relevant section of the 2-dimensional gels. Gel A shows the control; there is no significant label in the region of the two isoelectric variants of the small subunit. In gel B however,

there are two radioactive spots which exactly co-electrophorese with the small subunit. The small subunit from the chloroplasts acts as an internal unlabelled marker; the two radioactive spots can be placed exactly over the two staining spots on the gel. There are, in addition, two other spots which co-electrophorese with the small subunit in the second dimension but have different isoelectric points. Their relationship to the small subunit is unknown. It might be that they represent either intermediates in the processing reaction, which do not normally accumulate *in vivo*, or that they are artefacts of the *in vitro* reaction. Another interesting point is that, on the basis of staining intensity, the two isoelectric forms of the small subunit seen *in vivo* are present in approximately equal amounts. However, of the two labelled spots which correspond to the *in vivo* forms, the one with the lower isoelectric point seems to have significantly more label than the other. It is not clear whether this reflects the real situation *in vivo*, or whether this is a consequence of the processing reaction taking place *in vitro*. It might be that, *in vivo*, one form of the small subunit is derived from the other.

These experiments show that the new band which appears when chloroplasts and *in vitro*-synthesised polypeptides are incubated together, is the small subunit, as judged by immunological and electrophoretic analysis. Since this processing was obtained with a preparation of highly purified, intact chloroplasts, these results also confirm the suggestion that the activity is associated with the chloroplast.

To approach the problem of whether the processing activity is membrane-associated or soluble, it was decided to repeat the experiment shown in Figure 30 but to use two stromal

	processing activity
intact chloroplasts	100
intact chloroplasts + chloramphenicol (100µg/ml)	95
intact chloroplasts + cycloheximide (100µg/ml)	105
lysed chloroplasts	40
low-speed stroma	25
high-speed stroma	< 5

Table IX Processing activity of chloroplasts.

The [^{35}S]-methionine-labelled products of translation of poly(A)-containing RNA were incubated with chloroplasts or fractions thereof (section II2K). The products of such incubations were analysed by electrophoresis on slab polyacrylamide gels and autoradiographs obtained by suitable exposures, i.e. such that the radioactivity from the band corresponding to P20 had not saturated the film. The processing activity was determined by measurement of the area under the densitometer scan of the P20 band. After incubation with intact chloroplasts there was a 60% reduction in this area compared with the zero-time value; this was defined as 100% processing activity. This was repeated for the other incubations. The low-speed stroma was the supernatant obtained by a 4000g centrifugation of lysed chloroplasts. High-speed stroma was obtained by spinning the low-speed stroma at 30,000g_{ave} for 40 minutes.

fractions, one generated by a low-speed centrifugation of lysed chloroplasts, and the other by a high-speed centrifugation of the low-speed stroma. In this way it should be possible to see if the activity present in the stroma could be sedimented; if it is, this would imply that this activity is due to membrane fragments remaining after the low-speed centrifugation.

Table IX presents the data from an experiment of this sort. Processing activity was determined by microdensitometer scanning of autofluorographs. The area under the scan of P20 was determined for all the tracks. The reduction in label present in P20 was used as a measure of processing; the reduction of label in P20 after incubation with intact plastids (usually about 40 to 60%) was arbitrarily defined as 100% activity. It was not possible to use the amount of label which appeared in the small subunit as a measure of processing. The scans of this region show so many peaks that, for low activity fractions, it was not possible to measure the areas under the small subunit peaks with any accuracy. It can be seen from Table IX that removal of membrane fragments by stronger centrifugation also removes the processing activity from the stromal preparations.

It is important to establish whether or not the processing observed depends upon concomitant protein synthesis, as the signal hypothesis predicts. The fact that processing can occur after the wheat-germ incubations have stopped incorporating, suggests that it does not require protein synthesis by cytoplasmic ribosomes. Thus the mechanism operating here appears to be different from that described by the signal hypothesis. Chloramphenicol (D-threo isomer) and cycloheximide are inhibitors of chloroplast and cytoplasmic protein synthesis

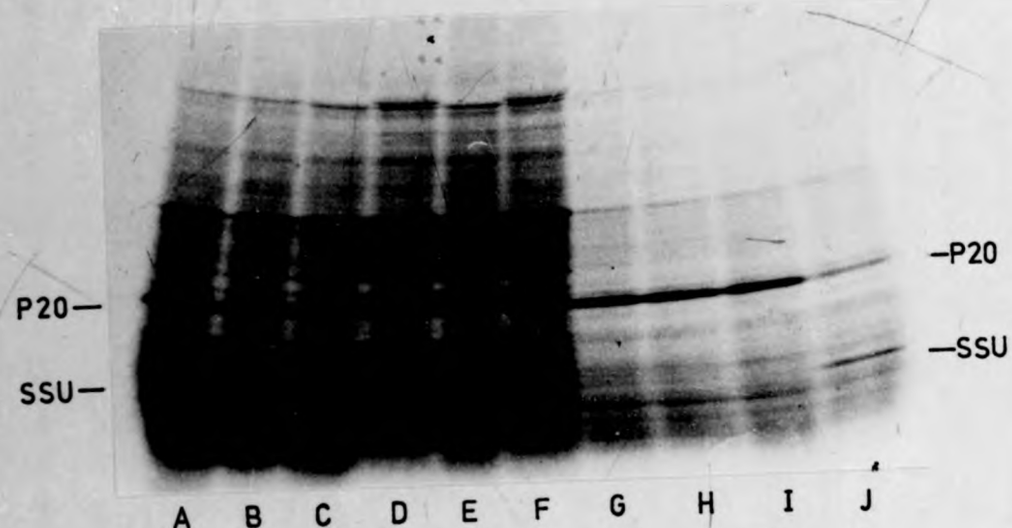

Figure 33 The effect of various treatments on processing of P20.

The [^{35}S]-labelled products of translation of poly(A)-containing RNA were treated as follows:-

Tracks A-F; aliquots (750,000 cpm) were incubated, in a final volume of 70µl, at 27°C in various ways

A. with intact plastid buffer , zero time,

B. with intact plastid buffer, for 60 minutes,

C. with intact chloroplasts (7.5µg of chlorophyll) for zero time,

D. as C but incubated for 60 minutes,

E. as D but in the presence of 100µg/ml chloramphenicol,

F. as D but in the presence of 100µg/ml cycloheximide,

Tracks G-J; the <u>in vitro</u> products were centrifuged at 100,000g_{ave} for 60 minutes. Aliquots equivalent to those used in tracks A-F (about 250,000 cpm) were incubated, in a final volume of 70µl, at 27°C in various ways

G. with intact plastid buffer, zero time,

H. with intact plastid buffer, for 60 minutes,

I. with intact chloroplasts (7.5µg of chlorophyll) zero time

J. as I but for 60 minutes.

respectively (Ellis, 1976). Addition of either of these compounds at 100µg/ml to the incubation did not affect the extent of processing (see Table IX and Fig. 33 tracks E&F). Thus the processing must be completely independant of protein synthesis by either cytoplasmic or chloroplast ribosomes.

The wheat-germ incubations which have been used so far have contained wheat-germ ribosomes; it is known that a large proportion of the in vitro-synthesised polypeptides remains associated with the ribosomes (Rosen, 1976). Thus it might be argued that the processing activity requires the presence of wheat-germ ribosomes, and that the P20 which is not processed represents those molecules which have been released from the ribosomes. To test this possibility, the wheat-germ incubations were centrifuged at $100000g_{ave}$, for 60 minutes before incubation of the supernatant fraction with intact chloroplasts. Figure 33 (tracks G-J) shows the result of this experiment. Removal of the polysomes by centrifugation reduces the TCA-precipitable radioactivity by about half. The main effect of this removal is to reduce the background radioactivity in the sample tracks; the amount of P20 is reduced by only 20%. This result is expected if the background results from incomplete polypeptide chains, which are present as peptidyl-tRNA and so remain bound to the ribosomes. The released P20 can still be processed to the small subunit in the absence of wheat-germ ribosomes (track J). Using the post-ribosomal supernatant for the processing reaction gives 50% processing of P20 (track J), compared with 35% processing when the complete wheat-germ incubation is used (tracks D-F). Such an increase could be due entirely to the fact that about 20% of the P20 pellets with the ribosomes. The amount of small subunit produced by the

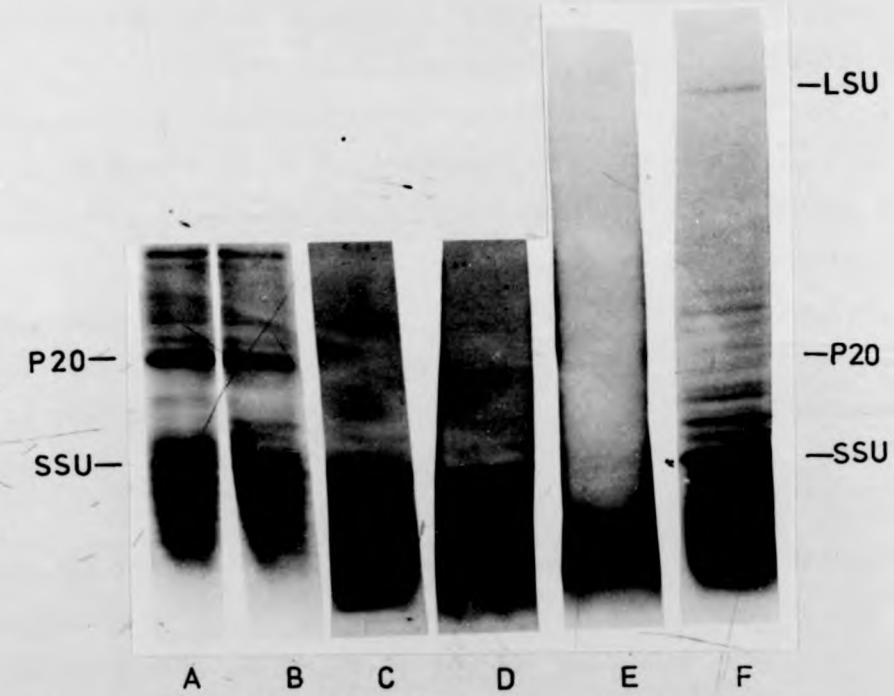
—LSU
P20—
—P20
SSU—
—SSU
A
B
C
D
E
F

Figure 34 Effect of trypsin on the products of processing.

A processing reaction was carried out using [^{35}S]-methionine-labelled *in vitro* products of poly(A)-containing RNA and isolated intact chloroplasts as described in section II2K. The final volume was 50µl and contained 5.5µg of chlorophyll. At the end of the 60 minutes of incubation at 27°C, trypsin 100µg/ml) or trypsin plus NP40 (1%) was added and incubation continued at 37°C for 30 minutes. The samples were then analysed by electrophoresis on a slab polyacrylamide gel

A. *In vitro*-synthesised products of poly(A)-containing RNA.

B. As A but incubated with intact chloroplasts for 60 minutes.

C. As B but incubated with trypsin for 30 minutes.

D. As B but incubated with trypsin and NP40 for 30 minutes.

E. As A but incubated with trypsin.

F. Track C exposed for three times longer.

A&B were exposed at room temperature for 3 days; C&D for 10 days and E&F for 30 days.

LSU:- large subunit of Fraction I protein

SSU:- small subunit of Fraction I protein

processing is approximately the same in both cases, but there is less P20 to start with after centrifugation. These observations suggest that the P20 associated with the ribosomes might not be able to take part in the processing reaction.

It should be noted that on no occasion has complete processing of P20 been achieved, also the extent of processing varied between different experiments. Processing was usually about 40-50% complete, but on two occasions only 10% processing was obtained. The standard conditions used were to incubate 10µl of wheat-germ incubation mixture with chloroplasts equivalent to about 5µg of chlorophyll at 27°C for 60 minutes.

The experiments described so far have shown that there is an activity associated with chloroplasts which can cleave P20 to the small subunit in the absence of protein synthesis. The next question to consider is whether this activity has any relevance to the entry of the small subunit into the chloroplast. Intact chloroplasts are resistant to digestion by trypsin (Bennett, J., personal communication), and trypsin-insensitivity has been used as a criterion for the uptake of proteins into membrane vesicles (Blobel and Dobberstein, 1975b). Chloroplasts were incubated with the in vitro-synthesised products, and then the subsequent effect of trypsin (100µg/ml) was investigated.

Figure 34 shows the results of such an experiment; the tracks were cut from the original autoradiographs and placed next to each other for ease of presentation. Track B shows the appearance of small subunit when processing occurs. In track C the incubation mixture had been treated with trypsin, and the main resistant band can be seen to be the small subunit. Tracks C&D were from longer autoradiographic exposure than tracks A&B.

Microdensitometer scanning of all the tracks shows that about 35% of the small subunit produced in the processing reaction is trypsin-resistant. The chloroplasts themselves carry out protein synthesis during the processing and the large subunit so produced serves as a useful control for the trypsin digestion. Any large subunit which is trypsin-sensitive must have either been synthesised by lysed chloroplasts or in chloroplasts which have lysed during the incubation. It was found that only 20% of the large subunit was trypsin-resistant. The fact that 65% of the small subunit is sensitive to trypsin, suggests that the cleavage is not necessarily associated with transport across a membrane. However, 80% of the large subunit, which must have been synthesised by the chloroplasts, was sensitive to trypsin. Thus there might be extensive lysis of the chloroplasts during the course of the incubation. Thus any small subunit taken into the stroma will be subsequently released and so become accessible to trypsin.

Addition of the non-ionic detergent NP40 at the same time as the trypsin causes the digestion of about 90% of the small subunit which was trypsin-resistant in the absence of detergent (Fig. 34, track D). This shows that the small subunit was previously protected from the trypsin by the presence of a membrane. Track E shows the result of adding trypsin to *in vitro*-synthesised products which were not incubated with chloroplasts. All the products are trypsin-sensitive.

If the autoradiograph of the trypsin-resistant polypeptides is allowed to expose for about four-times as long as track C, then bands other than the small subunit and large subunit are visible (track F). However, the amounts of radioactivity are very low; the possibility that a very small amount of the *in*

in vitro products are trypsin-resistant due to some adventitious association with the chloroplasts cannot be ruled out. Some of the bands correspond to major *in vitro* products but represent less than 0.1% of the original radioactivity. Some bands do not seem to correspond directly with any band visible in the *in vitro* products. If any of these polypeptides have been taken up into the chloroplasts, the amounts of radioactivity they represent are so small as to preclude further study.

It is significant that there is no P20 inside the chloroplasts as judged by trypsin resistance (Fig. 34, track F). This would imply either that processing occurs at the outer membrane of the chloroplasts, or that P20 enters the chloroplast and is rapidly cleaved.

B. Discussion

(i) Summary of results.

The results presented in this section have shown the following:-

1) P20 can be cleaved to the small subunit in a specific way; this confirms the suggestion made in section III2, that P20 is a precursor of the small subunit;

2) the processing activity is associated with chloroplasts and may be membrane-bound;

3) the processing activity is associated with the uptake of the small subunit by the chloroplast;

4) this uptake of the small subunit is independant of concomitant protein synthesis.

(ii) Independant work on the processing of small subunit

There is only one other report of the processing of P20

to the small subunit (Dobberstein et al,1977). These authors worked with the unicellular algae, Chlamydomonas reinhardtii. They reported that the translation of poly(A)-containing RNA in the wheat-germ system produces P20, which they identified by immunoprecipitation with anti-small subunit antibodies. However, they found that, when isolated C. reinhardtii polysomes were allowed to run-off in the wheat-germ system, only the small subunit, not P20, was synthesised. An activity capable of processing P20 to the small subunit was found in the post-ribosomal supernatant of Chlamydomonas: this processing was also independant of protein synthesis. They concluded that the processing activity associated with the polysomes was due to adventitious binding of the soluble processing activity to the polysomes.

