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PROTEIN SYNTHESIS IN ETIOPLASTS
AND
DEVELOPING CHLOROPLASTS

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

BY

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MAY 1976

TO HOBBLE

SUMMARY

1. The aim of this work was to study the function of plastid ribosomes in etioplasts and developing chloroplasts. The conclusions reached are interpreted in the context of plastid autonomy and biogenesis.
2. I have shown that isolated etioplasts and developing chloroplasts use added ATP as an energy source for amino acid incorporation into protein. Developing chloroplasts isolated from leaves after a minimum of 3 hours greening in continuous white light can also use light as an energy source for protein synthesis.
3. The relationship between ATP and light as an energy source for in vitro plastid protein synthesis has been studied in plastids isolated during greening.
4. The characteristics of amino acid incorporation in rapidly isolated etioplast preparations exclude the possibility that incorporation occurs on cytoplasmic ribosomes, in whole cells, nuclei or bacteria and are consistent with the known characteristics of in vitro plastid protein synthesis.
5. It has been shown that less than 3% of the amino acid incorporation into protein in etioplast suspensions is associated with mitochondria which contaminate the preparations.
6. The conditions for optimum incorporation of amino acids into protein were established in order to facilitate the identification of the polypeptides synthesized.
7. The products of in vitro protein synthesis were analysed by electrophoretic separation on SDS urea polyacrylamide gels.

8. Using ATP as the energy source and either L-(³⁵S) methionine, L-(³H) leucine or L-(³H) phenylalanine, as the protein precursor it has been shown that etioplasts synthesize seven major polypeptides.
9. These polypeptides electrophorese coincidentally with the products of protein synthesis in isolated chloroplasts.
10. As in chloroplasts only one of these polypeptides is present in a 150,000 g etioplast supernatant fraction. This product has been identified as the large subunit of Fraction I protein. Identity was established by comparing the tryptic and chymotryptic peptide map of the in vitro etioplast product labelled with L-(³⁵S) methionine with the tryptic and chymotryptic peptide map of authentic large subunit of Fraction I protein labelled with L-(³⁵S) methionine in vivo.
11. The same gel pattern of seven polypeptides is seen when plastids isolated from greening leaves are incubated with either added ATP or light as the energy source. The products of in vitro protein synthesis in developing chloroplasts also co-electrophorese with those synthesized in isolated chloroplasts.
12. The rates of synthesis of particular polypeptides are different in plastids isolated at different stages of the etioplast to chloroplast transition.
13. The results support the idea that plastid ribosomes synthesize only a small number of proteins and that the number and molecular weight of these proteins does not alter during the formation of chloroplasts from etioplasts.
14. The results are discussed in the context of plastid autonomy and biogenesis. The use of the plastid as a developmental system for studying the controls exerted upon the synthesis of particular polypeptides during differentiation is considered. Possible areas of research which might be undertaken as a result of the work described are outlined.

ACKNOWLEDGEMENT

I would like to thank my supervisor, Dr. John Ellis.

ABBREVIATIONS

\AA	angstrom (10^{-10} m)
A_{260}	absorbance at 260 nm
A_{280}	absorbance at 280 nm
A-T	adenine - thymine base pair
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BSA	bovine serum albumin
Ci	Curie (3.7×10^{10} disintegrations per second)
c. p. m.	counts per minute
CTP	cytidine triphosphate
DDSA	dodecanyl succinic anhydride
DEAE	diethylaminoethyl
DMP 30	tridimethylamine methylphenol
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
E	molar extinction coefficient (litre \cdot mol ⁻¹ \cdot cm ⁻¹)
E_x	Extinction (at wavelength x)
EDTA	ethylene diamine tetraacetic acid
g_{max} (av)	maximum (average) gravitational field
G-C	guanosine - cytosine base pair
GTP	guanosine triphosphate
HEPES	N 2 - hydroxyethylpiperazine - N - 2 - ethane sulphonic acid.
INT	2' - (p-iodophenyl) - 3 - (p-nitrophenyl) - 5-phenyl tetrazolium.
mA	milliampere
mRNA	messenger RNA
NMA	methyl endomethylene phthalic anhydride
pH	hydrogen ion concentration
PMSF	phenyl methyl sulphonyl fluoride
poly A	polyadenylic acid
poly U	polyuridylic acid
POPOP	1, 4 - bis - (5 - phenyloxazol - 2 - yl) benzene
PPO	2, 5 - phenyl oxazole.

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mRNA	messenger RNA
NMA	methyl endomethylene phthalic anhydride
pH	hydrogen ion concentration
PMSF	phenyl methyl sulphonyl fluoride
poly A	polyadenylic acid
poly U	polyuridylic acid
POPOP	1,4-bis-(5-phenyloxazol-2-yl) benzene
PPG	2,5 - phenyl oxazole.

$r_{\text{max}} (\text{av})$	maximum (average) radius of rotation of a column of liquid in a rotor tube.
RNA	ribonucleic acid
r. p. m.	revolutions per minute
RuBP	ribulose 1, 5-bisphosphate
RuBPCase	ribulose 1, 5-bisphosphate carboxylase (3-phospho-D-glycerate carboxylase E. C. 4.1.1.39)
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEMED	N, N, N, N'-tetramethylene diamine
TRICINE	N-tris (hydroxyethyl) methyl glycine
Triton X 100	octyl phenoxy polyethoxy ethanol
TRIZMA	2-amino-2-hydroxymethyl propanoic-1:3 diol
tRNA	transfer RNA
tRNA _{met}	methionyl charging transfer RNA
dTTP	deoxythymidine triphosphate
UTP	uridine triphosphate

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SECTION I - LITERATURE REVIEW

1. INTRODUCTION.

The hallmark of the eukaryotic cell is the compartmentalization of functions in membrane-limited organelles. In plant cells there is a group of organelles collectively termed the plastids. The function of the most intensely studied plastid form, the chloroplast, is to convert solar energy into chemical energy through the process of photosynthesis. This process is the primary energy transduction upon which all life ultimately depends and has therefore justifiably been the major aspect of plastid research for over 50 years. However, in the last 15 years a second area of investigation relating to the plastids has developed.

It has been known since the turn of the 20th century that the genetic determinants for some plastid characters are not transmitted according to the normal Mendelian rules of independent allelic segregation (Bauer, 1907), but are transmitted instead by an extranuclear, commonly uniparental, mode of inheritance. The significance of this observation was not however grasped until the realisation during the 1950's that DNA, and not protein, was the molecule through which genetic information is stored and transmitted (see Olby, 1974). Extranuclear inheritance must surely therefore indicate extranuclear DNA. Accordingly throughout the 1960's extensive study of the chloroplast revealed that it contains all the components theoretically necessary for autonomy i. e. DNA, DNA polymerase, RNA polymerase and a protein synthesizing system (Boulter *et-al*, 1972). These findings immediately provoked the idea that the chloroplast might be an autonomous organelle. To quote Kirk and Tilney-Bassett (1967) "just how much of the chloroplast protein is synthesized within the chloroplast is not known for certain, but the work with living cells suggests that most of it is, and indeed on general grounds, it seems likely that all of it is". The concept of plastid autonomy was however short-lived. Over the last five years it has become increasingly apparent that the chloroplast is not autonomous in the sense that it exerts complete control over its own replication and the biosynthesis of all its components. In addition to/

to numerous chloroplast characters which are known to be governed by nuclear genes (Kirk and Tilney-Bassett, 1967) it has now been established that a number of plastid components are encoded in nuclear DNA (Kawashima and Wildman, 1972; Kung *et al.*, 1972) and at least in one case (the small subunit of Fraction I protein) synthesized on cytoplasmic ribosomes (Criddle *et al.*, 1970; Gray and Kekwick, 1974b). At the same time however it has also been shown that the gene for the most abundant chloroplast polypeptide, the large subunit of Fraction I protein, is located in plastid DNA (Chan and Wildman, 1972) and expressed through translation on 70S chloroplast ribosomes (Blair and Ellis, 1973; Criddle *et al.*, 1970).

The general picture that emerges therefore is that the biosynthesis and replication of the plastid requires an integration between events occurring in the nucleus, cytoplasm and organelle. A considerable step towards understanding the nature of these integrative processes in the cell would be to identify the genes encoded in plastid DNA and the proteins synthesized on plastid ribosomes. The work presented in this thesis is concerned with the translational aspect of this problem.

Recently several reviews have appeared on the subjects of plastid structure, function, inheritance, autonomy and biogenesis (Kirk, 1971; Boardman *et al.*, 1971; Rosinski and Rosen, 1972; Pollock and Lee, 1972; Börner, 1973; Gillham *et al.*, 1974; Gillham, 1974; Tzagaloff, 1975; Thornber, 1975; Anderson, 1975b; Ellis, 1976a). The literature review presented here will therefore concentrate only on the more recent and interesting advances in particular areas. The emphasis will be to evaluate the extent to which the structure and function of the plastids can be related to the activity of their own nucleic acid and protein synthesizing systems. At the end of the introduction the limitations in our understanding of plastid autonomy are briefly discussed, and the problem investigated in this study and the approach adopted to elucidate this problem are stated.

2. PLASTID STRUCTURE AND FUNCTION

Kirk and Tilney-Bassett (1967) categorise seven, or possibly eight, different plastid forms whilst at the same time recognising that these forms are closely inter-related. To me it appears that a more consistent approach, and one that will be emphasized throughout this review, is that of Sironval (1975). The plastid is a single object which exists in any specific steady-state as a result of maintaining the plant in a particular set of environmental conditions. The normal steady-state of the plastid, apparent in most wild-type plants grown in their normal ecological environment, is the granal chloroplast. If the plant is grown in continued darkness then the plastids assume an abnormal steady-state, the etioplast. Etioplasts are essentially unstable and if exposed to visible light will develop through a continuum of transient forms into the granal chloroplast.

In the usual ecological situation chloroplasts do not normally develop from fully differentiated etioplasts. In the growing leaf the chloroplasts develop through a series of rather ill-defined transient forms, the earlier of which are termed proplastids. This terminology refers to higher plants. Dark-grown cells of *Euglena gracilis* contain steady-state plastid forms which are also referred to as proplastids. This section will consider some aspects of the structural and functional relationships between chloroplasts, etioplasts and proplastids.

A. STEADY-STATE FORMS

i. The granal chloroplast

In higher plant cells chloroplasts are generally lens-shaped and approximately 5 μm in diameter. The chloroplast is delineated from the cytoplasm by a double membrane, the so called chloroplast envelope. Purified preparations of chloroplast envelopes have been obtained from broad bean, spinach, pea and oats (Mackender and Leech, 1970; Poincelot, 1973; Douce *et al*, 1973; Joy and Ellis, 1975; Wellburn and Cobb, 1974; Poincelot and Day, 1974). The enzyme Mg^{2+} dependent ATPase, which is restricted to the plastid envelope (Sabris *et al*, 1970), provides/

provides a suitable biochemical marker throughout the purification procedure.

Chemical analysis of the envelope preparation from Vicia faba reveals that the lipid and fatty acid composition of the envelope membranes are in general qualitatively, but not quantitatively, similar to lamellar membranes isolated from the same plastids (Mackender and Leech, 1974). There may however be a number of minor lipid components which are unique to the envelope membrane. For example, Poincelot (1973) has shown that cerebroside, steryl glycoside and certain steryl esters are present in the envelope but not lamellar membranes of spinach chloroplasts. Recent studies suggest that the chloroplast envelope is the site at which some of its own component galactolipids are synthesized (Douce, 1974).

The proteins of oat, pea and spinach plastid envelopes have been analysed by polyacrylamide gel electrophoresis (Joy and Ellis, 1975; Mendiola - Morgenthaler and Morgenthaler, 1974; Pineau and Douce, 1974a; Cobb and Wellburn, 1974; Sprey and Laetsch, 1975). The fractionation pattern of envelope proteins is clearly distinct from that of the thylakoid proteins and shows that the envelope membranes contain up to 25 major polypeptides. Joy and Ellis (1975) conclude that at least two of these polypeptides are synthesized on chloroplast ribosomes.

One of the primary functions of the chloroplast envelope must be to control the flux of metabolites, and perhaps also macromolecules such as proteins (see Pineau and Douce, 1974b), between the chloroplast and the cytoplasm. Metabolite flux to and from the chloroplast has recently been excellently reviewed by Heber (1974). In broad terms it appears that whilst the outer membrane of the chloroplast envelope is unspecifically permeable to metabolites (Heldt and Sauer, 1971), the inner membrane is relatively impermeable (Stokes and Walker, 1971; Gimmler et al, 1974) but contains specific translocators (Walker and Crofts, 1970; Poincelot, 1975).

Chloroplasts which retain their envelope membranes during isolation (as do a high proportion of those isolated rapidly in isotonic medium) are classified as intact (Hall, 1972), and appear refractile if viewed /

viewed by phase contrast microscopy (Kahn and von Wettstein, 1961).

Within the delineation of the envelope membrane all chloroplasts conform to a basic plan. An outer compartment between the membranes of the envelope contains a finely granular continuum, the matrix or stroma, within which is supported a more or less elaborate system of interconnected lamellae. These lamellae themselves constitute an inner membranous compartment.

The stroma contains DNA (Ris and Plaut, 1962), ribosomes, tRNA and amino acid activating enzymes (Franki *et al.*, 1965). Also amongst the 50 or so soluble enzymes found in the stroma (Smillic and Scott, 1970) are those of the Calvin cycle (Trebst *et al.*, 1958). One of these Calvin cycle enzymes, ribulose 1, 5 bisphosphate carboxylase (RuBPCase) (3-phospho-D-glycerate carboxylase (dimerizing) E. C. 4.1.1.39), also referred to as Fraction I protein, can account for up to 50% of the soluble leaf protein (Kawashima and Wildman, 1970). Fraction I protein is the most intensively studied of all plant proteins (see reviews by Kawashima and Wildman, 1970; Ellis, 1973; McFadden, 1973).

Fraction I protein is found in all chemosynthetic and photosynthetic autotrophs (McFadden, 1973) and can be readily isolated and purified to homogeneity, commonly as judged by polyacrylamide gel electrophoresis. In tobacco species the protein can be easily crystallized in large yields from crude leaf extracts (Kawashima and Wildman, 1971a). The term Fraction I protein is used to describe a group of proteins which are isolated from autotrophic organisms their diagnostic feature being the enzyme activity of ribulose 1, 5 bisphosphate carboxylase. The molecular structure of Fraction I proteins varies between species, and on the basis of the molecular weight of the native protein three categories of protein can be recognised (McFadden, 1973).

Table A shows the distribution of the group types of Fraction I protein and also some of the physical characteristics of each group. In Table A the number of subunits in the Fraction I proteins of Groups II and III has been assumed from the molecular weight data, although in/

Table A. The subunit compositions of Fraction I proteins.

1. McFadden et al, 1975.
2. Akazawa et al, 1972.
3. Kawashima and Wildman, 1971b
4. Keuhn and McFadden, 1969.
5. Lord and Brown, 1975.
6. Moon and Thompson, 1969.
7. Sugiyama and Akazawa, 1970
8. Rutner and Lane, 1967.
9. Sugiyama et al, 1971
10. Gray and Kekwick, 1974a.
11. Iwanij et al, 1974.
12. Moon and Thompson, 1971
13. Tabita et al, 1974.
14. Anderson et al, 1968.
15. Akazawa et al, 1970
16. McFadden and Denund, 1972.
17. Anderson and Fuller, 1969.
18. Tabita and McFadden, 1974.
19. Baker et al, 1975.

SUBUNIT COMPOSITION

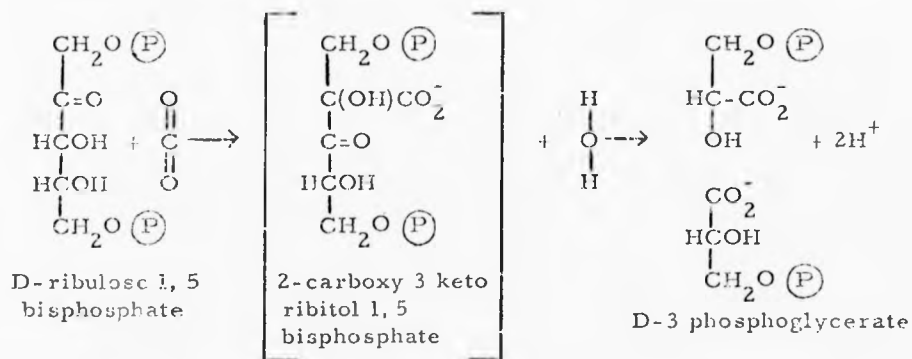
<u>Group</u>	<u>Mol. Wt. of Native Subunit (x10⁵)</u>	<u>Types of subunit</u>	<u>subunit mol. wt.</u>		<u>No. of subunits in native protein</u>		<u>Distribution</u>
			<u>large (x10⁴)</u>	<u>small</u>	<u>large</u>	<u>small</u>	
I	5.1 - 5.5	2 (6, 7, 8)	5.4 - 5.8 (9, 10)	1.2 - 1.8 (11, 12)	8 (19)	8 (?)	Higher plants green algae hydrogen bacteria Thiorhodaceae (1, 2, 3, 4, 5)
II	2.4 - 3.6	1	5.4 (13)			5 or 6 (?)	Thiobacillus denitrificans Chlorobium sulphatophilum Rhodospseudomonas palustris R. sphaeroides (13, 14, 15, 16)
III	1.2	1	5.6 (18)			2	Rhodospirillum rubrum (17)

in fact nothing is yet known of the quaternary structure of these enzymes. Group I Fraction I proteins contain eight subunits of molecular weight $5.4 - 5.8 \times 10^4$. This conclusion is strongly supported by electron microscope, x-ray diffraction and optical diffraction studies on crystalline tobacco Fraction I protein (Baker et al, 1975). This larger subunit has been shown to be the catalytic subunit of the enzyme (Nishimura and Akazawa, 1973, 1974a, 1974b). The number of small subunits (mol. wt. $1.2 - 1.8 \times 10^4$) in Group I proteins is also thought to be eight on the grounds that this is the simplest interpretation of the crystallographic data; also oligomeric proteins are almost always found to have a 1:1 binding ratio between non-identical subunits (Matthews and Bernhard, 1973). The majority of evidence suggests that the role of the small subunit is to modify or regulate the enzymic activity of the protein (Takabe and Akazawa, 1973; Kung and Marsho, 1976; Nelson and Surzycki, 1976a, 1976b). The evolutionary implications of varying subunit compositions in Fraction I proteins have been reviewed by McFadden (1973). A most interesting development in the study of Fraction I protein has been the recent finding by Kung et al (1974) that both the large and small subunits of crystalline tobacco Fraction I protein can be resolved into a number of discreet types by isoelectric focussing. The isoelectric points of these types differ interspecifically as well as intraspecifically. The nature of the charge differences within a population of subunits is unknown. There are no detectable differences in the tryptic peptides of polypeptides which exhibit charge differences (Wildman et al, 1974). These charge differences can however be used as phenotypic markers in the production of reciprocal interspecific hybrids (it should be borne in mind that these charge differences need not necessarily be a consequence of alterations in the gene(s) encoding the Fraction I protein subunits). Gray et al (1974) and Wildman et al (1974) have profitably employed this approach to determine evolutionary relationships within the genus Nicotiana. The same approach has also been used by Sakano et al (1974) to provide confirmatory evidence that the gene (s) encoding the large subunits/

subunits of Fraction I protein are located in chloroplast DNA (section B3Aii).

Comparisons of the Fraction I protein subunits have also been made at the intergeneric level. Studies on antigenic determinants (McFadden, 1973; Gray and Kekwick, 1974a), amino acid compositions (Iwanij *et al.*, 1974; Rabinowitz *et al.*, 1975) and tryptic peptides (Kawashima and Wildman, 1971a; Kawashima *et al.*, 1971; Kung *et al.*, 1974) all lead to the conclusion that the primary structure of the large subunit of Fraction I protein has been strongly conserved throughout evolution, whereas that of the small subunit has undergone considerable change.

The diagnostic enzymic activity of Fraction I protein is the catalysis of the carboxylation of ribulose 1, 5 bisphosphate to yield a six carbon intermediate which is rapidly hydrolysed to two molecules of phosphoglycerate (Sjodin and Vestermark, 1973).

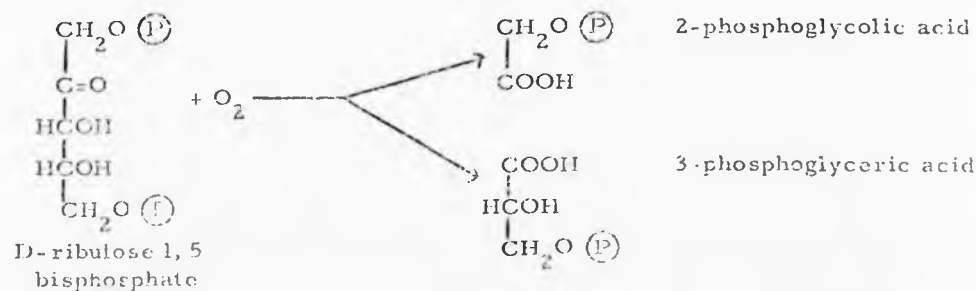


Carbon dioxide is the active species in the carboxylation reaction (Cooper *et al.*, 1969). There has been prolonged concern and discussion over an apparent paradox between the observed rates of CO_2 fixation *in vivo* and the kinetics of RuBPCase activity *in vitro*. It is increasingly clear that in fact no paradox exists. Lilley and Walker (1975) have shown that a crude preparation of RuBPCase isolated from spinach chloroplasts exhibits a V_{max} of between 500 and 900 $\mu\text{moles of CO}_2$ fixed/mg of chlorophyll/hour. A maximum reaction velocity of 1000 $\mu\text{moles of CO}_2$ fixed/mg of chlorophyll/hour is sufficient to account for the rates of CO_2 fixation observed/

observed in vivo or in isolated intact chloroplasts. These workers also demonstrate a $K_m(\text{CO}_2)$ for the in vitro CO_2 fixation reaction of $45 \mu\text{M}$, considerably higher than the value of $9 \mu\text{M}$ which results from equilibrating a solution with atmospheric CO_2 at a concentration of 0.03%. The enzyme must therefore operate in vivo at sub-optimal CO_2 concentrations.

A quite separate issue is the $K_m(\text{CO}_2)$ for the CO_2 fixation process in vivo, which has been determined as $9 \mu\text{M}$ (Goldsworthy, 1968; Buchanan and Schurman, 1973). Lilley and Walker (1975) maintain that this value is spuriously low and results from a limitation imposed by electron transport processes at high CO_2 concentrations. They maintain that the true $K_m(\text{CO}_2)$ for the primary fixation process is $45 \mu\text{M}$, the same CO_2 concentration that produces half maximal velocity for their RuBPCase activity in vitro. It is also increasingly clear that RuBPCase is under strong allosteric control by a number of metabolites (Chua and Bassham, 1972; 1973, 1974, 1975; Ryan et al., 1975; Laing et al., 1975). Allosteric regulation is most probably the cause of the wide variations in the measured kinetic parameters of the in vitro RuBPCase activities obtained using enzymes prepared in different ways and incubated under different conditions (Paulsen and Lane, 1966; Bahr and Jensen, 1974a; Laing et al., 1975). A detailed understanding of the regulation of RuBPCase activity in vivo seems far distant.

The full importance of Fraction I protein in the cell was not appreciated until the discovery by Bowes et al. (1971) that the enzyme also catalysed the oxygenation of D-ribulose 1,5 bisphosphate to yield one molecule of 3-phospho D glycerate and one molecule of phosphoglycolate.



The oxygenase activity has now been demonstrated in Fraction I protein isolated from soybean (Bowes et al, 1971), spinach (Badger and Andrews, 1974; Andrews et al, 1973; Nishimura and Akazawa, 1974a; Bahr and Jensen, 1974), tobacco (Chollet et al, 1975), the green alga Chlamydomonas reinhardtii (Berry and Bowes, 1973) and the obligate anaerobes Chromatium (Takabe and Akazawa, 1973a) and Rhodospirillum rubrum (McFadden, 1974). It is believed that both carboxylase and oxygenase activities are catalysed at the same active site on the protein for three reasons. Firstly antibodies raised specifically to the large subunit of spinach Fraction I protein will inhibit both activities (Nishimura and Akazawa, 1974b). Secondly there are close similarities in the kinetic parameters of the two activities (Ryan and Tolbert, 1975a), and thirdly CO_2 and O_2 are equally potent competitive inhibitors of the oxygenase and carboxylase activities respectively (Badger and Andrews, 1974).

The possible significance of the oxygenase reaction in cellular metabolism is that the phosphoglycolate produced may be dephosphorylated to yield glycolic acid (Bowes et al, 1971; Lorimer et al, 1973), and hence become available as the primary substrate of photorespiration, a process that can reconvert up to 50% of the carbon fixed in photosynthesis back into CO_2 (Zelitch, 1975). Thus the rate of synthesis of glycolic acid via the oxygenase reaction may be an important factor controlling photorespiration, although this idea is strongly contended (Zelitch, 1975; Bahr and Jensen, 1974; Kung and Marsho, 1976).

The inner membranes of the chloroplasts of higher plants and green algae contain the photosynthetic apparatus for the conversion of solar energy into chemical energy (Boardman, 1968). Electron microscopy has shown that the photosynthetic lamellae of the chloroplast consists of a system of flattened discs (thylakoids) superimposed to form an array, the granum. The number of thylakoids per granum appears to vary widely throughout the plant kingdom, from about 3 in Euglena gracilis to anything up to 100 in vascular plants (Kirk and Tilney-Bassett, 1967). Thylakoids also extend between and interconnect/

interconnect separate disc arrays.

The ultrastructure of the lamellar membrane has been investigated by two basic approaches. In the first approach physical methods such as conventional electron microscopy, freeze fracturing and x-ray diffraction are used. On the basis of these studies several models of membrane structure have been proposed. Essentially all models lie between two extremes. The earlier model, which was explicitly for chloroplast lamellae, envisages the membrane as a static bimolecular lipid leaflet into which proteins and pigments are rigidly embedded (Branton, 1969; Park and Pfeifhofer, 1969). This model has been critically reviewed by Kirk (1971a, 1972).

An alternative and more flexible model views the membrane as a lipid bilayer leaflet which provides a fluid matrix in which proteins or lipo-protein complexes either float (extrinsic or peripheral proteins) or are submerged (intrinsic or integral proteins) (Singer, 1974; Singer and Nicholson, 1972). The lipids in the matrix are also of two types; fluid, which make up the lipid domain and fixed boundary, which consist of macro molecular layers attached to the hydrophobic shells of penetrating intrinsic proteins (Jost *et al*, 1975; Trauble and Overath, 1973). This mosaic model of membrane structure has emerged from investigations not only on chloroplast membranes, but also on mitochondria, plasma membranes and animal cells, (Singer, 1974). The most recent interpretation of this model with respect to chloroplast lamellae is given by Anderson (1975a, 1975b). She envisages the bulk of the amphipathic chlorophyll molecules to be boundary lipids which lie at the surface of two major intrinsic protein complexes; giving rise to the Photosystems I and II chlorophyll - protein complexes when the membranes are solubilized by detergent (Thorner, 1975). This fluid model of the membrane would seem to accommodate more easily many of the conceptual difficulties associated with, for example, the step-wise insertion of molecules into the membrane either during development (Heise and Jacobi, 1973; Bogorad, 1975), or as a result of differential turnover rates for membrane components (Schimke and Doyle, 1970; Omura *et al*, 1967).

The second method of investigating the membrane ultrastructure has been to release from the membrane, by more or less drastic procedures, associations of components which together form complexes active in the partial reactions of photosynthesis, for example, photosynthetic phosphorylation, electron transport or light harvesting. By varying the severity with which the membrane is disrupted the relationships between the components of a complex can be investigated, and in time it is hoped that an understanding of the structure and function of the membrane will be built up. Mild procedures, such as gentle washing of spinach chloroplast lamellae with a narrow range of concentrations of dilute EDTA, release certain proteins, for example the latent Ca^{2+} activated ATPase (Jagendorf and Smith, 1962; Avron, 1963). This protein, also termed the photosynthetic coupling factor (CF_1), has been purified (Farron, 1970) and its molecular weight and subunit composition determined (Nelson *et al*, 1973; McEvoy and Lynn, 1973). This protein is clearly therefore a peripheral or extrinsic component of the membrane. In contrast, only prolonged treatment of chloroplast lamellae with the detergent digitonin is sufficient to release proteins which are intrinsic, for example cytochrome f (Nelson and Racker, 1972). Inbetween these two extremes partial dissolution of the lamellar membrane has been achieved by a variety of procedures including mechanical disruption, treatment with the ionic detergents sodium dodecyl sulphate (SDS) (Ogawa *et al*, 1966) and sodium dodecyl benzene sulphate (SDBS) (Thorner *et al*, 1967a; Thorner *et al*, 1966) or treatment with the non-ionic detergent Triton X 100 (Shiozawa *et al*, 1974).

Membrane solubilization studies indicate that 70% of the intrinsic protein mass of native chloroplasts is made up of the two complexes, chlorophyll-protein complex I (CPI) associated with Photosystem I and comprising some 20% of the protein mass and chlorophyll-protein complex II (CPII) associated with Photosystem II and comprising some 50% of the protein mass. The properties of these chlorophyll-protein complexes have been excellently reviewed by Thorner (1975) and a number of them are collated in Table B. Comparison of the chlorophyll a:b ratio of CPI with that of the Photosystem I particles isolated/

Table B. The chlorophyll-protein complexes.

1. Thornber, 1975.
2. Thornber et al, 1967b.
3. Ogawa et al, 1966
4. Thornber et al, 1967a
5. Dietrich and Thornber, 1971
6. Eaglesham and Ellis, 1974
7. Kung and Thornber, 1971
8. Thornber, 1969.
9. Shiozawa et al , 1974.
10. Lengis and Nelson, 1975.
11. Thornber and Highkin, 1974.
12. Alberte et al, 1974
13. Alberte et al, 1972
14. Argyroudi-Akoyunoglou and Akoyunoglou, 1973.
15. Thornber and Olsen, 1971.
16. Hiller et al, 1974.

<u>PROPERTY</u>	<u>CPI</u>	<u>CPII</u>
Synonyms	P700 chlorophylla protein (1)	Light-harvesting chlorophyll a:b protein (11)
Sedimentation coefficient	9S (2)	—
Chlorophyll a/b ratio	>7 (3) chl b not detected (4)	equimolar (12, 13, 14, 15)
Chlorophyll/P700 ratio	50:1 (5)	—
mol. wt.	1.1-1.5 x 10 ⁵ (6, 7)	2.7-3.5 x 10 ⁴ (6, 7, 16)
chlorophyll/protein ratio	14 moles/complex (8, 9)	6 moles/complex (1)
Carotenoid/chlorophyll ratio	—	3-7/1 (11)
activity	PSI photooxidation and reduction (9, 10)	Light-harvesting (1, 11)

isolated by Boardman and Anderson (1964), and the enrichment of SDS-treated digitonin Photosystem I particles with CPI (Thorner *et al.*, 1967a; Sironval *et al.*, 1967) leaves little doubt that CPI represents a smaller subunit of Photosystem I. Simple calculations from the data in Table B shows that not every CPI complex unit contains a molecule of P700, the reactive pigment species of Photosystem I. It has therefore been postulated, in analogy to the bacterial reaction centre protein (Clayton, 1973), that each P700 reaction centre protein is attached to only one of every three identical chlorophyll-protein complexes (Thorner, 1975).

As judged by NADP reduction, the most native CPI complex preparation yet isolated is obtained by Triton X 100 detergent treatment of digitonin-prepared Photosystem I complexes (Bengis and Nelson, 1975). This CPI complex is composed of five classes of polypeptides with apparent molecular weights of 70, 25, 20, 18 and 16×10^3 on SDS polyacrylamide gels. Both the P700 signal (primary oxidation) and NADP photoreduction (primary reduction) are associated with the 70×10^3 molecular weight polypeptide, although the presence of the minor molecular weight species seems necessary for photoreduction.

The second major chlorophyll-protein complex of the lamellae, CPII, has traditionally been obtained by polyacrylamide gel electrophoresis of anionic detergent extracts (Alberte *et al.*, 1974, 1972; Thorner and Highkin, 1974); although a new isolation procedure involving hydroxyapatite chromatography of SDS extracts (Kung and Thorner, 1971; Thorner and Olsen, 1971) yields a more homogeneous preparation.

The properties of a barley mutant which lacks chlorophyll b (Highkin, 1950) but still retains Photosystem II activities (Thorner and Highkin, 1974) suggests that unlike CPI, the CPII complex does not perform any function required for the operation of the Hill-Bendall % scheme (Hill and Bendall, 1960). The CPII complex is therefore thought to act simply as a light-harvesting component in the photosynthetic membrane. Consistent with this idea are the studies of/

of Chua and Bennoun (1975) on temperature-sensitive mutants of Chlamydomonas reinhardtii. These studies strongly implicate a polypeptide of molecular weight 47000, considerably higher than the CPII complex protein, as being involved in the activity of the Photosystem II reaction centre.

The organization of the 30% of chlorophylls and carotenoids in the plant that are not located in the two major chlorophyll-protein complexes (they are associated with a free-pigment zone upon the completion of electrophoresis) is obscure.

Also associated with chloroplast lamellae are DNA (Woodcock and Fernandez-Moran, 1968; Herrman *et al*, 1975), ribosomes (Chan and Wildman, 1970; Chua *et al*, 1973; Margulies and Michaels, 1974), DNA polymerase (Tewari and Wildman, 1967) and RNA polymerase activity (Spencer and Whitfield, 1967; Tewari and Wildman, 1969). These components will be discussed in more detail in later sections.

It is interesting to note that during the period in which it was considered plausible that chloroplasts were autonomous several attempts were made to maintain viable plastids in isolation from the rest of the cell (Ridley and Leech, 1970; Giles and Serafis, 1972; Wellburn and Wellburn, 1973; Rebeiz *et al*, 1973). These studies have principally been restricted to subjective interpretations of electron micrographic data. They suggest no more than that the dissolution of the structural and molecular integrity of the plastid can be delayed by incubation in the presence of specific cofactors. There is no evidence of plastid replication *in vitro*, nor is there any evidence of continued *de novo* nucleic acid or protein synthesis *in vitro*.

ii. Etioplasts and proplastids.

Etioplasts lack both chlorophyll and a thylakoid membrane system but they do have a definite and complicated structure (Kirk and Tilney-Bassett, 1967). However in comparison to the chloroplast our knowledge of this structure is rudimentary.

Etioplasts are usually irregularly ellipsoid and depending upon the age and species of tissue have a diameter of about 1 to 3 μm (Mego and Jagendorf, 1961; Bennet and Radcliffe, 1975). The etioplast is delineated/

delineated from the rest of the cell by a double membrane (Rebeiz *et al.*, 1973). The etioplast envelope membrane has been isolated from dark-grown *Avena* laminae and analysed by SDS polyacrylamide gel electrophoresis (Cobb and Wellburn, 1974). This membrane preparation contains 16 major polypeptides ranging in molecular weight from 25000 to 117000. There have been no reported analyses specifically on the non-protein components of the etioplast envelope although Tevini (1972) has shown that isolated barley etioplasts contain galactolipids, the plant sulpholipids and the phospholipids, phosphatidyl choline, phosphatidyl glycerol and phosphatidyl inositol. It would seem reasonable to conclude that at least some of these components are distributed within the envelope and inner lamellar membranes.

The permeability properties of the etioplast envelope membrane are also almost totally unknown. Smith and his colleagues have presented spectrophotometric evidence to suggest that the phytochrome associated with the etioplast is located primarily in the envelope membrane (Evans and Smith, 1976a, 1976b). On the basis of their observations they have presented the stimulating hypothesis that the phytochrome acts as a permease or transport factor to regulate the movement of one or more critical metabolites between the organellar and cytoplasmic compartments (Smith, 1970b; Evans and Smith, 1976a, 1976b).

As in chloroplasts two phases can be distinguished within the boundary of the etioplast envelope membrane. The stroma is a homogenous matrix which contains DNA (Jacobsen, 1968; Gyldenholm, 1968; Sprey and Gietz, 1973), ribosomes (Boardman, 1966; Brown and Guening, 1966; Dyer *et al.*, 1971), tRNA and amino acid activating enzymes (Guillemaut *et al.*, 1972; Burkard *et al.*, 1972) and all the enzymes of the Calvin cycle (Ireland and Bradbeer, 1971; Bradbeer, 1969; Bradbeer *et al.*, 1972; Bradbeer *et al.*, 1974b). Within the stroma there are a number of vesicles and lamellar systems, the most conspicuous of which is the prolamellar body (Hodge *et al.*, 1956).

Each etioplast contains usually one, but possibly up to four prolamellar/

prolamellar bodies, frequently interconnected by double lamellae (Kirk and Tilney-Bassett, 1967; Gunning, 1965). It appears that the structure of the prolamellar body may vary both within a tissue and also between species (Muhlethaler, 1971). Granick (1961) and Gunning (Gunning, 1965; Gunning and Jagoe, 1967) have presented a model of the prolamellar body structure based upon conventional electron microscopy of etiolated Avena coleoptile sections. The model envisages the prolamellar body as a three-dimensional cubic lattice of interconnected tubules in which the membranes of fusing tubules become smoothly confluent. The origin of the structure is believed to be the inner membrane of the plastid envelope (Virgin et al, 1963; von Wettstein, 1967). In contrast, the studies of Wehrmeyer (1965a, 1965b, 1965c, 1967), Ikeda (1968) and Weier and Brown (1970) indicate that in many cases the basic unit of the prolamellar body appears to be a tetrahedron (4 tubules meeting at one point) with the tubules forming five or six-membered rings. The centre of the lattice therefore has the shape of a pentagonal dodecahedron.

Independent studies by Bradbeer (1974a) on the structure of bean etioplast prolamellar bodies are equally consistent with both models.

Biochemical analysis of the prolamellar body is limited. Lutz (1975) has performed preliminary analyses on both the lipid and protein components of prolamellar bodies isolated from Avena etioplasts. After extraction with water and chelating agents the prolamellar body preparation is found to contain a number of lipids (mono and digalactosyl diglyceride, acylsteryl glycoside, cerebroside and phosphatidyl choline) and seven major SDS-soluble proteins. These proteins range in molecular weight from 22000 to 68000. The lowest molecular weight protein, SP1, is a glycoprotein. Similar analyses performed by Bogorad and his colleagues (Forger and Bogorad, 1973; Bogorad, 1975) show that prolamellar bodies isolated from Zea mays etioplasts also contain seven major proteins but in this case their molecular weights range from 25000 to 102000. In both studies comparisons are made with the protein components of photosynthetic lamellae and it is concluded that several, but not all, of/

of the polypeptides found in chloroplast lamellae are also present in etioplast prolamellar body membranes. A similar conclusion has been reached by Nielson (1975) who used acid-soaked polyacrylamide gels to electrophoretically characterize the plastid membrane proteins in greening barley seedlings.

This view differs however from those of Remy (1973) and Lagoutte and Duranton (1972). These authors conclude that there are no qualitative differences between lamellar proteins of etioplasts and those of plastids greened under intermittent light. They go as far as to claim that etioplasts must therefore contain reduced but significant amounts of, for example the CPI and II complex proteins. Inspection of the electrophoretic analyses upon which this claim is based indicates that this conclusion is however not justified upon the basis of the available data. A method of significantly higher resolution is required for separating plastid lamellar proteins (perhaps, for example, two-dimensional as opposed to one dimensional gel electrophoresis), before such questions can be answered.

Results of the type described above can be taken to suggest that the thylakoid lamellae of the mature chloroplast are assembled in a step-wise fashion, with components such as the membrane polypeptides, membrane lipids and photosynthetic pigments being added at different times. Evidence supporting this suggestion comes from a number of other studies. Lockshin *et al* (1971) and Gregory and Bradbeer (1975) have shown that the coupling factor CF_1 , which has the enzymic activity of Ca^{2+} -activated ATPase is found in both bean and maize etioplasts. These authors conclude that most or all of the coupling factor activity of a chloroplast may be present in the etioplast from which it develops. The relatively small light-triggered increase in CF_1 activity can be attributed to a modification of the enzymes properties (Gregory and Bradbeer, 1975). Bogorad (1975) has also shown that CF_1 particles isolated from maize etioplast membranes will restore photophosphorylating activity to maize chloroplast/

chloroplast membranes which have been stripped of CF_1 by washing in dilute EDTA solutions. Confirmation that a structurally as well as biochemically hybrid membrane has been reconstituted is provided by electron microscopy. Under the correct conditions 90\AA etioplast particles, in which the CF_1 activity resides (Howell and Moudriankis, 1967) become associated with EDTA stripped chloroplast membranes. These experiments clearly demonstrate that if there are any specific sites involved in the association of CF_1 particles with plastid membranes then they are already present in the etioplast membrane although it lacks chlorophyll and several other components necessary for photosynthesis.

Further support for the idea that etioplast membranes contain many components required in the functional chloroplast is provided by the demonstration that in addition to the enzymic coupling factors involved in phosphorylation, barley and bean etioplasts also contain three of the four cytochromes (namely f , b_{563} and b_{559LP}) which act as intermediates in photosynthetic electron transport (Plesničar and Bendall, 1972; Whatley *et al.*, 1972; Phung Nhu Hung *et al.*, 1972; Gregory and Bradbeer, 1973). In barley etioplasts these cytochromes, and also plastocyanin, are present on a fresh weight basis in amounts similar to those found in mature granal chloroplasts. It appears increasingly clear that the formation of the chloroplast thylakoid membranes is a multistep mechanism in which different components appear in the membrane independently of one another and at different times during its assembly.

The most obvious difference between etioplast and chloroplast membranes is the absence of chlorophyll molecules from the former. However it is possible to extract and partially purify from the prolamellar body membranes a photoreactive pigment-protein complex, the protochlorophyll-helochrome (Smith, 1960; Boardman, 1962; Schopfer and Scigelman, 1968). The spectroscopic studies of Kahn *et al.* (1970) indicate that there are three forms of protochlorophyll in the complex. Two of these forms PChl 650 and PChl 637 are photoreducible whilst/

whilst the third form PChl 628 is a relatively minor component and is not photoreducible. Statistical considerations upon the transfer of excitation energy from PChl 637 to both PChl 650 and chlorophyllide lead Kahn and his co-workers (Kahn *et al*, 1970; Boardman *et al*, 1972) to conclude that there are at least four chromatophores per protein molecule in the holochrome complex. The exact nature of the protochlorophyllide-protein relationships however remains to be resolved.

The remaining plastid form to be discussed in this section is the proplastid. The structure of the proplastid has been studied in the electron microscope by von Wettstein and Kahn (1960). The organelle exhibits a minimum amount of structure appearing to be little more than an oval stroma-filled sac approximately 1 μm in diameter. Commonly, irregularly shaped clear areas are evident in the matrix and they are often seen to contain fibrils which are speculated to be DNA (Whately *et al*, 1974). Occasionally starch grains are also evident within the matrix and in many cases they are associated with the restricted strands of internal membranes (Bradbeer *et al*, 1974a).

It is often implied that the relationship between the proplastid, the etioplast and the chloroplast is of a triangular nature (Kirk and Tilney-Bassett, 1967; see also Figure 17 in Robertson and Laetsch, 1974) in that proplastids develop either into etioplasts if the plant is maintained in the dark, or alternatively into granal chloroplasts if the plant is maintained in a light-dark regime. However, in many cases *in vivo* it appears that a high proportion of the proplastids initiate their development with the structural organization diagnostic of an etioplast. Only when environmental factors (presumably light being the major factor) instigate a developmental reorganization does the structural organization of the chloroplast result. Whately (1974) in an exacting study of plastid ultrastructure in bean, concludes that the transitory proplastid form which exists in the primary leaves immediately preceding hypocotyl emergence contain membrane arrays which are clearly prolamellar bodies, although they lack the highly ordered paracrystallinity of the steady-state etioplast structure. A similar/

similar phenomenon has also been observed by Leech et al (1972) who studied plastid membrane biogenesis in light/dark-grown leaves of Zea maia. This monocotyledonous species exhibits the basal meristematic organization which results in linear arrays of cells in the leaves, with the youngest cells at the base and the most senescent at the tip. Electron microscopy of thin sections from different regions of the leaf clearly shows that the maturing cells contain plastids which are differentiating in the direction of the normal chloroplast organization. However in the cells at the base of the leaf the majority of proplastids were found to contain at least one small prolamellar body.

These studies suggest that in the normal ecological situation a delineation between the proplastid-chloroplast transition and the proplastid-etioplast transition may at the early stages of development be extremely tenuous.

B. TRANSIENT FORMS : DEVELOPING CHLOROPLASTS

i. Ultrastructural changes and chlorophyll biosynthesis during greening.

The changing ultrastructure of the developing chloroplast has attracted the attention of electron microscopists for many years and is consequently a well described process in qualitative terms (von Wettstein, 1958; Virgin et al, 1963; Gyldenholm, 1968; Weier et al, 1970; Weier and Brown, 1970; Rosinski and Rosen, 1972; Gunning and Jagoe, 1967). However more recently quantitative approaches to describing the process of developing chloroplast membrane re-organization have been pursued. Bradbeer (1975) measured the decreasing volume of the prolamellar body and the increasing thylakoid area in developing chloroplasts of bean. He was able to conclude that the transformation of the prolamellar body membranes into porous lamellar sheets is essentially completed before the onset of increased thylakoid formation; which in turn correlates with the beginning of grana formation. These conclusions are in close agreement with those of Henningsen and Boynton (1970).

Robertson and Laetsch (1974) quantitated the process of membrane development/

development by determining the percentage of thylakoid appression (i. e. grana formation) in the plastids of greening barley leaves. As the dark-grown barley leaf also exhibits the basal meristematic type of organization these authors were able to investigate the process of plastid thylakoid development not only for the steady-state etioplast-chloroplast transition but also in relation to the degree of differentiation of the leaf. They conclude that the plastids in all regions of the leaf go through similar stages of development but they do not develop at the same rate. Younger regions require longer periods of illumination than older ones to form fully differentiated chloroplasts.

Concomitant with the morphological changes in the developing chloroplast during greening there are major alterations in the plastid pigments. The rapid changes which are evident directly after illumination are primarily due to the transformation of pigment molecules which represent the terminal stages of chlorophyll biosynthesis. Characterization of these transformations has principally involved monitoring the progression of spectroscopic forms of protochlorophyllide and chlorophyll in the leaf during greening (see review by Kirk, 1970). After longer periods of illumination light stimulates the *de novo* synthesis of chlorophyll. Identification of the earlier intermediates in this pathway has to date been achieved by enhancing their accumulation in the cell. Techniques such as prior feeding with the specific porphyrin precursor δ -amino levulinic acid (Gassman, 1973) or blocking of the normal biosynthetic pathway either genetically (Gough, 1972) or chemically (Duggan and Gassman, 1974) have been used. Recently however far more direct evidence has been provided by the development of cell-free systems in which partial reactions of the pathway can be demonstrated (Rebeiz *et al*, 1975a, 1975b, 1975c; Rebeiz and Castellfranco, 1973; Ellsworth and Hsing, 1973, 1974; Griffiths, 1975).

There is a considerable body of literature investigating the relationship between chlorophyll and protein synthesis during greening by examining the effects of antibiotics upon the formation of these two components/

components (see review by Kirk, 1974). The inhibitors of plant 70S ribosome function, streptomycin and D-threo chloramphenicol (Pestka 1971; Ellis, 1969) if administered for relatively short times to greening systems will cause a partial inhibition of chlorophyll synthesis (Kirk, 1962a, 1962b, 1968; Margulies, 1962). Similarly in short-term experiments cycloheximide, an inhibitor of 80S ribosomes in plants (Pestka, 1971), if administered in high doses (20-50 $\mu\text{g/ml}$) completely inhibits chlorophyll synthesis (Kirk and Allen, 1965; Hooper *et al*, 1969; Nadler and Granick, 1970). At lower dose levels inhibition by cycloheximide is less than complete and in some cases also transitory, perhaps due to inactivation of the antibiotic. In most of the systems investigated exogenously supplied δ -amino leavulinic acid (ALA) is able to cause a significant, but usually partial, diminution in the inhibition of chlorophyll (or protochlorophyll) synthesis by these drugs (Kirk, 1974; Nadler and Granick, 1970).

Two models have been postulated to account for these observations. The earlier model of Gassman and Bogorad (1967a, 1967b) suggested that the enzyme responsible for ALA synthesis (ALA synthetase) was labile and its resynthesis was prevented by both antibiotics. Inhibitors of ribosome function therefore indirectly inhibited chlorophyll synthesis during greening. This model now seems untenable as Duggan and Gassman (1974) have demonstrated that ALA synthetase is in fact a (relatively) stable enzyme with a half-life of about 16 hours.

An entirely different interpretation of the inhibitor data has been provided by Kirk (1974) in terms of chlorophyll carrier proteins and feedback inhibition of ALA synthesis. Several lines of evidence (see section 13Bii) indicate that a major part, but perhaps not all, of the protein complement of the CPI complex is synthesized on 70S plastid ribosomes. In contrast the single protein of the CPII complex is believed to be a product of 80S cytoplasmic ribosomes. Kirk postulates that the continued synthesis of chlorophyll requires the continued availability of chlorophyll/

chlorophyll acceptor sites which reside upon the CPI and CPII complex proteins. Inhibiting the synthesis of these proteins by drugs therefore leads to a build up of chlorophyll precursors which in turn suppress further chlorophyll synthesis by feedback inhibition of an essential enzyme (perhaps ALA synthetase). This model is consistent with the inhibitor data but to me it appears that an unjustified conclusion has been made to account for the partial reversal of the inhibitor effects by ALA. To postulate that the addition of exogenous ALA bypasses the feedback inhibition of ALA synthesis and therefore allows chlorophyll synthesis to continue requires that there is a reserve (perhaps only small) of chlorophyll acceptor proteins present in the greening plastid. This is equivalent to postulating that plastids in the early stages of greening have significant amounts of the CPI and CPII complex proteins. As is pointed out in section I2Aii I regard this conclusion as entirely speculative.

- ii. The development of plastid functions during greening.
 - a. Photosynthetic related functions.
 1. Photochemical activities.

The appearance of photosynthetic activity in greening tissue has been monitored from the level of oxygen evolution and carbon dioxide uptake and fixation (Oelze-Karow and Butler, 1972; Tamas *et al*, 1970) to the appearance and activity of individual components of the photosynthetic apparatus (Hiller and Boardman, 1971). Due to the different species, greening conditions and methods used, studies of this kind are inevitably of a correlative nature, relating the changes in photochemical activities to changes in, for example the concentration of electron carriers or the development of chloroplast structure.

More recently studies have emphasized the temporal development of separate activities of the two photosystems, PSI and PSII. The information obtained in a number of studies is collated in Table C. With the exception of the conclusions of Alberte *et al* (1972) it is clear that many photochemical activities associated with an active PSI complex are detected before the appearance/

Table C. The development of photochemical activities during greening.

Notes:

- I. phenazine methosulphate dependent
 - II. electron acceptor; methyl viologen
electron donor couple; NNN' N' tetra methyl-p-phenylene
diamine/ascorbate.
 - III. electron acceptor ; ferricyanide
 - IIIA. electron acceptor ; ferricyanide
electron donor ; water
 - IIIB. electron acceptor ; NADP
electron donor ; ascorbate or water
 - IV. electron acceptor ; AQ (anthraquinone-2-sulphonic acid)
electron donor couple ; NNN' N' tetra methyl-1, 4 phenylene
diamine hydrochloride/ascorbate
 - V. the appearance of oxygen evolution (Transient I) upon
illumination with 650 nm light
 - VI. NADP dependent
 - a. measured in plastids or plastid preparations isolated at
different times during greening.
 - b. measured in intact plants or whole leaf tissue
- DCMU 3-(3, 4 dichlorophenyl)-1, 1-dimethyl urea

1. Plesničar and Bendall, 1972
2. Plesničar and Bendall, 1973
3. Gregory and Bradbeer, 1975
4. Loshkin et al, 1971
5. Alberte et al, 1972
6. Egneus et al, 1972
7. Remy et al, 1972
8. Gyldenholm and Whately, 1968
9. Boardman et al, 1972
10. Oelze-Karow and Butler, 1972

ASSOCIATION WITH PHOTOCOMPLEX

<u>SPECIES</u>	<u>ACTIVITY</u>	<u>PSI</u>	<u>PSII</u>	<u>NONE</u>	<u>APPEARANCE</u>	<u>REF.</u>
Barley (Hordeum)	cyclic photophosphorylation I	+			0h ^a	1, 2
	O ₂ uptake II	+			0h ^a	1, 2
	non-cyclic photophosphorylation III		+		2½h ^a	1, 2
	O ₂ evolution III		+		2½-2¾h ^a	1, 2
Barley (Hordeum)	O ₂ uptake IV	+			5min ^a	6
	O ₂ evolution V		+		3h ^a	6
Barley (Hordeum)	cyclic photophosphorylation I	+			4h ^a	7
	non-cyclic photophosphorylation VI		+		10h ^a	7
Maize (Zea)	ATPase			+	0h ^a	4
Bean (Phaseolus)	ATPase light-triggered			+	30min ^a	3
Bean (Phaseolus)	cyclic photophosphorylation I	+			5h ^a	8
	non-cyclic photophosphorylation IIIA		+		10h ^a	8
	non-cyclic photophosphorylation IIIB		+		15h ^a	8
Bean (Phaseolus)	photooxidation of cytochrome f	+			1½-2h ^b	9
	photoreduction of cytochrome f		+		2-2½h ^b	9
Pea (Pisum)	photooxidation of cytochrome f	+			20-30 min ^b	9
	photoreduction of cytochrome f		+		1-2h ^b	9
Bean (Phaseolus)	DCMU-insensitive photophosphorylation	+			15 min	10
	DCMU-sensitive photophosphorylation		+		1h	10
Jack Bean (Canavalia)	oxygen evolution		+		45 min-1h	10
	oxygen evolution		+		2h ^b	5
	P700 photobleaching		+		6h ^a	5

appearance of reactions characteristic of the PSII complex. The precise temporal relationship appears to be a function of the species, the experimental conditions and the activity characterized. In the most exact study of this nature to date, Plesničar and Bendall (1973) were able to show that plastids began to develop grana at about the time that Photosystem II activity became detectable in greening barley leaves. Also the behaviour of particular electron carrier molecules correlated with the appearance of photochemical activities. On one hand the concentrations of plastocyanin, cytochrome *f*, cytochrome b563 and cytochrome b559LP either did not change or decreased during greening. In contrast, the concentrations of cytochrome b559HP and pigment P546 closely followed the activities of Photosystem II.

In addition to the photochemical reactions listed in Table C, Gregory and Bradbeer (1975) and Loshkin *et al* (1971) have demonstrated that etioplasts exhibit one of the important light-independent partial reactions of photosynthesis i. e. adenosine triphosphatase activity. This conclusion is consistent with the findings of Bogorad (1975). There is evidence to suggest however that other non light-requiring reactions are absent in etioplasts but develop along with the maturation of the thylakoid membrane. Forger and Bogorad (1973) and Bogorad (1975) have shown that in contrast to chloroplast thylakoid membranes, the membranes of maize etioplasts are unable to form ATP from ADP and inorganic phosphate when transferred from an acidic to an alkaline solution (acid/base (A/B) phosphorylation). Etioplasts lack either specific factors, or perhaps a functional organization required for A/B phosphorylation. Having already demonstrated that etioplast membranes did not lack adenosine triphosphatase activity these authors therefore postulated that the development of A/B phosphorylation might result from the construction of a particular organization in the developing chloroplast thylakoid. They subsequently were able to demonstrate that there is an almost perfect/

perfect correlation between the development of A/B phosphorylation, the development of osmotic responsiveness in plastid membrane preparations and the appearance of osmotically responsive vesicles in the thylakoid structure. The exact nature of the events which result in the development of intact vesicles is unknown. However Forger and Bogorad (1973) speculate upon the involvement of a 46000 molecular weight polypeptide which appears in the developing plastid membrane concomitant with the acquisition of A/B phosphorylating capacity.

2. The reactions of CO₂ fixation.

Although the development of carbon dioxide fixation cannot be detected until some 15 hours after illuminating dark-grown primary leaves of bean, it has been shown that bean etioplasts contain all the enzymes of the photosynthetic carbon cycle (Bradbeer, 1969; Bradbeer, 1975). During greening these enzymes exhibit a variety of responses in both their absolute and specific activities. In some cases, for example ribulose 1,5 biphosphate carboxylase, there is a short lag period which precedes an increased activity (on a per leaf basis); whilst in others there is for example no lag period or little rise in total activity.

These results indicate again that the etioplast already possesses several of the components which are required in the functional chloroplast. In many cases it appears that the effect of light stimulation is not to initiate the synthesis of plastid components but rather to induce an acceleration in their accumulation. Both specific mechanisms, for example phytochrome mediated responses (Graham *et al.*, 1971) and a non-specific stimulation of the rate of plastid division and growth (Bennet and Radcliffe, 1975) can be envisaged as being important in this process.

b. Protein synthesis.

It has been known for over 10 years that etioplasts have the components necessary for protein synthesis (Kirk and Tilney-Bassett, 1967). However there are still only a relatively small number of studies from which it can be inferred that etioplasts and developing chloroplasts synthesize proteins *in vivo*; even fewer which directly demonstrate/

demonstrate that the etioplast protein synthesizing system is functional either in vivo or in vitro; and none whatsoever which establish the identity of an etioplast or developing chloroplast ribosome product.

Smith (1970a) has shown that during growth in the darkness barley leaves accumulate Fraction I protein, the synthesis of which in green tissue has been unequivocally demonstrated to involve chloroplast ribosomes (Blair and Ellis, 1973). It seems reasonable to infer that the synthesis of this protein during growth in the dark is due in part to the activity of etioplast ribosomes. Similarly Bradbeer and his colleagues (Bradbeer, 1975) have, as has been stated, demonstrated that bean etioplasts contain significant amounts of cytochromes f, b563 and b559LP. Studies with ribosome inhibitors (see section I3Biiia) suggest that these proteins are also synthesized on plastid ribosomes and therefore the activity of the etioplast translational system can again be inferred.

