PROTEIN SYNTHESIS IN CHLOROPLASTS

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by

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TO MY MOTHER AND FATHER

SUMMARY

The function of chloroplast ribosomes was investigated by analysing the products of *in vitro* protein synthesis by isolated pea chloroplasts. Since previous attempts at identifying newly-synthesised proteins in isolated chloroplasts had either been unsuccessful or inconclusive, careful attention was paid both to the choice of chloroplast preparation and to the analytical techniques used to identify labelled proteins.

A rapid method of chloroplast isolation was used which gave a chloroplast preparation which contained about 50% intact chloroplasts and showed high rates of amino acid incorporation into protein. It was felt that the proteins synthesised by these chloroplasts would accurately reflect the nature and pattern of protein synthesis which occurs in chloroplasts *in vivo*. High rates of incorporation would also aid identification of newly-synthesised proteins. Amino acid incorporation was shown to be sensitive to selective inhibitors of 70S-type ribosomes, but was not affected by inhibitors of 80S-type ribosomes. In addition, incorporation was shown to by insensitive to ribonuclease, suggesting that protein synthesis was taking place in intact chloroplasts.

When the labelled chloroplasts were fractionated by differential centrifugation, approximately 25% of this incorporation was present in a 150 000 x g chloroplast supernatant fraction. Further analysis was confined to this supernatant fraction since only released, and therefore completed, polypeptides should be present in this fraction, thus aiding identification. The 150 000 x g supernatant fraction was analysed on polyacrylamide gels in the presence and absence of a denaturant, sodium dodecyl sulphate, and by gel chromatography on Sephadex G100 in a sodium dodecyl sulphate-containing buffer. Only one polypeptide was found to be labelled by all these procedures. This polypeptide was identified as the large subunit of Fraction 1 protein, a major protein constituent of the chloroplast.

Identity of the *in vitro* product present in the soluble phase of the chloroplast with the large subunit of Fraction I protein was established by comparing a two-dimensional tryptic peptide map of its [S^{35}] methionine-labelled peptides with a tryptic peptide map of the large subunit of Fraction I protein labelled *in vivo* with [S^{35}] methionine. It may therefore be concluded that only one of the many proteins present in the soluble phase of the chloroplast, namely the large subunit of Fraction I protein, is synthesised on chloroplast ribosomes.

PREFACE.

The work described in this thesis was performed between 1970 and 1973 and has been published in part (see Appendix). The work is entirely my own, with the exceptions acknowledged below.
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Finally, I allow my uncomplaining typist, Janet Welch, the luxury of self-congratulation on such excellent and rapid work.

ABBREVIATIONS.

ATPase	adenosine triphosphatase.
A_{280}	absorbance at 280nm.
BSA	bovine serum albumin.
CCCP	m-chlorocarbonyl cyanide phenylhydrazone.
Ci	Curie (3.7×10^{10} disintegrations per second).
c.p.m.	counts per minute.
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea.
DEAE	diethylaminoethyl.
EDTA	ethylenediamineteraacetic acid.
E_{280}	extinction at 280nm.
HEPES	N-2-hydroxyethylpiperazine-N^1-2-ethanesulphonic acid.
MES	2(N-morpholino)ethanesulphonic acid.
PAS	4-aminosalicylic acid.
PMSF	phenylmethyl sulphonyl fluoride.
POPOP	1,4-bis-(5-phenyloxazole-2-yl)benzene.
PPO	2.5-diphenyloxazole.
RNAase	ribonuclease.
SDS	sodium dodecyl sulphate.
TCA	trichloroacetic acid.
TEMED	N,N,N,N^1-tetramethylenediamine.
TES	N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid.
TNS	tri-isopropylnaphthalenesulphonic acid.
TPCK	L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone.
Tricine	N-tris(hydroxyethyl)methyl glycine.
Tris	2-amino-2-hydroxymethylpropane-1:3 diol.
Triton	octylphenoxypolyethoxy ethanol.

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SECTION I - LITERATURE REVIEW

1. INTRODUCTION.

It is now established that chloroplasts and mitochondria contain all the components of an autonomous, self-replicating system (Kirk and Tilney-Bassett, 1967). They contain their own DNA, a DNA polymerase to replicate the DNA, a DNA-dependent RNA polymerase to transcribe the DNA, and a complete protein-synthesising apparatus to translate messenger RNAs into proteins. In addition, the structure and function of these components bears a strong resemblance to those found in prokaryotes, generating speculation as to the possible common origin of plastids, mitochondria and prokaryotic organisms (Sagan, 1967; Raven, 1970). However, many lines of research, reviewed in this Section, show that chloroplasts are not autonomous in any real, functional sense, if by autonomy is meant complete control over the replication of the chloroplast and the biosynthesis of its components.

If both nucleus and cytoplasm exert a considerable measure of control over the synthesis and replication of chloroplasts, why is so much energy spent in the synthesis of an elaborate system of replication, transcription and translation within the chloroplast? No definitive answer can be given to this question since the precise functions of chloroplast DNA and chloroplast ribosomes are unknown. This problem has been approached in this thesis by directly studying the products of translation in isolated chloroplasts. Information can be obtained about the function of chloroplast ribosomes by studying <u>in vitro</u> chloroplast protein synthesis, free from direct control by nucleus or cytoplasm. Co-operation between nuclear and chloroplast genomes, and cytoplasmic and chloroplast ribosomes in the synthesis of chloroplast proteins can also be demonstrated.

The subject of chloroplast biogenesis has been well-reviewed recently (Kirk, 1970, 1972; Miller, 1970; Smillie and Scott, 1970; Levine and Goodenough, 1970; Boardman, Linnane and Smillie, 1971; Boulter, Ellis and Yarwood, 1972; Pollak and Lee, 1972). In this review a broad outline of the structure and function of chloroplast protein and nucleic acid synthesising systems will be given, with the emphasis on the major problems that remain to be solved.

2. CHLOROPLAST STRUCTURE AND REPLICATION.

A. Structure.

Chloroplasts/

Chloroplasts of higher plants appear lens-shaped with diameters around 4 to 6μm. Each chloroplast is surrounded by an outer double membrane, sometimes referred to as the chloroplast envelope, which is known to contain translocases for orthophosphate, certain sugar phosphates (such as 3-phosphoglycerate) and dicarboxylic acids (Walker and Crofts, 1970). The outer membranes of Vicia faba chloroplasts have been purified free from chlorophyll (Mackender and Leech, 1970) and have been observed by phase contrast and electron microscopy, although no marker enzymes, which might form the basis of a biochemical assay, were identified. No detailed analysis of the protein or lipid components of the purified outer membranes has yet been performed. When chloroplasts are rapidly isolated in isotonic medium a considerable proportion of the organelles retain their outer envelope and are classified as intact chloroplasts (Hall, 1972). Such chloroplasts appear bright and refractile when viewed under phase microscopy (Kahn and von Wettstein, 1961).

Electron micrographs of sections of chloroplasts show the interior to be composed of a rather electron-dense, granular phase usually referred to as the stroma, in which is embedded a complex array of membranes; this membrane system is usually referred to as the lamellae. The stroma contains DNA (Ris and Plaut, 1962), ribosomes, transfer RNA and amino acid activating enzymes (Francki, Boardman and Wildman, 1965) and intermediary metabolites such as sugars, amino acids, etc. The stroma also contains soluble enzymes, including those of the Calvin cycle (Trebst, Tsujimoto and Arnon, 1958); one of these enzymes can account for up to 50% of all soluble leaf protein (Kawashima and Wildman, 1970). This enzyme is ribulose diphosphate carboxylase [3-phospho-D-glycerate carboxylyase (dimerising), EC 4.1.1.39], frequently referred to as Fraction I protein on account of its high concentration in leaf extracts and its high sedimentation coefficient of 18S - other soluble leaf proteins (Fraction II) sediment at around 4S (Wildman and Bonner, 1947).

Fraction I protein is widely distributed, not only in the chloroplasts of green plants, but also in the blue-green algae and in photosynthetic and chemolithotrophic bacteria (Kawashima and Wildman, 1970). In other words, Fraction I protein is found in all organisms which fix carbon dioxide by the Calvin cycle. The reaction which it catalyses is:-

$$CO_2 + \text{D-ribulose-1,5-diphosphate} + H_2O \xrightarrow{Mg^{2+}} 2\ \text{3-phospho-D-glycerate.}$$

Recently/

Recently an addition oxygenase activity has been discovered in Fraction I protein preparations from soybean (Bowes, Ogren and Hageman, 1971), spinach (Andrews, Lorimer and Tolbert, 1973; Lorimer, Andrews and Tolbert, 1973) and an obligate anaerobe, Chromatium (Takabe and Akazawa, 1973b). This may mean that Fraction I protein functions in both photosynthesis and photorespiration.

The molecular weight of the native enzyme of higher plant chloroplasts is about 5.25×10^5 daltons (Ellis, 1973), and consists of two non-identical subunits of molecular weights 5.58×10^4 and 1.20×10^4 daltons respectively (Rutner and Lane, 1967; Rutner, 1970). Dissociation of the native enzyme into large and small subunits can be brought about by alkali, acetic acid, urea or sodium dodecyl sulphate (Kawashima and Wildman, 1970). The large subunits from a number of higher plant Fraction I proteins appear closely related, as judged by molecular weight, amino acid composition, tryptic peptide maps and immunological cross-reactivity (Kawashima and Wildman, 1970, 1971b). The large subunit appears to contain the active site both for the carboxylase activity (Gray and Kekwick, 1973; Takabe and Akazawa, 1973a; Sugiyama et al, 1970) and for the oxygenase activity (Takabe and Akazawa, 1973b). The small subunit, however, shows no homology with the large subunit of the same enzyme (Kawashima and Wildman, 1971c) or with the small subunit isolated from other higher plant Fraction I proteins (Kawashima and Wildman, 1970, 1971b; Gray and Kekwick, 1973).

The evolution of the structure and function of Fraction I protein presents a fascinating problem for the protein chemist interested in evolution. However, although Fraction I protein has been recognised for 26 years, no amino acid sequences of either of the subunit proteins are available. Even the N-terminal amino acids are unknown, although the partial C-terminal sequences of Chlorella ellipsoidea large and small subunit are known (Sugiyama, Ito and Akazawa, 1971). It would appear essential to gather much more basic, structural information on this major plant protein.

Chloroplast lamellae are composed of closely pressed sacs, termed thylakoids, which are arranged in a closely stacked fashion. These thylakoid stacks are called grana, and the number of thylakoids per granum appears to vary widely throughout the plant kingdom from about three in the case of Euglena gracilis to up to 100 in vascular plants, giving chloroplasts from different species a characteristic/

characteristic and striking appearance (Kirk and Tilney-Bassett, 1967). Many physical methods have been applied to the determination of the structure of thylakoid membranes - these have been well reviewed recently by Kirk (1971a) and will not be restated here. The specific protein components of chloroplast lamellae will now be considered.

The lamellae contain the pigments (chlorophylls, carotenoids, and quinones) and the enzymes, cytochromes and other factors associated with photosynthetic phosphorylation and electron transport (Boardman, 1968), arranged in a lipoprotein, unit membrane structure (Kirk, 1971a). Some of these proteins, such as cytochrome f, are tightly bound within the thylakoid membranes, requiring detergent treatment to free them (Nelson and Racker, 1972). Others are less tightly bound; the photosynthetic coupling factor (CF_1) can be dissociated from the lamellae by washing with EDTA. This protein has been purified (Farron, 1970) and has been shown to have a latent, Ca^{2+}-dependent ATPase activity which is expressed upon heat treatment (Farron and Racker, 1970). The molecular weight and subunit composition have been determined (Nelson *et al*, 1973), showing that both the number of subunits of CF_1, and their molecular weights bear a strong resemblance to those of the mitochondrial coupling factor (F_1). It would be interesting to test whether subunits could be exchanged, giving a functional, hybrid enzyme.

The precise molecular architecture of the lamellar membrane has been a matter of controversy for some years. Little information is available on the purification and properties of membrane-bound enzymes, of the sort now available for chloroplast CF_1. Until such information becomes available, little progress will be made either on the structure or biosynthesis of chloroplast membranes. The possible existence of a single structural protein to which all other membrane proteins and lipids bind (Criddle, 1969) has been questioned (Ashwell and Work, 1970; Senior and MacLennan, 1970). The rather extreme conditions of pH used to solubilise the membranes of chloroplasts and mitochondria might well denature membrane-bound enzymes and lead to the heterogeneity observed in preparations of structural protein. It can be argued that the structure of chloroplast membranes might result from protein-protein and protein-lipid interactions without the necessity for a single structural protein.

A more fruitful approach to the study of membrane proteins has been the/

the identification and purification of the protein-chlorophyll complexes associated with the two photosystems of photosynthesis (Kung and Thornber, 1971). Two major protein-chlorophyll complexes have been extracted from the lamellae of Antirrhinum majus (Herrmann and Meister, 1972) and from Beta vulgaris (Thornber et al, 1967a, 1967b). The characteristic chlorophyll a:b ratios of the two B. vulgaris complexes led Thornber et al (1967a) to conclude that they were derived from photosystems I and II. In addition, Gregory, Raps and Bertsch (1971) showed that in a mutant strain of Scenedesmus obliquus which lacked a functional photosystem I, the photosystem I protein-chlorophyll complex was absent when the lamellar proteins were solubilised in sodium dodecyl benzene sulphonate, and analysed by gel electrophoresis. Herrmann and Meister (1972), however, concluded from spectroscopic analysis that some of the minor protein-chlorophyll complexes in A. majus lamellae more closely correspond to photosystem I and II proteins. The en:alba-1 mutant of A. majus, deficient in photosystem I activity, was found to be devoid of the major and a minor protein-chlorophyll complex associated with photosystem I. These studies show that distinct proteins are associated with each photosystem in a complex with chlorophyll, although their functional role in electron transport or phosphorylation remains unknown.

Also associated with chloroplast lamellae are DNA (Woodcock and Fernandez-Moran, 1968), ribosomes (Chen and Wildman, 1970; Chua et al, 1973), DNA polymerase (Tewari and Wildman, 1967) and RNA polymerase activity (Spencer and Whitfeld, 1967; Tewari and Wildman, 1969). The partial purification of chloroplast DNA-dependent RNA polymerase has been reported from wheat leaves (Polya and Jagendorf, 1971a, 1971b) maize leaves (Bottomley, Smith and Bogorad, 1971) and from pea leaves (Bennett and Ellis, 1973). A similar purification of DNA polymerase has not been reported, although Spencer and Whitfeld (1969) obtained soluble DNA polymerase activity from spinach chloroplasts, after treatment with the detergent Triton X-100 and ammonium sulphate fractionation.

B. Division in vivo.

The process of chloroplast division is poorly understood. Although reports of division of chloroplasts by fission do exist, many experienced/

experienced workers have not observed this event in the light microscope (Honda et al, 1971). The difficulties in approaching this problem directly are numerous. For example, it is difficult to observe a single chloroplast in a living cell for long periods of time due to the streaming of the cytoplasm (Wildman, Hongladarom and Honda, 1962). Only a fraction of the chloroplasts of a palisade cell can be observed at one time since only one of the six faces of the cell can be viewed. More indirect methods of study have therefore been applied.

Honda et al (1971) adopted the approach of measuring the area of chloroplasts seen in sections of the leaves of a wide variety of plants. They found that the size range of chloroplasts within one species was extremely broad. The size distribution was skewed, leading the authors to conclude that the increase in chloroplast numbers per cell is due to division at a slow rate of small, equal-sized chloroplasts. Such chloroplasts are mature, i.e. have fully developed thylakoid systems, and may be identical to the constricted chloroplasts which are frequently observed in the living cell. It was also inferred that some control on the total number of chloroplasts in a cell must exist, since the fraction of a cell face occupied with chloroplasts was constant and independent of cell size and age, although the exact fraction varied between different species.

Cran and Possingham (1972) observed spinach chloroplasts presumed to be in the act of division, by examining serial sections in the electron microscope. In addition to the process of division by constriction, they also observed chloroplasts in which a central baffle segregated the organelle into two zones. It is impossible to be sure whether such plastids would have divided if left unfixed, and also whether the material in which the chloroplasts were situated (cultured leaf discs) really represents conditions in the whole leaf.