Thus, apart from the fact that P20 is a precursor to the small subunit and that processing takes place independently of protein synthesis, this report of Dobberstein et al (1977) reaches different conclusions from those presented here. However, there are no directly comparable experiments in the two pieces of work. All the experiments reported here used either isolated chloroplasts or fractions obtained from them, whereas Dobberstein et al used soluble protein from whole Chlamydomonas cells. It is not possible to isolate intact chloroplasts from this alga and so experiments analogous to those presented above can not be performed with this species. To break open the algal cells, a French pressure cell has to be used. It may be that this harsh treatment resulted in the solubilization of the processing activity. It is necessary to repeat the experiments of Dobberstein et al in the pea system to discover whether or not there really is a soluble processing

activity. If such a soluble processing activity were to be found, then its relationship with the chloroplast-associated activity must be investigated. It might be that one arises from the other during isolation, either by association or dissociation.

The strongest argument in favour of a membrane location of the processing activity is that the small subunit so produced is taken up by the chloroplasts. It is difficult to see what function a soluble, cytoplasmic processing step might serve. The findings of Dobberstein et al (1977) would suggest that the small subunit is synthesised as a higher molecular weight precursor, and that this is processed in the cytoplasm. This suggestion gives no indication as to how the small subunit enters the chloroplast or even why a precursor is necessary. The results presented here on the other hand, do suggest a possible mechanism both for the transport of the small subunit into the cytoplasm, and a function for the precursor.

(iii) Location of P20-synthesising polysomes.

Dobberstein et al (1977) report that the polysomes synthesising P20 are free in Chlamydomonas. However, they did not study the membrane-bound polysomes at all and so the distribution of the P20 mRNA between these two polysome populations is not known. Roy et al (1977) have studied the free and membrane-bound polysomes of pea leaves. They found that less than 10% of the total polysomes were membrane-bound. Moreover, when they allowed these different polysome fractions to "run-off" in a wheat-germ system, they found that both free and membrane-bound polysomes synthesised the same products. They concluded that the small subunit was synthesised predominantly on free polysomes.

The synthesis of P20 on free polysomes presents no problems for a model explaining the synthesis and uptake of the small subunit if it could be shown that soluble P20 molecules could specifically reach the chloroplast envelope with a sufficiently high frequency. This frequency is a function of the surface area of the envelope and the concentration of P20. Forde and Steer (1976) have measured the relative areas of particular membranes present in developing cucumber leaf cells by electron microscopic techniques. The point to consider in the present context is the percentage of cellular membrane which is due to the chloroplast envelope. If the areas of the chloroplast thylakoid membrane and mitochondrial cisternal membrane are ignored, because they do not have direct contact with the cytoplasm, then about 50% of the cellular membrane is due to the chloroplast envelope (Forde and Steer, 1976).

If P20 is synthesised by free polysomes then it is reasonable to assume that the frequency with which P20 molecules approach a particular membrane is related to the volume of the cytoplasm occupied by the membrane. It was not possible to find any figures in the literature for the relative volumes of the cytoplasm and chloroplasts in a mature pea leaf cell. To estimate the percentage of cytoplasmic volume occupied by the chloroplasts in pea leaf cells, it was necessary to measure the size of the leaf palisade cells. These cells have a distinctive cylindrical appearance and also contain most of the chloroplasts of the leaf. A suspension of pea leaf cells was obtained from Dr. B. R. Barraclough; the cells had been prepared by Macerase-digestion (3% solution in 0.3M sorbitol, 2% polyvinylpyrollidone, 12.5mM K_2SO_4, 20mM MES-KOH, pH5.8.) of chopped-up pea leaves. Aliquots of this suspension were examined in the light microscope and the dimensions of the

θ	N	A(um^2)	a(um)	V(um^3)	total V(um^3)	percentage of cytoplasm
30°	20	109.5	8.20	29.5	590	64
	25	87.6	7.34	21.1	528	57
	50	43.8	5.12	7.45	377	41
45°	25	87.6	5.05	31.25	800	85
	50	43.8	3.57	11.05	552	60

Table X Calculations concerning the amount of cytoplasm occupied by chloroplasts in pea leaf palisade cells.

A suspension of pea leaf cells was obtained by Macerase-digestion of leaf tissue and was examined in the light microscope. The dimensions of the palisade cells were measured by calibration against an eye-piece graticule. The average over 15 measurements are:-

height(h) = 24.7$\pm$ 4.8 um radius(r) =7.5 $\pm$ 0.9um

If the cells are assumed to be cylindrical then

volume(V) $=\pi r^2 h$ and surface area(A) $=2\pi r(r+h)$

i.e. $V = 4360um^3$ $A = 1516um^2$

Forde and Steer (1976) provides a relationship between the areas of various membranes in cucumber leaf cells. Using their data:-

$A(tonoplast) = A(cell).\ 0.85 = 1296um^2$

Assuming the vacuole to be cylindrical and its height slightly shorter than that of the cell, if h(tonoplast) is 23um, then r(tonoplast) = 6.9um

and so $V(tonoplast) = 3439um^3$

Thus the cytoplasmic volume V(cytoplasm) is equal to the difference between V(cell) and V(tonoplast)

i.e. $4360-3439 = 921um^3$

From Forde and Steer (1976) total A(chloroplast) equals $A(cell).1.43 = 2176um^2$

therefore A(chloroplast) = 2176/N um where N is the total number of cloroplasts in a cell.

Assuming the chloroplasts to be represented by a section through a sphere, and that this section subtends a half-angle θ at the centre, then:-

Table X $A(\text{chloroplast}) = \pi(a.\sin\theta)^2 - 2\pi a^2.(\cos\theta - 1)$

$V(\text{chloroplast}) = a^3\pi[\,2/3 - \cos\theta + (\cos\theta)^3/3]$

where a = the radius of the sphere

If $= 30^\circ$ then $A = 0.518\pi a^2$

and $V = 0.017\pi a^3$

If $= 45^\circ$ then $A = 1.08\pi a^2$

and $V = 0.077\pi a^3$

These formulae were used to derive the values give in the table; A(chloroplast) can be calculated from the data of Forde and Steer (1976) for any value of N and so the radius of the sphere, a, is obtained, V(chloroplast) can then be calculated and compared with the volume of the cytoplasm.

palisade cells measured using a calibrated eye-piece graticule. From these dimensions, assuming a regular cylindrical shape for the cells, it is possible to calculate the cell volume and cell surface area. The results of Forde and Steer (1976) provide a basis for relating the area of the plasma membrane to the areas of other cellular membranes, assuming that pea and cucumber leaf cells are comparable in this regard.

Table X presents the data concerning cell size, and also the calculations required to derive the volume of the cytoplasm and chloroplasts. The chloroplasts are assumed to approximate in shape to a tangential section through a sphere. The calculations indicate that the chloroplasts account for at least 40%, and up to 85%, of the cytoplasmic volume. There are several assumptions inherent in this analysis, but the general picture which emerges fits with observations of leaf cells by light and electron microscopy, i.e. a large vacuolar space with large numbers of chloroplasts occupying the cytoplasm. According to Bradbeer et al (1974), palisade cells of Phaseolus contain around 25 chloroplasts each. On this basis (N=25) the calculations in table X would suggest that over 50% of the cytoplasmic volume is occupied by chloroplasts. The shape of the chloroplasts plays a major role in determining the chloroplast volume for a given surface area. In fact if it were assumed that the chloroplasts were hemispherical, they would occupy over 100% of the cytoplasmic volume. This cannot be the case and so the chloroplasts must be represented by spherical sections smaller than hemispheres.

The high proportion of total cytoplasmic volume which is occupied by chloroplasts explains how P20 molecules can reach the chloroplasts efficiently. To explain the specificity of

this transport it is necessary to look at the composition of the chloroplast envelopes. In section I2 the distinctive lipid and protein composition of the envelopes was described. Recently it has been shown that one of these proteins has a role in the translocation of inorganic phosphate through the envelope (Flugge and Heldt, 1977). The specificity of the processing and transport of P20 to the small subunit could be due to the presence of a protein, or lipo-protein complex, on the surface of the chloroplasts which recognises some feature of P20. This recognition leads to the cleavage of P20 to the small subunit and the appearance of the small subunit inside the envelope. This is the basis of the membrane carrier hypothesis for the transport of cytoplasmically-synthesised polypeptides into the chloroplast (Highfield and Ellis, 1978).

Once P20 has reached the chloroplast envelope, it is cleaved to the small subunit and becomes inaccessible to trypsin. From the experiments presented here it is not possible to say more than this. It is not known how the processing and transport steps are related to each other either temporally or physically, nor is it known whether inaccessibility to trypsin reflects the passage of small subunit into the stroma, or its sequestration inside the envelope membranes. However, it is intended to present a plausible model and to describe experiments which might be performed to test this model.

(iv) Model for the uptake of the small subunit by chloroplasts

The molecular weight of P20 is about 6,000 larger than the small subunit, which means that it contains about an extra 50 amino acids. In Figure 32 it can be seen that P20 has an isoelectric point about 1.0 to 1.5 units lower than the two

isoelectric variants of the small subunit. This difference must mean that some of these extra amino acids are acidic in nature. There may also be basic amino acids present, but the net effect of the extra amino acids is to lower the pI of P20. Thus, when P20 is cleaved to the small subunit, this results in the removal of a large number of amino acids some of which are charged. It is highly likely that a conformational change accompanies this cleavage and it is suggested that the newly-formed small subunit moves through the membrane and into the stroma as a result of this change. This is obviously just the bare outline of a mechanism for the uptake of the small subunit but it does make certain predictions. The systems described in section III3A can be used to test these predictions and to elucidate the mechanism in more detail.

This model would suggest that processing can take place after synthesis of the polypeptide has finished, because it is the change in conformation of the whole polypeptide chain which allows movement across the chloroplast envelope. The model might be taken to an extreme form whereby processing and uptake can only take place after synthesis is complete, so that nascent polypeptide chains, whilst they might be processed, would not be transported. The results presented earlier show that processing and transport do take place after polypeptide synthesis has finished. To test whether or not it is essential for processing to have full-length chains of P20, it would be necessary to obtain a preparation containing progressively longer fragments of P20. Puromycin will discharge the nascent chains of polysomes (Suhadolnik,1970) and so, by puromycin discharge of wheat-germ polysomes, it should be possible to obtain P20 fragments of different sizes, all containing the

NH_2-terminus. Incubating these fragments with chloroplasts should show whether or not there is a minimum size below which transport does not occur.

The proposed mechanism also requires that the processing activity is present in the chloroplast envelope. There are published procedures for the isolation of highly purified chloroplast envelopes from pea leaves (Joy and Ellis, 1975), and so it would be possible to look for processing activity associated with isolated envelopes. However, this approach might not provide a straight-forward result. In table IX it was shown that lysis of the chloroplasts caused a large reduction in processing activity. Thus the large-scale organization of the membrane might be important. If this is the case, it may be that a preparation of envelopes would have no processing activity because of the incorrect orientation of the processing enzyme in the membrane. It has recently been shown that the enzyme which removes the signal peptide from polypeptides can be solubilised with deoxycholate, and is active in this form (Jackson and Blobel, 1977). It may be necessary to solubilise the chloroplast processing activity in this way to study it *in vitro*.

In the foregoing discussion it has been assumed that the small subunit produced by cleavage of P20 is transported into the stromal compartment of the chloroplast. The chloroplast envelope consists of two membranes and so it is possible that the small subunit is present only in the intramembrane space. The outer membrane of the envelope is freely permeable to small molecules while the inner membrane appears to have specific transport mechanisms for small molecules (Heldt, 1976). It is clearly necessary to know how the small subunit gets across

both membranes. There are several possibilities:-

1) P20 can diffuse through the outer membrane, and processing and transport occurs at the inner membrane;

2) there are two processing and transport steps, one at each membrane; with intact chloroplasts the intermediate produced by the first step would not be seen;

3) perhaps P20 can be processed and transported across both membranes at once, this might be at points where the two membranes have fused and so have formed a "gate" through which proteins can pass; although there is no evidence for such gates,they might be transient and also represent only a small fraction of the total area of the chloroplast envelope and, therefore,would not be easily detecable in the electron microscope.

At the moment, it is not possible to distinguish between these possibilities; this distinction requires the separation of the inner and outer envelope membranes. However, it should be possible to show that the small subunit had entered the stroma. The chloroplasts are known to be synthesising the large subunit during the processing reaction assays (Fig. 34). It is likely that the large and small subunit polypeptides so produced might associate into native Fraction I protein. Analysis of the chloroplast proteins, after incubation of the chloroplasts with <u>in vitro</u>-synthesised polypeptides, on non-denaturing gels (section II2Eia) should show whether or not this association has occurred. Failure of the small subunit to appear in the Fraction I protein might mean only that the assembly process was damaged, and not indicate that the small subunit was absent from the stroma.

The systems described in section III3A could be used to

study the assembly of Fraction I protein from its subunits. There are only a few reports of the dissociation of Fraction I protein into its subunits, and subsequent re-association into an active enzyme, and all these reports are from one group (Nishimura and Akazawa, 1973,1974; Nishimura *et al*,1973). No other group has reported that it is possible to reconstitute Fraction I protein from the dissociated subunits, and several attempts in this laboratory to repeat the observations of Nishimura and Akazawa have been unsuccessful (Ballantine, personal communication). It may be that assembly of native Fraction I protein *in vivo* does not require large and small subunits, but rather precursor forms of one or both. The assembly step may result in a cleavage of the precursor. Such a process would explain the great difficulty observed in re-associating the subunits *in vitro*. If such a mechanism does exist, then P20 cannot be the precursor involved in this step because the stromal protein does not contain any processing activity; such an assembly might be expected to take place in the stroma. However, the assembly may also take place as part of the processing and transport of P20; in which case the large subunit may be associated, at least transiently, with the processing enzyme. An alternative idea is that P20 is processed to some other intermediate, which enters the stroma, and this second intermediate is cleaved to the small subunit when assembled into Fraction I protein. All these suggestions could be investigated using the systems described in this section.

(v) Relationship to the signal hypothesis.

It is obvious that the mechanism involved in the transport of the small subunit into the chloroplast is different from

the signal mechanism of Blobel and Sabatini (1972). This difference raises several important points:-

1) Did this mechanism arise independantly of the signal mechanism or is it a modified version of it?

2) Why is a different mechanism required for the transport of proteins into the chloroplast?

3) Is there only one mechanism by which proteins enter the chloroplast?

Signal peptides have been found at the NH_2-terminus of a large number (at least 18) of *in vitro*-synthesised polypeptides (Campbell and Blobel, 1976). These polypeptides are precursors of proteins which are exported from the cell, e.g. uteroglobin (Beato and Nieto, 1976); proinsulin (Chan *et al*, 1976); and lysozyme (Palmiter *et al*, 1977). In some cases it has been possible to show that the signal peptide is involved in transport of the polypeptide across the membrane *in vitro* (Boime *et al*, 1977; Dobberstein and Blobel, 1977; Lingappa *et al*, 1977; Rothman and Lodish, 1977). Thus the signal hypothesis would appear to be generally applicable to proteins destined for export from the cell. However, there are some exported proteins for which such precursors have not been described, e.g. vitellogenin (Tata, 1976) and collagen (Benveniste *et al*, 1976). These are large polypeptides and so any small differences due to the presence of a signal sequence may go undetected by the gel electrophoretic methods normally used. The standard proteins used are also normally isolated in the mature form, i.e. after all the post-translational modifications, such as glycosylation, have occurred; the presence of such modifications affects the electrophoretic mobility of the standards and so makes comparison with the *in vitro* products difficult. In the case

of the G-protein of vesticular stomatitis virus, the precursor polypeptide, which has a signal peptide, has an apparently lower molecular weight than the fully glycosylated final product, which has had the signal removed (Katz *et al*, 1977). The only certain criterion which can be used to detect a signal peptide is to compare the sequence of the NH_2-terminus of the *in vitro* product with that of the *in vivo* standard. There is one report that ovalbumin, which is the main protein exported from the cells of the chick oviduct, is not synthesised as a precursor (New Scientist, 1977). It is claimed that there is no signal sequence at the NH_2-terminus of the protein but, because this was just a meeting report, no data were presented. However, it is apparent that work in progress in the laboratories of Palmiter and O'Malley confirms this finding (Lodish, H. and Smith, A.E., personal communication). It is not clear how ovalbumin *is* transported across the membrane of the endoplasmic reticulum but it is obviously different from the signal mechanism and may well be different from that described in this thesis.