More direct attempts to demonstrate the activity of etioplast and developing chloroplast ribosomes in vivo have been made. However these studies, involving inhibitor and double-labelling techniques (Kaveh and Harel, 1973) have met with only limited success. In addition to the practical difficulties encountered there is also a strong theoretical objection when this approach is used to characterize the products of developing chloroplast protein synthesis. The specificity of any ribosome inhibitor is always questionable in vivo because of the possibility that polypeptides synthesized on one set of ribosomes, for example 80S ribosomes, may control the synthesis of polypeptides upon another ribosome group, for example 70S ribosomes. A precedent for this situation has been discussed in relation to the synthesis of Fraction I protein (Ellis 1975a).

A more direct approach to establishing the functionality of etioplast and developing chloroplast ribosomes is to maintain and analyse physiological translation in isolated plastids. There are a few reports which indicate that etioplasts isolated from algae (Reger *et al.*, 1972b) and higher plants (Reger *et al.*, 1972a; Drumm and Margulies/

Margulies, 1970; Smillie *et al.*, 1972) are able to carry out *in vitro* protein synthesis. The characteristics of such amino acid incorporation appear very similar to those of *in vitro* chloroplast protein synthesis in regard to both duration and antibiotic inhibition (Reger *et al.*, 1972a, 1972b; Drumm and Margulies, 1970). The capacity for *in vitro* protein synthesis also appears to increase in developing chloroplasts, the magnitude of the increase varying with both the species and experimental conditions used.

Beyond these very basic observations there is no further information upon the characteristics of etioplast and developing chloroplast protein synthesizing systems during development.

3. CHLOROPLAST TRANSCRIPTION AND TRANSLATION

A. CHLOROPLAST TRANSCRIPTION.

i. The components of chloroplast transcription

a. Chloroplast DNA

In all higher plant species examined to date chloroplast DNA has a buoyant density of $1.697 \pm 0.002 \text{ g cm}^{-3}$ and a base composition of $37.5 \pm 2\%$ GC (Kirk, 1976). The invariance of chloroplast DNA in this respect contrasts with nuclear DNA which may have a smaller, larger or similar density to chloroplast DNA from the same plant, depending upon the species (Wells and Birnstiel, 1969; Wells and Ingle, 1970; Whitfeld and Spencer, 1968). Chloroplast DNA may also be distinguished from nuclear DNA in that it contains no detectable 5-methylcytosine (Whitfeld and Spencer, 1968; Tewari and Wildman, 1970) and is not combined with basic proteins (Tewari and Wildman, 1969).

After denaturation with either heat or alkali chloroplast DNA renatures extremely rapidly (Bastia *et al.*, 1971) in comparison to nuclear DNA which will renature to only a slight extent over a period of several hours (Kung and Williams, 1969). The rapid renaturation of a chloroplast DNA sample implies that homologous sequences are present in high concentration, and therefore that the number of unique sequences are small. This fact can be expressed quantitatively by determining the kinetic complexity of the DNA molecules in a sample. The kinetic complexity is a measure of the size/

size of the unique set of nucleotide sequences in a DNA sample and it is determined from the rate at which the DNA renatures (Britten and Kohne, 1968; Wetmur and Davidson, 1968). A number of the values for the kinetic complexity of chloroplast DNA which have been published (Wells and Sager, 1971) and still are being published (Kirk, 1976) are incorrect because they are estimated by reference to the unrevised molecular weight of 1.3×10^8 for bacteriophage T₄ DNA (Dubin *et al.*, 1970). However using the corrected value of 1.06×10^8 it is striking that the kinetic complexities of chloroplast DNA from the small number of algae and higher plants examined so far are all in the range $0.9 - 1.0 \times 10^8$. Intuitively it would seem unlikely that this is coincidental. If extended to a wider spectrum of organisms then this observation might be taken to indicate that at least the amount, and possibly also the type of information residing in chloroplast DNA is invariant throughout evolution.

It is interesting to compare the analytical complexities of chloroplast DNA i. e. the amount of DNA per chloroplast, with the apparent kinetic complexity. The analytical complexity varies throughout the plant kingdom (Kirk and Tilney-Bassett, 1967), ranging from about 10^9 to 10^{10} daltons per chloroplast (Ellis and Hartley, 1974). The kinetic complexities are thus always much lower than the analytical complexities, indicating that there must be between 10 and 100 copies of the DNA sequences in each chloroplast. Herrman *et al.* (1975) has shown that the number of copies is related to the size of the chloroplast; the larger the chloroplast the more copies of DNA it contains.

It is not possible to demonstrate by renaturation kinetics that there is any intermolecular heterogeneity in chloroplast DNA but it is becoming clear that intramolecular base-sequence heterogeneity exists. This is most clearly seen in the modality of the differential melting curves of certain algal DNA preparations (Dalmon and Bayen, 1975). The same approach with higher plant chloroplast DNA is not able/

able to detect any gross intramolecular heterogeneity (Kolodner and Tewari, 1975a) but the presence of AT and GC rich regions in circular pea chloroplast DNA can be demonstrated by denaturation mapping (Kolodner and Tewari, 1975c).

More recently it has been shown that the sequences in chloroplast DNA are not present in a highly reiterated molecule but exist in the form of multiple copies. If precautions are taken to minimise shearing a proportion of chloroplast DNA can be isolated as circles. Circular DNA has been reported from the chloroplasts of pea, spinach, corn, lettuce, oats, bean (Kolodner and Tewari, 1972, 1975a; Manning *et al.*, 1972), *Euglena* (Gray and Kekwick, 1974b) and *Antirrhinum majus*, *Oenothera hookeri* and *Beta vulgaris* (Herrman *et al.*, 1975). In all these species the contour length of the majority of the circles is in the range 37-45 μm ; this is the expected length of a double-stranded DNA molecule having a molecular weight of $0.85 - 1.0 \times 10^8$, a figure remarkably close to the apparent kinetic complexity of chloroplast DNAs. This correspondence of length and kinetic complexity therefore suggests that the genetic information carried by the chloroplast DNA is accommodated by the length of a circular molecule. Direct evidence for chloroplast polyploidy has come from electron microscopic and autoradiographic studies, which show that each chloroplast can have up to 32-DNA containing regions, the number depending upon the size of the chloroplast (Kowallik and Herrman, 1972).

It seems likely that the circular chloroplast DNA molecules exist *in vivo* in the form of circular supercoils. Manning *et al.* (1972) and Kolodner and Tewari (1972) reported the presence of supercoiled molecules in their preparations and Kolodner and Tewari (1974a) report that a maximum of 80% of the chloroplast DNA can be isolated as circular molecules, and as much as 30-40% of the total chloroplast DNA could be isolated in the supercoiled form.

The same authors, Kolodner and Tewari (1975b) have shown that chloroplast DNA isolated from both pea and corn contains both Cairns/

Cairns type and rolling circle intermediates. They conclude that the replication of chloroplast DNA involves a round of Cairns type replication which precedes a round of rolling circle replication.

b. Chloroplast DNA polymerase

The replication of chloroplast DNA is mediated by the activity of chloroplast DNA polymerase. DNA polymerase activity has been detected in chloroplast preparations from spinach and tobacco (Spencer and Whitfeld, 1969; Tewari and Wildman, 1967) and Euglena (Scott *et al*, 1968). The activity is dependent upon the presence of all four deoxynucleoside triphosphates and Mg^{2+} , and incorporation is sensitive to DNase. This suggests that the polymerase activity was associated with lysed chloroplasts. Bohnert *et al* (1974) has reported the incorporation of (3H) thymidine into DNA in isolated intact chloroplasts incubated in the presence of DNase I. This unequivocally demonstrates the activity of a chloroplast-located DNA polymerase.

The products of DNA synthesis in isolated chloroplasts show great similarity to chloroplast DNA. The labelled product has the same buoyant density as chloroplast DNA, it hybridizes to a much larger extent with chloroplast DNA than with nuclear DNA and it has a similar base composition to chloroplast DNA (Tewari and Wildman, 1967). The product of DNA synthesis in isolated spinach chloroplasts also exhibits the rapid renaturation characteristic of chloroplast DNA (Spencer and Whitfeld, 1969). It should be remembered that with this data alone it is not possible to distinguish whether the incorporation activity is associated with an enzyme carrying out either a replicase or a repair function.

The chloroplast DNA polymerase is membrane-bound, although at least in the case of Euglena it can be solubilized by treatment with high concentrations of salt (Keller *et al*, 1973). The purified enzyme is inhibited by ethidium bromide, which possibly correlates with the observation that the treatment of Chlamydomonas cells with ethidium bromide induces a selective and reversible inhibition of/

of chloroplast DNA replication (Fletcher and Sager, 1973).

Chloroplast DNA in Euglena and Chlamydomonas has been shown to replicate in a semi-conservative fashion, the synthesis of chloroplast and nuclear DNA taking place at different times in the cell cycle (Chiang and Sueoka, 1967). The factors controlling the time and rate of synthesis of chloroplast DNA are unknown.

c. Chloroplast RNA polymerase

DNA-dependent RNA polymerase activity has been demonstrated in chloroplast preparations from both algal and higher plant species (Surzyki, 1969; Tewari and Wildman, 1969; Bottomley, 1970). In most cases these studies have been performed using lysed plastids, principally because the nucleoside triphosphates used as substrates penetrate the chloroplast envelope at slow rates. Lysed preparations do not however synthesize discrete species of RNA; instead a polydisperse pattern of products ranging in size from 5S to 23S is obtained (Spencer and Whitfeld, 1967). On the other hand intact isolated chloroplasts, which are able to use light to phosphorylate (^3H) uridine (Hartley and Ellis, 1973), synthesize a small number of discrete RNA products (section 13Aiid). In contrast to the lysed chloroplast preparation RNA polymerase activity in intact chloroplasts is light-dependent, does not require the addition of exogenous nucleoside triphosphates and is not sensitive to ribonuclease.

Chloroplast RNA polymerase is bound to the thylakoid membranes (Tewari and Wildman, 1967) but it can be relatively easily removed by washing with distilled water (Bennet and Ellis, 1973) or a low salt buffer containing EDTA (Bottomley et al, 1971). RNA polymerase solubilized from wheat thylakoids by high salt buffers (Polya and Jagendorf, 1971) is found to be totally insensitive to rifampicin and only under specific ionic conditions were Bogorad et al (1973) able to demonstrate even a partial (30%) inhibition of in vitro polymerase activity by Rifampicin-SV. It can be argued that the failure of this drug (which is a specific inhibitor of RNA initiation in bacteria (Wehrli and Stahelin, 1971)) to inhibit chloroplast RNA polymerase/

polymerase activity in vitro is because in these assays incorporation is due only to chain elongation. However I believe that conclusions based upon the action of this inhibitor in vivo (section I3Aiiia) must nevertheless be regarded with extreme caution.

Smith and Bogorad (1974) have analysed the polypeptide composition of purified RNA polymerase preparations from Zea mays by SDS polyacrylamide gel electrophoresis. They conclude that the enzyme preparation is composed of 2 major and 4 minor polypeptide components. It is not yet possible to determine which if any of these proteins are true components of the polymerase.

ii. The function of chloroplast DNA

A 40 μm circle of double-stranded DNA of unique base sequence is sufficient in principle to code for about 125 proteins of molecular weight 50000. The most direct and unequivocal manner in which the function of chloroplast DNA could be elucidated would be to reconstitute a transcriptional system containing purified chloroplast DNA in circular form and purified chloroplast RNA polymerase complete with regulatory subunits. Such a system would theoretically transcribe the genes in chloroplast DNA into a form in which they could be identified either as primary gene products (for example, ribosomal and tRNAs) or, by translation in a protein synthesizing system, as secondary gene products i. e. protein. However such studies must for the time being await upon, at least, the purification of the chloroplast RNA polymerase enzyme. Meanwhile four methods have been used, with different degrees of success, to determine the function of chloroplast DNA.

a. The selective inhibition of chloroplast DNA transcription.

Rifampicin is reported to be a potent inhibitor of the initiation of RNA synthesis in bacteria (Wehrli and Stahelin, 1971). This drug will also inhibit chloroplast RNA polymerase in extracts of Chlamydomonas (Surzyki, 1969). When applied to whole organisms it has been reported to inhibit the incorporation of labelled precursors into the chloroplast, but not cytoplasmic, ribosomal RNA of Chlamydomonas /

Chlamydomonas (Surzyki, 1969), Chlorella (Brandle and Zetsche, 1971) and Acetabularia (Galling, 1971). These results are consistent with the idea that the functional genes for chloroplast ribosomal RNA are located in chloroplast DNA and are transcribed by the chloroplast RNA polymerase. The specificity of this drug must however be questioned. As has been pointed out (section I3Aic) the chloroplast RNA polymerase(s) from higher plants as normally prepared and assayed, appears to be insensitive to rifampicin. It has also proved difficult to repeat the observations of the algal systems in higher plants (Bottomley et al., 1971). It is quite possible that the inhibition of chloroplast ribosomal RNA synthesis by rifampicin in algae is a secondary effect of the antibiotic.

b. Genetic analysis of mutants.

The majority of studies on mutations which affect chloroplast components and are believed to reside in chloroplast DNA have been performed using the alga Chlamydomonas and species of the genus Nicotiana.

In Chlamydomonas many mutations which affect chloroplast ribosomes and photosynthetic capacity are inherited in a uniparental fashion as a single circular linkage group (Gillham et al., 1974; Harris et al., 1974; Sager, 1972) and are therefore assumed to reside in chloroplast DNA. The basis for believing that the extranuclear linkage group being mapped is indeed chloroplast DNA is that the linkage group is circular, the mapped mutations affect the chloroplast, and mitochondrial DNA is usually inherited in a non-Mendelian, but biparental manner (Alexander et al., 1974). The inexactness of these criteria has however recently been demonstrated by the discovery that there exists two Chlamydomonas mutants which can grow heterotrophically or mixotrophically in the presence of antibiotics which can be demonstrated to have inhibited chloroplast ribosome function (Boynton et al., 1973; Conde et al., 1975). The mutation conferring antibiotic resistance (presumably therefore to mitochondrial ribosome function) is however inherited as both a circular/

circular linkage group and in a uniparental fashion. These observations can be accommodated by postulating that the mutation does in fact alter a chloroplast DNA encoded ribosomal protein which only however confers antibiotic resistance when it is assembled into a mitochondrial ribosome. Clearly these anomalies ought to be resolved.

There is also uncertainty over the physical basis for uniparental inheritance in Chlamydomonas. It has been suggested that when like gametes fuse there is a selective destruction of the chloroplast DNA of one of the gametes by a modification-restriction system of the type known in bacteria (Sager and Lane, 1972; Sager and Ramanis, 1974). Sager (1972) also claims that the extranuclear linkage group in Chlamydomonas recombination also behaves as if it were diploid in vegetative cells. As the chloroplast genome is polyploid (section 13A1a) this implies that there must be a reduction of the number of chloroplast DNA molecules in the gamete to one. There is no experimental evidence to support this implication. An alternative model of extranuclear recombination in Chlamydomonas argues the data are equally consistent with the transmission of multiple genome copies at gamete fusion and seems more attractive (Gillham et al, 1974).

Bearing in mind these reservations the simplest interpretation of the existence of at least six extranuclear gene loci mutations affecting the resistance of chloroplast ribosomes to antibiotics is that six proteins of chloroplast ribosomes are encoded in chloroplast DNA. In a number of cases the nature of the mutation has been further defined. Changes in the sedimentation coefficient and altered binding capacities of the chloroplast ribosomes from antibiotic-resistant mutants have been reported (Gillham et al, 1970; Mets and Bogorad, 1971; Burton, 1972) and in a few cases the presence of an alteration in a specific ribosomal protein has been detected (Mets and Bogorad, 1972; Ohta et al, 1975; Brügger and Boschetti, 1975).

Good evidence that a mutation inherited in a maternal or uniparental fashion actually alters the amino acid sequence of a protein has only been obtained in one case. Chan and Wildman (1972) studied the inheritance/

inheritance of a mutation in the large subunit of Fraction I protein in Nicotiana tabacum. By analysing tryptic peptide fingerprints of the polypeptide present in reciprocal hybrids involving parent plants which exhibited differences in the primary structure of the protein, they were able to conclude that the mutation was inherited via the maternal line only. In higher plants it is believed that maternal uniparental inheritance is a consequence of the absence of plastids from the pollen. Consequently these authors demonstrated unequivocally that the gene for the large subunit of Fraction I protein resides in chloroplast DNA. The studies of Kung et al (1974) (section I2Ai) corroborate this conclusion although their criteria are less exacting than those of Chan and Wildman. Whereas an altered tryptic peptide fingerprint is accepted to indicate a change in the primary sequence of a protein (Ingram, 1958) it seems that small charge differences, resolvable by isoelectric focussing, are equally likely to arise from post-translational modifications.

c. DNA-RNA hybridization studies.

If RNA isolated from chloroplasts can be shown to hybridize to chloroplast DNA but not to nuclear DNA, this is good grounds for believing that such RNA is both encoded in and transcribed from chloroplast DNA. Attempts to quantitate the proportion of the chloroplast genome that is transcribed in vivo have been made by hybridizing a vast excess of cellular RNA to the chloroplast DNA, and determining how much of the DNA is present in DNA/RNA duplexes. Estimates varying between 16 and 60% of the single-stranded chloroplast genome have been obtained by Rawson (1975) and Howell and Walker (1974). The higher figure may indicate that, at least in part, transcription of chloroplast DNA may be symmetrical.

A number of hybridization studies have been carried out more specifically with the larger (23S and 16S) chloroplast ribosomal RNAs. In the most exacting study to date Thomas and Tewari (1974a) have used only circular, and presumably therefore only intact, chloroplast DNA for hybridization to chloroplast rRNA. For a number of higher plant/

plant species (pea, bean, lettuce, spinach and corn; Thomas and Tewari 1974a, 1974b) they conclude that each circle of chloroplast DNA contains two cistrons for 16S ribosomal RNA and two cistrons for 23S ribosomal RNA. Chloroplast rRNA has also been shown to hybridize to chloroplast DNA in tobacco (Tewari and Wildman, 1968), swiss chard (Ingle et al, 1970) and Euglena (Scott, 1973). There has been only a preliminary report of the hybridization of 5S chloroplast rRNA to chloroplast DNA in Euglena (Phillips and Carr, 1975).

There are several reports that chloroplast ribosomal RNA will also hybridize to nuclear DNA (Tewari and Wildman, 1970; Ingle et al, 1970) raising the question of the functionality of chloroplast ribosomal DNA cistrons in vivo. The significance of these reports awaits further study but it cannot yet be discounted that there are two types of chloroplast ribosomal RNA, one encoded in chloroplast DNA and the other encoded in nuclear DNA.

Hybridization studies have also shown that 0.4 - 0.7% of the chloroplast DNA contains sequences complementary to tRNAs (Tewari and Wildman, 1970; Schwartzbach et al, 1975). This amount of DNA would be sufficient to code for about 20-30 tRNA molecules each of molecular weight 25000 (Tewari and Wildman, 1970). This work used unfractionated tRNA however and more studies need to be done to establish the site of encoding of individual tRNA species.

To date no chloroplast mRNA has been purified. However RNA isolated from chloroplasts has been shown to direct the synthesis of the large subunit of Fraction I protein in an Escherichia coli cell-free protein synthesizing system (Hartley et al, 1975). Purification of this messenger and its hybridization to chloroplast DNA would confirm the genetic evidence as to the coding site of this protein.

d. Identification of RNA and protein molecules synthesized in isolated chloroplasts.

If translation coupled to transcription could be obtained in isolated chloroplasts then identification of the products would at the same time determine both the structural genes present in chloroplast DNA/

DNA and the function of chloroplast ribosomes. However to date it has not been possible to demonstrate coupled transcription and translation in isolated chloroplasts. Isolated chloroplasts do however synthesize discrete species of RNA (and also protein, section 13Bib) and therefore at least some of the primary gene products of chloroplast DNA can be identified by this method.

The major product of RNA synthesis in isolated intact chloroplasts is a species of molecular weight 2.7×10^6 (Hartley and Ellis, 1973). This molecule has been shown by competitive hybridization to chloroplast DNA to be a precursor to chloroplast ribosomal RNA (C. Head, personal communication; Ellis 1976a). Minor species of 1.2×10^6 and 0.47×10^6 are also synthesized in isolated intact chloroplasts (Hartley and Ellis, 1973) but their identities are unknown. All three of the *in vitro* synthesized RNA species coelectrophorese with *in vivo* synthesized species (Hartley and Ellis, 1973).

Summarizing the studies described in this section it is possible to state that the known genes in chloroplast DNA are; some chloroplast tRNA, 23S and 16S chloroplast ribosomal RNA, several chloroplast ribosomal proteins, and the large subunit of Fraction I protein.

B. CHLOROPLAST TRANSLATION

i. The components of chloroplast translation.

Chloroplasts contain their own protein synthesizing system, which differs markedly from that of the cytoplasm, but bears a strong resemblance to prokaryotic systems (Ellis, 1970). For example chloroplasts and bacteria show similarities in the size of their ribosomes, the sensitivity of protein synthesis to particular antibiotics and in the mechanism of initiation of protein synthesis (Boulter *et al*, 1972). These similarities provide a basis for suggesting that there is a possible common origin of prokaryotes and cellular organelles.

a. Ribosomes.

The existence of two classes of ribosomes in green plants was first shown by Lyttleton (1962). Ribosomes of the 70S class have been isolated from chloroplasts from several algae and higher plants (Hoerber/

(Hooper and Blobel, 1969; Scott *et al*, 1971; Leaver and Ingle, 1971; Boardman *et al*, 1965). Chloroplast ribosomes thus resemble those from prokaryotic cells in their S value.

The molecular weights and base compositions of the chloroplast ribosomal RNAs have been determined for a number of species (Ellis and Hartley, 1973). The small subunit of chloroplast ribosomes (30S) contains one RNA species of molecular weight 0.56×10^6 (16S) (Ingle *et al*, 1970); which is smaller than the corresponding cytoplasmic 40S ribosomal subunit RNA which has a molecular weight of 0.7×10^6 (18S). The larger subunit of chloroplast ribosomes (50S) contains two RNA species. The larger species has a molecular weight of 1.1×10^6 (23S) and is unstable, giving rise to breakdown products which are characteristic for each species (Leaver and Ingle, 1971). The 60S subunit of cytoplasmic ribosomes contains an analogous 25S RNA species. Also present in the large chloroplast ribosomal subunit is a 5S RNA (mol. wt. 0.04×10^6). This RNA can be distinguished from an analogous 5S RNA in the cytoplasmic ribosome by fractionation on methylated albumin kieselguhr columns or polyacrylamide gels (Dyer and Leech, 1968; Payne and Dyer, 1971). Recently evidence from DNA/RNA hybridization studies (Phillips and Carr, 1975) indirectly indicate that the chloroplast 5S rRNA does not show extensive homology with cytoplasmic 5S rRNA in *Euglena*. A much higher degree of homology exists between the *Euglena* chloroplast 5S rRNA and the 5S RNAs of a number of blue-green algae.

The proteins of chloroplast ribosomes have been analysed by gel electrophoresis (Hooper and Blobel, 1969; Odintsova and Yurina, 1969; Vasconcelos and Bogorad, 1971; Hanson *et al*, 1974; Freyssnet and Schiff, 1974) and by immunological cross-reactivity tests (Wittman, 1970). Using the technique of two-dimensional gel electrophoresis, which affords high resolution, it has been demonstrated that in both *Euglena* and *Chlamydomonas* the total number of chloroplast ribosomal proteins is in the range of 35 to 48 (Hanson *et al*, 1974; Freyssnet and Schiff, 1974). This number is similar to those obtained for prokaryotic/

prokaryotic ribosomes (Bickle and Traut, 1971) but differs from the figure of approximately 60 to 70 which appears to be more characteristic of the proteins isolated from both algal cytoplasmic ribosomes (Hanson *et al*, 1974; Freyssnet and Schiff, 1974) and other eukaryotic cytoplasmic systems (Sherton and Wool, 1972; Wolfe *et al*, 1971).

However although there appears to be a numerical similarity between chloroplast and prokaryotic ribosomal proteins the protein complements are quite different as judged by polyacrylamide gel electrophoresis (Hooper and Blobel, 1969). The degree of similarity in the electrophoretic behaviour of ribosomal proteins from the chloroplast and cytoplasm of *Chlamydomonas* is small. Only two pairs of proteins of the small subunits and four pairs of the large subunits were found to have similar electrophoretic mobilities in both directions.

Some of the chloroplast ribosomes are bound to internal membranes (Chen and Wildman, 1970; Tao and Jagendorf, 1973) and polyribosomes can be isolated from both the 'free ribosome' fraction of the stroma (Chen and Wildman, 1967) and from thylakoid membranes treated with the detergent Nonidet P40 (Margulies and Michaels, 1975). The characteristics of polyribosomes isolated by the detergent procedure appear anomalous in several respects. For instance treatment with proteases as well as ribonuclease is required to dissociate these polyosomes to the monomeric ribosomes. The significance of these properties is not clear. There is evidence that the two classes of polyribosomes in the chloroplast synthesize different types of protein. Ellis, (1976b) has shown that the large subunit of Fraction I protein is synthesized only by the free ribosomes of the chloroplast and conversely Margulies *et al* (1975) postulate that there is a vectorial discharge of nascent polypeptides towards the membrane by polyribosomes attached to thylakoids.

It is well established that protein synthesis by chloroplast ribosomes is inhibited by the same antibiotics which inhibit protein synthesis by prokaryotic ribosomes (Boulter *et al*, 1972; Ellis *et al*, 1973). D-three chloramphenicol (but not other isomers), spectinomycin, lincomycin/

lincomycin and erythromycin all inhibit chloroplast and bacterial ribosome function in vitro (Ellis, 1970). As the protein complements of the chloroplast and bacterial ribosomes are different the similarity between their function with respect to inhibition by antibiotics cannot be attributable to any property of the proteins as revealed by gel electrophoresis or immunology. There is no reason to believe that there is any similarity in the mechanism of antibiotic inhibition in the two cases, nor even in the relatedness of the antibiotic binding site on the two types of ribosome.

On the other hand, cycloheximide inhibits protein synthesis on 80S ribosomes of green (Ellis, 1969) and non-green tissue (Ellis and McDonald, 1967) but has no effect on in vitro protein synthesis by chloroplasts or bacterial ribosomes. With regard to the studies described in section 13Bii it should be remembered that all these findings relate only the activity of isolated sub-cellular systems. There are numerous examples to show that inhibitors such as cycloheximide and chloramphenicol have a variety of non-specific effects in whole tissues (Ellis, 1963; Ellis and McDonald, 1970). It cannot be assumed that treating intact cells with these compounds specifically inhibits protein synthesis in one cellular compartment alone with no other effects.

b. Amino acid activation and the initiation of chloroplast protein synthesis.

The existence of plastid specific tRNAs and amino acyl tRNA synthetases has been demonstrated in chloroplasts from a range of algae and higher plants (Ellis et al., 1973). In tobacco leaves a comparatively straight-forward relationship appears to exist between the compartmentalization of tRNAs_{leu} and charging by their homologous amino acyl tRNA_{leu} synthetase enzymes. Six isoaccepting leucine specific tRNAs are found (Guderian et al., 1974). Two of these are exclusively located in the chloroplasts and could only be charged by the homologous chloroplast amino acyl tRNA synthetase preparation. Of/

Of the remaining four tRNA_{leu} species two are exclusively located in the mitochondria and two in the cytoplasm. In bean leaves a more complex situation is apparent. (Burkard et al, 1970)

The cytoplasm of leaves of Phaseolus contains a leucyl-transfer RNA synthetase that can add leucine only to the two leucyl transfer RNA species that are common to the cytoplasm and chloroplast, but is not able to recognise the three leucyl transfer RNA species found only in the chloroplast. The chloroplast enzyme on the other hand can aminoacylate all five leucyl transfer RNA species. The functional significance of these findings is obscure. Boulter et al (1972) have made the suggestion that this apparently complex situation with regard to the charging specificity may in fact be one way in which the control of cellular differentiation may be effected at the translational level. Modifications in transfer RNA species which endow a specificity for involvement in chloroplast mRNA translation may allow for the integration of protein synthesis in the cytoplasm and chloroplast.

Reger et al (1970) have demonstrated that in Euglena chloroplast-specific isoleucyl and phenylalanyl tRNA synthetases are induced by light. Studies involving the use of inhibitors and the aplastidic Euglena mutant W₃BUL indicate that at least one (phenylalanyl tRNA synthetase) of these enzymes is encoded in nuclear DNA and synthesized on cytoplasmic ribosomes (Hecker et al, 1974). It is interesting to note that Parthier et al (1972) conclude that chloramphenicol inhibits the light-induced synthesis of this enzyme. One hypothesis compatible with this data is that light induces the synthesis of a protein on chloroplast ribosomes which in turn regulates the synthesis of phenylalanyl tRNA synthetase in the cytoplasmic compartment. Alternatively the inhibitor data of Parthier et al (1972) might be considered suspect for reasons already stated.

By analogy to the bacterial situation the initiation of protein synthesis on chloroplast ribosomes could be expected to involve N-formyl methionine and its cognate tRNA. Schwartz et al (1967) have shown that chloroplast ribosomes from Euglena translate RNA from bacteriophage f₂ into viral coat protein with N-formyl methionine/

methionine at the amino-terminus. The existence of a methionyl tRNA which can be formylated by either endogenous or *E. coli* transformylase has been demonstrated in chloroplasts (Leis and Keller, 1970, 1971; Merrick and Dare, 1971; Burkard *et al.*, 1969; Guillemaut *et al.*, 1972). Two additional methionyl tRNAs are present in bean chloroplasts, both of which cannot be formylated and are therefore probably involved in peptide chain elongation (Guillemaut *et al.*, 1973). Highfield (1975) has extended the earlier studies of Bianchetti *et al.* (1971) and has shown that isolated pea chloroplasts form N-formyl methionyl puromycin when incubated with light, methionine and puromycin. The initiation of protein synthesis in chloroplasts appears similar therefore to prokaryotic systems.

c. Chloroplast mRNA

The major remaining component involved in chloroplast protein synthesis which has not been mentioned is chloroplast messenger RNA (mRNA). This is because no specific chloroplast mRNA has been purified sufficiently to allow any investigation of its nature. The earliest biochemical evidence to support the idea that chloroplast contain mRNA came from Brawerman and Eisenstadt (1964). They demonstrated that *Euglena* chloroplast RNA was able to stimulate amino acid incorporation in a cell-free protein synthesizing system from *E. coli*. Subsequently Hartley *et al.* (1975) was able to show that the total RNA fraction isolated from spinach chloroplasts was able to stimulate amino acid incorporation into protein, also in a cell-free protein synthesizing system from *E. coli*. In contrast to Brawerman and Eisenstadt (1964), Hartley *et al.* (1975) proceeded to identify one of the *in vitro* products by chymotryptic peptide mapping. The polypeptide identified is the large subunit of Fraction I protein. A second discrete product is synthesized in the cell-free system but this polypeptide remains unidentified.

It does not appear that the method of poly-U Sepharose affinity chromatography, which is commonly used to assist in the purification of animal cytoplasmic mRNA (Adesnik *et al.*, 1972) will be of any use in the case of the Fraction I protein large subunit mRNA. /

mRNA. Wheeler and Hartley (1975) conclude that chloroplast mRNA lacks poly-A and it will not therefore bind to poly-U Sepharose.

Rosner *et al* (1975) have demonstrated the light-induced synthesis of an RNA species in greening *Spirodella* fronds. This RNA has the same molecular weight (approximately 0.5×10^6) as the fraction of total chloroplast RNA which is most effective in stimulating amino acid incorporation in a cell-free protein synthesizing system. The identity of this light-induced species has not been established and it may or may not be a mRNA.

ii. The function of chloroplast ribosomes.

The large number of plastid ribosomes raises several questions as to their function. Do chloroplasts synthesize any of their own proteins and, if so, which proteins? Is the abundance of plastid ribosomes necessary to produce a few proteins in larger amounts or many proteins in smaller amounts? The problem of identifying which proteins are synthesized by chloroplast ribosomes has been tackled in two ways.

a. Evidence from *in vivo* inhibitor studies.

The rationale of this approach is that if the synthesis of a chloroplast protein decreases in the presence of an inhibitor of chloroplast ribosomes, then synthesis must take place on chloroplast ribosomes *in vivo*. Conversely, if synthesis is inhibited in the presence of an inhibitor of cytoplasmic ribosomes then synthesis must occur in the cytoplasm. It is clear that the validity of this rationale depends however upon making a number of assumptions.

The first assumption is that the action of the inhibitor *in vivo* is to specifically inhibit ribosome function. There is considerable evidence accumulating to suggest that the most commonly used inhibitors, chloramphenicol and cycloheximide both have effects upon systems other than protein synthesis in some higher plants (see Table 3, Ellis, 1976a). For chloramphenicol at least this objection can be partially removed by relating the effects of D-threo chloramphenicol, which specifically inhibits protein synthesis by isolated chloroplast ribosomes, to the effects of other isomers of the drug which are not ribosomal/

ribosomal inhibitors in vitro (Ellis, 1969).

A second assumption made is that the appearance in the chloroplast of any particular protein in an active state does not depend upon additional protein (s) which are synthesized by a different class of ribosomes. A third problem often associated with, but not inherent in the use of inhibitors, arises if the increase in a protein is measured (often of necessity) in terms of enzymic activity. In this case the increased activity of the protein may be due either to de novo synthesis or activation of a precursor protein. These possibilities can be distinguished by the D_2O labelling technique (Filner and Varner, 1967). Bearing in mind these reservations Table D lists the chloroplast proteins which the use of inhibitors suggest are either the products of chloroplast ribosomes or whose synthesis involves plastid ribosomes. The small number of proteins listed in Table D implies that the bulk of the chloroplast proteins are synthesized on cytoplasmic ribosomes, a conclusion supported by numerous studies involving the use of cycloheximide (Boulter et al, 1972; Ellis, 1975b). Chloroplast ribosomes appear therefore to synthesize relatively few protein species, although one of their products, the large subunit of Fraction I protein, is the major plastid polypeptide.

b. Evidence from in vitro studies.

Analysing the products of protein synthesis in isolated chloroplasts provides the most direct method of elucidating the function of chloroplast ribosomes. The method is free of the difficulties encountered with inhibitor studies and provides unambiguous results. However it must be remembered that the results obtained by studying in vitro plastid protein synthesis must always be related back to the function of plastid ribosomes in vivo.

Prior to 1973 the characteristic of amino acid incorporation by isolated chloroplasts and the components of the protein synthesizing system of chloroplasts had been studied (Boulter et al, 1972). However no unequivocal demonstration of the synthesis of a chloroplast protein in vitro had been achieved (Kirk, 1970; Woodcock and Bogorad, 1971). In retrospect the difficulties seem to have arisen from a combination of the inability to isolate chloroplasts which would carry out protein synthesis at high rates and with fidelity, and the use of poor /

Table D. The products of chloroplast ribosomes.

1. Surzycki et al, 1970
2. Margulies, 1971
3. Armstrong et al, 1971
4. Smillie et al, 1971
5. Schiff, 1970
6. Ireland and Bradbeer, 1971
7. Ellis and Hartley, 1971
8. Hoerber, 1972
9. Criddle et al, 1970
10. Horak and Hill, 1972
11. Smillie et al, 1967
12. Bradbeer, 1975
13. Gregory and Bradbeer, 1973
14. Smith-Johannsen and Gibbs, 1972
15. Sirevag and Levine, 1972
16. Machold and Aurich, 1972
17. Apel and Schweiger, 1972
18. Eytan and Ohad, 1970
19. Goodenough, 1971
20. Thomson and Ellis, 1972

<u>PLASTID COMPONENT</u>	<u>SPECIES</u>	<u>INHIBITOR</u>	<u>REF.</u>
Ribulose 1, 5 biphosphate carboxylase	Chlamydomonas	spectinomycin	1
	Chlamydomonas	(spectinomycin (chloramphenicol*	2
	Chlamydomonas	spectinomycin	3
	Euglena	chloramphenicol*	4
	Euglena	streptomycin	5
	Phaseolus	(D-threo chloramphenicol (L-threo chloramphenicol	6
	Pisum	lincomycin	7
Ribulose 1, 5 biphosphate carboxylase (large subunit)	Chlamydomonas	chloramphenicol*	8
	Hordeum	chloramphenicol*	9
ATPase	Phaseolus	(D-threo chloramphenicol (L-threo chloramphenicol	10
Cytochrome f	Euglena	streptomycin	5
	Euglena	D-threo chloramphenicol	11
	Phaseolus	(D-threo chloramphenicol (L-threo chloramphenicol	12, 13
Cytochrome b ₅₆₃	Euglena	D-threo chloramphenicol	11
	Phaseolus	(D-threo chloramphenicol (L-threo chloramphenicol	12, 13
	Chlamydomonas	(spectinomycin (chloramphenicol*	3

Cytochrome b ₅₅₉ LP	Phaseolus	(D-threo chloramphenicol (L-threo chloramphenicol	12, 13
Ribosomal proteins	Pisum	lincomycin	7
Fatty acid synthetase	Ochromonas	D-threo chloramphenicol	14
	Chlamydomonas	spectinomycin	15
Unidentified thylakoid protein(s)	Vicia	D-threo chloramphenicol	16
	Acetabularia	chloramphenicol*	17
	Chlamydomonas	D-threo chloramphenicol	18
	Chlamydomonas	(spectinomycin (chloramphenicol*	19
	Chlamydomonas	chloramphenicol*	8
	Pisum	lincomycin	20
	Phaseolus	(D-threo chloramphenicol (L-threo chloramphenicol	12
	Ochromonas	D-threo chloramphenicol	14

* denotes isomer unspecified.

poor methods for analysing the products. In the majority of cases preparations contained principally broken chloroplasts (Parenti and Margulies, 1967; Spencer, 1965; Boardman et al, 1966). Consequently a massive dilution of chloroplast components, including any controlling factors for protein synthesis, must have taken place during isolation.

The significant innovation in this field was introduced by Blair and Ellis (1973) who realised that in order to obtain identifiable products in isolated chloroplasts, conditions in which correct elongation, termination and release of polypeptides occurred, must be used. Such conditions were most likely to be met in intact chloroplasts. These authors were accordingly able to demonstrate that isolated intact pea chloroplasts were able to synthesize discrete proteins using light as the source of energy. Even in intact plastids it was found that translation was not coupled to transcription. By the use of tryptic peptide fingerprinting Blair and Ellis were able to conclude that the only detectable soluble product of in vitro chloroplast protein synthesis was the large subunit of Fraction I protein. This was the first unequivocal identification of a protein that is synthesized by chloroplast ribosomes. This result also fully confirmed the conclusions from inhibitor studies on whole cells.

Analysis of the membrane-bound proteins in pea chloroplasts incubated under identical conditions (Eaglesham and Ellis, 1974) revealed that chloroplast ribosomes also synthesize five proteins of the chloroplast membrane. However the difficulties in analysing lipophilic proteins of this type has to date precluded their identification. Refining the analysis, Joy and Ellis (1975) have shown that two proteins uniquely associated with the chloroplast envelope membrane are the products of plastid ribosomes.

The study of protein synthesis in isolated chloroplasts has been extended to other species (Ellis, 1976a, Bottomley et al, 1974) with essentially similar results. It does not seem unlikely that the pattern seen in pea may turn out to be universal. Ellis (1976a) has also demonstrated that there is a division of labour in the chloroplast protein/

protein synthesizing system in that only free ribosomes are involved in the synthesis of the large subunit of Fraction I protein.

Assuming therefore that the polyacrylamide gel fractionation used in these studies is resolving individual protein species it is possible to conclude that only 8 proteins are known to be synthesized on the ribosomes of isolated chloroplasts.

In conjunction with the results of in vivo inhibitor studies these results lead to the conclusion that the high proportion of chloroplast ribosomes found in the leaf is required, not because they synthesize a wide range of proteins, but because one of their products, the large subunit of Fraction I protein, is part of the major chloroplast protein.

4. THE NATURE OF THE PROBLEM AND THE APPROACH ADOPTED IN THIS THESIS.

Clearly the outstanding problem in the field of plastid autonomy is that, assuming chloroplast DNA is transcribed asymmetrically, the function of up to 80% of the chloroplast genome remains obscure. Intuitively it seems unlikely that this proportion of the chloroplast DNA plays a solely structural role. Indeed the DNA/RNA (in excess) hybridization studies (section IBAiic), although preliminary, suggest that at least a high proportion if not all of the chloroplast DNA is transcribed into RNA at some time in the cell cycle. The failure to identify the function of the majority of chloroplast DNA probably therefore reflects limitations in the methods that have been used so far. These limitations may be theoretical ones, limitations of practicality, or perhaps limitations in the application of the methods.

At the present time it would seem that the most direct way to circumvent these limitations will be to develop either a homologous or heterologous system for the in vitro transcription of chloroplast DNA. Such a task may not be as daunting as it may at first appear. For example, it is now possible to asymmetrically transcribe the entire genome (mol. wt. 3.6×10^6) of the DNA tumour virus, polyoma, in vitro, using *E. coli* RNA polymerase (Kamen and Ziff, 1975). The complementary RNA synthesized is able to direct a cell-free protein/

protein synthesizing system from wheatgerm in the synthesis of all the structural proteins of the virus and also a protein related to the so called A gene product (Smith *et al*, 1975). With the improved methods of extracting chloroplast DNA in its native configuration (Kolodner and Tewari, 1975a) similar studies with chloroplast DNA ought to follow.

The role of the plastid ribosomes in the autonomy of the organelle seems more clear cut. Both *in vivo* and *in vitro* studies independently lead to the same conclusion. Only a very limited spectrum of proteins are synthesized on plastid ribosomes and their abundance is necessary only because they synthesize one particular polypeptide, the large subunit of Fraction I protein, in massive amounts. There is however a major reservation to be made, which provides the rationale for the work presented in this thesis.

It is quite clear from the literature that the etioplast already contains many of the components, although not all the functions, of the photosynthetic chloroplast. It seems quite probable therefore that plastids at stages of development before they have acquired the functionality of the mature organelle may nevertheless synthesize a wide and different spectrum of proteins to the chloroplast. The object of this thesis was to investigate exactly this possibility by identifying the products of the etioplast and the developing chloroplast protein synthesizing systems.

Pea plants provide a convenient experimental situation for these studies because they can be grown from seed in the dark for extended periods. The etioplasts which accumulate in the leaves can then be converted to chloroplasts by illumination under experimentally controlled conditions. It was decided to adopt the approach of *in vitro* etioplast protein synthesis because the method is direct and unambiguous. Also the only unequivocal reports on the identity of plastid ribosome products have resulted from using this approach with photosynthetic chloroplasts.

It was decided to also extend the investigation to studying the products of ribosome function in isolated developing chloroplasts. The possibility exists that there may be a number of proteins that are synthesized/

synthesized on plastid ribosomes only transiently during greening. Neither the in vivo inhibitor, nor the in vitro protein synthesizing studies to date have been designed to investigate this possibility. In addition if plastid translation is at any time coupled to plastid transcription (in the sense that the synthesis of plastid protein is simultaneously dependent upon the transcription of chloroplast DNA) then it seems most likely that this time will be during the greening phase when the synthesis of plastid proteins is maximally stimulated. Consequently therefore a study of plastid translation during greening may also give insights into the function of plastid DNA.

SECTION II - MATERIALS AND METHODS

1. MATERIALS

A. PLANT MATERIALS

i. Source.

Pea seeds (*Pisum sativum* L. var. Meteor) were purchased from S. Dobie, Chester, U.K.

ii. Growth conditions.

a. Etiolated seedlings.

Pea seeds were surface sterilised by soaking in 2% (v/v) sodium hypochlorite solution for 5 minutes. The seeds were then washed in running tap water for 24 hours. The imbibed seeds were sown in plastic trays containing moist vermiculite ('Micafil' from Dupre Vermiculite Ltd., Hertford, U.K.). The trays were incubated in darkness for 9 days at 22-24°C. The vermiculite was kept moist by occasional watering. Illumination for the handling of etiolated tissue was provided by a hand torch fitted with a green filter (Wratten Gelatin filter No. 54, Kodak Ltd., Hemel Hempstead, U.K.) (Wilkins, 1965). Etioplasts were isolated from the apical buds of dark-grown seedlings.

b. Normal seedlings.

Normal seedlings were grown by sterilising, imbibing and sowing seeds as described above. The seedlings were grown for 9 days at approximately 24°C under a 12 hour photoperiod. The illumination used was white light of 2000 lux intensity provided by Phillips 'Warmwhite' fluorescent tubes. The trays were watered each day. Chloroplasts were isolated from the youngest leaves.

c. Greening seedlings.

Greening seedlings were obtained by transferring 9 day old etiolated seedlings, grown as described above, into the light. The seedlings were illuminated by continuous white light of 2800 lux intensity ('Warmwhite' tubes). During greening of the seedlings the air temperature was kept at 22-24°C. The seedlings were watered each day. Plastids from greening seedlings were isolated from the first-formed leaflets.

B. CHEMICALS AND RADIOISOTOPES

i. Chemicals

The majority of chemicals and solvents used were of 'Analar' grade quality. Chemicals of specific purity and their source are listed below:

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

N-2-(hydroxyethylpiperazine-N'-yl) ethane sulphonic acid (HEPES)

N-tris (hydroxymethyl) methyl glycine (TRICINE)

2-amino-2-hydroxymethylpropane-1:3 diol (tris, TRIZMA base)

D-threo chloramphenicol

cycloheximide

blue dextran

glutaraldehyde

dansyl chloride

phenyl methyl sulphonyl fluoride (PMSF)

octyl phenoxy polyethoxy ethanol (TRITON X-100)

bovine serum albumin (BSA, crystallized and lyophilized)

ovalbumin

myoglobin (whale)

haemoglobin (sheep)

transferrin (human)

BDH Chemicals Ltd., Dorset, U. K.

bromophenol blue

sodium dodecyl sulphate (SDS, especially pure)

methanol (Aristar)

hydrogen peroxide (100 volumes)

pyridine (Analar)

butanol

Fisons Ltd., Loughborough, U. K.

osmium tetroxide

formic acid (98-99%)

acetic acid (glacial, Analar)

Polaron Equipment Ltd., Watford, U. K.

Epon 812 resin.

dodecenyl succinic anhydride (DDSA)

tri dimethylamine methyl phenol (DMP-30)

methyl endomethylene phthalic anhydride (MNA)

uranyl acetate

Koch-Light Ltd., Buck's, U. K.

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

2'-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-tetrazolium (INT)

Nuclear Enterprises (GB) Ltd., Edinburgh, U. K.

2, 5-diphenyloxazole (PPO)

1, 4-bis-(5-phenyloxazole-2-yl) benzene (POPOP)

Searle Diagnostic, Buck's, U. K.

coomassie brilliant blue R.

Fluka AG, Buchs, Switzerland

acrylamide

Eastman Organic Chemicals, N. Y., U. S. A.N, N¹ - methylene bisacrylamide.Hopkins and Williams Ltd., Essex, U. K.

Folin-Ciocalteu reagent

Whatman Ltd., Kent, U. K.

DEAE-cellulose (DE52 grade)

GF/C (2.5cm glass fibre discs)

Merke, Sharpe and Dohme, N. J., U. S. A.

actinomycin D (supplied free)

Parke Davis and Co., Wales, U. K.

L-threo chloramphenicol (supplied free)

Biorad, Kent, U. K.

Aminex A5 resin

Macherey-Nagel and Co., Düren, Germany

Polygram Sil G; 0.025mm layer (pre-coated 20cm x 20cm silica gel plastic t.l.c. plates)

Chicopee Mills Inc., N. Y., U. S. A.

Miracloth

Sephadex G-types were supplied by Pharmacia Ltd., London, U. K.

Photographic materials, including Kodirex X-ray film, were obtained from Kodak Ltd., Hert's, U. K.

ii. Radioisotopes

The following radioisotopic compounds were obtained from The Radiochemical Centre, Amersham, Buck's, U. K. :

L-(³⁵S) methionine (SJ 204, 200-300 Ci/mmole, 3-5 mCi/ml in aqueous solution containing 0.04% 2-mercapto-ethanol)

L-(³H) leucine (TRK 170, 50 Ci/mmole, 1 mCi/ml in sterilised aqueous solution containing 2% (v/v) ethanol)

L-phenyl (2, 3H) alanine (TRK 266, 20 Ci/mmole, 1 mCi/ml in sterilised aqueous solution containing 2% (v/v) ethanol)

Sodium (¹⁴C) bicarbonate (CFA 3, 56.5 mCi/mmole, 656 μ Ci/mg in dilute sterilised aqueous solution)

Iodine-125 (IMS 30, carrier-free 100 mCi/ml in NaOH solution)

n-(1-¹⁴C) hexadecane (CFA 101, 30-60 mCi/mmole in benzene solution)

C. ENZYMES AND SUBSTRATES

chloro-4-phenyl-3-L-tolucene-p-sulphonamido-butan-2-one (TPCK) treated trypsin was supplied by Worthington Biochemical Corp., N. J., U. S. A.

Pronase (Grade B) was obtained from Calbiochem, London, U. K.

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

creatine phosphokinase (rabbit muscle)

pancreatic ribonuclease A (bovine)

α -chymotrypsinogen A (bovine)

alcohol dehydrogenase (yeast)

carboxypeptidase A (bovine)

trypsin (bovine)

lactoperoxidase (from milk)
 L-methionine (Sigma grade)
 L-leucine (Sigma grade)
 L-phenylalanine (Sigma grade)
 ribulose 1, 5-bisphosphoric acid (Ru BP) (tetra-sodium salt)
 creatine phosphate (crystalline)
 ATP (grade 1)
 GTP (type 11S)
 dTTP
 CTP (type 111)
 UTP (type 1V)

2. METHODS

A. PLASTID ISOLATION

In order to minimize bacterial contamination of the plastid preparations two precautions were taken:

- i. Sterile media and glassware were used.
- ii. Plastic gloves were worn at all stages of the isolation procedure.

Plastids were isolated by the method of Ramirez *et al* (1968) as modified by Blair and Ellis (1973). The plastid isolation was performed rapidly (less than 10 minutes) and where possible solutions were kept at, or near, 4°C. Excised tissue (10-20g) was homogenized in 100 mls of semi-frozen isolation medium. The isolation medium contained,

0.35 M sucrose
 25 mM HEPES-NaOH (pH 7.6)
 2 mM EDTA
 2 mM sodium isoascorbate

The tissue was homogenized in a Willens Polytron (Northern Media Supply Co. Ltd., Hull, U.K.) at speed setting 7 (probe PC 20). The homogenate was strained through two lots of 8 layers of absorbent muslin and centrifuged at 2500 x g for 1 minute at 4°C (r_{max} 14.6cm) in an MSE 6L centrifuge. The supernatant fraction was discarded and the pellet carefully resuspended by gentle swirling in/

in 1-5 mls of KCl resuspension medium. The KCl resuspension medium contained,

0.2 M KCl
66 mM Tricine-KOH (pH 8.3)
6.6 mM $MgCl_2$

Tissue excision and plastid isolation were routinely carried out in normal daylight. However, for particular etioplast preparations the complete procedure was performed under dim green light (see Section III 2Bib).

A preparation containing only broken plastids was obtained by modifying the procedure described above as follows. After discarding the supernatant fraction the plastid pellet was re-suspended in 0.75-2.5 mls of sterile distilled water. After 1 minute an equal volume of 2 x 'KCl resuspension medium' containing 0.4 M KCl, 0.132 M Tricine-KOH (pH 8.3) and 13.2 mM $MgCl_2$ was added.

The protein concentration varied between 0.3-1.0 mg/ml for etioplast preparations and 4.0-9.0 mg/ml for chloroplast preparations.

A further modification of the isolation procedure was used to obtain certain etioplast preparations. After homogenization and filtration 5 ml aliquots of the brei were centrifuged individually. The homogenate was centrifuged at $2500 \times g$ for 1.5 minutes at $4^\circ C$ (r_{av} 21.08 cm). After discarding the supernatant fraction the pellets were resuspended in 0.5 ml of resuspension medium. The compositions of these resuspension media are described in the appropriate figure legends. The protein content of these etioplast preparations varied between 1.4 and 1.8 mg/ml.

B. MICROSCOPIC ANALYSIS OF PLASTID PREPARATIONS

i. Phase microscopy

a. Plastid counts

The number of plastids in a given volume of suspension was estimated by direct counting using a haemocytometer as described by Norris and Powell (1961). The haemocytometer grid was viewed at a magnification of x320 using a Gillet and Sibert 'Lab Lynx' phase microscope. The ratio of intact to broken plastids was determined by a method which makes use of the fact that when viewed by phase contrast microscopy/

microscopy intact plastids appear highly refractile whilst broken plastids appear non-refractile (Kahn and Von Wettstein, 1961; Wellburn and Wellburn, 1971). The precision schedules of Cassell (1965) were used to calculate the number of plastids which had to be counted in order to ensure (with 95% confidence) that the estimated ratio was no more than 15% in error.

b. Photomicrography

Plastid preparations were photographed at a magnification of $\times 725$ under phase optics in a Leitz Orthoplan microscope.

ii. Electron microscopy

The fixation, post-fixation and dehydration procedures were a modification of those used by Wellburn and Wellburn (1972). Etioplast pellets were prepared as described in Section IIA. The pellets were resuspended in 5 mls of 3% (v/v) glutaraldehyde in 0.3 M sucrose - 0.1 M phosphate buffer (pH 7.0) and left for 3 hours at 0°C. The plastids were then pelleted at 750 x g for 3 minutes, and given 3 successive 30 minute washes (5 mls each) in 0.1 M phosphate buffers (pH 7.0) containing 0.26, 0.23 and 0.2 M sucrose respectively. Post-fixation was carried out for 3 hours in 2% osmium tetroxide in 0.1 M phosphate buffer containing 0.14 M sucrose. The excess osmium was removed by three 30 minute washes in 0.14 M sucrose solution. The samples were then partially dehydrated by a graded alcohol series containing decreasing amounts of sucrose; i. e. 30 minutes in 5 mls each of 25, 40 and 50% ethanol which contained 0.08, 0.04 and 0.015 M sucrose respectively. Dehydration was completed by washing the pellets for 30 minutes in 5 mls each of 50, 75, 90 and 100% (twice) ethanol. The pellets were then transferred into 100% acetone and stored overnight. The samples were embedded in Epon resin next day. Fresh Epon resin was prepared by mixing 9 parts of mixture A (1.0 g of Epon 812 and 0.81 g of DDSA) with 1 part of mixture B (1.0 g of Epon 812 and 0.96g of NMA). A small amount (25 μ l) of DMP-30 was added for each 3 mls of resin mixture. The pellets were embedded by infiltrating for 12 hours in acetone/Epon (1:1, v/v), acetone/Epon (1:3, v/v) and Epon (twice). Finally/

Finally the resin was polymerized at 60°C for 48 hours and subsequently at room temperature for 3 days.

The pellets were sectioned on a Reichert OMU2 ultramicrotome, mounted on uncoated grids and double-stained in uranyl acetate and lead citrate (Reynolds, 1963). The sections were then examined in an AEI Corinth electron microscope.

C. AMINO ACID INCORPORATION IN ISOLATED PLASTIDS

i. Incubation

As a routine incubation mixtures using added ATP as the energy source had a final volume of 500 μ l and contained

100 μ mol.	KCl
33 μ mol.	Tricine-KOH (pH 8.3)
3.3 μ mol.	MgCl ₂
0.5 μ Ci	L-(³⁵ S) methionine (200 Ci/mmol)
1 μ mol.	ATP
plastids	

Antibiotics, inhibitors and enzymes were added to the reaction mixture by replacing a portion of the KCl resuspension medium with an equal portion of KCl resuspension medium plus the addition. All the compounds tested were soluble in KCl resuspension medium at the concentrations used. An ATP-generating system was added to the incubation by including 2.5 μ mol of creatine phosphate and 100 μ g of creatine phosphokinase in the reaction components. The components of incubations using light as the source of energy were identical except that ATP and the ATP-generating system were omitted. Plastids in the reaction mixture usually contained between 40 and 400 μ g of protein.

Reaction components were mixed at 2°C and the incubation was started by increasing the temperature of the incubation mixture to 20°C. This temperature was maintained during the incubation. A Churchill circulating water-cooler was used to control the temperature of the glass water bath in which protein synthesis incubations were performed. Light was excluded from the incubations using added ATP as the energy source by wrapping the reaction vessel in aluminium foil. For the light-driven system samples were illuminated with filtered red light (4000 lux) which was provided by an air-cooled Philips Photoflood/

Photoflood lamp. The incubations were terminated, usually after 1 hour, by the addition at 2°C of 100 μ l of a saturated aqueous solution of L-(³²S) methionine and 1 ml of 20% (w/v) trichloroacetic acid. All assays were carried out in duplicate.

ii. Assay

Amino acid incorporation was measured essentially according to the method of Sicevitz (1952). Terminated reaction mixtures were left to stand at 2°C for 30 minutes and then heated to 90°C for 15 minutes. The precipitate was transferred quantitatively to glass-fibre discs which had been previously washed with 5% (w/v) trichloroacetic acid and placed on to a Millipore 3025 sampling manifold. Each disc was washed by suction filtration with 100 mls of ice-cold 5% (w/v) trichloroacetic acid, 60 mls of ethanol (to remove carotenoids and chlorophyll) and finally with 60 mls of diethyl ether. The filters were dried at 60°C for 30 minutes and then placed in scintillation vials containing 8 mls of a toluene-based scintillation fluid. The scintillation fluid contained,

0.5% (w/v) 2,5 diphenyloxazole

0.03% (w/v) 1,4-bis-(5-phenyloxazol-2-yl) benzene

dissolved in toluene.