C. Division in vitro.

One of the logical consequences of the theory that organelles may be autonomous, is that the organelle should be able to survive and replicate when removed from the rest of the cell. Accordingly, several studies of this type have been performed for chloroplasts (Ridley and Leech, 1970; Giles and Serafis, 1972; Rebeiz et al, 1973), etioplasts (Wellburn and Wellburn, 1973) and plant mitochondria (Romani and Ozelkok/

Ozelkok, 1973). Although survival of organelles can be demonstrated, evidence of development or differentiation has been confined to inevitably subjective electron micrographs. Ridley and Leech (1970) showed the presence of constricted chloroplasts *in vitro*, and suggested that division might therefore occur *in vitro*. Whether these chloroplasts were actually dividing or were responding in that fashion to the growth conditions is unknown. Certainly, in this, and in all other *in vitro* studies, no good evidence of new protein or nucleic acid synthesis has been presented. In spite of the use of sophisticated growth media and aseptic techniques, no good evidence of chloroplast replication *in vitro* has come to light. In view of the large amount of genetic evidence which suggests that many genes controlling chloroplast functions are nuclear in location (Section I4Ci), it is not surprising that attempts to culture isolated chloroplasts *in vitro* have been unsuccessful.

3. THE CHLOROPLAST PROTEIN-SYNTHESISING SYSTEM.

Chloroplasts contain their own protein-synthesising system, which differs markedly from that of the cytoplasm, but bears a strong resemblance to prokaryotic systems (Ellis, 1970). The characteristics of protein synthesis by isolated chloroplasts have been described for numerous higher plant and algal species (Boulter *et al*, 1972). A problem which is frequently encountered in studies of *in vitro* chloroplast protein synthesis is the contamination of chloroplast preparations by bacteria. Indeed much early work on chloroplast protein synthesis has been invalidated due to subsequent demonstration of heavy bacterial contamination of chloroplast preparations (Gnanam, Jagendorf and Ranalletti, 1969). More recent work has confirmed the essential similarity between chloroplast and bacterial protein synthesis (Boulter *et al*, 1972), providing a basis for the possible common origin of prokaryotes and cellular organelles. 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. However, differences between the two systems do exist, indicating that while both may have had a common ancestor some divergence has almost certainly taken place...

A/

A. Ribosomes.

Ribosomes may be divided into two major categories according to their ultracentrifugal properties : 70S ribosomes, present in bacteria, blue-green algae and chloroplasts, and 80S ribosomes present in the cytoplasm of plant and animal cells. The existence of two classes of ribosomes in green plants was first shown by Lyttleton (1962). Chloroplast ribosomes from several species of higher plants and algae have been isolated, and all have been found to fall into the 70S class, although minor variations from 70S have been noted (Boulter et al, 1972). No massive reductions of the size of chloroplast ribosomes have been found of the sort known to exist in animals, where mitochondrial ribosomes sediment at 55S (Borst and Grivell, 1971; Hernandez, Burdett and Work, 1971).

Protein synthesis on isolated chloroplast ribosomes is inhibited by chloramphenicol, specifically by the D-*threo* isomer of chloramphenicol, which also inhibits protein synthesis by bacterial ribosomes (Ellis, 1969). Chloroplast and bacterial ribosome function is also inhibited by the antibiotics spectinomycin, lincomycin and erythromycin (Ellis, 1970). Sensitivity to these chemically unrelated antibiotics argues for a similarity between chloroplast and prokaryote ribosomes. Cycloheximide, on the other hand, inhibits protein synthesis on 80S ribosomes of green (Ellis, 1969) and non-green tissue (Ellis and MacDonald, 1967), but has no effect on protein synthesis by chloroplast or bacterial ribosomes.

i. Ribosomal proteins.

The proteins of chloroplast ribosomes are very different from those of bacterial ribosomes, when analysed by gel electrophoresis (Hoober and Blobel, 1969; Odintsova and Yurina, 1969; Vasconcelos and Bogorad, 1971) and by immunological cross-reactivity tests (Wittman, 1970). In addition, the protein composition of chloroplast ribosomes varies widely, even between closely related species, much more so than the proteins of cytoplasmic ribosomes (Lyttleton, 1968; Gualerzi and Cammarano, 1970). The proteins of chloroplast and cytoplasmic ribosomes from the same plant species show characteristic differences when analysed by gel electrophoresis (Vasconcelos and Bogorad, 1971; Gualerzi and Cammarano, 1969; Jones *et al*, 1972). It would appear that the protein composition of chloroplast ribosomes has undergone considerable change, perhaps at a faster rate than the proteins of cytoplasmic ribosomes. The result is that chloroplast ribosomes now show little similarity to either cytoplasmic or bacterial ribosomes/

ribosomes at least in regard to their protein components. It must also be added that great variation also exists in the ribosomal proteins both between and within families of bacteria (Wittman, 1970).

ii. Ribosomal RNAs.

The molecular weights and base compositions of the chloroplast ribosomal RNAs have been determined for a number of plant species (Ellis and Hartley, 1973). The small subunit of chloroplast ribosomes contains one RNA species of molecular weight 0.56×10^6 daltons in most higher plants, often referred to as 16S RNA (Ingle et al, 1970). This RNA species has the same molecular weight as the RNA from the small subunit of E. coli ribosomes, but is smaller than the corresponding RNA from cytoplasmic ribosomes. The single RNA species of the 40S subunit of cytoplasmic ribosomes has a molecular weight of 0.70×10^6 daltons.

The larger ribosomal subunit of chloroplast ribosomes contains two RNA species of similar molecular weight to those of the E. coli 50S subunit. They are referred to as 23S RNA and 5S RNA. The 60S subunit of cytoplasmic ribosomes contains 25S and 5S RNAs. The 5S RNA of broad bean chloroplast ribosomes shows different properties to the cytoplasmic 5S RNA when analysed by chromatography on methylated-albumin kieselguhr columns (Payne and Dyer, 1971). The partial nucleotide sequences of both 5S RNAs also show considerable differences; no conclusions can yet be made as to the possible homology of chloroplast 5S RNA with bacterial 5S RNA (P.I. Payne, personal communication). The 23S RNA has been found to be unstable, giving rise to breakdown products which are characteristic for each species (Leaver and Ingle, 1971). However, the 23S RNA can be stabilised by divalent cations or by extracting and fractionating at 4°C (Leaver, 1973). Newly-synthesised 23S RNA appears to be more stable (Ingle et al, 1970); this indicates that the 23S RNA is synthesised as a complete, continuous sequence, but is 'nicked' later in its life-time, probably when it is incorporated into the ribosome. No information is available on the possible homology of chloroplast and bacterial 23S and 16S RNA at the level either of partial sequences or oligonucleotide fingerprints.

iii. Organisation of ribosomes.

Up to 50% of the ribosomes in a chloroplast can be tightly bound

to/

to the thylakoid membranes (Chen and Wildman, 1970). The nature of the binding of the ribosome to the membrane is unknown, although the ribosomes can be released by detergent treatment in a similar manner to the membrane-bound ribosomes of the endoplasmic reticulum (Campbell and Sargent, 1967). Polyribosomes have been isolated both from the total chloroplast ribosomes of Euglena gracilis by deoxycholate treatment (Avadhani and Buetow, 1971) and from the 'free' ribosomes of the stroma of tobacco chloroplasts by osmotic lysis (Chen and Wildman, 1967). Falk (1969) has observed polysomes attached to the thylakoid membranes of Phaseolus vulgaris chloroplasts by electron microscopy. Both membrane-bound chloroplast polysomes and polysomes attached to the endoplasmic reticulum showed similar conformations, of whorls, spirals and rosettes. Chloroplast polysomes are highly active in protein synthesis, both as isolated polysomes (Avadhani and Buetow, 1971) and inside isolated chloroplasts, at least during the initial stages of incubation (Harris and Eisenstadt, 1971).

The function of membrane-bound chloroplast ribosomes is unknown. It is possible to speculate that a division of protein synthetic activities takes place in the chloroplast, with membrane-bound ribosomes synthesising membrane proteins while soluble proteins are synthesised on free ribosomes. Chua et al (1973) find that in synchronous cells of Chlamydomonas reinhardi about to enter the light phase, there is a net movement of ribosomes from the stroma to the thylakoid membranes. It is also known that at this time, chloroplast membrane proteins are being synthesised (Hoober, 1972). The connection between the two events remains to be demonstrated.

B. Chloroplast tRNA and aminoacyl-tRNA synthetases.

The existence of plastid-specific tRNAs and aminoacyl-tRNA synthetases has been demonstrated in chloroplasts from tobacco (Guderian, Pulliam and Gordon, 1972), bean (Burkard, Guillemaut and Weil, 1970), cotton (Merrick and Dure, 1972) and Euglena gracilis (Reger et al, 1970; Kislev et al, 1972) and in bean etioplasts (Burkard, Vaultier and Weil, 1972). In tobacco leaves, six isoaccepting leucine-specific tRNAs were found (Guderian et al, 1972). Two were found exclusively in the chloroplasts and could only be charged by the homologous chloroplast aminoacyl-tRNA synthetase preparation/

preparation. No overlapping of the distribution of leucine tRNAs within the tobacco leaf cell appears to exist; two of the remaining four leucine tRNAs were found exclusively in the mitochondrial fraction and the last two appeared to be cytoplasmic species. In bean leaves, a more complex situation was encountered (Burkard et al, 1970). Bean chloroplasts appear to contain five leucine tRNAs, two of which are similar to the two cytoplasmic leucine tRNAs. These two tRNAs can be charged by both cytoplasmic and chloroplast synthetases - however the remaining three chloroplast-specific leucine tRNAs can be charged only by chloroplast synthetases.

The functional significance of these findings is difficult to assess. Fundamental differences appear to exist with respect to the distribution of leucine tRNA, and the specificity of the leucyl-tRNA synthetases in two species of higher plants. It is possible that translation on chloroplast ribosomes might be controlled either by the level of plastid-specific versus shared tRNAs or by the activity of the synthetase complex. Such points may be resolved by in vitro studies on reconstituted chloroplast protein-synthesising systems using purified components.

The synthesis of plastid-specific tRNAs and aminoacyl-tRNA synthetases appears to be stimulated by light. The level of plastid-specific leucyl-tRNA and valyl-tRNA was found to be higher in chloroplasts than in etioplasts of Phaseolus vulgaris (Burkard et al, 1972). In Euglena gracilis, chloroplast-specific isoleucyl- and phenylalanyl-tRNA synthetases which are induced by light have been identified (Reger et al, 1970). The chloroplast phenylalanyl-tRNA synthetase is also found in a bleached mutant which lacks both chloroplasts, and chloroplast DNA. This suggests that the enzyme is coded on nuclear DNA and synthesised on cytoplasmic ribosomes. However, Parthier et al 1972) found that the light-induced synthesis of phenylalanyl-tRNA synthetase (and several other chloroplast synthetases) could be inhibited by both naladixic acid and chloramphenicol, indicating that the enzyme is coded on chloroplast DNA and synthesised on chloroplast ribosomes. The results obtained by Reger et al (1970) using the bleached mutant are perhaps more trustworthy, casting doubt on the results obtained using inhibitors of DNA and protein synthesis.

C./

C. The mechanism of protein synthesis.

Chloroplast ribosomes (Grivell and Groot, 1972) and chloroplast polysomes (Section I3Aiii) active in protein synthesis have been isolated. However, only poly(U)-directed and endogenous translation has been studied. No chloroplast messenger RNA, or plant cytoplasmic or mitochondrial messenger RNA has yet been isolated. Until a natural messenger can be identified and isolated, advances in the understanding of the protein-synthesising system of the chloroplast will be limited. One of the main aims of any study of *in vitro* protein synthesis in isolated chloroplasts must be the identification of specific products of translation. This then points the way to the isolation of a specific chloroplast messenger RNA.

Chloroplast ribosomes can accept exogenous messenger RNA and translate it with a certain degree of fidelity. Schwartz *et al* (1965) reported that chloroplast ribosomes from *Euglena gracilis* would use f2 RNA as template to synthesise the coat protein of f2 phage. The N-terminal amino acid of the *in vitro* product was shown to be N-formyl methionine, showing similarity to the process of initiation of protein synthesis on *E. coli* ribosomes (Schwartz *et al*, 1967). The existence of a methionyl-tRNA which can be formylated by either endogenous or *E. coli* transformylase has been demonstrated in chloroplasts of wheat (Leis and Keller, 1970, 1971), cotton (Merrick and Dure, 1971) and bean (Burkard, Eclancher and Weil, 1969; Guillemaut, Burkard and Weil, 1972). Two additional methionyl-tRNAs are present in bean chloroplasts, both of which cannot be formylated. Their function is presumably to direct methionine into internal positions of the polypeptide chain (Guillemaut *et al*, 1973). Two non-formylated methionyl-tRNAs are found in bean cytoplasm, in common with those found in the cytoplasm of animal cells (Smith and Marcker, 1970). In mitochondria from yeast and rat liver (Smith and Marcker, 1968) and bean (Guillemaut *et al*, 1973), two methionyl-tRNAs exist, one of which can be formylated.

It would seem therefore that initiation of protein synthesis is similar in bacteria, chloroplasts and mitochondria in using a formylated methionyl-tRNA. However, little additional information is available on the molecular requirements for chloroplast protein synthesis. No initiation, elongation or termination factors of chloroplast ribosomes have been identified. A crude preparation of *E. coli* initiation factors/

factors stimulates the AUG-directed binding of fMet-tRNA, and the synthesis of fMet-puromycin by washed ribosomes isolated from Euglena gracilis chloroplasts, Neurospora crassa mitochondria, and a blue-green alga Nostoc (Sala, Sensi and Parisi, 1970; Sala, Kuntzel, Parisi and Ciferri, 1970). This possible interchangeability of factors demonstrates another similarity between organelle and prokaryotic ribosomes.

4. CHLOROPLAST DNA.

A. Isolation and properties.

Chloroplast DNA was first isolated in 1963 (Kirk, 1963; Chun, Vaughan and Rich, 1963) although previously microscopy and autoradiography had strongly suggested that chloroplasts contained their own DNA (Kirk and Tilney-Bassett, 1967). Most of the information gained since that time has come from physical and chemical studies of isolated DNA. A major controversy, resolved only recently (Kirk, 1971b), has centred on the true identity of isolated chloroplast DNA. The controversy began with the initial isolation of chloroplast DNA. Kirk (1963) showed by chemical analysis that the chloroplast DNA of broad bean had a base composition (as judged by content of G + C) of 37.4%. This was close to but significantly less than the GC content of nuclear DNA (39.4%). Chun et al (1963) came to opposite conclusions. They found that chloroplast DNA isolated from both spinach and beet consisted of two species, both of which had much higher GC contents (46% and 60%) than nuclear DNA (36%). There followed many reports of chloroplast DNA which consisted of multiple species with higher buoyant densities and higher GC contents than the respective nuclear DNAs (Kirk, 1971b). However, more recent work has supported the original work of Kirk. Whitfeld and Spencer (1968) found that DNA isolated from the chloroplast fraction of spinach leaves gave a single component, when centrifuged in a CsCl gradient, of buoyant density 1.696 g cm^{-3}; in tobacco chloroplasts the single DNA species had a density of 1.697 g cm^{-3}. The corresponding nuclear DNAs had densities of 1.694 and 1.697 g cm^{-3} respectively. Wells and Birnstiel (1969), working with spinach, lettuce, sweet pea and broad bean came to similar conclusions. The consensus at the moment appears to be that the chloroplast DNA of higher plants has a buoyant density in CsCl of 1.697 ± 0.001 g cm^{-3} and a base composition of about 37.5 ± 1% GC (Kirk/

(Kirk 1971b). Nuclear DNA varies widely in its physical and chemical parameters between different species, having for example a buoyant density higher, lower or the same as the chloroplast DNA from the same species. Much of the early work on chloroplast DNA has been shown to be suspect due to contamination by other subcellular fractions (nuclei and mitochondria) and by bacteria. The use of improved techniques of base analysis (Kirk, 1967), the discovery of other distinctive properties of chloroplast DNA and the careful isolation of subcellular fractions has resolved many of the earlier uncertainties.