Recently there have been reports which suggest that the signal mechanism might be used by bacterial cells for the synthesis and secretion of extracellular proteins. Inouye and Beckwith (1977) have shown that *E. coli* alkaline phosphatase can be synthesised *in vitro* as a polypeptide which is larger than the mature protein. They were able to convert this polypeptide to the size of the mature protein *in vitro*, by adding cellular fractions; the processing activity was present in the outer membrane fraction of the cells. Smith *et al* (1977) found that membrane-bound polysomes of *E. coli* could be labelled by adding AMMP (acetyl-[^{35}S]-methionyl methyl phosphate sulphone)

to whole cells. This observation suggests that the nascent chains of the membrane-bound polysomes are accessible on the outside of the cell, i.e. that the polypeptides were being exported from the cell as they were being synthesised. Using antibodies, Smith *et al* (1977) obtained evidence that alkaline phosphatase was amongst these labelled polypeptides. Inouye *et al* (1977) sequenced the NH_2-terminus of the *in vitro*-synthesised prolipoprotein of *E. coli* and found that there were an extra 20 amino acids present. This extra sequence is highly hydrophobic, as are the sequences so far determined for eukaryotic signals (Burstein and Schechter, 1977; Devillers-Thiery *et al*, 1975).

All this evidence taken together suggests that the signal mechanism is present in *E. coli*, and so might have appeared early in evolution. It may be that with the appearance of eukaryotic cells, which have internal organelles, the signal mechanism was retained for export of proteins but that a second mechanism evolved for the transport of proteins from the cytoplasm in to organelles. Whether or not this second mechanism evolved from the signal mechanism can be determined only when the precise nature of the extra amino acids present in P20 are known, and can be compared with those present in signal sequences.

Ellis (1977) has suggested two principles which might govern the synthesis of both chloroplast and mitochondrial proteins. These principles state:-

1) that organelles synthesise only some of the subunits of multisubunit proteins, and

2) that cytoplasmically-synthesised proteins control organellar protein synthesis.

Combining these two principles leads to the suggestion that for a given multisubunit protein, the cytoplasmically-synthesised subunits control the synthesis of the subunits in the chloroplast. Thus, any polypeptide, which is synthesised in the cytoplasm but functions in the chloroplast, has to encompass a number of functions within its sequence. The polypeptide must be able to cross the chloroplast envelope, to combine with polypeptides synthesised within the chloroplast to produce an active enzyme, and to control some aspect of chloroplast gene expression. It may be that these requirements are not compatible with the signal mechanism of transport across membranes.

It is important to establish whether or not the other cytoplasmically-synthesised chloroplast proteins enter the chloroplast in the same way as the small subunit. From the results presented in Figure 34 it was concluded that the main effect of incubating the in vitro-synthesised polypeptides with chloroplasts was to cleave P20 to the small subunit, which subsequently became trypsin-resistant. On very long exposures of the autofluorograph, bands other than the Fraction 1 protein subunits could be seen and could be regarded as trypsin-resistant (see Fig. 34, track F). However, the amount of radioactivity in the bands was so low relative to the small subunit that it was not really possible to say whether they represented polypeptides taken up by the chloroplasts, whether they were just residues from the trypsin-digestion, or whether they were synthesised inside the chloroplasts. These bands were rendered trypsin-sensitive by addition of NP40 which makes it unlikely that they represent residues of trypsin-digestion. Since it was not possible to identify any chloroplast protein

in the *in vitro* products, apart from P20, it is impossible to say whether or not other cytoplasmically-synthesised chloroplast proteins enter the chloroplasts in the experiments described.

In section III2D it was argued that the chlorophyll *a*/*b*-binding protein was a chloroplast protein whose synthesis might be studied by means of the *in vitro* systems. It was suggested that this protein ought to be present in the *in vitro* synthesised products of poly(A)-containing RNA. By analogy with P20, which is the precursor to the small subunit, it was tentatively suggested that P32 might be a precursor to the chlorophyll *a*/*b*-binding protein. The fact that there is no detectable decrease in the label associated with P32 when chloroplasts are added might be due to one of two reasons:-

1) P32 is not related to any chloroplast protein, and so is not affected by incubation with chloroplasts;

2) P32 is a precursor to the chlorophyll *a*/*b*-binding protein but it is processed and transported into the chloroplasts by a mechanism different from P20, perhaps even by a signal-type of mechanism.

This argument emphasises that it is not possible to extend the observations on P20 to all other chloroplast proteins until some other proteins have been studied. The second alternative mentioned above could be investigated by having the chloroplasts present in the protein-synthesising reaction from zero-time. If some chloroplast proteins do enter the chloroplasts by a signal-type of mechanism, then processing should be detected.

The chlorophyll *a*/*b*-binding protein is synthesised in the cytoplasm, yet is part of the thylakoid membrane system of the chloroplast. The incorporation of this protein into the

PAGE

MISSING

thylakoid membrane may have a profound effect on the membrane morphology and can be correlated with the appearance of granal stacks (Armond *et al.* 1977). Thus the protein must cross the double membrane of the chloroplast envelope, become associated with chlorophyll, and enter the thylakoid membrane. Study of the synthesis and transport of this protein could provide important information about the assembly of proteins into membranes. With antibodies to the chlorophyll *a*/*b*-binding protein, which are necessary to identify any precursor forms, the methods described in the present work could be used for this sort of study.

4. ATTEMPTS TO ISOLATE THE MESSENGER RNA FOR P20.

One of the aims of this project was to isolate the mRNA coding for the small subunit of Fraction I protein. During the course of the work, it became apparent that the small subunit was synthesised *in vitro* as a precursor, P20 (section III2). In this section it is intended to present the results of several attempts to isolate the mRNA for P20. None of the approaches used were successful; however, they give pointers to the development of a potentially successful strategy.

A. Immune precipitation of polysomes from pea leaves.

It has proved possible, in some situations, to isolate the polysomes synthesising a specific polypeptide by reaction with antibodies against that polypeptide (Shapiro *et al*, 1974; Schechter, 1974; Scott and Wells, 1975; Jost and Pehling, 1976). The procedure used in this work was based on that described by Shapiro *et al* (1974). This method uses antibodies which have been purified by chromatography on an antigen-affinity column (section II2Ciia) and by chromatography on DEAE/CM cellulose (section II2Biiic). As described in section III2B, the antibodies used throughout this work were raised against whole Fraction I protein rather than against isolated small subunit.

It is necessary to isolate polysomes which still have nascent polypeptides attached because the antibodies must bind to these nascent chains (Palmiter *et al*, 1972). The inclusion of cycloheximide in the buffers used to isolate polysomes should keep the polysomes intact, because it inhibits the elongation reactions of protein synthesis (Pestka, 1974).

Excised pea shoots (12 days old) were placed in a glass vial containing 1mCi of [^{35}S]-methionine in a final volume of

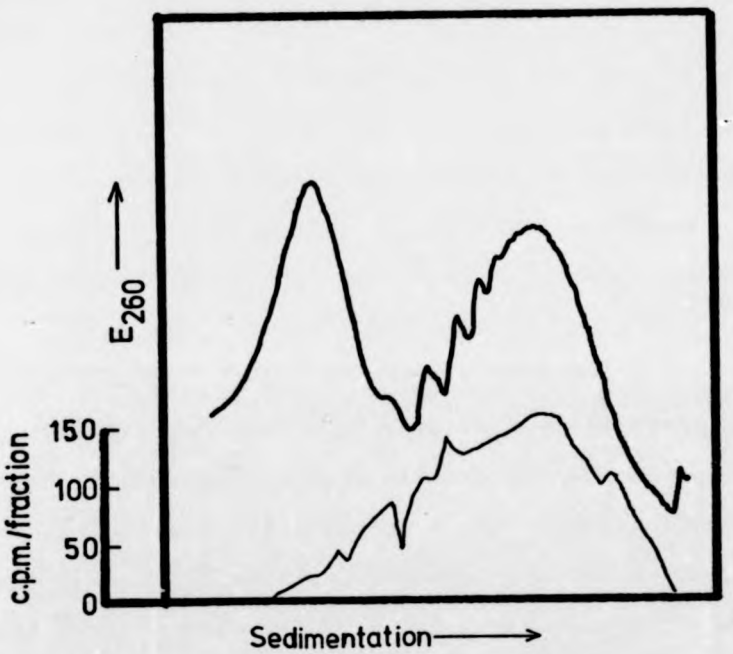
E_{260}
c.p.m./fraction
150
100
50
0
Sedimentation

Figure 35 Sedimentation profile of [^{35}S]-methionine-labelled pea leaf polysomes on a sucrose gradient.

Polysomes were isolated from pea shoots which had been labelled with [^{35}S]-methionine for 4hours (sections II2Fi and II2Ni). An aliquot of these polysomes (2.5 E_{260} units, 4,800 TCA-precipitable cpm), was analysed by centrifugation on a 15 to 50% sucrose gradient as described in section II2Fii. The E_{260} profile along the gradient was monitored continuously and fractions (0.15ml) were collected for measurement of TCA-precipitable radioactivity. BSA (100µg) was added to the fractions which were then made 5% in TCA. The precipitates were filtered onto Whatman GFC discs, washed with 5% TCA, ethanol and finally ether before being dried. Scintillation fluid (4ml; 0.5% PPO, 0.03% POPOP in toluene) was added and the filters were counted in a Packard Tricarb Scintillation counter at 12% gain, open window.

0.5ml of water. The shoots took up the label through their cut ends. After 4 hours under illumination (10,000 lux, white light), the apices were removed from the shoots, and polysomes isolated as described (section II2Fi). The polysomes so obtained were analysed on sucrose gradients (section II2Fii), and 0.15ml fractions collected. TCA-precipitable radioactivity present in the fractions was measured, on duplicate 20µl aliquots, by the filter-strip method described in section II2Jiia.

Figure 35 shows the profiles of absorbance at 260nm and TCA-precipitable radioactivity plotted together. There is no detectable radioactivity on top of the gradient, which suggests that there were no released polypeptides present in the polysome preparation. The TCA-precipitable radioactivity profile closely follows the absorbance profile, which suggests that the polysomes do still carry nascent polypeptide chains. There is no radioactivity associated with the 80S monomers. This shows that the labelling time was too short for a significant amount of incorporation into ribosomal proteins; this re-inforces the conclusion that the radioactivity associated with polysomes is due to nascent chains. The lack of labelling of the 80S monosomes also suggests that there has not been any extensive degradation of the polysomes. The only way 80S monomers, which carry nascent polypeptides, could arise is by nuclease cleavage of polysomes during isolation. It is clearly important, when attempting to isolate a messenger RNA, to establish that the methods used do not result in degradation of the messenger.

This analysis does not show that all the nascent chains present <u>in vivo</u> have remained attached to the polysomes. The polysomes were centrifuged through a sucrose pad, and this procedure would separate the polysomes from any released

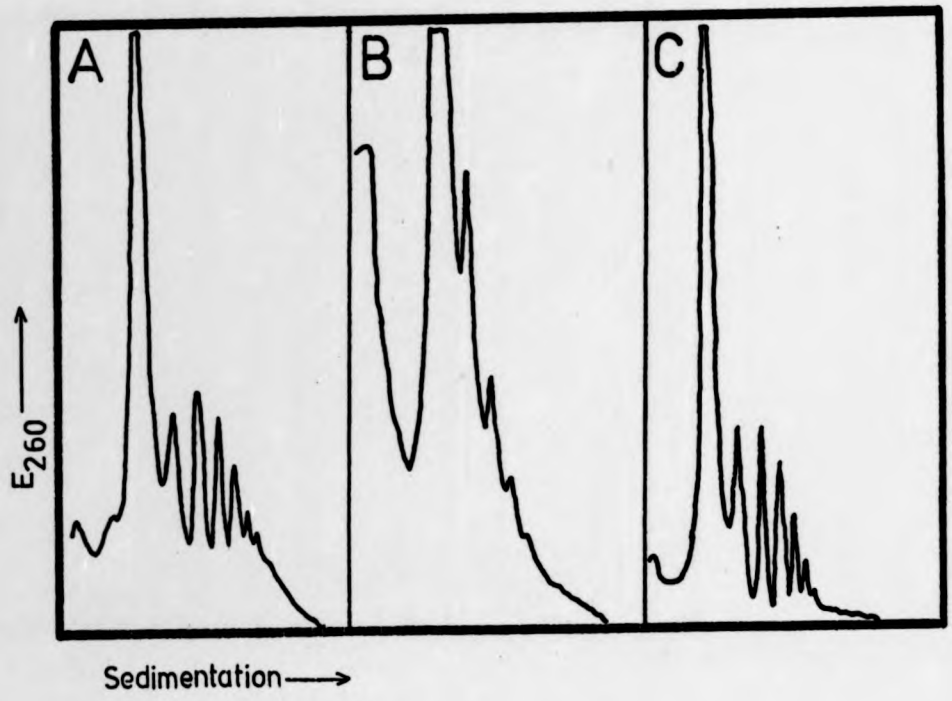
A
B
C
E_{260}
Sedimentation

Figure 36 Effect of immunoglobulins on the sedimentation profile of pea leaf polysomes.

Polysomes were isolated from 10 day old pea apices (section II2Fi) and aliquots ($1E_{260}$ unit) were incubated with 30µg of rabbit immunoglobulin at 37°C for 1 hour. The samples were then analysed by sedimentation through 15 to 50% sucrose gradients (section II2Fii).

A. Profile of polysomes incubated in the absence of immunoglobulin.

B. Profile of polysomes incubated with untreated immunoglobulin.

C. Profile of polysomes incubated with immunoglobulin treated by chromatography on DEAE/CM cellulose (section II2Biiib).

polypeptides and from the free, radioactive amino acid. Nascent chains lost at any stage before the centrifugation through sucrose would not be detected. Figure 35 does show that at least some nascent chains are present on the polysomes.

It was intended to incubate the polysomes with antibodies as part of the immune precipitation procedure. It is thus necessary to determine whether the antibody preparation contains nuclease activity. Anti-Fraction I antibody (50µg), which had been obtained by binding to the Fraction I protein-Sepharose 4B column (section II2Ciia), was incubated at 37°C for 90 minutes with 1 E_{260} unit of pea polysomes. Two preparations of antibody were used, one of which had been further purified on DEAE/CM cellulose, while the other had not. The polysomes were analysed on sucrose gradients (section II2Fii).

Figure 36A shows that incubation of the polysomes in the absence of antibody does not cause any significant degradation; polysomes up to heptamers are visible as distinct peaks. The antibody which had been further purified by chromatography on DEAE/CM cellulose appeared to be free of RNAase activity since the profiles of Figure 36A&C are practically identical. However the other antibody preparation caused extensive degradation of the polysomes (Fig. 36B). Thus chromatography on DEAE/CM cellulose efficiently removes RNAase activity from antibody preparations.

The next stage was to optimise the various immune reactions required for the immune precipitation of polysomes. The polysomes are first reacted with specific anti-Fraction I antibodies, and then sufficient goat anti-rabbit antibody is added to precipitate all the rabbit antibody. Only those polysomes bearing nascent chains of P20 should be precipitated. It was

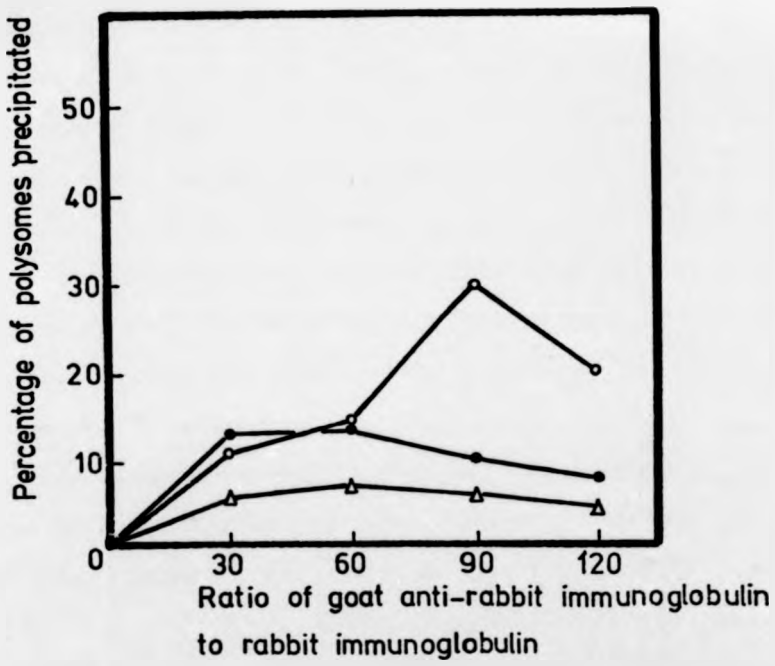
Percentage of polysomes precipitated
50
40
30
20
10
0
30
60
90
120
Ratio of goat anti-rabbit immunoglobulin
to rabbit immunoglobulin

Figure 37 Quantitation of immune precipitation of polysomes.