The radioactivity was determined in a Packard Tri-Carb model 3320 scintillation spectrophotometer. At 12% gain, with an open window, a counting efficiency of 70% was determined by comparison with an internal standard of (¹⁴C) hexadecane.

D. SOLUBILIZATION OF RADIOACTIVELY LABELLED ETIOPLASTS BY TRITON X-100 DETERGENT

An ATP-driven protein synthesis incubation mixture (Section II2Ci) was terminated after 1 hour by the addition of an equal volume (500 μ l) of 4% (v/v) Triton X-100 in KCl resuspension medium which had been saturated with L-(³²S) methionine. The final concentration of Triton X-100 was therefore 2%. The mixture was incubated at 20°C for 15 minutes and then centrifuged at 12500 \times g for 10 minutes at 4°C (r_{av} 6.7 cm, MSE 50). The supernatant and pellet fractions were then separated. An equal volume (1 ml) of 20% (w/v) trichloroacetic acid was added to the supernatant fraction and the pellet was resuspended /

resuspended in 1 ml of 10% (w/v) trichloroacetic acid. The protein in each fraction was precipitated overnight at 4°C. The flocculated precipitates were heated to 90°C for 15 minutes, quantitatively transferred to glass-fibre discs and assayed for amino acid incorporation as described in Section III.Cii.

E. ORGANELLE PURIFICATION

One technique which was used to study the site of amino acid incorporation in etioplast preparations was a procedure for subcellular fractionation involving a combination of rate and equilibrium sedimentation in sucrose gradients. This procedure also allows the one-step purification of mitochondria from whole leaf homogenates.

i. Gradient conditions

The gradient conditions were those described by Miflin and Beevers (1974) for the isolation of intact chloroplasts and proplastids. Density gradients were composed of sucrose solutions made up in 0.1 M Tricine-KOH (pH 7.6) buffer. The gradients contained, in sequence, 1 ml of 1.75 M sucrose, 9 mls of a linear gradient from 1.46 M to 0.73 M sucrose and a final 1.5 mls of 0.73 M sucrose. The gradients were formed and loaded at 4°C and used immediately. The gradients were loaded with 5 mls of sample. The sample was either 5 mls of whole leaf homogenate or 4 mls of whole leaf homogenate plus 1 ml of incubated ATP-driven protein synthesis mixture.

The protein synthesis mixture was prepared and incubated as described for the analysis of polypeptides synthesized in isolated etioplasts (Section II2Fi) with the exception that the reaction was terminated by the addition of L-(³²S) methionine alone. The whole leaf homogenate was prepared by grinding 10g of etiolated tissue for 4 seconds in the Polytron homogenizer in 100 mls of semi-frozen sterile grinding medium. The grinding medium contained,

0.70 M	Sucrose
25 mM	HEPES-NaOH (pH 7.6)
2 mM	EDTA
2 mM	Sodium isoascorbate
1 mM	2-mercaptoethanol

The sucrose concentration was increased to 0.875 M for
 • homogenates/

homogenates that were to be diluted one-sixth by addition of the incubated protein synthesis mixture. Gradients providing fractions to be assayed for succinate dehydrogenase activity were loaded with homogenates prepared using the basic grinding medium without 2-mercaptoethanol and sodium isoascorbate; these reductants interfere in the assay of succinate dehydrogenase. The homogenate was filtered through 8 layers of absorbent muslin and then 8 layers of Miracloth.

The loaded gradients were placed in a 3 x 23 ml rotor (r_{av} 9.37 cm) in an MSE 50 centrifuge with a controlled bowl temperature of 2°C. As soon as vacuum permitted the gradients were centrifuged at 1600 x g for 5 minutes and then at 10500 x g for a further 10 minutes. The rotor was allowed to decelerate to 5000 r.p.m. (2621 x g) before applying the rotor brake. The gradients were fractionated into samples of 0.5 ml using a Buchler Auto Densi-Flow gradient unloader run at 1 ml/min and a Gilson Microcol TCL 80 fraction collector. The pelleted fraction was resuspended in 0.5 ml of 1.75 M sucrose in 0.1 M Tricine-KOH (pH 7.6) buffer.

The appearance of the density gradient after centrifugation, and the appearance, under the phase contrast microscope, of particular fractions off the gradient are shown in Plate 1. After centrifugation a discrete opaque white band (band M) was clearly visible within the gradient (Plate 1. A). Microscopic inspection of fractions obtained from this region of the gradient (fractions No. 15 to 23) show a homogeneous population of subcellular organelles (Plate 1. B). The size, quantity and appearance of these organelles suggests they are mitochondria.

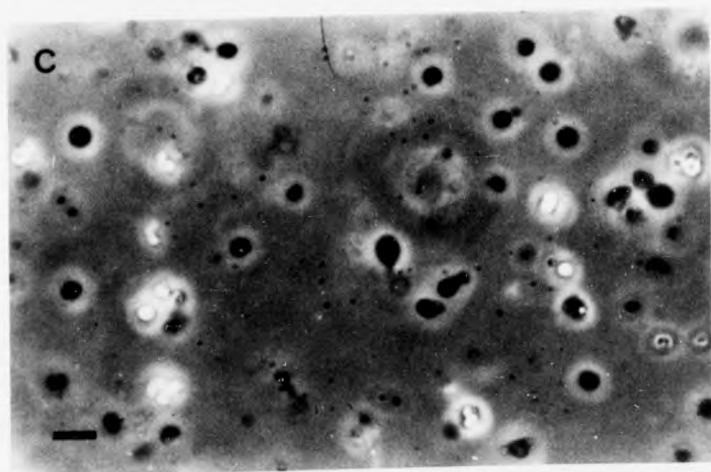
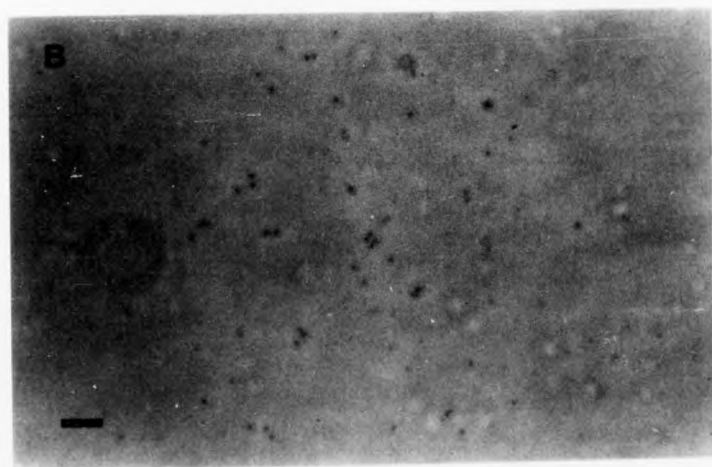
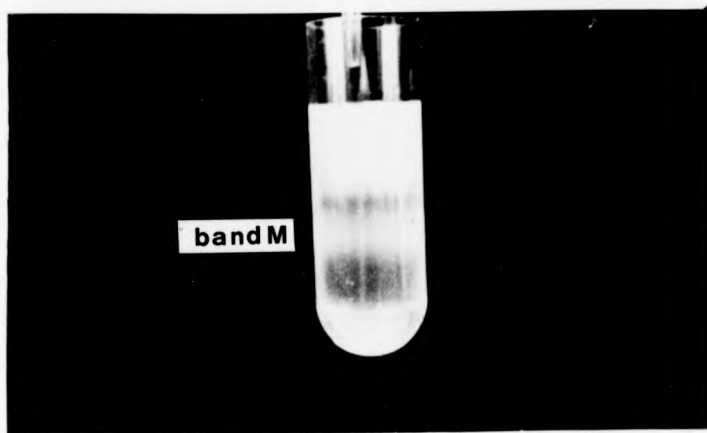
A second discrete fraction is visible as a yellow pellet in the bottom of the centrifuge tube. When viewed with the phase contrast microscope this fraction appeared to contain at least five types of subcellular body (Plate 1. C). The appearance and relative frequencies of these species suggested that this fraction contains both intact and broken plastids, nuclei, starch grains and mitochondria. To confirm the separation of subcellular organelles the gradient fractions were assayed/

Plate 1. Sucrose density gradient fractionation of whole leaf homogenates; visual analysis.

Whole leaf homogenates of etiolated tissue were fractionated as described in Section II2Ei. Photomicrography was performed as described in Section II2Bib.

- A. Total gradient after centrifugation.
- B. Phase contrast microscope appearance of band M fractions.
- C. Phase contrast microscope appearance of the resuspended pellet fraction.

The bar represents 10 μ m



assayed for succinate dehydrogenase and ribulose 1, 5 biphosphate carboxylase activity. These two enzymes are established markers for mitochondria and intact plastids respectively (Hallaway, 1965).

ii. Assay for succinate dehydrogenase

The assay for succinate dehydrogenase was performed according to Porteous and Clark (1965). Portions (100 μ l) of each gradient fraction were incubated for 15 minutes at 37°C in an incubation mixture containing,

100 μ l	0.2 M phosphate buffer (pH 7.3), containing 10 mM EDTA
50 μ l	0.3 M sucrose, adjusted to pH 7.0
100 μ l	0.5% (w/v) 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium (INT)
100 μ l	0.2 M sodium succinate in phosphate buffer

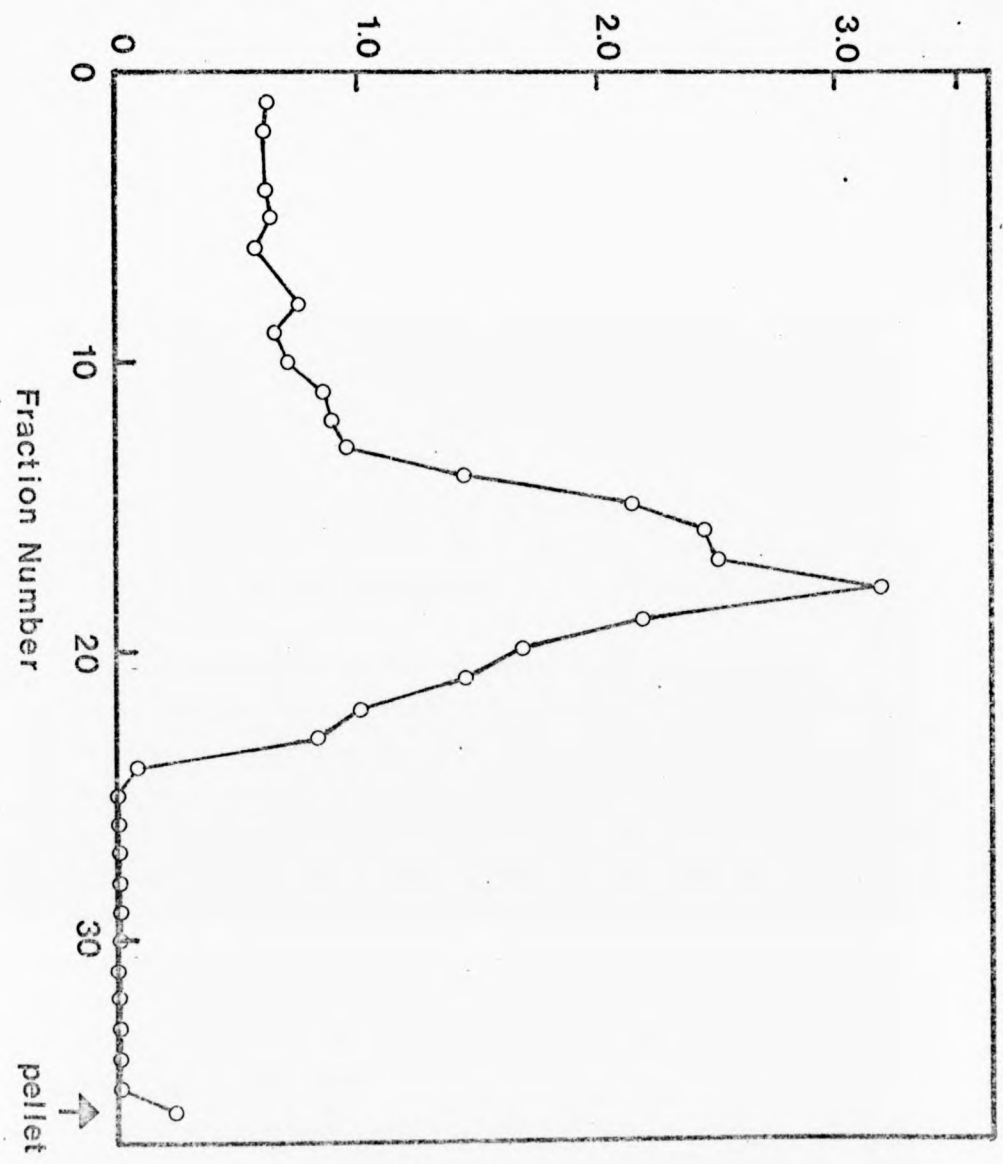
For each gradient fraction control incubations were performed in which the succinate or the enzyme preparation were replaced by water. Reaction components were mixed at 4°C and the reaction initiated by raising the temperature of the incubation to 37°C. The reaction was terminated by the addition, at 4°C, of 500 μ l of 20% (w/v) trichloroacetic acid. The reduced formazan produced in the assay was extracted into 1.5 ml of ethyl acetate and the extinction of the solution measured at 490 nm. A molar extinction coefficient (ϵ) for formazan in ethyl acetate of 20.1×10^3 litre . mol⁻¹ . cm⁻¹ is assumed (Pennington, 1961). The measured activities are considered qualitative as the kinetic parameters of the reaction were not investigated.

Figure 1. shows the distribution of succinate dehydrogenase activity in the gradient after fractionation of a whole leaf homogenate. A peak of succinate dehydrogenase activity is seen in the region of the gradient between fractions 15 and 24. This result confirms the microscopical evidence which suggested that free mitochondria sediment in this region of the gradient. Less than 1% of the succinate dehydrogenase activity distributed within the gradient is associated with the pelleted fraction. This amount of succinate dehydrogenase activity in the pelleted fraction is most likely due to a small percentage of mitochondria which bind to other cellular components.

Figure 1. Sucrose density gradient fractionation of a whole leaf homogenate; the distribution of succinate dehydrogenase activity in the gradient.

A whole leaf homogenate was prepared, fractionated and assayed for succinate dehydrogenase as described in Sections II2Ei and II2Eii.

succinate oxidation - nmoles/min/fraction



iii. Assay for ribulose 1, 5 biphosphate carboxylase (RuBPCase)

The assay for RuBPCase is essentially that described by Paulsen and Lane (1966). This assay is based on the fixation of $^{14}\text{CO}_2$ into acid-stable products.

Gradient fractions were added to an equal volume (500 μl) of 2 x assay buffer plus 0.2% (v/v) Triton X-100. The assay buffer contained,

25 mM Tris-HCl (pH 7.8)

1 mM EDTA

10 mM MgCl_2

10 mM 2-mercaptoethanol

A 100 μl aliquot of this enzyme preparation was then mixed with 10 μl of 10 mM sodium (^{14}C) bicarbonate (5 μCi) in an open reaction vessel. After 10 minutes pre-incubation at 25°C the reaction was initiated by the addition of 100 μl of 2 mM ribulose 1, 5 biphosphate dissolved in assay buffer. The incubation was continued for 20 minutes at 25°C and was then terminated by the addition of 100 μl of 1N HCl. After termination of the reaction the incubation vessels were flushed with nitrogen to remove unreacted $^{14}\text{CO}_2$.

Aliquot portions (100 μl) of the reaction mixture were transferred onto Whatman GF/C glass-fibre discs and the filters were dried at 60°C for 30 minutes. Radioactivity on the filters was determined by liquid scintillation counting as described in Section II2Cii. The efficiency of counting carbon-14 was assumed to be 70%.

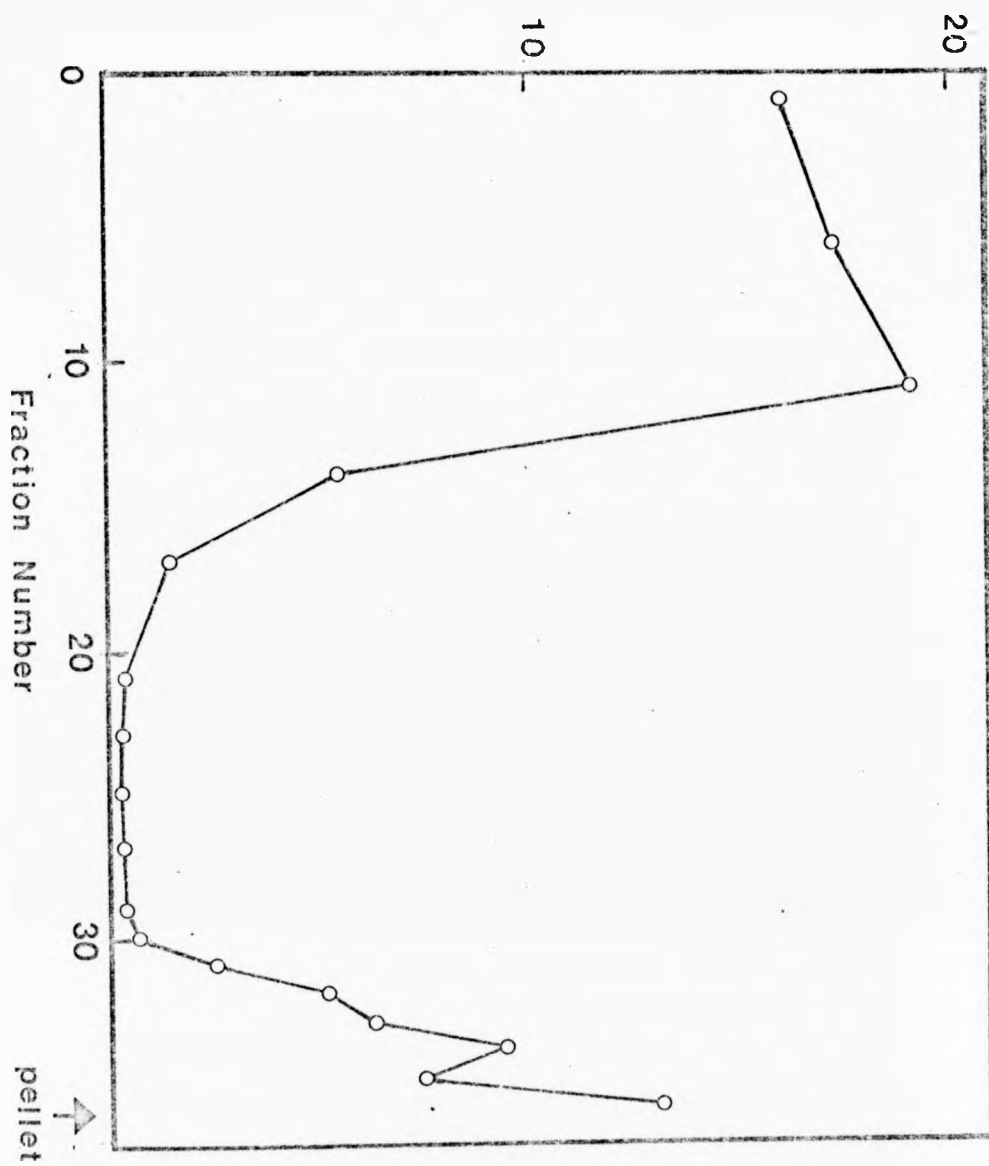
Controls were performed by replacing the ribulose 1, 5 biphosphate solution by an equal volume of assay buffer. Although it was established that the measured activities were proportional to the enzyme concentration the activities must be considered qualitative since other kinetic parameters of the reaction were not taken into account (see Section I2Ai).

Figure 2 shows the distribution of RuBPCase activity within the density gradient after fractionation of a whole leaf homogenate. Two areas within the gradient show RuBPCase activity. Approximately half of the activity is found in fractions 1 to 14. These fractions represent/

Figure 2. Sucrose density gradient fractionation of a whole leaf homogenate; the distribution of RuBPCase activity in the gradient.

A whole leaf homogenate was prepared, fractionated and assayed for RuBPCase activity as described in Sections II2Ei and II2Eiii.

CO₂ fixed - nmoles/min/fraction



represent the volume and position of 0.70 M and 0.73 M sucrose solutions in the gradient. This RuBPCase activity is presumably due to enzyme which is released upon breakage of the plastids during their isolation. Subcellular bodies were not seen in fractions taken from this region of the gradient. Virtually no RuBPCase activity is detected in fractions 15 to 29 of the gradient. The remaining RuBPCase activity is distributed between the pellet fraction and fractions 30 to 35. This activity is presumed to be enzyme located in intact plastids. This location of the RuBPCase enzyme marker confirms the microscopical evidence which suggested that one type of subcellular body which sediments at the bottom of the gradient is intact plastids.

Figures 1 and 2, taken together, show that a clear separation of mitochondria and intact plastids can be achieved by this method.

F. ELECTROPHORETIC ANALYSIS OF POLYPEPTIDES SYNTHESIZED IN ISOLATED PLASTIDS.

i. Pre-electrophoresis

a. Incubation

Plastids isolated from 40g of tissue were resuspended in 3 mls of KCl resuspension medium (Section II2A). Light-driven protein synthesis incubation mixtures which were used for product analysis had a final volume of 1 ml and contained,

200 μ mol.	KCl
66 μ mol.	Tricine-KOH (pH 8.3)
6.6 μ mol.	MgCl ₂
100 μ Ci.	radioactively labelled amino acid (L-(³⁵ S) methionine, 300 Ci/mmol) (L-(³ H) leucine, 50 Ci/mmol) (L-phenyl 2,3-(³ H) alanine, 20 Ci/mmol)
	plastids (1-6mg of protein)

ATP-driven protein synthesis incubation mixtures contained, in addition to the above components, 2 μ mol of ATP, 5 μ mol of creatine phosphate and 100 μ g of creatine phosphokinase. The reaction components were mixed at 2^oC and the incubations were started by raising the temperature/

temperature of the incubation mixture to 20°C. This temperature was maintained during the incubation. The ATP-driven system was incubated in the dark, whereas incubations with light as the energy source were illuminated with filtered red light as described in Section II2Ci. After 1 hour the incubations were terminated by the addition, at 4°C, of 200 µl of a saturated aqueous solution of L-(³²S) methionine and 120 µl of a 20% (w/v) solution of sodium dodecyl sulphate. In preparation for electrophoresis the terminated incubation mixtures were boiled for 3 minutes and then exhaustively dialysed at room temperature against 5 litres of dialysis buffer. The dialysis buffer contained,

- 2.5 mM Tris-19 mM glycine (pH 8.5)
- 100 mM 2 mercaptoethanol
- 0.2% (w/v) sodium dodecyl sulphate

At room temperature an insoluble precipitate of potassium dodecyl sulphate forms when sodium dodecyl sulphate is added to the incubation mixtures. This precipitate disappears during dialysis.

b. Preparation of 150000 x g etioplast supernatant fraction.

Etioplasts were isolated and incubated with ATP as the energy source for the analysis of the products as described in Sections II2A and II2Fi. The incubation was terminated by the addition of L-(³²S) methionine alone. The incubation mixture was then centrifuged at 2,500 x g for 2 minutes at 2°C (r_{max} 14.6 cm). The supernatant fraction and pellet were separated and the pellet was resuspended in 1.2 mls of buffer containing 2.5 mM Tris-glycine (pH 8.5) and 4 mM 2-mercaptoethanol. This buffer lyses the plastids. The supernatant fraction was added to the lysed etioplast preparation and centrifuged at 150000 x g for 100 minutes at 2°C (r_{av} 7.62 cm) using a 3 x 5 mls swing-out rotor fitted with 1 ml tube adaptors in an MSE 50 centrifuge. After centrifugation of the sample the clear supernatant was removed and 240 µl of an aqueous solution of 20% (w/v) sodium dodecyl sulphate was added. The 150000 x g supernatant fraction was boiled for 3 minutes, cooled to room temperature and exhaustively dialysed at room temperature against 5 litres of dialysis buffer.

c. Pronase and ribonuclease A treatment of polypeptides synthesized in isolated etioplasts.

Incubation for the analysis of the products formed in isolated etioplasts was performed as described in Section II2Fia. The incubation was terminated by the addition of L-(³²S) methionine alone and a lysed etioplast preparation was prepared as described in Section II2Fib. After the supernatant fraction had been added to the lysed etioplast preparation the protein concentration was determined and aliquots (600 μ l) were taken for enzymic treatment.

Pronase and pancreatic ribonuclease A were freshly prepared at a concentration of 1 mg/ml in 50 mM Tris-380 mM glycine (pH 8.5) containing 10 mM 2-mercaptoethanol. The enzymes, in a volume of 250 μ l, were added to the lysed etioplast preparations to give protein to enzyme ratios of 10:1 (pronase) and 20:1 (ribonuclease) respectively. An equivalent volume of buffer was added to a third aliquot to act as a control. The samples were incubated at 37°C for 3 hours. The incubations were terminated by the addition of 85 μ l of 20% (w/v) sodium dodecyl sulphate and boiling for 3 minutes. The solubilized digests were then exhaustively dialysed at room temperature against 5 litres of dialysis buffer.

d. Acetone extraction of radioactively labelled plastid preparations.

Incubation mixtures for the analysis of the products of plastid protein synthesis in vitro were performed as described in Section II2Fia. After dialysis 1 ml aliquots of the preparation were mixed for 1 minute at room temperature with 9 mls of acetone. The sample was centrifuged at 1000 \times g for 5 minutes and the pellet and supernatant fractions separated. The supernatant was discarded and the pellet resuspended in 10 mls of 90% (v/v) acetone. This extraction procedure was repeated twice. The final precipitate was dried at 60°C for 10 minutes and resuspended in 1 ml of 2.5 mM Tris-19 mM glycine (pH 8.5) containing 100 mM 2-mercaptoethanol and 2% (w/v) sodium dodecyl sulphate. The sample was boiled for 3 minutes and exhaustively dialysed at room temperature against 5 litres of dialysis buffer.

ii. Analytical SDS-polyacrylamide gel electrophoresis.

a. Disc gel electrophoresis.

The polyacrylamide gel electrophoresis system used contained sodium dodecyl sulphate and urea in the buffers. Electrophoresis was performed at room temperature. Both acrylamide and bis-acrylamide were used without recrystallization.

Gels consisted of 15% (w/v) acrylamide-0.3% bis-acrylamide made up in a buffer containing 0.375 M Tris-HCl (pH 8.5), 0.1% (w/v) sodium dodecyl sulphate and 3% (w/v) urea. The gel components were mixed in a round-bottomed flask. The mixture contained,

5 mls	3 M Tris-HCl (pH 8.5)
5 mls	24% (w/v) urea / 0.8% (w/v) SDS
10 mls	60% acrylamide - 1.2% bis-acrylamide
20 mls	0.14% (w/v) ammonium persulphate (freshly prepared)
23 μ l	TEMED (N, N, N, N'-tetramethylene diamine)

This mixture was rapidly degassed and transferred to glass tubes (6mm x 100mm) which were sealed at one end with moist dialysis tubing. The acrylamide solution was gently overlaid with 200 μ l of distilled water using an Eppendorf pipette. This produces a flat top to the gel upon polymerization. The gels polymerized in 15 minutes. The gels were transferred to electrophoresis tanks containing running buffer. The running buffer contained,

50 mM	Tris-380 mM glycine (pH 8.5)
	0.1% (w/v) sodium dodecyl sulphate
10 mM	2-mercaptoethanol
	or
8 mM	cysteine

The top gel-tank compartment contained the cathode and proteins electrophoresed down through the gel towards the anode in the bottom gel-tank compartment. The gels were prerun at 10 mA/gel for 3 hours to remove excess ammonium persulphate. Samples (20-200 μ l, containing 20-400 μ g of protein) were layered directly on the gels with sucrose (10%, w/v) and bromophenol blue (0.005%, w/v). The samples were/

were concentrated by applying a current of 0.5 mA/gel for 30 minutes and then electrophoresis was carried out at 5 mA/gel. The bromophenol blue was used as a marker to determine the duration of electrophoresis which achieved maximum separation of proteins.

After electrophoresis of the proteins the gels were simultaneously fixed and stained in a solution of coomassie brilliant blue R (0.1%, w/v) in 50% (v/v) methanol and 7.5% (v/v) acetic acid. After staining for 12 hours the excess stain was removed by washing in 50% methanol containing 7.5% acetic acid. The gels were scanned at 620 nm in a Joyce-Loebl Chromascan. The distribution of radioactivity within the gel was determined in the following manner.

Firstly the gels were swollen in 7.5% (v/v) acetic acid to remove the methanol. The gels were then frozen at -90°C for 30 minutes and the frozen gels were sliced into 1 mm slices with a Mickle gel slicer. The slices were solubilized in 200 μl of H_2O_2 (100 vols) for 3 hours at 80°C in capped scintillation vials. After cooling the vials 8 mls of Triton X-100 - toluene scintillation fluid was added. This scintillation fluid contained,

0.4% (w/v) 2,5-diphenyloxazole

0.05% (w/v) 1,4-bis-(5-phenyloxazol-2-yl) benzene
in toluene - Triton X-100 (2:1, v/v).

The samples were counted for radioactivity in a Packard Tri-Carb model 3320 liquid scintillation spectrophotometer. In samples containing L- (^{35}S) methionine alone the machine settings were 15% gain and open window. The radioactivity was counted at an efficiency of 88% as shown by comparison with internal standards of (^{14}C) hexadecane. In gel slices containing both L- (^{35}S) methionine and L- (^3H) leucine the radioactivity due to each isotope was estimated by the channels ratio method of Hendler (1964).

b. Slab gel electrophoresis; calibration for molecular weights estimated on 15% polyacrylamide gels.

The relationship between the molecular weight of a protein and its electrophoretic mobility in 15% SDS-polyacrylamide gels was determined using a slab gel electrophoresis system. The principal advantage of using this system is that variations between individual gels/

gels, both in their uniformity (e. g. pore size) and in the conditions of electrophoresis, are minimized. A disadvantage of the system is that the small amount of protein which can be electrophoresed in each sample necessitates the use of radioactively-labelled calibration proteins. In addition the resolution of the slab gel system was found to be poor by comparison with disc gel electrophoresis.

The slab gel apparatus was essentially that explicitly described by Studier (1973). The gel system used, i. e. the composition of the gel and running buffers, is exactly as described for disc gel electrophoresis in the preceding section. The following proteins were used for calibration and their molecular weights are assumed to be the values given in brackets:-

human transferrin (78000), bovine serum albumin (68000 for the monomer), ovalbumin (43000), yeast alcohol dehydrogenase (37000), carboxypeptidase A (34600), chymotrypsinogen (25000), bovine trypsin (23300), whale myoglobin (17200) and sheep haemoglobin (15000).

Each of these proteins was iodinated with iodine-125 as described in Section II2H, and prepared for electrophoresis by adding sucrose and bromophenol blue. The samples were diluted with appropriate volumes of running buffer to give a radioactive concentration of 50000 c. p. m. / 15 μ l.

Aliquots (15 μ l) of each sample were co-electrophoresed at 2 volts/cm for approximately 24 hours. After electrophoresis the position of the bromophenol blue marker dye was noted, and the gel was dried between silicone rubber sheets under vacuum in a water bath at 90°C. The dried gel was exposed to Kodirex X-ray film for 4 days. The mobility of each protein was determined from the resulting autoradiograph.

Figure 3A shows an autoradiograph of a calibration gel and from this data the known molecular weights of the calibration proteins can be plotted as a function of their relative mobilities, (R_m), (figure 3B). Although there appears to be considerable degradation of the marker proteins, possibly occurring during iodination, the major radioactive species in each sample is assumed to be the authentic marker protein. The calibration curve is found to be biphasic. This is in agreement with/

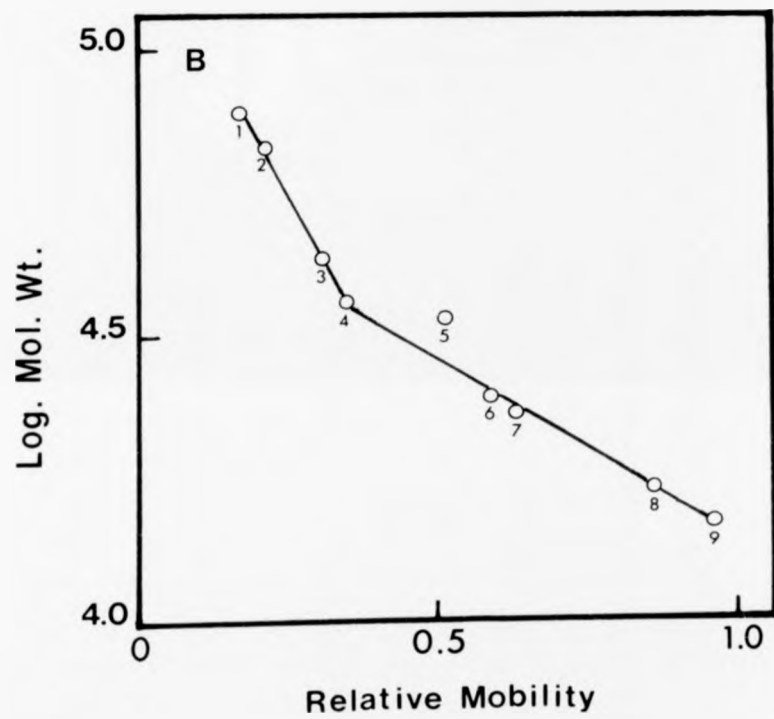
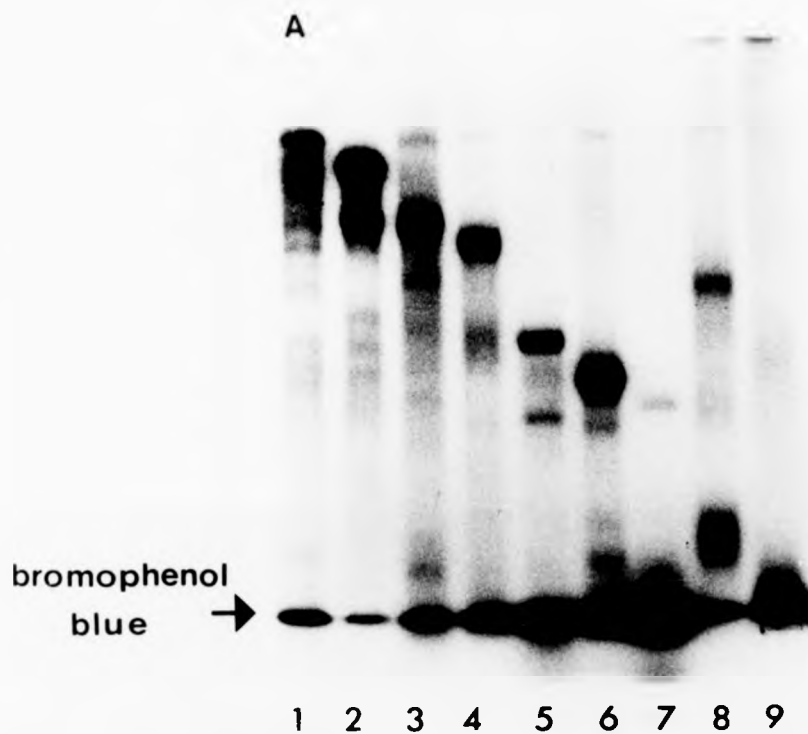
Figure 3 Calibration of 15% SDS-polyacrylamide gels.

The mobilities of purified proteins of known molecular weight were determined as described in Section II2Fii.

A. Calibration gel autoradiograph

1. transferrin
2. B. S. A.
3. ovalbumin
4. alcohol dehydrogenase
5. carboxypeptidase A
6. chymotrypsinogen
7. trypsin
8. myoglobin
9. haemoglobin

B. A semi-log plot of molecular weight against relative mobility for 9 proteins of known molecular weight.



with the results of Neville (1971) and Eaglesham and Ellis (1974). The calibration curve was used to estimate the molecular weights of proteins fractionated by 15% SDS-polyacrylamide gel electrophoresis.

iii. Preparative gel electrophoresis of the 150000 x g supernatant fraction from isolated etioplasts.

The dialysed, sodium dodecyl sulphate-treated 150000 x g supernatant fraction prepared as described in Section II2Fib was further fractionated by the preparative polyacrylamide gel electrophoresis method of Moore and Burke (1974). Electrophoresis was performed on cylindrical columns (18 mm x 220 mm) of 15% (w/v) acrylamide - 0.3% (w/v) bisacrylamide. The gels were prepared and run in the same sodium dodecyl sulphate-containing buffer system described in Section II2Fiia, adapting the volumes of the components to the requirements of the preparative system. The gels were prerun at 5 mA/gel for 20 hours. Sucrose crystals (0.2 mg) were added to 2 mls of dialysed preparation (containing 4 mg of protein) and the samples were loaded directly onto the gels using a disposable hypodermic syringe. The samples were concentrated on top of the gel by applying a current of 3 mA/gel for 3 hours before electrophoresis at 5 mA/gel for 30 hours. The gels were electrophoresed at room temperature. After electrophoresis a single area of the gel was excised for recovery of the radioactive sample. This area corresponded to the position of the large subunit from Fraction I protein. This position in the gel was determined from the mobility of dansylated pea Fraction I protein fractionated in parallel gels. Pea Fraction I protein was prepared as described in Section II2Gii. After excision from the gel cylinder the radioactive protein was eluted from the gel section in 0.1% (w/v) sodium dodecyl sulphate containing 1 mM phenyl methane sulphonyl fluoride at 37°C for 16 hours. After elution the gel pieces were removed by centrifugation at 1500 x g for 10 minutes at room temperature. The supernatant fraction was then passed through a Whatman GF/C glass-fibre disc by centrifugal filtration at 2500 x g for 15 minutes to ensure removal of all polyacrylamide gel pieces. Finally the eluted protein was precipitated by the addition of 0.4 volumes of 100% (w/v) trichloroacetic acid (final concentration of 20% (w/v) trichloroacetic/

trichloroacetic acid) for 30 minutes at 4°C. The precipitated protein was pelleted by centrifugation at 10000 x g for 30 minutes at 2°C and the pellet was washed by resuspension and centrifugation (at 10000 x g for 20 minutes) in ethanol-diethyl ether (3:1, v/v) (twice) and ether (once). This material was dried at 30°C for 30 minutes and then stored at 4°C until used. The homogeneity of this material is discussed in Section III3B.

G. PURIFICATION OF PEA FRACTION I PROTEIN

i. Purification of unlabelled pea Fraction I protein

Unlabelled Fraction I protein was purified from 10-15 day-old pea seedlings grown as described in Section IAiib. The method is described by Blair and Ellis (1973) and is a modification of a procedure of Kawashima and Wildman (1971). All operations were performed at 4°C. Leaves (200g) were blended in 300 mls of ice-cold buffer A in an Atomix blender for 60 seconds. Buffer A contained,

0.025 M	Tris-HCl (pH 7.4)
0.05 M	NaCl
1 mM	MgCl ₂
0.5 mM	EDTA
0.04 mM	2-mercaptoethanol
2 mM	phenyl methane sulphonyl fluoride

The homogenate was strained through 8 layers of muslin and centrifuged at 10000 x g for 30 minutes (r_{av} 14.4 cm) in an MSE 18 centrifuge. The supernatant was removed and further centrifuged at 105000 x g for 60 minutes (r_{av} 7.62 cm) in an MSE 50 centrifuge. The yellowish 105000 x g supernatant fraction was passed through a column of coarse grade Sephadex G-25 (6.5 cm x 50 cm) at a flow rate of 20 mls/minute using buffer B as the eluting buffer. Buffer B contained,

0.025 M	Tris-HCl (pH 7.4)
0.05 M	NaCl
0.5 mM	EDTA
10 mM	2-mercaptoethanol

The protein which was eluted in the void volume (low molecular /

molecular weight contaminants were retarded on the column) was collected. Solid $(\text{NH}_4)_2\text{SO}_4$ was added and the precipitate which appeared between 35% and 45% saturation was collected by centrifugation at 10000 x g for 10 minutes. The pellet was resuspended in 2 mls of Buffer C. Buffer C contained,

0.05 M Tris-HCl (pH 8.0)
 0.5 mM EDTA
 10 mM 2-mercaptoethanol

The protein solution was dialysed overnight against 1 litre of buffer C.

The dialysed solution was absorbed onto a column (1.5 cm x 15 cm) of DEAE-cellulose, previously equilibrated with buffer C. The column was thoroughly washed with more buffer C and Fraction I protein was then eluted with buffer C containing 100 mM NaCl. Protein was precipitated by adding solid ammonium sulphate to 50% saturation, and the precipitate was spun down at 10000 x g for 10 minutes and resuspended in 5 ml of buffer B. Finally the protein was applied to a Sephadex G-200 column (2.5 cm x 90 cm) and eluted with buffer B at a flow rate of 2.5 mls/hour. Fraction I protein elutes just after the void volume. Fractions from this part of the elution profile (with A_{280}/A_{260} of 1.8 or greater) were pooled and the protein precipitated from them with 50% saturated ammonium sulphate. The precipitate was centrifuged to a pellet at 10000 x g for 10 minutes. The pellet was resuspended in a volume of sterile distilled water and dialysed against 1 litre of sterile distilled water overnight. The dialysed sample was freeze-dried.

The purity of the material prepared by this procedure has been established by Blair and Ellis (1974). For use as a marker protein the purity of the material was not critical and was therefore not independently verified.

ii. Dansylation of purified unlabelled pea Fraction I protein.

Pea Fraction I protein prepared as described in Section II2Gi was dansylated essentially as described by Talbot and Yphantis (1971). Freeze-dried protein (3 mg) was dissolved in 400 μl of phosphate-buffered saline (PBS). Phosphate-buffered saline contained,

239 mM NaCl
 2.8 mM KCl

9 mM Na_2HPO_4
 1.5 mM KH_2PO_4 (pH 7.4)

The protein solution was mixed with 200 μl of an aqueous 10% (w/v) solution of sodium dodecyl sulphate and 20 μl of 10% (w/v) dansyl chloride in acetone. The dansyl chloride solution must be freshly prepared. The mixture was vigorously agitated and then boiled for 3 minutes at 100°C. After cooling the mixture, 5 μl of 2-mercaptoethanol was added and the mixture was boiled for 1 minute. The sample was applied to a short (0.8 cm x 10 cm) column of Sephadex G-25 (fine grade) which had been previously equilibrated with phosphate-buffered saline containing 1% (w/v) sodium dodecyl sulphate and 1% (v/v) 2-mercaptoethanol (elution buffer). Elution of the dansylated protein from the column was visualized by viewing the column under ultraviolet illumination (334 nm). Unreacted dansyl chloride was slightly retarded in elution from the column. The faster moving band of dansylated protein had a characteristic yellow fluorescence whilst the dansyl chloride appeared blue. The dansylated Fraction I protein solution (approximately 2 mls) was collected and stored at -20°C.

Before electrophoresis the sample was boiled again for 1 minute and 2-mercaptoethanol was readded to a final concentration of 1% (v/v). Sucrose (10%, w/v) and bromophenol blue (0.005%, w/v) were also added to the sample before electrophoresis. The sample was loaded onto the preparative gels as a phosphate-buffered saline solution.

iii. Purification of in vivo radioactively labelled pea Fraction I protein.

Labelled Fraction I protein was prepared by the method of Blair and Ellis (1973) from the leaves of excised pea seedlings that had been fed with L-(³⁵S) methionine during greening. The method of Fraction I protein purification is essentially as described in Section II2Gii except that chromatography columns of smaller dimensions were used to minimize losses.

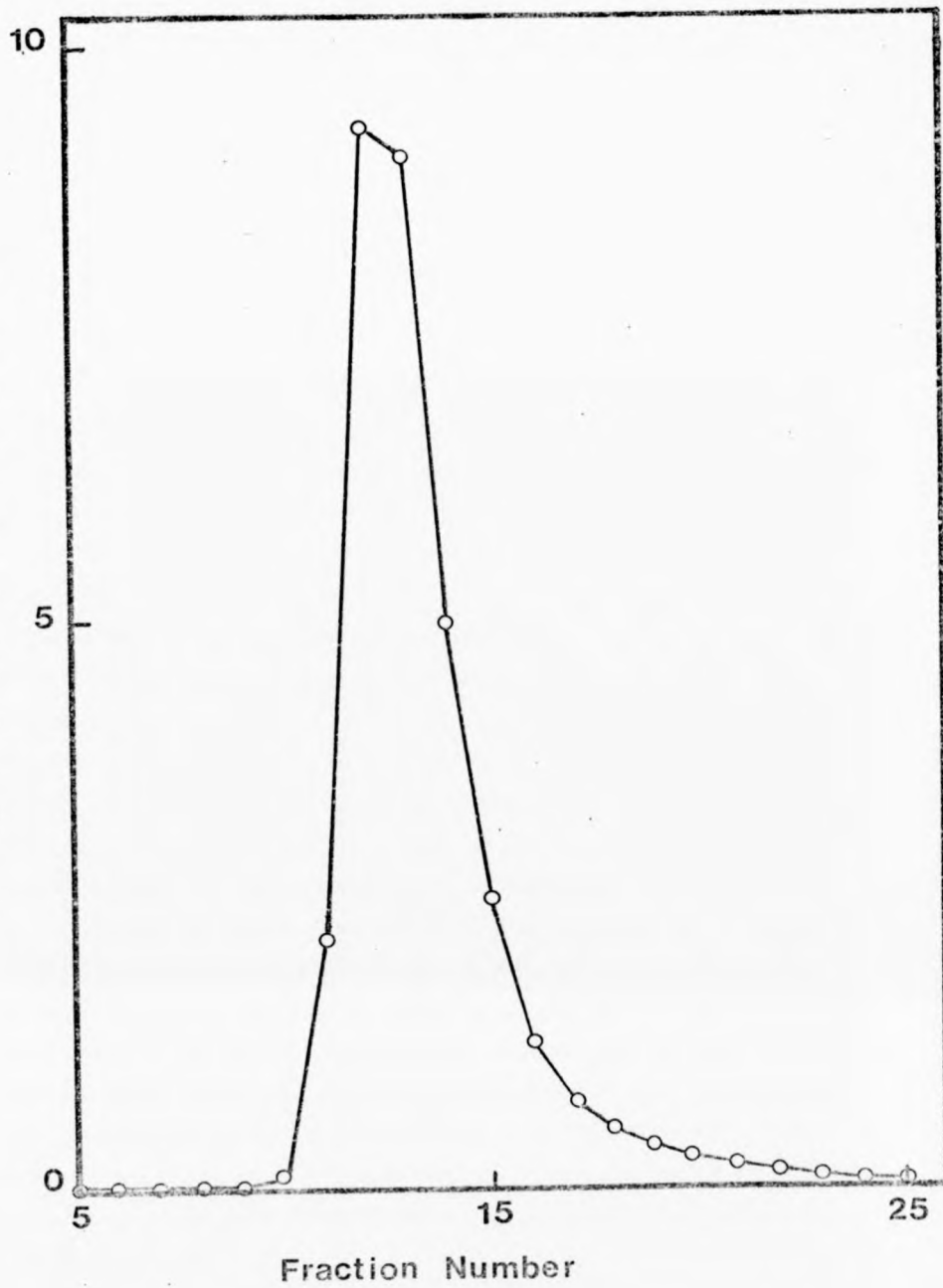
Etiolated pea seedlings were grown as described in Section IAiia. A total of 80 apices were excised under water about 5 cm below the apex. The cut apices were placed in 20 small vials, each containing/

containing 0.5 ml of sterile distilled water with 10 μCi of L-(^{35}S) methionine (0.56 μM). The shoots were then illuminated with light of 4000 lux intensity provided by "Warmwhite" fluorescent tubes. After 24 hours illumination a further 10 μCi of L-(^{35}S) methionine was added to each vial, and the illumination was then continued for a further 48 hours. The vials were regularly topped up with sterile distilled water during the greening period. After feeding with radioactively labelled amino acid the green apices were harvested (approximately 6 g fresh weight) and ground in 20 ml of buffer A in a chilled mortar. The homogenate was filtered through absorbent muslin and centrifuged at 10000 x g for 30 minutes (r_{av} 9.37 cm). The supernatant fraction was collected and further centrifuged at 105000 x g for 60 minutes (r_{av} 5.61 cm). The 150000 x g supernatant was passed through a column (2.5 cm x 30 cm) of Sephadex G-25 (coarse grade) at a flow rate of 15 ml/hour. Protein was eluted at the void volume with buffer B. Ammonium sulphate fractionation was performed as described in Section II2Gii and the precipitate (obtained between 35% and 45% saturation) was centrifuged, resuspended in 2 ml of buffer and dialysed overnight against 1 litre of buffer C. DEAE-cellulose chromatography of the dialysed sample was performed as described earlier using a 0.8 cm x 15 cm column. The eluted Fraction I protein was precipitated by adding solid ammonium sulphate to 50% saturation. The precipitate was spun down and resuspended in 1 ml of buffer B. The protein solution was applied to a Sephadex G-200 column (1.5 cm x 40 cm) and eluted with buffer B at a flow rate of 5 ml/hour. Aliquots (100 μl) were removed from each fraction, added to 8 ml of Triton-toluene scintillation fluid and counted for radioactivity at 12% gain, open window, in a Packard Tri-Carb spectrophotometer. Figure 4 shows the elution of labelled Fraction I protein from Sephadex G-200. Peak fractions were collected and ammonium sulphate added to 50% saturation. The precipitate was centrifuged and either dissolved directly in alkaline phosphate buffer (see next section) or was resuspended in a small volume of 2.5 mM Tris-19 mM glycine (pH 8.5) containing 10 mM 2-mercaptoethanol and stored at 4°C in solution. The yield of purified/

Figure 4. Purification of in vivo radioactively-labelled
pea Fraction I protein; chromatography on Sephadex G-200.

The experimental details are given in Section II2Giii.

Radioactivity - c.p.m. $\times 10^{-4}$ / 100 μ l aliquot



purified radioactively-labelled Fraction I protein was approximately 12 mg.

iv. Preparation of large and small subunits from in vivo radioactively-labelled pea Fraction I protein.

Purified L-(³⁵S) methionine-labelled Fraction I protein was prepared as described in Section II2Giii. The protein was dissociated into its constituent subunits by solubilization in 1.0 ml of 50 mM Na₂HPO₄/NaOH buffer (pH 11.2, alkaline phosphate buffer) (Kawashima and Wildman, 1971). The subunits were separated by applying the sample to a column (1.5 cm x 90 cm) of Sephadex G-100 (superfine grade) previously equilibrated with alkaline phosphate buffer. The subunits were eluted with the alkaline phosphate buffer at a flow rate of 5 mls/hour. Aliquots (50 µl) were removed from the eluted fractions (1.5 mls), added to 8 mls of Triton-toluene scintillant and counted for radioactivity at 15% gain and open windows on a Packard Tri-Carb spectrophotometer. A typical elution profile of labelled Fraction I protein subunits is shown in Figure 5.

It can be seen that the large and small subunits are well separated. Peak fractions of protein, corresponding to large and small subunits, were precipitated by adding, at 4°C, an equal volume of 40% (w/v) trichloroacetic acid. The precipitated protein was pelleted after 30 minutes at 4°C by centrifugation at 10000 x g for 30 minutes at 2°C. The pellet was washed by resuspension and centrifugation (at 10000 x g for 20 minutes) in ethanol-diethyl ether (3:1, v/v) (twice) and ether (once). This material was dried at 30°C for 30 minutes and then stored at 4°C until used.

The purity of the radioactively-labelled large subunit prepared in this way was established by the criteria of homogeneity of the protein, at high loadings, on polyacrylamide gels. A known weight (500 µg) of large subunit protein was resuspended in 500 µl of 2.5 mM Tris-19 mM glycine (pH 8.5) containing 100 mM 2-mercaptoethanol and 2% (w/v) sodium dodecyl sulphate. The sample was boiled for 5 minutes and sucrose and bromophenol blue were added as described previously. Aliquots (200 µl) were electrophoresed on 15% SDS-polyacrylamide/

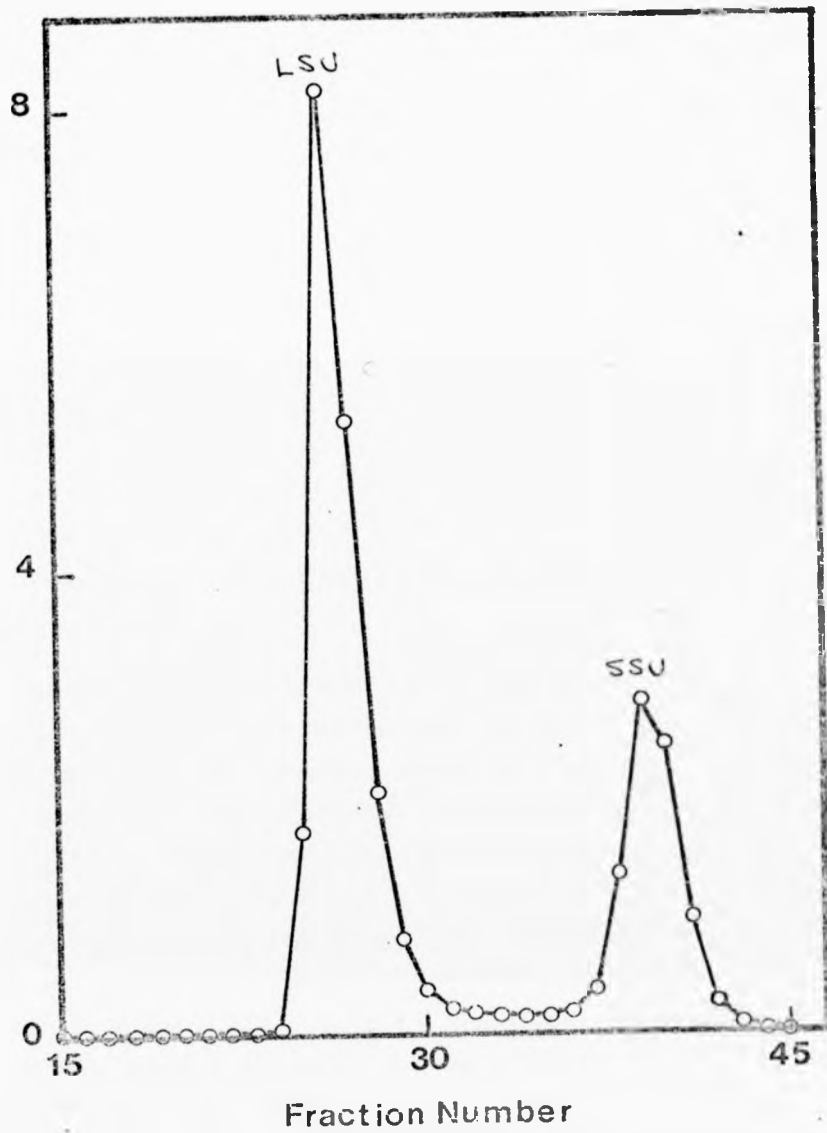
Figure 5. Elution profile of alkali-denatured,
radioactively-labelled pea Fraction I protein on
Sephadex G-100.

Purified L-(³⁵S) methionine-labelled Fraction I protein
was dissociated into subunits and fractionated as
described in Section II2Giv.

LSU, large subunit

SSU, small subunit

Radioactivity - c.p.m. $\times 10^{-4}$ / 50 μ l aliquot



polyacrylamide gels which were then stained, sliced and counted for radioactivity as described in Section IIFia.

Figure 6 shows the optical density profile of a stained gel and also the distribution of radioactivity within the gel. A major radioactive peak is found to coincide with the single discrete protein band which has migrated into the gel. A second high molecular weight protein species, which migrated into only the first 5 mm of the gel was also labelled. This high molecular weight species is assumed to be an aggregated form of the large subunit of Fraction I protein for two reasons. Firstly incubation of the material for longer times or in higher concentrations of 2-mercaptoethanol prior to electrophoresis results in a decrease in the relative amount of this species and secondly, if protein eluted from the region of the gel containing the higher mobility species is re-electrophoresed, then a proportion remains in the top few millimetres of the gel (Section III3B).

The fact that only one radioactively-labelled polypeptide could be detected even at high (200 μ g) loadings of protein testifies to the purity of the in vivo labelled large subunit of Fraction I protein used in subsequent work.

H. IODINATION OF PROTEINS WITH IODINE-125.

The iodination of proteins followed the procedure of Stanley and Haslam (1971). Protein solution (500 μ l, 1 mg/ml) in phosphate buffered saline was mixed with 10 μ l of lactoperoxidase (0.1% w/v), 10 μ l of Iodine-125 (100 μ Ci) and 10 μ l of dilute H_2O_2 (0.04 vols). The mixture was shaken vigorously for 1 minute and then allowed to react at room temperature for 30 minutes. The reaction was terminated by the addition of 5 μ l of 0.05 M cysteine in PBS. The sample was then exhaustively dialysed at 4°C against 2.5 mM Tris-19 mM glycine (pH 8.5), 100 mM 2-mercaptoethanol and 0.2% (w/v) SDS in order to remove unreacted Iodine-125.

J. TRYPTIC AND CHYMOTRYPTIC DIGESTION AND PEPTIDE ANALYSIS.

i. Performic acid oxidation and enzymic digestion.