Several features distinguish chloroplast DNA from that found in the nucleus. Chloroplast DNA renatures readily after heat or alkali treatment (Bastia et al, 1971); the extent of renaturation of nuclear DNA being slight (Kung and Williams, 1969). DNA from bacteria (Lark, 1968), nuclear DNA from animals (Sneider and Potter, 1969) and plants (Tewari and Wildman, 1970) contains the methylated base 5-methyl cytosine. Chloroplast DNA from both algae and higher plants contains no detectable amounts of this modified base (Kirk and Tilney-Bassett, 1967; Tewari and Wildman, 1970), and this fact can be used as negative evidence for establishing the chloroplast nature of a DNA sample (Whitfeld and Spencer, 1968).

The ease of renaturation of chloroplast DNA has been used to estimate its kinetic complexity by well-established techniques (Britten and Kohne, 1968; Wetmur and Davidson, 1968). The kinetic complexity is a measure of the size of the unique base sequences present in the DNA. The kinetic complexities of the chloroplast DNA from several higher plants and two algae, Euglena gracilis and Chlamydomonas reinhardi are all of the same order, around 1×10^8 daltons (Ellis and Hartley, 1973). It is interesting to compare this figure with the analytical complexity of chloroplast DNA, i.e. the amount of DNA per chloroplast. The analytical complexity varies throughout the plant kingdom, ranging from 10^{-14} g to 10^{-16} g per chloroplast (Kirk and Tilney-Bassett, 1967). This figure represents about 6×10^8 to 6×10^{10} daltons of DNA per chloroplast (Wells and Birnstiel, 1969). Kinetic complexities are thus always much lower than analytical complexities, indicating that chloroplast DNA is extensively reiterated or consists of multiple copies.

The conformation and molecular size of chloroplast DNA has been studied/

studied by electron microscopy. Woodcock and Fernandez-Moran (1968) found lengths of DNA between 40 and 160μm when osmotically shocked spinach chloroplasts were observed by electron microscopy. When DNA was isolated from the chloroplasts, the lengths of the chloroplast DNA molecules were found to be between 2 and 20μm. Using improved techniques to avoid degradation of DNA, circular chloroplast DNA has been demonstrated in both purified DNA and in lysates of pea (Kolodner and Tewari, 1972a), spinach (Manning et al, 1972) and Euglena gracilis (Manning et al, 1971) chloroplasts. Circular DNA molecules are found as a discrete size class in high yield in all these species, with contour lengths ranging between 39μm (for pea) and 44μm (for spinach). There are also reports of circular chloroplast DNA in corn (Manning et al, 1972) of contour length 43μm, and in spinach, lettuce and bean (Kolodner and Tewari, 1972a) of contour length 39μm. Both Kolodner and Tewari (1972a) and Manning et al (1971) have calculated the molecular size of the circular chloroplast DNA molecules of pea and Euglena gracilis using their data obtained from electron microscopy. They find very close agreement between the molecular size obtained by electron microscopy and the molecular size as judged by kinetic complexity. This suggests that the DNA of the chloroplast is organised in a number of circular molecules rather than a single, highly reiterated molecule. By directly comparing the kinetic and analytical complexities of a given chloroplast DNA, an estimate of the number of molecules of DNA per chloroplast can be obtained. On this basis, Tewari and Wildman (1970) have concluded that there are about 20 molecules of DNA per tobacco chloroplast.

Can chloroplast DNA be distinguished from plant mitochondrial DNA? DNA isolated from the mitochondrial fraction of lettuce (Wells and Birnstiel, 1969) and pea (Kolodner and Tewari, 1972b) renatures rapidly, as does the chloroplast DNA from these species. However, mitochondrial DNA possesses a higher buoyant density (1.706 g cm^{-3}) than chloroplast DNA in both these species, and can therefore be distinguished by centrifugation in a CsCl gradient. Although mitochondrial DNA is assumed to lack 5-methyl cytosine (Borst, 1970), as chloroplast DNA does, Evans and Evans (1970) have detected 5-methyl cytosine as a level of 2% of the mitochondrial DNA of the slime mould Physarum polycephalum. Plant mitochondria also contain circular DNA molecules smaller than those found in chloroplasts, but much larger than/

than mitochondrial DNA from animal cells. Pea mitochondrial DNA is a circular molecule of length 30μm, almost as large as pea chloroplast DNA (Kolodner and Tewari, 1972b). Similarly, yeast mitochondrial DNA is found as circles of length 25-30μm; animal mitochondrial DNA, by contrast, exists as small circles of 5μm in length (Borst, 1970). Higher plant mitochondria therefore contain DNA of greater size and genetic complexity than animal mitochondrial DNA, implying that they may enjoy greater autonomy than animal mitochondria. This provides a good reason for studying protein and nucleic acid synthesis in mitochondria of higher plants.

Chloroplast DNA appears to be very similar in molecular size, conformation, and genetic complexity throughout the plant kingdom. Quantitatively, but not necessarily qualitatively, the same amount of genetic information appears to be present in a 40μm circle of chloroplast DNA from species diverse in evolution, such as Euglena gracilis and pea. Do these diverse chloroplast genomes code for the same proteins and (or) RNA molecules? A 40μm circle of DNA can code for the amino acid sequences of about 280 polypeptides of molecular weight 20 000 (Manning et al, 1971). This coding capacity is enormous when compared to the 15 000 base pairs present in a 5μm animal mitochondrial DNA circle (Borst 1970), equivalent to about 30 polypeptides of molecular weight 20 000. This constitutes a major reason for the study of chloroplasts as a model cellular organelle system.

B. DNA replication in vivo and in vitro.

Chloroplast DNA replicates in vivo in a semi-conservative fashion in synchronous cultures of both Chlamydomonas reinhardi (Chiang and Sueoka, 1967) and Euglena gracilis (Manning and Richards, 1972). In both organisms, synthesis of chloroplast and nuclear DNA takes place at different times in the cell cycle. Manning and Richards (1972) have also shown that, while nuclear DNA doubles once per generation, chloroplast DNA replicates about 1.5 times as fast. Nuclear DNA appears highly stable, while chloroplast DNA shows a turnover rate in Chlamydomonas equivalent to a half-life of about two cell doublings. These results show that both the timing and the rate of DNA synthesis in the chloroplast is different to that in the nucleus. Some form of control must exist in order to regulate DNA synthesis in the two cell compartments (and also in mitochondria). At the moment, the precise nature/

nature of this control system is unknown. Could control be exerted by different DNA polymerases in the nucleus and chloroplast?

DNA polymerase activity has been demonstrated in tobacco (Tewari and Wildman, 1967), spinach (Spencer and Whitfeld, 1969) and Euglena gracilis chloroplasts (Scott, Shah and Smillie, 1968). 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 when analysed on CsCl gradients, hybridises to chloroplast but not to nuclear DNA, and has a similar base composition to chloroplast DNA (Tewari and Wildman, 1967). The product of DNA synthesis in isolated spinach chloroplasts also renatures readily (Spencer and Whitfeld, 1969).

No attempts at purification of the chloroplast DNA polymerase have yet been reported. Consequently, it is impossible at this moment to compare nuclear and chloroplast DNA polymerases. Spencer and Whitfeld (1969) found that both spinach chloroplast DNA polymerase and chloroplast DNA remained associated with the lamellae after two hypotonic washes of the chloroplast preparation; addition of plant or animal DNAs caused no stimulation in this system. When spinach chloroplasts were prepared in a medium which preserves the structure of the chloroplasts (Honda, Hongladarom and Laties, 1966), a soluble DNA polymerase activity was detected which responded to added DNA, preferably native DNA (Spencer and Whitfeld, 1969). Tewari (1971) has discussed these results by analogy with DNA synthesis in bacteria, and suggests that the membrane-bound DNA polymerase may be the true replicase, while the soluble enzyme may have a repair function. Equally, Spencer and Whitfeld's results may be interpreted in other ways. For example, since the demonstration of soluble polymerase activity depended on the method of chloroplast isolation, perhaps the isolation conditions have the effect of solubilising part of the membrane-bound polymerase. If the soluble polymerase is a repair enzyme, it should have a preference for double-stranded, nicked DNA if it resembles the Kornberg DNA polymerase (Kornberg, 1969). The template specificity of the chloroplast DNA polymerase has not been thoroughly investigated; such studies must await purification of the enzyme.

C./

C. The function of chloroplast DNA.

i. Genetic studies.

The existence of mutations which could not be assigned to nuclear linkage groups was recognised long before the demonstration of unique organelle DNAs (Sager, 1972). Some of these cytoplasmic mutants possessed chloroplasts which were altered either in their structure or in the amounts of certain chloroplast components (Levine and Goodenough, 1970 ; Kirk and Tilney-Bassett, 1967). Not only were these mutations inherited in a non-Mendelian way (i.e. they did not show independent assortment), they were also passed *via* the maternal parent. This has been explained for higher plants by assuming that the pollen cell contains no chloroplasts or plastid precursors while the egg cell contributes all the extra-nuclear genetic information. This explanation has not been rigorously proved. In *Chlamydomonas reinhardi*, a molecular basis of maternal inheritance has been shown (Sager and Lane, 1972). Using a density-labelling technique, it was shown that the chloroplast DNA from the 'male' parent was destroyed soon after zygote formation; the DNA from the 'female' parent persisted, although a slight shift towards a lighter buoyant density was noted. It is known that in *Chlamydomonas* the single chloroplasts from each parent fuse in the zygote (Cavalier-Smith, 1970). Selective degradation of the chloroplast DNA may then follow. Although these mechanisms might occur in higher plants, there is no biochemical evidence, as yet, to support them.

Great difficulties have been experienced in identifying the precise lesion caused by cytoplasmic gene mutations, especially in higher plants (Kirk and Tilney-Bassett, 1967). It is also difficult to prove rigorously that a cytoplasmic mutation which affects chloroplast structure or function necessarily resides in the chloroplast DNA, and not in some other extranuclear DNA, although this seems the simplest hypothesis. In this connection, Wong-Staal and Wildman (1973) have isolated a satellite DNA from the chloroplast fraction of a cytoplasmic mutant of *Nicotiana tabacum*, which showed white leaf variegation. A 1% difference in GC content was demonstrated between the satellite and the normal chloroplast DNA. In addition, a region of 500 to 1 000 base pairs was mismatched when alkali-denatured normal and satellite DNAs were annealed and observed by electron microscopy. Although the statistical significance of these results can be questioned/

questioned, they do possibly provide a link between a change in the structure of chloroplast DNA and a lesion in the structure of the chloroplast. Respiratory-deficient (petite) strains of yeast contain no detectable amounts of cytochromes a, a_3, b and c, but also show massive deletions in mitochondrial DNA (Sager, 1972); such large deletions have not been detected in chloroplast DNA from organisms with mutated chloroplasts.

In the alga Chlamydomonas reinhardi, several non-Mendelian mutants have been identified which show such phenotypes as resistance to the antibiotics streptomycin, erythromycin, carbomycin, and neamine, and a requirement for acetate for growth. These mutants have been mapped into a single circular linkage group (Sager, 1972). Further analysis has shown that some of these mutants have altered ribosomal phenotypes (Gillham et al, 1970). Schlanger, Sager and Ramanis (1972) have described a non-Mendelian, carbomycin-resistant mutant. Resistance was shown to reside in the chloroplast ribosome, by comparing the carbomycin sensitivity of poly(U)-directed protein synthesis in mutant and wild-type S-30 extracts, and in purified preparations of mutant and wild-type ribosomes and cell sap. Mets and Bogorad (1972) were able to identify the nature of erythromycin resistance in a non-Mendelian mutant with greater precision. They showed that a single ribosomal protein of the large ribosomal subunit had been replaced in the mutant strain by several proteins of higher molecular weight. However, another erythromycin-resistant mutant which showed Mendelian inheritance was also shown to contain an altered protein composition of the large ribosomal subunit. Nuclear genes have also been shown to control both the amount of ribosomes in the chloroplast (Levine and Goodenough, 1970) and the assembly of the small ribosomal subunit (Boynton et al, 1970). Many other components of Chlamydomonas chloroplasts appear to be coded in the nuclear genome : plastocyanin, cytochromes 553 and 559, Q (the quencher of fluorescence of photosystem II), P700, an unidentified component of photosynthesis M, and the enzyme phosphoribulokinase (Levine and Goodenough, 1970).

In higher plants, the use of inter-specific hybrids of Nicotiana species has provided useful information on the relative roles of chloroplast and nuclear genomes in chloroplast development. Many species of tobacco are available which form viable hybrids and which originate/

originate from continents that have been geographically isolated for long periods on an evolutionary time scale, i.e. Australia and America. Several alterations in the primary structure of chloroplast proteins have been discovered when proteins have been isolated from different species. This provides a direct way of determining the location of the structural genes for specific chloroplast proteins. Chan and Wildman (1972) studied the inheritance of the large subunit of Fraction I protein by comparing the tryptic peptide maps of this protein, isolated from several American and Australian species of tobacco. The Australian species possessed an extra peptide which was not present in any American species. This extra peptide appeared in the large subunit of an F_1 hybrid only when an Australian species was the maternal parent. Consequently, it was concluded that chloroplast DNA contains the structural gene for the large subunit of Fraction I protein. In contrast, the gene for the small subunit of Fraction I protein was found to be located in the nucleus (Kawashima and Wildman, 1972) since in several species, peptides unique to the small subunit were inherited in a Mendelian manner.

These methods can be extended to study other chloroplast proteins. For example, Kung, Thornber and Wildman (1972) have shown by tryptic peptide mapping that the gene for the photosystem II chlorophyll-protein complex of tobacco chloroplasts is coded in nuclear DNA. Perhaps the study of F_1 hybrids might also be extended to analysis of chloroplast ribosomal RNAs, especially 5S RNA which can be readily fingerprinted.

The results of genetic studies on both algae and higher plants show that chloroplast DNA codes for rather few chloroplast proteins. We may conclude that both chloroplast and nuclear genomes co-operate in the biosynthesis of specific chloroplast proteins (such as Fraction I protein).

ii. DNA/RNA hybridisation.

Hybridisation of radioactively-labelled RNA species with chloroplast DNA can provide good evidence for the existence in the chloroplast of cistrons coding for ribosomal RNA, tRNA and messenger RNA. Such studies do not conclusively demonstrate that these cistrons act as templates for the synthesis of RNA species. Hybridisation studies are/

are therefore best complemented by *in vivo* and *in vitro* studies of RNA synthesis.

a. Ribosomal RNA.

Scott and Smillie (1967) first demonstrated the existence of chloroplast ribosomal RNA genes in the chloroplast DNA of *Euglena gracilis*. Up to 1% of chloroplast DNA was occupied by bound P^{32}-labelled chloroplast ribosomal RNA. This binding was specific since hybrid formation was reduced in the presence of unlabelled ribosomal RNA from autotrophically-grown cells (which contain much chloroplast RNA) and chloroplasts, but was not affected by the presence of unlabelled ribosomal RNA from either dark-grown cells or from a bleached mutant which lacks chloroplasts, and chloroplast RNA. Chloroplast DNA species from *Euglena*, enriched for ribosomal RNA cistrons by shearing the DNA into smaller fragments (around 10^6 daltons), have been shown to hybridise chloroplast ribosomal RNA up to 1.9% of the chloroplast genome (Rawson and Haselkorn, 1973). Most of the ribosomal RNA hybridised to the heavy strand of alkaline-denatured *Euglena* chloroplast DNA (Stutz and Rawson, 1970) showing similarity with the arrangement of ribosomal RNA cistrons on mitochondrial DNA (Borst, 1970).

Chloroplast DNA hybridises with chloroplast ribosomal RNA isolated from tobacco (Tewari and Wildman, 1968, 1970) and swiss chard (Ingle *et al*, 1971). Although 0.5% to 1.5% of chloroplast DNA hybridised to chloroplast ribosomal RNA in both species, considerable cross-hybridisation was observed (0.1% to 0.3%) between nuclear DNA and chloroplast ribosomal RNA. This raises the possibility that there are genes for chloroplast ribosomal RNA in both nuclear and chloroplast DNA. This possibility has been further investigated in both tobacco and swiss chard, with conflicting results. Tewari and Wildman (1968) showed that chloroplast ribosomal RNA did not compete with cytoplasmic ribosomal RNA for sites on nuclear DNA. In swiss chard, however, some evidence of competition was obtained (Ingle *et al*, 1971). This point needs further study with attention being paid to the stability of the hybrids formed.