The [^{35}S] -methionine-labelled polysomes (4.5 E_{260} units, 9,600 cpm), as described in Fig.35, were incubated in a final volume of 400µl with either 500µg/ml of pre-immune immunoglobulin or either 250 or 500µg/ml of anti-Fraction I immunoglobulin. After 1 hour at 37°C, goat anti-rabbit immunoglobulin was added at the indicated ratio to the rabbit immunoglobulin. After a further hour at 37°C, the reaction mixtures were left at 4°C overnight. The radioactivity present in the precipitates was determined as described in section II2Fiiia and is expressed relative to the input radioactivity (9,600 cpm)

●——● 250 µg/ml anti-Fraction I immunoglobulin

o——o 500 µg/ml anti-Fraction I immunoglobulin

Δ——Δ 500 µg/ml pre-immune immunoglobulin

necessary to find the proportions of polysomes to specific antibody to goat anti-rabbit antibody which gave good yields of polysomes in the final precipitate yet gave only low non-specific absorbtion of polysomes.

Labelled polysomes were prepared as described above, except that the pea shoots were labelled for 24 hours. The polysomes were then used in immune precipitation reactions as described in section II2Fiiia. All the antibodies had been chromatographed on DEAE/CM cellulose and so did not contain any RNAase activity. The polysomes (11 E_{260} units/ml) were incubated at 37°C, with various concentrations of either anti-Fraction I IgG or pre-immune IgG, for 60 minutes. Then goat anti-rabbit IgG was added; the amount added depended upon the concentration of rabbit IgG already present in any particular incubation mixture. In section III2B, it was shown that a 30:1 ratio (by weight) of goat anti-rabbit to rabbit IgG gave complete precipitation of the rabbit IgG. In this experiment the ratio of goat anti-rabbit to rabbit IgG was varied from 30 to 120:1.

Figure 37 shows the results of this experiment. The amount of radioactivity present in the immune precipitate was determined as described in section II2Fiiia. The results are expressed as percentages of the input radioactive polysomes (4.5 E_{260} units, 9,600 cpm). The maximum precipitation occurs with 500µg/ml of anti-Fraction I antibody and at a ratio of goat anti-rabbit to rabbit antibody of 90:1. The amount of radio-activity present in the pre-immune controls presumably represents non-specific interactions between the polysomes and antibodies. After subtracting the pre-immune control, the maximum precipitation obtained was about 25% of the added polysomes.

The tissue used in this experiment was etiolated, and had

been greened for 24 hours in the presence of [^{35}S]-methionine. The greening experiment described in section III1Biii shows that the mRNA for P20 starts to increase in amount by 24 hours. However, from the *in vitro* translation products of poly(A)-containing RNA from this tissue, P20 does not appear to constitute as much as 25% of the total products synthesised (see Fig. 17). This discrepancy may mean that either the polysomes precipitated by this method are synthesising other polypeptides as well as P20, or that the *in vitro* translation assay does not accurately represent the relative proportions of the various mRNAs.

Attempts to repeat this immune precipitation on a preparative scale, using about 100 E_{260} units of polysomes per reaction, were unsuccessful. The procedure described in section II2Fiiib was used. Initially, phenol extraction of the immune precipitates was used to extract the polysomal RNA (section II2Gi). However, heparin, which was present in all the buffers used in the immune precipitation procedure, remains associated with RNA isolated in this way. Heparin is a potent inhibitor of protein synthesis, probably because it has a high affinity for the initiation factors (van der Mast *et al.* 1977). All the RNA isolated in this way was inhibitory in the wheat-germ system.

The LiCl-precipitation procedure (section II2Gii) was used to extract the polysomal RNA from the immune precipitates; this procedure gave RNA preparations which were active in the wheat-germ system. Three separate experiments were performed to attempt to specifically immunoprecipitate the P20-synthesising polysomes from a preparation of total polysomes. Two experiments used goat anti-rabbit antibody as the second precipitant; the

Antibody used for precipitation	Total polysomal RNA (mg)	RNA in precipitate	RNA in supernatant	% RNA recovered	% RNA precipitated
anti-Fraction I	1.25	0.04	o.48	41.5	8
pre-immune	1.25	0.01	0.50	40.8	2

Table XI Quantitation of the immune precipitation of polysomes.

The immune precipitation of polysomes, using goat anti-rabbit IgG as the second precipitant, was performed as described in section II2Fiiib. The amount of polysomal RNA present in the immune precipitation was determined by extraction of the RNA from an aliquot of the polysome preparation. The supernatant of the first sucrose gradient was removed and the RNA extracted. The RNA was extracted from this supernatant fraction and from the immune precipitate by the LiCl-precipitation procedure (section II2Gii). The amount of RNA in each fraction was determined from the total E_{260} units assuming 20 E_{260} units are equivalent to 1 mg of RNA.

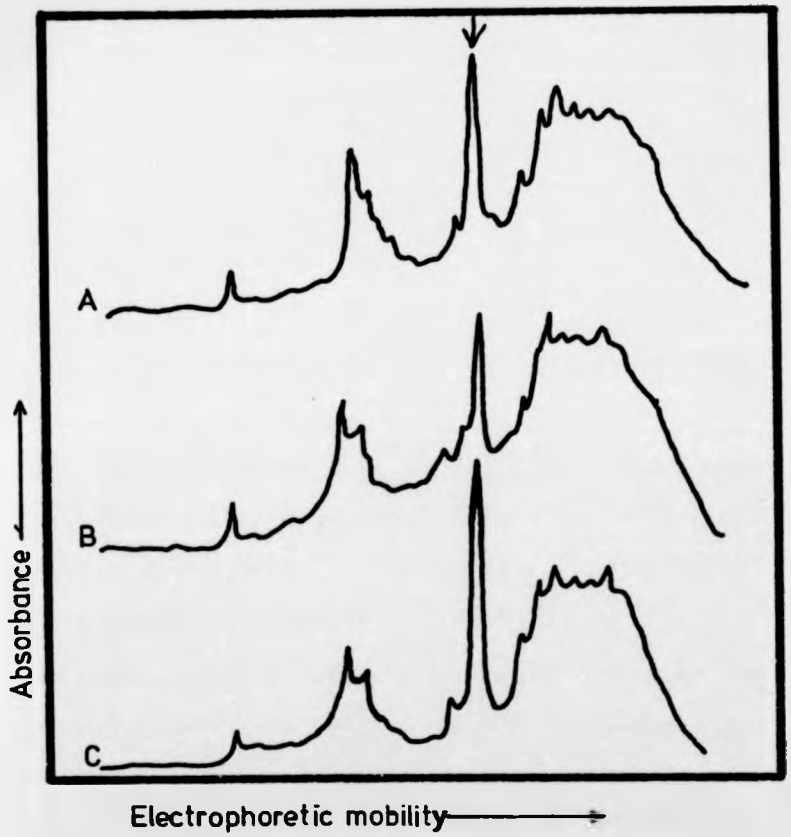
A
B
C
Absorbance
Electrophoretic mobility

Figure 38 Products of translation of RNA from immune precipitated polysomes.

RNA was isolated from polysomes obtained by immune precipitation as described in section II2Fiiib. This RNA was translated in the wheat-germ system (section II2Jiia) and the products analysed on polyacrylamide gels (section II2Jiib). The auto-radiograph of the dried gel was scanned on the Joyce-Loebl Chromoscan.

A. Products of RNA isolated from polysomes from etiolated pea shoots greened for 48 hours.

B. Products of RNA isolated from polysomes, as in A, precipitated with pre-immune antibody

C. Products of RNA isolated from polysomes, as in A, precipitated with anti-Fraction I anti-body.

third used heat-inactivated Staphylococcus aureus in this role. The results of all three experiments were essentially similar; table XI and Figure 38 show the results of one experiment.

Table XI shows that the recovery of RNA was poor, and that the proportion of recovered RNA present in the immune precipitate was low. From the titration curve (Fig. 37), about 25% of the added polysomes should have been precipitated by the conditions used in this experiment (500µg/ml anti-Fraction I, 90:1 excess of goat anti-rabbit IgG). Indeed, it would not have been suprising if a much higher proportion of the polysomes had been precipitated. The polysomes used to construct the curve in Figure 37 were isolated from tissue which had greened for 24 hours; in the experiment shown in Table XI the tissue had greened for 48 hours. From the experiment described in section III1Biii, it is known that the proportion of polysomal RNA represented by the mRNA for P20 increases between 24 and 48 hours of greening (see Fig. 17). It would be expected that the proportion of polysomes present in the immune precipitate should increase if the proportion of P20-synthesising polysomes increases.

However, the two experiments are not directly comparable because different methods were used to measure the extent of precipitation of the polysomes. In the analytical procedure (section II2Fiiia), the immune precipitates were washed by two cycles of resuspension and pelleting in the reaction vessel. The immune precipitates from the preparative procedure (section II2Fiiib) were washed by two cycles of centrifugation through sucrose pads. It may be that the differences observed here result from this procedural difference. Material was observed at the interface between the 0.5M and 1.0M sucrose pads. This

material may represent at least part of the difference found in the immune precipitates obtained by the different methods.

Although the amount of RNA found in the immune precipitate was lower than expected, the precipitation appears to be specific. The amount of RNA in the immune precipitate was four times greater than that in the pre-immune control. The RNA isolated from the various fractions was assayed for mRNA activity in a wheat-germ, cell-free system (section II2Jiia). Figure 38 shows a microdensitometer trace of the autofluorograph of the *in vitro* products, analysed on a slab polyacrylamide gel(section II2Eic). The position of P20 is indicated by an arrow. The most obvious point to note is that the RNA isolated from the immune precipitate directs the synthesis of polypeptides other than P20 (trace C). The proportion of P20 relative to the other products has increased compared with the products of total polysomal RNA (trace A). This increase appears to be only slight. The RNA from the pre-immune precipitate directs the synthesis of a range of products very similar to that of total polysomal RNA (compare traces A&B). This result implies that the presence of polysomes in the pre-immune precipitate is due to a non-specific interaction between the polysomes and the IgG.

It would appear from these results that this method suffers from high losses, and does not give a great enrichment of the P20 mRNA. In section III1Biii, it was stated that it was difficult to quantify the amount of any particular polypeptide amongst a large number of *in vitro* products. This is true especially when the polypeptide is minor. However, in Figure 38 P20 constitutes a major product. It was therefore possible to obtain an estimate of the proportion of *in vitro* products represented by P20; the areas under the microdensitometer

RNA source	amt. (µg)	mRNA activity cpm/µg	mRNA activity total	P20-mRNA (%)	P20-mRNA total activity	Recoveries total mRNA	Recoveries P20 mRNA	enrichment	Percent of recovered P20-mRNA
total polysomes	1250	2000	2.5×10^6	10	250×10^3	100	100	1.0	----
pre-immune precipitate	10	3000	0.03×10^6	9	2.7×10^3	1.2	1.1	0.9	2.3
pre-immune supernatant	500	2300	1.15×10^6	10	115×10^3	46	46	1.0	97.7
immune precipitate	40	3000	0.12×10^6	15	18×10^3	4.8	7.2	1.5	15.8
immune supernatant	480	2000	0.96×10^6	10	96×10^3	38.4	38.4	1.0	84.2

Table XII Messenger enrichment by immune precipitation of polysomes.

The RNA extracted from the various fractions obtained as described in Table XI were translated in a wheat-germ extract. The specific activity of the RNA was determined as the TCA-precipitable counts incorporated per ug of added RNA. The products of translation were analysed as described in Figure 38. From the densitometer scans of the relevant tracks, it was possible to estimate the percentage of total translation products represented by P20; it is assumed that this a measure of P20 mRNA. The enrichment is calculated by dividing the percentage of total P20 mRNA activity by the percentage of total mRNA activity present in each fraction.

traces were measured.

Table XII shows the result of such an analysis. The data show that the immune precipitation procedure results in an enrichment for P20 messenger activity in the RNA preparation by only 1.5-fold. To obtain such a meagre enrichment only 16% of the total P20 messenger activity was recovered in the immune precipitate. These data must be contrasted with other reports on the immune precipitation of polysomes.

Houdebine and Gaye (1976) report that ewe β-casein-synthesising polysomes were quantitatively precipitated by the indirect immune precipitation method. The mRNA obtained from the immune precipitate was 75-80% specific for the ewe β-casein; most of the rest was ewe α-casein mRNA. The β-casein-synthesising polysomes accounted for about 25-30% of the starting material.

Jost and Pehling (1976) found that 65% of vitellogenin-synthesising polysomes could be precipitated in an immune reaction. This resulted in a 4.2-fold enrichment of the vitellogenin messenger. The vitellogenin-synthesising polysomes accounted for about 30% of the total polysome preparation used.

Both these reports show that the immune precipitation procedure can result in good yields of specific polysomes. A modification of the procedure was therefore tried in the hope of improving the enrichment. This modification involved the use of S. aureus cells as the second precipitant. Mueller-Lantzsch and Fan (1976) used S. aureus, instead of sheep anti-rabbit antibody, in attempts to carry out immune-precipitation of polysomes specific for Moloney murine leukemia virus mRNAs. They found that this procedure gave much better recoveries of RNA, and improved the specificity of the precipitation. They

used rabbit antibody against a viral structural protein as the specific antibody.

The immune precipitation was repeated using 100:1 excess (by weight) of the heat-inactivated S. aureus (section II2D) instead of the goat anti-rabbit antibody. The procedure was exactly as before (section II2Fiiib) except that, after addition of the S. aureus, the reaction mixtures were left for only 1 hour at 4°C. The final pellet was resuspended in 2ml of 100mM NaCl, 10mM tris-HCl, pH 7.6, 1mM EDTA and 0.5% SDS and incubated at 37°C for 30 minutes. This treatment should dissociate any polysomes present on the surface of the bacterium (Mueller-Lantzsch and Fan, 1976). The bacteria were removed by a low-speed centrifugation (300g) and RNA was extracted from the supernatant solution as described in section II2Gii.

The results obtained using this procedure were essentially the same as those presented in Tables XI and XII, i.e. low recovery of polysomal RNA and very poor enrichment of P20 messenger activity.

Thus it was concluded that the immune precipitation of specific polysomes was not a feasible step to use in the isolation of the mRNA for P20.

B. Physical sizing of RNA

In section I5D various methods for isolating specific mRNAs were discussed. Many mRNAs can be obtained in high purity by sizing the mRNA population on either sucrose gradients or polyacrylamide gels. Only when the mRNA constitues a major fraction of the total mRNA population can a completely pure RNA be obtained solely by these techniques, e.g. chick ovalbumin mRNA (Rosen et al. 1975).