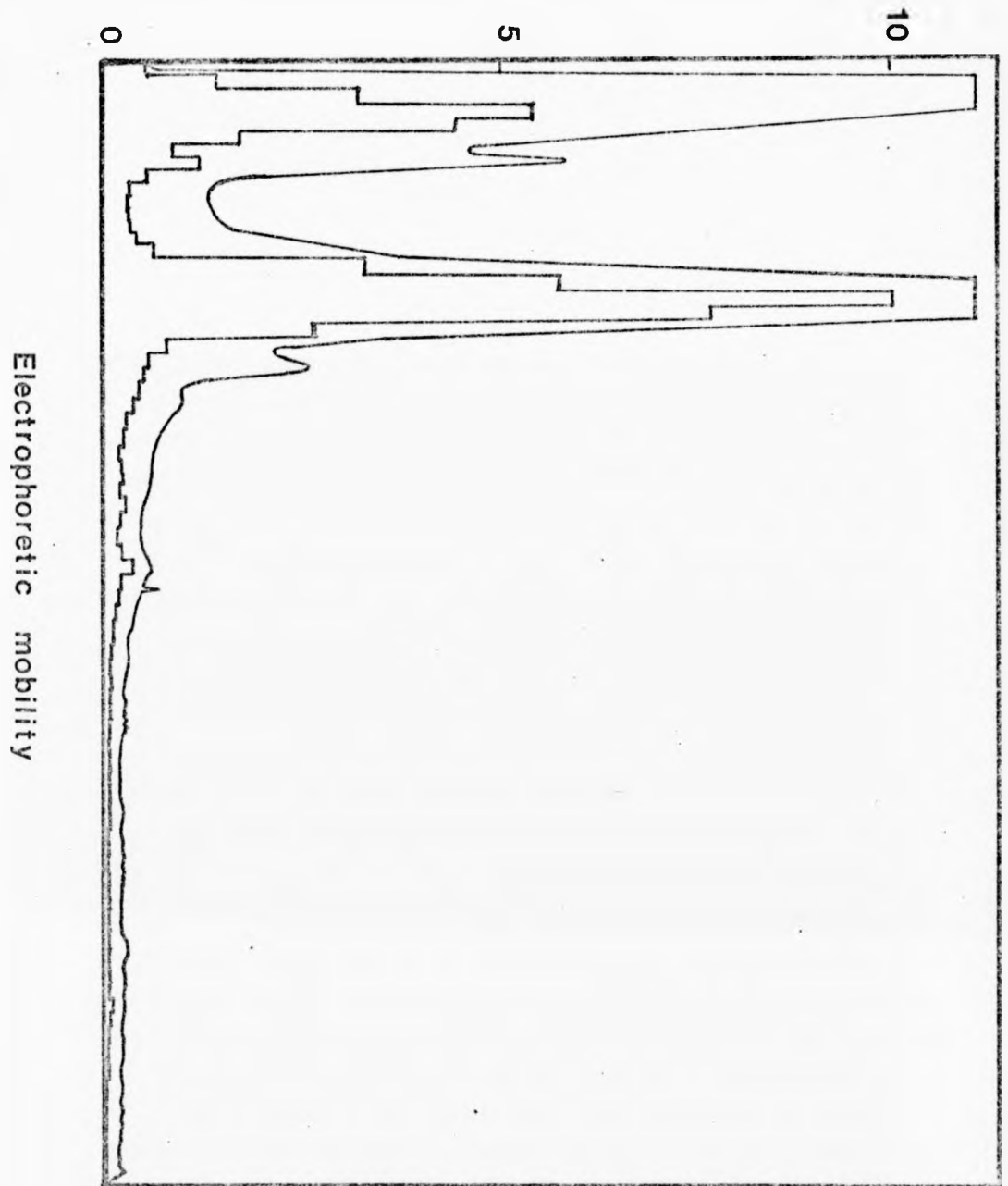
The samples for tryptic or chymotryptic analysis were oxidized with /

Figure 6. Polyacrylamide gel electrophoresis of L-(³⁵S) methionine-labelled large subunit from pea Fraction I protein purified by Sephadex G-100 chromatography.

The large subunit was prepared as described in Section II2Giv. A 1 mg/ml solution of the large subunit was prepared in 2.5 mM Tris-19 mM glycine, 100 mM 2-mercaptoethanol, 2% (w/v) SDS and analysed by electrophoresis as described in Section II2Fiia.

The solid line represents the absorbance at 620 nm and the histogram shows the radioactivity in each 1 mm gel fraction.

Radioactivity - c.p.m. $\times 10^{-3}$ /gel fraction



with performic acid as described by Bray and Brownlee (1973). Performic acid was prepared by mixing 1 ml of 30% H_2O_2 (100 vols) with 19 mls of formic acid. This mixture was kept at 20°C for 3 hours before use.

Purified protein (1 mg) was re-suspended in 200 μl of a mixture of formic acid and methanol (4:1, v/v). The samples were cooled to -8°C by immersion in a semi-frozen, 20% (v/v) solution of ethanol and 50 μl of performic acid was added. Oxidation was continued for two hours at -8°C. The oxidized samples were transferred quantitatively to freeze-drying tubes and evaporated to dryness under a stream of nitrogen. To remove all traces of performic acid the samples were resuspended in distilled water and freeze-dried twice ('Speedivac' centrifugal freeze-dryer).

Tryptic digestion was performed by dissolving aliquots of the samples (250 μg of protein) in 50 mM NH_4HCO_3 (pH 8.0) containing 1 mM CaCl_2 and by adding 12.5 μg of TPCK (1-chloro-4-phenyl-3-L-toluene-p-sulphonamido-butan-2-one) - inactivated trypsin dissolved in 50 mM NH_4HCO_3 (pH 8.0) - 1 mM CaCl_2 . The trypsin: protein ratio was therefore 1 : 20. Incubation was at 37°C for 7 hours.

Chymotryptic digestion was performed as described by Hartley *et al* (1975). The reaction mixture (1 ml) contained,

250 μg	Sample protein
5 μg	α -chymotrypsin
0.5 μg	ovamucoid trypsin inhibitor
100 μmol	NH_4HCO_3 (adjusted to pH 8.2 with NH_4OH)

The α -chymotrypsin and ovamucoid trypsin inhibitor were pre-incubated together for 30 minutes at room temperature before adding the protein sample. Digestion was performed for 3 hours at 37°C and was terminated by freeze drying the samples. All digested samples were stored at 4°C in a desiccator under vacuum.

ii. Peptide separation by cation-exchange chromatography.

The peptides in the tryptic digest were separated by cation-exchange chromatography on a column (0.9 cm x 60 cm) of Aminex A5 resin (Atassi *et al*, 1972). Peptide separation was performed using a Biocal/

Biocal automatic amino acid analyser fitted with an LKB Ultrograd gradient former. The tryptic digest, prepared as described in Section II2Ji, was re-suspended in 1.5 mls of starting buffer (0.1 M pyridine acetate, pH 3.5) and applied to the column. The sample was washed into the column with a further 10 mls of starting buffer. Peptides were eluted from the column, at a flow rate of 0.4 ml/minute, with a biphasic linear gradient of pyridine acetate buffer. The buffer elution profile was a 60 mls gradient from 0.1 M buffer, pH 3.5, to 1.0 M buffer, pH 5.0, followed by a 120 mls gradient from 1.0 M buffer, pH 5.0 to 2.0 M buffer, pH 6.5. Eluted fractions (1 ml) were collected directly into liquid scintillation vials using a Gilson Fraction collector. Triton-toluene scintillant (9 mls) was added to each vial and the radioactivity was determined in a Packard Tri-Carb spectrophotometer. The counting efficiency was not determined.

iii. Peptide separation by two-dimensional mapping.

The digested samples were prepared for peptide separation as follows. The samples (250 μ g of protein, 50-100 $\times 10^3$ c. p. m.) were dissolved in 150 μ l of distilled water and centrifuged at 1500 g for 5 minutes to remove any particulate matter. The samples were then freeze-dried again and re-dissolved in 15 μ l of water. Peptide separation was then carried out by the procedure of Sargent and Vadlamudi (1968). This procedure allows two peptide mixtures to be 'fingerprinted' simultaneously and under identical conditions.

The two samples for comparison were spotted onto a single 20 cm \times 20 cm silica gel plastic thin layer chromatography plate. The plate was then moistened by aerosol with electrophoresis buffer, i. e. acetic acid-formic acid - water (4:1:45 by vol), pH 2.1. After removal of excess moisture from the plates with a warm air flow the samples were electrophoresed for 2.5 hours at 12.5 volts/cm. The peptides migrated towards the cathode. Electrophoresis was performed in a modified Shandon U77 electrophoresis tank at room temperature. After electrophoresis of the peptides the plates were dried at 60°C for 30 minutes and then cut into two halves, each half containing one of the peptide mixtures being compared. The peptides were separated in the second dimension by ascending chromatography in/

in butan-1-ol-acetic acid - water (3:1:1 by vol) for 30 minutes. After chromatography the plates were dried and placed in contact with Kodirex X-ray film for 8-12 days. The X-ray film was developed by normal procedures.

K. ESTIMATION OF PROTEIN

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

The following solutions were used;

Solution A 0.5% (w/v) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 1% (w/v) sodium potassium tartrate.

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

Solution C 1M Folin-Ciocalteu reagent.

A standard curve was prepared by taking aliquots (5-500 μl) of a standard (1 mg/ml) solution of BSA in 1N NaOH. The aliquots were made up to 0.7 ml with 1M NaOH. Solution B (7.0 mls) was added and the mixture was left to stand for 15 minutes. Solution C (0.7 ml) was added and the mixture was allowed to stand for a further 30 minutes. The extinction of each sample was measured at 750 nm.

Aliquots of unknown protein samples were precipitated by adding an equal volume of 10% (w/v) trichloroacetic acid at 4°C for 1 hour. The precipitated protein was centrifuged at 2500 x g for 5 minutes and the pellet was re-suspended in 0.7 ml of 1N NaOH. The sample was then treated as described above for the standards. The protein concentration was determined by reference to the standard curve.

L. ESTIMATION OF CHLOROPHYLL AND CAROTENOID.

Chlorophyll was measured by the method of Arnon (1949). A known fresh weight of tissue (approximately 1 g) was ground in a mortar in a small (5-10 mls) volume of 80% acetone. Precipitated protein and cellular debris was removed by centrifugation at 2500 x g for 5 minutes. The extinction of the supernatant fraction was measured at 645 nm and 663 nm and the chlorophyll concentration in this solution, and hence by calculation in the tissue, was determined from the formula,

$$(\text{Chlorophyll})_{\text{mg/l}} = (20.2 \times E_{645} + 8.02 \times E_{663}).$$

Carotenoid concentrations in suspension of etioplasts were measured as follows. Aliquots (0.1 - 0.5 ml) of etioplast suspension were /

were made 80% (v/v) with respect to acetone in a final volume of 5 ml. The extracts were filtered to remove precipitated protein and the extinction was measured at 473 nm. The carotenoid concentration was calculated assuming the extinction of a 1% solution of carotenoid in 80% acetone, with a light-path of 1 cm, to be 2500 at 473 nm (Bottomley, 1970).

SECTION III - RESULTS AND DISCUSSION

1. THE CHARACTERISTICS OF AMINO ACID INCORPORATION IN ISOLATED ETIOPLASTS.

A. INTRODUCTION.

The arguments presented in Section I3Biib imply that it would be advantageous to use intact rather than broken plastids in the study of in vitro plastid protein synthesis. Plastids which retain their outer membrane should exhibit high rates of amino acid incorporation, thus facilitating the identification of newly-synthesized polypeptides. In addition, the fidelity of translation may be higher in intact plastids and the polypeptides synthesized in vitro should therefore more closely reflect the nature and pattern of protein synthesis in vivo.

In this section experiments are described which establish whether the methods of isolation successfully used to produce chloroplast preparations exhibiting high rates of amino acid incorporation can also be used to prepare etioplasts capable of rates of in vitro protein synthesis sufficient to allow product analysis. Although isolated etioplasts are able to carry out phenazine methosulphate - dependent cyclic photophosphorylation (Plesničar and Bendall, 1973) there is no evidence that light can act as an energy source for in vitro etioplast protein synthesis. Consequently energy must be supplied as added ATP. Although the outer chloroplast membrane is relatively impermeable to adenylates the transfer of ATP into the chloroplast has been demonstrated (Heldt, 1969).

Using added ATP as the energy source for in vitro etioplast protein synthesis reinforces the need to establish that etioplasts are the only site of amino acid incorporation in the relatively crude preparations which result from the use of rapid isolation procedures. Experiments are presented in this section which investigate the possibility that cytoplasmic or non-plastid 70S ribosomes are the site of amino acid incorporation. These experiments involve the use of specific inhibitors of ribosome function, detergent and procedures that allow for the separation of sub-cellular components.

The extent of amino acid incorporation due to ribosomes situated in either intact or broken plastids was assessed by the use of ribonuclease. The outer plastid membrane is impermeable to ribonuclease (Margulies et al, 1968) which can therefore be used to selectively destroy mRNA- ribosome complexes unprotected by a plastid /

plastid envelope. Consequently it is possible to distinguish between amino acid incorporation at the the two sites.

The remaining experiments in this section describe the characteristics of amino acid incorporation in isolated etioplasts. A number of physical parameters of the in vitro system (pH, temperature, ion and energy requirements) were investigated in order to determine the optimal conditions for protein synthesis.

B. PLASTID ISOLATION

i. Phase ^{contrast} and electron microscopy of etioplast preparations.

The etioplast preparations used in these studies were obtained using techniques of rapid isolation in aqueous buffers. These techniques were originally developed to yield chloroplast preparations capable of high rates of CO₂ fixation (Kalburer et al., 1967; Walker and Crofts, 1970). It was therefore necessary to establish whether the isolation procedure could, without extensive modification, yield etioplast preparations containing a reasonable percentage of intact plastids but without gross non-plastid contamination. Phase and electron microscopy were used to assess the quality of the etioplast preparation and the results are shown in Plate 2 A-E.

Plate 2 A shows the appearance of a typical etioplast preparation under the phase contrast microscope. Although much of the detail is not visible on the photograph it is possible to clearly distinguish the phase-bright intact etioplasts (IE) from the phase-dark broken etioplasts (BE). The fraction of intact etioplasts in these preparations varied between 35% and 60% as determined by quantitative phase contrast microscopy (Section II2Bia). The nature of the etioplast preparation is more clearly shown in Plate 2B, which shows a low-power electron micrograph of a preparation. It can be seen that etioplasts, both intact and broken, are a major component of the preparation. The most diagnostic feature of the etioplast, the prolamellar body (PLB) (Gunning, 1965) can be seen in some cases.

Plates 2C to E show that the etioplast preparation contains a mixture of plastids in early, but well defined, stages of light-induced development. The conclusion that the initial stages of light-induced etioplast/

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Plate 2. Phase and electron microscopy of pea etioplast preparations.

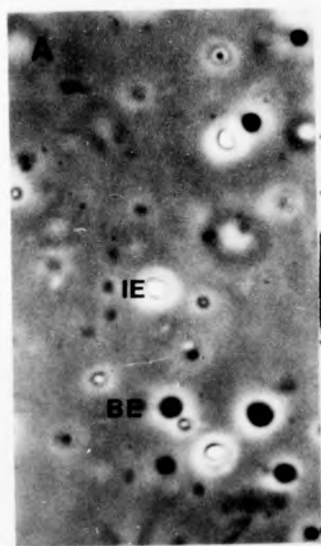
Pea etioplasts were isolated as described in Section II2A. Phase and electron microscopy were performed as described in Sections II2Bib and II2Bii.

A. Phase contrast microscopy of a pea etioplast preparation (magnification x 725).

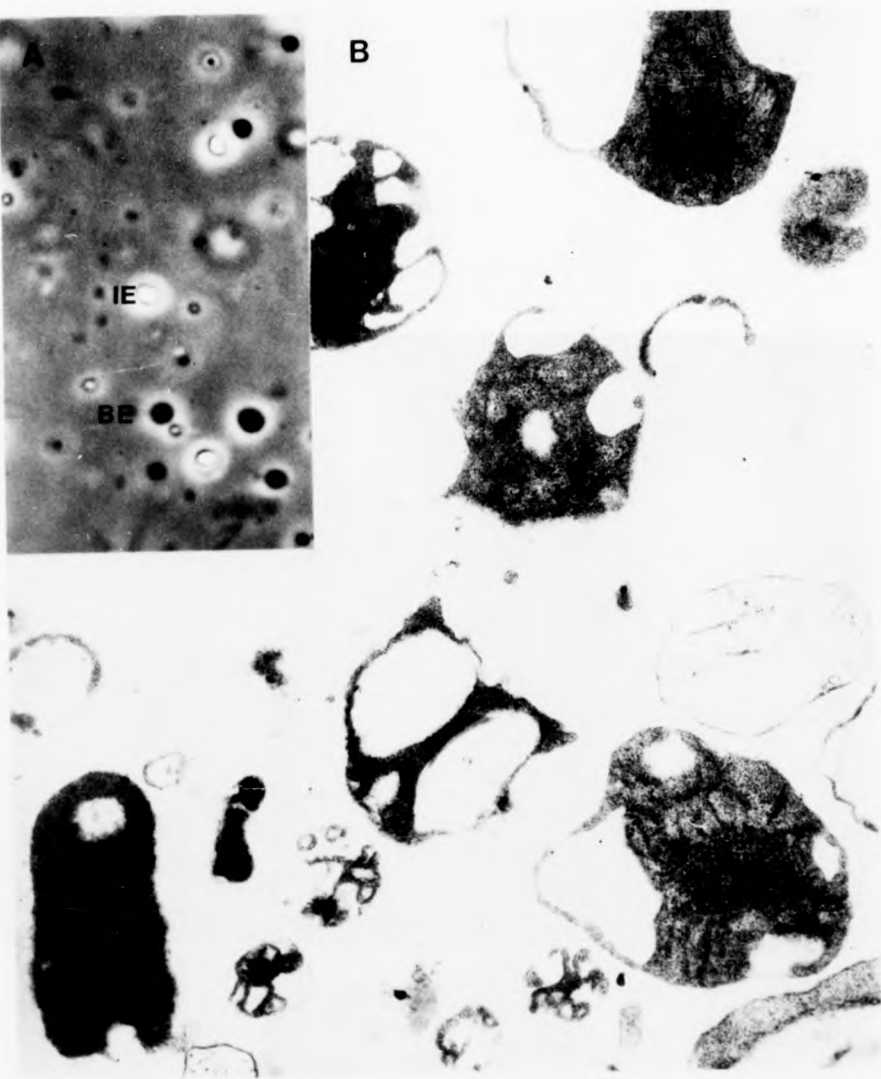
B. Low-power electron micrograph of pea etioplast preparation (magnification x 25000).

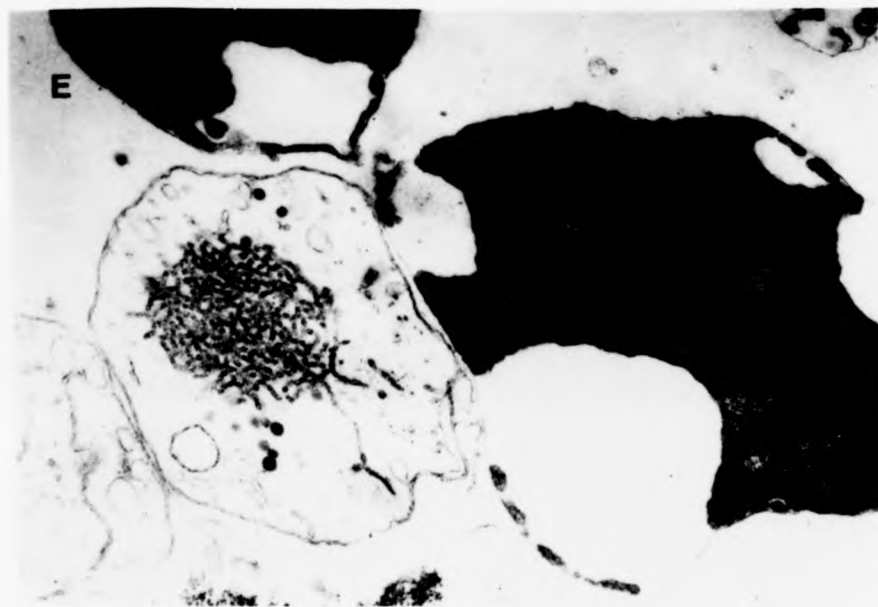
C., D. and E. Electron micrographs showing isolated etioplasts. All magnifications are x 40000.

PLB, prolamellar body; Pt, perforated thylakoid; Bt, bithylakoid; S, starch inclusion; IE, intact etioplast; BE, broken etioplast.



B





etioplast transformation occur in these preparations is in accordance with their routine method of preparation, which involved exposure to light. Plate 2C shows an etioplast in which a paracrystalline, or tight (Wellburn and Wellburn, 1973), prolamellar body is evident. A further stage of light-induced development is shown in Plate 2D. In this plastid the paracrystallinity of the prolamellar body tubules has dispersed and the outgrowth of perforated thylakoids (Pt) has started (Wellburn and Wellburn, 1971). Plate 2E shows two plastids. Bithylakoid (Bt) formation (Wellburn and Wellburn, 1973) can be clearly seen in one whilst in the other the process of tube transformation in the prolamellar body is clearly seen. This process is maintained by Henningsen and Boynton (1969) to be the first step in the dispersal of the prolamellar body tubules into primary lamellar layers.

This limited study with the phase contrast and electron microscopes suggest that the rapid isolation procedure does yield an etioplast preparation containing a reasonable percentage of intact plastids. The level of non-plastid contamination in these preparations seems acceptable.

C. THE SOURCE OF ENERGY FOR AMINO ACID INCORPORATION

i. The energy source.

The ability of etioplast preparations to use different energy sources for the incorporation of L-(^{35}S) methionine into a hot TCA-insoluble product was investigated. The results are shown in Table 1.

It is evident that ATP will, but light will not, act as an energy source for protein synthesis in isolated etioplasts. In contrast to Hearing (1973) no light stimulation of ATP-driven incorporation could be detected. Addition of an ATP-generating system, as well as ATP, to the incubation mixture did not cause a marked increase in the incorporation. Apart from ATP none of the nucleoside triphosphates tested produced any stimulation of incorporation above the level of the controls incubated in the absence of added nucleotides.

ii. L-(^{35}S) methionine incorporation as a function of ATP concentration.

The effect of varying the ATP concentration in the incubation mixture/

Table 1. Effect of the energy source on the incorporation of L-(³⁵S) methionine into a hot TCA-insoluble product in isolated etioplasts.

Etioplasts were isolated and incubated as described in Sections II2A and II2Ci. Amino acid incorporation was determined as described in Section II2Cii. The results are expressed as a percentage of the incorporation by the complete ATP-driven system. The initial rate of incorporation in this system varied between 0.25-0.8 pmoles of L-(³⁵S) methionine/h per mg of protein.

<u>Energy Source</u>	<u>Treatment</u>	<u>% Incorporation</u>
ATP	Complete	100
ATP + ATP generating system	Complete	112
None	Zero time	6
None	Complete	7
Light	Complete	7
ATP + Light	Complete	100
UTP	2 mM	10
GTP	2 mM	7
CTP	2 mM	7
TTP	2 mM	8

mixture is shown in Figure 7. The experimental details are given in the figure legend. L-(³⁵S) methionine incorporation is stimulated by increasing the ATP concentration up to a value of 2 mM, but no further stimulation occurs up to 4 mM - ATP. The concentration of ATP which produces maximum stimulation of incorporation (2mM) was used in subsequent incubation mixtures. A concentration of 2 mM - ATP is similar to that used by other workers for studying *in vitro* chloroplast protein synthesis (Spencer and Wildman, 1964; Bottomley *et al.*, 1974).

D. THE TIME COURSE FOR AMINO ACID INCORPORATION

i. Initial reaction velocities as a function of temperature.

Figure 8 illustrates the time course for the incorporation of L-(³⁵S) methionine into hot-TCA-insoluble material by isolated etioplasts at different temperatures. The dependence of the incorporation on added ATP (Table 1) is also confirmed. The experimental details are given in the figure legend.

Several features of the time course are noteworthy. Firstly, the incorporation rate is not constant during the incubation period. At 20°C the rate of incorporation falls to zero after about 30 minutes. Incubation at higher or lower temperatures alters the duration of incorporation. Hyperbolic time-courses of this nature are characteristic of *in vitro* plastid protein synthesis (Blair and Ellis, 1973; Bottomley *et al.*, 1974; Drumm and Margulies, 1970). Secondly, the initial rate of incorporation is dependent upon the temperature at which the assay is performed. This relationship is illustrated in Table 2. The initial rates calculated from incubations at 10°C and 20°C show that the incorporation rate is approximately halved when the incubation temperature was lowered by 10°C. Incorporation in the absence of added ATP, at any temperature, was less than 10% of the complete ATP-driven rate throughout the incubation.

ii. Comparison with ATP-driven protein synthesis in isolated chloroplasts.

Several reasons make it difficult to compare the rates of amino acid incorporation measured in the etioplast preparations used in these studies with those reported in the literature. For example, most incorporation rates are quoted as pmoles of radioactive amino acid incorporated/

Figure 7. The effect of ATP concentration on the incorporation of L-(³⁵S) methionine into a hot-TCA-insoluble product in isolated etioplasts.

Etioplasts were prepared by the procedure described in Section II2A. Incubation mixtures (final volume 500 μ l) contained 300 μ l of etioplast suspension (300 μ g of protein), 100 μ l of KCl resuspension medium (pH 8.3) containing 0.5 μ Ci of L-(³⁵S) methionine (200 Ci/mmol) and 100 μ l of ATP suspension medium (rebuffered to pH 8.3). Incubations were terminated after 1 hour and assayed for amino acid incorporation as described in Section II2Cii.

$10^{-3} \times$ ^{35}S methionine incorporated
c.p.m./incubation

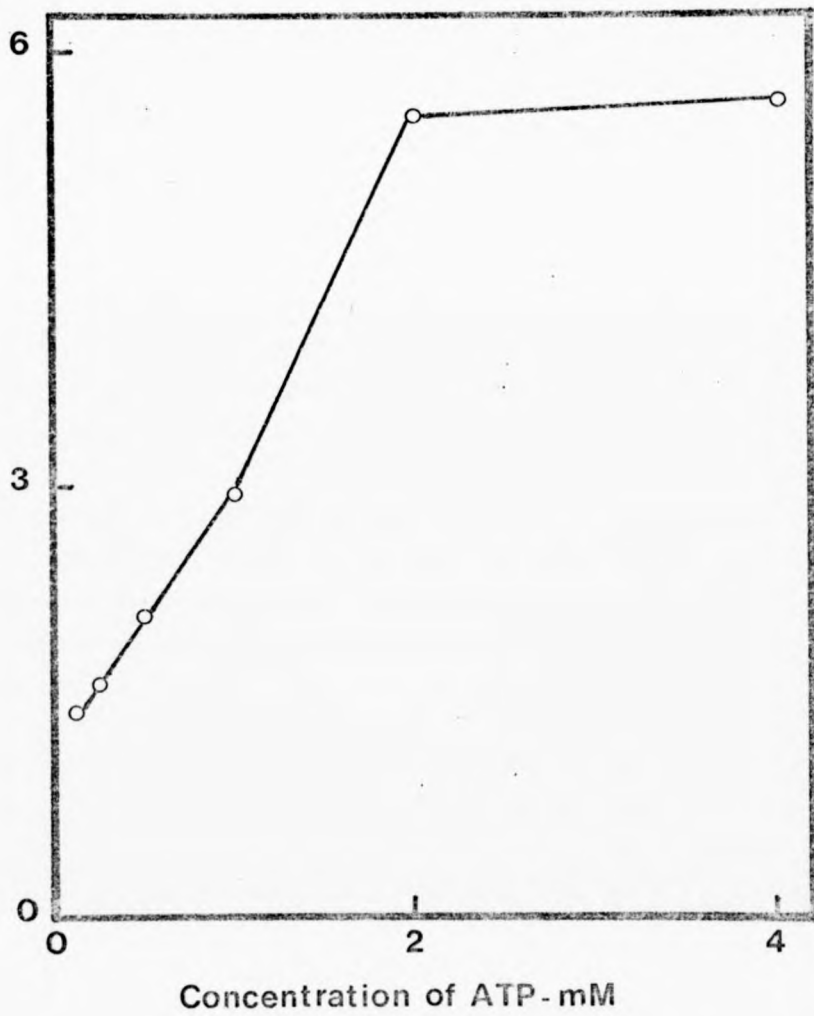


Figure 8. The effect of ATP and temperature on the incorporation of L-(³⁵S)methionine into a hot-TCA-insoluble product in isolated etioplasts.

Etioplasts were prepared by the procedure described in Section II2A. . Incubation mixtures (final volume 500 μ l) contained 300 μ l of etioplast suspension (190 μ g of protein), 100 μ l of KCl resuspension medium (pH 8.3) containing 0.5 μ Ci of L-(³⁵S) methionine (200 Ci/mmol) and either 100 μ l of KCl resuspension medium containing 10 mM ATP (\square , \circ , \triangle) or 100 μ l of KCl resuspension medium alone (\square , \circ , \triangle). Incubations were terminated and assayed for amino acid incorporation as described in Section II2Cii.

\circ , 10^oC, +ATP; \circ , 10^oC, -ATP; \square , 20^oC, +ATP;
 \square , 20^oC, -ATP; \triangle , 30^oC, +ATP; \triangle , 30^oC, -ATP.

$10^{-3} \times$ ^{35}S methionine incorporated
c.p.m. / incubation

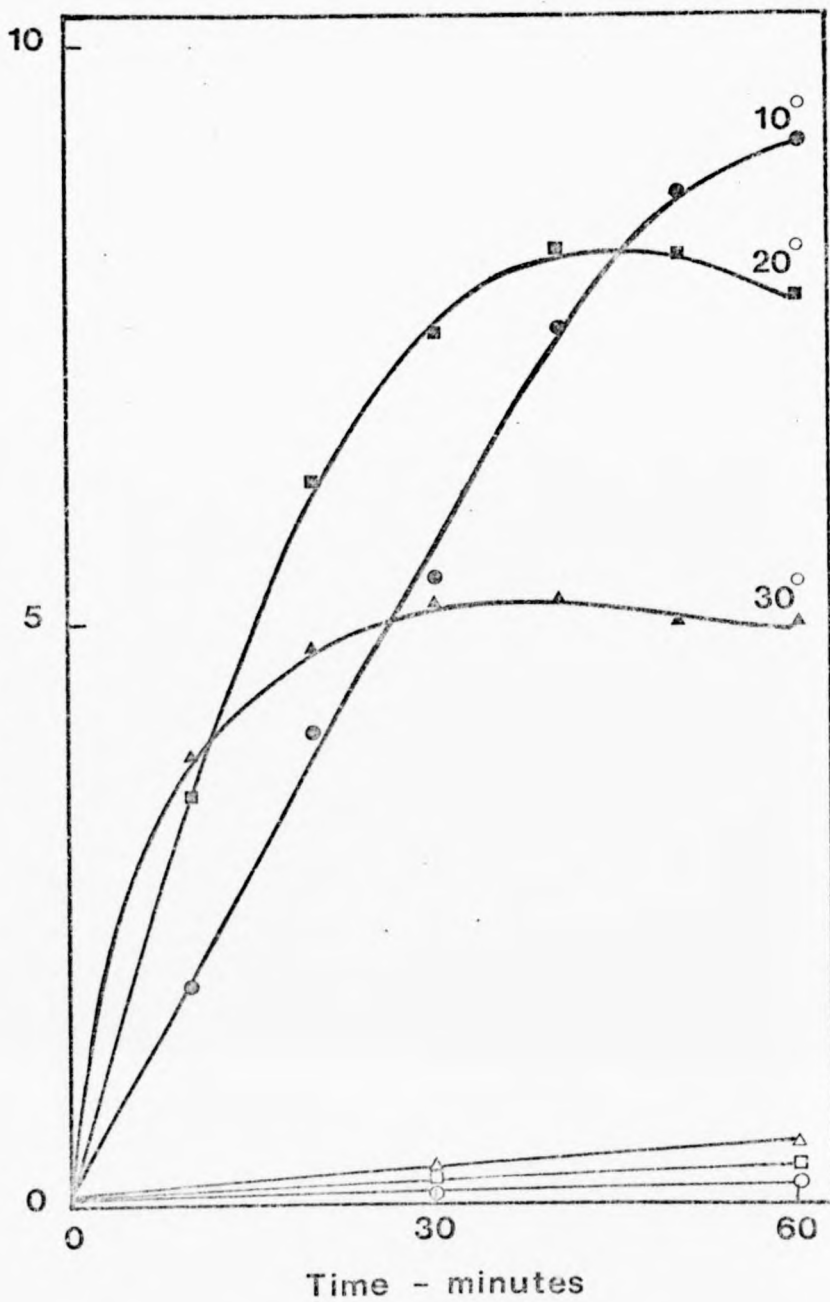


Table 2 The initial rate of amino acid incorporation
as a function of incubation temperature.

The experimental details are given in the legend
to Figure 8.

Temperature °C

Initial rate of incorporation
(pmoles of L-(³⁵S) methionine
/h per mg of protein).

10

0.14

20

0.27

incorporated/h per mg of protein (or RNA) (Drumm and Margulies, 1970; Reger *et al*, 1970). The calculation of such a rate requires a knowledge of the specific activity of the radioactively labelled amino acid. Most workers simply use the figures quoted in the manufacturers specification and ignore the alteration in the specific activity of the radioactively-labelled amino acid precursor due to free amino acid pools inside the plastid. A further complication is that, as will be shown in Section IIIHiv, the measured rate is most likely an average of two different rates of incorporation occurring in intact and broken plastids respectively.

A more practical approach is to determine whether these etioplast preparations incorporate sufficient radioactive amino acid into protein to allow analysis of the products. Comparison should therefore be with a system in which product analysis is known to be feasible, for example, light (or ATP) - driven protein synthesis in isolated chloroplasts (Blair and Ellis, 1973). Figure 9 illustrates the time course for protein synthesis, using added ATP as the energy source, in isolated chloroplasts. Also illustrated, for direct comparison, is a time course of *in vitro* etioplast protein synthesis which was determined in the same experiment. From the data presented in Figure 9 the initial rates of incorporation can be calculated:- isolated chloroplasts, 0.69 p moles of L-(³⁵S) methionine/h per mg of protein; isolated etioplasts, 0.27 p moles of L-(³⁵S) methionine/h per mg of protein. Comparison of these rates indicates that the etioplast preparations are sufficiently active in protein synthesis to allow analysis of the newly synthesized polypeptides.

E. THE EFFECT ON AMINO ACID INCORPORATION OF:-

i. Increasing L-(³⁵S) methionine concentration.

The relationship between the amount of L-(³⁵S) methionine incorporated into protein and the amount of L-(³⁵S) methionine added to the incubation mixture is shown in Figure 10. A linear relationship is found. The simplest interpretation of this finding is that the effect of increasing the amount of radioactive amino acid added to the incubation is to proportionally increase the specific activity of the L-methionine/

Figure 9. Time course for protein synthesis in isolated pea chloroplasts.

Plastids were isolated and incubated as described in Section II2A and II2Ci. All incubations were performed in the dark at 20°C. Incubations were terminated and assayed for amino acid incorporation as described in Section II2Cii.

- ⊙ chloroplasts, 40 μg of protein/incubation
- chloroplasts, 20 μg of protein/incubation
- etioplasts, 240 μg of protein/incubation
- etioplasts, 120 μg of protein/incubation

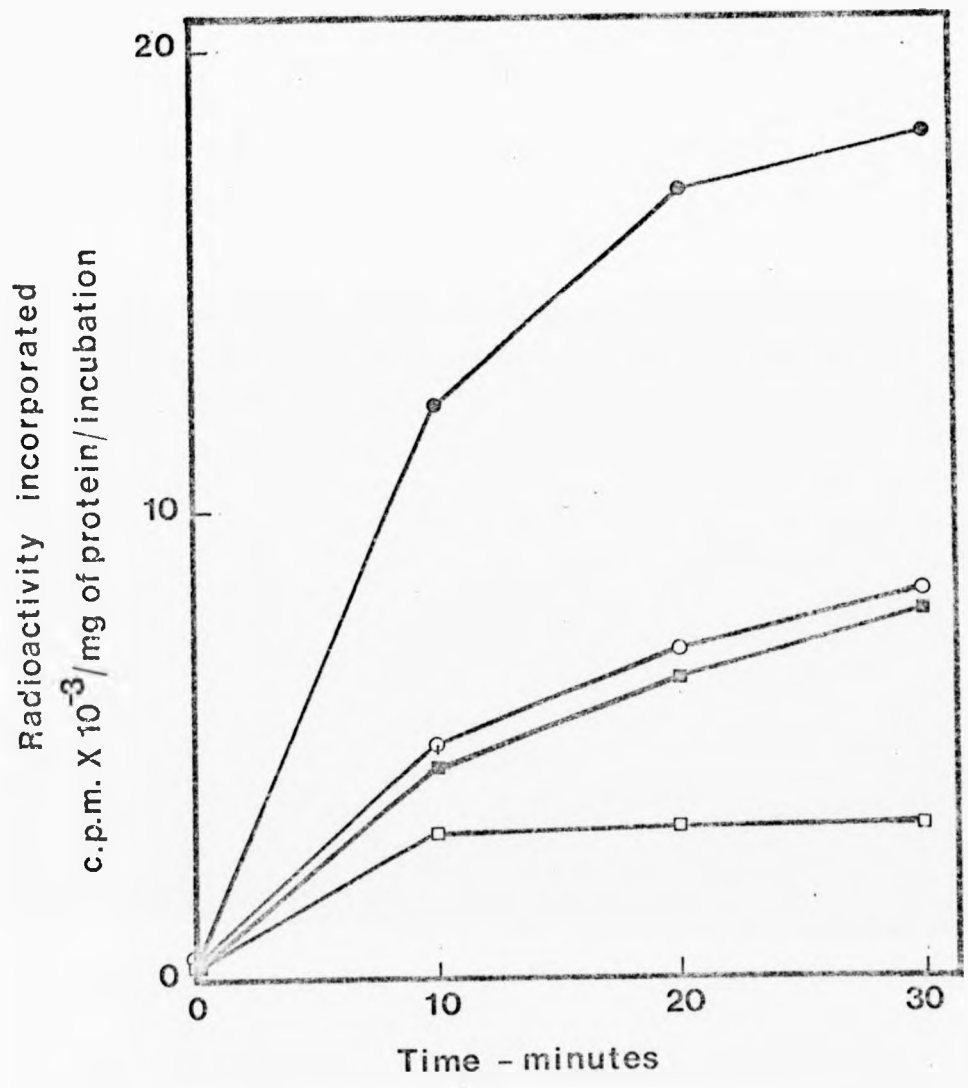
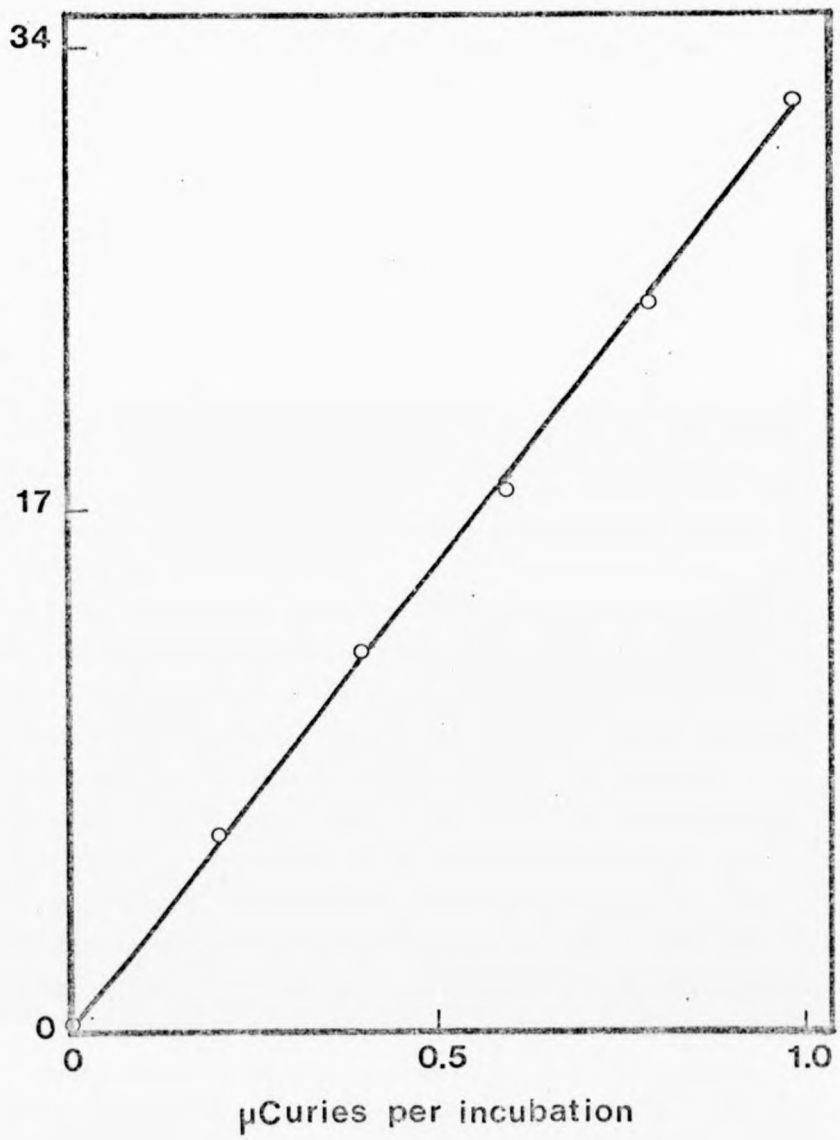


Figure 10. The relationship between L-(³⁵S) methionine incorporation and the amount of added L-(³⁵S) methionine.

The experimental procedures are as given in Figure 7, except that the incubations contained 2 mM - ATP, 180 μg of protein and varying amounts (0 to 1.0 μCi) of L-(³⁵S) methionine.

Radioactivity in protein
c.p.m. X 10⁻³ / incubation



L-methionine pool at the site of incorporation. Less than 2% of the added L-(^{35}S) methionine is incorporated into protein, indicating that there is only a small decrease in the L-methionine concentration during the incubation.

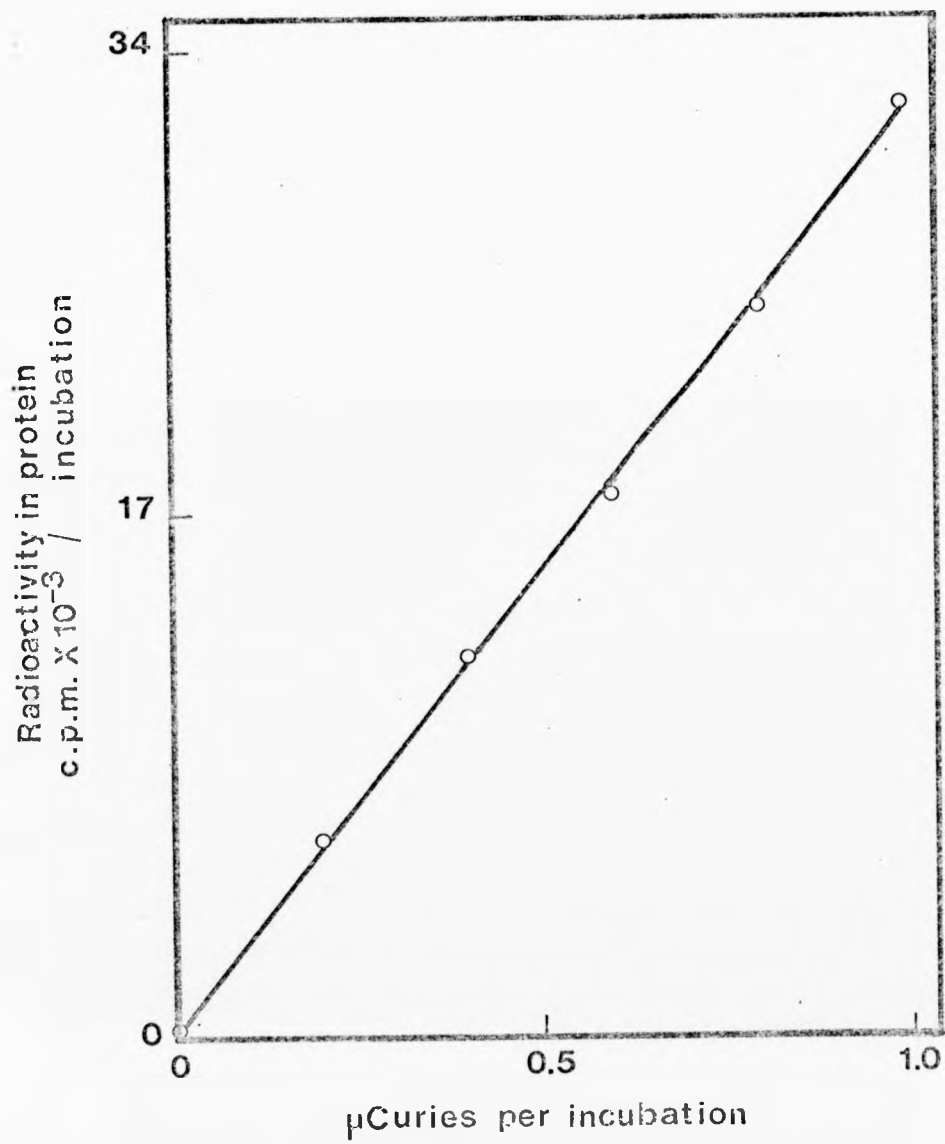
ii. Increasing the concentration of etioplasts.

The relationship between the L-(^{35}S) methionine incorporation and the carotenoid or protein concentration (i. e. plastid concentration) is shown in Figure 11. Under the conditions of the assay a maximum level of incorporation is reached when the incubation contains approximately 250 μg of protein or 3 μg of carotenoid. The nature of the limitation upon incorporation above these concentrations is unknown. The data in Figure 9 shows that at subsaturating concentrations of protein the incorporation rate is directly proportional to the protein concentration throughout the incubation period.

F. THE K^+ ION AND pH REQUIREMENT FOR AMINO ACID INCORPORATION

In order to facilitate the study of the polypeptides synthesized in isolated etioplasts, the incorporation of radioactivity into newly synthesized protein should be maximal. To this end various parameters of the incubation conditions were investigated in order to empirically determine the optimal conditions for amino acid incorporation. One such parameter was the K^+ ion concentration.

The effect of varying the K^+ ion concentration on the incorporation of L-(^{35}S) methionine is shown in Figure 12. The experimental conditions are given in the figure legend. Although it is clear that L-(^{35}S) methionine incorporation in isolated etioplasts is stimulated by a high K^+ ion concentration the degree of stimulation is not as great as in isolated chloroplasts (Blair and Ellis, 1973). Bottomley *et al* (1974) have conducted an extensive study of the cation requirements for both light and ATP-driven protein synthesis in isolated spinach chloroplasts. They conclude that NH_4^+ (at approximately 80 mM) best fills the requirement for monovalent cations in the ATP-driven system which uses broken plastids. In the light-driven system, which requires intact, photophosphorylating plastids/



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Figure 11. The relationship between L-(³⁵S) methionine incorporation and the concentration of etioplasts.

The details of the experimental procedure are given in Figure 7, except that the incubations contained 2 mM-ATP, 0.5 μ Ci of L-(³⁵S) methionine and varying amounts of carotenoid (0 to 9.0 μ g/ml) and protein (0 to 840 μ g/ml).

○, carotenoid

■, protein

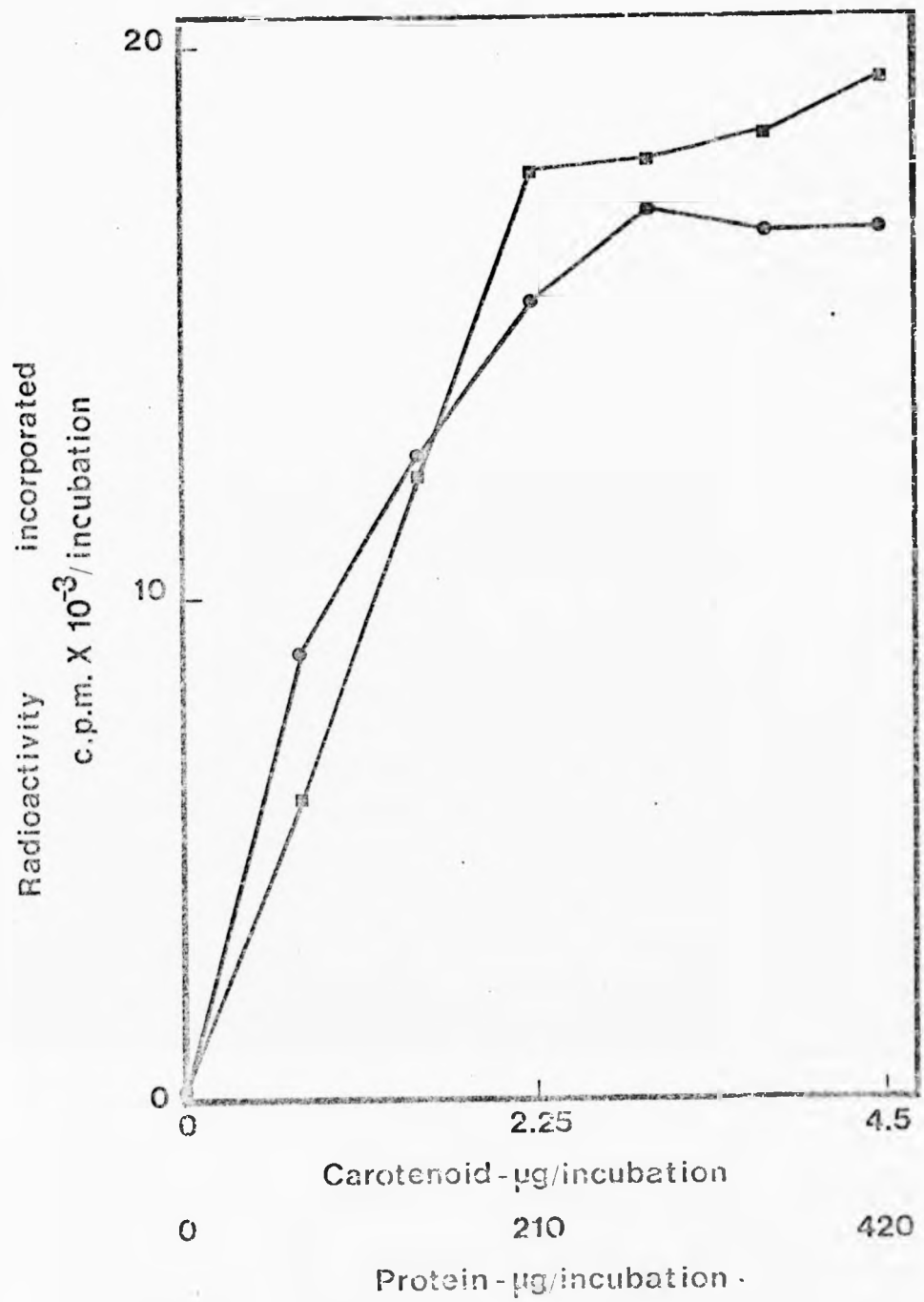
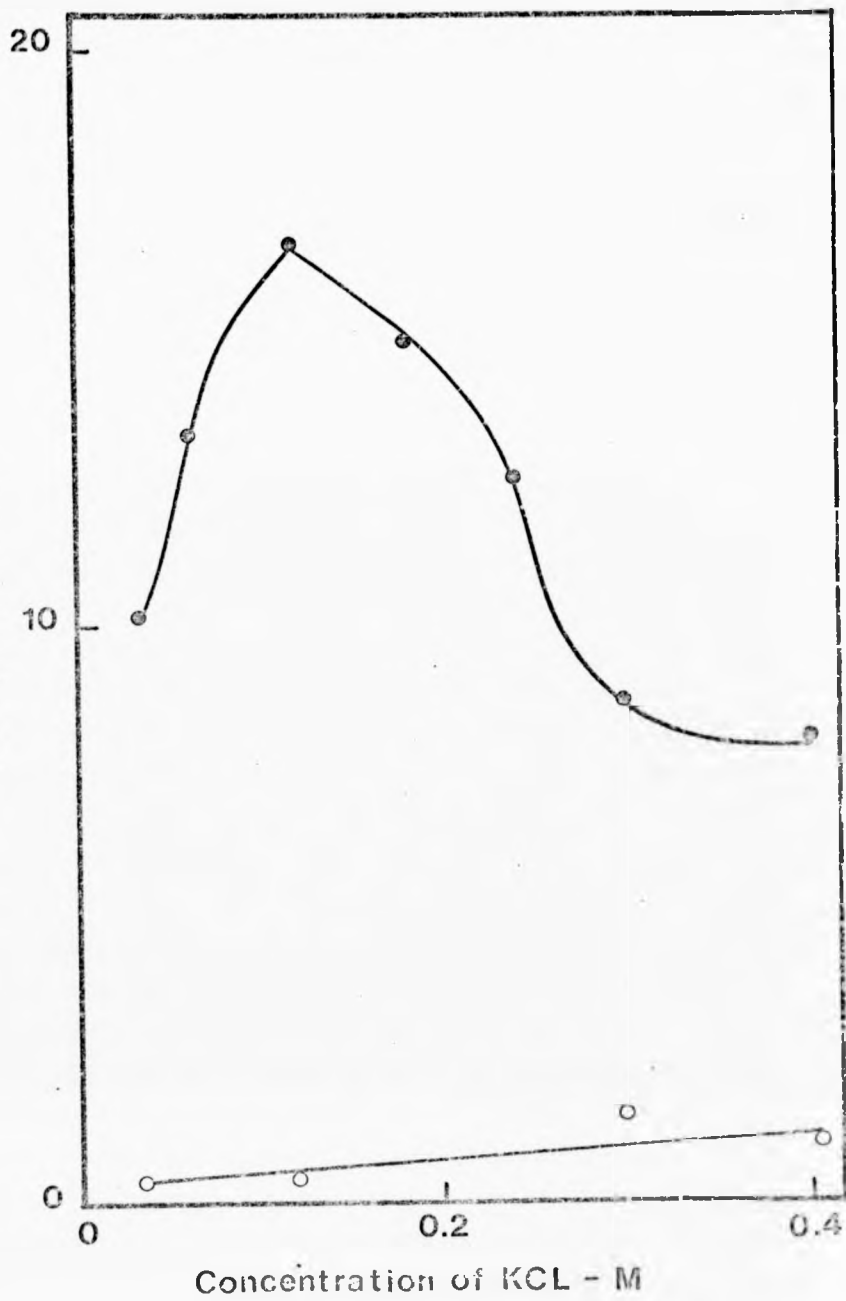


Figure 12. The effect of K^+ ion concentration on the incorporation of L- (^{35}S) methionine into a hot-TCA-insoluble product in isolated etioplasts.

Etioplasts were prepared by the modified procedure described in Section II2A in which 5 ml portions of the homogenized tissue were centrifuged individually. The etioplast pellets (900 μ g of protein) were resuspended in 500 μ l of modified KCl resuspension medium in which the total K^+ ion concentration varied from 33 to 453 mM (pH 8.3), containing either 0.5 μ Ci of L- (^{35}S) methionine (200 Ci/mmol) and 2 mM ATP (\odot) or 0.5 μ Ci of L- (^{35}S) methionine (200 Ci/mmol) alone (O). Incubations were terminated and assayed as described in Section II2Cii.

$10^{-3} \times$ 35 Smethionine incorporated
c.p.m./incubation



plastids, K^+ ion (approximately 30 mM) is the most effective monovalent cation.

The K^+ ion stimulation of incorporation in etioplasts confirms the suggested role of this ion as a co-factor for protein synthesis in isolated chloroplasts rather than a factor that stimulates incorporation by an involvement in, for example, photophosphorylation (Blair and Ellis, 1973).

The effect of varying the hydrogen ion concentration (pH) of the incubation was also investigated. The results are shown in Figure 13. A maximum level of incorporation is achieved at pH 8.3. This pH is also the optimal value for protein synthesis in isolated chloroplasts.

G. THE EFFECT OF ACTINOMYCIN D ON AMINO ACID INCORPORATION.

It has already been stated that the study of *in vitro* plastid protein synthesis would constitute a direct approach to elucidating the function of plastid DNA if a system of coupled transcription and translation could be developed. The evidence from using inhibitors of RNA synthesis, such as actinomycin D or rifampicin (Kahan *et al*, 1963; Weisblum and Davies, 1968) suggests that isolated chloroplasts, broken or intact, translate only mRNA synthesized before the plastids are isolated (Blair and Ellis, 1973; Bottomley *et al*, 1974; Harris *et al*, 1973; Spencer, 1965). This may not however be the case in isolated etioplasts. Etioplasts may be actively synthesizing mRNA species, in order to fulfil the role, suggested by Kirk and Tilney-Bassett (1967), of a potentiated, readily convertible precursor to the chloroplast. For this reason the effect of actinomycin D on amino acid incorporation in isolated etioplasts was studied. Table 3 shows the effect of actinomycin D over a range of concentrations from 0.02 $\mu\text{g/ml}$ to 20 $\mu\text{g/ml}$.

Actinomycin D clearly does not inhibit incorporation at these concentrations. As equivalent concentrations of actinomycin D inhibit light-driven incorporation of (^3H) uridine into RNA in isolated chloroplasts (Hartley, M. R., quoted in Blair and Ellis, 1973) there is no reason to suppose the inhibitor cannot penetrate into the plastid. It must/

Figure 13. The effect of pH on the incorporation of L-(³⁵S) methionine into a hot-TCA-insoluble product in isolated etioplasts.

Etioplasts were prepared by the modified procedure described in Section II2A, in which 5 ml portions of the homogenized tissue were centrifuged individually. The etioplast pellets (770 µg of protein) were resuspended in 500 µl of KCl resuspension medium (buffered to the indicated pH values with either KOH or HCl) containing 0.5 µCi of L-(³⁵S) methionine (200 Ci/mmol) and 2 mM-ATP. Incubations were terminated and assayed as described in Section II2Cii.