Various estimates of the number of unique sequences coding for chloroplast ribosomal RNA in each DNA molecule have been made. These estimates are made difficult by a lack of knowledge of the specificity and efficiency of hybrid formation (Rawson and Haselkorn, 1973), but figures are usually one to three chloroplast ribosomal RNA cistrons per chloroplast DNA molecule in both algae and higher plants (Tewari and Wildman, 1970; Rawson and Haselkorn, 1973). It has also been estimated that there is approximately 1 000 times as much coding

information/

information for chloroplast ribosomal RNA in the nucleus, than in a single tobacco chloroplast (Tewari and Wildman, 1968).

b. tRNA and messenger RNA.

Radioactively-labelled chloroplast tRNAs hybridise to between 0.4% and 0.7% of tobacco chloroplast DNA (Tewari and Wildman, 1970). This would be equivalent to between 20 and 30 tRNAs per chloroplast DNA molecule, at least one for each amino acid. The number of messenger RNA sequences present in chloroplast DNA cannot be determined, since no chloroplast (or plant) messenger RNA has been isolated.

Molecular hybridisation thus shows that one function of chloroplast DNA may be to code for chloroplast ribosomal RNA and some chloroplast tRNAs. Hybridisation data alone cannot resolve the question as to whether these cistrons (or the chloroplast ribosomal RNA cistrons present in nuclear DNA) are expressed during the development of the plastid.

iii. Studies on transcription of chloroplast DNA, *in vivo* and *in vitro*.

The function of chloroplast DNA has been investigated by studying the effect of inhibitors of chloroplast DNA-dependent RNA polymerase. Rifampicin, a specific inhibitor of bacterial but not eukaryotic RNA polymerases (Wehrli and Staehelin, 1971), has been frequently used in such studies (Surzycki, 1969; Surzycki *et al*, 1970). A concentration of 250µg/ml rifampicin was found to inhibit phototrophic but not heterotrophic growth in *Chlamydomonas reinhardi*. Plastid development continued in the presence of rifampicin, although chloroplast membrane formation appeared disorganised (Surzycki *et al*, 1970). In the presence of rifampicin, synthesis of chloroplast ribosomal RNAs (23S, 16S and 5S) was inhibited; the amount of chloroplast ribosomes also decreased. Rifampicin was also shown to inhibit the increase in cytochromes 553 and 563 during prolonged growth experiments (Armstrong *et al*, 1971). The level of ribulose diphosphate carboxylase was not affected by rifampicin.

It may be concluded from rifampicin inhibition experiments that the chloroplast DNA of *Chlamydomonas* contains the information for chloroplast ribosomal RNA. A similar conclusion cannot be drawn for cytochromes 553 and 563, since the results could be due to the lack of chloroplast/

chloroplast ribosomes which might translate messenger RNAs synthesised in the nucleus. The information for the remaining chloroplast components which were assayed (ferredoxin, ferredoxin-NADP reductase, phosphoribulokinase and ribulose diphosphate carboxylase) is therefore assumed to reside in the nucleus (Armstrong et al, 1971). However, negative results obtained with rifampicin do not necessarily mean that the genes for such proteins lie in the nucleus. To conclude this, one must assume that the messenger RNA for the protein has a much shorter half-life than the time-period of the experiment, and also that several cell divisions have taken place in order to 'dilute out' any pre-existing proteins. These points were not adequately considered in the work of Armstrong et al (1971).

The effects of rifampicin on chloroplast RNA polymerases is controversial. Inhibition of activity by rifampicin has been reported for other algae, Chlorella (Galling, 1971) and Acetabularia (Brandle and Zetsche, 1971). In higher plants, however, no inhibition was found for chloroplast RNA polymerases assayed from several species (Bottomley et al, 1971). The application of rifampicin to analysis of chloroplast DNA transcription in higher plants is therefore open to question. No specific inhibitor of the chloroplast RNA polymerase of higher plants has been demonstrated (Bottomley et al, 1971). For these reasons, investigation of the synthesis of RNA by isolated chloroplasts may be a more informative way of assessing the transcription of chloroplast DNA than inhibitor experiments on intact cells.

Isolated chloroplasts from a number of species of algae and higher plants have been shown to possess DNA-dependent RNA polymerase activity (Smillie and Scott, 1970). The products of in vitro RNA synthesis by isolated chloroplasts of higher plants have frequently been shown to be heterogeneous in size (Tewari and Wildman, 1969; Spencer et al, 1971) although complementary to chloroplast DNA (Tewari and Wildman, 1970). However, discrete chloroplast RNAs have been synthesised by isolated chloroplasts of three plant species.

Berger (1967) showed that labelled nucleosides and nucleoside triphosphates were incorporated into discrete peaks of RNA synthesised in chloroplasts isolated from enucleated Acetabularia mediterranea. The labelled peaks co-sedimented with E. coli 23S and 16S ribosomal RNA markers on sucrose gradients. In addition, peaks of radioactivity at 9S and 4S were also observed. This would appear to be good evidence in/

in favour of specific transcription of chloroplast ribosomal RNA and possibly tRNA genes. However, these results, and all studies on isolated Acetabularia chloroplasts, must be treated with caution in view of the cytoplasmic contamination found in preparations of Acetabularia chloroplasts (Bidwell, 1972).

Similar indications of chloroplast ribosomal RNA synthesis in isolated tobacco chloroplasts was obtained by Wollgiehn and Munsche (1972). The important aspect of this study was the addition of bentonite (a nuclease inhibitor) to the incubation medium. This converted a previously heterogeneous profile of products of RNA synthesis into one which suggested that labelling of chloroplast ribosomal RNA had taken place. Perhaps the best, but as yet incompleted study of RNA synthesis in isolated chloroplasts has been performed by Hartley and Ellis (1973). Isolated spinach chloroplasts were shown to use light energy to incorporate [H^3]uridine into a major discrete product of molecular weight 2.7×10^6 daltons. Two minor products of molecular weight 1.2×10^6 and 0.47×10^6 daltons were also obtained. In contrast to the studies of Berger (1967) and Wollgiehn and Munsche (1972), no label was incorporated into chloroplast ribosomal RNA. Elucidation of the exact nature of the major RNA product awaits further analysis by molecular hybridisation and oligonucleotide fingerprinting. One possibility might be that the labelled high molecular weight RNA is a precursor RNA containing sequences for both 23S and 16S chloroplast ribosomal RNA.

5. THE SITES OF SYNTHESIS OF CHLOROPLAST PROTEINS.

This problem has been approached in two ways. One approach is to study the effects of selective inhibitors of chloroplast and cytoplasmic ribosomes on the synthesis of chloroplast enzymes in vivo. The other approach, which is technically more demanding, is to study the synthesis of proteins by isolated chloroplasts.

A. The use of inhibitors of protein synthesis in vivo.

The rationale of the first approach is that if the synthesis of a chloroplast enzyme decreases in the presence of an inhibitor of chloroplast ribosomes, then synthesis must take place on chloroplast ribosomes in vivo. There are several requirements which must be met when this method is used. The specificity of the inhibitor used on the intact cells must be evaluated. Good evidence exists which shows that/

that both cycloheximide and chloramphenicol affect processes in plant cells other than protein synthesis (Ellis, 1963; Ellis and MacDonald, 1970). All four stereoisomers of chloramphenicol (D- and L-threo,D- and L-erythro) affect such processes as ion uptake and oxidative phosphorylation in higher plants. Only the D-threo isomer specifically inhibits protein synthesis on chloroplast ribosomes (Ellis, 1969). This stereospecificity test should be applied to in vivo studies using chloramphenicol.

It is also important to be sure that the plant cells which are treated with the inhibitor are actively synthesising chloroplasts. The amount of a particular enzyme must be shown to be strictly dependent on light, i.e. it must be present at a low level in etiolated tissue. In experiments with synchronous cultures of algae it is important to be sure that several cell divisions take place during the course of the experiment. In addition to these practical considerations, several theoretical objections can be made against the use of inhibitors in vivo. It may be difficult to be sure that the inhibitor stops de novo synthesis, and not the conversion of a proenzyme to an active enzyme, or the synthesis of a cofactor or subunit essential for activity. For example, in yeast mitochondria, the apoprotein of cytochrome oxidase is synthesised on cytoplasmic ribosomes but requires the synthesis of an additional protein on mitochondrial ribosomes for full activity to be expressed (Ashwell and Work, 1970). The use of chloramphenicol would lead to the erroneous conclusion that cytochrome oxidase is synthesised on mitochondrial ribosomes. This sort of objection can be answered by the use of immunological methods (to detect possible apoproteins) or by the use of the density-labelling technique which can provide good evidence for or against de novo synthesis (Filner and Varner, 1967); however, this technique has not been applied to the synthesis of chloroplast enzymes. Perhaps a more basic objection is that one is disrupting a complex cellular control system by selectively stopping the synthesis of one of its components. The level of one component may control the level of another; for example in Chlamydomonas reinhardi the level of chlorophyll is believed to control the synthesis of certain chloroplast lamellar proteins (Eytan and Ohad, 1970). For these reasons, the use of inhibitors of protein synthesis in vivo can rarely give results which are more than suggestive. Strictly interpreted, the results of such experiments never say more than that chloroplast/

chloroplast ribosomal activity is necessary for a given protein to accumulate in the chloroplast, which is not the same as saying that this protein is synthesised on chloroplast ribosomes.

Table 1 shows the proposed sites of synthesis of some chloroplast proteins based on studies of the effects of various 70S ribosomal inhibitors on greening cells of several algae and higher plants. Results obtained by different groups working on the same organisms can be contradictory. For example, Smillie et al (1967) found that the synthesis of NADP-triose phosphate dehydrogenase was inhibited by chloramphenicol but not by cycloheximide in greening cells of Euglena gracilis. Schiff (1970), however, found no inhibition of the synthesis of the same enzyme when a greening Euglena culture was treated with streptomycin. It must be added that Smillie et al (1967) did not show that inhibition was stereospecific for the D-threo isomer of chloramphenicol.

In all the studies shown in Table 1 there seems to be general agreement that the synthesis of Fraction I protein is inhibited by 70S ribosomal inhibitors. The synthesis of membrane-bound cytochromes and some membrane proteins also appears to require 70S ribosomal activity. The results of Armstrong et al (1971) show that the synthesis of cytochromes 553 and 563 is also inhibited by cycloheximide. Perhaps a membrane protein, synthesised by cytoplasmic ribosomes, is required for insertion of the cytochromes into the thylakoid membranes. Ellis and Hartley (1971) inferred that some ribosomal proteins may be synthesised on chloroplast ribosomes since lincomycin did not inhibit either the synthesis or the activity of pea chloroplast RNA polymerase. However, the amount of 70S ribosomes declined in the presence of the inhibitor. A defect in the assembly of ribosomes, possibly due to the absence of certain ribosomal proteins, could explain these results.

The major exception to this relatively unanimous view of the sites of synthesis of proteins in higher plant chloroplasts is provided by the work of Graham et al (1970). These workers found that in maize, sorghum, oat and wheat, the synthesis of several enzymes of the Calvin cycle (including NADP-triose phosphate dehydrogenase) and the C_4 dicarboxylic acid pathway, was inhibited by chloramphenicol. These results must be considered exceptional since the stereospecific nature of the inhibition was not tested.

Armstrong et al (1971) found that both cytoplasmic and chloroplast ribosomes/

Table 1. Suggested sites of synthesis of chloroplast proteins (modified after Boulter et al, 1972).

[1] Ellis & Hartley (1971).
[2] Ireland & Bradbeer (1971).
[3] O. Wara-Aswapatti (personal communication).
[4] Gregory & Bradbeer (1973).
[5] Graham et al (1970).
[6] Smillie et al (1967).
[7] Schiff (1970).
[8] Armstrong et al (1971).
[9] Hoober et al (1969).
[10] Hoober (1970).
[11] Hoober (1972).

* confirmed by Haslett et al (1973) using lincomycin.

** LP, low potential (+70 mV).

*** HP, high potential (+370 mV) - see Bendall et al (1971).

Protein	Species					
	Pisum sativum[1]	*Phaseolus vulgaris*[2,3,4]	*Zea mays*[5]	*Euglena gracilis*[6]	*Euglena gracilis*[7]	*Chlamydomonas reinhardi*[8,9,10,11]
	Ribosomal Inhibitor Used					
	Lincomycin	D-*threo*- & L-*threo*-chloramphenicol	D-*threo*-chloramphenicol, cycloheximide	D-*threo*-chloramphenicol, cycloheximide	Streptomycin	D-*threo*-chloramphenicol, cycloheximide, spectinomycin
Ribosephosphate isomerase	80S	80S	-	-	-	-
Phosphoribulokinase	80S	80S	-	-	-	80S
Ribulose diphosphate carboxylase (Fraction I)	70S	70S	70S	70S	70S	70S & 80S
Phosphoglycerate kinase	80S	80S	-	-	-	-
Triosephosphate dehydrogenase (NADP)	80S	80S	70S	70S	80S	-
Triosephosphate isomerase	-	80S	-	-	-	-
Fructose diphosphate aldolase	-	80S	-	70S	-	-
Transketolase	-	80S	-	-	-	-
Pyruvate Pidikinase	-	-	70S	-	-	-
Ferredoxin	-	80S*	-	-	-	80S
Ferredoxin-NADP reductase	-	80S	-	70S	-	-
Cytochrome f	-	70S	-	70S	70S	70S & 80S
Cytochrome b-563	-	70S	-	70S	-	70S & 80S
Cytochrome $b\text{-}559_{LP}$**	-	70S	-	-	-	-
Cytochrome $b\text{-}559_{HP}$***	-	80S	-	-	-	-
RNA polymerase	80S	-	-	70S	-	-
Ribosomal proteins	70S	-	-	-	-	-
Membrane proteins	-	-	-	-	-	70S & 80S

ribosomes were required to synthesise Fraction I protein in Chlamydomonas reinhardi. Criddle et al (1970) analysed the radioactive labelling in vivo of the large and small subunits of barley Fraction I protein, in the presence of 70S and 80S ribosomal inhibitors. They found that chloramphenicol preferentially inhibited the synthesis of the large subunit, whereas cycloheximide preferentially inhibited the labelling of the small subunit. Kawashima (1970) found that $C^{14}O_2$ was incorporated into the amino acids of the large subunit of Fraction I protein to a greater specific activity than the small subunit. These results suggest that the synthesis of Fraction I protein takes place in two cellular compartments, the large subunit being synthesised in the chloroplast and the small subunit in the cytoplasm.

In vivo studies of the synthesis of chloroplast enzymes suggest that most enzymes are synthesised outside the chloroplast, on cytoplasmic ribosomes. The major exception to this scheme is Fraction I protein. However, even in this instance, one of the two subunits of the enzyme appears to be synthesised on cytoplasmic ribosomes. Two conclusions may be drawn from these studies. Firstly, mechanisms must exist to transport enzymes from the cytoplasm into the chloroplast. This implies that some sort of protein translocase exists in the outer membrane of the chloroplast which recognises and transports proteins destined for the chloroplast. Secondly, the rates of synthesis of individual enzymes, and of their subunits (e.g. of Fraction I protein) must be regulated in both chloroplast and cytoplasmic compartments. The elucidation of these control mechanisms provides work for the future.

B. The study of in vitro protein synthesis in isolated chloroplasts.

The characteristics of amino acid incorporation by isolated chloroplasts have been established for a number of species (Boulter et al, 1972), and the components of the protein-synthesising system of chloroplasts have also been widely studied (see Section I3). However, no unequivocal demonstration of the synthesis of a chloroplast protein in vitro has been made (Kirk, 1970; Woodcock and Bogorad, 1971). The distribution of incorporated radioactivity between particulate and soluble fractions of the chloroplast has been determined. Between 50% and 75% of radioactivity incorporated in vitro is associated with the particulate/

particulate fraction (Bamji and Jagendorf, 1966; Spencer, 1965). This fraction would include not only membrane proteins but also ribosomes and attached nascent polypeptides.