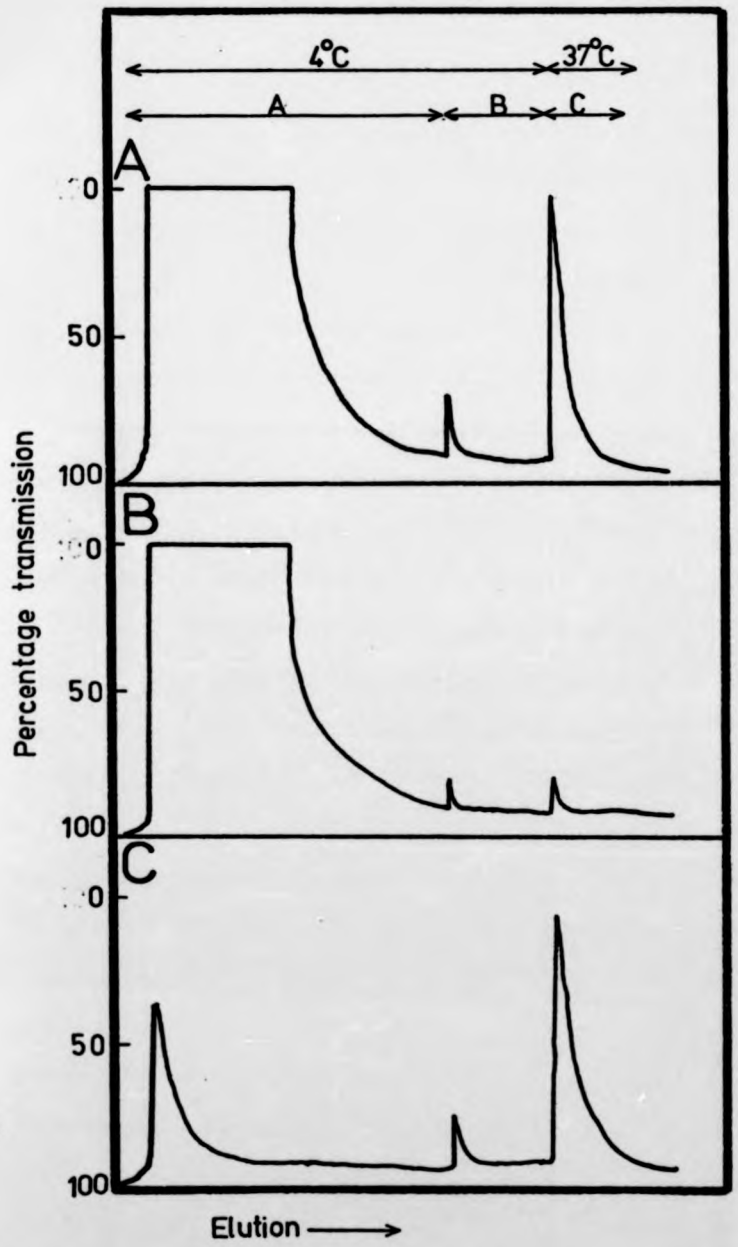
4°C
37°C
A
B
C
A
B
C
0
50
100
Percentage transmission
Elution

Figure 40 Repeated chromatography of polysomal RNA on oligo-dT cellulose.

The conditions used for chromatography were exactly as described in Fig. 12; buffer A (10mM tris-HCl, pH 7.6, 0.4M LiCl, 0.4% SDS), buffer B (10mM tris-HCl, pH 7.6, 0.1M LiCl, 0.4% SDS), buffer C (10mM tris-HCL, pH 7.6, 0.4% SDS).

A. Chromatography of total polysomal RNA (155 E_{260} units)

B. Re-chromatography of the RNA which did not bind in A.

C. Re-chromatography of poly(A)-containing RNA obtained in A, after dissociation by heating in DMSO as described in section II2Hi.

The difficulties encountered with the immune precipitation of polysomes made it necessary to consider using sizing techniques to isolate the mRNA for P20. Even if the immune precipitation technique had been successful, the mRNA obtained would not necessarily have been pure, and so a sizing step would still have been necessary.

Polysomes were isolated from pea shoots which had been greened for 16 hours in continuous light. The RNA was extracted from these polysomes by the phenol/SDS procedure (section II2Gi). There is no difficulty in obtaining large amounts of plant tissue for such extractions; in this experiment, 60g of pea shoots were used and this amount of material yielded 645 E_{260} units of polysomal RNA.

The poly(A)-containing RNA was isolated from the polysomal RNA by chromatography on oligo dT-cellulose (section II2Hi). A column containing 0.5g of oligo dT-cellulose was used; this was saturated by about 200 E_{260} units of polysomal RNA, and so the polysomal RNA was passed through the column in batches each containing 150 E_{260} units. Figure 39 shows the results of this procedure. Figure 39A shows a typical elution profile for polysomal RNA. All the RNA which had not bound to the oligo dT-cellulose was pooled and re-chromatographed. Figure 39B shows that very little RNA binds to the column; thus all the poly(A)-containing RNA has been removed from the polysomal RNA by the first elution.

Bantle et al (1976) have shown that poly(A)-containing RNA obtained in this way can still contain some rRNA; this is because there are aggregates formed between the mRNA and rRNA. These workers developed a method for disrupting these aggregates, followed by re-chromatography on oligo dT-cellulose, to obtain

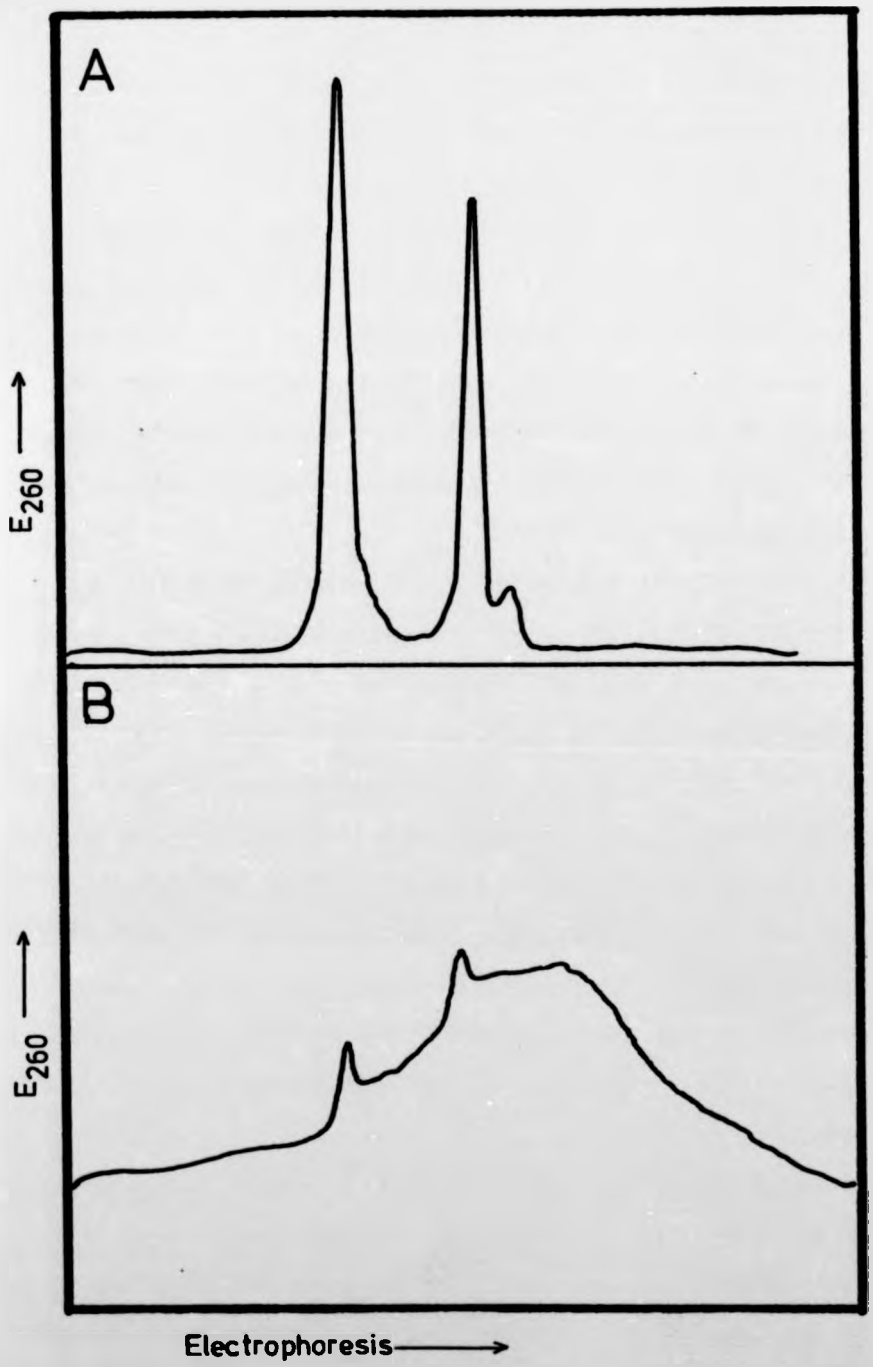
A
B
E_{260}
E_{260}
Electrophoresis

Figure 40 Electrophoretic analysis of RNA fractions obtained by chromatography on oligo-dT cellulose.

The non-poly(A)-containing and poly(A)-containing RNA fractions, obtained as described in Fig. 39, were analysed by electrophoresis on 2.4% polyacrylamide gels. The E_{260} profile was obtained using a Gilford gel scanner.

A. Non-poly(A)-containing RNA; this is the RNA fraction which did not bind to the oligo-dT cellulose in Fig.39B

B. Poly(A)-containing RNA; this is the RNA fraction which bound to the oligo-dT cellulose even after denaturation of the RNA(Fig. 39C).

pure poly(A)-containing RNA. Therefore all the pea RNA which had bound to the oligo dT-cellulose was pooled and heated in DMSO as described by Bantle et al (1976). Figure 39C shows the chromatography of this RNA on oligo dT-cellulose; the denaturation step resulted in some RNA, which had previously bound to the oligo dT-cellulose, no longer binding.

Figure 40 shows the analysis of the RNA fractions on polyacrylamide gels (section II2Eii). The RNA which had not bound to oligo dT-cellulose contains only the cytoplasmic rRNAs plus a small amount of chloroplast rRNA (Fig. 40A). The RNA which bound to the oligo dT-cellulose after denaturation in DMSO (i.e. the fraction which was eluted by no-salt buffer at 30°C in Fig. 39C), exhibits a heterogeneous size distribution on the gel (Fig. 40B). This RNA contains only a very small amount of rRNA, probably due to a small amount of re-annealing between the rRNA and mRNA after denaturation. The RNA absorbance profile is maximal at around 15S; this profile of poly(A)-containing RNA is similar to that obtained previously, except that the amount of rRNA contamination is much lower (Fig. 13A).

The final poly(A)-containing RNA preparation contained 6.5 E_{260} units of RNA, which represents about 1% of the total polysomal RNA used as starting material.

The two RNA samples were fractionated further by centrifugation through a sucrose gradient (section II2Ii). The method used exponential gradients of sucrose from 7 to 25% (w/v), and was designed for use with the MSE 8x25 titanium fixed-angle rotor. The resolution achieved using this method depends upon the acceleration/deceleration characteristics of the centrifuge used, and so the centrifugation time must be determined for any particular centrifuge. This method was

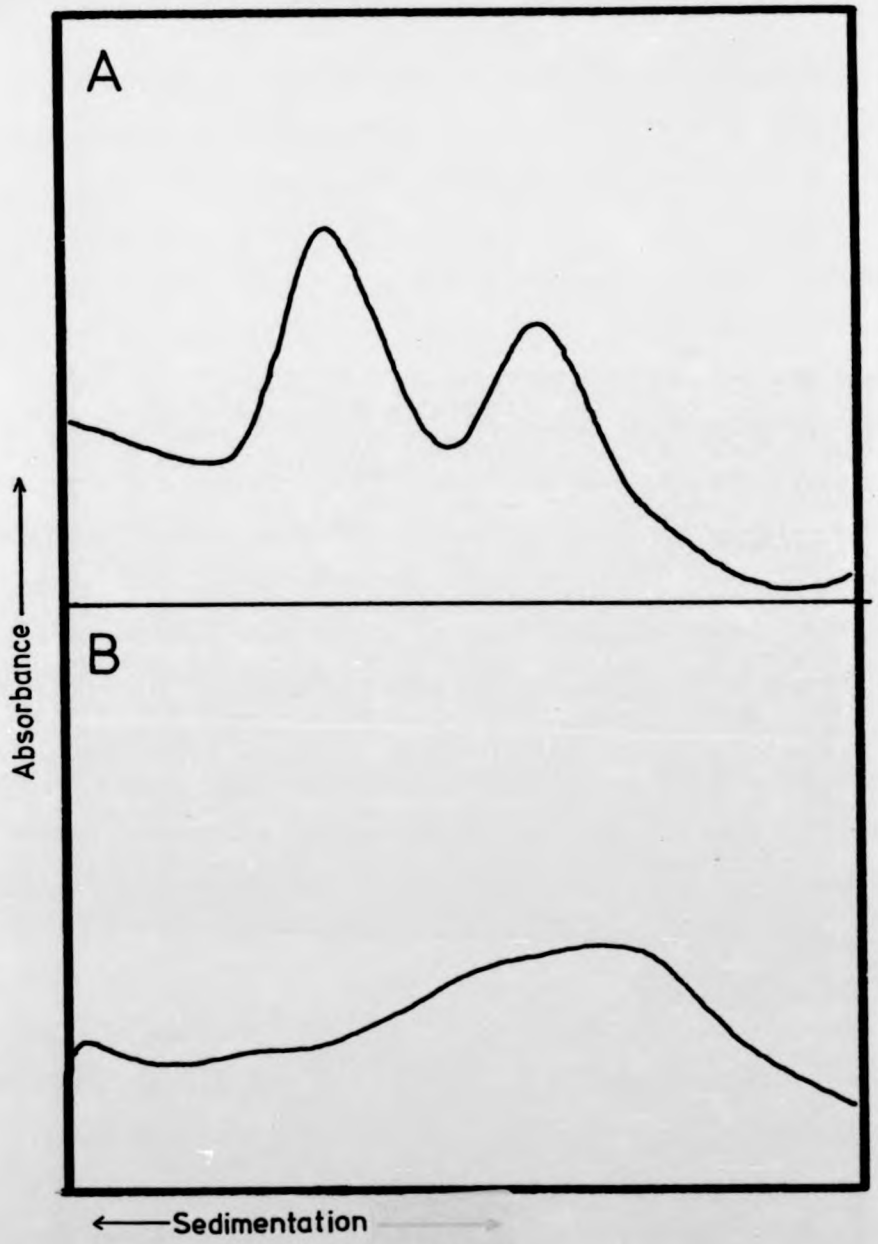
A
B
Absorbance
Sedimentation

Figure 41 Analysis of RNA fractions, obtained by chromatography on oligo-dT cellulose, on sucrose gradients.

The RNA fractions obtained as described in Fig. 39, were analysed by sedimentation through a 7 to 25% exponential sucrose concentration gradient using the fixed-angle rotor method (section II2Ii). Centrifugation was for 60 minutes at $230,000g_{ave}$.

A. Non-poly(A)-containing RNA; this is the RNA fraction which did not bind to oligo-dT in Fig.39B

B. Poly(A)-containing RNA; this is the RNA fraction which bound to oligo-dT cellulose even after denaturation of the RNA. (Fig. 39C)

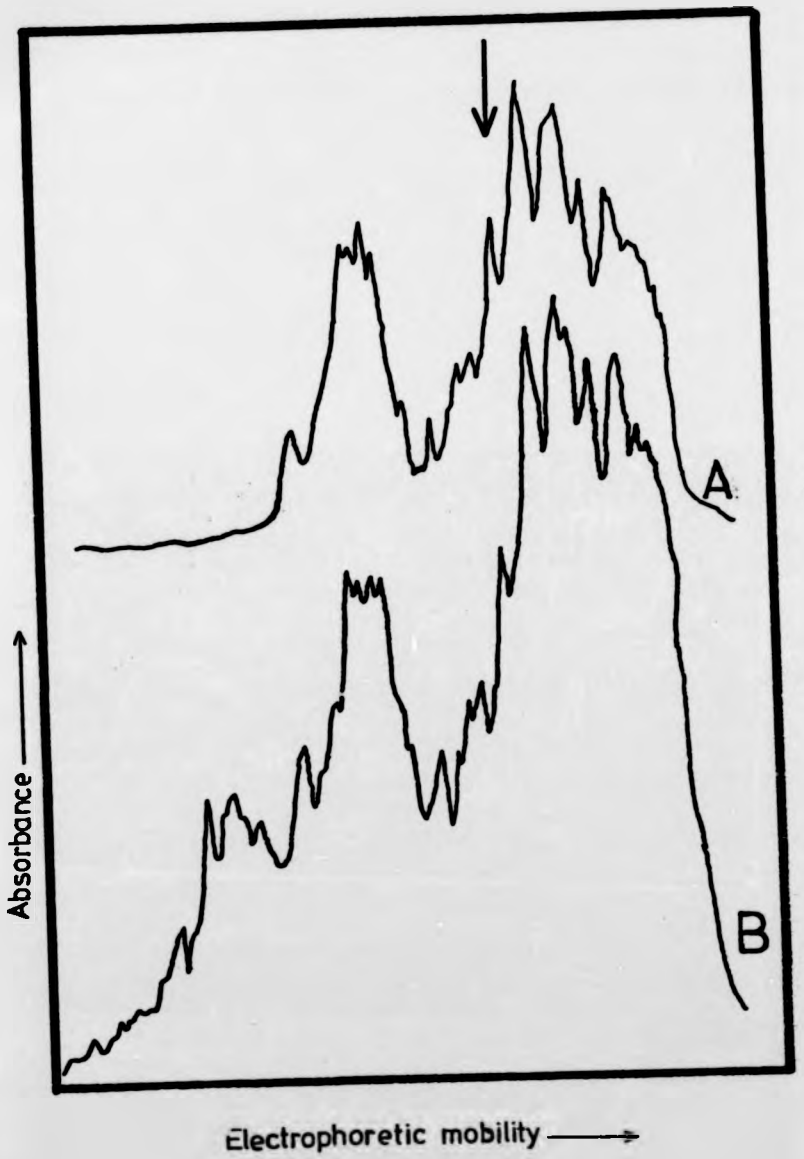
A
B
Absorbance
Electrophoretic mobility

Figure 42 Translation products of RNA fractionated on sucrose gradients.