$10^{-3} \times$ ^{35}S methionine incorporated
c.p.m./incubation

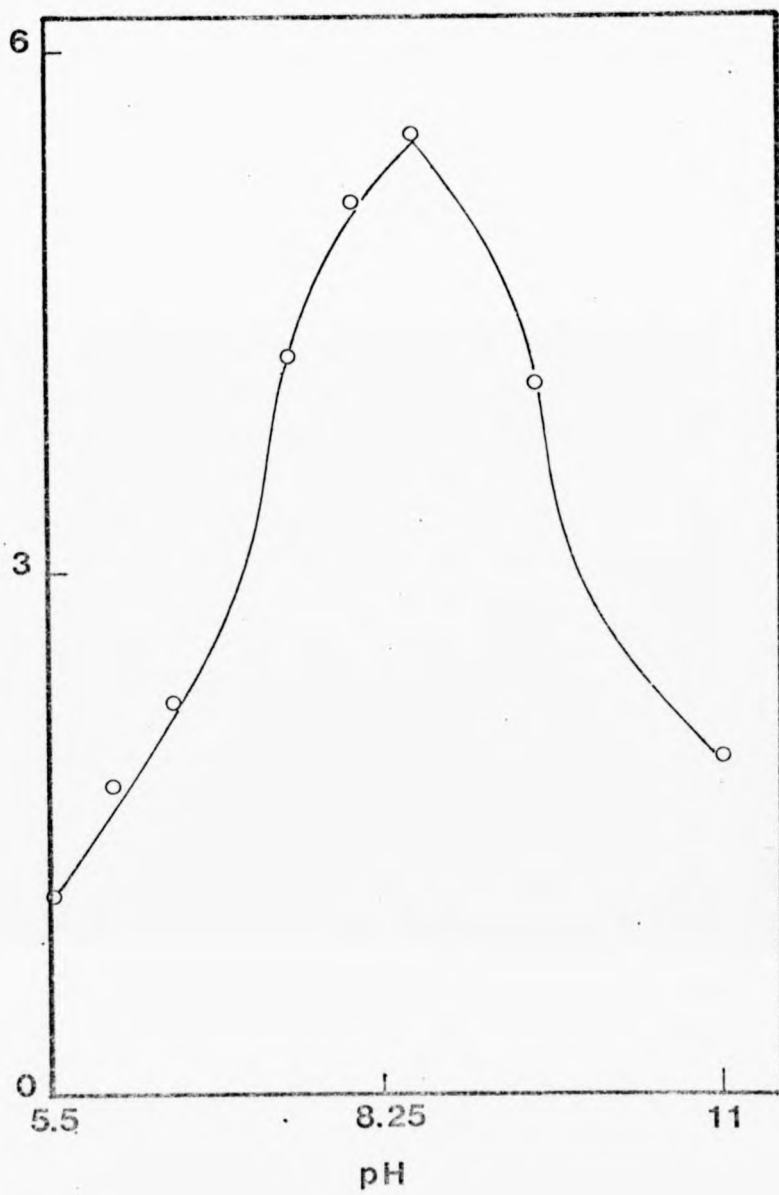


Table 3. The effect of actinomycin D on amino acid incorporation in isolated etioplasts.

The experimental details are given in Figure 7. All incubations contained 2 mM-ATP, 240 μ g of protein and 0.5 μ Ci of L-(³⁵S) methionine. The results are expressed as a percentage of the incorporation by the complete ATP-driven system incubated in the absence of actinomycin D.

<u>Energy Source</u>	<u>Treatment</u>	<u>% Incorporation</u>
ATP	Complete	100
None	Complete	8
ATP	Actinomycin D 0.02 $\mu\text{g}/\text{ml}$	81
ATP	Actinomycin D 0.2 $\mu\text{g}/\text{ml}$	103
ATP	Actinomycin D 2 $\mu\text{g}/\text{ml}$	94
ATP	Actinomycin D 20 $\mu\text{g}/\text{ml}$	100

must be concluded therefore that mRNA translated in isolated etioplasts is most likely present inside the plastids before they are isolated.

H. THE SITE OF AMINO ACID INCORPORATION IN ETIOPLAST PREPARATIONS.

In the results presented so far it has been tacitly assumed that amino acid incorporation is occurring in the etioplast. In this section the evidence to support this assumption is presented.

i. Effect on amino acid incorporation of inhibitors of ribosome function; chloramphenicol and cycloheximide.

Inhibitors which specifically block protein synthesis on either 70S or 80S ribosomes can be used to provide strong evidence that amino acid incorporation in etioplast preparations occurs on 70S ribosomes rather than on contaminating cytoplasmic ribosomes. Cycloheximide is the most frequently used and studied inhibitor of 80S ribosomes (Baliga *et al.*, 1969). The antibiotic chloramphenicol exists as four stereo-isomers, only one of which, D-threo chloramphenicol, inhibits protein synthesis on 70S ribosomes (Ellis 1969). The effect of these two inhibitors on amino acid incorporation by etioplast preparations is shown in Figure 14.

The results show that amino acid incorporation is over 90% inhibited by D-threo chloramphenicol at a concentration of 40 $\mu\text{g/ml}$. The L-threo isomer of chloramphenicol, at a concentration of 50 $\mu\text{g/ml}$, produces no inhibition of amino acid incorporation. Incorporation was also unaffected by cycloheximide up to a concentration of 50 $\mu\text{g/ml}$. Above this concentration inhibition of amino acid incorporation occurs, perhaps due to non-specific effects (Ellis and MacDonald, 1970).

These results indicate that amino acid incorporation in the etioplast preparation does not occur on contaminating 80S cytoplasmic ribosomes.

ii. Solubilization of labelled etioplast products by Triton X-100.

Parenti and Margulies (1967) reported that the detergent, Triton X-100, at specific concentrations, selectively solubilizes chloroplasts and mitochondria, but not nuclei, bacteria or whole leaf cells. This criteria has therefore been widely used to assess the degree/

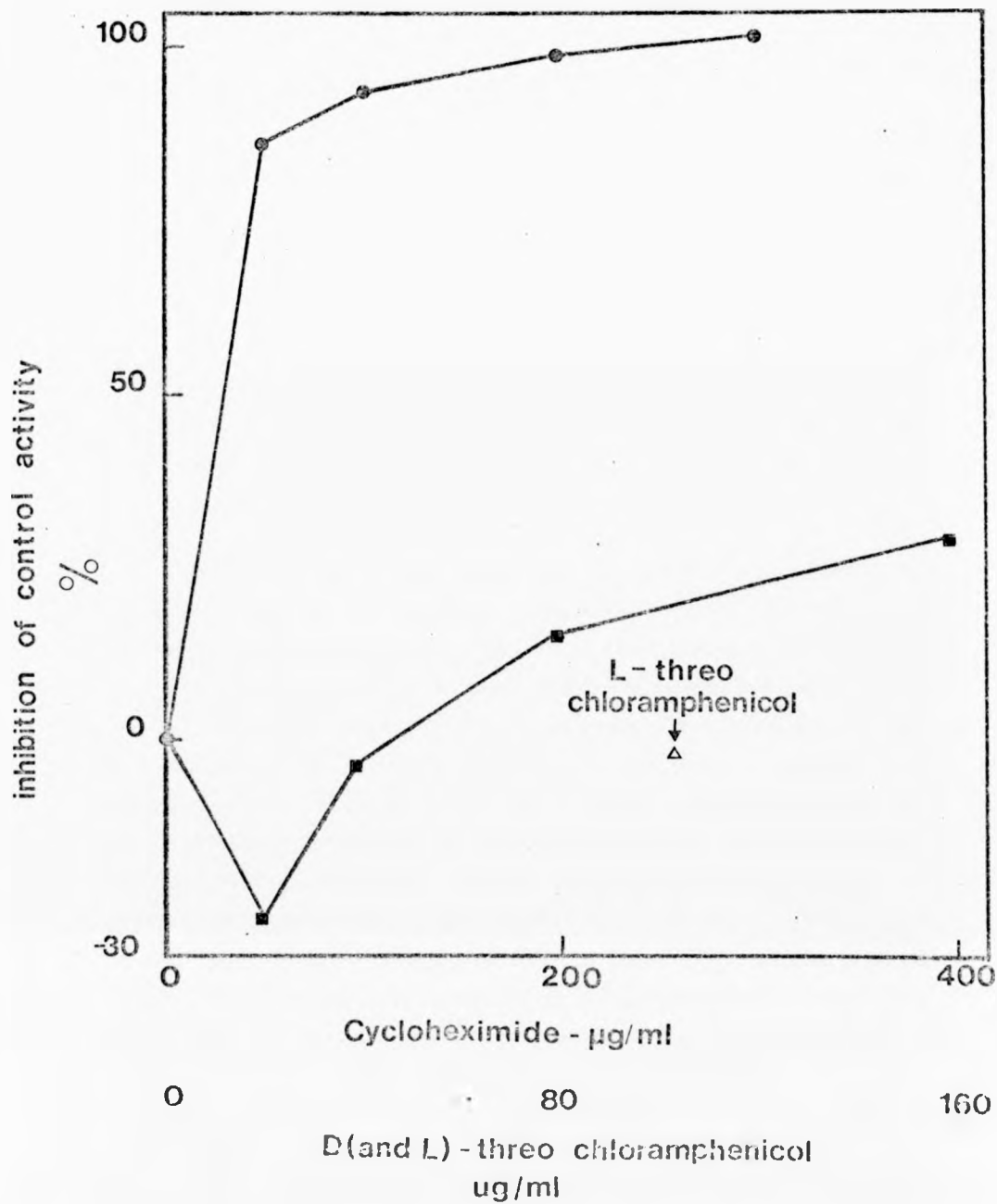
Figure 14. The effect of cycloheximide and D-threo chloramphenicol on amino acid incorporation in etioplast preparations.

The experimental details are given in Figure 7. All incubations contained 2 mM-ATP and 0.5 μ Ci of L-(³⁵S) methionine. Incubations with D-threo chloramphenicol contained 189 μ g of protein. Incubations with cycloheximide contained 150 μ g of protein.

○, + D-threo chloramphenicol.

□, + cycloheximide.

△, + L-threo chloramphenicol.



degree of amino acid incorporation in chloroplast preparations which is attributable to contaminating nuclei, bacteria or whole cells (Blair and Ellis, 1973; Harris *et al*, 1973). However Bottomley (1970) concluded that although treatment of pea etioplasts with Triton X-100 caused membranes to disappear and pigments to solubilize, a considerable quantity of plastid material could be pelleted after treatment by a low-speed (2000 x g) centrifugation. To minimize this apparent difference between etioplasts and chloroplasts solubilization of etioplast preparations should be performed at high pH (pH 8.5) and low magnesium concentration (1-2 mM).

The result of solubilizing an etioplast preparation at the end of an incubation with L-(³⁵S) methionine is shown in Table 4. The result shows that less than 5% of the radioactivity incorporated into protein is present in a 12500 x g pellet. This strongly suggests that amino acid incorporation is taking place in etioplasts or mitochondria.

iii. Mitochondria as the site of amino acid incorporation in etioplast preparations.

The results presented so far cannot exclude the possibility that amino acid incorporation in these preparations occurs on mitochondrial ribosomes. The most convincing way to show that amino acid incorporation is occurring on plastid and not mitochondrial ribosomes is to show that the products of *in vitro* protein synthesis are associated with the plastid and not the mitochondrial fraction. A method for obtaining a pure fraction of either mitochondria or plastids from the crude etioplast preparation is therefore required. The method of hybrid rate and equilibrium density centrifugation described in Section II2Di fulfills this requirement.

Figure 15 shows the distribution of hot-TCA-insoluble material within a gradient that has been used to fractionate the components of an etioplast preparation after incubation with L-(³⁵S) methionine. The experimental details are given in the figure legend. It is clear that the majority (73%) of the *in vitro* synthesized protein is located in the pellet and in gradient fractions 30-35. These fractions principally contain etioplasts (fig. 1.). Less than 3% of the newly-synthesized/

Table 4. Solubilization of a labelled etioplast preparation by Triton X-100.

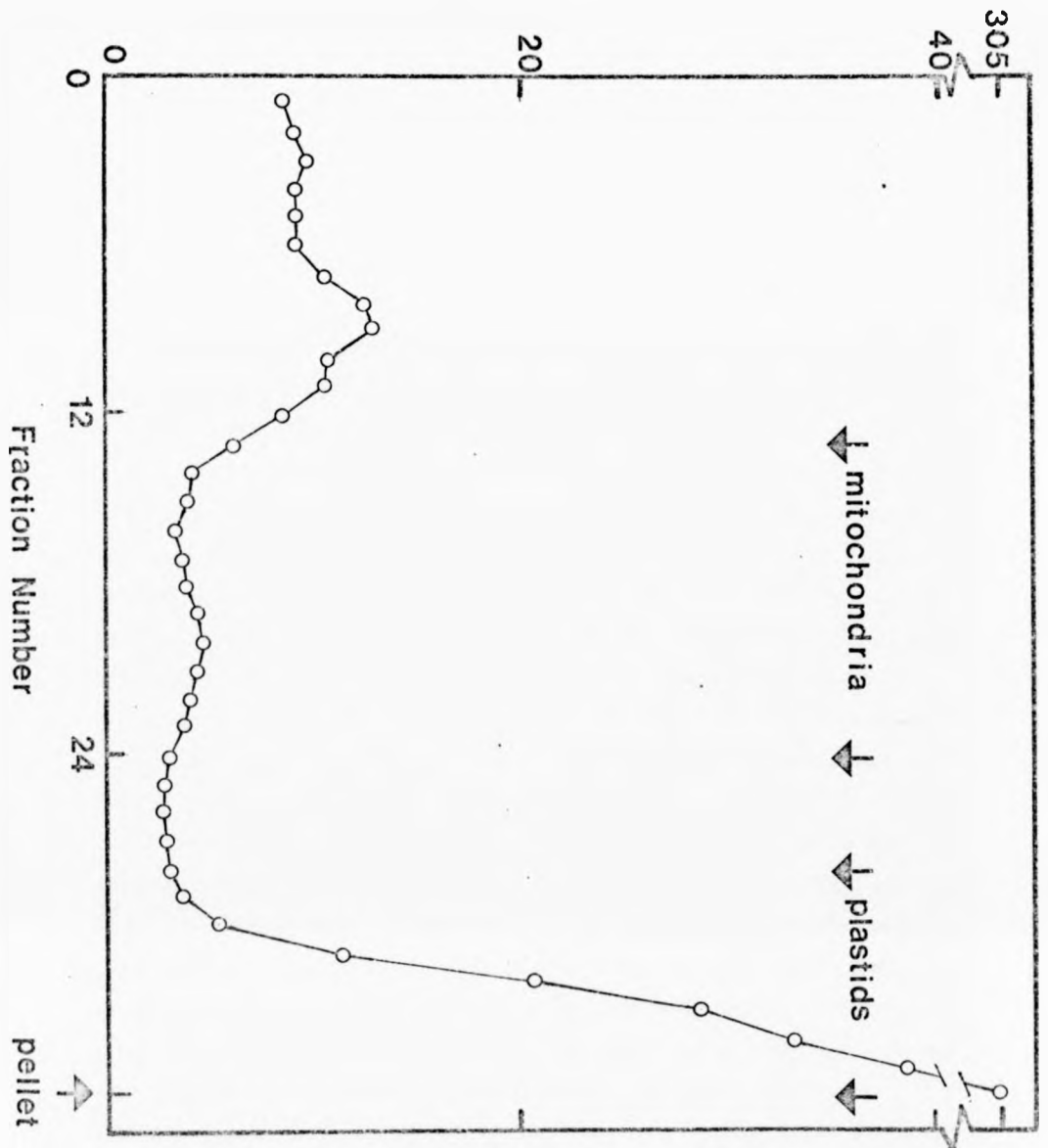
Solubilization of the etioplast preparation was performed as described in Section II2D. The results are expressed as a percentage of the incorporation by the unsolubilized etioplast preparation.

<u>Treatment</u>	<u>L-(³⁵S) methionine incorporation c. p. m.</u>	<u>%</u>
Unsolubilized etioplasts	16,611	100
12500 x g supernatant	11,758	70.7
12500 x g pellet	628	3.7
losses	4,225	25.6

Figure 15. Sucrose density gradient fractionation of etioplast preparations incubated with L-(³⁵S) methionine; the distribution of hot-TCA-insoluble material in the gradient.

An ATP-driven protein synthesis incubation mixture was prepared, incubated and fractionated as described in Section II2Ei. Each fraction was assayed for labelled protein by mixing at 4°C with an equal volume (0.5 ml) of 20% (w/v) TCA. After precipitation over-night at 4°C the protein was quantitatively transferred to glass-fibre discs and assayed for amino acid incorporation as described in Section II2Cii.

$10^{-3} \times {}^{35}\text{S}$ methionine incorporated
c.p.m./fraction



synthesized protein is associated with the band of mitochondria located in the centre of the gradient (fig. 2.). The remaining labelled protein is in the top 7.5 mls of the gradient (approximately 0.73 M sucrose). This material may be polypeptide chains that are synthesized on ribosomes in broken plastids and are therefore released into the incubation medium on completion. Alternatively the material may originate from intact etioplasts which are broken during the gradient centrifugation procedure.

This result, in conjunction with those described in Sections II2Hi and ii, conclusively establish the etioplast as the site of amino acid incorporation in these preparations.

iv. Sensitivity of amino acid incorporation to ribonuclease A

Ribonuclease does not cross the chloroplast envelope membrane (Margulies et al, 1968). This enzyme can therefore be used to distinguish between amino acid incorporation occurring on ribosomes in intact and broken plastids. ATP-driven amino acid incorporation in etioplast preparations is likely to be occurring at both sites; Bottomley et al (1974) has shown that broken spinach chloroplasts are superior to intact plastids in their ability to synthesize protein, providing they are supplied with ATP and GTP. Accordingly ATP-driven L-(³⁵S) methionine incorporation was assayed in the presence of a range of ribonuclease concentrations. The sensitivity of incorporation to inhibition by ribonuclease was also determined in etioplast preparations containing either a mixture of intact and broken plastids, or broken plastids alone.

The results in Table 5 show that ribonuclease inhibits amino acid incorporation in etioplast preparations by between 29% and 35%. The same inhibition is observed when the incubation contains an ATP-generating system in addition to ATP. This result suggests that approximately 35% of the incorporation is due to the activity of ribosomes not bounded by a plastid envelope. This implies that the remaining 65% of the incorporation occurs in intact etioplasts. This implication is supported by the complete ribonuclease inhibition of incorporation in etioplast preparations that have been deliberately lysed. The significant incorporation (49%) observed in lysed etioplast preparations/

Table 5. The sensitivity of ATP-driven amino acid incorporation by isolated etioplast preparations to ribonuclease A.

Etioplasts were prepared as described in Section II2A. All incubations contained plastids (180 μ g of protein) and 0.5 μ Ci of L-(³⁵S) methionine. Incubations were performed at 20°C in the dark. Incubations were terminated and assayed for amino acid incorporation as described in Section II2Cii. The results are expressed as percentages of the complete ATP-driven system incubated without added ribonuclease.

<u>Energy Source</u>	<u>Treatment</u>	<u>% Incorporation</u>
ATP	Complete	100
None	Complete	8
ATP + ATP- generating system	Complete	93
ATP	Ribonuclease 12 $\mu\text{g/ml}$	71
ATP	Ribonuclease 24 $\mu\text{g/ml}$	65
ATP	Ribonuclease 48 $\mu\text{g/ml}$	66
ATP + ATP- generating system	Ribonuclease 24 $\mu\text{g/ml}$	67
ATP	Lysed	49
None	Lysed	4.5
ATP	Lysed + ribonuclease (12 $\mu\text{g/ml}$)	7.1

preparations in the absence of ribonuclease indicates that lysis does not destroy ribosome activity. In contrast to chloroplasts, (Bottomley *et al.*, 1974), lysed etioplast preparations are not as active in protein synthesis as preparations which contain intact organelles.

J. DISCUSSION

The results presented in this section show that L-(³⁵S) methionine is incorporated into protein in isolated etioplasts. In general, protein synthesis in isolated etioplasts has the same requirements, and exhibits the same characteristics as protein synthesis in isolated chloroplasts. In contrast to earlier reports of protein synthesis in isolated etioplasts (Reger *et al.*, 1972a and 1972b; Drumm and Margulies, 1970; Hearing, 1973) the majority of amino acid incorporation in these preparations occurs in intact plastids. The inclusion of ribonuclease in the incubation mixture causes only a 35% decrease in the level of incorporation (Table 5). If necessary, ribonuclease could be used to ensure that incorporation occurs only in intact etioplasts. In view of Bottomley's report (1974) that broken chloroplast provided with ATP and GTP synthesize discreet polypeptides at higher rates than the light-driven intact system, it is not surprising that, provided ATP is supplied, preparations of broken etioplasts are also capable of protein synthesis at significant rates (Table 5).

The rate of incorporation of L-(³⁵S) methionine in non-lysed preparations (0.2 to 0.8 pmoles of L-(³⁵S) methionine/h per mg of protein) is difficult to compare with other published values. The rate of amino acid incorporation has been measured in etioplasts isolated from pea (*Pisum sativum*), wheat (*Triticum aestivum*) and bean (*Phaseolus vulgaris*) (Reger *et al.*, 1972a; Drumm and Margulies, 1970). In most cases the rates are expressed as c. p. m. /mg of RNA/minute (Reger *et al.*, 1972). However Drumm and Margulies (1970) quote a rate of approximately 60 pmoles of L-(¹⁴C) leucine incorporated/mg of protein/hour in etioplasts isolated from *Phaseolus*. This figure is directly compatible with the rates reported in this thesis. The apparently higher rate of Drumm and Margulies (1970) can be explained by two factors. Firstly, L-leucine is a more commonly occurring amino acid in normal cellular proteins than is L-methionine and/

and secondly, the incubations in which Drumm and Margulies (1970) performed their assays for in vitro protein synthesis contained 10 μCi of radioactively-labelled amino acid as compared to the 0.5 μCi normally used in these studies. Irrespective of the absolute rate, it is clear from direct comparison with the rates of amino acid incorporation in isolated chloroplasts (Figure 9), that sufficient radioactive amino acid is incorporated into protein in isolated pea etioplast to allow analysis of the products.

The incorporation of amino acids into protein by isolated pea etioplasts is dependent upon added ATP (Table 1, Figure 7). Incorporation is stimulated 15-fold by the addition of 2 mM-ATP to the incubation mixture (Table 1). This compares with the 2 $\frac{1}{2}$ -fold stimulation observed by Reger et al (1972a) in isolated pea etioplasts. As the majority of incorporation occurs in intact plastids ATP (phosphate energy) must cross the outer plastid membrane in amounts sufficient to allow protein synthesis to proceed at the measured rates. The transfer of phosphate energy across the chloroplast membrane in vivo may not involve the direct transfer of ATP, but rather an energy shuttle involving, for example, dihydroxyacetone phosphate (DHAP) and phosphoglyceric acid (PGA) (Heber, 1974). The rate of export of phosphate energy from the chloroplasts of Elodea densa can be deduced from the measured light-dependent increase in cytoplasmic ATP. The energy flux is calculated to be equivalent to a transfer of 7 to 9 μmoles of ATP/mg chlorophyll/hour (Heber and Santavius, 1970). Heldt (1969) has made direct measurements of adenylate transfer in isolated spinach chloroplasts. He concludes that by analogy to mitochondria (Pfaff et al, 1969) chloroplasts contain a specific translocator which facilitates exchange of adenylates. As the adenine nucleotide translocator has a higher affinity for external ATP than for ADP, and its capacity (2.03 μmoles influx of ATP/mg of chlorophyll per hour) is too low to account for the observed light-dependent fluctuations in cytoplasmic adenylate levels, its physiological role has been questioned (Heber, 1974). However simple calculation shows that an influx of ATP into the isolated etioplasts at a rate of 2.0 μmoles /mg of chlorophyll (assumed to be equivalent to 0.1 mg of protein) per hour/

hour will equilibrate the ATP level within the plastid with the external medium (2 mM) in less than 1 minute. This concentration of ATP can easily account for the rates of amino acid incorporation measured in isolated etioplasts. In agreement with Drumm and Margulies (1970), who worked with etioplasts isolated from *Phaseolus vulgaris*, no light stimulation of ATP-driven protein synthesis could be detected (Table 1).

At 20°C the time-course for ATP-driven amino acid incorporation is hyperbolic, with the rate of incorporation dropping to zero after about 30 minutes. Raising or lowering the temperature of the incubation caused both an alteration in the initial rate of incorporation (Table 2), and a change in the duration of incorporation (Figure 8). A hyperbolic time-course is characteristic of plastid protein synthesis (Blair and Ellis, 1973; Drumm and Margulies, 1970). However no explanation has yet been provided as to why incorporation ceases after a relatively short time. The hyperbolic time-course is observed irrespective of whether light or added ATP is used as the energy source, suggesting that the cessation of amino acid incorporation is not simply due to a limitation in energy supply. The inability of an ATP-generating system to significantly increase the level of incorporation in the etioplast system (Table 1) supports this suggestion, although the presence of an active ATP phosphohydrolase (ATPase) in the preparation cannot be ruled out.

A more complex explanation is that a component of the particular incubation mixture in which amino acid incorporation is assayed is inhibiting continued protein synthesis. This explanation arises from an analogous situation for light-driven RNA synthesis in isolated chloroplasts. Hartley and Ellis (1973) find that incorporation of (³H) uridine into RNA in isolated spinach chloroplasts incubated in KCl resuspension medium ceases after 30 minutes. Bohnert *et al* (1974) however, find that isolated spinach chloroplasts continue to synthesize RNA for up to 150 minutes if they are incubation in a medium containing sorbitol/HEPES as the osmoticum/buffer system. This explanation is however rendered unlikely by the finding of Bottomley *et al* (1974) that isolated spinach chloroplasts incubated not in KCl resuspension medium, but/

but in 0.33 M sorbitol - 80 mM Tricine (pH 8.4) medium, also cease to incorporate amino acids into protein after about 30 minutes.

Another possibility is that amino acid incorporation ceases because a factor involved in the mechanism of protein synthesis, e. g. an elongation factor or initiation factor, is lost from the plastids during the incubation. This explanation also seems unlikely as a lysed preparation, in which all factors are presumably at maximum dilution, still exhibits significant rates of amino acid incorporation. Direct observation of etioplasts purified after they have been incubated (Plate 1C) shows they do not lose their outer membrane during incubation. The new finding presented in this section is that there is a correlation between the initial incorporation rate and the duration of incorporation (Figure 8). This might suggest that some factor involved in ATP-driven protein synthesis is present in a limited amount in the isolated plastids and this factor is temperature-sensitive. Alternatively an inactivating factor, for example endogenous nuclease activity, is synthesized or activated during the incubation. Clearly this question remains unresolved.

In these studies considerable effort was devoted to establishing the etioplast ribosome as the site of amino acid incorporation. All possible sources of non-plastid ribosomes that might contaminate the preparation must be considered and excluded. Active ribosomes could be derived from nuclei, bacteria, whole cells, mitochondria and cell cytoplasm. In the majority of studies on protein synthesis in isolated plastids contamination by active cytoplasmic ribosomes does not appear to be a problem. One exception has been the studies on protein synthesis in plastids isolated from Acetabularia (Apel and Schweiger, 1972, 1973). Incorporation of labelled amino acid into protein was found to be inhibited, up to 60%, by both chloramphenicol and cycloheximide. Cycloheximide completely inhibited the labelling of one of the two in vitro synthesized proteins. Cytoplasmic 26S RNA was also found in these chloroplast preparations. This indirect evidence for cytoplasmic contamination was confirmed by Winkenbach et al (1972) who showed that chloroplasts isolated from Acetabularia are enclosed in a droplet of cytoplasm which is itself bounded by a membrane. /

membrane. Contamination of the etioplast preparations used in the present studies by active cytoplasmic ribosomes is excluded by the insensitivity of incorporation to cycloheximide, an inhibitor considered to be specific for 80S ribosomes. Incorporation is not inhibited at concentrations of cycloheximide 100 times greater than those required to inhibit amino acid incorporation by 75% in a cell-free protein synthesizing system from rat liver (Baliga *et al.*, 1969). In contrast, D-threo chloramphenicol (20 µg/ml) inhibits amino acid incorporation in etioplast preparations by over 85% (Figure 14); strongly suggesting that protein synthesis is occurring on 70S ribosomes.

Nuclei isolated from rapidly growing tobacco cell cultures have been reported to incorporate (^{14}C) lysine into protein at rates of up to 3nmoles/mg of protein per hour (Flamm *et al.*, 1963). This incorporation is insensitive to chloramphenicol. However, conflicting reports in the literature suggest it would be unwise to rely on inhibitor data to assess the contamination of the etioplast preparations by nuclear ribosomes. Whilst Zimmerman *et al.* (1969) report that amino acid incorporation in nuclei, isolated from HeLa cells was completely insensitive to inhibition by chloramphenicol, Helmsing (1970), studying protein synthesis in nuclei isolated from *Drosophila melanogaster*, reported that amino acid incorporation was specifically inhibited by chloramphenicol and not by cycloheximide. A more reliable method for assessing the contribution of nuclei to amino acid incorporation in crude etioplast preparations is to determine the radioactivity present in a 12500 x g pellet prepared after labelled etioplasts are treated with Triton X-100. This pellet would be expected to contain nuclei but not plastids (Parenti and Margulies, 1967). As less than 4% of the incorporated radioactivity is present in the Triton X-100-insoluble fraction (Table 4), nuclear incorporation in these preparations can be excluded. The same evidence can be used to exclude the possibility that bacteria and whole cells contribute to the observed amino acid incorporation. Bacteria and whole cells would also be expected to resist solubilization by low concentrations of Triton X-100 detergent (Parenti and Margulies, 1967).

This conclusion is particularly important with regard to bacterial contamination. /

contamination. Bacteria were found to ^{be} responsible for a high percentage of the amino acid incorporation observed in early studies on in vitro plastid protein synthesis (Gnanam et al., 1969). Several other features of amino acid incorporation in these preparations support the conclusion that bacteria are not the site of protein synthesis. Firstly there is a strict dependence on an added energy source. This is uncharacteristic of bacterial amino acid incorporation which commonly proceeds in the absence of an added energy source. Secondly the time course for amino acid incorporation in the etioplast preparations is hyperbolic. This is in contrast to the time course for bacterial amino acid incorporation which normally shows a constant or increasing rate of incorporation extending over several hours. In conjunction with the precautions taken to minimise bacterial contamination these results exclude bacteria as the site of amino acid incorporation in the etioplast preparations.

The only remaining non-plastid source of active ribosomes which could arguably contribute to the observed amino acid incorporation is the mitochondrion. The in vitro protein synthesizing activity of mitochondrial ribosomes (mitoribosomes) is evidenced by the major research effort into the nature of protein synthesis in isolated mitochondria (Poynton and Groot, 1975; Burke and Beattie, 1974). The similarity of the mitochondrial and plastid protein synthesizing system - for example, they are both specifically inhibited by D-threo chloramphenicol (Ellis, 1969; Borst and Grivell, 1971) - makes it difficult to easily assess the contribution of each organelle to the amino acid incorporation of crude preparations. In addition, the need to add ATP as the energy source in the study of in vitro etioplast protein synthesis further increases the possibility of incorporation occurring on contaminating mitoribosomes. Some workers have attempted to exclude this possibility by the following approach (Parenti and Margulies, 1967). Firstly the amino acid incorporating activity of a mitochondrially-enriched fraction is measured. The contamination of the plastid enriched fraction is then assessed by measuring the activity of a marker enzyme specific for mitochondria. From this information the contribution of mitochondrial/

mitochondrial protein synthesis to the amino acid incorporation in the crude plastid preparation is deduced. The validity of this approach rests however on the unproven assumption that mitochondria in the mitochondrially-enriched fraction exhibit the same rate of incorporation as mitochondria which contaminate the plastid preparation.

A more rigorous approach adopted in this study is to determine the percentage of radioactive protein that is associated with a mitochondrial fraction which is purified from the crude etioplast preparation after incubation in the protein synthesis assay. The method of purification used was a mixture of rate and equilibrium sedimentation in sucrose density gradients (Miflin and Beavers, 1974). The absence of radioactively-labelled protein in the position at which mitochondria band in these gradients (Figures 1, 2 and 15) show that mitoribosomes are not contributing significantly to amino acid incorporation in these preparations.

The remaining experiments described in this section were devoted to optimizing the conditions for protein synthesis in isolated etioplasts. Figure 12 shows that the incorporation of L-(³⁵S) methionine in isolated etioplasts is stimulated by a high K⁺ ion concentration in the incubation mixture. The effect of mono- and divalent cations on light and ATP-driven protein synthesis in intact and broken spinach chloroplasts has been studied by Bottomley (1974). It is clear that the relationship between the rate of *in vitro* chloroplast protein synthesis and the presence of cations in the external medium is complex. For example, high rates of protein synthesis can be obtained with intact chloroplasts isolated and assayed for amino acid incorporation in buffers completely devoid of Mg²⁺ ion. The addition of as little as 1 mM MgCl₂ to the incubation medium results in a 60% inhibition of amino acid incorporation. This marked inhibition is surprising in view of the report by Gimmler *et al* (1974) which strongly suggests that intact spinach chloroplasts maintain an internal Mg²⁺ ion concentration of about 27 mM, even against a concentration gradient produced by resuspending the plastids in a medium containing 0.02 mM Mg²⁺ ion. Gimmler *et al* (1974) also conclude /

conclude that the cation permeability of the plastid envelope is low, although a specific K^+ ion transport into the plastid can be induced by light. The light induced K^+ ion flux is specifically brought about by exchange translocation and is linked to H^+ extrusion. However the magnitude of the light-induced K^+ ion exchange reaction is small when compared with anion fluxes. The apparent ability of chloroplasts to maintain their internal composition in spite of changes in the external medium suggests that the concentration of the potassium ion inside the plastid may well be different from that present in the KCl resuspension medium. Estimates of the K^+ ion concentration in chloroplasts vary dramatically. Larkum (1968) estimated the concentration of potassium in chloroplasts isolated from the alga Tolypeicella intricata. Using non-aqueous methods of plastid isolation a value of 340 mM K^+ ion was reported. This contrasts sharply with the value of 19-36 mM reported by Gimmler et al (1974), who used aqueous buffers to isolate spinach chloroplasts. In view of the very limited understanding of the ion relations of isolated plastids, the maximal stimulation of amino acid incorporation in etioplasts by approximately 130 mM K^+ ion should be considered as an empirical observation. The effects of varying the pH and the ATP concentration in the protein synthesis incubation mixture were also investigated in an empirical manner (Figures 13 and 8). Maximum incorporation is obtained at pH 8.3 and 2 mM-ATP.

The results discussed in this section show that intact etioplasts can be isolated which exhibit rates of ATP-driven amino acid incorporation sufficient to allow analysis of the in vitro products of etioplast protein synthesis. This analysis will now be described.

SECTION III - RESULTS AND DISCUSSION

2. ANALYSIS OF THE PRODUCTS OF IN VITRO
ETIOPLAST PROTEIN SYNTHESIS.

A. INTRODUCTION

The study of the in vitro products of plastid protein synthesis can be separated into two phases. The first phase involves determining the number of products and classifying these products on the basis of their physical or chemical characteristics, e. g. molecular weight and solubility. This limited information is sufficient to allow for meaningful comparative studies. For example, it is of interest to compare the products of in vitro protein synthesis in plastids isolated from different species (Boulter et al., 1972) or, as in this study, the products of protein synthesis in plastids isolated from one species at different stages of development. In some cases, information from the first phase of analysis leads directly to the second, which is to identify the products of in vitro protein synthesis in terms of known proteins. However although the gross physical characteristics of an in vitro synthesized protein may give a strong clue to its identity, the unequivocal identification must rely on more rigorous criteria e. g. tryptic peptide mapping. In other cases the gross physical characteristics of a protein give no clue to its identity. These cases more often than not reflect a limitation in our basic knowledge of the chloroplast components, in particular of the membrane-bound proteins (Eaglesham and Ellis, 1974).

Any method used for comparative product analysis must allow for comparison with the results of previous studies on in vitro plastid protein synthesis. Electrophoresis in polyacrylamide gels has been the method of analysis adopted in all those studies which have been able to demonstrate discrete proteins as the products of in vitro synthesis (Blair and Ellis, 1973; Eaglesham and Ellis, 1974; Bottomley et al., 1974). Polyacrylamide gel electrophoresis in 15% SDS-gels was therefore the method of analysis used in this study. SDS-gel electrophoresis provides a method of high resolution for analysing mixtures of proteins (Laemmli, 1970). The fractionation of protein mixtures by this method is based solely on the different molecular weights of the constituent proteins (Fish et al., 1970; Shapiro et al., 1967).

In the first part of this section experiments are described which/

which provide the preliminary characterization of the products of in vitro etioplast protein synthesis. The number and molecular weight of the products is determined, using three different radioactive amino acids as protein precursor. A direct comparison is made with the products of in vitro chloroplast protein synthesis by coelectrophoresis and the solubility characteristics of the etioplast products are investigated.

The second phase in the analysis of one of the products of in vitro etioplast protein synthesis; i. e. identification, is reported in the latter part of this section. This analysis has been restricted for two reasons to the soluble phase of the etioplast. Firstly the identification of an in vitro synthesized protein is more easily accomplished the closer the polypeptide resembles its related authentic protein. By examining proteins which are present in a ribosome free supernatant fraction, only those polypeptides that have been released from the ribosome are studied. These proteins will contain their full complement of amino acids and should therefore be more easily identified. Their identification may however still be hindered by the absence of post-translational modifications which might normally alter the in vivo synthesized protein. The second reason for restricting analysis to the soluble fraction is the dearth of information on the identities and physical characteristics of the polypeptide components which make up the membranes of plastids. The number of chloroplast membrane polypeptides which have been characterized has only recently increased as a result of innovations in the methods of analysing lipophilic proteins (Garewal and Wasserman, 1974a; 1974b). In view of these recent advances in the study of plastid membrane components it may soon be possible to expand the analysis of the in vitro products of plastid protein synthesis to membrane-bound proteins.

A method frequently chosen to establish the identity of an in vitro synthesized polypeptide is to compare the tryptic peptides of the product labelled in vitro with those of authentic protein labelled in vivo (Campbell and Kernot, 1962). The peptide mapping of enzymic digests of protein was originally used to detect differences in the amino acid sequences of abnormal human haemoglobins (Ingram, 1958).
However/

However the technique does not always detect slight differences between proteins, for example the replacement of a single amino acid, or certain post-translational modifications. It can however be used to show close homologies in the primary sequences of two related proteins (Kawashima *et al.*, 1974; Clegg and Kennedy, 1975). The methods of peptide mapping chosen for this study involved both tryptic and chymotryptic digestion, and separation of the resulting peptides by either cation-exchange chromatography or two-dimensional mapping.

The experiments described in the later part of this section compare the L-(³⁵S) methionine-labelled tryptic and chymotryptic peptides of the soluble *in vitro* etioplast product with firstly, the L-(³⁵S) methionine-labelled tryptic peptides of the soluble product of protein synthesis in isolated chloroplasts, and secondly with the tryptic and chymotryptic peptides of the large subunit of Fraction I protein labelled *in vivo* with L-(³⁵S) methionine.

B. ELECTROPHORETIC ANALYSIS ON SDS-POLYACRYLAMIDE GELS

i. Total products.

a. Etioplasts isolated in the light.

The isolation of etioplasts was normally performed in the light. The fractionation of the *in vitro* synthesized proteins present in a solubilized preparation of etioplasts which had been incubated with L-(³⁵S) methionine and added ATP is shown in Figure 16A. Figure 16B shows an analysis of the *in vitro* products synthesized in the absence of added ATP. Figure 16C shows an analysis of the *in vitro* products synthesized in the absence of added ATP, but in this case the protein synthesis incubation mixture was illuminated as described in Section II2c. All other experimental details are given in the figure legend.

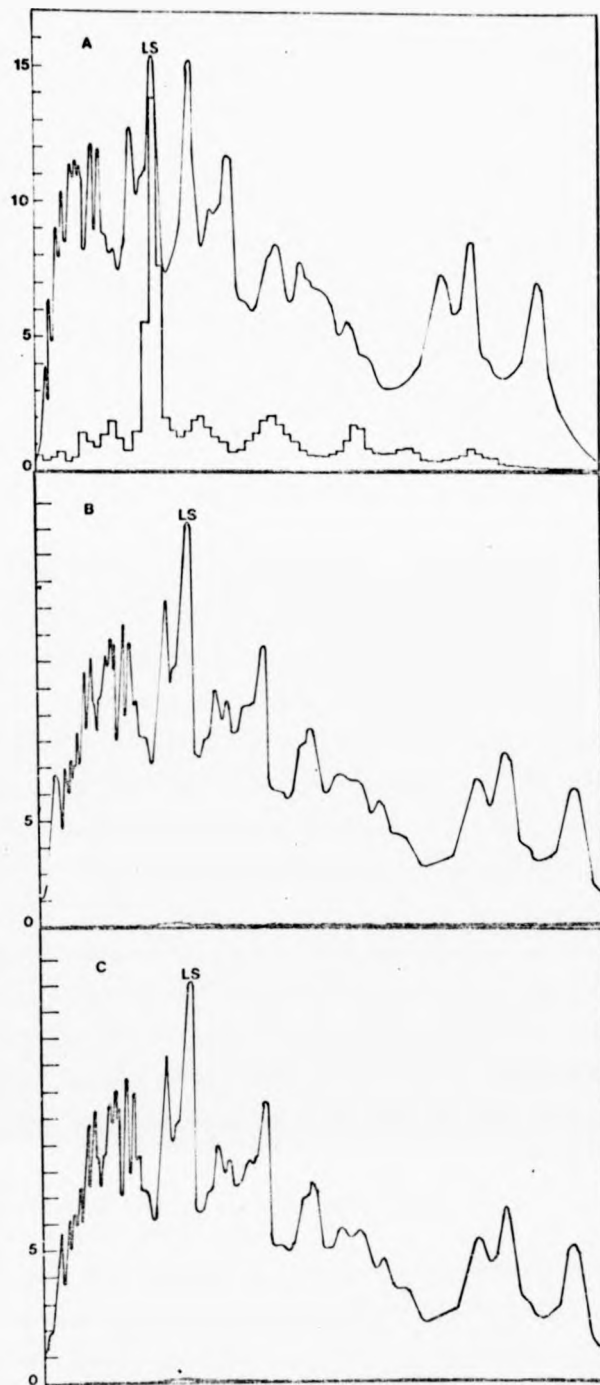
Staining of the gels with Coomassie Brilliant blue R shows many protein bands. The majority of these bands must represent some of the major protein components of the etioplast. However the heterogeneity of the crude etioplast preparation (see Plate 2B) suggests that some of these proteins might not be of plastid origin. The relative mobility of the most intensely staining band indicates that it electrophoreses to a position coincident with the large subunit of pea Fraction I protein. This conclusion is confirmed in Section III2Big. The/

Figure 16. SDS polyacrylamide gel electrophoresis of the polypeptides synthesized in vitro in etioplasts; the dependence of protein synthesis on the energy source.

Etioplasts were isolated and incubated with L-(³⁵S) methionine as described in Section II2Fia. Terminated incubation mixtures were solubilized and electrophoresed as described in Section II2Fia and II2Fiaa. Gels were loaded with 100 μ l of solubilized incubation mixture (130 μ g of protein). After electrophoresis the gels were stained, sliced and counted for radioactivity as described in Section II2Fiaa. The solid line represents the absorbance at 620 nm and the histogram shows the radioactivity in each 1 mm gel slice.

- A, incubated in the dark with added ATP.
- B, incubated in the dark.
- C, incubated in the light.

10^{-3} X Radioactivity c.p.m./1mm slice



A 620

Electrophoretic
mobility

The molecular weight of this polypeptide, established by reference to the calibration curve (Figure 3), is approximately 6×10^4 .

Figure 16A shows that the products of ATP-driven protein synthesis in isolated etioplasts are reproducibly fractionated into seven discrete radioactive peaks. The absence of radioactive peaks in the analysis of incubation mixtures that did not contain added ATP, whether illuminated or not, confirms the conclusion that only added ATP can act as an energy source for protein synthesis in isolated etioplasts (Table 1). The number of polypeptides synthesized in isolated etioplasts is the same as the number of pronase-digestible proteins synthesized in isolated chloroplasts (Eaglesham and Ellis, 1974).

b. Etioplasts isolated in the dark.

It is possible that the isolation of etioplasts in the light might affect the number or nature of proteins synthesized *in vitro*. Therefore the *in vitro* products of protein synthesis in etioplasts which had been isolated, incubated and solubilized in dim green light were analysed. Figures 17A and B show the analyses of the proteins synthesized in dark-isolated etioplasts incubated in the presence or absence of added ATP. The experimental details are given in the figure legend.

Although the radioactive bands are less discretely separated in this gel fractionation (Figure 16A) it is clear that there is no gross difference in the number and nature of proteins synthesized in etioplasts isolated either in the light or in the dark. Figure 17B confirms the dependence of *in vitro* etioplast protein synthesis on added ATP as an energy source.

c. Synthesized using L-(³H) leucine or L-phenyl(2, 3H) alanine as protein precursor.

Several proteins, for example baboon milk lysozyme (Herman *et al*, 1973) and soybean leghaemoglobin (Ellfolk and Sierers, 1971), do not contain any methionine residues. It is therefore necessary to use at least two and preferably three different labelled amino acid precursors to be reasonably confident that the full spectrum of proteins synthesized in isolated etioplasts are detected.

Figure 18 shows the SDS polyacrylamide gel electrophoresis fractionation/

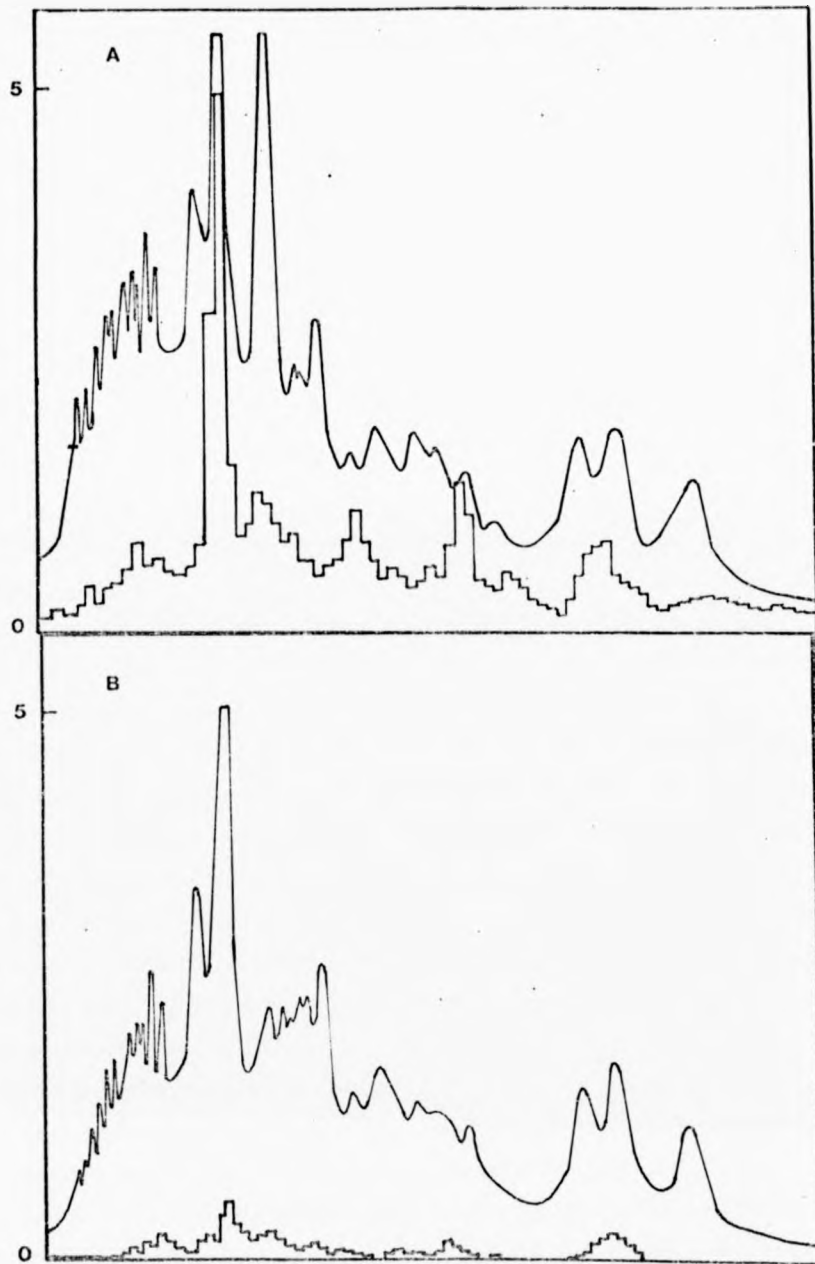
Figure 17. SDS polyacrylamide gel electrophoresis of the polypeptides synthesized in vitro in etioplasts; proteins synthesized in dark-isolated etioplasts.

The experimental details are as described in Figure 16, except that all procedures up to and including the solubilization of the terminated protein synthesis incubation mixture were performed under dim green light. The gels were loaded with 100 μ l (50 μ g of protein) of solubilized incubation mixture.

A, incubated in the dark with added ATP.

B, incubated in the dark.

10^{-3} X Radioactivity c.p.m./1mm slice



A₆₂₀

Electrophoretic mobility

fractionation of the in vitro products of etioplast protein synthesis using either L-(^3H) leucine (Figure 18A) or L-phenyl(2, ^3H) alanine (Figure 18B) as protein precursor. The qualitative differences between the fractionation patterns obtained are minor (cf. also with Figure 16A). Firstly L-(^{35}S) methionine was the only labelled amino acid precursor which reproducibly gave two peaks of radioactivity electrophoresing with a lower mobility than the most highly labelled product (Figure 16A). Often, using even the highest specific activity L-(^{35}S) methionine available, it was difficult to resolve two peaks from the background of radioactivity on the gel. Secondly, the use of L-(^3H) ^{phenyl}alanine as the labelled amino acid precursor (Figure 18A) results in an additional radioactive peak in the fractionation pattern. This peak has the highest electrophoretic mobility of all the in vitro products.

The gel fractionation pattern shown in Figure 18B is atypical and serves to illustrate a radioactive peak that was occasionally but not reproducibly found in the fractionation pattern of the in vitro etioplast products. This peak electrophoresed with a slightly greater mobility than the radioactive peak which coelectrophoreses with the large subunit of Fraction I protein. Taken together Figures 16A, 18A and 18B illustrate the complete spectrum of proteins synthesized in isolated etioplasts. Quantitative differences between the radioactivity incorporated into each product using different amino acid precursors presumably reflects the amino acid composition of the in vitro synthesized proteins.

d. Molecular weight estimation.

The apparent molecular weights of the proteins synthesized in isolated etioplasts were estimated from their mobility, relative to bromophenol blue, in 15%, SDS polyacrylamide gels. The estimates are given in Table 6. The experimental details are given in the legend to the table.

e. Digestion by pronase and ribonuclease A.

The sensitivity of the in vitro labelled etioplast products to digestion by pronase and ribonuclease A was investigated. The results are shown in Figure 19. Incubation of the in vitro products in the absence/

Figure 18. Polyacrylamide gel electrophoresis of the in vitro products of etioplast protein synthesis using L-(³H) leucine and L-phenyl(2, 3H) alanine as labelled amino acid precursor.

The experimental details are as described in Figure 16, except that the incubation mixture contained either 100 μ Ci of L-(³H) leucine (specific activity 50 Ci/mmol) or 100 μ Ci of L-phenyl (2, 3H) alanine (specific activity 20 Ci/mmol). The gels were loaded with 100 μ l (160 μ g of protein) of solubilized incubation mixture.

A, incubated with L-(³H) leucine

B, incubated with L-phenyl(2, 3H) alanine

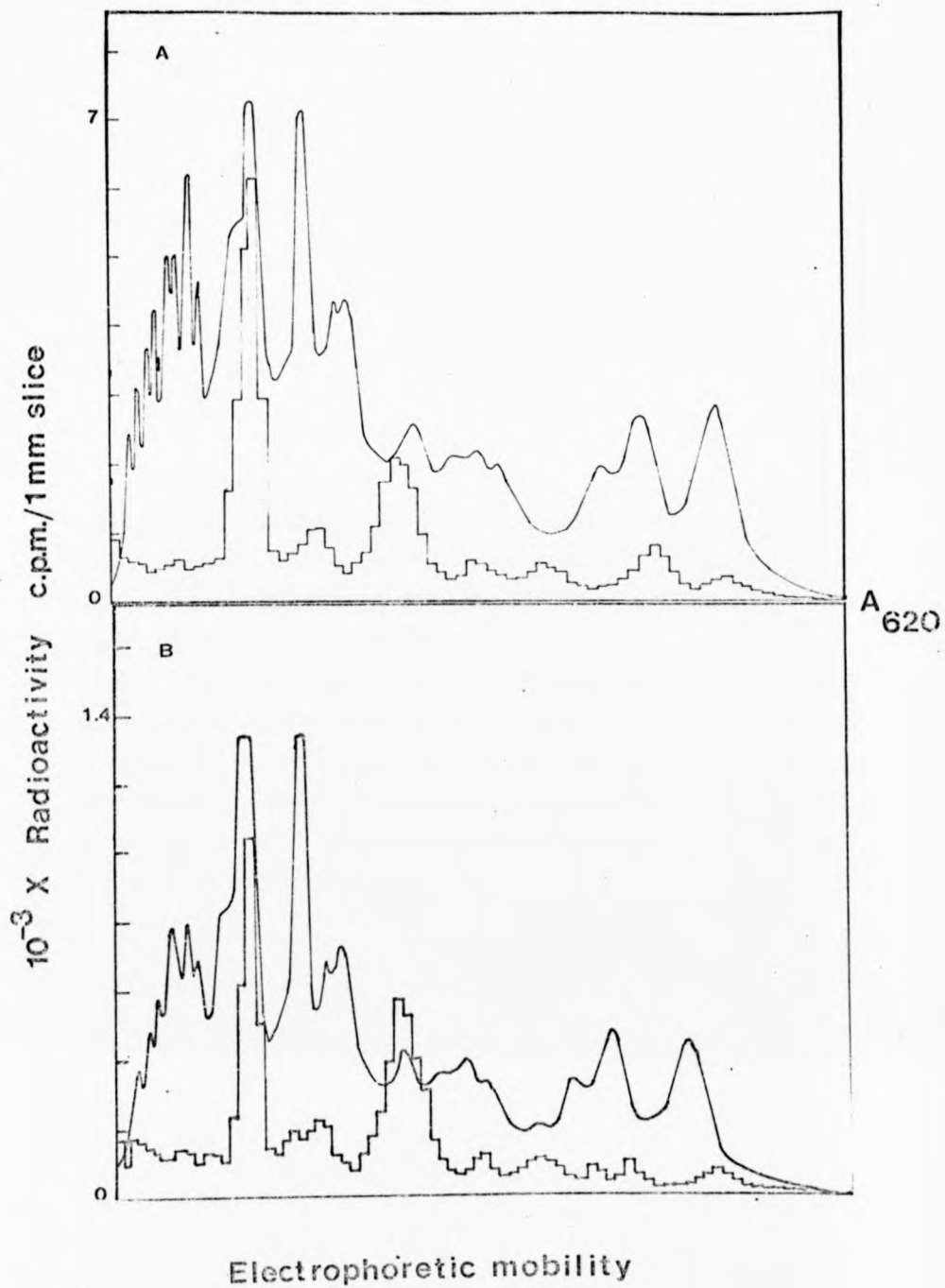


Table 6. The molecular weights of the proteins synthesized in isolated etioplasts.

Etioplast preparations were isolated, incubated and electrophoresed as described in Sections H2A, H2Fia and H2Fiia. The mobility of the bromophenol blue was recorded and the gels were sliced into 1 mm slices without staining for protein. The mobility of the in vitro synthesized products was determined from the distribution of radioactivity within the gel. The molecular weights were estimated from the calibration curve (Figure 3).

<u>Relative Mobility</u>			<u>Mol. wt.</u>
L-(³⁵ S) methionine	L-(³ H) leucine	L-phenyl (2, 3H) alanine	
0. 109	0. 109	0. 107	112000
0. 150	-	-	84000
0. 236	0. 230	0. 232	60000
* -	-	0. 275	49000
0. 310	0. 300	0. 300	43500
0. 450	0. 450	0. 450	32000
0. 610	0. 590	0. 590	25000
0. 710	0. 700	0. 700	21000
0. 920	0. 910	0. 910	16000
-	-	0. 960	15000

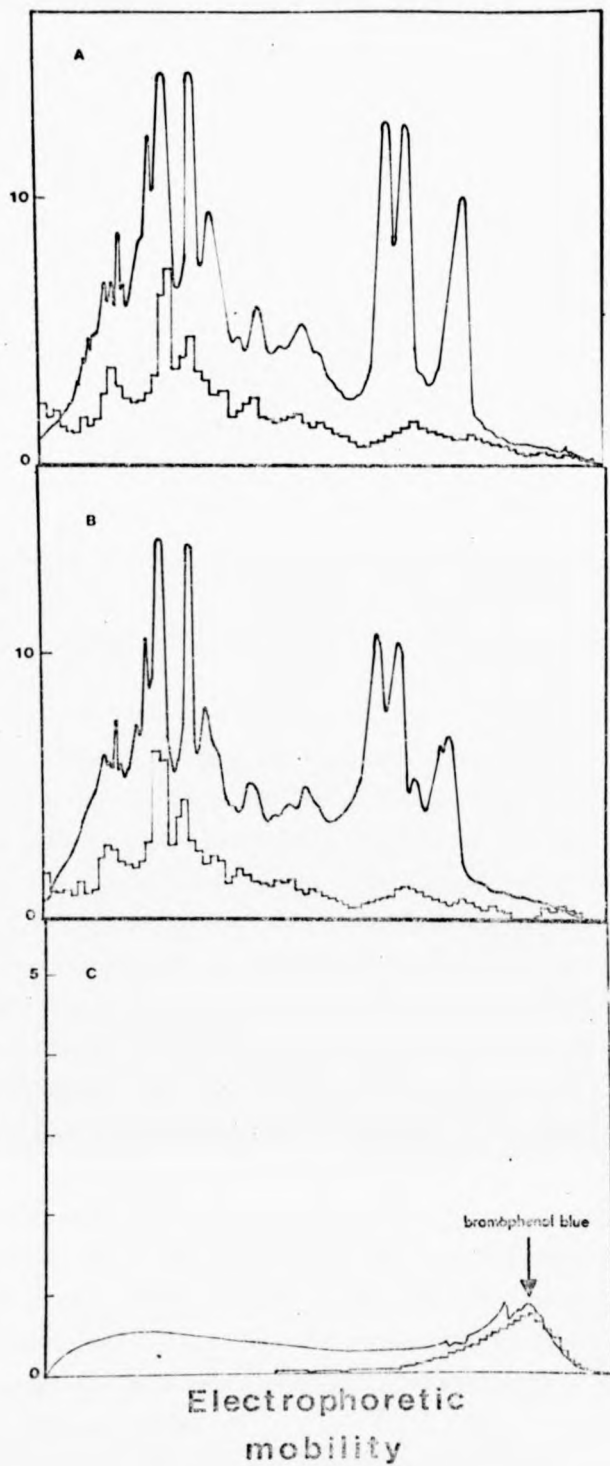
* not reproducible

Figure 19. The stability of the products of in vitro etioplast protein synthesis to digestion by pronase and ribonuclease A.