Attempts at the further characterisation of the products of *in vitro* protein synthesis have been inconclusive. Margulies (1970) found some indication of labelling of Fraction I protein in isolated bean chloroplasts. Spencer *et al* (1971) found that ferredoxin might by synthesised in isolated spinach chloroplasts. Ranaletti, Gnanam and Jagendorf (1969) found that chloroplast coupling factor (Ca^{2+}-dependent ATPase) purified from isolated wheat chloroplasts appeared to be labelled. Conversely, Chen and Wildman (1970) found no evidence of *in vitro*-labelling of Fraction I protein, or any other chloroplast protein in isolated tobacco chloroplasts. Most of the incorporated radioactivity was associated with ribosomes, presumably as nascent polypeptides.

The literature on *in vitro* studies of chloroplast protein synthesis therefore presents a picture of failure to identify products conclusively. However, the *in vitro* method does provide in principle the most direct way of studying the protein synthetic capability of the chloroplast, freed from direct control by nucleus or cytoplasm. *In vivo* studies provide good suggestions as to which proteins are synthesised on chloroplast ribosomes : Fraction I protein, either complete or one of its subunits, and perhaps some membrane proteins, ribosomal proteins and membrane-bound cytochromes. The aim of the results presented in this thesis is to define the conditions for optimal and physiological translation in isolated chloroplasts, and to attempt to identify definitely the products of *in vitro* chloroplast protein synthesis.

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

The research presented in this thesis attempts to answer some basic questions about the biosynthesis of chloroplast proteins and the function of chloroplast ribosomes. Do chloroplasts synthesise any of their own proteins? And, if so, which proteins? The use of selective inhibitors of chloroplast ribosomes has produced conflicting results, and is also open to theoretical objections. Therefore a study of *in vitro* protein synthesis by isolated chloroplasts, although beset by intrinsic biochemical difficulties was performed in order to provide some definitive answers to such questions. Previous uses of the/

the *in vitro* approach have either been unconvincing or have failed due to the following difficulties:-

i. low protein synthetic activity of chloroplast preparations. This is a general problem, whether structurally-preserved (Honda *et al*, 1966) or broken chloroplasts are used. This difficulty was overcome by using a rapid method of chloroplast isolation which gave a good yield of intact chloroplasts. Protein synthesis proceeded at a high rate in such preparations, using light as the energy source.

ii. the unphysiological nature of the chloroplasts. It is difficult to believe that a faithful translation process takes place either in broken chloroplasts (where a massive dilution of all chloroplast components takes place) or in chloroplasts whose structure is preserved by adding ficoll, dextran or BSA to the medium (Honda *et al*, 1966). In this thesis intact chloroplasts were used since none of the enzymes, tRNAs or factors necessary for protein synthesis should be lost; the photochemical systems essential for the generation of ATP from light should not be destroyed.

iii. poor methods of analysing the products of *in vitro* protein synthesis. All analytical methods operate at the limit of their sensitivity if the protein-synthesising system is inactive, and therefore difficulty i. above has also a direct bearing upon this problem. In this work, the newly-synthesised proteins were analysed by polyacrylamide gel electrophoresis under both denaturing and non-denaturing conditions. Sucrose gradients were not used, in contrast to previous research, since it was felt that they did not provide either the sensitivity or resolution given by polyacrylamide gels. However, elution of radioactively-labelled proteins from Sephadex G100 was also performed as an additional method.

iv. adequate criteria of identification of *in vitro*-synthesised proteins. It was felt that the existence of discrete, labelled peaks on gels was not in itself an adequate criterion of identity, even when they exactly co-electrophoresed with known marker proteins, since this merely gives a molecular weight comparison. The primary structure of *in vitro*-synthesised proteins was analysed by tryptic peptide fingerprinting and compared with the fingerprint of authentic, *in vivo*-labelled protein.

Using these approaches, results were obtained which make a direct comparison possible between *in vitro* studies and *in vivo* inhibition data/

data. They also provide a basis for comparison with the protein synthesising-system of mitochondria, and for speculation on the possible functions of chloroplast ribosomes; the biochemical relationship between chloroplast, nucleus and cytoplasm; and the evolution of plastid protein synthesis.

SECTION II - MATERIALS AND METHODS

1. MATERIALS

A. PLANT SOURCES

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

Spinach seeds (Spinacea oleracea var. Monstrous Viroflay) were obtained from Thompson and Morgan Ltd., Ipswich, Suffolk.

B. GROWTH OF PLANTS

1. Pisum sativum

a. Seeds were surface sterilised by soaking in a 10% (v/v) solution of sodium hydrochlorite for three minutes, and were then imbibed in running tap water for 36 hours. These seeds were planted in plastic seed trays containing J. Arthur Bowers compost (made by Lindsay and Kesteven Fertilisers Ltd., Saxilby, Lincoln). The trays were kept in a well-ventilated room at approximately 24°C under a 12 hour photoperiod of 2 000 lux white light, obtained from Philips 'Warmwhite' fluorescent tubes. The trays were watered with tap water each day. Pea seedlings were grown by this method for 10 days. The youngest leaves were used for chloroplast isolation.

b. Etiolated pea seedlings were grown by sterilising and soaking the pea seeds as detailed above. The seeds were then planted in either J. Arthur Bowers compost or in vermiculite ('Nicafil' from Dupre Vermiculite, Tamworth Road, Hertford). The trays were then incubated in the dark in seed incubators at 22°-24°C for 9 days. The compost or vermiculite was kept moist by occasional watering with distilled water, care being taken to exclude light.

2. Spinacea oleracea

Spinach seeds were sown in J. Arthur Bowers compost and germinated for 7 days in covered plastic trays. Seedlings were then removed, their roots washed free from compost and transferred to aerated Huntner's medium (Huntner, 1953) adjusted to pH 6.4 (see below). Seedlings were grown under a 12 hour photoperiod of 10 000 lux obtained from 'Warmwhite' fluorescent tubes, at 22°-24°C for 14 days. The first-formed leaf pair were used for chloroplast isolation.

Huntner's/

Huntner's medium. Seven concentrated stock solutions were made up, at the concentrations shown below. To make 1 litre of medium, 20ml Solution 1 + 10ml Solution 2 + 1ml Solution 3 + 1ml Solution 4 + 1ml Solution 5 + 10ml Solution 6 + 10ml Solution 7 were made up to 1 litre with distilled water, and adjusted to pH 6.4 with 1N NaOH.

Solution 1 (50 times concentrated)

KH_2PO_4	34g/l
KNO_3	75.7g/l

Solution 2 (100 times concentrated)

H_3BO_3	28.6mg/l
$ZnSO_4.7H_2O$	2.2mg/l
$Na_2MoO_4.2H_2O$	1.2mg/l
$CuSO_4.5H_2O$	0.8mg/l
$MnCl_2.4H_2O$	3.62mg/l

Solution 3 (1 000 times concentrated)

0.9g Na_2EDTA + 5ml 5M KOH

Solution 4 (1 000 times concentrated)

$FeCl_3.6H_2O$	0.540g/100ml

Solution 5 (1 000 times concentrated)

$K.C_4H_4O_6$ (tartaric acid) 470mg/100ml

Solution 6 (100 times concentrated)

$MgSO_4.7H_2O$	50g/l

Solution 7 (100 times concentrated)

$Ca(NO_3)_2.4H_2O$	118g/l

C. CHEMICALS AND RADIOISOTOPES

All chemicals were Analar grade, and all solvents were either Analar or chromatography grade.

Ammonium persulphate; glycine; ninhydrin; 4 - aminosalicylic acid, (PAS); sodium dodecyl sulphate, (SDS); trichloroacetic acid, (TCA); octylpheroxypolyethoxy ethanol, (Triton X-100) were obtained from British Drug/

Drug Houses Ltd.

m- chlorocarbonyl cyanide phenylhydrazone, A grade (CCCP) was obtained from Calbiochem.

N,N^1-methylenebisacrylamide and tri-isopropylnaphthalenesulphonic acid, sodium salt (TNS) were obtained from Eastman Organic Chemicals, Rochester, N.Y., U.S.A.

Osmium tetroxide was obtained from Fisons Ltd.

Acrylamide was obtained from Fluka AG, Buchs, Switzerland.

Amidoblack and phenol red dyes were obtained from G.T. Gurr.

Bromophenol blue dye, hydrogen peroxide (100 volumes) and Folin-Ciocalteau reagent were obtained from Hopkin and Williams Ltd.

Hyamine hydroxide and N,N,N,N^1-tetramethylenediamine, (TEMED) were obtained from Koch-Light Laboratories.

2,5-diphenyloxazole, (PPO) and 1,4-bis-(5-phenyloxazol-2-yl) benzene, (POPOP) were obtained from Nuclear Enterprises (GB) Ltd., Sighthill, Edinburgh.

Sephadex G25 (coarse grade), G100 (medium grade) and G200 (medium grade) were obtained from Pharmacia (GB) Ltd.

Bovine serum albumin, Grade III (BSA); blue dextran; D-threo-chloramphenicol; erythromycin; glutaraldehyde, Grade V; N-2-hydroxyethylpiperazine-N^1-2-sulphonic acid, (HEPES); 2-mercaptoethanol; 2 (N-morpholino)ethanesulphonic acid, (MES); phenylmethyl sulphonyl fluoride, (PMSF); sodium isoascorbate; sorbitol; sodium pyrophosphate; N-tris (hydroxymethyl)-2-aminoethanesulphonic acid, (TES); N-tris (hydroxyethyl)methyl glycine, (tricine); 2-amino-2-hydroxymethylpropane-1:3 diol/

diol, (tris, TRIZMA base); octylphenoxypolyethoxy ethanol,(Triton X-100) were obtained from Sigma Ltd.

DEAE-cellulose (DE52 grade) and 3MM paper were obtained from Whatman Ltd.

The following chemicals were kindly supplied gratis:
3-(3,4-dichlorophenyl)-1,1-dimethylurea,(DCMU) from Fisons Ltd.
Lincomycin from Upjohn Ltd.
Spectinomycin from Dr. D. Suttie, University of Aberdeen.

[^{14}C]hexadecane (specific activity 1.06μCi/g, density 0.7731), [^{14}C] leucine (specific activity 331mCi/mmole, radioactive concentration 50μCi/ml), [^{14}C]phenylalanine (specific activity 513mCi/mmole, radioactive concentration 50μCi/ml) and [^{35}S]methionine (specific activity 25-270Ci/mmole, radioactive concentration 0.5-1.0mCi/ml) were obtained from the Radiochemical Centre.
[^{35}S]methionine (specific activity 65-126Ci/mmole, radioactive concentration 0.8-5.0mCi/ml) was obtained from New England Nuclear.

D. ENZYMES AND SUBSTRATES.

Pronase (B grade) was obtained from Calbiochem.

Creatine phosphokinase and pancreatic ribonuclease A (Type 1A) were obtained from Sigma Ltd.

Trypsin, inactivated by L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone, (TPCK) was obtained from Worthington Corporation.

dATP (Grade I), dGTP (Type II-S), creatine phosphate, L-leucine, L-methionine and L-phenylalanine were obtained from Sigma Ltd.

2. METHODS AND GENERAL ANALYTICAL PROCEDURES.

A. CHLOROPLAST ISOLATION.

In all methods of chloroplast isolation, sterile media and glassware were used in order to minimise bacterial contamination of the chloroplast/

chloroplast preparations. Chloroplast pellets were gently resuspended initially in a small volume (0.5ml) of medium using a small piece of cotton wool and a glass rod.

i. The method of Jensen and Bassham (1966).

Media. 1. Basic medium.

0.33M sorbitol
2mM $NaNO_3$
2mM EDTA
2mM sodium isoascorbate
1mM $MnCl_2$
1mM $MgCl_2$
0.5mM K_2HPO_4

2. Medium A.
Basic medium containing 0.05M MES-NaOH (pH 6.1) and 0.02M NaCl.

3. Medium B.
Basic medium containing 0.05M HEPES-NaOH (pH 6.7) and 0.02M NaCl.

Method. Leaves (20g) were homogenised with 80ml Medium A in an MSE Atomix for 5 seconds at top speed, the leaves being packed round the blades of the atomix before homogenisation was started. The container was chilled before use, and the Medium A was used semi-frozen. The homogenate was squeezed through 6 layers of cheesecloth and centrifuged at 2 000 x g for 50 seconds at 0°C (r_{av} 14.6cm) in an MSE 6L centrifuge. The chloroplast pellets were immediately resuspended in 4.5ml Medium B; the supernatant fluid was discarded. The preparations had chlorophyll concentrations of about 0.2mg/ml.

ii. The method of Walker (1968).

Media. 1. Isolation medium.

0.33M sorbitol
10mM $Na_4P_2O_7.10H_2O$
0.1% (w/v) $MgCl_2$

The pH was adjusted to pH6.5 with concentrated HCl. This medium cannot/

cannot be autoclaved, due to the formation of a heavy precipitate of $Mg_3(PO_4)_2$. The medium was therefore made up immediately before use, using sterile water to minimise bacterial contamination.

2. Resuspension medium.

0.33M sorbitol
0.05M HEPES-NaOH (pH 7.6)
1mM $MgCl_2$
1mM EDTA

Method. Leaves (25g) were homogenised with 100ml semi-frozen isolation medium in an Atomix for 4 seconds at top speed. The homogenate was squeezed through 2 layers of cheesecloth. The liquid was centrifuged at 4 000 x g for 60 seconds at 0°C (r_{av} 14.6cm) in an MSE 6L. The supernatant fluid was discarded and the pellet resuspended in 4ml of resuspension medium. The chlorophyll concentration of the preparation was 0.20-0.27mg/ml.

iii. The method of Nobel (1967).

Medium. 0.2M sucrose
0.02M TES-NaOH (pH 6.9)

Method. Leaves and stems (20g) were harvested and cut into approximately $1cm^2$ pieces. They were then transferred into a two layer thick nylon bag (mesh size 80µ, 33% open area, obtained from Henry Simon Ltd., Stockport, England) and placed in a chilled mortar. Chilled medium (20ml) was added, and the bag was then ground firmly for 10 seconds. The contents were then squeezed into the mortar, poured into a chilled centrifuge tube, and centrifuged at 1 000 x g for 60 seconds at 0°C (r_{av} 14.6cm) in an MSE 6L. The pellet was resuspended in 4ml of medium, giving a chlorophyll concentration of 0.15mg/ml.

iv. The method of Ramirez, del Campo and Arnon (1968).

Media. 1. Sucrose isolation medium.

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

2. KCl resuspension medium.

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

3. Sucrose resuspension medium.

0.35M sucrose
66mM tricine-KOH (pH 8.3)
6.6mM $MgCl_2$

Method. Leaves (20g) were homogenised for 4 seconds in a Willems Polytron homogeniser (speed setting 7, probe No. PC 20, obtained from Northern Media Supply Ltd., Hull, Yorks.) in 100ml of semi-frozen medium. The homogenate was immediately strained through 8 layers of cheesecloth and centrifuged at 2 500 x g for 1 minute at 4°C (r_{av} 14.6cm) in an MSE 6L. The supernatant fluid was decanted and the pellet resuspended in 4ml of either KCl or sucrose resuspension medium. Chlorophyll concentrations were between 0.13 and 0.40mg/ml.

v. A method which yields only broken chloroplasts.

Chloroplasts were isolated as described in iv. above, ie. by the method of Ramirez et al (1968). The pellet obtained was resuspended in 4ml of 25mM tricine-KOH, 10mM $MgSO_4$, 5mM 2-mercaptoethanol (pH 8.0). (This medium is referred to as TMS resuspension medium). The chlorophyll concentration of preparations was about 0.16mg/ml.

B. MICROSCOPIC ANALYSES OF CHLOROPLAST PREPARATIONS.

i. Light microscopy.

a. Estimation of the percentage of intact chloroplasts in chloroplast preparations by phase microscopy.

The percentage of intact chloroplasts was determined by quantitative microscopy using a haemocytometer grid. The haemocytometer was thoroughly cleaned before use. A drop of the chloroplast suspension was flooded underneath the coverslip. A square of the grid was examined under phase optics using the x 40 objective of a Gillett and Sibert microscope. (Total magnification was x 320). The total number of chloroplasts was counted; those which appeared highly refractile were scored as intact, and those which had dark granal structures were scored/

scored as broken (Kahn and von Wettstein, 1961). Another square was also so examined, until at least 200 chloroplasts had been counted. The number of intact chloroplasts was expressed as a percentage of the total number of chloroplasts.

b. Chloroplasts were photographed at a magnification of x 725 under phase optics in a Leitz Orthoplan microscope. Photography was kindly performed by Mr. C.S. Dow.

ii. Electron microscopy.