The poly(A)-containing RNA analysed on sucrose gradients in Fig. 41B was split into two fractions; that larger and that smaller than 18S rRNA. The RNA was recovered from the gradient by ethanol precipitation and the products of translation in the wheat-germ system were analysed by electrophoresis on a slab polyacrylamide gel (section II2Jii). The autoradiograph of the dried gel was scanned in a Joyce-Loebl Chromoscan.

A. Products directed by RNA smaller than 18S.

B. Products directed by RNA larger than 18S.

The arrow indicates the position of P20.

devised by Dr. S. Covey in this department; the conditions of centrifugation used in this experiment were those which he had found suitable for resolution of the 18S and 23S rRNAs from spinach leaves.

Figure 41A shows the resolution of the two cytoplasmic rRNAs present in the non-poly(A)-containing RNA. These two RNAs are almost completely resolved; the absorbance does not quite fall to zero between the two RNAs. The advantage of this method, compared with standard sucrose gradient procedures, is that the two rRNAs are almost completely resolved after 2 hours of centrifugation (including the run-up and run-down times) whereas convential procedures employ centrifugations of 16 hours and longer. The short centrifugation time causes some problems, because the individual centrifuge characteristics become important; several trial runs must be performed to find the optimal conditions.

The poly(A)-containing RNA appears as a heterogeneous population (Fig. 41B). Sucrose gradients have poorer resolution than polyacrylamide gels, and so the rRNA contamination is not visible. Again the absorbance profile seems to peak at around 15S. The RNA from this gradient was split into two fractions, RNA larger than 18S, and RNA smaller than 18S. The parallel, non-poly(A)-containing RNA gradient was used to determine the position of 18S RNA.

The two RNA fractions were assayed for messenger activity in the wheat-germ cell-free system (see section II2Jiia); Figure 42 shows a microdensitometer scan of the products. Trace A represents the products of translation of RNA smaller than 18S; trace B, the products of RNA larger than 18S. The pattern of polypeptides is very similar in each case except

that the larger RNA directs the synthesis of some larger products. The largest polypeptide detectable has a molecular weight of about 30,000 in trace A and 60,000 in trace B. The position of P20 is marked with an arrow. There is relatively little P20 present because the RNA was isolated from tissue which had greened for only 16 hours.

This result shows that it is possible to recover RNA from sucrose gradients which is still active in a translation assay and that a certain amount of fractionation can be achieved. The fact that the heavier RNA fraction contained all the messengers present in the lighter RNA fraction, as well as some additional ones, would suggest that the RNA was aggregated. The problem of RNA aggregation could be overcome by heat-denaturing the RNA before loading onto the gradient and/or using much more denaturing conditions on the gradient. For example, the presence of formamide, at high concentration, in a sucrose gradient will denature RNA (Brown and Suzuki, 1974).

In spite of the problems with aggregation, the fact that the larger RNA directs the synthesis of larger polypeptides indicates that the method can resolve RNA into size classes. In particular, the mRNA for P20 probably has a sedimentation coefficient less than 18S. This is what would be expected, because ovalbumin has a molecular weight of 40,000 and its mRNA sediments at about 15S on a sucrose gradient (Haines *et al*, 1974).

Zonal centrifugation has been used to isolate a specific mRNA on a large scale (Williamson *et al*, 1971). In this particular case the mRNA was for mouse globin, and it could be visualised as a discrete peak of absorbance separate from the rRNAs and tRNA. When handling very large amounts of polysomal

RNA, zonal centrifugation is a convenient bulk fractionation method. The result presented earlier would suggest that the mRNA for P20 is smaller than 18S. With a more accurate determination of the size of the mRNA, it would be possible to pool fractions from the zonal centrifugation covering a range of sedimentation coefficients about the size of this mRNA. This would remove all of the 23S rRNA and probably some of the 18S rRNA from the preparation, and so represent a useful purification step.

The results of an attempted zonal separation of pea polysomal RNA were presented in section III1Bii, where the problems with RNA aggregation were discussed. In this case the aggregation was caused primarily by the high LiCl concentration used to precipitate the RNA. However, aggregation of RNA was a problem throughout this work, and is probably to be expected for RNA which has been precipitated out of solution by high ethanol concentrations. It would seem that, before any fractionation procedure, it is advisable to denature the RNA by heating in denaturing conditions, i.e. with urea, DMSO or formamide present.

Comparing Figure 40 with Figure 41, it can be seen that polyacrylamide gels give better resolution of different RNA species than do sucrose gradients. Attempts were, therefore, made to fractionate RNA on polyacrylamide gels, and to extract the RNA out of the gel for use in messenger assays.

Dolja _et al_ (1977) have described a procedure for extracting RNA from segments of polyacrylamide gel. It is claimed that this procedure results in good yields (about 50%) of RNA which is not contaminated with acrylamide. A major problem, when extracting RNA from gel segments, is that acrylamide is also

extracted and is difficult to subsequently remove. Various attempts to obtain pea RNA from polyacrylamide gels which was active in protein synthesis were unsuccessful. The RNA preparations obtained were always heavily contaminated with other material. This material was soluble in aqueous buffers; insoluble in ethanol and did not absorb at 260 or 280nm; this was assumed to be the case because the spectrum of the solution was that of pure RNA. This extracted RNA was inhibitory when added to the wheat-germ system.

The RNA, which was fractionated on polyacrylamide gels, was poly(A)-containing i.e. had bound to oligo dT-cellulose. Therefore, re-chromatography on oligo dT-cellulose of the RNA extracted from the gel should have separated the RNA from contaminants. However, with every sample tested, all the RNA passed straight through the oligo dT-cellulose column at high-salt concentrations. This observation may indicate that the contaminating material interfered with the hybridization reaction. Alternatively, the RNA may have been hydrolysed by the extensive handling necessary to extract the RNA from the gel.

C. Discussion.

The results presented in this section are disappointing but serve to emphasise the difficulties encountered when trying to apply published procedures to a new situation. As a result of the problems encountered, it is possible to make suggestions as to how to modify the approach and make it more likely to be successful.

(i) Discussion of results.

It is instructive to consider why the immune precipitation of polysomes did not work. Similar techniques have been used to

isolate polysomes synthesising albumin (Taylor and Tse, 1976), α_s and β-caseins (Houdebine and Gaye, 1976); immunoglobulins (Schechter, 1974); ovalbumin (Shapiro et al, 1974), and vitellogenin (Jost and Pehling, 1976). Therefore, this technique would appear to be widely applicable.

Berridge et al (1976) investigated immune precipitation as a method for the isolation of vitellogenin-synthesising polysomes from Xenopus liver. They isolated the polysomes from liver at pH 8.5 in the presence of heparin, a procedure very similar to that used for isolating pea polysomes in this work. They found that, if the polysomes had been pelleted through a sucrose pad, the immune precipitation reaction was unsuccessful. Whereas, if the polysomes were obtained without pelleting, then the immune precipitation reaction was successful. They claimed that the pelleted polysomes were aggregated.

The polysomes used throughout this work were isolated by pelleting through a sucrose cushion (section II2Fi).It is possible that these polysomes might be unsuitable for immune precipitation reactions, but it is unlikely. Berridge et al (1976) do not say what characteristics their pelleted polysomes had when assayed by immune precipitation, only that they were not suitable. From their description of the polysome pellets, it would appear that they had extensive contamination with non-polysomal material. These pellets may also have been difficult to solubilise. The polysome pellets obtained from pea shoots were free from obvious contamination and could be readily resuspended. The polysome suspension was clarified by a 30 minute centrifugation at $30,000g_{ave}$; there was only a small amount of insoluble material. The $E_{260}:E_{280}$ ratio was about 1.5 as expected for polysomes. These observations suggest that the

aggregation of polysomes, by pelleting through sucrose, might not be a general phenomenon.

This conclusion is re-inforced by the findings of Jost and Pehling (1976a), who prepared polysomes from chick liver by a method which included a step where the polysomes were pelleted through a sucrose pad. These polysomes were used successfully in an immune precipitation reaction, and the vitellogenin-synthesising polysomes were specifically isolated. This would suggest that the aggregation was peculiar to the system used by Berridge *et al* (1976). Polysomes prepared in this way have also been used successfully in immune precipitation reactions specific for other proteins, e.g. ovalbumin (Rhoads, 1975).

In section I5E, the background to the development of immune precipitation as a technique for isolating specific polysomes, was discussed. The important point to emerge was that specific precipitation of polysomes only became feasible when it was possible to obtain highly purified antibody preparations. Immunoglobulin molecules are able to interact, non-specifically, with ribosomes via the Fc-portion of the molecule (Holme *et al*. 1971). With highly-purified antibody preparations, the amount of immunoglobulin protein which needs to be used is greatly reduced. A consequence of this reduction is that the specific antibody-antigen reaction with the nascent polypeptide chains of the polysomes predominates over the non-specific interactions.

Bearing this in mind, a possible reason for the failure of the immune precipitation reaction becomes apparent. From Figure 37 the optimum immune precipitation conditions were 500µg of antibody for every 10 E_{260} units of polysomes. This is much greater than any other published value. In the literature, about 40 to 100µg of antibody for every 10 E_{260} units of

polysomes is usual (e.g. Jost and Pehling, 1976; Taylor and Tse, 1976; Schechter, 1974; Rhoads, 1975). Shapiro et al (1974) used 300µg of anti-ovalbumin for every 10 E_{260} units of polysome from chick oviduct, but then ovalbumin-synthesising polysomes do account for about 60% of the total polysome population. Considering that P20-synthesising polysomes may account for a relatively lower percentage of the total polysomes, the amount of antibody used was a factor of 5 to 10-fold higher. This means that any specific interaction between the antibodies and the nascent chains could be masked by non-specific interactions.

However, the reason for the lack of specificity, as judged by the products synthesised in response to RNA isolated from the immune precipitate, cannot be due entirely to this non-specific interaction. The anti-Fraction I antibody precipitates more polysomes than an equal amount of pre-immune antibody (Table XII). If the main cause of the lack of specificity was a reaction involving the Fc-portion of IgG, the both antibodies should precipitate the same amount of material. Thus the anti-Fraction I antibody must be recognising an antigenic site present on all or most, of the polysomes isolated from the pea shoots. Eschenfeldt and Patterson (1975) reported that antibodies could bind to isolated ribosomal subunits as well as to nascent chains, but only when the subunits had been in contact with antigen. They postulated that it was possible for the antigen to become associated with the ribosomes. Thus antibodies would recognise the adsorbed antigen and so precipitate polysomes which would not necessarily be synthesising that antigen. The adsorption of antigens onto polysomes cannot be a widespread phenomenon otherwise the immune precipitation technique would never work. Eschenfeldt and Patterson (1975) were working with

myeloma cell lines and so this interaction of the antigen with the ribosomes may be identical to the interaction of IgG molecules and ribosomes.

When pea shoots are ground in extraction buffer at the start of the polysome preparation (section I12Fi), the non-ionic detergent present lyses the chloroplasts. Thus the polysomes will be in contact with a large amount of Fraction I protein before being pelleted through the sucrose pad. It may be that some Fraction I protein adsorbs onto the polysomes and so affects the subsequent immune precipitation reaction.

It would be possible to check whether or not Fraction I protein was associating with the polysomes during isolation by adding labelled Fraction I protein at the grinding stage. If it proved to be the case that Fraction I protein was associating with the polysomes, then it is difficult to see what could be done to prevent it. Washing ribosomes in high-salt buffers will remove peripheral proteins (Nomura, 1970), and so increasing the salt concentration in the extraction buffer and in the sucrose pad might prevent this association taking place.

The above explanation suggests why the immune precipitation was not specific, but also suggests that a high proportion of the polysomes ought to have been precipitated. Yet only about 8% of the total RNA recovered was present in the immune precipitate, i.e. only about 4% of the starting material. It is suggested that some losses occured at the interface between the 0.5M and 1.0M sucrose pads when washing the immune precipitate. Another situation where losses might have occurred was during the isolation of the RNA from the immune precipitate. Rhoads (1975) comments that the LiCl-precipitation procedure was unsatisfactory for isolating RNA from immune precipitates

because of the large amount of protein present. He advises a phenol extraction step to remove most of the protein before using the LiCl-precipitation procedure. This may well explain the apparently low yield of polysomes in the immune precipitate.

Thus the poor results obtained with the immune precipitation procedure can be explained as follows. The poor yield was due to losses of RNA because of the large amount of protein present in the immune precipitate. The lack of specificity was probably due to the large amount of IgG which had to be used as well as the possible non-specific adsorption of Fraction I protein onto the polysomes. In the light of this explanation it is possible to suggest what might be tried to improve the procedure.

The most important point to investigate is the postulated association of Fraction I protein with polysomes. If it can be shown that this does happen, then immune precipitation may not be a feasible method for isolating P20-synthesising polysomes. It would be necessary to adapt the isolation procedure in some way to prevent this happening, perhaps by increasing the salt concentration.

The next point to consider is the specificity of the antibodies. It was not possible to obtain antibodies against the small subunit and so antibodies against whole Fraction 1 protein had to be used. This meant that only a subset of the IgG molecules would be specific for the small subunit and so proportionately more antibody had to be used. The antibody which bound most tightly to the Fraction I protein-Sepharose 4B was irreversibly denatured by the conditions used to elute from the column (section III2B). Thus the anti-Fraction 1 preparation contained only those antibodies which eluted from the antigen

column at pH 4.8 and only 30% of those were specific for Fraction I protein (Table VIII).

An improvement would be to make another attempt to raise antibodies against the small subunit. The reasons why the previous attempts failed are unknown. However, a different approach might be successful. Proteins can be isolated from SDS-polyacrylamide gels and used to raise antibodies in rabbits. Dobberstein et al (1977) used such a procedure to prepare antibodies against the small subunit from Chlamydomonas. The subunits of Fraction I protein can be isolated by chromatography of the protein on Sephadex G100 in an SDS-containing buffer (section II2Bii). It may be that small subunit isolated in this way would be suitable for immunization of rabbits. With antibodies against the small subunit, the immune precipitation reaction may be successful.

The low yields of RNA can probably be avoided by following the procedure of Rhoads (1975) and carrying out a phenol extraction of the immune precipitate before the LiCl-procedure.

Even if all these modifications can be made to the procedure, there is no guarantee that it will be possible to specifically isolate the polysomes which are synthesising P20. One possible difficulty is that even antibodies specific for the small subunit will not recognise the nascent chains of P20.