Details of the digestion procedure are given in Section II2Fic. Electrophoresis was performed as described in Section II2Fiia. The gels were loaded with 100 μ l of digest.

- A, control incubation (90 μ g of protein)
- B, ribonuclease A treated (90 μ g of protein)
- C, pronase treated

$10^{-3} \times$ Radioactivity c.p.m./1mm slice



A
620

absence of either enzyme (Figure 19A) results in considerable degradation of the newly-synthesized polypeptide chains. This degradation may be due to the action of endogenous proteases. However the higher molecular weight products in particular remain as discrete peaks. Incubation with ribonuclease (Figure 19B) produces a fractionation pattern essentially similar to the control. The labelled peaks and the stained protein bands were stable to the action of ribonuclease. In contrast all the radioactive peaks and all the stained protein bands are hydrolysed by incubation of the preparation with pronase (Figure 19C). After pronase digestion the only material containing radioactively labelled amino acid electrophoreses to a point coincident with the bromophenol blue marker dye, and therefore presumably represents oligo-peptides resulting from enzymic hydrolysis.

f. Coelectrophoresis with the products of in vitro chloroplast protein synthesis.

A direct comparison between the products of protein synthesis in isolated etioplasts and isolated chloroplasts can be made on the basis of their electrophoretic behaviour. Coelectrophoresis of the samples was used to give the most accurate comparison. As the disc gel electrophoresis system was chosen to give high resolution, it was necessary to label the etioplast and chloroplast in vitro products with different isotopes. Double-label counting was then used to separate the radioactivity determined in each gel slice into its isotopic components. Figure 20 shows the simultaneous gel fractionation of the etioplast and chloroplast products before and after they have been extracted with acetone as described in Section II2Fid. All other experimental procedures are given in the figure legend.

Before extracting the samples with acetone the in vitro etioplast and chloroplast products electrophoresed with similar, but reproducibly different, mobilities (Figure 20A). However after acetone extraction the mobilities of the polypeptides synthesized in isolated etioplast are identical with the mobilities of the polypeptides synthesized in isolated chloroplasts (Figure 20B).

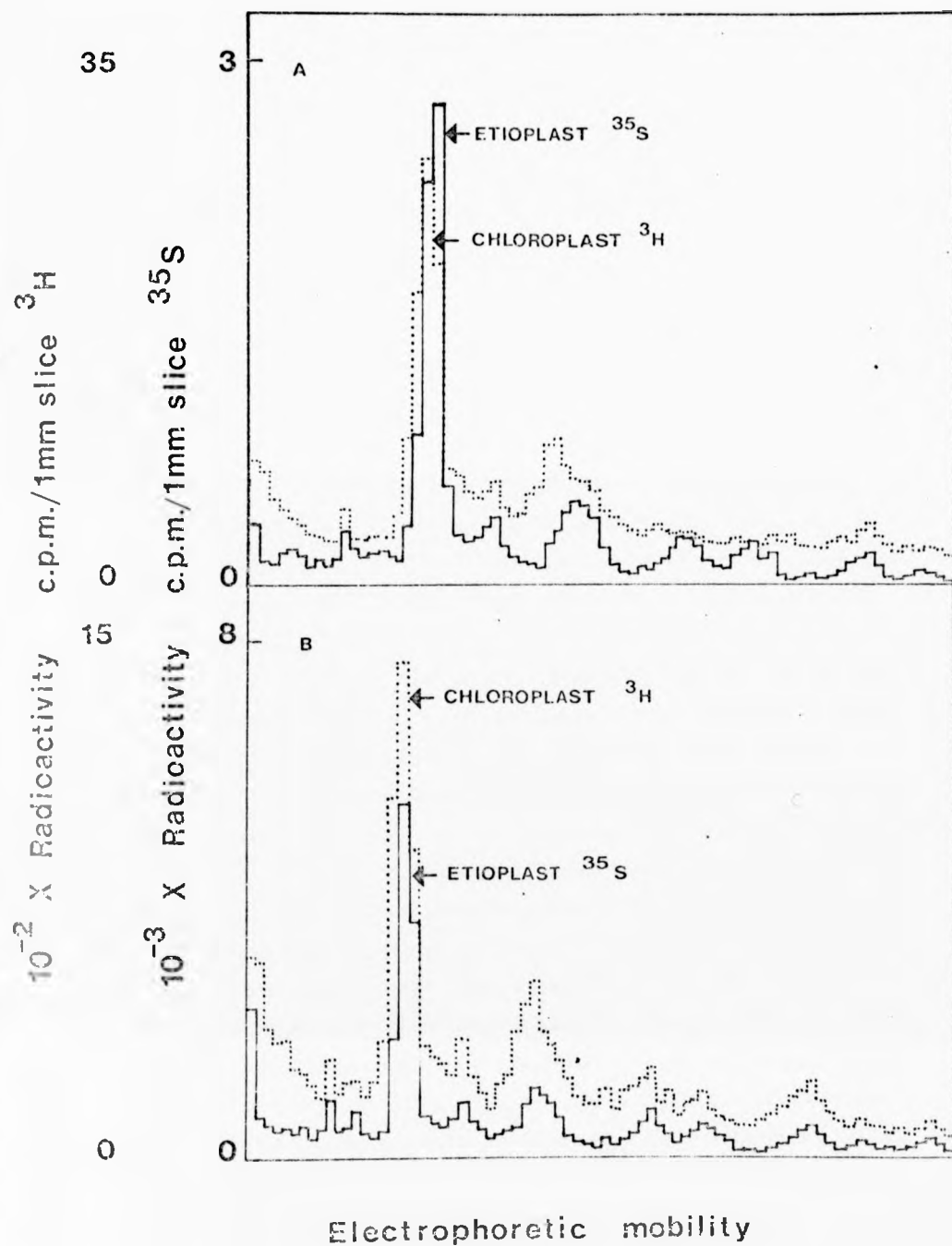
g. Comparison with the electrophoretic mobility of pea Fraction I protein.

The electrophoretic mobilities of the in vitro etioplast products and/

Figure 20. Coelectrophoresis of in vitro synthesized etioplast and chloroplast products in SDS polyacrylamide gels.

Etioplasts and chloroplasts were isolated and incubated as described in Sections II2A and II2Fia. Etioplast products were labelled in vitro with L-(³⁵S) methionine and chloroplast products labelled in vitro with L-(³H) leucine. The etioplast samples contained 150 µg of protein (3×10^3 c. p. m.) and the chloroplast sample 10 µg of protein (3×10^3 c. p. m.). Light was the energy source for isolated chloroplast protein synthesis. Samples were extracted with acetone as described in Section II2Fid. Electrophoresis and related procedures were performed as described in Section II2Fia.

- A, samples coelectrophoresed before acetone extraction.
- B, samples electrophoresed after acetone extraction.



and pea Fraction I protein were compared. A sample of purified pea Fraction I protein was denatured by boiling for 2 minutes in 2.5 mM Tris-19 mM glycine pH 8.5, containing 10 mM 2-mercaptoethanol and 2% (w/v) SDS. An aliquot of the denatured protein was mixed with a solubilized preparation of radioactively labelled in vitro etioplast products. The mixed sample was then electrophoresed. Figure 21 shows the optical density trace and the distribution of radioactivity in the resulting gel. The gel was scanned in the Chromoscan at a low sensitivity to allow an accurate positioning of the peaks enhanced by the addition of Fraction I protein. The staining of two bands on the gel was found to be enhanced. The lower mobility band is the large subunit of Fraction I protein. The distribution of radioactivity in the gel shows that one of the in vitro etioplast products (approximate molecular weight 6×10^4) coelectrophoreses, and therefore has the same molecular weight (Shapiro et al., 1967) as the large subunit of Fraction I protein.

ii. Soluble products.

a. Products in the 150000 g etioplast supernatant fraction.

The products of in vitro etioplast protein synthesis can be characterized on the basis of their solubility. High-speed centrifugation of a lysed preparation of etioplast proteins (Section H2Fib) yields two fractions. The pelleted fraction would be expected to include membrane proteins and both membrane-bound and free ribosomes with attached nascent polypeptides. The supernatant fraction would be expected to contain only complete polypeptides which are destined in vivo for the soluble phase of the plastid. Figure 22 shows the fractionation pattern obtained when a labelled 150000 g etioplast supernatant fraction is electrophoresed in SDS-polyacrylamide gels. The experimental details are given in the figure legend. Only a single product of in vitro etioplast protein synthesis is present in the 150000 g supernatant fraction. This polypeptide has been shown (Figure 21) to coelectrophorese with the large subunit of pea Fraction I protein and has a molecular weight of approximately 6×10^4 (Table 6).

Figure 21. Comparison of the electrophoretic mobility of pea Fraction I protein with the in vitro products of etioplast protein synthesis.

Etioplasts were isolated and incubated as described in Sections II2A and II2Fia. Pea Fraction I protein was purified as described in Section II2Gi and solubilized by boiling in 2% SDS for 2 minutes. 50 μ l (90 μ g of protein) of solubilized preparation of in vitro etioplast products were mixed with 50 μ l (90 μ g) of denatured Fraction I protein. The mixed sample was electrophoresed as described in Section II2Fia.

LS, large subunit of Fraction I protein.

SS, small subunit of Fraction I protein.

$10^{-3} \times$ radioactivity c.p.m./1mm slice

Electrophoretic mobility

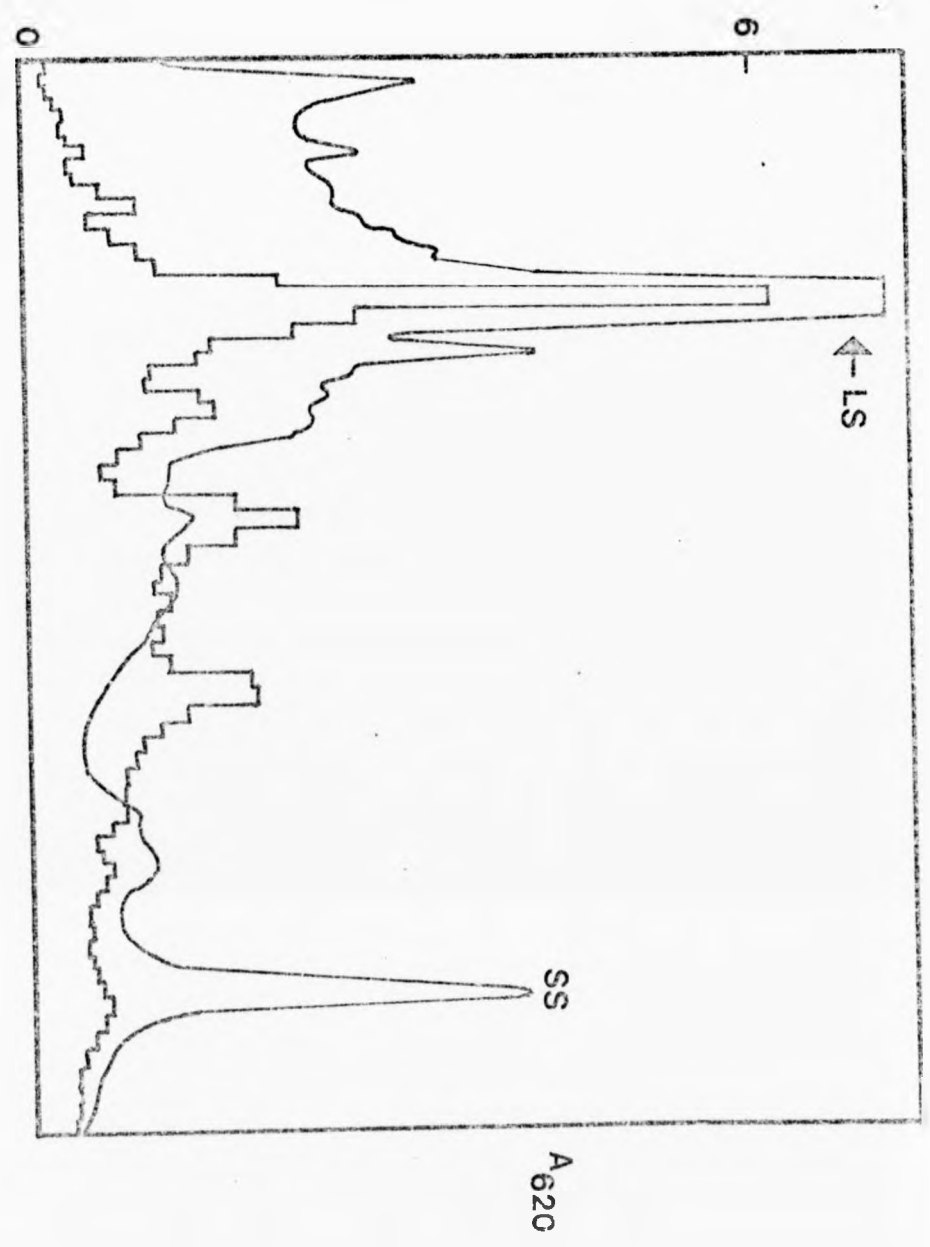
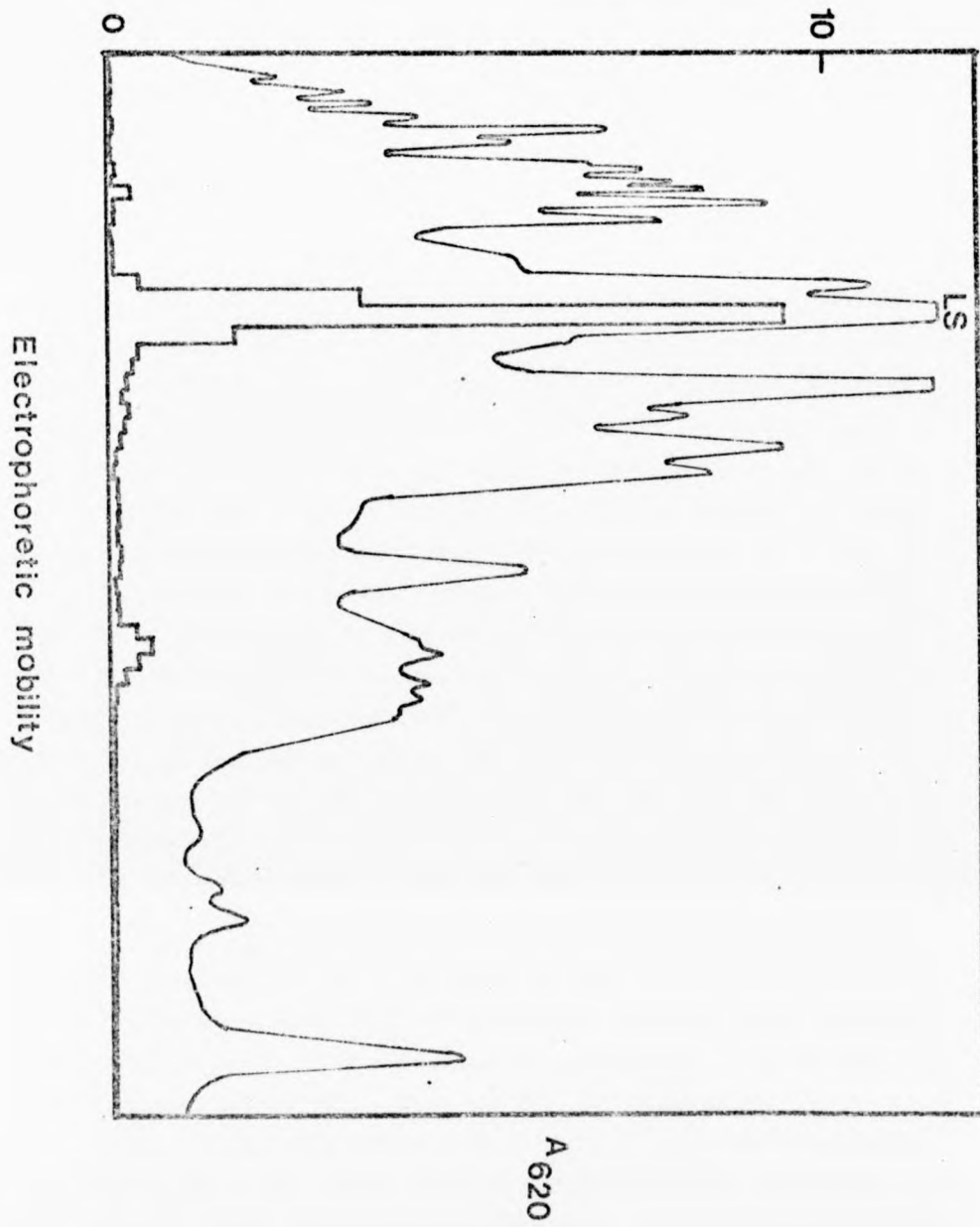


Figure 22. Electrophoresis of the labelled 150000 g etioplast supernatant fraction in SDS-polyacrylamide gel.

Etioplasts were isolated and incubated as described in Sections II2A and II2Fia. A 150000 g supernatant fraction was prepared as described in Section II2Fib. Electrophoresis and related procedures were performed as described in Section II2Fia. The gel was loaded with 100 μ l of dialysed sample (220 μ g of protein).

L.S, large subunit of Fraction I protein.

$10^{-3} \times {}^{35}\text{S}$ radioactivity c.p.m./1mm slice



C. IDENTIFICATION OF THE SOLUBLE IN VITRO
ETIOPLAST PRODUCT.

i. Electrophoresis of the purified in vitro synthesized
soluble product.

The homogeneity of the purified in vitro synthesized soluble product of etioplast protein synthesis was established by electrophoresis in SDS polyacrylamide gels. Although the material used for enzymic digestion was purified by preparative gel electrophoresis (Section II2Diii) and was the only discrete radioactively labelled protein in the 150000 g etioplast supernatant (Figure 22), this precaution was nevertheless necessary as any partial breakdown of this material during purification could give rise to many peptides not specifically resulting from tryptic or chymotryptic hydrolysis. Figure 23 shows that only a single radioactive peak is found to have migrated into the gel. A higher molecular weight species of protein which does not enter the gel as a discrete band but penetrates into the first two millimeters of the gel is also radioactively labelled. This higher molecular weight species is assumed to be an aggregated or insoluble form of the 60000 molecular weight in vitro product and arises during the purification procedure perhaps due to the use of organic solvents. The absence of any radioactively labelled protein species of lower molecular size than 60000 molecular weight product indicates that there has not been significant degradation of the in vitro labelled material during its purification. A similar conclusion has already been reached for the purified large subunit of Fraction I protein which has been labelled with L-(³⁵S) methionine in vivo (Section II2Giv).

ii. Peptide mapping; tryptic digestion.

a. Cation-exchange chromatography.

1. soluble in vitro etioplast product.
2. soluble in vitro chloroplast product.

The analysis of the L-(³⁵S) methionine labelled tryptic peptides of the soluble in vitro chloroplast product presented in this section was performed by Dr. R. J. Ellis and Ms. E. Forrester.

Figure 24 shows a comparison of the L-(³⁵S) methionine labelled tryptic peptides of the soluble in vitro etioplast and chloroplast products separated by cation-exchange chromatography. This comparison can be used to indirectly establish the identity of the soluble in vitro etioplast product/

Figure 23. Electrophoresis of the purified soluble in vitro product of etioplast protein synthesis in SDS-polyacrylamide gels.

Etioplasts were isolated and incubated as described in Sections II2A and II2Fia. A 150000 g supernatant fraction was prepared as described in Section II2Fib. The soluble product was purified by preparative polyacrylamide gel electrophoresis as described in Section II2Fiii. A 1 mg/ml solution of the soluble in vitro product was prepared in 2.5 mM Tris-19 mM glycine (pH 8.5), containing 100 mM 2-mercaptoethanol and 2% (w/v) SDS and analysed by electrophoresis as described in Section II2Fia. The gel was loaded with 100 μ l (10^5 c. p. m.) of sample.

$10^{-3} \times {}^{35}\text{S}$ radioactivity c.p.m./1mm slice

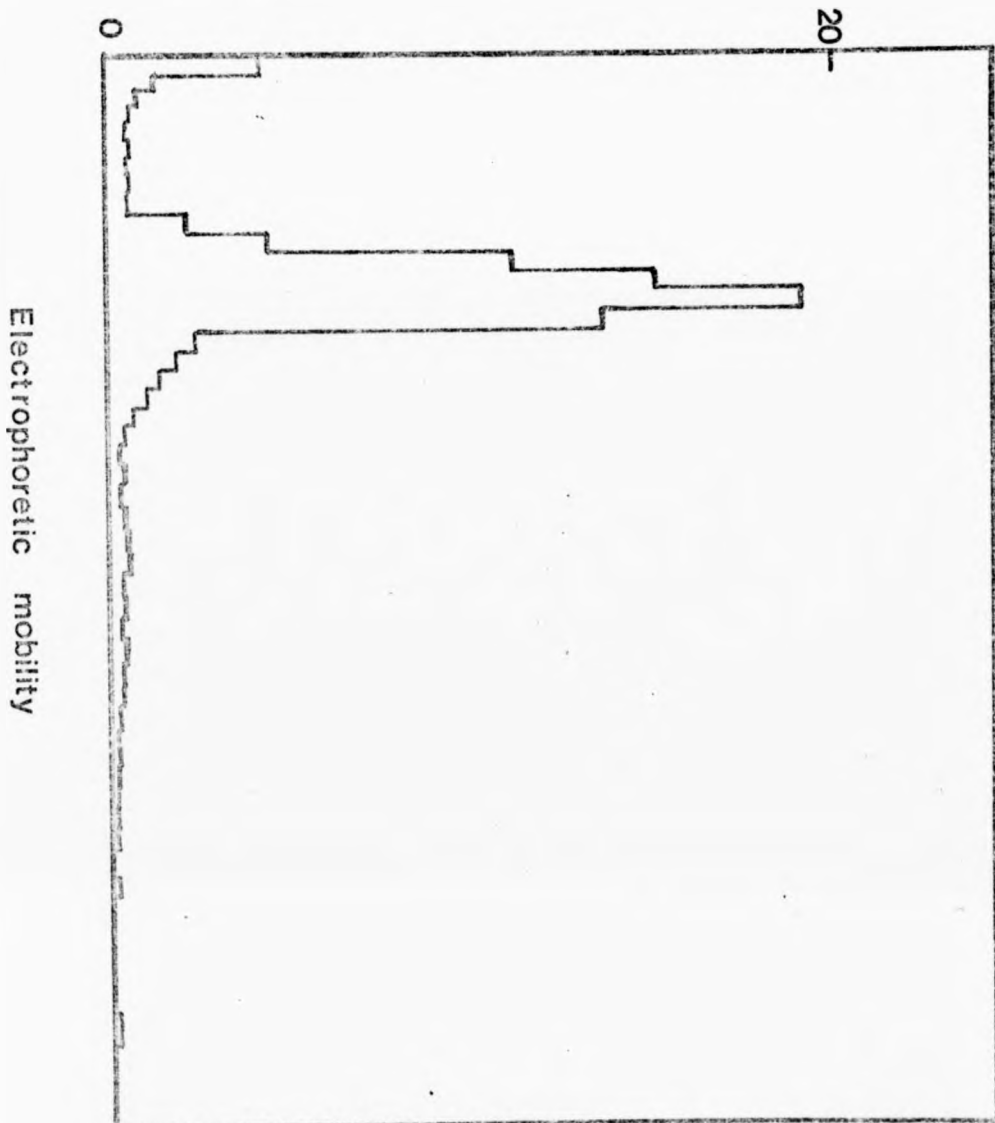
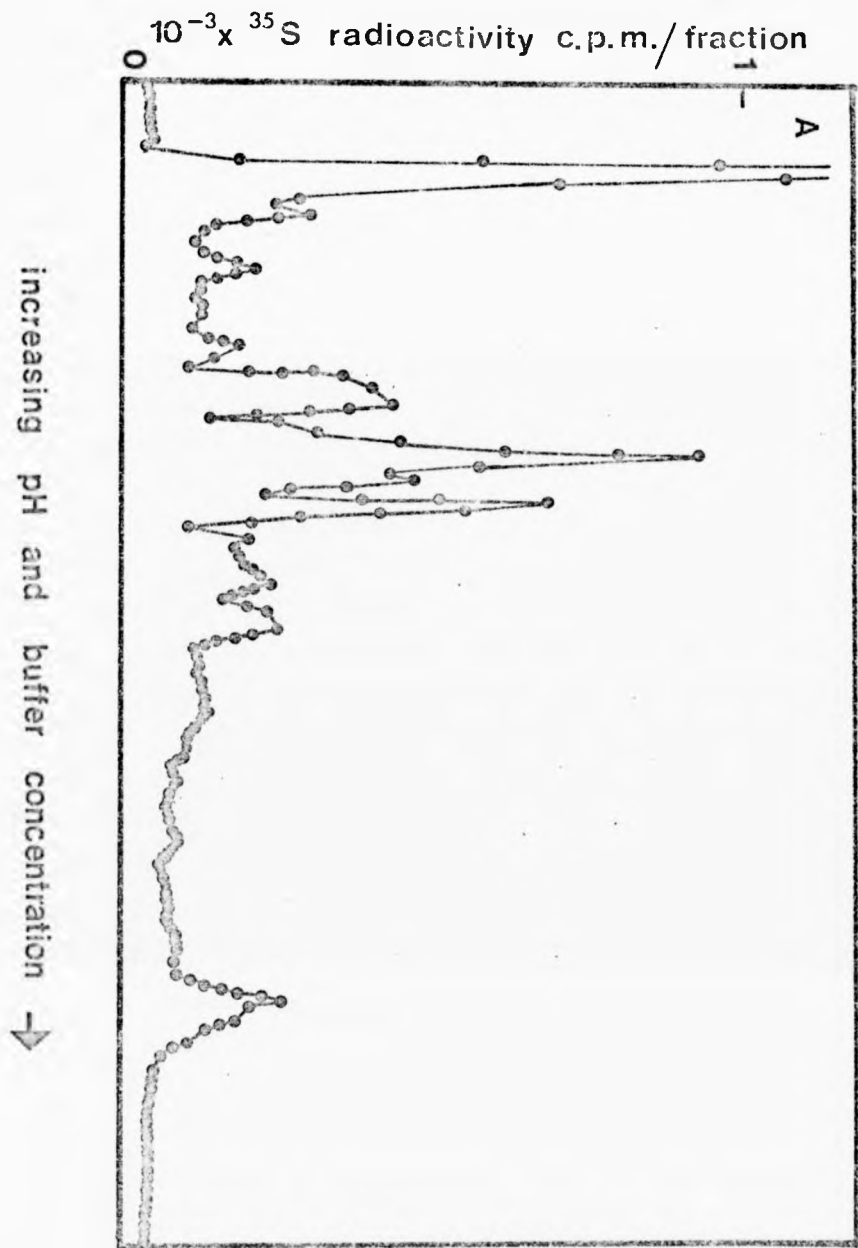


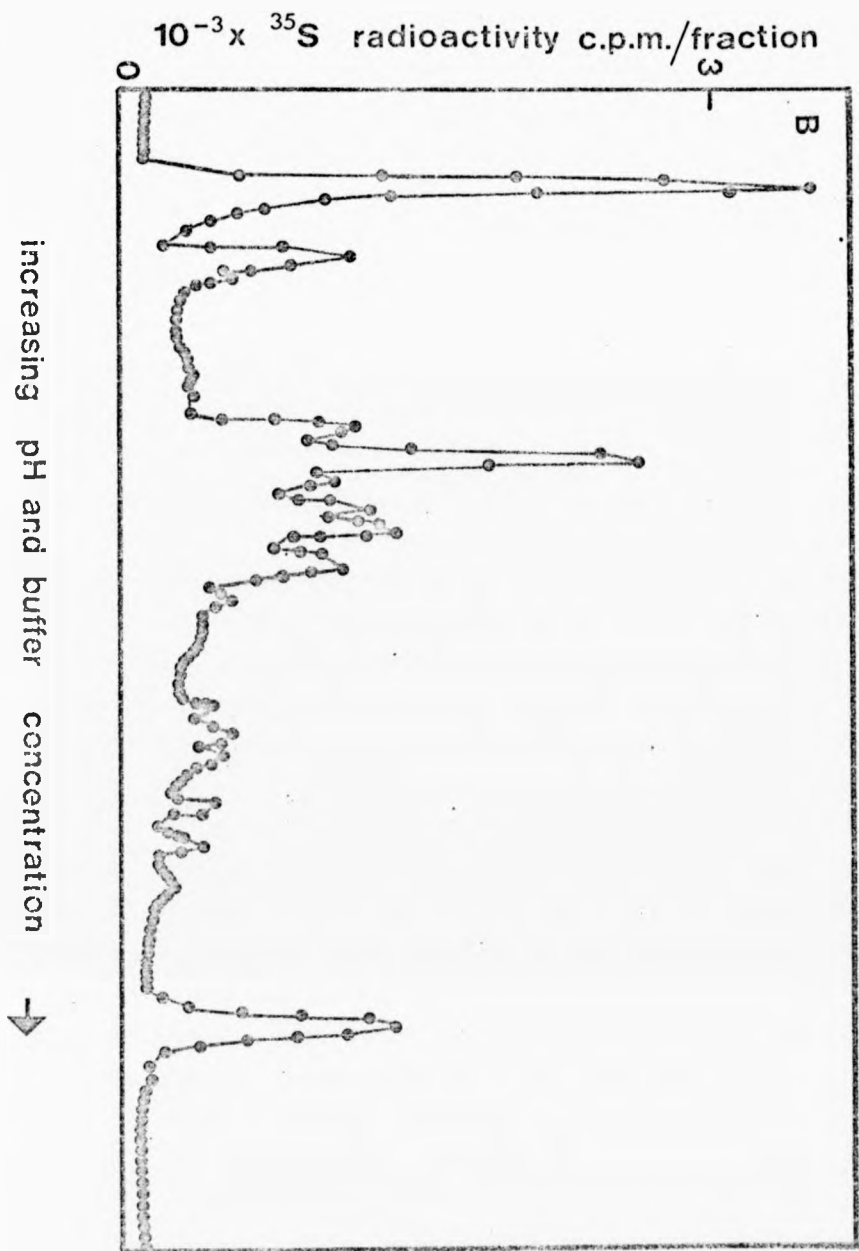
Figure 24. Cation-exchange chromatography of the soluble in vitro product of light-driven chloroplast protein synthesis and the soluble in vitro product of ATP-driven etioplast protein synthesis.

Plastids were isolated and incubated as described in Sections II2A and II2Fia. 150000 g supernatant fractions were prepared and purified as described in Sections II2Fib and II2Fiii. Enzyme digestion and peptide separation by cation-exchange chromatography were performed as described in Sections II2Ji and ii. Both samples contained approximately 2×10^5 c. p. m. and 250 μ g of protein.

A, the soluble in vitro product of light-driven chloroplast protein synthesis.

B, the soluble in vitro product of ATP-driven etioplast protein synthesis.





product as the soluble in vitro product of chloroplast protein synthesis has been identified as the large subunit of Fraction I protein (Blair and Ellis, 1973). The elution profiles show that several methionine-labelled tryptic peptides are common to both polypeptides. The result is therefore consistent with the identification of the soluble in vitro etioplast product as the large subunit of Fraction I protein. However the comparison is inconclusive. The majority of tryptic peptides elute from the column under similar conditions of pH and buffer concentration. Thus, although the specificity of the enzymic hydrolysis has resulted in relatively few methionine labelled peptides, the poor resolution of the separation technique does not allow them to be identified as characteristic of a particular protein. A more effective method of peptide separation was therefore sought.

- b. Two-dimensional mapping.
 - 1. soluble in vitro etioplast product.
 - 2. in vivo radioactively-labelled purified pea Fraction I protein large subunit.

To provide a conclusive identification of the soluble in vitro product of etioplast protein synthesis, a two dimensional map of the L-(³⁵S) methionine-labelled tryptic peptides of the in vitro synthesized product was compared with a map of the L-(³⁵S) methionine-labelled tryptic peptides of the in vivo labelled large subunit of Fraction I protein. Plate 3 shows that the in vitro etioplast product shares 7 major L-(³⁵S) methionine-labelled tryptic peptides with the large subunit of Fraction I protein labelled with L-(³⁵S) methionine in vivo. Some minor peptides may also be common to both proteins. This number of methionine tryptic peptides for the large subunit of Fraction I protein agrees with the amino acid analyses reported for bean (Phaseolus) and spinach beet (Beta) by Gray and Kekwick (1974a, 1974b)

- iii. Peptide mapping - chymotryptic digestion.
 - a. Two-dimensional mapping.
 - 1. soluble in vitro etioplast product
 - 2. in vivo radioactively labelled purified pea Fraction I protein large subunit.

Chymotryptic digestion of the in vitro etioplast product and purified Fraction I protein large subunit labelled in vivo with L-(³⁵S) methionine/

Plate 3 Autoradiographs of two-dimensional tryptic peptide maps of the soluble in vitro product of ATP-driven etioplast protein synthesis and in vivo labelled large subunit of Fraction I protein.

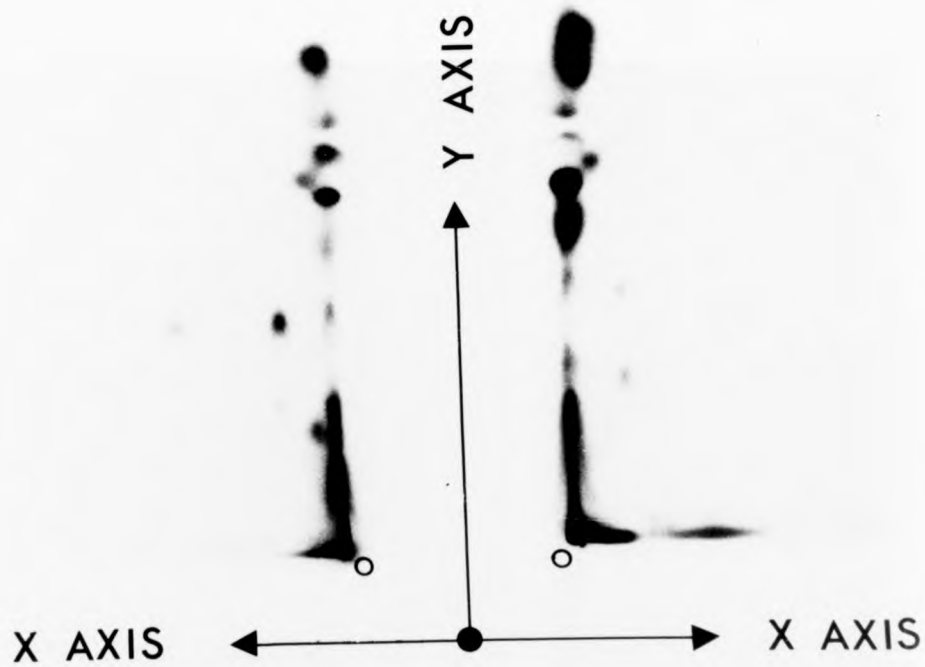
Etioplasts were isolated and incubated as described in Sections II2A and II2Fia. A 150000 g supernatant fraction was prepared and purified as described in Sections II2Fib and II2Fiii. In vivo labelled large subunit of Fraction I protein was prepared as described in Sections II2Giii and iv. Enzymic digestion and peptide separation by two-dimensional mapping was performed as described in Sections II2Ji and iii. Both samples contained 7.5×10^4 c. p. m. Electrophoresis was performed in the direction of the Y axis and chromatography in the direction of the X axis.

A, the soluble in vitro etioplast product

B, in vivo labelled large subunit of Fraction I protein

A

B



methionine was performed to substantiate the conclusion reached using trypsin. Although the intrinsic specificity of chymotrypsin is broader than trypsin, it can, particularly if used in conjunction with a trypsin inhibitor, give rise to a set of oligopeptides which are indicative of a specific protein. Plate 4 shows a comparison between the two dimensional maps of the L-(³⁵S) methionine labelled chymotryptic peptides of the soluble in vitro etioplast product and the large subunit of Fraction I protein labelled with L-(³⁵S) methionine in vivo. The majority of the peptides can be clearly recognised in both maps, indicating close homology in the primary sequence of the two proteins.

D. DISCUSSION

The results presented in this section constitute the first analysis of proteins unequivocally synthesized in isolated etioplasts. Fractionation of a solubilized preparation by electrophoresis in 15% SDS-polyacrylamide gels separates over 20 discrete protein bands which stain with Coomassie Brilliant blue R (Figure 16A). The majority of these bands are most likely to be protein components of the etioplast, although some of the stained bands may originate from nuclei or other subcellular organelles which contaminate the crude etioplast preparation (Section III1Bi). One of the most intensely staining bands, which electrophoreses with a slightly higher mobility than the large subunit of pea Fraction I protein, is absent from fractionation patterns of etioplast preparations that were incubated without an ATP generating system (Figure 16E). This protein band is therefore presumed to be the enzyme creatine phosphokinase (subunit molecular weight 4.2×10^4 ; Eppenberger et al., 1967). The identities of the majority of the other protein components of the fractionated etioplast preparation are unknown. The most intensely staining band has an estimated molecular weight of 6×10^4 , and coelectrophoreses with the large subunit of Fraction I protein (Figure 21). A molecular weight of 6×10^4 is in general agreement with the known molecular weight of the large subunit of Fraction I protein from other higher plant sources (Futner and Lane, 1967; Kawashima, 1969; Moon and Thompson, 1969; Ellis, 1973). Fraction I protein has been found in the etioplasts of many species (Graham et al., 1971; Smith et al., 1970). Indeed etioplasts isolated from certain Avena /

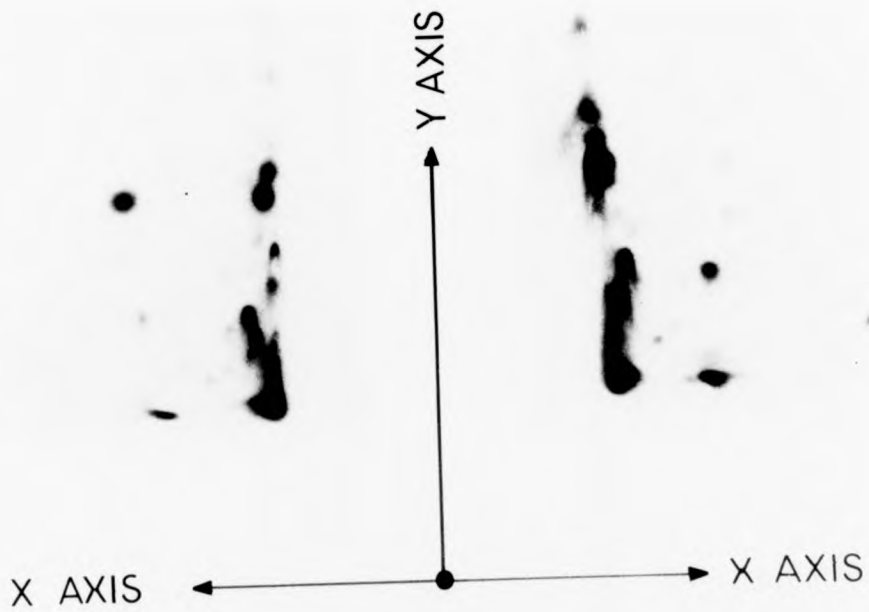
Plate 4. Autoradiographs of two-dimensional chymotryptic peptide maps of the soluble in vitro product of ATP-driven etioplast protein synthesis and in vivo labelled large subunit of Fraction I protein.

The experimental details are described in Plate 3. Both samples contained 2×10^4 c. p. m.

- A, the soluble in vitro etioplast product
- B, in vivo labelled large subunit of Fraction I protein.

A

B



Avena species contain macromolecular structures, the so-called stromacentres (Gunning, 1965). Stromacentres in plastids have been suggested to represent semi-crystalline linear aggregates of Fraction I protein; although this suggestion is based on tentative evidence (Gunning *et al.*, 1968; Murakami, 1974; Wrischer, 1973). This intensely staining protein band can therefore be tentatively identified as the large subunit of Fraction I protein.

The fractionation pattern of the pea etioplast preparation (Figure 16A) contrasts with the reported fractionation pattern of purified oat (Avena) etioplast proteins (Cobb and Wellburn, 1973). For example, the pea etioplast contains a number of proteins with an estimated molecular weight greater than 6×10^4 (Figure 16A). In contrast, oat etioplasts, purified by a method involving macromolecular filtration (Wellburn and Wellburn, 1971), contain only 8 protein components, the largest with an estimated molecular weight of 5.4×10^4 (Cobb and Wellburn, 1973). This discrepancy most probably stems from practical difficulties due to the enormous variation in amounts of different etioplast proteins. This suggestion is supported by the findings of a subsequent analysis on an oat etioplast fraction containing pure envelope membranes (Cobb and Wellburn, 1974). This fraction is found to contain 15 protein species, six of which have molecular weights greater than 6×10^4 . Clearly any comparative analyses of etioplast proteins by polyacrylamide gel electrophoresis must be quantitative as well as qualitative. The number and range of etioplast proteins revealed by gel fractionation argues against considering the etioplast as a structurally simple and undifferentiated organelle. This point is discussed further in the general discussion (see also Kirk and Tilney-Bassett, 1967).

Etioplasts that have been isolated in the light and incubated with L-(^{35}S) methionine in the presence of ATP incorporate radioactive amino acid into a small number of discrete products (Figure 16A). Amino acid incorporation does not take place in the absence of added ATP as an energy source (Figures 16B and 16C). These conclusions closely reflect the results shown in Table 1. The dependence of incorporation on an added energy source indicates that the labelled peaks/

peaks present in the gel fractionation cannot be explained by the binding of L-(³⁵S) methionine to specific proteins. This conclusion is important as the gel slices were not subject to treatment with hot trichloroacetic acid. The possibility that at least one of the radioactively labelled products is (³⁵S) methionyl charged tRNA is excluded by the insensitivity of the in vitro products to digestion with ribonuclease A (Figure 19B). The conclusion that the labelled amino acid is incorporated intramolecularly into protein is verified by the sensitivity of the fractionated products to digestion with pronase (Figure 19C).

Performing the isolation and incubation of the etioplasts in dim green light did not alter the nature of the in vitro synthesized products (Figure 17A). Light does not therefore stimulate the incorporation of amino acids into specific proteins in isolated pea etioplasts. The only report of such a specific light stimulation is based on studies of in vitro protein synthesis in maize etioplast preparations (Hearing, 1973). It was not however demonstrated that amino acid incorporation in these maize etioplast preparations was dependent upon an added energy source, nor was the etioplast established as the only site of protein synthesis. Indeed the incorporation of amino acid into four proteins in the presence of 300 µg/ml chloramphenicol is strong evidence of cytoplasmic contamination in these preparations. For these reasons the conclusions of Hearing must be considered tenuous.

In addition to providing further evidence that amino acids are incorporated into protein in isolated etioplasts, the use of three different protein precursors (L-(³⁵S) methionine, L-(³H) leucine and L-phenyl(2, 3H) alanine) suggests that the full spectrum of in vitro synthesized proteins are likely to be detected (Figures 16A, 18A and 18B). The incorporation of radioactivity into protein is greatest using L-(³⁵S) methionine. This labelled amino acid has the highest specific activity (300 Ci/mmol) of the three protein precursors used. Decreased incorporation of radioactivity occurs with the lower specific activity tritium-labelled amino acids, L-(³H) leucine and L-phenyl (2, 3H) alanine. Factors which will affect the level of incorporation of each amino acid are the relative pool sizes of different amino acids
in/

in the etioplast and the differential permeability of the plastid envelope to individual amino acids (Nobel and Wang, 1970).

The products of protein synthesis in isolated pea etioplasts can be identified as discrete proteins, and categorized on the basis of their molecular weights (Table 6). The synthesis of seven polypeptides (molecular weights 1.6 to 11.2×10^4) can be reproducibly observed. The use of specific amino acid precursors allows the detection of a further two products (molecular weights 8.4×10^4 and 1.5×10^4). A further polypeptide (molecular weight 4.9×10^4) is labelled on occasions but is not reproducibly detected. The discrete labelling of in vitro synthesized protein testifies to the normal functioning of the protein synthesizing machinery in isolated intact etioplasts. The processes of chain elongation and termination must function correctly if the products of in vitro synthesis are detected as discrete proteins. Evidence for the initiation of polypeptide chain formation in isolated spinach chloroplasts has also been presented (Bianchetti et al., 1971).

The proteins synthesized in isolated etioplasts can be compared directly and indirectly with the products of light-driven protein synthesis in isolated pea chloroplasts (Blair and Ellis, 1973; Eaglesham and Ellis, 1974). The more indirect comparison, based on the estimated molecular weights of the in vitro products, suggests that all the products of protein synthesis in isolated chloroplasts have a counterpart in the spectrum of in vitro synthesized etioplast proteins. This conclusion is only tentative and takes into account the error inherent in estimating the molecular weight of a protein by SDS-polyacrylamide gel electrophoresis (Fish et al., 1970). Isolated etioplasts synthesize, in addition, three polypeptides which are not synthesized in isolated chloroplasts. However the detection of specific proteins synthesized in isolated etioplasts borders on the resolution of the analytical techniques used. The use of large quantities of three different protein precursors of the highest radioactive specific activity available ensures that any proteins that are being synthesized in isolated etioplasts, but have not been detected, must be produced in very small amounts.

It/

It seems unlikely that the proteins synthesized in isolated etioplasts are produced by cleavage or proteolysis of a higher molecular weight species. As the samples were boiled in SDS at the termination of the protein synthesis incubation the processing of any hypothetical precursor molecule must have been rapid and concomitant with or immediately following translation. This possibility could be tested by analysing the products of protein synthesis incubations of short duration.

A direct comparison of pea etioplast and chloroplast *in vitro* synthesized proteins can be made by co-electrophoresis (Figure 20B). The mobilities of the polypeptides synthesized in isolated etioplasts are identical with the mobilities of the polypeptides synthesized in isolated chloroplasts. To obtain a double-labelled gel pattern in which the *in vitro* synthesized etioplast and chloroplast proteins electrophoresed with identical rather than similar mobilities (Figure 20A) it was necessary to extract the samples with acetone before electrophoresis. This suggests that acetone-soluble components of the solubilized plastid preparation slightly affect the mobility of polypeptides in the electrophoresis system used.

The most highly labelled product of *in vitro* etioplast protein synthesis (molecular weight 6×10^4) is also common to the fractionation pattern of proteins synthesized in isolated chloroplasts (Figure 20B). The identity of this polypeptide has been established in the light-driven chloroplast system as the large subunit of Fraction I protein (Blair and Ellis, 1973). Therefore by inference from the chloroplast situation, the results suggest that the 6×10^4 molecular weight polypeptide synthesized in isolated etioplasts is the large subunit of Fraction I protein. This suggestion is supported by the coelectrophoresis of purified large subunit of Fraction I protein with the 6×10^4 molecular weight etioplast product (Figure 21). Figure 22 shows that this polypeptide is also the only product of *in vitro* etioplast protein synthesis that is present in a 150000 g supernatant fraction. The single soluble product of light-driven protein synthesis in isolated intact chloroplasts is the large subunit of Fraction I protein (Blair and Ellis, 1973). Figure 22 also shows that no/

no labelling of the small subunit of Fraction I protein is observed when the soluble products of in vitro etioplast protein synthesis are analysed by SDS-gel electrophoresis. This observation further strengthens the conclusion that the small subunit of Fraction I protein is synthesized on cytoplasmic ribosomes (Gray and Kekwick, 1974).

The results so far discussed in this section suggest that the products of in vitro protein synthesis in both pea etioplasts and chloroplasts are qualitatively similar. They also show that the etioplast ribosome is required to translate only a restricted number of mRNA species. The validity of this conclusion is, as in all cases, limited by the sensitivity of the techniques employed. The most highly labelled etioplast product can be tentatively identified as the large subunit of Fraction I protein on the basis of its molecular weight and solubility. The remaining etioplast in vitro products are membrane-bound. The discrete nature of the membrane-bound products and their insensitivity to treatment with ribonuclease makes it unlikely that they represent nascent polypeptide chains which are bound to free or membrane-bound ribosomes. The identities of the membrane-bound products of in vitro plastid protein synthesis are at present unknown. They are however clearly minor components of the etioplast and chloroplast membranes as they do not coincide with any of the major plastid protein bands revealed by staining.

The remaining experiments reported in this section were aimed at providing an unequivocal identification of the soluble product of protein synthesis in isolated etioplasts. Peptide mapping provides a direct comparison of the primary structures of proteins, and can be used to conclusively establish the identity of a protein. The purity of the samples used for peptide mapping was established by gel electrophoresis and the results are shown in Figures 6 and 23. It was concluded that the soluble in vitro etioplast product and the in vivo labelled large subunit of Fraction I protein had been purified sufficiently to allow peptide mapping.

Separation of the L-(³⁵S) methionine-labelled tryptic peptides of the soluble etioplast in vitro product and the authentic large subunit of Fraction I protein was performed by cation-exchange chromatography and/

and two-dimensional mapping. Column chromatography did not separate the peptides sufficiently to allow a conclusive identification (Figure 24). However qualitative comparison of the two-dimensional tryptic peptide maps showed that there is a high degree of similarity between the primary structures of the two proteins (Plate 3).

Quantitative differences in the radioactivity incorporated into methionine containing tryptic peptides of the proteins may result from, for example, the inability of the isolated etioplast to initiate the synthesis of the large subunit of Fraction I protein during the period of amino acid incorporation. Confirmation that the large subunit of Fraction I protein is the sole detectable soluble product of protein synthesis in isolated etioplasts is provided by a comparison of the L-(³⁵S) methionine-labelled chymotryptic peptides derived from the in vitro etioplast product and authentic large subunit of Fraction I protein (Plate 4).

SECTION III - RESULTS AND DISCUSSION

3. THE CHARACTERISTICS AND PRODUCTS OF
AMINO ACID INCORPORATION IN PLASTIDS
ISOLATED FROM GREENING LEAVES

A. INTRODUCTION

The results discussed in Sections III 1 and 2 show that not only the characteristics and requirements, but also the products, of in vitro protein synthesis in pea etioplasts and chloroplasts are qualitatively the same. This conclusion is based, for one of the products (the large subunit of Fraction I protein) on an unequivocal identification. For the remaining in vitro products, all of which are membrane-bound proteins, this conclusion is based on less rigorous grounds i. e. their electrophoretic behaviour and solubility characteristics.

The remaining question set out in the objectives to this study is whether or not additional proteins are synthesized by plastid ribosomes during the conversion of etioplasts into chloroplasts. Various inhibitor experiments have suggested that particular proteins which appear during greening are synthesized by plastid ribosomes, for example, the protein component of the Photosystem I - chlorophyll complex (Machold and Aurich, 1972). However, as has already been stated, these experiments are inconclusive in that they show no more than the functioning of the plastid ribosomes is required for the accumulation of a particular protein in the plastid.

The most dramatic change during the transition from the etioplast to the chloroplast is the development of photosynthetic competence (Egneus *et al.*, 1972). However, in addition to being able to fix CO₂ (Lilley and Walker, 1975), isolated chloroplasts can also use light as an energy source for in vitro protein synthesis (Blair and Ellis, 1973). The energy source for light-driven protein synthesis is assumed to be ATP produced by photophosphorylation (Blair and Ellis, 1973). Therefore the development of photophosphorylation during chloroplast development should correlate with the appearance of light-driven protein synthesis in plastids isolated during greening. Consequently the ability of plastids isolated from greening leaves to use ATP and light as energy sources for in vitro protein synthesis was investigated. This analysis was restricted to the study of amino acid incorporation in intact plastids by the use of ribonuclease in the incubation mixture. Whereas ATP can act as an energy source for/

for in vitro protein synthesis in both intact and broken plastids (Table 5; Blair, 1974), light will act as an energy source for in vitro protein synthesis only in intact chloroplasts (Blair and Ellis, 1973).

The products of both ATP and light-driven protein synthesis in plastids isolated from greening leaves were again analysed by SDS polyacrylamide gel electrophoresis. This allows both an indirect and a direct comparison with the products of protein synthesis in isolated etioplasts and chloroplasts. The products of in vitro protein synthesis using both ATP and light as the energy source were studied, as minor differences have been reported (Bottomley *et al.*, 1974).

B. THE ENERGY SOURCE FOR PROTEIN SYNTHESIS IN PLASTIDS ISOLATED FROM GREENING LEAVES.

i. Chlorophyll accumulation during greening.

Measurements on the accumulation of chlorophyll in the leaves of etiolated seedlings which had been exposed to light are shown in Figure 25. References to the experimental details are given in the figure legend. After an initial lag period of 5 hours, the accumulation of chlorophyll was maximal between 10 and 70 hours after the onset of illumination.

ii. The time course of amino acid incorporation in plastids isolated during greening.

- a. ATP-driven incorporation.
- b. Light-driven incorporation.

The initial rate of either ATP-driven or light-driven amino acid incorporation in plastid isolated during greening can be determined from the time course of the incorporation, providing the rate of incorporation is limited by the number of plastids in the incubation. Figure 26 shows the time course for energy-dependent amino acid incorporation in plastids isolated from greening tissue at various times after the onset of illumination. The time course was determined over a 45 minute incubation period, under conditions of direct proportionality between the initial incorporation rate and the number of plastids in the incubation. Proportionality was established by performing the experiment at two different concentrations of plastids.

The/

Figure 25. Chlorophyll accumulation in greening seedlings.

Etiolated seedlings were grown as described in Section II1Aii and after 9 days they were transferred to illumination by continuous white light of 2800 lux intensity (Warmwhite fluorescent tubes) (Section II1Aii). Chlorophyll accumulation in the leaves was estimated on a fresh weight basis as described in Section II2L.