The fixation procedure used was a modification of that of Wellburn and Wellburn (1972) for etioplasts. Solutions in this procedure are made up in 0.33M phosphate buffer, pH 7.5.

An equal volume of 5% (v/v) glutaraldehyde in 0.5M sucrose - 0.33M phosphate buffer was added to the chloroplast suspension and left for 2 hours at 0°C. The plastids were then spun down at 3 000 x g for 2 minutes at 0°C (r_{av} 11.2cm) in round-bottomed polythene tubes in the MSE 18 centrifuge.

The pellet was then washed with 5ml of 0.5M sucrose, 0.35M sucrose and 0.2M sucrose (in 0.33M phosphate buffer) successively for 5 minutes each. A volume of 1ml of 2% (w/v) OsO_4 in 0.15M sucrose-phosphate buffer was added to the pellet and left for 2 hours at room temperature. After removal of the osmium, 1ml 30% (v/v) acetone containing 0.1M sucrose was added and left for 30 minutes.

The sample was then dehydrated with a series of acetone treatments, i.e. 30 minutes in 5ml each of 30%, 50%, 70% and 90% acetone. The sample was left in 100% acetone overnight; this was replaced with fresh 100% acetone 2 hours before embedding. Samples were embedded in Spurr's resin in the original polythene tubes. The tubes were left overnight at room temperature, and then left in a 70°C oven for 8 hours. Each pellet was subsequently cut and re-embedded in gelatine capsules. The embedding, sectioning and microscopy was kindly performed by Dr. Rachel Leech and Miss Helen Prior of the University of York.

C. INCUBATION OF CHLOROPLASTS AND ASSAY OF AMINO ACID INCORPORATION.

i. Assay of total amino acid incorporation.

For routine assays of amino acid incorporation by chloroplast preparations/

preparations, 300µl or 400µl of chloroplast suspension were incubated in a final volume of 500µl with 0.25µCi of either [C^{14}] leucine (3µM) or [S^{35}] methionine (36nM). The difference in volume was made up with resuspension medium, or with an ATP-generating system, or some other addition e.g. an antibiotic or inhibitor. In light-driven protein synthesis, samples were illuminated with filtered red light at 4 000 lux (as measured by a Megatron lightmeter type E1) from a Philips Photoflood lamp, mounted underneath the glass water bath. The temperature was maintained at 20°C by a Churchill circulating water cooler, and the Photoflood was cooled by a fan. In ATP-driven protein synthesis, the same water bath was used; however the tubes containing the reaction mixtures were carefully covered with aluminium foil to exclude light. The ATP-generating system contained 2mM ATP, 5mM creatine phosphate and 100µg/ml creatine phosphokinase. Chloroplasts resuspended in TMS resuspension medium (see Section II2Av) were similarly incubated at 20°C with 100mM KCl, 1.25mM ATP, 0.125mM GTP, 5mM creatine phosphate and 200µg/ml creatine phosphokinase. The components of both ATP-generating systems, and any other additions to the reaction mixture, such as antibiotics, were always dissolved in the resuspension medium in order to keep the osmolarity of the reaction mixture constant. The radioisotope was added in a small (1-10 microlitre) volume from the stock vial.

Reactions were stopped by standing tubes on ice and adding 0.5ml of a saturated solution of unlabelled amino acid, 0.1ml of a 20mg/ml solution of BSA (bovine serum albumin) to act as a carrier protein, and 1.0ml of 10% (w/v) TCA (trichloroacetic acid). Protein was allowed to precipitate overnight at 4°C before the amino acid incorporation assay was performed.

Amino acid incorporation was measured essentially according to the method of Siekevitz (1952). Throughout the procedure, all precipitates were spun down at 1 000 x g for 10 minutes at 4°C (r_{av} 21.6cm) and all supernatant fluids were discarded. The initial precipitate was spun down, washed once with 8ml of 5% TCA and twice with 5ml of 5% TCA. The pellet was resuspended in 5ml of 5% TCA and heated for 15 minutes at 90°C. The tubes were left to cool, the precipitate spun down, and then resuspended in 8ml of absolute ethanol (to remove chlorophyll). After centrifugation, the pale yellow pellet was resuspended in 5ml ether, to remove water which interferes with scintillation counting. The precipitate/

precipitate was again spun down, and the remaining traces of ether were removed from the pellet by evaporation. As soon as the pellet was dry, it was resuspended in 1.2ml hyamine hydroxide - 0.5M methanol. An aliquot (1.0ml) of this suspension was pipetted into a scintillation vial containing 8ml toluene - 0.5% PPO (2,5-diphenyloxazole) scintillant. The vials were counted at 15% gain, open window on a Packard Tricarb scintillation spectrometer. Counting efficiency was found to be 70%, by comparison with standard [C^{14}] hexadecane.

ii. Analysis of the labelled products of protein synthesis.

When a highly-labelled preparation was required, e.g. for tryptic peptide analysis, several modifications were introduced. During isolation of chloroplasts, the pellets were resuspended in smaller volumes of resuspension medium (0.5-1.0ml) so that increased amino acid incorporation could be obtained. Chloroplasts were incubated with 50-100uCi of [S^{35}] methionine per incubation. Incubation was for one hour, by which time amino acid incorporation had ceased (fig. 9); therefore no labelled amino acid was added so that a highly-labelled, concentrated chloroplast supernatant fraction could be prepared.

D. ELECTROPHORETIC ANALYSIS OF RADIOACTIVELY-LABELLED CHLOROPLAST SUPERNATANT FRACTION.

i. Preparation of supernatant fraction.

A 150 000 x g chloroplast supernatant fraction was prepared as follows. After incubation as described in Section II2Cii, the chloroplast preparation was dialysed overnight against 1 litre of 2.5mM tris - 19mM glycine, 10mM 2-mercaptoethanol (pH 8.5) at 4°C. When samples were to be denatured with sodium dodecyl sulphate (SDS), the 2-mercaptoethanol concentration was increased to 100mM. The dialysed preparations were centrifuged at 150 000 x g for 60 minutes at 4°C (r_{av} 7.62cm) on an MSE Superspeed 50, with a 3x5ml swing-out head fitted with 1ml tube adaptors. The clear supernatant liquid was removed and its protein concentration was determined (see Section II2Mi). Some supernatant fractions were denatured by adding SDS such that the SDS:protein ratio was at least 2:1 (w/w) (Reynolds and Tanford, 1970), and 2-mercaptoethanol was added to a final concentration of 100mM. This mixture was incubated for 60 minutes at 37°C.

ii./

ii. Polyacrylamide gel electrophoresis.

a. Sodium dodecyl sulphate-denatured supernatant proteins.

Polyacrylamide gel electrophoresis was performed at room temperature using 6mm x 100mm gels. SDS-denatured supernatant fractions were fractionated on 8.0%, 10.0% and 12.0% acrylamide gels. The bis-acrylamide concentration in all gels was 0.2%. Both acrylamide and bisacrylamide were used without recrystallisation.

The gels were polymerised as follows. In strict order, 3ml tris gel buffer [3M tris-HCl; 0.03% SDS; 0.46% (v/v) TEMED (N,N,N,N^1-tetramethylenediamine) added fresh; pH 8.5)], 3ml distilled water, 6ml acrylamide solution and 12ml 0.14% (w/v) fresh ammonium persulphate solution were gently mixed in a flask, without de-aeration. The mixture was transferred (by Pasteur pipette) into perspex running tubes, closed at the bottom with dialysis tubing and a rubber grommet. The tubes stood in a Petri dish containing distilled water. A small layer of distilled water, several millimetres thick, was placed on top of the acrylamide mix with a finely drawn-out Pasteur pipette, without disturbing the acrylamide. This ensures that the gel has a uniformly flat top, so that proteins migrate as bands, and not as semi-circles. Polymerisation was complete in about 15 minutes.

The gels were transferred to electrophoresis tanks containing buffer (50mM tris - 380mM glycine, 10mM 2-mercaptoethanol, 0.03% SDS, pH 8.5) with the cathode in the top gel tank compartment and the anode in the lower compartment. The gels were pre-run at 100V for at least 30 minutes before use, in order to remove excess ammonium persulphate which might bind to proteins. The sample (100µl containing 100-200µg protein and approximately 10% sucrose) was layered directly on to the top of the gel, and electrophoresis was performed at 100V (5mA/gel) for 2.5 hours. Bromophenol blue was used as a marker dye, and was mixed with sample to a final concentration of 0.1% (w/v).

After electrophoresis, gels were fixed in 7% (v/v) acetic acid for at least 30 minutes, and then stained in 0.5% (w/v) amidoblack dye in 7% acetic acid for one hour. Destaining was performed either by washing the gel with several changes of 7% acetic acid (this method was used for destaining 4.0% gels) or by an electrophoretic method whereby a current of 1A was passed across the length of the gels which were immobilised in slits in a tank containing 7% acetic acid. The current was passed for 30 minutes.

Each/

Each gel was scanned at 620 nm in a Joyce-Loebl Chromoscan recording densitometer (Joyce-Loebl Ltd., Gateshead, England) frozen on powdered dry ice and then sliced into 1mm fractions using a Mickle gel slicer (Mickle Engineering Co., Gomshall, Surrey). The fractions were solubilised on 0.1ml H_2O_2 (100 volumes) for 1 to 2 hours in a 70°C oven. A volume of 8ml of Triton-toluene scintillant [0.4% (w/v) 2,5-diphenyloxazole, (PPO); 0.05% (w/v)1,4-bis-(5-phenyloxazol-2-yl) benzene,(POPOP) in toluene-Triton X-100 (2:1,v/v)] was added, and radioactivity was measured in the Packard Tricarb spectrometer at 15% gain, open window. Counting efficiency was estimated at 90% by comparison with standard [C^{14}]hexadecane.

b. Non-denatured supernatant proteins.

The procedure for non-denatured supernatant proteins was the same as for SDS-denatured proteins, except that SDS was absent from both the tris gel buffer and the electrophoresis buffer. In addition, supernatant proteins were fractionated on 5.0% and 4.0% acrylamide gels. The bisacrylamide concentration was 0.2% in all cases. Gels were fixed, stained, scanned and counted as described above.

iii. Enzymic digestion of the supernatant fraction by pronase and ribonuclease A.

Pronase and pancreatic ribonuclease A were freshly made up at a concentration of 1mg/ml in 50mM tris-380mM glycine, 10mM 2-mercapto-ethanol (pH 8.5). A total of 10µg of each enzyme (or an equivalent volume of buffer to act as a control) was added to 700µg of chloroplast supernatant protein, and incubated at 37°C for 4 hours. The digests were then denatured with SDS as described above (Section II2Di) and the products were separated by electrophoresis on 10.0% SDS gels.

E. SOLUBILISATION OF RADIOACTIVELY-LABELLED CHLOROPLASTS BY TRITON X-100 DETERGENT.

At the end of the incubation (see Section II 2Ci) 0.5ml unlabelled amino acid was added to the 0.5ml reaction mixture. A volume of 1ml of 10% (v/v) Triton X-100 detergent was added, followed by 3ml of KCl resuspension medium. The final concentration of Triton X-100 was therefore 2% (v/v). This mixture was incubated at 20°C for 15 minutes, and/

and then centrifuged at 10 000 x g for 10 minutes at 20°C (r_{av} 7.14cm) on the MSE 50. The supernatant was discarded, and the pellet was resuspended in 0.5ml distilled water. This was transferred, with washings, to a conical glass centrifuge tube; 0.2ml of a 20mg/ml solution of BSA and 5ml 10% TCA were added. Amino acid incorporation into this fraction was determined as described in Section 2Ci.

F. PURIFICATION OF PEA FRACTION I PROTEIN.

Fraction I protein was purified from leaves of 10-15 day pea seedlings following a procedure modified from that of Kawashima and Wildman (1971a). The modifications were:-

a. the inclusion of 2-mercaptoethanol in all buffers at a concentration of at least 10mM. This addition prevents the formation of very high molecular weight aggregates of Fraction I protein.

b. the use of DEAE-cellulose chromatography before Sephadex chromatography rather than vice-versa, as Kawashima and Wildman (1971a) reported. This procedure was adopted since it was found to give greater purification. Subsequently chromatography on Sephadex was carried out and the high molecular weight protein eluting at the void volume was collected.

c. the use of a step-wise elution of Fraction I protein from DEAE-cellulose, rather than a gradient elution.

d. the inclusion of 2mM phenylmethyl sulphonyl fluoride (PMSF) in the extraction buffer. This inactivates plant serine proteases which cause slow degradation of Fraction I protein during storage (Gray and Kekwick, 1973).

e. the use of a structural assay for Fraction I protein on polyacrylamide gels, namely its very low characteristic mobility on non-denaturing gels, and its recognisable subunit composition on SDS-gels rather than the enzyme assays or ultracentrifugal analyses used by Kawashima and Wildman (1971a).

All operations were performed at 4°C. Leaves (200g) were blended with 300ml of ice-cold 0.025M tris-HCl, 0.05M NaCl, 1mM $MgCl_2$, 0.5mM EDTA, 0.04M 2-mercaptoethanol and 2mM PMSF, pH 7.4 (Buffer A) in an Atomix blender at top speed. The homogenate was strained through 8 layers of cheesecloth and centrifuged at 10 000 x g for 30 minutes (r_{av} 14.4cm) in an MSE 18. The supernatant liquid was removed and centrifuged at 105 000/

105 000 x g for 60 minutes (r_{av} 7.62cm) in an MSE 50. The yellowish 105 000 x g supernatant fraction was passed through a column of coarse grade Sephadex G25 (6.5cm x 50cm) at a flow rate of 20ml/min in order to remove low molecular weight contaminants such as phenols. Protein was eluted in the void volume with 0.025M tris-HCl, 0.05M NaCl, 0.5mM EDTA and 10mM 2-mercaptoethanol, pH 7.4 (Buffer B). Approximately 400ml of protein solution was collected. Solid ammonium sulphate was added according to Dixon's nomogram and table in Green and Hughes (1955), and the precipitate which appeared between 35% and 45% saturation was collected by centrifugation at 10 000 x g for 10 minutes (r_{av} 14.4cm) and resuspended in 2ml of 0.05M tris-HCl, 0.5mM EDTA and 10mM 2-mercaptoethanol, pH 8.0 (Buffer C). The protein was dialysed overnight against 1 litre of Buffer C.

The protein was adsorbed on to a column (1.5cm x 15cm) of DEAE-cellulose, previously equilibrated with Buffer C. The column was thoroughly washed with more Buffer C, and Fraction I protein was eluted with Buffer C to which NaCl had been added to 100mM. Protein was precipitated by adding solid ammonium sulphate to 50% saturation, and the precipitate was spun down at 10 000 x g for 10 minutes (r_{av} 10.7cm) and resuspended in 5ml of Buffer B. Finally, the protein was applied to a Sephadex G200 column (2.5cm x 90cm) and eluted with Buffer B at a flow rate of 20ml/hr. Fig. 1 shows the elution of Fraction I protein on Sephadex G200. The peak which elutes before Fraction I protein has an $E_{280}:E_{260}$ ratio of less than one, and might therefore be high molecular weight nucleic acid. Fraction I protein peak fractions with $E_{280}:E_{260}$ of 1.8 or greater were pooled, precipitated with 50% saturated ammonium sulphate, centrifuged, resuspended in 5ml of 2.5mM tris - 19mM glycine, 10mM 2-mercaptoethanol, pH 8.5 and dialysed overnight against 1 litre of that same buffer. Fraction I protein was stored in solution at 4°C.

The purity of the Fraction I protein was assessed by polyacrylamide gel electrophoresis. Gels were loaded with up to 200µg protein per gel, to maximise the chance of detecting any impurities which might not be visible at lower loadings. In addition both non-denaturing and SDS-gels were used. If a very basic protein, which would be positively charged at pH8.5, were present as a contaminant in the protein sample, this contaminant would not be shown by non-denaturing gel electrophoresis since the protein would migrate upwards through the gel tank to the cathode; it would not enter the gel. By assessing purity on SDS-gels, all proteins are/

Figure 1. Purification of pea Fraction I protein : chromatography on Sephadex G200.