Palmiter et al (1972) were able to show that the smallest nascent chain which could be detected by anti-ovalbumin antibodies had a molecular weight of about 10,000. The actual figure itself need not be very accurate (polyacrylamide gels are not accurate at such low molecular weights) but it serves to emphasise that , before an antibody can bind to a polysome, the nascent chain must be long enough to be recognised as part

of the antigen. Sabatini and Blobel (1970) have shown that, until a nascent chain is longer than 39 amino acids, it is resistant to proteolytic digestion. This would suggest that it is "buried" inside the large ribosomal subunit. Therefore it is unlikely that antibodies could bind to this portion of the polypeptide chain. Assuming a molecular weight of 115 for an average amino acid, this argument indicates that, for ovalbumin at least, the minimum-sized polypeptide which binds an antibody is about 85 amino acids long, and 46 of those are exposed outside the ribosome.

P20 contains 50 additional amino acids when compared with the small subunit and these may be present as one sequence at either end of the P20 polypeptide. If these extra amino acids are at the NH_2-terminus, which they might be if they are analogous to the signal sequences present on secreted proteins, then they could have a profound effect on the ability of the anti-small subunit antibodies to recognise the nascent chain. The mature small subunit does not contain the extra sequence and so the antibodies should not bind to this sequence. In the absence of any other data, the ovalbumin situation can be used as a model to explore the possibilities.

The nascent P20 chain would have to reach a critical length before sufficient of the small subunit part of the molecule were outside the ribosome for an antibody to bind. The nascent chain would have to contain the 50 amino acids which are peculiar to P20, about 45 amino acids to be recognised by an antibody, and a further 39-40 amino acids buried inside the ribosome, i.e. 135 amino acids in all. P20 contains only 180 amino acids in total; thus about 75% of the whole molecule may have to be synthesised before an antibody can bind to the

nascent chain. Expressed another way, only 25% of the ribosomes in P20-synthesising polysomes carry a nascent chain sufficiently long to be recognised by an antibody. It is not known how many ribosomes there are, on average, in a P20-synthesising polysome but a plausible estimate can be made.

It is usually assumed that ribosomes pack onto an mRNA at a density of one per 90 nucleotides, i.e. one for every 30 amino acids in the protein (Staehlin et al, 1964). This would mean that polysomes synthesising P20 would contain 6 ribosomes and on average only 1.5 of them carry a site for an antibody. This small number of potential sites for antibody binding may also have an effect on the efficiency of immune precipitation. Palmiter et al (1972) suggest that cross-linking of nascent chains by antibody molecules is important; when the ovalbumin-synthesising polysomes were split into moncsomes by RNAase-digestion, then the number of ribosomes precipitated was reduced by a third.

This result can be contrasted with that obtained with rat albumin-synthesising polysomes, where immune precipitation has been used as a first step in the isolation of the mRNA for albumin (Taylor and Tse, 1976). This protein is about 560 amino acids long (molecular weight about 65,000) and has a signal 18 amino acids long (Yu and Redman, 1977). Using the same assumptions as above, an albumin-synthesising polysome would contain about 18-19 ribosomes. Only 103 amino acids would have to be synthesised before an antibody site was available (18 for the signal, 45 for the binding site and 40 hidden); this is only about 18% of the molecule. Thus, about 14 ribosomes on every albumin-synthesising polysome have a nascent chain long enough to potentially bind an antibody. This is about 10-fold more

than for P20-synthesising polysomes.

If the extra amino acids present in P20 were at the C-terminus of the molecule, then calculations similar to those above show that about 3 ribosomes per polysome could potentially bind an antibody. Again this is not many when compared to albumin-synthesising polysomes.

All these calculations are based on assumptions; one of the most important is that at least 45 amino acids have to be present in the exposed part of the nascent chain before an antibody can bind. If this were reduced to only 10 amino acids then all the figures for the numbers of ribosomes capable of binding antibodies could be increased by one.

These calculations show that for small proteins, i.e. around 20,000 molecular weight, there may be reasons why immune precipitation might not work. This is especially true when the proteins are synthesised with extra peptides at the NH_2-terminus.

(ii) Techniques for the purification of mRNA.

The foregoing discussion shows that isolation of the mRNA for P20 may have to be accomplished by fractionation of the RNA on the basis of size and poly(A)-content. Even if the immune precipitation of polysomes were successful, the mRNA would have to be removed from the rRNA by chromatography on oligo dT-cellulose. The purity of the mRNA preparation would have to be checked by some sort of sizing technique, e.g. by means of polyacrylamide gel electrophoresis.

Some mRNAs have been isolated purely by fractionation of cellular RNA on the basis of size and/or poly(A)-content, but these have been highly abundant mRNAs, e.g. globin (Williamson *et al*, 1971) and ovalbumin (Rosen *et al*,1975). The mRNA for

P20 is not particularly abundant (about 15% of the total poly(A) containing mRNA as judged by the proportion of P20 in total products of polysomal RNA) and so it might not be possible to purify the mRNA by this means. However, it might be possible if the mRNA for P20 had extensive secondary structure. Ovalbumin mRNA has an apparent sedimentation coefficient of 15S on sucrose gradients, yet on agarose-urea gels it has a lower mobility than the 18S rRNA (Rosen et al, 1975). This change in mobility relative to the 18S rRNA on two different fractionation systems is a result of changes in the secondary structure of the mRNA with different ionic conditions. This use of different sizing techniques might result in the purification of P20 mRNA, if a number of fortuitous changes in relative mobilities occur.

The main problem encountered with the procedures used in this work was aggregation of the RNA. However, the use of more denaturing conditions should solve this.

Polyacrylamide gels have greater resolving power than sucrose gradients but recovering the RNA from them presents problems. All attempts made during this work were unsuccessful. The main problems are poor recovery from the gel itself and contamination of the RNA with acrylamide.

Recently a method has been described for the use of a gel cross-linker which contains a disulphide linkage (Hanson, 1976). This can be used instead of bisacrylamide to form the gel. The use of this cross-linker has the advantage that the gel is soluble in thiol reagents, e.g. 2-mercaptoethanol. In this way the recovery is improved tremendously. The RNA will, however, now be contaminated with linear polyacrylamide as well as acrylamide. These can be removed by subsequent extraction with phenol.

A recent paper, which reports the isolation of leghaemoglobin mRNA from soybean nodules in a pure form, illustrates the problems associated with mRNA isolation and determination of purity (Sidloi-Lumbroso and Schulman, 1977). These authors isolated poly(A)-containing, polysomal RNA from soybean root nodules and fractionated it on a sucrose gradient. They took the fraction of RNA with a sedimentation coefficient of 10S and translated it in a wheat-germ extract; the major product was leghaemoglobin. This RNA was claimed to be pure because it gave a single absorbance peak on formamide-polyacrylamide gels,

The RNA had been isolated by sizing on a sucrose gradient, so using another sizing technique as a criterion of purity is not necessarily valid. It was mentioned earlier that two RNAs can have different relative mobilities when compared in two different systems. Thus it might be argued that the appearance of a single species, on both sucrose gradients and formamide gels, is a good test of purity. However, two RNAs need not necessarily behave in this way. This illustrates the main problem when a message has been isolated by a sizing technique; what criterion can be used to test its purity?

Sidloi-Lumbroso and Schulman (1977) translated the mRNA in a cell-free system, yet only 72% of the total incorporation was into leghaemoglobin. This result would suggest that the mRNA was not pure. Yet, even if all the incorporation had been into leghaemoglobin. This is not necessarily sufficient to show that the mRNA is pure. Any RNA which was not a messenger would not have been detected.

Thus, it is unlikely that the RNA preparation obtained by Sidloi-Lumbroso and Schulman contains only leghaemoglobin mRNA. This conclusion illustrates the problems associated with this

simple approach to isolating an mRNA; only if the mRNA in question forms a high proportion of the total mRNA, or if it has an unusually high or low molecular weight, can this approach give a pure mRNA.

(iii) Suggested approach to the isolation of P20 mRNA.

The arguments presented in this Discussion lead to the following suggestions which may be tried in future attempts to isolate the mRNA for P20:-

1) the question of whether or not Fraction I protein associates with pea polysomes during isolation must be resolved, if such association does occur, methods must be devised for eliminating, or at least reducing, it;

2) antibodies specific for the small subunit of pea Fraction I protein should be obtained;

3) the immune precipitation of polysomes should be repeated using these antibodies; the technique may well be successful if much lower amounts of antibody are found to be necessary than was the case in the present work;

4) the RNA should be extracted from the immune precipitate by the procedure of Rhoads (1975);

5) the poly(A)-containing RNA should be isolated using oligo dT-cellulose chromatography; the final traces of rRNA can be removed by denaturation followed by rechromatography;

6) the mRNA should be fractionated on the basis of size by either sucrose gradients or polyacrylamide gels under a variety of conditions; these methods serve to both remove any contaminating RNA and to check the purity of the RNA.

Throughout this procedure, translation of the RNA in a wheat-germ extract will serve to locate the P20 mRNA and also

to determine the purity of the RNA in terms of messenger activity. This must be coupled with the techniques mentioned in step 6 to determine the purity of the RNA with respect to both translatable and non-translatable RNAs.

SECTION IV - GENERAL DISCUSSION

The principal aim of the work reported in this thesis was to purify the mRNA for the small subunit of Fraction I protein from pea leaves. It did not prove possible to achieve this aim. However, it was established that the small subunit is synthesised as a precursor (P20) and that this precursor is processed to the mature size and transported into isolated chloroplasts. Thus, the results presented in this thesis have two separate aspects; one concerns the possible isolation of P20 mRNA and mRNAs for other chloroplast proteins, the other concerns the transport of proteins across the chloroplast envelope and perhaps protein transport in general. These two aspects will now be discussed.

1. mRNAS FOR CHLOROPLAST PROTEINS.

A. Cytoplasmic mRNAs.

In section II4C, the reasons why P20 mRNA proved difficult to isolate were discussed. It was argued that improvements in the immune precipitation technique used in this thesis might enable purification of P20 mRNA to be achieved. However, the rapid advances which have been made in the field of recombinant DNA research in the last year, have probably outmoded such an approach.

At the time this research was started it was envisaged that the mRNA would be used as a template for the synthesis of a cDNA probe, and that this probe would be used to quantitate the amount of mRNA present in samples of RNA, isolated from different stages of the greening process. This type of approach is destructive for the mRNA. When the cDNA probe has been formed by the reverse transcriptase reaction, the mRNA used as template is hydrolysed (Verma et al, 1972). The cDNA produced cannot be used more than once. So if an

extensive study using this approach were contemplated, it would be necessary to prepare relatively large amounts of mRNA. It was for this reason that methods capable of yielding milligram quantities of specific mRNA were investigated (Schechter, 1974; Taylor and Tse, 1976).

The repeated isolation of highly purified mRNA on a large scale is obviously an expensive proposition. In some situations it may be both technically and economically difficult to obtain large amounts of starting material, e.g. tissue culture cells. With plant mRNAs starting material might not pose a problem, but isolation of the mRNA would require the preparation of large amounts of monospecific antibodies. Thus, even in favourable cases, the repeated isolation of a pure mRNA would not be routine.

In recent months techniques have been described whereby it is possible to construct bacterial plasmids which contain an inserted piece of double-stranded DNA(dsDNA) which contains at least part of the sequence of globin mRNA (Rougeon *et al*, 1975; Rabbits, 1976; Efstratiadis *et al*, 1976; Rougeon and Mach, 1977). The basic techniques are as follows:-

1) reverse transcriptase is used to produce a DNA copy of the mRNA;

2) this DNA is then used as a template for *E. coli* DNA polymerase I which generates a dsDNA molecule;

3) the 3'-ends of each of the DNA strands are extended by addition of either adenylate or cytosine residues;

4) the circular plasmid which is to be used as the cloning vehicle is converted into its linear form by cleavage with a restriction enzyme;

5) the 3'-ends of the linear plasmid DNA are also extended this time by addition of the nucleotide complementary to that

used for the DNA to be inserted;

6) the plasmid and dsDNA are mixed together and form a circular molecule consisting of the plasmid DNA and globin DNA inserted; a ligase enzyme is used to covalently link the two sequences;

7)these chimeric plasmids are then used to transfect E. coli; the plasmid must carry some easily detected marker, such as drug resistance, so that the transfected bacteria can be detected.

In this way it has proved possible to obtain plasmids which carry DNA specific for globin mRNA; plasmids carrying DNA specific for ovalbumin RNA have also been constructed in this way (McReynolds et al,1977). The DNA present in the plasmid does not represent the entire mRNA molecule because the reverse transcriptase step generates a series of different sized DNA molecules. However, plasmids which contain a large proportion of the mRNA sequence have been obtained.

The methods described above still require that the mRNA be very pure. If it were not pure, then plasmids containing DNA specific for a variety of mRNAs would be generated. In the method described, any clone of E. coli which has received the plasmid probably also contains an insert of DNA specific for the mRNA used as initial template. If a mixture of mRNAs were used then it would be necessary to screen every plasmid-containing clone to determine which were carrying the DNA specific for a particular mRNA. Methods for screening large numbers of clones by hybridization with radioactive cDNA have been described (Grunstein and Hogness, 1975). This method requires a cDNA probe specific for the mRNA, which again means that the mRNA must be obtained in a reasonably pure form to

generate such a probe.

Very recently, Seeburg et al (1977) have described methods whereby plasmids containing DNA specific for rat growth hormone mRNA can be constructed and isolated. The most important point is that the mRNA for this protein constituted only 10% of the poly(A)-containing RNA used to prepare the cDNA. They circumvented all the problems of generating a specific probe for the growth hormone mRNA by a novel procedure.

The heterogeneous population of plasmids that can be generated when total poly(A)-containing RNA is used as template for the dsDNA inserts, does not make it difficult to eventually obtain single plasmids containing one specific insert. Repeated cloning of the transfected bacteria is all that is required. The difficult part of the procedure is to obtain a probe specific for the particular insert. The cDNAs produced by transcription of the original poly(A)-containing RNA form a heterogeneous population and cannot be used to probe for specific plasmids. However, digestion of these cDNAs with restriction enzymes can generate discrete fragments of cDNA. These fragments must arise by cleavage of the whole range of cDNA molecules transcribed from one mRNA at specific points. Seeburg et al (1977) were able to obtain a cDNA fragment specific for growth hormone mRNA in this way, even though this mRNA was only about 10% of the total mRNA. With a probe it is then possible to screen clones, and so obtain the plasmid(s) containing the DNA insert specific for growth hormone mRNA.

This technique will enable many more mRNA sequences to be cloned as DNA inserts in plasmids because it has the great advantage that there is no need to purify the mRNA. The method has been shown to work for an mRNA which accounts for only 10%

of the total poly(A)-containing RNA, and may work for an mRNA of even lower abundance. The only caveat on the general applicability of this technique is that the cDNA fragment, produced by the restriction enzyme digestion of the total cDNA, must be identified. Seeburg et al (1977) used the rapid DNA sequencing method of Maxam and Gilbert (1977) to sequence their cDNA fragment. They were able to compare the DNA sequence with the known sequence of rat growth hormone, and so could show that the fragment was derived from growth hormone mRNA. If the sequence of the protein of interest is not known, then some other technique must be used , e.g. the use of poly(A)-containing RNA from similar tissue which is not synthesising the protein in question.

This technique can be applied to the cloning of P20 mRNA. This mRNA accounts for more than 10% of the total mRNA, as judged by translation assays, and so it should be possible to generate a cDNA probe specific for P20 mRNA. Experiments are at present underway to examine the possibility of cloning this mRNA (Bedbrook, personal communication). The complete sequence of the small subunit of Fraction I protein is not known; however, it has been shown in this thesis (section III1Biii) that polysomal, poly(A)-containing RNA from etiolated tissue contains very much less P20 mRNA than does RNA isolated from tissue greened for 48 hours. Thus, comparison of fragments generated by digestion of cDNA transcribed from these two RNA samples should help to identify a fragment which might be complementary to P20 mRNA. Once such a probe has been obtained, it should be relatively straight-forward to isolate a plasmid carrying DNA specific for P20 mRNA.

The advantage of having a cloned plasmid which is specific

for P20 mRNA is that it is then simple to obtain large amounts of specific probe. All the experiments which could be done using the conventional cDNA approach would then be possible.