$10^{-3} \times$ Chlorophyll $\mu\text{g/g.f.w.}$

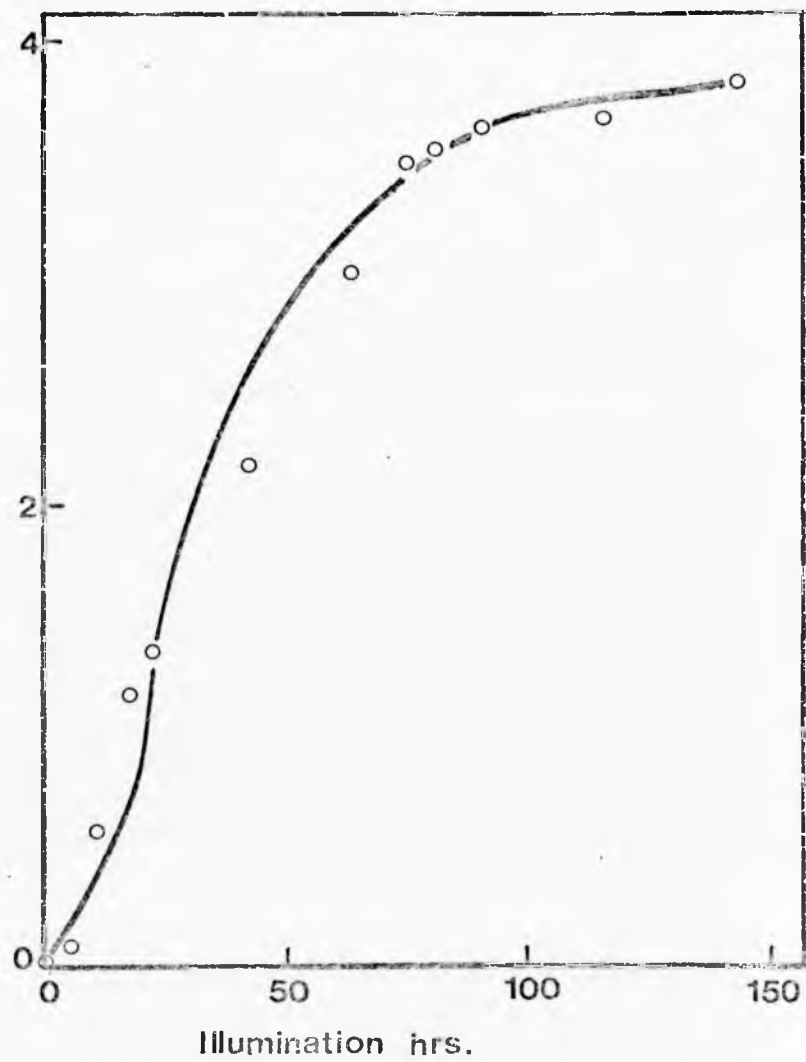


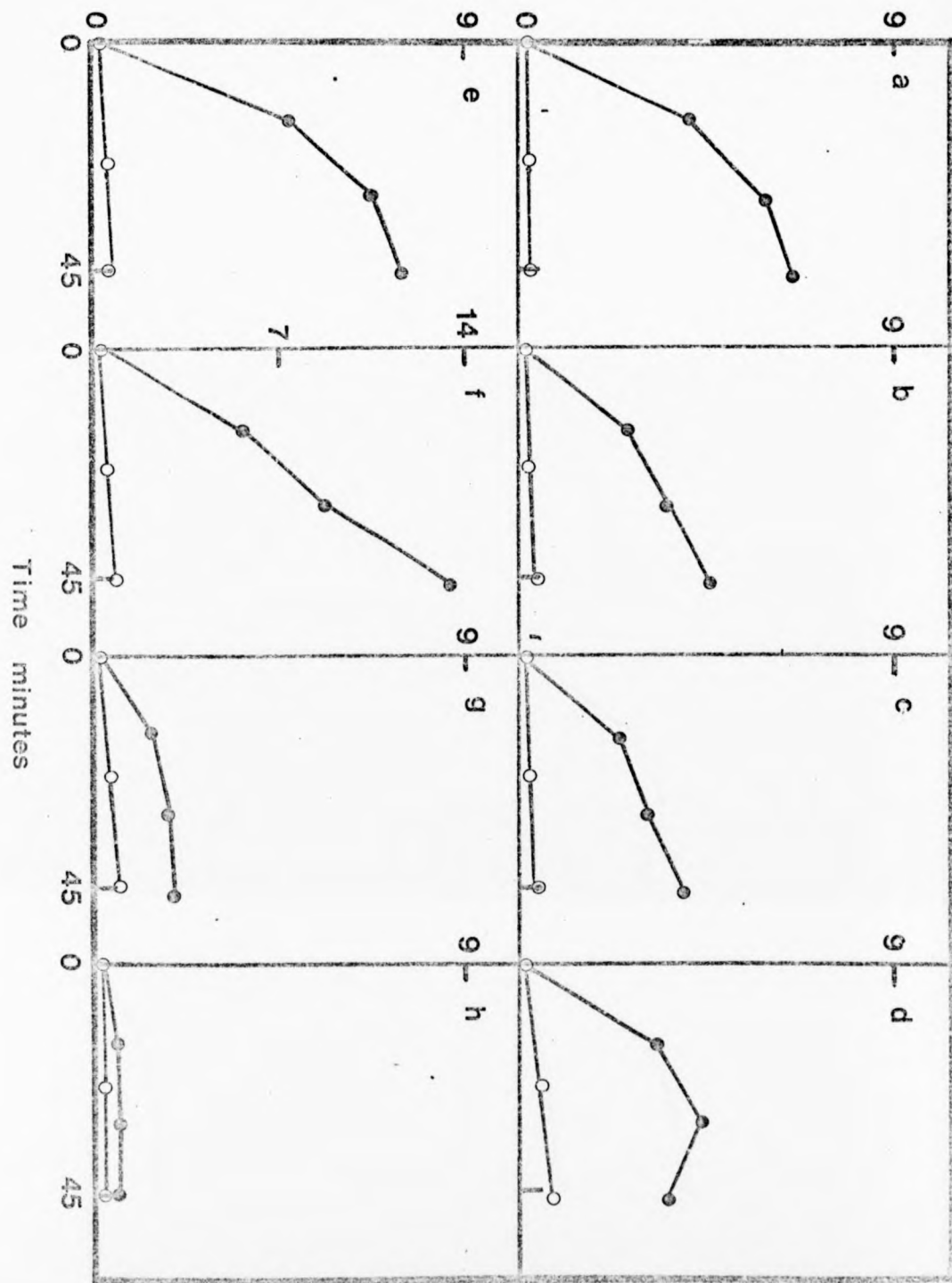
Figure 26. The time course for ATP-driven and light-driven amino acid incorporation in plastids isolated from greening tissue.

Plastids were isolated from etiolated and greening seedlings which had been grown and illuminated as described in Sections II1Aia and II1Aic. Plastid isolation and incubation was performed as described in Sections II2A and II2Ci. The number of intact plastids in each incubation was determined by quantitative phase contrast microscopy as described in Section II2Bia. All incubations were performed at 20°C. ATP-driven incubations were performed in the dark. Ribonuclease was included in the incubations to prevent amino acid incorporation on ribosomes associated with broken plastids. Incubations were terminated and assayed for amino acid incorporation as described in Section II2Cii.

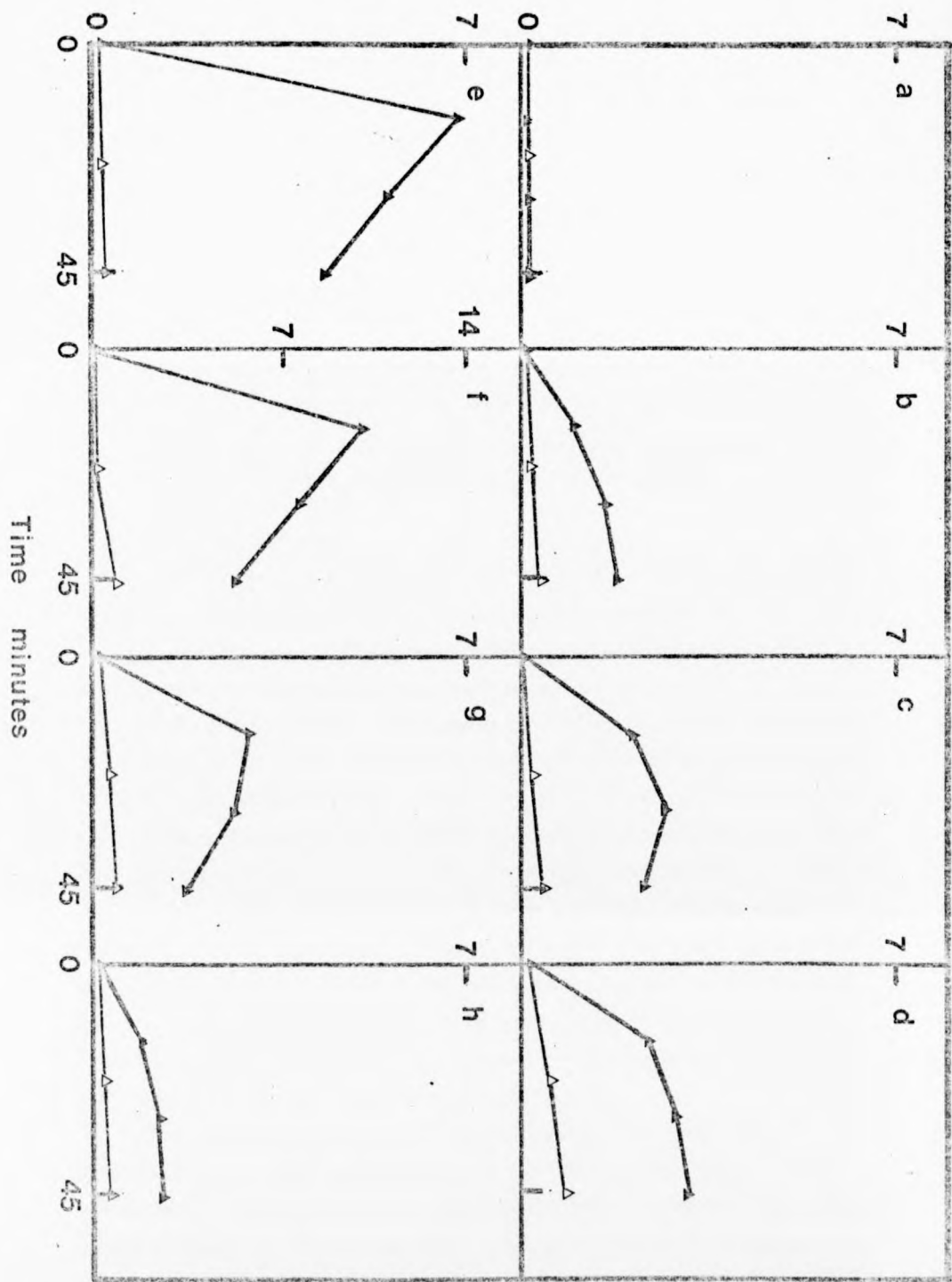
Key O - ATP
 ● + ATP
 △ - light
 ▲ + light

a	0 h greening,	7.92×10^6	intact plastids/incubation
b	3 h greening,	6.1×10^6	intact plastids/incubation
c	6 h greening,	8.48×10^6	intact plastids/incubation
d	12 h greening,	4.4×10^6	intact plastids/incubation
e	24 h greening,	7.44×10^6	intact plastids/incubation
f	48 h greening,	10.8×10^6	intact plastids/incubation
g	96 h greening,	8.52×10^6	intact plastids/incubation
h	120 h greening,	2.88×10^6	intact plastids/incubation

$10^{-2} \times {}^{35}\text{S}$ Radioactivity incorporated
c.p.m. per 10^6 intact plastids



$10^{-2} \times {}^{35}\text{S}$ Radioactivity incorporated
c.p.m. per 10^6 intact plastids



The result of only one series of experiments is shown.

In the majority of cases the time course of amino acid incorporation is found to be hyperbolic. However the time courses for light-driven incorporation in plastids isolated after 24 hours, 48 hours and 96 hours of greening are unusual. After an initial period of incorporation the amount of L-(³⁵S) methionine in hot trichloroacetic acid-insoluble material in these incubations decreases. The reason for the observation was not investigated but the observation may indicate, for example, a change in the endogenous protease activity in the plastids during development. The time courses illustrated in Figure 26 can be used to calculate the initial rates of ATP and light-driven amino acid incorporation in plastids isolated from greening tissue. The calculations are collated in Figure 27. The interpretation of these results is discussed in Section III D.

C. ELECTROPHORETIC ANALYSIS OF THE PROTEINS SYNTHESIZED IN PLASTIDS ISOLATED FROM GREENING LEAVES.

i. Total products.

L-(³⁵S) methionine was the only protein precursor used in this analysis. The data presented in this section are representative of several experiments. The products of both ATP and light-driven protein synthesis in plastids isolated from leaves after 0, 3, 6, 12, 24, 48, 96 and 218 hours of greening have been analysed. However, the overall conclusions of these experiments can be drawn from the analyses performed after 0, 24, 48, 96 and 218 hours of greening alone.

Figure 28 shows the product analyses performed at these times. Both ATP and light-driven systems have been investigated and control incubations in the absence of an added energy source have been included in each experiment. The results immediately suggest that although quantitative differences exist, qualitatively the products of protein synthesis are the same in plastids isolated throughout the transition from etioplast to chloroplast. This conclusion applies to both the ATP and light-driven systems.

An indirect comparison between the proteins synthesized in plastids isolated during greening can be made on the basis of their estimated molecular weights. The estimated molecular weights are shown in Table 7. These molecular weight estimations confirm that the/

Figure 27 The initial rate of ATP and light-driven amino acid incorporation in plastids isolated from greening tissue.

The experimental details are referred to in Figure 26.

- ⊙ ATP-driven amino acid incorporation
- △ light-driven amino acid incorporation

Initial rate of ^{35}S methionine incorporation
pmoles/ 10^9 intact plastids/h

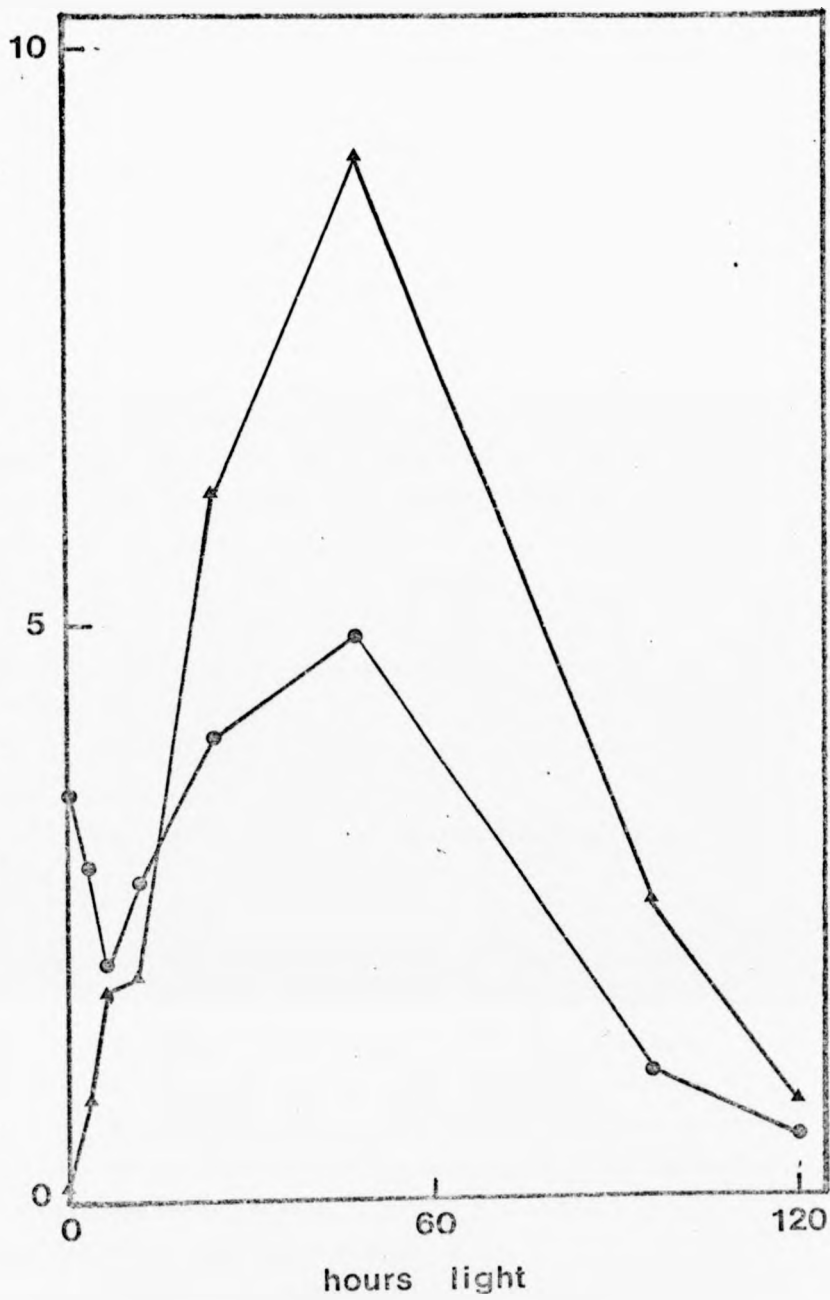


Figure 28. SDS-polyacrylamide gel electrophoresis of the polypeptides synthesized in vitro in plastids isolated from leaves during greening.

Plastids were isolated and incubated with L-(³⁵S) methionine as described in Sections II2A and II2F*i*. Electrophoresis and related procedures were performed as described in Section II2F*ii*. The solid lines represent the absorbance at 260 nm, and the histogram shows the radioactivity in each 1 mm gel slice. PSII, photosystem II chlorophyll-protein complex; LS, large subunit of Fraction I protein; SS, small subunit of Fraction I protein.

Gels labelled A were preparations incubated with added ATP in the dark.

Gels labelled B were preparations incubated in the dark without an energy source.

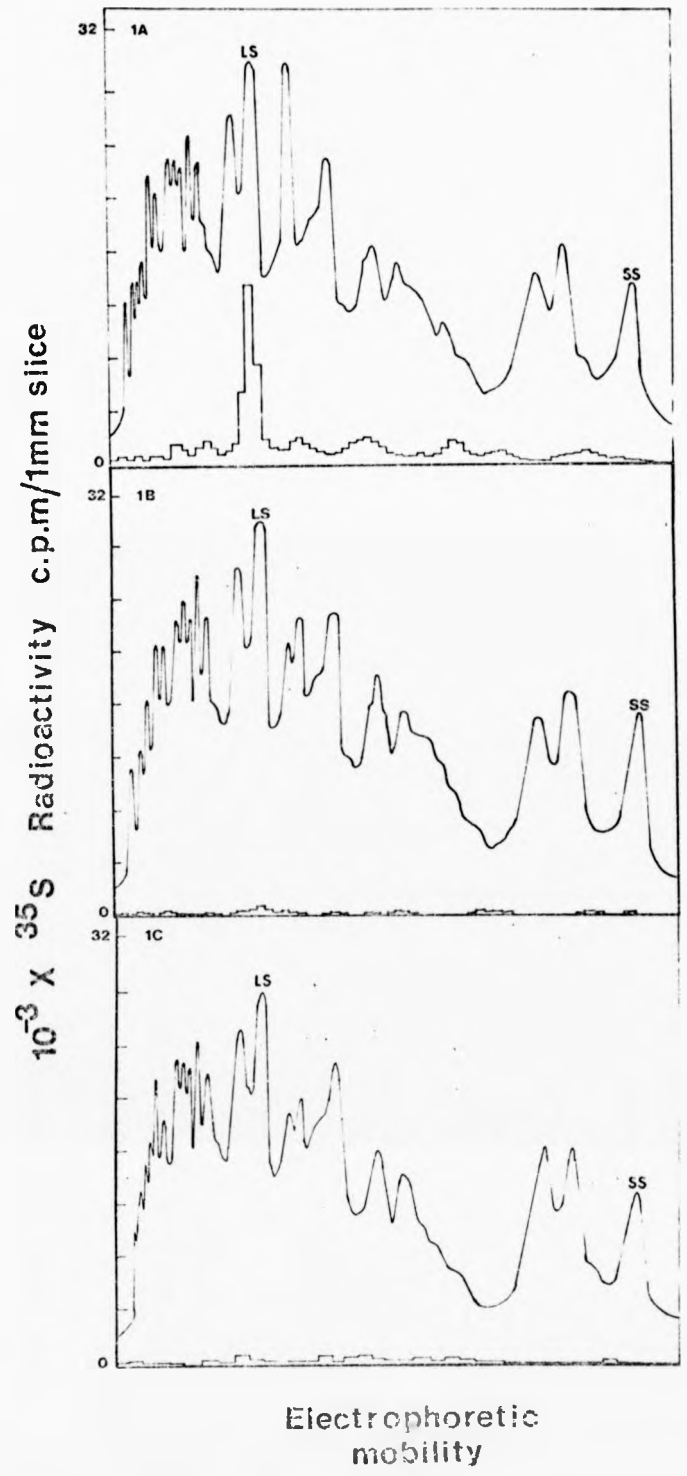
Gels labelled C were preparations incubated in the light.

Series	1	Plastids isolated after	0 hours greening
	2	Plastids isolated after	24 hours greening
	3	Plastids isolated after	48 hours greening
	4	Plastids isolated after	96 hours greening
	5	Plastids isolated after	218 hours greening

All gels were loaded with 100 μ l of solubilized incubation mixture.

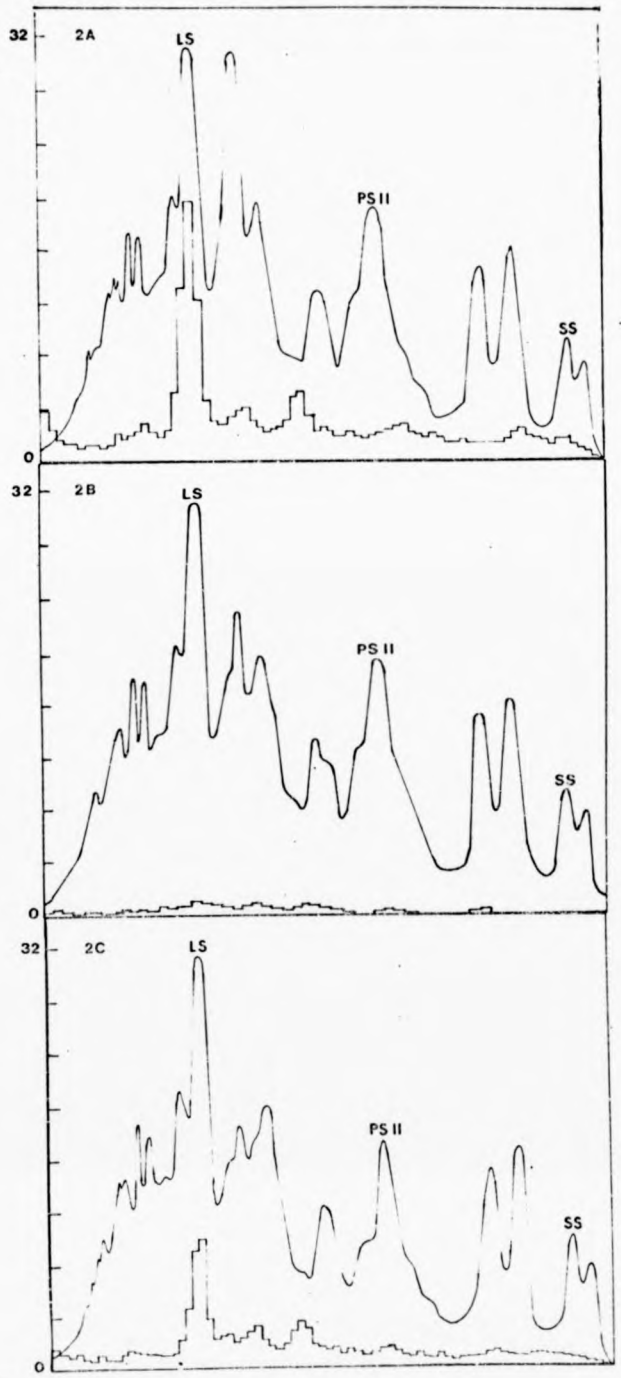
The protein loadings were,

Series	1	130 μ g of protein/gel
	2	130 μ g of protein/gel
	3	200 μ g of protein/gel
	4	400 μ g of protein/gel
	5	440 μ g of protein/gel



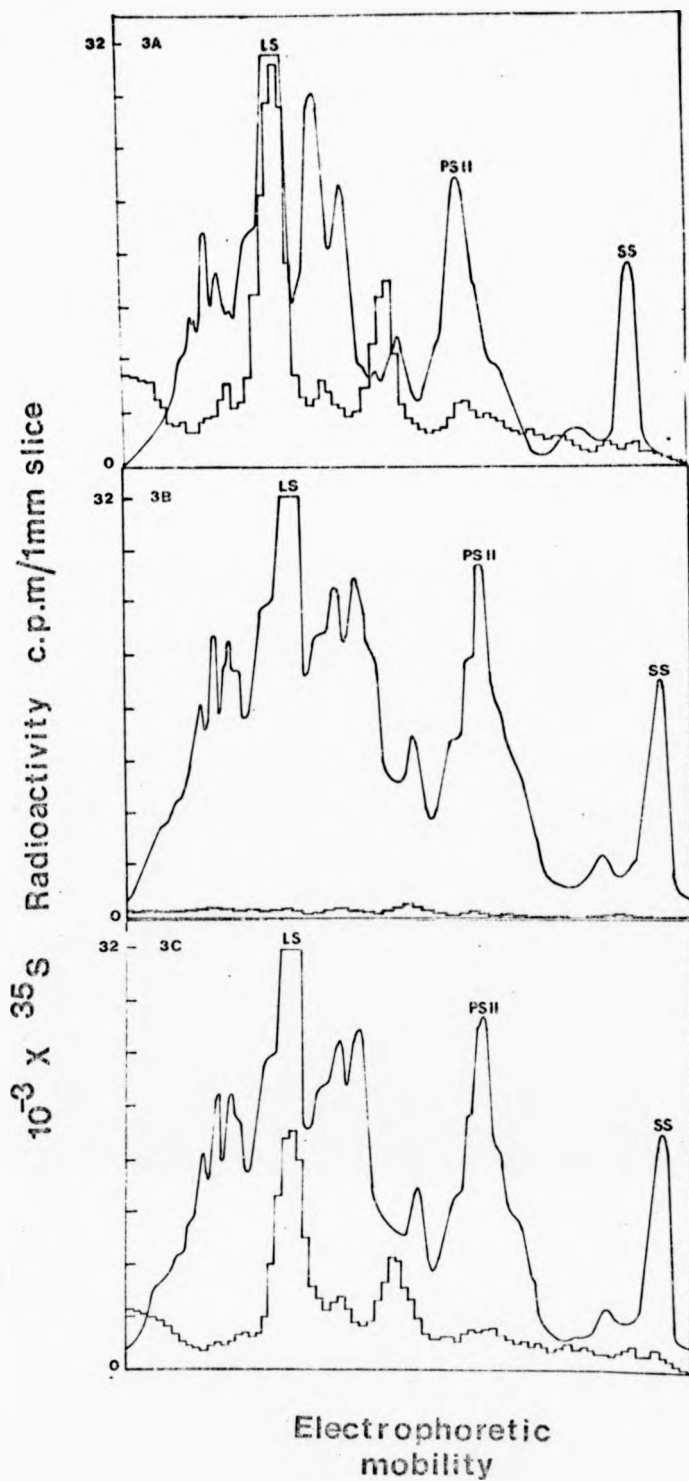
A 620

$10^{-3} \times 35\text{S}$ Radioactivity c.p.m./1mm slice

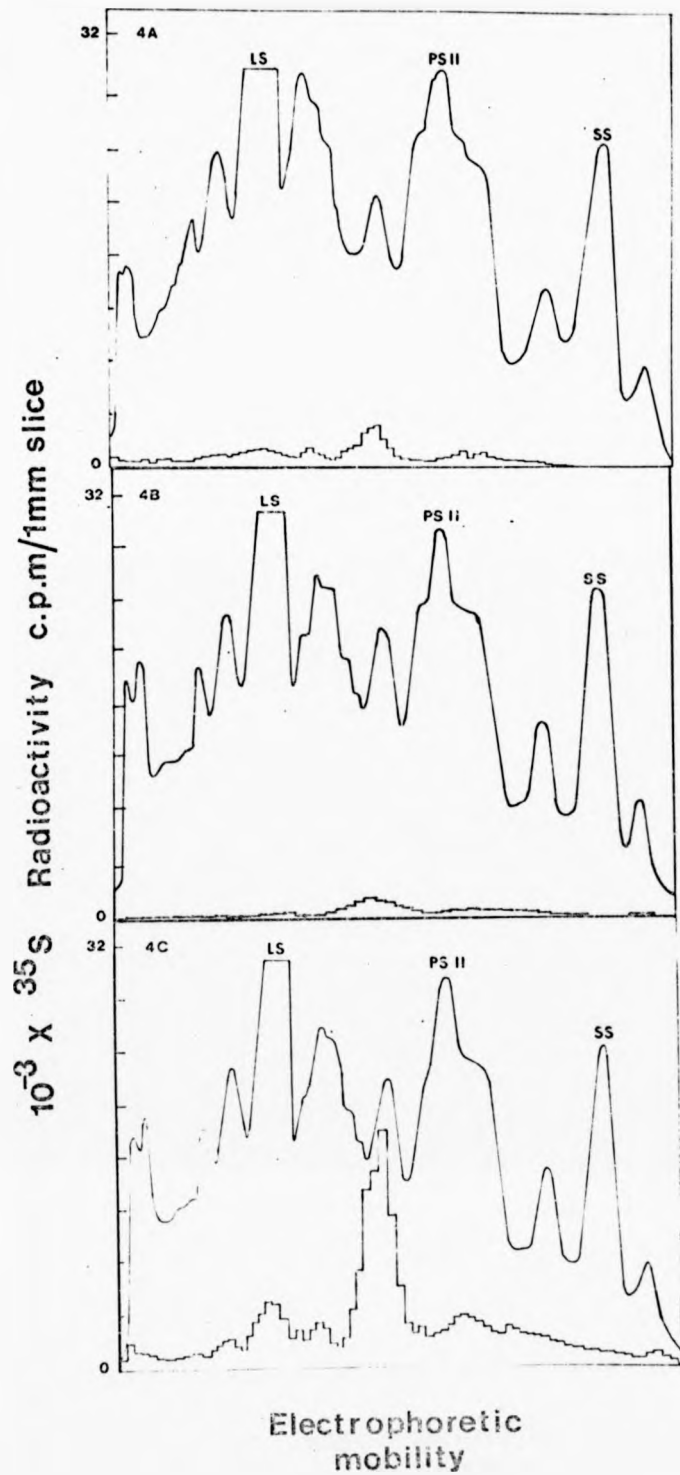


A 620

Electrophoretic mobility



A
620



A 620

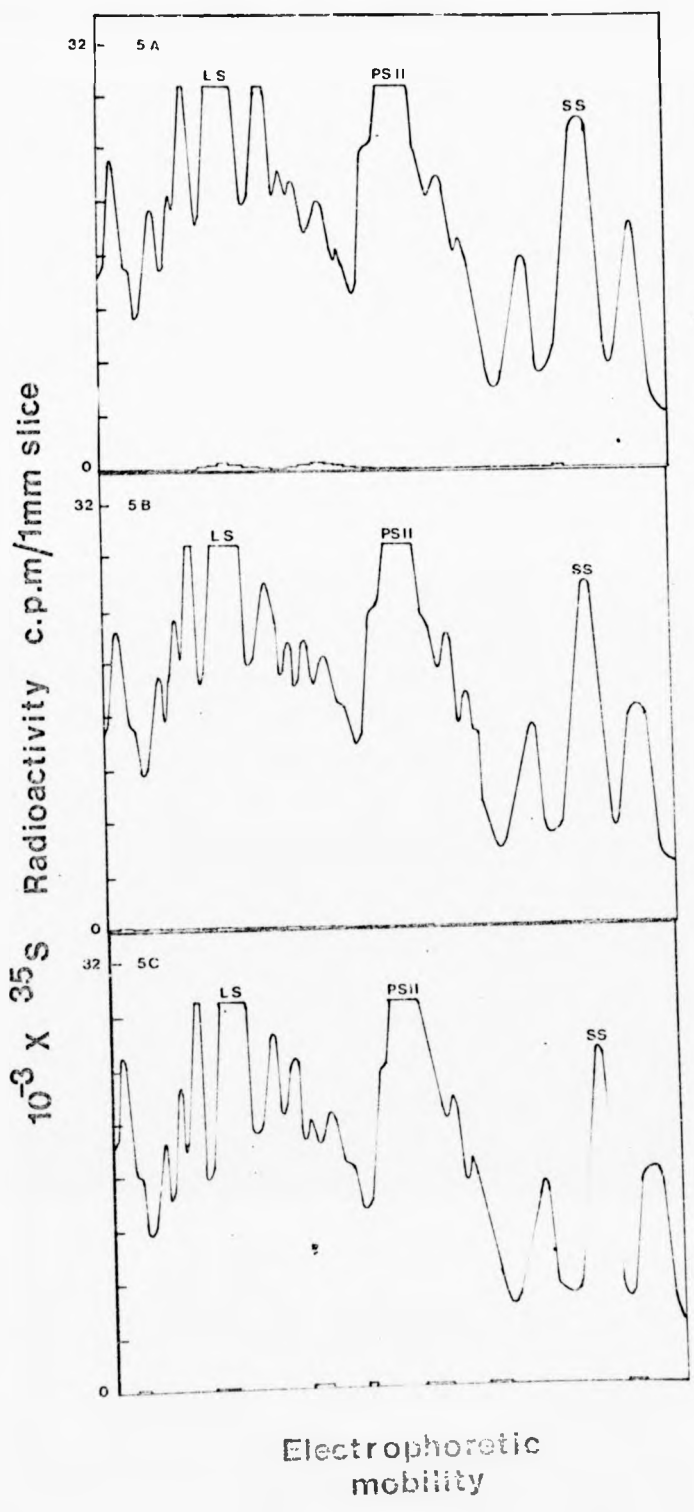


Table 7. The molecular weights of the proteins synthesized in plastids isolated during greening.

The experimental details are given in Figure 28. The molecular weights of the proteins were estimated from their relative mobility and by reference to the molecular weight calibration curve (Figure 3).

the same spectrum of proteins are synthesized in plastids isolated throughout the greening process. Minor alterations in the estimated molecular weights of the proteins synthesized in plastids isolated during greening are within the recognised 5% error associated with molecular weight determinations by SDS-polyacrylamide gel electrophoresis (Fish *et al.*, 1970). Although the pattern of products does not change during greening, the product analysis does suggest that there is a major alteration in the relative rates of synthesis of particular products in plastids isolated at different stages of development. Comparison of the products synthesized in isolated etioplasts with those synthesized in plastids isolated after 96 hours of greening shows a radical difference in the major product (c. f. Figures 28, 1A and 28, 4C).

ii. Coelectrophoresis with the products of *in vitro* etioplast and chloroplast protein synthesis.

A direct comparison between the products of protein synthesis in isolated etioplasts and chloroplasts, and the products synthesized in plastid isolated from leaves during greening can be made on the basis of their electrophoretic behaviour. Figure 29 shows the result of co-electrophoresing the etioplast and chloroplast *in vitro* products with the products synthesized in plastids isolated from leaves after 48 hours of greening. The experimental details are given in the figure legend. It is clear that the mobilities of the proteins synthesized in isolated etioplasts and chloroplasts are identical to the mobilities of the proteins synthesized in developing plastids.

iii. The products of protein synthesis in isolated intact etioplasts and isolated intact developing plastids.

The analysis of the proteins synthesized in plastids isolated from greening tissue described in the previous section was performed using preparations that may have contained active ribosomes associated with broken and intact plastids. Ribonuclease was not routinely added to the incubation mixture. It is possible that the products of intact and broken plastids are not identical. To study this point the products of ATP-driven *in vitro* protein synthesis in intact plastids isolated from both etiolated tissue and tissue which had been greened for 72 hours were investigated. The results are shown in Figure 30. The fractionation/

Figure 29. Coelectrophoresis of etioplast and chloroplast products synthesized in vitro, with the products of protein synthesis in plastids isolated from greening leaves after 48 hours of illumination.

Etioplasts, chloroplasts and developing plastids were isolated and incubated as described in Sections II2A and II2Fia. Etioplast and chloroplast products were labelled in vitro with L-(³⁵S) methionine and L-(³H) leucine respectively. Two preparations of the products of protein synthesis in developing plastids were used, one labelled with L-(³⁵S) methionine and the other labelled with L-(³H) leucine. The etioplast sample contained 100 µg of protein (8×10^4 c. p. m.). The developing plastid samples both contained 50 µg of protein (3.5×10^4 c. p. m. of ³H; 4.8×10^4 c. p. m. of ³⁵S). The chloroplast sample contained 30 µg of protein (1×10^5 c. p. m.). Light was the energy source for protein synthesis in isolated developing plastids and isolated chloroplasts. Samples were extracted with acetone as described in Section II2Fid. Electrophoresis and related procedures were performed as described in Section II2Fia.

- A, Coelectrophoresis of developing plastid products with in vitro etioplast products.
- B, Coelectrophoresis of developing plastid products with in vitro chloroplast products.

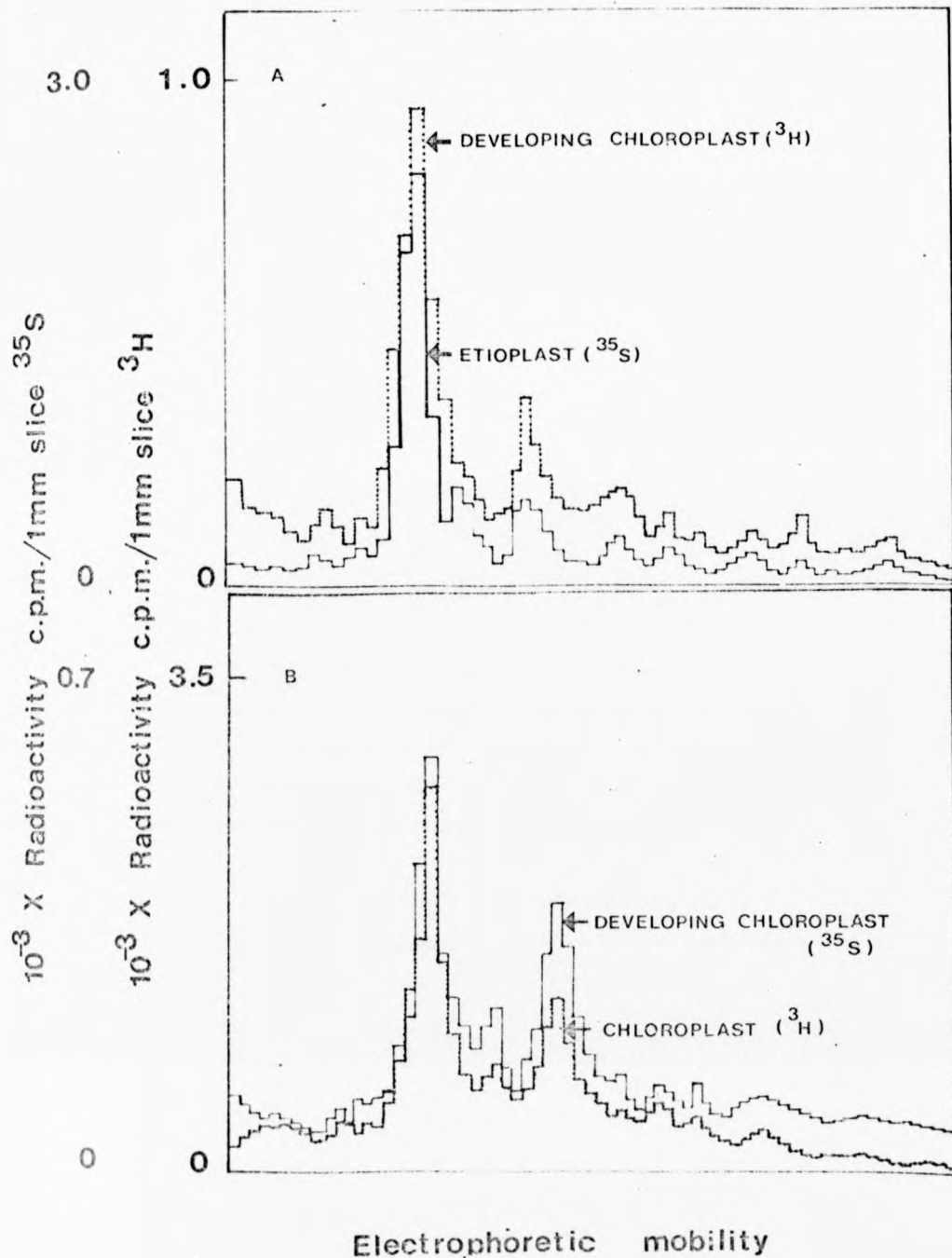
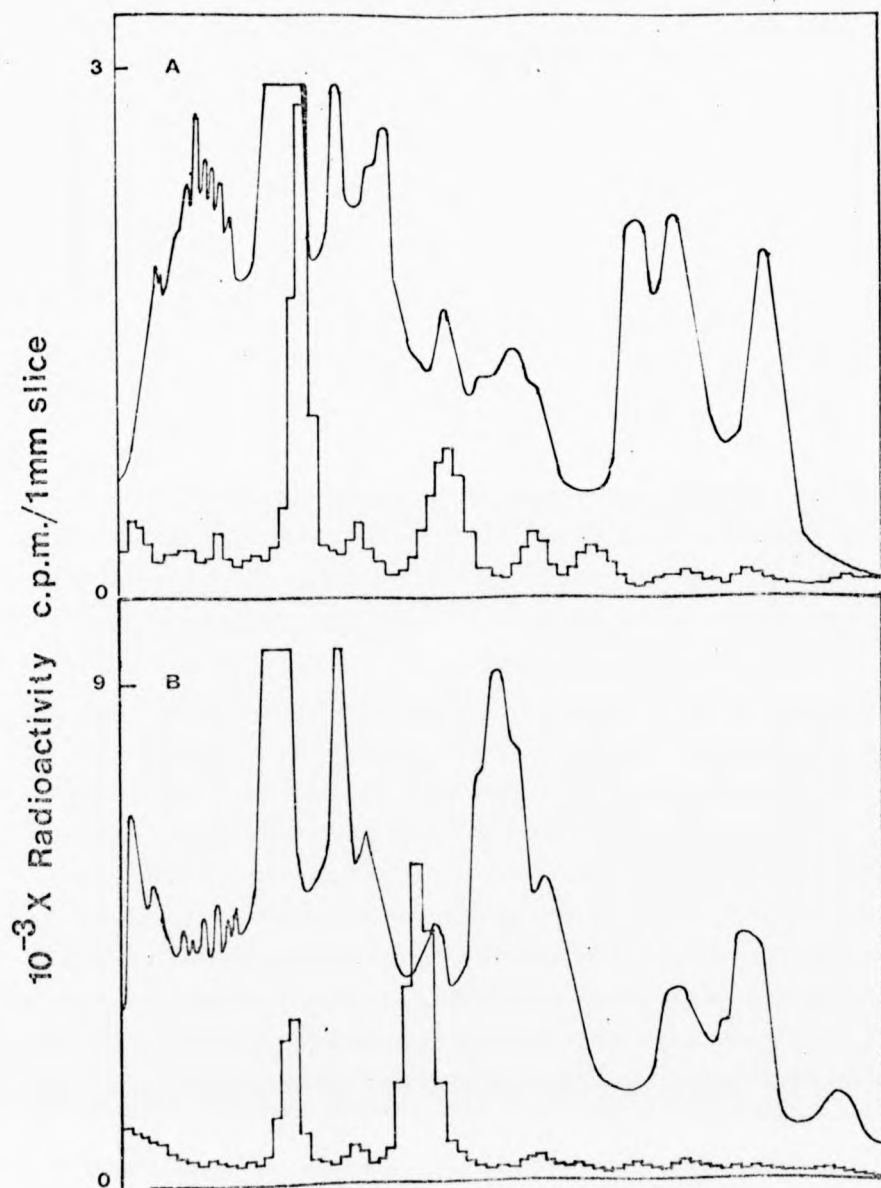


Figure 30. SDS-polyacrylamide gel electrophoresis of the polypeptides synthesized in vitro in intact etioplasts and intact developing plastids isolated from tissue after 72 hours of greening.

Plastids were isolated and incubated as described in Sections II2A and II2Fia except that ribonuclease A was added to the protein synthesis incubation mixture at a concentration of 50 $\mu\text{g}/\text{ml}$. ATP was the energy source for in vitro protein synthesis. Electrophoresis and related procedures were performed as described in Section II2Fii.

- A, intact etioplasts (170 μg of protein/gel)
- B, intact developing plastids, 72 hours illumination (220 μg of protein/gel)



A
620

Electrophoretic mobility

fractionation pattern of the proteins synthesized in intact etioplasts is very similar to that obtained using a mixture of intact and broken etioplasts (c. f. Figure 16A). The fractionation of the products of ATP-driven protein synthesis in plastids isolated from leaves after 72 hours of greening (Figure 29B) shows the previously noted alteration in the major labelled product (c. f. Figure 28, 4C).

D. DISCUSSION

The results presented in this section are the first report of the characteristics and products of protein synthesis in plastids isolated from greening leaves. Under the experimental conditions used chlorophyll accumulation in the greening seedlings is completed 90 to 100 hours after the onset of illumination (Figure 25). There is an initial lag in chlorophyll synthesis. Henningsen and Boynton (1970) have demonstrated that the lag phase of chlorophyll accumulation normally seen in greening barley seedling (Egneus *et al.*, 1972) can be abolished by maintaining the relative humidity of the atmosphere above 80% during illumination. A similar conclusion has been reached for the greening of jack bean leaves (Alberte *et al.*, 1972; Alberte *et al.*, 1975). No attempts were made to control the relative humidity during the greening of pea seedlings. Conditions of water stress were unlikely to occur due to the frequent watering of greening material.

The initial rates of energy dependent protein synthesis in plastids isolated during greening are shown in Figures 26 and 27. These results confirm that light cannot be used as a source of energy for protein synthesis in isolated etioplasts. Light-driven protein synthesis can first be detected in plastids isolated 3 hours after the onset of illumination. This indicates that ATP derived from photophosphorylation can be used as an energy source for *in vitro* protein synthesis at this time. The development of the partial reactions of photosynthesis during greening has been widely studied (Remy *et al.*, 1972; Plesničar and Bendall, 1973; Gyldenholm and Whatley, 1968; Egneus, 1972; Alberte *et al.*, 1972). There remains however a lack of precise information on the order in which specific photosynthetic components appear during chloroplast development. For instance, Alberte *et al.* (1972) have studied the appearance of the protein chlorophyll complexes derived from/

from Photosystems I and II in the chloroplasts of greening jack bean leaves. The complexes are easily identified in polyacrylamide gel electrophoresis fractionations of chloroplast lamellar material solubilized in SDS (Thorner *et al.*, 1967). It was concluded that the protein-chlorophyll complex associated with Photosystem II is first detectable after 2 hours of greening. The Photosystem I derived protein-chlorophyll complex cannot be detected until 6 hours after the onset of illumination. The appearance of both complexes was associated with the development of photochemical activities characteristic of each. In contrast, Plesničar and Bendall (1973) have shown that ATP can be produced in isolated etioplasts by cyclic photophosphorylation providing an electron donor (sodium ascorbate) and an electron carrier (phenazine methosulphate) are included in the reaction mixture. They were unable to detect the chlorophyll molecule, P700, which constitutes the reaction centre pigment of Photosystem I (Clayton, 1966). Clearly many components of the photochemical apparatus are present in the etioplast, whilst others remain to be synthesized during greening. It should be borne in mind that the appearance of complex photochemical activities or ultrastructural systems may only be dependent on the synthesis of the final component which completes a perhaps lengthy series of biosynthetic events.

In contrast to light-driven protein synthesis, the initial rate of ATP-driven amino acid incorporation decreases in plastids isolated in the early stages of greening (Figure 27). The reason for this apparent decrease has not been investigated. However, Cockburn and Wellburn (1974) have reported that there is a flux of specific amino acids from the cytoplasm into the plastid during the early stages of light-induced greening of etiolated oat laminae. The decreased initial amino acid incorporation rate in pea plastids isolated in the early stages of greening may therefore simply reflect changes in the specific activity of the radioactive amino acid precursor at the site of incorporation. Upon further illumination of the leaves, the initial rate of amino acid incorporation in isolated plastids increases for both ATP and light-driven protein synthesis. Drumm and Margulies (1970) reported a similar increase for ATP-driven protein synthesis in plastids isolated/

isolated from greening Phaseolus leaves. These authors have concluded that this increase represents a real alteration in the protein-synthesizing ability of the plastids, rather than an alteration in the amino acid pool sizes or decreased levels of inhibitory factors.

After 48 hours illumination, the rate of light-driven incorporation in isolated pea plastids is approximately twice that of the ATP-driven system. The time courses for light-driven incorporation in plastids isolated after 24, 48 and 96 hours of illumination are unusual (Figure 26). The decreasing amount of radioactively labelled protein after incubation periods more than 30 minutes most likely results from endogenous protease activity. It is unclear why the same phenomenon is not seen in the ATP-driven system. Plastids isolated from leaves which have been illuminated for longer than 48 hours show increasingly less capacity for both ATP and light-driven incorporation. However plastids isolated after 96 hours of illumination apparently maintain a greater capacity for light-driven incorporation than for ATP-driven protein synthesis. This difference may however simply reflect an alteration in the permeability of the plastid envelope to ATP during greening rather than any difference in the intrinsic protein synthesizing capacities of the isolated plastids.

The analysis of the products synthesized in plastids isolated from greening tissue is shown in Figure 28. The absorbance profile of the stained protein bands reflects the qualitative and the quantitative changes in the proteins of the etioplast and the developing chloroplast. As only three of the protein bands detected on these gels can be identified, the interpretation of these data must remain limited. There is an increase in the absorbance of the stained protein bands during greening. Since equal volumes of incubation mixture were placed on each gel, this indicates that there is an increase in the total plastid protein during greening. Direct measurement of protein in plastid pellets from greening pea shoots confirms this indication (Ellis, 1975a). The two to three-fold increase in the total plastid protein observed during the greening of etiolated pea seedlings is in good agreement with the results of Cobb and Wellburn (1974) who studied changes in the proteins/

proteins of oat chloroplasts during development. The most noticeable change in the absorbance profile is the appearance during greening of the protein component associated with the Photosystem II complex (Thornber *et al.*, 1967; Thornber and Highkin, 1974). This polypeptide electrophoreses with an apparent molecular weight of 2.7×10^4 - 2.8×10^4 (Eaglesham and Ellis, 1974). The appearance of the Photosystem I complex was not seen in these fractionation patterns because the preparations had been boiled in sodium dodecyl sulphate. This treatment reduces the Photosystem I complex to several protein components which electrophorese with a higher mobility than the complex itself (Anderson and Levine, 1974). The remaining two protein-staining bands which can be recognised are the large and small subunits of Fraction I protein. The increased staining intensity of these bands in preparations of plastids isolated progressively later during greening confirms the reports of Fraction I protein synthesis in developing chloroplasts (Smith *et al.*, 1970; Kleinkopf *et al.*, 1970; Bennett, 1975).

The products synthesized in plastids isolated during their development from etioplasts into chloroplasts were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (Figure 28). This analysis leads to the conclusion that irrespective of the energy source used, plastids isolated during greening synthesize the same polypeptides that are synthesized in both isolated etioplasts and chloroplasts. The evidence for this conclusion is firstly based on the estimated molecular weights of the proteins synthesized in plastids isolated during greening. These are found to be the same as the molecular weights of the products of *in vitro* etioplast protein synthesis (Table 7). Strong support for this conclusion is provided by a direct comparison of the electrophoretic behaviour of proteins synthesized in isolated etioplasts and chloroplasts with plastids isolated at one particular stage of greening. Figure 29 shows that the proteins synthesized in plastids isolated after 48 hours of greening coelectrophorese with the products of both *in vitro* etioplast and *in vitro* chloroplast protein synthesis. In some cases the synthesis of specific proteins cannot be convincingly demonstrated in plastids isolated at particular stages of greening. However at no time during greening/

greening have plastids been isolated which synthesize proteins not identifiable as products of in vitro etioplast protein synthesis.

Although there are no significant qualitative changes in the nature of the products synthesized in plastids isolated during greening, the analysis of the proteins synthesized in plastids isolated after 96 hours of illumination suggests a radical change in the major product. This change is most clearly seen in the light-driven system which is more active at this time (Figure 28, 4C). Whereas the major product in isolated etioplasts is the large subunit of Fraction I protein (Section III2D), almost the only polypeptide synthesized in plastids isolated after 96 hours of greening is an unidentified membrane-bound protein with a molecular weight of about 3.2×10^4 . Whether this alteration in the major product synthesized in vitro of plastid ribosomes reflects a similar alteration in vivo is unknown. It is possible for example that during the isolation of chloroplasts, but not etioplasts, there is a significant loss of free 70S ribosomes, the class of ribosomes that Ellis (1976b) has shown to be the site of Fraction I protein large subunit synthesis. Indeed Tao and Jagendorf (1973) have shown that aqueously isolated pea chloroplasts, which are judged to be intact by phase contrast microscopy and other criteria, contain four times less free 70S ribosomes than equivalent non-aqueously isolated plastids. The numbers of bound 70S ribosomes in both types of plastid were approximately the same.

The same alteration in the in vitro rate of synthesis of particular proteins during greening is seen if the analysis of the products is restricted entirely to intact plastids by the use of ribonuclease (Figure 30). This analysis was performed with plastids isolated after 72 hours of illumination because ATP will not act as an efficient energy source for protein synthesis in plastids isolated at later stages of greening. These results suggest that there are controls exerted on the production of proteins in isolated plastids, and clearly if this is the case then the mechanism of these controls deserves further study.

Taken together the results discussed in this section support the idea that plastid ribosomes synthesize only a small number of proteins and that the number and molecular weight of these proteins does not alter during the formation of chloroplasts from etioplasts. However the rates/

rates of synthesis of particular polypeptides are different in plastids isolated at different stages of the etioplast to chloroplast transition.

rates of synthesis of particular polypeptides are different in plastids isolated at different stages of the etioplast to chloroplast transition.

SECTION IV - GENERAL DISCUSSION

A. THE ETIOPLAST VIEWED IN THE CONTEXT OF PLASTID AUTONOMY.

The results presented in this thesis show that isolated pea etioplasts incorporate labelled amino acids into protein. They also suggest that isolated pea etioplasts synthesize only a small number of proteins; one of these, the large subunit of Fraction I protein, has been unequivocally identified. Moreover the results show that the number and molecular weight of these proteins does not alter during the light-induced formation of chloroplasts from etioplasts in Pisum sativum.

Two general conclusions can be drawn from these findings. Firstly, the ribosomes of both etioplasts and developing chloroplasts synthesize a few proteins in larger amounts rather than many proteins in smaller amounts. This conclusion supports the idea that the occurrence of large numbers of plastid ribosomes in the leaf (Boardman et al, 1966) can be explained by the requirement to synthesize one of the plastid products, the large subunit of Fraction I protein, in massive amounts (Blair and Ellis, 1973). Fraction I protein can represent up to 50% of the soluble protein in the mature chloroplast (Kawashima and Wildman, 1970). Secondly, it is clear that the transformation of the etioplast into the chloroplast does not require the synthesis of a novel spectrum of protein on the plastid ribosome.

These conclusions support the view that the plastid is not autonomous in any meaningful sense (Ellis, 1975b). The accumulated evidence of both in vitro and inhibitor studies on plastid protein synthesis now indicate that only a very limited spectrum of proteins is synthesized on plastid ribosomes (Ellis, 1975b; Bärner, 1973). The inference is that most of the plastid components are synthesized on cytoplasmic ribosomes and then transported into the plastid. In the case of one plastid component, the small subunit of Fraction I protein, the site of synthesis has been established as the cytoplasmic ribosomes by both inhibitor (Criddle et al, 1970) and in vitro (Gray and Kekwick, 1974) studies. It is increasingly evident that the formation of the plastid results from a complex interplay between nuclear and plastid genomes and the cytoplasmic and plastid protein-synthesizing machinery. This discovery/

discovery points to areas of plastid research which may provide insights of much wider interest than the question of plastid autonomy alone. For example the plastid appears to offer a unique opportunity for studying the transport of proteins across a biological membrane. This process is central to many questions in biology and deserves immediate and extensive study. In addition the plastid-cytoplasmic relationship provides an opportunity to study the mechanisms which regulate and integrate the synthesis of proteins in different cellular compartments, another question of central importance (see next section).

The results presented in this thesis have not been able to provide any additional information about the number and nature of plastid components which are translated on plastid ribosomes. They do however testify to the usefulness of the *in vitro* approach in elucidating the question of plastid ribosome function. If a system of coupled transcription and translation could be developed for isolated plastids or plastid components then the same *in vitro* approach may also yield information in the future about the identity of the genes encoded in plastid DNA.

The conclusion that plastid ribosomes synthesize only a small range of proteins would seem to make the challenge of identifying these proteins easier. There still remains however a major reservation regarding this conclusion. There is a strong possibility that many, as yet unknown, protein components of the plastid are synthesized on plastid ribosomes. It may be that these components are synthesized during the earliest stages of plastid development i. e. during the conversion of the proplastid to the etioplast or chloroplast. If this is the case, then none of the experiments performed to date would have detected the synthesis of these proteins.

The reasons for considering this possibility are basically the same as those outlined in the introduction to the work presented in this thesis. Briefly, it remains a striking fact that chloroplasts isolated from a wide range of organisms contain DNA which has approximately the same kinetic complexity (Boulter *et al*, 1972; Kolodner and Tewari, 1975). The amount of uniquely sequenced DNA
in/

in the chloroplasts of all the species so far examined is sufficient to encode approximately 100 proteins each of molecular weight 50000; yet, assuming chloroplast DNA is asymmetrically transcribed, the known genes in chloroplast DNA still account for less than 20% of this potential coding capacity. As stated already it would seem surprising if 80% of the chloroplast DNA was redundant i. e. did not code for protein, or had a purely structural function. Indeed the recent hybridization studies (Howell and Walker, 1974; Haff, 1975; Rawson, 1975) seem to suggest that the amount of chloroplast DNA that is transcribed into RNA in both algal and higher plant species represents almost the complete potential coding capacity of the chloroplast DNA. These preliminary indications must however be supported by more exacting confirmatory evidence.

As there has been no reported evidence for the transport of nucleic acids across the plastid envelope, it seems reasonable to assume at the current state of knowledge, that if the information encoded in chloroplast DNA is translated into protein then this process will occur on plastid ribosomes. The failure of the work presented in this thesis to increase the number of, albeit unidentified, plastid ribosome products provides even stronger reason for considering the possibility that a number of proteins are synthesized on plastid ribosomes during the development of the etioplast or chloroplast from the proplastid.

A direct method for testing this possibility would be to adapt the in vitro approach to studying the spectrum of proteins synthesized in proplastids isolated during their development into chloroplasts or etioplasts. Three alternative systems seem feasible. Firstly, it is known that successive sections from the base to the tip of normal light-grown monocotyledonous leaves represent successive steps in cell and plastid differentiation (Leech et al, 1973). Young maize leaves could therefore be used to provide plastids isolated at different stages of development between the proplastid and the chloroplast. The biosynthetic capabilities of isolated maize proplastids and developing chloroplasts is almost completely unknown; the only reported activity to date refers to the synthesis of fatty acids (Hawke et al, 1974). However these plastids could presumably be readily assayed for in vitro protein synthesizing/

synthesizing activity by methods similar to those described in this thesis.

An alternative higher plant system, which would seem more attractive for comparative purposes, would be to develop a method of isolating developing pea proplastids. By far the most detailed knowledge on the products of in vitro plastid protein synthesis have been obtained using this species (Blair and Ellis, 1973; Eaglesham and Ellis, 1974; Siddell and Ellis, 1975). The methods of the isolation of proplastids from root tissue have already been reported (Thomson et al, 1972) and it has recently been demonstrated that the roots of particular varieties of pea will accumulate chlorophyll, presumably due to plastid differentiation, upon illumination (R. M. Leech, personal communication). It would seem feasible therefore to isolate pea proplastids in different stages of light-induced transformation into chloroplasts, and these plastids could be studied with regard to in vitro protein synthesis. This system could also be used in other areas of research into plastid development.

A third possible alternative is to study the proteins synthesized in plastids isolated from the algal species, Euglena gracilis. In this species the development of the chloroplast from the proplastid (which structurally resembles a rudimentary etioplast) can be experimentally controlled by the light-induced conversion of dark-adapted (heterotrophic) cells to normal autotrophic growth (Kirk and Tilney-Bassett, 1967). It has already been shown that both proplastids and chloroplasts isolated from this species can incorporate labelled amino acids into protein (Reger et al, 1972b). However, the analysis of the chloroplast products (Harris et al, 1973) reveals that no discrete membrane-bound proteins were synthesized, although labelled amino acids are incorporated into a soluble fraction which co-migrates with Fraction I protein on agarose column chromatography.