Details of this procedure are given in Section II2F. The sample volume was 5ml, and the fraction volume was 4ml. The extinction of each fraction was measured at 280 nm and 260 nm, and the E_{280} : E_{260} ratio was determined. E_{280} ▲——▲; E_{260} ●——●; E_{280} : E_{260} ■——■.

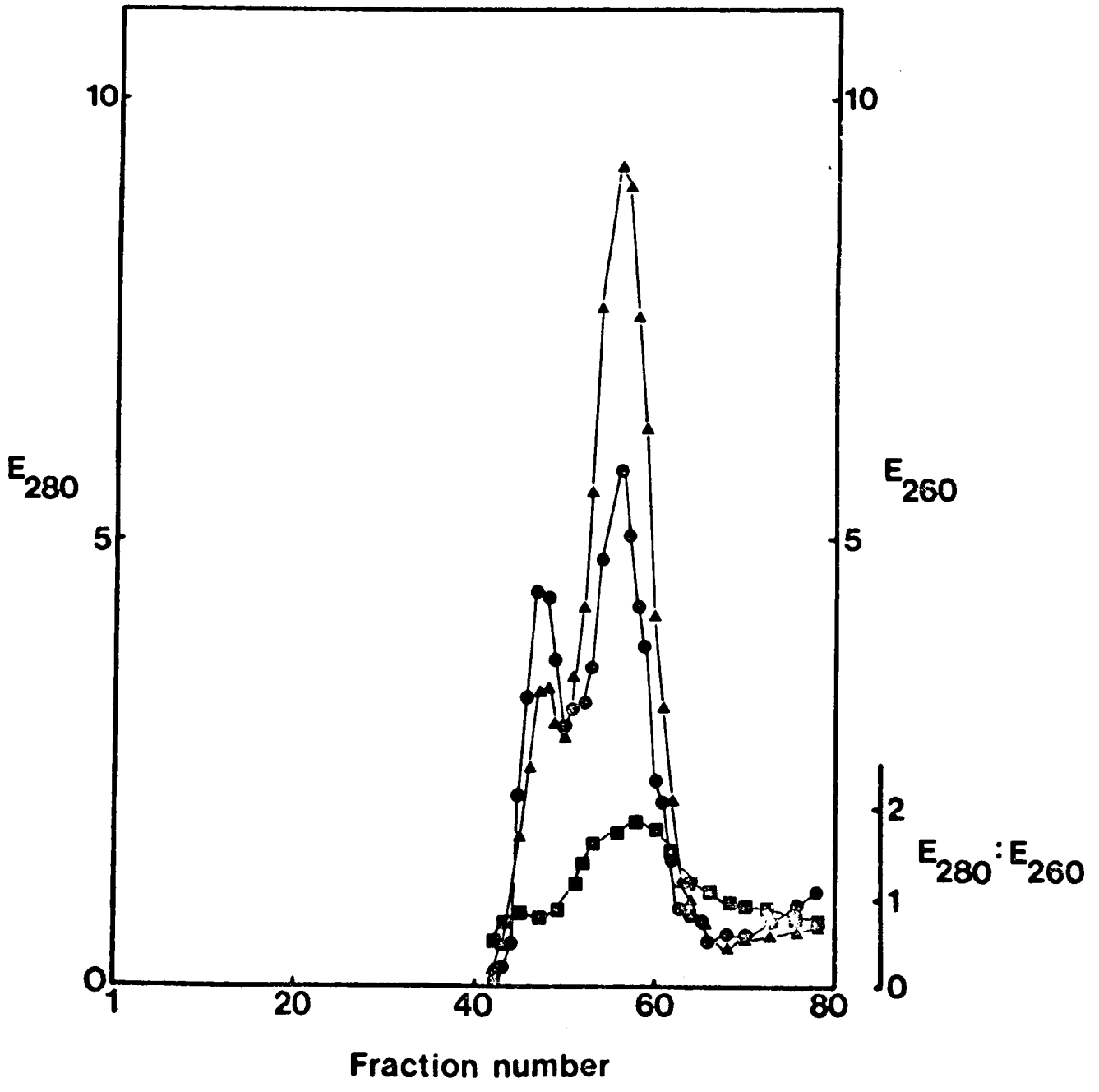
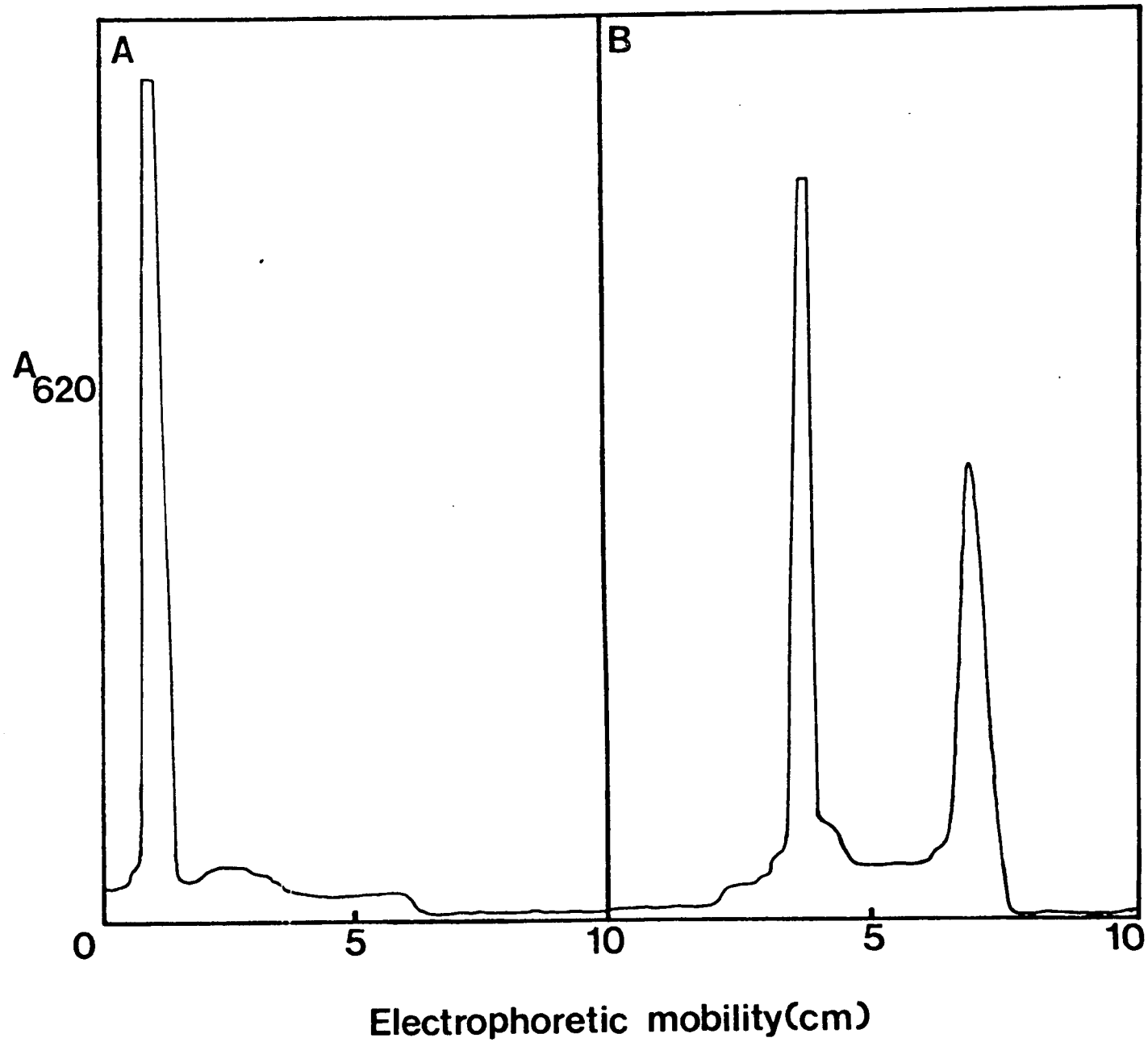

10
5
0
E_{280}
10
5
E_{260}
2
1
0
$E_{280}:E_{260}$
1
20
40
60
80
Fraction number

Figure 2. Polyacrylamide gel electrophoresis of purified pea Fraction I protein.

Purity was assessed by loading 200µg of protein A. on 5.0% acrylamide gels in the absence of SDS and B. on 10.0% acrylamide SDS-gels. The conditions of electrophoresis and staining are described in Section II2Dii. The gels were scanned at maximum sensitivity to allow the detection of contaminants. This produces the flat top to the main protein bands.


A
B
A_{620}
0
5
10
5
10
Electrophoretic mobility(cm)

are fractionated since they all have a high negative charge due to bound SDS. In addition if a protease introduces a small number of breaks in the polypeptide chain, the protein may still run as a single band on non-denaturing gels if the molecule is stabilised by weak, non-covalent bonds. However on SDS-gels, several protein species would be seen. Indeed in early work on the purification of Fraction I protein when PMSF was not used and technique was not well developed, this effect was observed, viz. an apparently homogeneous preparation when fractionated on non-denaturing gels appeared highly heterogeneous when analysed on SDS-gels. This point is especially important when tryptic peptide analysis is performed since one must be sure that the peptides obtained are due to hydrolysis by trypsin only and not by trypsin and some other protease.

Some typical gel scans of purified pea Fraction I protein are shown in fig. 2. These scans show that the Fraction I is highly pure on both non-denaturing (fig. 2A) and SDS-gels (fig. 2B). Traces of other components can be seen when the gels are scanned at the maximum sensitivity obtained in the Chromoscan.

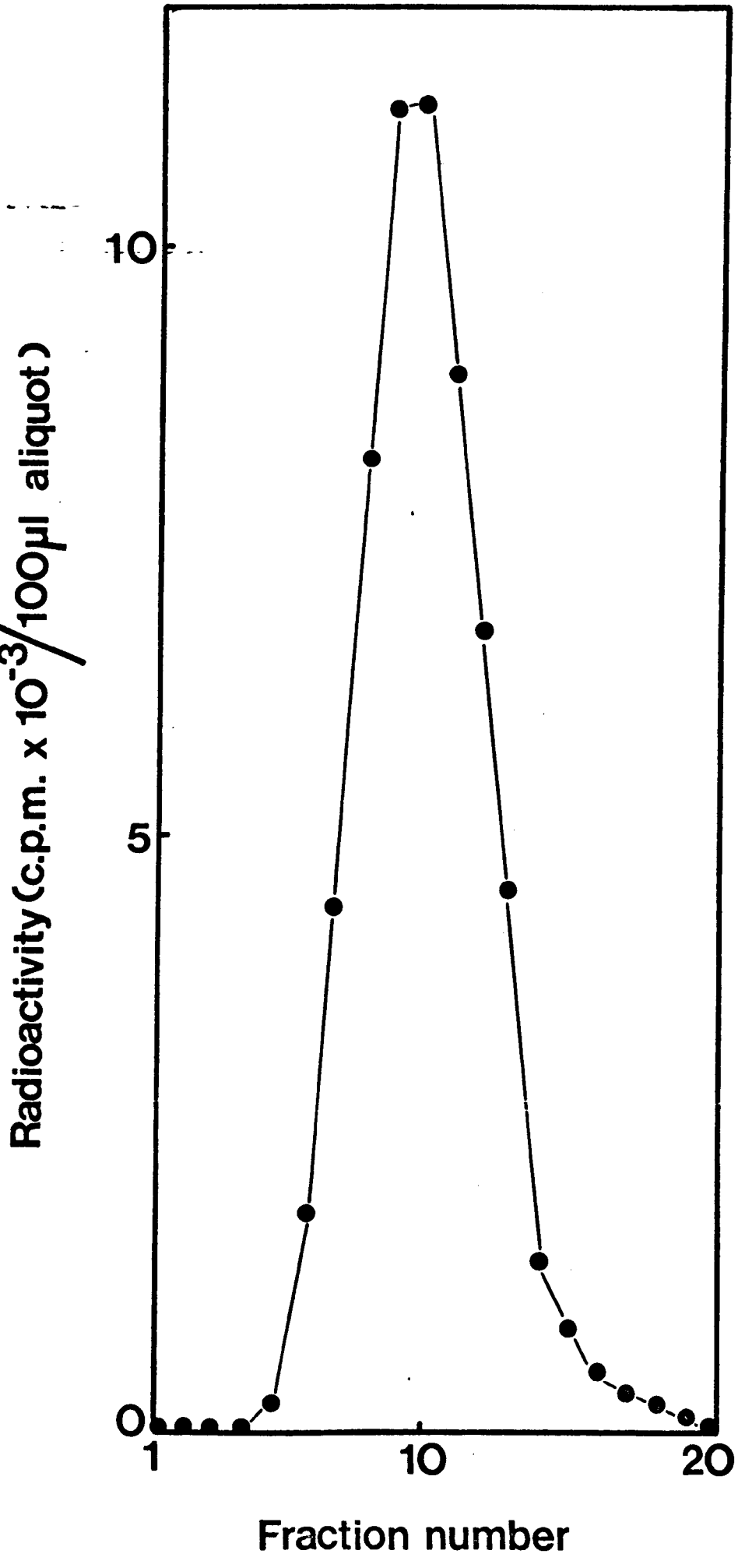
G. PURIFICATION OF IN VIVO, RADIOACTIVELY-LABELLED FRACTION I PROTEIN.

Etiolated pea seedlings were grown as described in Section I1B1b A total of 40 shoots were excised about 5cm below the apex and the cut ends placed in 10 small vials, each containing 0.5ml sterile distilled water with 16μCi [S^{35}] methionine (0.9μM). The shoots were illuminated for 3 days with 12 000 lux from 'Warmwhite' fluorescent tubes. The vials were regularly topped up with sterile distilled water.

Fraction I protein was purified from these green apices by a method similar to that described above (Section II2F) except that chromatography columns of smaller dimensions were used to minimise losses. All the apices were harvested (2.5g fresh weight) and ground in 15ml Buffer A in a chilled mortar. The homogenate was filtered through cheesecloth and centrifuged at 10 000 x g for 30 minutes (r_{av} 10.7cm). The supernatant liquid was removed and centrifuged at 105 000 x g for 60 minutes (r_{av} 7.62cm). The 105 000 x g supernatant fluid was passed through a small column (1.5cm x 10cm) of coarse grade Sephadex G25. The flow rate was 10ml/hr. Protein was eluted at the void volume with Buffer B; approximately 40ml of protein solution was collected. Ammonium sulphate fractionation was performed as described above, and the precipitate (obtained/

Figure 3. Purification of *in vivo*, radioactively-labelled Fraction I protein : chromatography on Sephadex G200.

All experimental details are given in Section II2G.


Radioactivity (c.p.m. x 10^{-3}/100µl aliquot)
10
5
0
1
10
20
Fraction number

(obtained between 35% and 45% saturation) was centrifuged, resuspended in 1ml of Buffer C and dialysed overnight against 1 litre of Buffer C.

DEAE-cellulose chromatography was performed as previously described, except that a smaller column (0.9cm x 7cm) was used. A volume of 20ml of protein solution was collected and protein precipitated by adding solid ammonium sulphate to 50% saturation. The precipitate was spun down and resuspended in 1ml of Buffer B. The protein solution was applied to a Sephadex G200 column (1.5cm x 20cm) and eluted with Buffer B at a flow rate of 7ml/h. Aliquots (100µl) were removed from each fraction, added to 8ml of Triton-toluene scintillant and counted at 15% gain, open window in a Packard Tricarb spectrometer. Fig. 3 shows the elution of labelled Fraction I protein on Sephadex G200. Peak fractions were selected and ammonium sulphate added to 50% saturation. At this stage approximately 5mg of cold, purified pea Fraction I protein was added. The precipitate was centrifuged and resuspended in 1ml of 2.5mM tris-19mM glycine, 10mM 2-mercaptoethanol (pH 8.5) and dialysed overnight against 1 litre of that same buffer. The protein was stored at 4°C in solution.