Using labelled plasmid DNA it would be possible to measure the concentration of P20 mRNA in any RNA preparation. In this way, the changes in P20 mRNA concentration during any developmental situation could be followed. By examining the polysomal and non-polysomal RNA fractions it should also be possible to detect whether or not there is "storage" of P20 mRNA before it enters the polysomes. By combining this sort of analysis with *in vitro* protein synthesis assays, it should be possible to determine whether increases in small subunit synthesis are due to increased mRNA synthesis, to translation of previously untranslated mRNA, or to more efficient utilization of the existing mRNA. It is necessary to use translation assays because hybridization will only detect mRNA sequences, and cannot determine whether or not these sequences are active in translation.

The ability to produce large amounts of DNA complementary to P20 mRNA makes some experiments feasible which could not be contemplated previously. Merkel *et al* (1976) used a cellulose column, with globin cDNA bound to it, to study changes in the globin mRNA during mouse erythroid development. This approach is only feasible when large amounts of pure mRNA can be obtained. However, using plasmid DNA, it should be possible to preparatively isolate P20 mRNA. In this way the physical properties of the RNA could be investigated; in particular, the existence of a cap, and the nature of the poly(A)-tail of the mRNA, could be determined.

Using the rapid sequencing techniques available at present

(Maxam and Gilbert, 1977) it should be possible to seqence the DNA inserted into the plasmid. This should give details of the non-coding region at the 3'-end of the mRNA, the amino acid sequence of the small subunit of Fraction I protein, and the extra amino acids present in P20. All this information would be very useful to know; the sequence of the P20-specific amino acids may give some insight into how the transport and processing mechanism works, the complete sequence of the small subunit has not been obtained by conventional means.

The availability of a probe for the mRNA sequence will enable investigation of the organisation of the small subunit gene(s) within the nuclear DNA. Using this type of approach, it has been shown that the genes for globin and ovalbumin contain long pieces of DNA inserted into the sequence found in the mRNA in the cytoplasm (Jeffreys and Flavell, 1977; Breathnach et al, 1977). The function of these sequences is not known but they may be important in the control of the expression of the gene.

B. Chloroplast mRNAs.

The availability of purified restriction enzymes has permitted a new approach to the study of the chloroplast genome. The chloroplast DNA can be cleaved by restriction enzymes into a series of specific fragments. By using combinations of different restriction enzymes, it is possible to obtain a physical map of the chloroplast genome (Bedbrook and Bogorad, 1976; Gray and Hallick, 1976; Herrmann et al, 1976). Individual fragments can be cloned using suitable plasmids as cloning vehicles. Bedbrook et al (1977) used this technique to show that the rRNA genes are located within a 22,000 base-pair

inverted repeat on chlDNA from *Zea mays*. These workers used the restriction enzyme EcoR1 to generate the original restriction fragments, and then used further restriction enzymes to generate smaller fragments and so improve the accuracy of the localization. They have been able to determine the relative orientation of the two sets of rRNA genes within the chlDNA, and the relative orientation of the 23S and 16S rRNA cistrons.

It has been possible to locate the genes for some other chloroplast components on the restriction map of chlDNA (Bedbrook, personal communication). The tRNAs can be located by hybridization to isolated restriction fragments. A different approach must be used to locate the genes for chloroplast proteins. A transcription-translation system using *E. coli* RNA polymerase and either a wheat-germ or rabbit reticulocyte cell-free protein-synthesising system has been developed (Bedbrook, personal communication). Using this system, it has been possible to locate the gene coding for the large subunit of Fraction I protein on part of the chlDNA circle almost diametrically opposite to the rRNA genes. By using more restriction enzymes, it is possible to obtain a fragment, about 1700 base-pairs long, which still contains the large subunit gene; this is similar to the size expected of a gene coding for a protein of 55,000 molecular weight.

Although this approach has been very successful, and has removed the need to isolate chloroplast mRNAs by conventional techniques, it cannot directly answer the question as to what genes are encoded in the chlDNA. Theoretically, every chlDNA restriction fragment could be used in the transcription-translation assay system, and so the coding potential of the entire

genome could be determined. However, until more is known about the protein chemistry of the many enzymes found in the chloroplast, these *in vitro* products will remain unidentified. Apart from the large subunit, only the subunits of CF_1 are known to be synthesised within the chloroplast. The protein known as peak D has not yet been unequivocally identified, although it may be involved in binding CF_1 to the thylakoid membrane (Ellis *et al*, 1978).

Thus a growing problem in the elucidation of the function of chlDNA is the lack of information about the identity of the majority of chloroplast proteins. Until this information becomes available, it will be possible to map the genes of the chlDNA only in terms of the molecular weight of the proteins for which they code.

However, the fact that it is now possible to consider mapping the genes for chloroplast proteins on the chloroplast genome testifies to the ability of the new techniques to solve problems which have proved refractory to solution by conventional methods.

2. TRANSPORT OF PROTEINS.

In section III3B, it was argued that the mechanism of transport of the small subunit of Fraction I protein into the chloroplast differs from that found for proteins which are secreted from the cell. It is necessary to consider how widespread might be the mechanism of transport outlined for P20.

The synthesis of mitochondrial proteins has been extensively studied, and the hypothesis advanced that mitochondrial proteins are synthesised on membrane-bound polysomes. Butow *et al* (1975) have shown that, in yeast, there

are polysomes present on the surface of the mitochondria. These polysomes are claimed to be synthesising mitochondrial proteins, and Butow et al postulate that the proteins enter the mitochondrion by a signal-type of mechanism. Shore and Tata (1977a&b) have shown that, in rat liver, membrane-bound polysomes are the site of synthesis of mitochondrial proteins. These polysomes are bound to the endoplasmic reticulum and not to the outside of the mitochondrion. However, electron micrographs of rat liver cells show close apposition of the rough endoplasmic reticulum and the mitochondria, so Shore and Tata argue that the situation in yeast and rat liver may be practically the same. In fact the outer membrane of the mitochondrion may be contiguous with the rough endoplasmic reticulum (Morre et al, 1971).

These observations suggest that the mechanism of synthesis and transport of proteins into the mitochondrion differs from that which has been proposed for chloroplast proteins. They certainly do not support the idea that the mechanism of transport of the small subunit into the chloroplast might be generally applicable to other chloroplast and mitochondrial proteins. However, Poyton and Kavanagh (1976) were able to show that soluble, cytoplasmic proteins are able to stimulate protein synthesis by isolated mitochondria from yeast. In particular, they were able to show a specific stimulation of the synthesis of subunits I,II and III of cytochrome c oxidase. The stimulatory activity could be removed from the cytoplasmic preparation by reaction with antibodies to subunits IV and VI of cytochrome c oxidase. This treatment specifically precipitated a 55,000 molecular weight polypeptide; the relationship between this polypeptide and subunits IV and VI is not known.

This situation is what might be expected from the principles of organelle protein synthesis described earlier in section III3B. The 55,000 polypeptide is soluble, and yet able to control the synthesis of mitochondrial proteins. If this protein has to enter the mitochondrion to exert this control, then the mechanism of transport cannot be of the signal type but may well be similar to that postulated for the small subunit.

Recently two reports have appeared from one group which suggest that, in Neurospora crassa , mitochondrial proteins are synthesised on membrane-bound polysomes, and are released into the cytoplasm before being transported into the mitochondria (Hallermayer et al, 1977; Harmey et al, 1977). These workers concluded that this was the case from data obtained using both whole cells and cell-free homogenates. In whole cells, the cytosol and mitochondrial fractions were labelled with a pulse of [^{3}H]-leucine at a lower rate than the rest of the cell. More importantly, the labelling of these fractions continued during a chase period when the labelling of the other fractions fell. Thus the labelled proteins produced by the cytoplasmic polysomal and microsomal fractions must pass into the cytosol and mitochondrial fractions. The labelling of particular mitochondrial proteins increases even after cytoplasmic protein synthesis has been inhibited by cycloheximide. Thus mitochondial proteins must be synthesised outside the mitochondria, and subsequently enter the mitochondria, even in the absence of continued protein synthesis.

Harmey et al (1977) were able to confirm this idea from studies of protein synthesis by cell-free homogenates of Neurospora crassa. When the nascent polypeptide chains on the cytoplasmic polysomes were discharged by puromycin, there was

a dramatic increase in the labelled protein present in the cytosol followed by a gradual decrease as the labelled protein in the mitochondria increased. When protein synthesis was inhibited by cycloheximide, the label associated with both the cytoplasmic ribosome and microsomal fractions remained constant, whilst that of the cytosol fell as that of the mitochondria rose. These observations suggest that the cytosol fraction contains mitochondrial proteins which can enter the mitochondria after synthesis and release from the ribosomes. By the use of immune precipitation, it was shown that individual mitochondrial proteins behaved as did the total mitochondrial protein. In particular, the cytosol fraction was shown to contain mitochondrial matrix proteins, which could not have resulted simply from leakage of the mitochondria during isolation.

In work of this type, it is necessary to ensure that artefacts due to the isolation procedures do not result in changes in the location of components. These workers have carried out several controls to ensure that such artefacts did not occur. However, the cytoplasmic polysome fraction seems to contain a lot of labelled protein which must be adventitiously bound to the ribosomes. When puromycin is added, only about 30% of the label in this fraction is released. Harmey _et al_ (1977) do show that about 50% of added mitochondrial matrix protein can become associated with the ribosomal and microsomal fractions, this non-specific association would explain the apparently low discharge of labelled protein from the polysomes by puromycin.

Addition of labelled, mitochondrial matrix proteins to the cell-free homogenate did not result in any label appearing in the mitochondria. Thus only newly-synthesised polypeptides can enter the mitochondria.

These results are consistent with the conclusions of Poyton and Kavanagh (1976), but are directly in disagreement with those of Butow et al (1975). Hallermayer et al (1977) report that they found no evidence for the association of polysomes with the outer membrane of the mitochondria even using the preparation coditions of Butow et al (1975).

These results suggest that mitochondrial proteins are synthesised on cytoplasmic ribosomes, are released into the cytosol and then are transported into the mitochondria. The mechanism by which this transport occurs is not known, but after the proteins have crossed the mitochondrial envelope they have been modified such that they cannot move across the envelope in the same direction again.

This model for the synthesis and transport of proteins into mitochondria agrees well with that outlined in this thesis for the synthesis and transport of the small subunit of Fraction I protein into chloroplasts. There is no evidence for any mitochondrial protein being synthesised as a precursor but the techniques used to examine the newly-synthesised proteins are not sufficiently accurate to detect small molecular weight differences. Processing of precursors would explain why the isolated matrix proteins could not cross the mitochondrial envelope.

The data presented in this thesis do not give any indication as to the exact mechanism by which P20 crosses the chloroplast envelope; it is suggested that cleavage to the small subunit is probably involved. In section III3B, approaches which should lead to a clearer understanding of the transport mechanism were outlined. Until further data become available it is only possible to speculate.

Dobberstein et al (1977) suggested that the transport of diphtheria toxin into a mammalian cell might provide a useful analogy to the transport of the small subunit of Fraction I protein into the chloroplast. Diphtheria toxin consists of two polypeptide chains, A and B, joined by disulphide bonds; the A polypeptide has a molecular weight of 24,000 and is enzymically active, the B polypeptide has a molecular weight of 39,000 and serves to bind the whole toxin to the cell surface (Collier, 1975). The transport of the A polypeptide across the cell membrane is achieved via the binding of the B polypeptide to a receptor in the cell membrane; the receptor-toxin complex then undergoes a rearrangement, which moves the A polypeptide across the membrane; the disulphide bonds holding the A and B polypeptides together are then cleaved (Boquet and Pappenheimer, 1976). The toxin is initially synthesised as one polypeptide of molecular weight 63,000, and it is not clear when the cleavage into the A and B polypeptides takes place; most toxin preparations contain both precursor and mature forms (Collier, 1975). The A polypeptide is at the NH_2-terminus, the B polypeptide at the C-terminus.

Using radioactive Triton X-100, Boquet et al (1976) have been able to study the distribution of hydrophobic and hydrophilic regions in diphtheria toxin, and in the isolated A and B polypeptides. Their conclusions are as follows:-

1) there are no hydrophobic regions present on the whole toxin molecule in solution or on the A polypeptide; the B polypeptide has a hydrophobic region at the NH_2-terminus of the chain which is the region bound to the A polypeptide;

2) the hydrophilic part of the B polypeptide interacts with the cell-surface receptor;

3) this interaction changes the conformation of the toxin such that the hydrophobic end of the B polypeptide is brought into contact with the membrane lipids;

4) because the A polypeptide is attached to this hydrophobic region it passes through the membrane;

5) once through the membrane, the A and B polypeptides separate.

This suggestion may also serve as a model for the transport of glycopeptide hormones into cells. These hormones also consist of two chains; the α-chain, molecular weight about 10,000, has the hormone activity, while the β-chain, molecular weight about 15,000, provides the tissue specificity. It has been reported that the β-chain binds to the cell surface, and that the α-chain enters the cell (Tager and Steiner, 1974).

Dobberstein et al (1977) suggest that the fragment, F, removed in the processing of P20 to the small subunit, functions like the B-polypeptide of diphtheria toxin and binds to the chloroplast envelope, whilst the small subunit moves through the envelope and into the chloroplast. The analogy between the two systems is worth considering because the transport of diphtheria toxin is the only other suggested model for the post-translational transport of proteins. However, the analogy might not be very strong. The B and β-chains described above are much bigger than fragment F. This size difference may well be critical. According to the model of Boquet et al (1976) fragment must have both a hydrophilic region to interact with a hypothetical receptor on the surface of the chloroplast, and a hydrophobic region to enable the small subunit to pass through the membrane. Since this fragment can only be about 50 amino acids long, or less by the estimates of Dobberstein et al

(1977), and would have to facilitate the transport of a protein more than twice its size, it seems unlikely that the transport occurs in this fashion. Thus the analogy with diphtheria toxin may only be superficial.

The large change in molecular weight and pI which occurs in the processing of P20 to the small subunit must cause a large conformational change, and this change is crucial to the model for transport proposed in this thesis. In this context it is interesting to note an incidental observation of Bingham and Campbell (1972), made during a study of the synthesis of malate dehydrogenase in rat liver. The enzyme was labelled in an *in vitro* protein synthesis system, and was identified by its co-purification with purified enzyme. The main finding of this work was that this enzyme was synthesised on the rough endoplasmic reticulum; however, it was also noted that the *in vitro*-synthesised enzyme was more acidic than the enzyme isolated from mitochondria. P20 has a lower pI than the small subunit and so this difference between the two forms of malate dehydrogenase may be significant. This observation was not followed up and so remains no more than suggestive.

Just how important the change in pI might be to the transport mechanism of chloroplasts can only be judged when more data becomes available. Dobberstein *et al* (1977) did not analyse their small subunit precursor by isoelectric focussing. This point is important, because the pI of small subunits from different sources is known to differ, sometimes quite markedly (Wildman *et al*, 1975). It would be interesting to know whether the pI of P20 follows these variations,i.e. whether or not there is always a similar pI change on processing.

P20 appears as a single spot on two-dimensional gels yet

gives rise to two isoelectric forms of the small subunit when processed (Fig.33). In tobacco the two isoelectric forms of the small subunit are thought to be derived from different genes (Wildman et al, 1975; Gray et al, 1978). For pea, this would not appear to be the case. For the two variants of the small subunit to be the products of different genes, it would be necessary that the extra amino acids present in their respective P20s were such that the differences in pI were cancelled out. This seems unlikely.

It is postulated that the two forms of the small subunit might arise from some property of the processing reaction. The A polypeptide of diphtheria toxin has three isoelectric variants which cover a range of 0.3 pH units (Collier, 1975). Each of the variants differs by a lysine residue, and these differnces are a result of variation in the site of cleavage from the B polypeptide. Thus it is possible that the two forms of the small subunit might arise in a similar way.

The most important finding reported in this thesis is that the small subunit of Fraction I protein can enter isolated chloroplasts. This system can be used to elucidate how this transport occurs, and so provides a model which may be generally applicable to the transport of proteins into organelles. Furthermore, the assembly of the large and small subunits of Fraction I protein into the native enzyme can also now be studied. Although the attempts to purify the P20 mRNA were unsuccessful, the assay systems developed here for studying the changes in the mRNA population during greening can be used for other studies. Thus the systems developed during this work, and the results obtained with them, point the way

to obtaining a greater understanding of chloroplast development, and of the mechanisms involved in maintaining the rapport between chloroplast and cytoplasm.

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