The inability to synthesize discrete proteins in this system may be due to the method of plastid isolation which involves the use of high pressures to break open the cells. If this is the case, modification of the isolation procedure may result in a valuable system for studying protein synthesis in developing plastids isolated from a species widely separated/

separated from the higher plants in evolution. Only when the protein synthesizing capabilities of developing proplastids is known can the full contribution of plastid ribosome products to the structure and function of the organelle be satisfactorily evaluated.

If it is the case that both etioplasts and chloroplast acquire a large proportion of their components in the early stages of development, then the etioplast should be viewed as a readily convertible chloroplast precursor, rather than a relatively undifferentiated progenitor to the chloroplast (Kirk and Tilney-Bassett, 1967). Evidence to support this view is discussed in the literature review. It is clear that etioplasts contain a significant number of components and biochemical activities of the mature chloroplast. For example, bean etioplast contain a large number of structural and functional components in common with the chloroplast, although in reduced amount (Bradbeer 1975). It is also clear that etioplasts possess extensive biosynthetic capabilities.

For example the presence within the etioplast of the machinery required to synthesize nucleic acids is evidenced by both in vivo and in vitro studies. As judged by the accumulation of plastid DNA, pea etioplasts are able to synthesize DNA at half the rate of chloroplasts of similar age (Bennet and Radcliffe, 1975). The capacity for RNA synthesis in etioplasts is demonstrated by the presence of an active DNA-dependent RNA polymerase in isolated pea etioplasts (Ellis and Hartley, 1971; Bottomley, 1970). The results presented in this thesis show that isolated etioplasts are able to synthesize proteins, a process involving a complex and intricate machinery composed of many components.

If the etioplast is ^a readily convertible chloroplast precursor then how should the phenomenon of de-etiolation, or greening, be viewed? The most important event during the greening of etioplasts is the development of photosynthesis. In this framework the development of photosynthetic competence can be viewed as the accumulation within the plastid of only proteins, or the products of proteins, whose synthesis/

synthesis is stimulated by light. To quote Griffiths (1975a), "Studies on light-dependent acquisition of photosynthetic activity by higher plants has been carried out using both in vivo assays on greening etiolated leaves (Bonner and Hiller, 1963; Hiller and Boardman, 1971) and assays in vitro on plastids isolated from leaves at different stages of greening (Gyldenholm and Whatley, 1968; Plesničar and Bendall, 1973; Forger and Bogorad, 1973; Remy, 1973). The picture emerging from this work is that of a stepwise mechanism for the greening process with most of the components required for photosynthesis being already present in the etioplast systems: development involves the light-dependent addition of chlorophyll and possibly some other functional (Plesničar and Bendall, 1973) and structural (Forger and Bogorad, 1973) components enabling photosynthetic reactions to proceed".

It remains to consider the evidence on the site of synthesis of those proteins which appear in the plastid during greening. The results of inhibitor studies, although not conclusive, do suggest that with some notable exceptions (e. g. the large subunit of Fraction I protein and the cytochromes F, b_{563} and b_{559LP}) the majority of components which accumulate in the greening plastid are synthesized on cytoplasmic ribosomes (Bradbeer, 1975; Börner, 1973; Ellis and Hartley, 1971). For example the studies of Ireland and Bradbeer (1971) suggest that the majority of Calvin cycle enzymes whose activity increases during de-etiolation are synthesized on 80S cytoplasmic ribosomes. A similar conclusion has been reached for numerous other plastid proteins, for example, ferredoxin, and chloroplast glyoxalate reductase (Bradbeer, 1975). Although there has been no direct evidence regarding the site of synthesis, it is clear from genetic studies that the majority of the enzymes involved in chlorophyll biosynthesis are also nuclear gene products (Kirk and Tilney-Bassett, 1967) and are therefore likely to be synthesized on cytoplasmic ribosomes.

If the etioplast and the process of de-etiolation are viewed in this context, then it does not seem surprising that the results presented in this thesis show that plastids isolated during the etioplast to chloroplast transition do not synthesize an extensive array of novel proteins. One of the objectives of future research into plastid development should be to provide/

provide concrete rather than circumstantial evidence to support the view of the etioplast and of de-etiolation that is presented throughout this thesis. It is my belief that such evidence will be gained by the biochemical and genetic approach, rather than the structural approach that has only recently ceased to dominate this area of plastid research.

B. FRACTION I PROTEIN SYNTHESIS DURING GREENING AS A MODEL FOR GENE REGULATION.

One of the major goals in present day biology is to understand, in molecular terms, the processes of cellular development and differentiation. The current dogma maintains that both these processes must ultimately be a consequence of controlling the expression of specific genes within the cell. The many possible forms of gene control are divisible into two broad categories; transcriptional controls which alter the spectrum and amounts of mRNA species within the cell, and translational controls which alter the frequency with which any particular mRNA is translated by ribosomes.

The relative importance of transcriptional and translational controls in the developmental process has however been almost exclusively assessed by studies in mammalian and avian systems. Of particular note are the studies on the selective activation of the globin gene during erythroid cell development (Ramirez *et al*, 1975; Marks *et al*, 1974). Gene regulation in animals has also been investigated in model systems involving the induction of a specific protein in response to hormonal stimulation. Particular attention has been paid to the oestrogen induction of sex steroid-dependent proteins (e. g. ovalbumin) in the chick oviduct (Chan *et al*, 1973) and the glucocorticoid-induced synthesis of hepatic tryptophan oxygenase in rat liver (Schutz *et al*, 1975). These studies strongly suggest that it is transcriptional rather than translational controls that are the major regulator of gene expression in higher organisms. The role of translational control appears to be to augment rather than duplicate the fundamental control of protein synthesis which occurs at transcription (Kerr, 1975).

At the present time there are very few reports on the regulation of mRNA species in plants (Tuan Hua and Varner, 1974; Verma *et al*, 1975). In the most detailed study Verma *et al* (1975) conclude that the mRNA/

mRNA for the enzyme cellulase, increases as a result of treating pea epicotyls with a synthetic auxin ((2, 4 dichlorophenoxy) acetic acid). In contrast to the general picture emerging from animal studies these authors conclude that translational as well as transcriptional controls are equally important in regulating the increased activity of this enzyme. Clearly there is a pressing need for more extensive studies on the control of gene expression in plants.

The results presented in this thesis have shown that one of the proteins synthesized in isolated etioplasts and developing chloroplasts is the large subunit of Fraction I protein. The expression of the gene(s) for the large subunit of Fraction I protein during the etioplast to chloroplast transition provides an ideal opportunity for studying gene regulation. The exposure of etiolated pea apices to continuous illumination for 48 hours results in a five-fold increase in the amount of Fraction I protein relative to apices maintained in the dark (Ellis and Hartley, 1971; Bennet, 1975). By analogy to the animal situation this large increase in the level of Fraction I protein during greening is presumably preceded by an increase in the level of Fraction I protein large subunit mRNA (LS mRNA). If this is the case, then the greening pea apex should provide an ideal experimental system for studying light-induced changes in the transcription of the gene for the large subunit. It should be relatively easy to monitor the translational capacity of the LS mRNA isolated from developing plastids in a heterologous cell-free translation system (Hartley *et al*, 1975). This information could be correlated with the rate of large subunit accumulation in plastids at an equivalent stage of development *in vivo*. If these two parameters rose in parallel it would suggest that the rate of large subunit synthesis *in vivo* is determined by the availability of mRNA. However, this suggestion could only be confirmed by demonstrating that the samples of LS mRNA isolated from plastids at different stages of development do not initiate peptide chain formation in the cell-free system with different frequencies. In order to test this point it would be essential to purify the LS mRNA's isolated from developing plastids.

With the availability of pure LS mRNA the synthesis of a highly radioactive/

radioactive complementary DNA strand could be achieved with avian myoblastosis virus reverse transcriptase (Ross *et al.*, 1972). This complementary DNA could then be used to make a quantitative estimation of the LS mRNA sequences in RNA from plastids isolated at different stages of development. This approach would allow a comparison to be made between the total number of LS mRNA sequences and the number of translatable LS mRNA molecules in a developing plastid. Any differences between these two parameters would perhaps indicate the extent of translational control *in vivo*.

An added incentive for studying the expression of the large subunit gene is that the model which has been developed for the co-operation of nuclear and plastid genomes in the synthesis of Fraction I protein (Ellis, 1975a) postulates that co-ordinate control may be exerted over the synthesis of the two subunits. If this is the case, then an understanding of the processes which regulate the expression of the large subunit gene may provide a paradigm for many other integrated processes in the cell.

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APPENDIX - PUBLISHED PAPER

Protein Synthesis in Chloroplasts

CHARACTERISTICS AND PRODUCTS OF PROTEIN SYNTHESIS *IN VITRO* IN ETIOPLASTS AND DEVELOPING CHLOROPLASTS FROM PEA LEAVES

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The function of plastid ribosomes in pea (*Pisum sativum* L.) was investigated by characterizing the products of protein synthesis *in vitro* in plastids isolated at different stages during the transition from etioplast to chloroplast. Etioplasts and plastids isolated after 24, 48 and 96 h of greening in continuous white light, use added ATP to incorporate labelled amino acids into protein. Plastids isolated from greening leaves can also use light as the source of energy for protein synthesis. The labelled polypeptides synthesized in isolated plastids were analysed by electrophoresis in sodium dodecyl sulphate urea-polyacrylamide gels. Six polypeptides are synthesized in etioplasts with ATP as energy source. Only one of these polypeptides is present in a 150000g supernatant fraction. This polypeptide has been identified as the large subunit of Fraction I protein (3-phospho-D-glycerate carboxylase EC 4.1.1.39) by comparing the tryptic 'map' of its L-[³⁵S]methionine-labelled peptides with the tryptic 'map' of large subunit peptides from Fraction I labelled with L-[³⁵S]methionine *in vitro*. The same gel pattern of six polypeptides is seen when plastids isolated from greening leaves are incubated with either added ATP or light as the energy source. However, the rates of synthesis of particular polypeptides are different in plastids isolated at different stages of the etioplast to chloroplast transition. The results support the idea that plastid ribosomes synthesize only a small number of proteins, and that the number and molecular weight of these proteins does not alter during the formation of chloroplasts from etioplasts.

Chloroplasts contain all the components theoretically necessary for autonomy, i.e. DNA, DNA polymerase, RNA polymerase and a protein-synthesizing apparatus (Boulter *et al.*, 1972). The chloroplast DNA is sufficient to encode about 125 polypeptides each of molecular weight 5×10^4 , whereas chloroplast ribosomes can represent up to 50% of the total ribosomes in a leaf (Ellis & Hartley, 1974). These quantities might be thought to support the idea of chloroplast autonomy, but recent evidence suggests that this idea is not correct. Studies of the effects on chloroplast development of antibiotic inhibitors specific for chloroplast ribosomes suggest that most chloroplast proteins are made by cytoplasmic ribosomes (Boulter *et al.*, 1972; Ellis *et al.*, 1973). This conclusion is supported by the finding that only one soluble protein is synthesized by isolated intact pea chloroplasts (Blair & Ellis, 1973). This protein is the large subunit of Fraction I protein (3-phospho-D-glycerate carboxylase EC 4.1.1.39), which is the most abundant protein in leaves. Five membrane-bound proteins are also synthesized in isolated chloroplasts; these are unidentified, but are clearly minor components of the chloroplast membranes (Eaglesham & Ellis, 1974). Similarly, genetic studies suggest

that many chloroplast proteins are encoded in nuclear rather than chloroplast DNA (Kirk, 1972); an exception is the large subunit of Fraction I protein, which is encoded in chloroplast DNA (Chan & Wildman, 1972).

This evidence suggests that the abundance of chloroplast ribosomes is required, not because they synthesize a wide range of different proteins, but because one of their products, the large subunit of Fraction I protein, occurs in much larger quantities than any other protein. However, two criticisms can be made of this conclusion. The first stems from the fact that our previous experimental conditions ensure that only chloroplasts that are able to carry out photophosphorylation can incorporate amino acids into protein (Blair & Ellis, 1973), and the second is that the known genes in chloroplast DNA account for less than 10% of its potential coding capacity (Ellis & Hartley, 1974). It occurred to us that plastids isolated at a stage of development before they have acquired the ability to carry out photophosphorylation may synthesize a different spectrum of proteins from mature chloroplasts. Pea plants provide a convenient experimental situation to test this possibility because when they are raised from seed in the dark

they accumulate plastids termed etioplasts which lack chlorophyll and thylakoid membranes (Kirk & Tilney-Bassett, 1967); illumination of dark-grown plants results in a rapid conversion of etioplasts into chloroplasts. The present paper describes the characteristics and products of protein synthesis in isolated etioplasts and in plastids isolated from greening tissue.

Experimental

Materials

Pea seeds (*Pisum sativum* L. var. Meteor) were purchased from S. Dobic, Chester, U.K. Vermiculite (Micafil) was bought from Dupre Vermiculite Ltd., Herts., U.K. Creatine phosphokinase, ATP, UTP, GTP, CTP, dGTP, Hepes [2-(N-2-hydroxyethyl)-piperazin-N'-yl]ethanesulphonic acid, Tricine [N-tris(hydroxymethyl)methylglycine], phenylmethanesulphonyl fluoride, *n-threo*-chloramphenicol, cycloheximide and Triton X-100 [octylphenoxypolyethoxyethanol] were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Pronase (B grade) and carbonyl cyanide *m*-chlorophenylhydrazide were purchased from Calbiochem, London W11 1AS, U.K. Coomassie Brilliant Blue R was from Searle Diagnostic, High Wycombe, Bucks., U.K., Bromophenol Blue and sodium dodecyl sulphate (especially pure) were from BDH Chemicals Ltd., Poole, Dorset, U.K., acrylamide was from Fluka A.G., Buebs, Switzerland, and *NN'*-methylenebisacrylamide was from Eastman Organic Chemicals, Rochester, N.Y., U.S.A.

L-[³⁵S]Methionine (SJ 204, 200–300 Ci/mmol, 3–5 mCi/ml in aqueous solution containing 0.04% 2-mercaptoethanol) and L-[³H]leucine (TRK 170, 50 Ci/mmol, 1 mCi/ml in sterilized aqueous solution containing 2% v/v ethanol) were supplied by The Radiochemical Centre, Amersham, Bucks., U.K. 1-Chloro-4-phenyl-3-*t*-toluene-*p*-sulphonamidobutan-2-one treated trypsin was supplied by Worthington Biochemical Corp., Freehold, N.J., U.S.A. Precoated 20 cm × 20 cm silica gel plastic (i.e. plates (Polygram Sil G; 0.25 mm layer) were obtained from Macherey-Nagel and Co., Duren, Germany. The following chemicals were kindly supplied gratis: 3-(3,4-dichlorophenyl)-1,1-dimethylurea by Isons Ltd., Loughborough, Leics., U.K., actinomycin D from Merck, Sharpe and Dohme, Rahway, N.J., U.S.A., tentoxin (cyclohexyl-*N*-methylalanyl-glycyl-*N*-methylidihydrophenylalanyl) by Dr. R. D. Durbin, University of Wisconsin, U.S.A., and *n-threo*-chloramphenicol by Parke Davis and Co., Pontypool, Wales, U.K.

Methods and procedure

Plant tissue. Pea seeds were surface sterilized in 2% (w/v) sodium hypochlorite solution for 5 min and

then washed in running tap water for 24 h. The imbibed seeds were sown in moist vermiculite and grown at 22–24 °C for 9 days. Mature chloroplasts were isolated from the youngest leaves of seedlings grown under a 12 h photoperiod of 2000 lx provided by white fluorescent tubes. Etioplasts were isolated from the apical buds of plants grown in darkness. Etiolated seedlings were greened under continuous white light of 2800 lx at 22–24 °C. Plastids from greening seedlings were isolated from the first-formed leaflets.

Plastid isolation. Plastids were isolated by the method of Ramirez *et al.* (1968) as modified by Blair & Ellis (1973). Plant tissue was excised and 20 g homogenized for 4 s in a Polytron homogenizer (Northern Media Supply Ltd., Hull, U.K.) in 100 ml of semi-frozen sterile isolation medium [0.35 M-sucrose-25 mM-Hepes-NaOH buffer (pH 7.6)-2 mM-EDTA (disodium salt)-2 mM-sodium isoascorbate]. The homogenate was strained through two lots of eight layers of absorbant muslin and centrifuged at 2500 g for 1 min at 4 °C. The supernatant fraction was discarded and the pellet carefully resuspended in sterile KCl resuspension medium [66 mM-Tricine-KOH buffer (pH 8.3)-0.2 M-KCl-6.6 mM-MgCl₂]. Plastid isolations were performed in the light. Experiments were performed which showed that no differences could be found with regard to these studies between plastids isolated in the light and plastids isolated in the dark. About 50–60% of the plastids isolated by this method were highly refractive when viewed with the phase-contrast microscope, suggesting they possessed a completely limiting envelope. Studies with the electron microscope indicate that nuclei are present in these preparations to the extent of one nucleus for twelve plastids in the etioplast preparations and one nucleus for 50 plastids in the chloroplast preparations. Protein was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Assay for L-[³⁵S]methionine incorporation into hot trichloroacetic acid-insoluble material in isolated etioplasts. The etioplasts isolated from 20 g of tissue were resuspended in 5 ml of sterile KCl resuspension medium. As a routine, incubation mixtures with a final volume of 500 μl contained 100 μmol of KCl, 33 μmol of Tricine-KOH buffer (pH 8.3), 3.3 μmol of MgCl₂, 0.5 μCi of L-[³⁵S]methionine (200 Ci/mmol), 1 μmol of ATP and etioplasts containing 150–300 μg of protein. All the inhibitors tested were soluble in KCl resuspension medium at the concentrations used, and were added to the incubation mixture by replacing a portion of KCl resuspension medium with an equal portion of KCl resuspension medium plus inhibitor. Reaction components were mixed at 2 °C, and the incubation was started by increasing the temperature of the incubation mixture to 20 °C. This temperature was maintained during the incubation.

Incubations were performed in the dark. After 1 h the incubations were terminated by the addition of 100 μ l of a saturated aqueous solution of L-[35 S]methionine and 1 ml of 20% (w/v) trichloroacetic acid. The suspensions were left to stand at 2°C for 30 min and then heated to 90°C for 15 min. The precipitate was transferred quantitatively to glass-fibre discs (Whatman GF/C, 2.5 cm) and each disc was washed with 100 ml of ice-cold 5% (w/v) trichloroacetic acid, 60 ml of ethanol and finally with 60 ml of diethyl ether. Filters were dried at 60°C for 30 min and placed in scintillation vials containing 8 ml of a toluene-based scintillation fluid [0.5% (w/v) 2,5-diphenyloxazole, 0.03% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene]. The radioactivity was determined in a Packard Tri-Carb model 3320 liquid-scintillation spectrometer at a counting efficiency of 70% as determined by internal standardization.

Incubation of plastids for analysis of synthesized polypeptides. The plastids isolated from 40 g of tissue were resuspended in 3 ml of KCl resuspension medium. Light-driven protein synthesis incubation mixtures with a final volume of 1 ml contained 200 μ mol of KCl, 66 μ mol of Tricine-KOH buffer (pH 8.3), 6.6 μ mol of Fig 1₂, 100 μ Ci of L-[35 S]methionine (700 Ci/mmol) or 100 μ Ci of L-[3 H]leucine (50 Ci/mmol) and plastids containing 1–6 mg of protein. ATP-driven protein synthesis incubation mixtures contained in addition 2 μ mol of ATP, 5 μ mol of creatine phosphate and 100 μ g of creatine phosphokinase. Reaction components were mixed at 2°C, and the incubations were started by raising the temperature of the incubation mixture to 20°C. This temperature was maintained during the incubation. The ATP-driven system was incubated in the dark, whereas incubations with light as the energy source were illuminated with filtered red light of 4000 lx intensity as measured by the Megatron Light Meter Type E1. After 1 h the incubations were terminated by the addition of 200 μ l of a saturated aqueous solution of L-[35 S]methionine and 120 μ l of an aqueous solution of 20% (w/v) sodium dodecyl sulphate. The incubation mixtures were boiled for 3 min and then dialysed at room temperature against 5 litres of 2.5 mM-Tris-glycine buffer (pH 8.5), 100 mM-2-mercaptoethanol and 0.2% (w/v) sodium dodecyl sulphate. At room temperature an insoluble precipitate of potassium dodecyl sulphate forms when sodium dodecyl sulphate is added to the incubation mixtures. This precipitate disappears during dialysis.

Preparation of a 150000g etioplast supernatant fraction. Etioplasts were isolated and incubated for the analysis of the products *in vitro* as described above. The incubation was terminated by the addition of L-[35 S]methionine and the incubation mixture was then centrifuged at 2500g for 2 min at 2°C. The supernatant fraction and pellet were separated, and the pellet was resuspended in 1.2 ml of a buffer containing

2.5 mM-Tris-glycine (pH 8.5) and 4 mM-2-mercaptoethanol to lyse the etiooplasts. The supernatant fraction was added to the lysed etioplast preparation and centrifuged at 150000g for 100 min at 2°C. The clear supernatant was removed, 240 μ l of an aqueous solution of 20% (w/v) sodium dodecyl sulphate was added and the sample boiled for 3 min. The 150000g supernatant fraction was dialysed at room temperature against 5 litres of a buffer containing 2.5 mM-Tris-glycine (pH 8.5), 100 mM-2-mercaptoethanol and 0.2% (w/v) sodium dodecyl sulphate.

Pronase digestion of the polypeptides synthesized in isolated etiooplasts. Incubation for the analysis of the products synthesized *in vitro* was performed as described. The incubation was terminated by the addition of L-[35 S]methionine and a lysed etioplast preparation was prepared as described above. After the supernatant fraction had been added to the lysed etioplast preparation, a 600 μ l sample was taken for pronase digestion. Pronase, at a concentration of 1 mg/ml in a buffer containing 50 mM-Tris-glycine (pH 8.5) and 10 mM-2-mercaptoethanol, was added to the sample to give a protein/pronase ratio of 10:1 (w/w) and the sample was incubated at 37°C for 3 h. The incubation was terminated by the addition of 65 μ l of an aqueous solution of 20% (w/v) sodium dodecyl sulphate and boiling for 3 min. The solubilized digest was dialysed at room temperature against 5 litres of a buffer containing 2.5 mM-Tris-glycine (pH 8.5), 100 mM-2-mercaptoethanol and 0.2% (w/v) sodium dodecyl sulphate, and the dialysed sample was then analysed by electrophoresis as described below.

Analytical sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The polyacrylamide gel electrophoresis system used contained sodium dodecyl sulphate and urea in the buffers. Gels were polymerized in glass tubes (6 mm \times 100 mm) and consisted of 15% (w/v) acrylamide, 0.3% bisacrylamide made up in a buffer containing 0.25 M-Tris-HCl (pH 8.5), 0.1% (w/v) sodium dodecyl sulphate and 3% (w/v) urea. The running buffer contained 50 mM-Tris-glycine (pH 8.5), 0.1% (w/v) sodium dodecyl sulphate and 10 mM-2-mercaptoethanol or 8 mM-cysteine. Gels were prerun at 10 mA/gel for 3 h. Samples (100 μ l), containing 100–400 μ g of protein, were layered directly on the gels with sucrose (10%, w/v) and Bromophenol Blue (0.005%, w/v). The samples were concentrated by applying a current of 0.5 mA/gel for 30 min. Electrophoresis was then carried out at 5 mA/gel at room temperature; to obtain maximum separation of the polypeptides the electrophoresis was continued 30% longer than the time taken for the marker dye to reach the bottom of the gel. Experiments were performed to ensure that this did not cause protein bands or radioactive peaks to be lost from the bottom of the gel. Gels were stained in Coomassie Brilliant Blue R [0.1% (w/v) in 50% (v/v) methanol and 7.5% (v/v) acetic acid] and the excess

of stain was removed by washing in 50% (v/v) methanol containing 7.5% (v/v) acetic acid. The gels were calibrated for molecular weight as described (Eggleham & Ellis, 1974). The gels were scanned at 620 nm in a Joyce-Loebl Chromoscan, swollen in 7.5% (v/v) acetic acid to remove the methanol, frozen at -90°C for 30 min and then sliced into 1 mm slices with a Mickle gel slicer. The slices were solubilized in 200 μl of H_2O_2 (100 vol.) for 3 h at 80°C in capped scintillation vials and, after cooling the vials, 8 ml of Triton X-100-toluene scintillation fluid [0.1% (w/v) 2,5-diphenyloxazole, 0.65% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene Triton X-100 (2:1, v/v)] was added. Samples were counted for radioactivity in a Packard Tri-Carb model 3320 liquid-scintillation spectrometer. L-[^{35}S]Methionine alone was determined at a counting efficiency of 88% as determined by internal standardization. In gel slices containing both L-[^{35}S]methionine and L-[^3H]leucine the radioactivity due to each isotope was estimated by the channels-ratio method of Hundler (1967).

Preparative gel electrophoresis of the 150000g supernatant fraction from isolated etioplasts. The dialysed sodium dodecyl sulphate treated 150000g supernatant fraction prepared as described above was further fractionated by the preparative polyacrylamide-gel electrophoresis method of Moore & Burke (1974). Sucrose crystals were added to 2 ml of dialysed preparation (4.4 mg of protein) and the samples were loaded directly on to cylindrical columns (18 mm \times 220 mm) of 15% (w/v) acrylamide-0.3% bisacrylamide. Gels were prepared and run in the sodium dodecyl sulphate-containing buffer described above. The gels were prerun at 5 mA/gel for 20 h and samples were concentrated on top of the gel by applying a current of 3 mA/gel for 3 h. Electrophoresis of the sample was carried out at 5 mA/gel at room temperature for 30 h. The position in the gel of the large subunit from Fraction I protein was determined from the mobility of that from dansylated pea Fraction I protein fractionated in parallel gels. Pea Fraction I protein was prepared as described by Blair & Ellis (1973), and dansylated as described by Talbot & Yphantis (1971). The appropriate section of the gel containing the radioactive sample was excised and the protein eluted in 0.1% (w/v) sodium dodecyl sulphate containing 1 mM-phenylmethanesulphonyl fluoride at 37°C for 16 h. The gel pieces were removed by centrifugation at 1500g for 10 min and the supernatant fraction was passed through a Whatman GF/C disc by centrifugal filtration at 2500g for 15 min. The protein was precipitated from the filtrate with 20% (w/v) trichloroacetic acid at 4°C for 30 min and centrifuged at 10000g for 30 min at 2°C . The protein was washed by resuspension and centrifugation in ethanol-diethyl ether (3:1, v/v) (twice) and ether (once). This material was stored at 4°C and was used for the tryptic peptide analysis.

Preparation of the large subunit from pea Fraction I protein labelled *in vivo* with L-[^{35}S]methionine. Labelled Fraction I protein was prepared by the method of Blair & Ellis (1973) from the leaves of excised pea seedlings that had been fed with L-[^{35}S]methionine during greening. Purified labelled Fraction I protein was dissociated into its constituent subunits by solubilization in 50 mM- Na_2HPO_4 -NaOH buffer (pH 11.2). The subunits were separated by chromatography on Sephadex G-100 (superfine grade) with 50 mM- Na_2HPO_4 -NaOH buffer (pH 11.2) as the eluting buffer. The three peak fractions containing the large subunit from Fraction I protein were pooled, and the protein was precipitated with 20% (w/v) trichloroacetic acid at 4°C for 30 min. The protein was washed by resuspension and centrifugation in ethanol-ether (3:1, v/v) (twice) and ether (once). This material was stored at 4°C and used for tryptic peptide analysis.

Tryptic peptide analysis. The samples for analysis were resuspended in a mixture of formic acid and methanol (4:1, v/v). Performic acid oxidation was performed as described by Bray & Brownlee (1973). The oxidized samples were dried with N_2 , resuspended in water, and freeze-dried twice to remove all traces of performic acid. Tryptic digestion was performed by dissolving the samples (250 μg of protein) in 50 mM- NH_4HCO_3 (pH 8.0) containing 1 mM- CaCl_2 and by adding 12.5 μg of TPCK (1-chloro-4-phenyl-3-(1-toluene-*p*-sulphonamyl)butan-2-one)-inactivated trypsin dissolved in 50 mM- NH_4HCO_3 (pH 8.0)-1 mM- CaCl_2 (protein/trypsin, 20:1, w/w). Incubation was at 37°C for 7 h. After digestion, samples were freeze-dried and then dissolved in 150 μl of water. The samples were centrifuged at 1500g for 5 min to remove particulate matter. Finally, the samples were freeze-dried again and dissolved in 15 μl of water. Samples contained 50×10^3 - 100×10^3 c.p.m. The tryptic peptides were separated by the two-dimensional 'mapping' procedure of Sargent & Vadlamudi (1968). This technique allows two peptide mixtures to be 'fingerprinted' simultaneously and under identical conditions. Separation occurs on thin layers of silica gel with acetic acid-formic acid-water (4:1:45, by vol.) as the electrophoresis vector, and butan-1-ol-acetic acid-water (3:1:1, by vol.) for the chromatography vector. After drying, the thin-layer plates were exposed to Kodirex X-ray film for 8-12 days.

Results and Discussion

Characteristics of protein synthesis in isolated etioplasts

Fig. 1 illustrates the time-course of the incorporation of L-[^{35}S]methionine into hot-trichloroacetic acid-insoluble material by isolated etioplasts at different temperatures, and the dependence of this

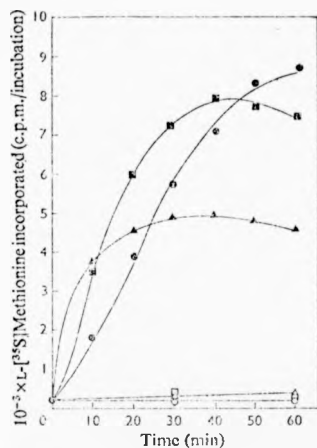


Fig. 1. Effect of ATP and temperature on the incorporation of L-[³⁵S]methionine into a hot-trichloroacetic acid-insoluble product in isolated etioplasts

Etioplasts were prepared as described under 'Methods and procedure'. Incubation mixtures (final vol. 500 μ l) contained 300 μ l of etioplast suspension (120 μ g of protein), 100 μ l of KCl resuspension medium (pH 8.3) containing 0.5 μ Ci of L-[³⁵S]methionine (200 Ci/mmol) and either 100 μ l of KCl resuspension medium containing 10 mM-ATP (\square , \circ , Δ) or 100 μ l of KCl resuspension medium alone (\square , \circ , Δ). Incubations were terminated and assayed for incorporation as described under 'Methods and procedure'. \square , 10°C, +ATP; \circ , 10°C, -ATP; Δ , 20°C, +ATP; \square , 20°C, -ATP; Δ , 30°C, +ATP; Δ , 30°C, -ATP.

incorporation on added ATP. At 20°C the rate of incorporation falls to zero rapidly after about 30 min; a similar time-course was reported for isolated chloroplasts (Blair & Ellis, 1973). The duration of incorporation by etioplasts can be extended by decreasing the incubation temperature but the initial incorporation rate is halved when the incubation temperature is lowered by 10°C. The initial rate of incorporation at 20°C is in the range 0.35–0.7 pmol of L-[³⁵S]methionine/h per mg of protein. This rate is 5–10 times less than that quoted by Blair & Ellis (1973) for ATP-driven protein synthesis in isolated chloroplasts.

To find optimum incubation conditions, the effect of changing some parameters of the incubation were investigated. The effect of changing the ATP concentration is shown in Fig. 2. L-[³⁵S]Methionine incorporation is stimulated by increasing the ATP concentration up to a value of 2 mM, but no further stimulation occurs up to 4 mM-ATP. Under our conditions the addition of an ATP-generating system

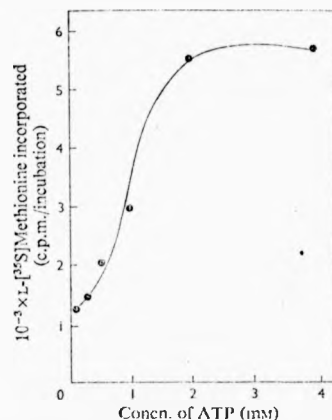


Fig. 2. Effect of ATP concentration on the incorporation of L-[³⁵S]methionine into a hot-trichloroacetic acid-insoluble product in isolated etioplasts

Etioplasts were prepared as described under 'Methods and procedure'. Incubation mixtures (final vol. 500 μ l) contained 300 μ l of etioplast suspension (300 μ g of protein), 100 μ l of KCl resuspension medium (pH 8.3) containing 0.5 μ Ci of L-[³⁵S]methionine (200 Ci/mmol) and 100 μ l of ATP in KCl resuspension medium (buffered to pH 8.3). Incubations were terminated after 1 h and assayed for incorporation as described under 'Methods and procedure'.

did not alter the total incorporation in etioplasts. The effect of altering the incubation pH is shown in Fig. 3. Maximum incorporation is obtained at a pH of 8.3. This pH is also the optimum value for protein synthesis in isolated chloroplasts. Although L-[³⁵S]methionine incorporation in isolated etioplasts is stimulated by a high K⁺ ion concentration in the incubation medium (Fig. 4), the degree of stimulation is not as great as in isolated chloroplasts. The K⁺ ion stimulation of incorporation in etioplasts confirms the suggested role of this ion as a cofactor for protein synthesis in isolated chloroplasts rather than as a factor that stimulates incorporation by an involvement in, for example, photophosphorylation (Blair & Ellis, 1973).

Some of the characteristics of protein synthesis in isolated etioplasts are given in Table 1. ATP will, but light will not, act as an energy source for protein synthesis in isolated etioplasts. This suggests that our etioplast preparations cannot carry out photophosphorylation. Neither GTP, UTP, CTP nor dTTP, each at 2 mM, will act as an energy source for protein

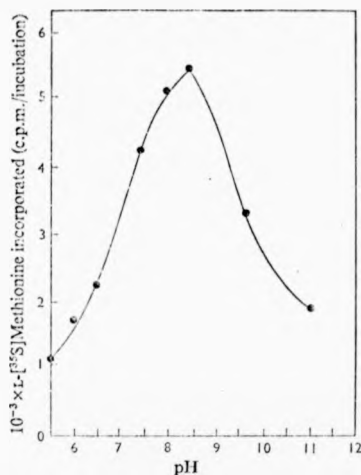


Fig. 3. Effect of pH on the incorporation of 1- ^{35}S methionine into a hot-trichloroacetic acid-insoluble product in isolated etioplasts

Etioplasts were prepared as described under 'Methods and procedures', except that 5 ml portions of the homogenized tissue were centrifuged individually. The etioplast pellets (770 μg of protein) were resuspended in 500 μl of KCl resuspension medium (buffered to the indicated pH values with either KOH or HCl) containing 0.5 μCi of 1- ^{35}S methionine (200 Ci/mmol) and 2 mM-ATP. Incubations were terminated after 1 h and assayed for incorporation as described under 'Methods and procedure'.

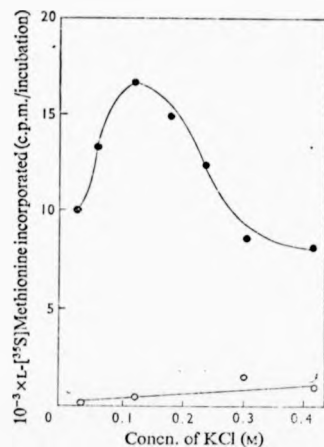


Fig. 4. Effect of K^+ ion concentration on the incorporation of 1- ^{35}S methionine into a hot-trichloroacetic acid-insoluble product in isolated etioplasts

Etioplasts were prepared as described under 'Methods and procedure', except that 5 ml portions of the homogenized tissue were centrifuged individually. The etioplast pellets (900 μg of protein) were resuspended in 500 μl of modified KCl resuspension medium in which the total K^+ ion concentration varied from 33 to 453 mM (pH 8.3), containing either 0.5 μCi of 1- ^{35}S methionine (200 Ci/mmol) and 2 mM-ATP (●) or 0.5 μCi of 1- ^{35}S methionine (200 Ci/mmol) alone (○). Incubations were terminated and assayed as described under 'Methods and procedure'.

Table 1. Effect of the energy source and inhibitors on the incorporation of 1- ^{35}S methionine into a hot-trichloroacetic acid-insoluble product in isolated etioplasts

Etioplasts were isolated and incubated as described under 'Methods and procedure'. The Triton X-100 solubility of the material containing the incorporated 1- ^{35}S methionine was determined by addition of an equal volume of 4% (v/v) Triton X-100 to the incubation after 1 h, centrifuging the solubilized preparation at 12,500g for 10 min at 4°C, separating the supernatant fraction and pellet and precipitating the protein in these fractions with 10% (w/v) trichloroacetic acid. Incorporation was assayed as described under 'Methods and procedure'. Incorporation by the complete ATP-driven system is called 100.

Energy source	Treatment	Incorporation (%)
ATP	Complete	100
None	Complete	7
None	Zero time	6
Light	Complete	7
ATP	Triton X-100-insoluble	5
ATP	Ribonuclease (25 $\mu\text{g}/\text{ml}$)	65
ATP	D-threo-Chloramphenicol (25 $\mu\text{g}/\text{ml}$)	8
ATP	1-threo-Chloramphenicol (40 $\mu\text{g}/\text{ml}$)	101
ATP	Cycloheximide (25 $\mu\text{g}/\text{ml}$)	98
ATP	Actinomycin D (30 $\mu\text{g}/\text{ml}$)	100
ATP	Tentoxin (2 $\mu\text{g}/\text{ml}$)	105
ATP	3-(3,4-Dichlorophenyl)-1,1-dimethylurea (1 μM)	80
ATP	Carbonyl cyanide <i>m</i> -chlorophenylhydrazone (5 μM)	78

synthesis in etioplasts. If the preparations are solubilized at the end of the incubation in 2% Triton X-100, only about 5% of the radioactivity incorporated into protein is present in a 12500g pellet. This result suggests that incorporation is occurring in either plastids or mitochondria, rather than in whole leaf cells, nuclei or bacteria, which are not solubilized by the detergent at this concentration (Parenti & Margulies, 1967). Studies on the t -[35 S]methionine-incorporating activity of a mitochondrially enriched fraction isolated from etiolated pea leaves, and the effect of this fraction on incorporation by the etioplast-enriched pellet, exclude the possibility that incorporation is due to the activity of mitochondrial ribosomes. In contrast with the 80% inhibition observed by Reger *et al.* (1972) with isolated wheat etioplasts, ribonuclease, over a concentration range 10–50 μ g/ml, inhibits incorporation in our system by 35%. Blair & Ellis (1973) have concluded that in the light-driven system there is a strong correlation between increasing ribonuclease sensitivity and decreasing intactness of the chloroplasts in the preparation. Our results with isolated etioplasts suggest that 35% of the incorporation is due to the activity of ribosomes not bounded by a plastid envelope. We suggest that the remaining 65% of the incorporation occurs in intact etioplasts, and that the inclusion of ribonuclease in the incubation mixture can be used, if necessary, to ensure that incorporation occurs only in intact etioplasts.

L-[35 S]Methionine incorporation in this system is completely and stereospecifically inhibited by the *D*-*threo* isomer of chloramphenicol, but is not affected by cycloheximide (Table 1). This result suggests the incorporation cannot be attributed to contaminating 80S ribosomes. The activity of the system is not inhibited by actinomycin D at a concentration of 30 μ g/ml. At this concentration actinomycin D inhibits the incorporation of [3 H]uridine into RNA by 70% in identical preparations of etioplasts (results not shown). It seems therefore that, as in isolated chloroplasts (Blair & Ellis, 1973), the mRNA translated in isolated etioplasts is most likely present inside the plastids before they are isolated. The commonly used inhibitors of photophosphorylation such as carbonyl cyanide *m*-chlorophenylhydrazone and 3-(3,4-dichlorophenyl)-1,1-dimethylurea, and the less commonly used tentoxin, which is thought to act as an inhibitor of coupled electron transport (Arnten, 1972), have little or no inhibitory effect in this system. By contrast, light-driven protein synthesis in isolated chloroplasts is greatly inhibited by these compounds (Blair & Ellis, 1973).

These results taken together indicate that t -[35 S]-methionine is incorporated into protein in isolated etioplasts, and that protein synthesis in isolated etioplasts has the same requirements and exhibits the same characteristics as protein synthesis in isolated chloroplasts.

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Analysis of the polypeptides synthesized in isolated etioplasts by sodium dodecyl sulphate-polyacrylamide gel electrophoresis

The fractionation pattern obtained by sodium dodecyl sulphate-polyacrylamide-gel electrophoresis of the polypeptides synthesized in isolated etioplasts is shown in Fig. 5. Staining the gels with Coomassie Brilliant Blue R shows many protein bands, the most

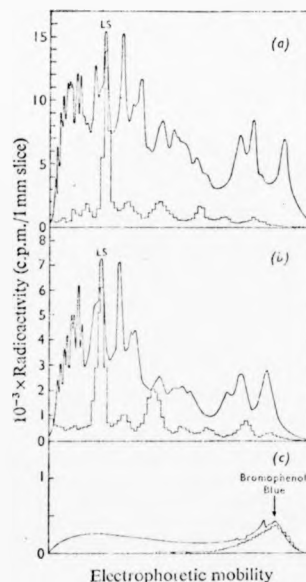


Fig. 5. Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis of the polypeptides synthesized *in vitro* in etioplasts and the sensitivity of these polypeptides to Pronase

Etioplasts were isolated from 20–30g of tissue and incubated with t -[35 S]methionine (300 Ci/mmol) (a) and (c), or t -[3 H]leucine (50 Ci/mmol) (b). Plastid isolation and incubation, and Pronase digestion of the products synthesized *in vitro*, was performed as described under 'Methods and procedure'. Gels were loaded with 100 μ l of solubilized incubation mixture: (a), 130 μ g of protein; (b), 160 μ g of protein; (c) 100 μ l of the Pronase digest. Electrophoresis and related procedures were performed as described under 'Methods and procedure', except for (c) in which the electrophoresis was stopped when the marker dye reached the bottom of the gel. —, F_{620} ; —□— (histogram), radioactivity; LS, large subunit of Fraction I protein.

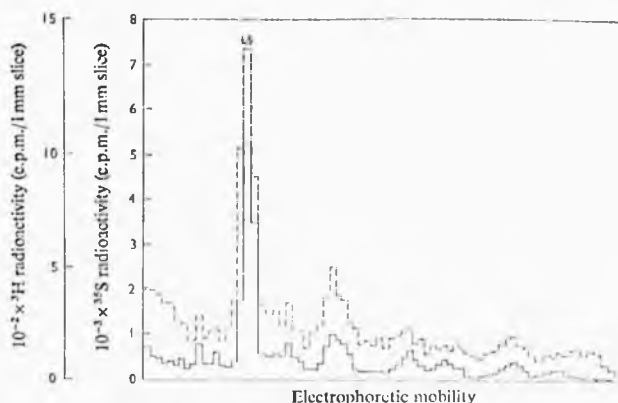


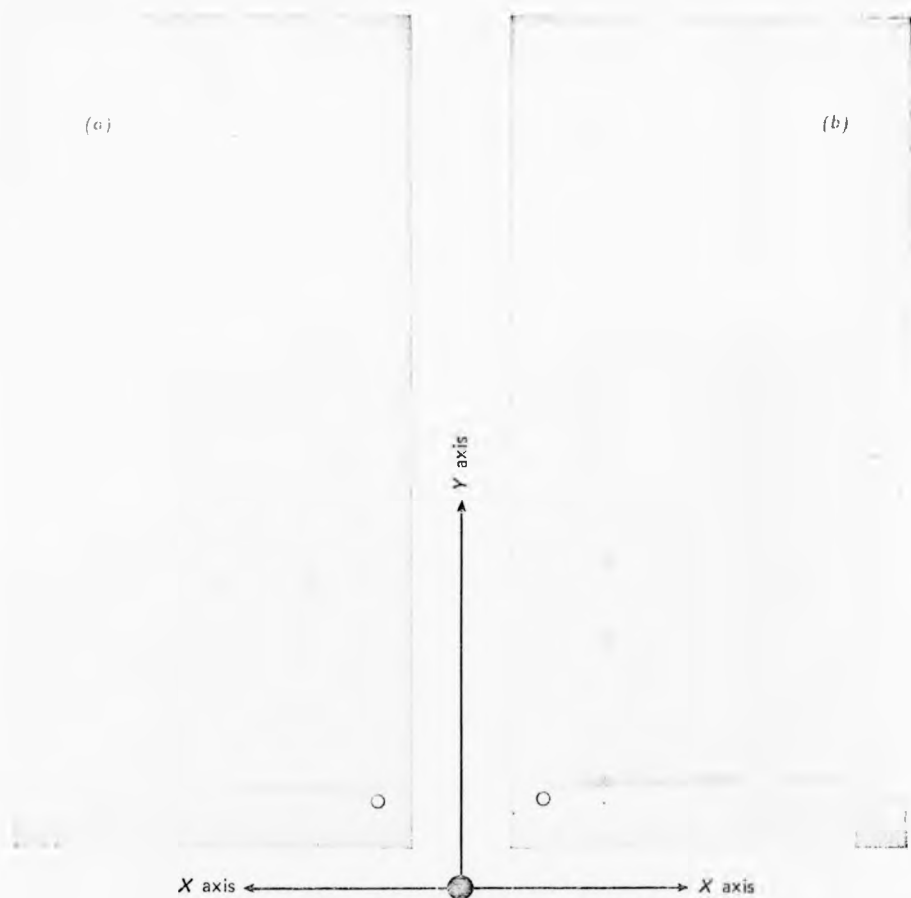
Fig. 6. Co-electrophoresis of etioplast and chloroplast products synthesized *in vitro* in sodium dodecyl sulphate-polyacrylamide gels

Etioplasts and mature chloroplasts were isolated and incubated as described under 'Methods and procedures'. Etioplast products were labelled *in vitro* with L-[^{35}S]methionine (—) and chloroplast products labelled *in vitro* with L-[^3H]leucine (---). The etioplast sample contained 150 μg of protein (3×10^5 c.p.m.) and the chloroplast sample 10 μg of protein (3×10^5 c.p.m.). Light was the energy source for isolated chloroplast protein synthesis. Both solubilized plastid preparations were extracted three times with acetone (90% v/v) by resuspension and centrifugation before electrophoresis. Electrophoresis and related procedures were performed as described under 'Methods and procedures'. LS, Large subunit of Fraction I protein.

intensely staining of which electrophoreses to a position coincident with the large subunit of pea 1 fraction I protein. Fraction I protein has been found in the etioplasts of many species (Graham *et al.*, 1971; Smith *et al.*, 1970). Fig. 5 shows that the products of protein synthesis in isolated etioplasts can be fractionated into six discrete radioactive peaks in sodium dodecyl sulphate gels. All the radioactive peaks are digested by Pronase (Fig. 5c). The same six polypeptides are labelled when either L-[^{35}S]methionine or L-[^3H]leucine is used as the precursor (Figs. 5a and 5b). A double-labelling experiment showed that the mobilities of the six polypeptides synthesized in isolated etioplasts are identical with the mobilities of the polypeptides synthesized in isolated chloroplasts (Fig. 6). To obtain a double-labelled gel pattern in which the six etioplast and chloroplast products *in vitro* electrophoresed with identical, rather than similar, mobilities, it was necessary to extract the samples with acetone before electrophoresis. This suggests that acetone-soluble components of the solubilized plastid preparation slightly affect the mobility of polypeptides in our electrophoresis system.

These results suggest that not only the characteristics and requirements, but also the products of protein synthesis *in vitro* in both pea etioplasts and

chloroplasts are qualitatively the same. One of the peaks of activity common to the fractionation pattern of both the etioplast and chloroplast products synthesized *in vitro* is more highly labelled than the others, and runs with an apparent molecular weight of about 6×10^5 . The identity of this polypeptide has been established in the light-driven chloroplast system as the large subunit of Fraction I protein (Blair & Ellis, 1973). This polypeptide is the only product present *in vitro* in the 150 000g supernatant fraction of chloroplasts, and Fig. 7 shows that this polypeptide is also the only product of etioplasts *in vitro* that is present in this fraction. By inference from the chloroplast situation, these results suggest that the 6×10^5 -mol. wt. polypeptide synthesized in isolated etioplasts is the large subunit of Fraction I protein; this suggestion has been confirmed by comparing the L-[^{35}S]methionine-labelled tryptic peptides of the product labelled *in vitro* with those of the large subunit from Fraction I protein labelled *in vivo* with L-[^{35}S]methionine. Plate 1(a) shows that the etioplast product *in vitro* shares seven major L-[^{35}S]methionine-labelled tryptic peptides with the large subunit of Fraction I protein labelled with L-[^{35}S]methionine *in vivo* (Plate 1b). Some minor peptides may also be common to both proteins. This number of methionine tryptic peptides for the large subunit of Fraction I



EXPLANATION OF PLATE I

Radioautograms of tryptic peptide maps of soluble product labelled *in vitro* and large subunit of Fraction I protein labelled *in vivo*

Experimental details are given in the 'Methods and procedure' section. Electrophoresis (Y axis) was performed for 2.5 h at 250 V. Chromatography (X axis) was performed for 30 min. The origin is labelled O. Both samples contained 7.5×10^4 c.p.m. (a) Product labelled *in vitro*; (b) large subunit of Fraction I protein labelled *in vivo*.

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protein agrees with the amino acid analyses reported for bean (*Phaseolus*) and spinach beet (*Beta*) by Gray & Kekwick (1974).

The remaining question to be studied is whether additional proteins are synthesized by plastid ribosomes during the conversion of etioplasts into chloroplasts in greening tissue. Machold & Aurich (1972) have suggested from inhibitor experiments that the protein of the photosystem I-chlorophyll complex, which appears during greening, is synthesized by plastid ribosomes, but other interpretations of such inhibitor data are not ruled out (Ellis, 1975).

Analysis of the products synthesized in vitro in developing chloroplasts by sodium dodecyl sulphate-polyacrylamide-gel electrophoresis

The analysis of the products synthesized in plastids isolated from greening tissue is shown in Fig. 8. Samples were taken from tissue exposed to continuous light for up to 96 h. Electron-microscopic examination of the tissue shows that fully differentiated chloroplasts are formed within this period. By 24 h light, as well as added ATP, can be used as an energy source for protein synthesis. The data are representative of many experiments performed at frequent intervals during the greening process.

The absorbance profile of the stained protein bands reflects the qualitative and quantitative changes in the proteins of the etioplast and developing chloroplast. As only three of the protein bands detected on these gels can be identified, the interpretation of these data must remain limited. There is an increase in the absorbance of the stained protein bands during greening (Fig. 8). Since equal volumes of incubation mixture were placed on each gel, this indicates that there is an increase in the total plastid protein during greening. Direct measurement of protein in plastid pellets from greening pea shoots confirms this indication (Ellis, 1975). The most noticeable change in the absorbance profile is the appearance during greening of the protein component associated with the photosystem II complex (Thornber *et al.*, 1967; Thornber & Highkin, 1974). This polypeptide electrophoreses with an apparent molecular weight of 2.7×10^4 – 2.8×10^4 (Eaglesham & Ellis, 1974). The appearance of the photosystem I complex is not seen because these preparations have been boiled in sodium dodecyl sulphate; this treatment reduces the photosystem I complex to several protein components which electrophorese with a higher mobility than the complex itself (Anderson & Boardman, 1974).

Several conclusions can be drawn from the results shown in Fig. 8. First, light cannot be used as the source of energy for protein synthesis in isolated etioplasts; this confirms the picture suggested by Table 1. Under our conditions light-driven protein synthesis can first be detected in plastids isolated 3 h

after the onset of illumination. Secondly, in the first 48 h of greening there appears to be an increase of about threefold in the protein-synthesizing capacities of the isolated plastids. Drumm & Margulies (1970) reported a similar increase for plastids from *Phaseolus vulgaris* L., and have concluded that this represents a real increase in the protein-synthesizing ability rather than alterations in the amino acid pool sizes or concentrations of inhibitors. Thirdly, it is clear that whereas light-driven protein synthesis occurs in plastids isolated after 96 h of greening (Fig. 8h), added ATP will no longer stimulate protein synthesis in these plastids (Fig. 8d). This observation has not been further investigated, but may represent an alteration in the permeability of the plastid envelope to ATP. Fourthly, the fractionation of the polypeptides synthesized in plastids isolated after 96 h of illumination shows a radical change in the major product. Whereas the major product in isolated etioplasts is the large subunit of Fraction I protein, almost the only polypeptide synthesized in plastids isolated after 96 h of greening is an unidentified membrane-bound protein with a molecular weight of about 3.2×10^4 (Fig. 8h). Whether this alteration in the major product synthesized *in vitro* of plastid ribosomes reflects a similar alteration *in vivo* is not known. However, there are clearly controls exerted on the production of proteins in isolated plastids, and the mechanisms of these controls deserve further study.

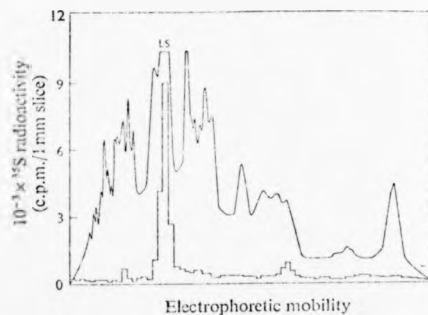


Fig. 7. Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis of labelled 150000g etioplast supernatant fraction

Etioplasts were isolated and incubated as described under 'Methods and procedure'. The products labelled *in vitro* were fractionated by centrifugation at 150000g, the supernatant fraction was electrophoresed and the radioactivity determined as described under 'Methods and procedure'. The gel was loaded with 100 μ l of dialysed sample (220 μ g of protein). —, E_{266} ; —, 15 S (histogram), radioactivity; 15S, large subunit of Fraction I protein.

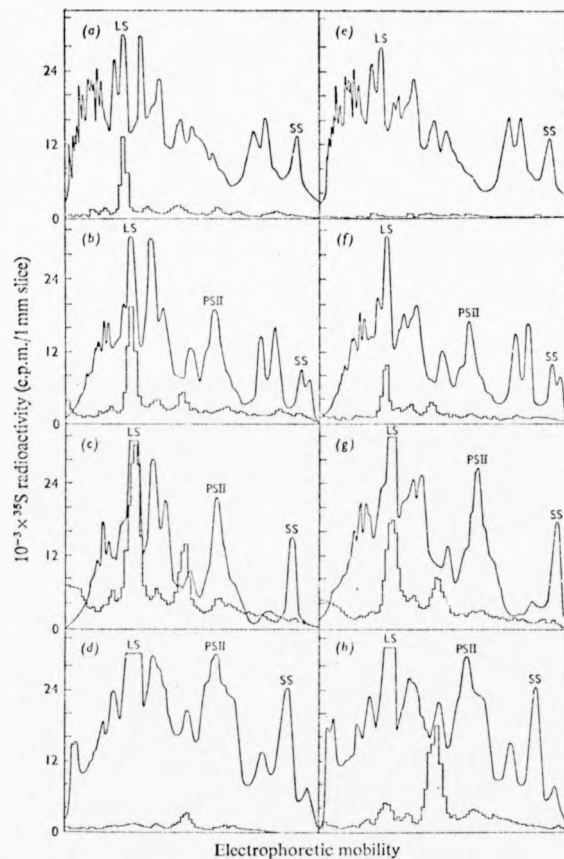


Fig. 8. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of the polypeptides synthesized *in vitro* in plastids isolated during greening

Plastids were isolated from 40 g of tissue and incubated with 1- 35 S]methionine (300 Ci/nmol) as described under 'Methods and procedure'. Either added ATP [gels (a), (b), (c) and (d)] or light [gels (e), (f), (g) and (h)] was used as the energy source. Gels (a) and (e) are solubilized incubation mixtures containing plastids isolated after 0 h greening; gels (b) and (f), 24 h greening; gels (c) and (g), 48 h greening; and gels (d) and (h), 96 h greening. Gels were loaded with 100 μ l of solubilized incubation mixtures; (a), (b), (e) and (f), 130 μ g of protein; (c) and (g), 200 μ g of protein; (d) and (h), 400 μ g of protein. Electrophoresis and related procedures were performed as described under 'Methods and procedure'. —, E_{620} ; ▭ (histogram), radioactivity; PSII, photosystem II chlorophyll protein complex; LS, large subunit of Fraction I protein; SS, small subunit of Fraction I protein.

The results presented in this paper lead to the conclusion that, irrespective of the energy source used, plastids isolated during greening synthesize the same

polypeptides that are synthesized in both isolated etioplasts and chloroplasts. This conclusion provides support for the idea that only a very limited spectrum

of proteins is synthesized on plastid ribosomes. The abundance of plastid ribosomes therefore seems necessary to produce a few proteins in large quantities rather than many proteins in smaller amounts. This conclusion has been important in formulating the experiments which have led to the identification of the major polypeptide synthesized in both isolated etioplasts and isolated chloroplasts (Blair & Ellis, 1973) and the isolation of the mRNA coding for this polypeptide (Hartley *et al.*, 1975). It may also suggest that the plastid offers a developmental system for studying the controls exerted on the synthesis of particular polypeptides during differentiation.

One area of plastid development remains uninvestigated at the biochemical level. The transition of the proplastid to the chloroplast is the most commonly encountered developmental sequence of the plastid *in vivo*, and until the range of proteins synthesized on the ribosomes of plastids undergoing this differentiation is known, our knowledge of the function of plastid ribosomes cannot be regarded as complete. It is possible that the unidentified genes in chloroplast DNA encode proteins that are synthesized during the development of chloroplasts from proplastids; if this is the case, studies of isolated developing proplastids may reveal that they synthesize a different spectrum of proteins from either chloroplasts or etioplasts.

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