H. PREPARATION OF LARGE AND SMALL SUBUNITS FROM PEA FRACTION I PROTEIN.

Large and small subunits of pea Fraction I protein were purified on Sephadex G100 essentially according to the method of Rutner and Lane (1967). A chloroplast supernatant fraction was prepared as described in Section II2Di, mixed with 5 to 10mg of purified pea Fraction I protein and denatured with SDS as previously described. The protein was applied to a Sephadex G100 column (2.5cm x 45cm) and eluted with 50mM tris-HCl, 0.1mM EDTA, 0.5% SDS, 10mM 2-mercaptoethanol (pH 8.6) at a flow rate of 10ml/h at room temperature. Fractions (2ml) were collected, the extinction read at 280nm, and 100µl aliquots analysed for radioactivity in Triton-toluene scintillant. A typical elution profile of unlabelled Fraction I protein is shown in fig. 4. It can be seen that good separation of large and small subunits is obtained. Peak fractions corresponding to large and small subunits were precipitated in 90% (v/v) acetone by stirring at room temperature for at least 30 minutes. The precipitates were washed twice in 20ml of 90% acetone, to remove SDS. At each stage the precipitates were spun down at 1 000 x g for 10 minutes (r_{av} 21.6cm) in an MSE 6L centrifuge. Finally, the precipitates were dispersed in distilled water (both isolated subunits were rather insoluble/

Figure 4. Elution profile of SDS-denatured pea Fraction I protein on Sephadex G100.

Purified pea Fraction I protein (20mg) was denatured with SDS as described in Section II2Di. The volume of denatured protein sample applied to the column was 2ml. Other experimental details are given in Section II2H. LSU, large subunit; SSU, small subunit.

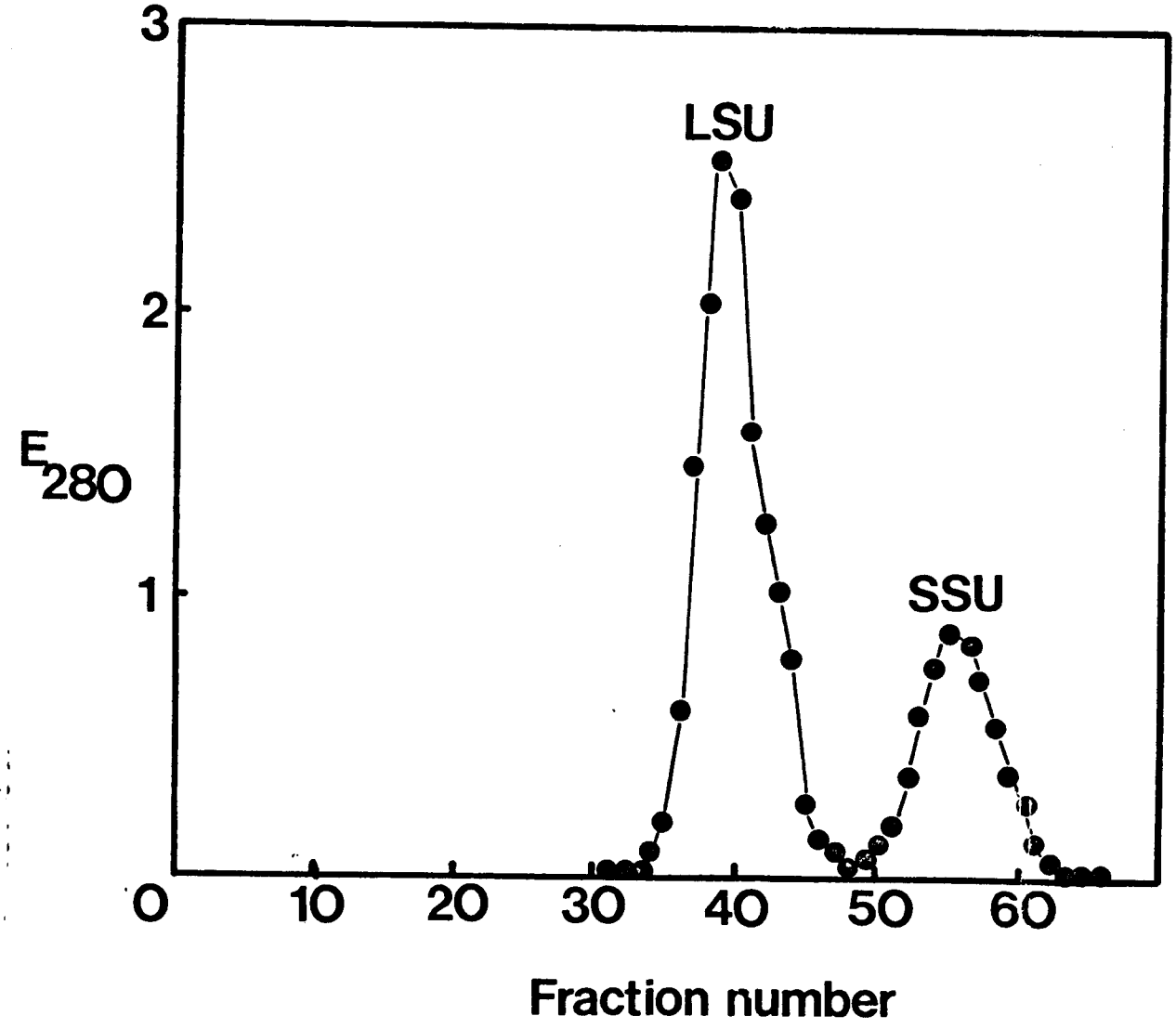
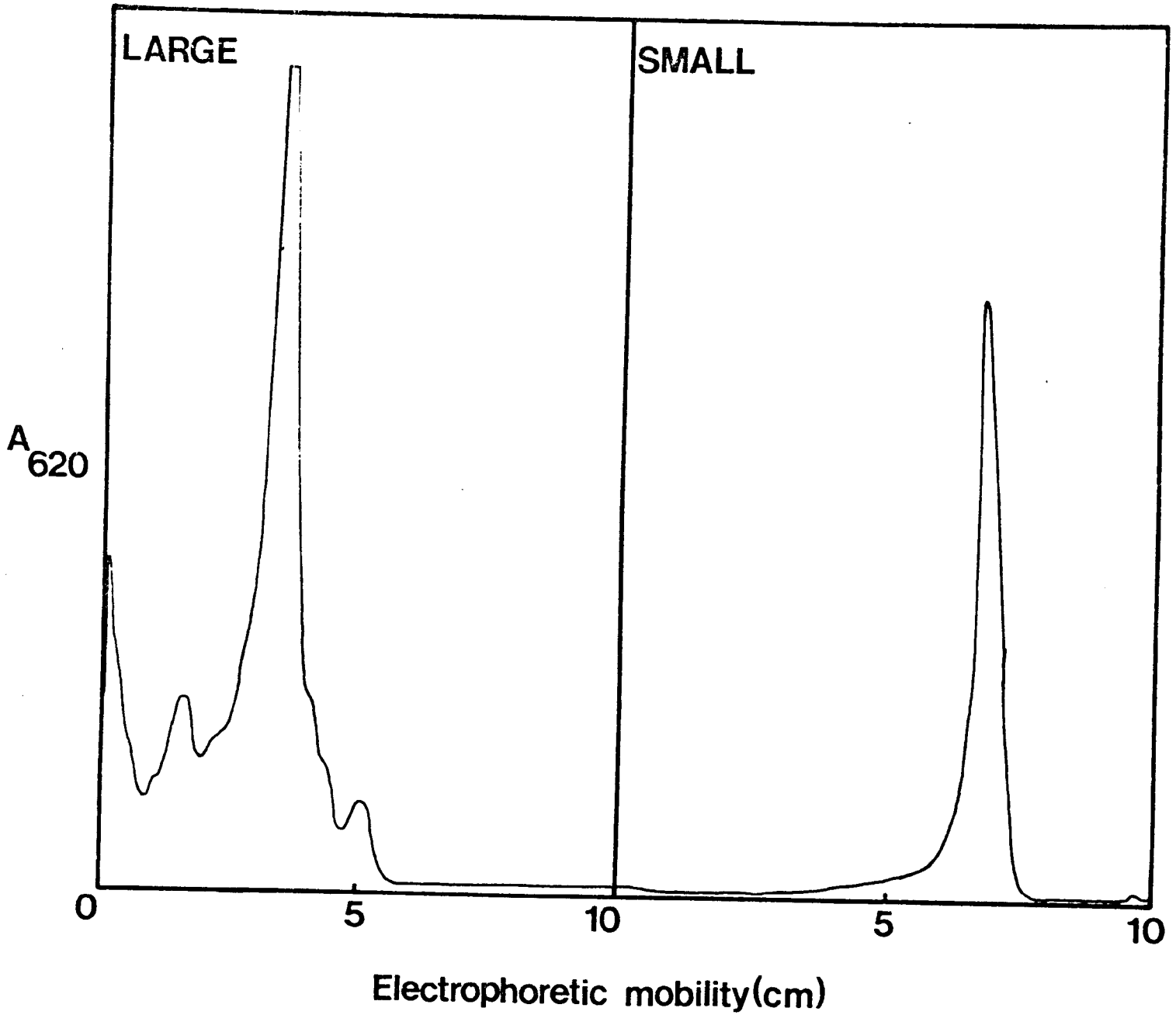

3
2
1
0
E_{280}
LSU
SSU
10
20
30
40
50
60
Fraction number

Figure 5. Polyacrylamide gel electrophoresis of large and small subunits of pea Fraction I protein purified by Sephadex G100 chromatography.

Subunits were purified as described in Section II2H. After drying *in vacuo*, 5mg/ml solutions of each subunit were prepared in 2.5mM tris-19mM glycine, 100mM 2-mercaptoethanol, 1% (w/v) SDS (pH 8.5). They were then incubated for one hour at 37°C and 50µg of each subunit were analysed on 10.0% SDS-gels.


LARGE
SMALL
A620
0
5
10
5
10
Electrophoretic mobility(cm)

insoluble in water, the large subunit being much less soluble than the small subunit), transferred to an acid-washed ampoule and dried *in vacuo* in an Edwards High Vacuum drier.

The purity of each subunit was assessed by resuspending a small known weight of each protein in a small volume of 2.5mM tris-19mM glycine, 100mM 2-mercaptoethanol, 1% SDS (pH 8.5) and running an aliquot on 10.0% SDS-gels. Fig. 5 shows gel scans of purified subunits obtained by this procedure.

I. TRYPTIC DIGESTION AND PEPTIDE MAPPING.

The protein (2mg) was resuspended in 500µl of 0.2M ammonium acetate buffer (pH 8.5), and 100µg of L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone, (TPCK)-inactivated trypsin was added. The trypsin: protein ratio was therefore 1:20. Incubation was at 37°C for 4 hours. The digest was acidified with 5% formic acid and dried *in vacuo*. Peptides were resuspended in 50µl of 0.1M ammonia and the whole digest was spotted on to a sheet of Whatman 3MM paper. The peptides were separated in the first dimension by descending paper chromatography in n-butanol - acetic acid - water (3:1:1 by volume) for 15 hours; phenol red was used as a marker dye. The paper was then dried at 40°C for one hour. The paper was then cut to dimensions suitable for fitting on to a Shandon High-voltage flat plate electrophoresis kit. This involved cutting the paper at the phenol red marker, and reducing the width to about 25cm. (No radioactively-labelled peptides moved faster than the marker in the first dimension. This was shown by exposing the discarded piece of paper to X-ray film - no labelled material was observed). The final dimensions of the maps were about 25cm square. The second dimension was electrophoresis in pyridine - acetic acid - water (1:10:89 by volume) at pH 3.5 at 2KV for 1.5 hours. The paper was dried at 100°C for 30 minutes. It was then placed in contact with Kodak Blue-Brand X-ray film for 2-4 weeks, and then the film was developed.

J. EXTRACTION OF CHLOROPLAST NUCLEIC ACIDS.

Chloroplast nucleic acids were extracted by a procedure based on the phenol - detergent method of Parish and Kirby (1966). All operations were performed at 4°C and acid-washed glassware was used to minimise contamination by ribonuclease.

To each 0.5ml reaction mixture of chloroplasts, another 0.5ml of KCl resuspension /

resuspension medium was added. An equal volume (1ml) of 20mM tris-HCl (pH 7.6); 100mM KCl; 2% (w/v) tri-isopropylnaphthalenesulphonic acid, (TNS); 12% (w/v) 4-aminosalicylic acid, (PAS); 6% (v/v) phenol-cresol was added and the suspension mixed thoroughly. The organic and aqueous phases were separated by centrifugation at 1 000 x g for 10 minutes (r_{av} 21.6cm). The aqueous layer was removed and re-extracted with an equal volume of phenol-cresol solution. Nucleic acid in this layer was precipitated by adding 2.5 volumes of absolute ethanol (stored at -20°C) and leaving overnight at 4°C. The precipitate was collected by centrifugation at 1 000 x g for 10 minutes (r_{av} 21.6cm), and was then washed twice by resuspending in 5ml 80% (v/v) ethanol containing 50mM NaCl. Traces of ethanol in the pellet were removed by standing the tubes, inverted, over paper tissue for 30 minutes. The pellet was then dissolved in 100µl of E buffer (Bishop et al, 1967) containing 36mM tris, 30mM NaH_2PO_4, 1mM EDTA (disodium salt), 0.2% (w/v) SDS and 7% (w/v) sucrose, pH 7.8.

The concentration of nucleic acid was assessed by the ultraviolet absorption spectrum obtained in a Unicam SP800 recording spectrophotometer. It was assumed that a 1mg/ml solution of nucleic acid has an absorption of 20 absorbance units at 260nm near neutrality.

K. FRACTIONATION OF CHLOROPLAST NUCLEIC ACIDS BY POLYACRYLAMIDE GEL ELECTROPHORESIS.

The methods used are essentially those of Loening (1967), as modified by Leaver (1973). Polyacrylamide gels were prepared as follows. A stock solution of acrylamide containing 15%(w/v) acrylamide and 0.75% (w/v) bisacrylamide was prepared and stored at 4°C. Before use, the acrylamide was recrystallised from chloroform, and the bis-acrylamide from acetone. Electrophoresis was performed in E buffer, the composition of which is described in Section II2J, except that sucrose was omitted from the buffer. The buffer was made up 5 fold concentrated, and stored at 4°C.

In the preparation of 2.4% acrylamide gels, 5.0ml stock acrylamide solution, 6.25ml 5 fold concentrated buffer and 19.75ml distilled water were pipetted into a round-bottomed flask. This solution was de-gassed with a vacuum pump for 30 seconds. To this solution, 25µl TEMED followed by 0.25ml of freshly prepared 10% (w/v) ammonium persulphate were added and mixed by gentle swirling. The solution was transferred into perspex tubes (internal diameter 6mm) with a Pasteur pipette. The top surface/

surface of the gel column was carefully overlaid with a small volume of distilled water, delivered from a Pasteur pipette with a finely drawn-out tip. The gel tubes were held vertically in a rubber rack, and had short lengths of glass rod surrounded by polypropylene 'O' rings inserted in the bottom of the gel tube; this kept the polymerisation mix in the running tube. After polymerisation was complete (about 30 minutes at room temperature) the short lengths of glass rod were removed, the geltubes inserted into the electrophoresis tank, E buffer was added, and the gels were then pre-run at a potential of 50V for at least 30 minutes. Both the pre-running and the electrophoresis of the nucleic acid samples were performed at 4°C.

Nucleic acid samples were dissolved in E buffer plus 7% sucrose and the nucleic acid concentration was then determined (Section II2J). A total of 40µg of nucleic acid was loaded on each gel with a microsyringe. Electrophoresis was performed at 50V, 5mA/gel for 5 hours at 4°C. After electrophoresis, gels were removed from their running tubes by inverting the tubes over test tubes containing distilled water. Gentle air pressure was applied to the lower end of the gel tubes.

Gels were soaked in distilled water for one hour before scanning, in order to wash out ultraviolet-absorbing background material. The gels were then scanned at 265nm in a Joyce-Loebl Chromoscan recording densitometer.

L. ESTIMATION OF CHLOROPHYLL.

Chlorophyll was measured by the method of Arnon (1949). Aliquots (0.1-0.5ml) of chloroplast suspensions were made 80% (v/v) with respect to acetone, in a final volume of 5ml. The extracts were filtered to remove precipitated protein, and the extinction was read in a Unicam SP500 spectrophotometer at 640nm and 655nm against an 80% acetone blank. The chlorophyll concentrations were calculated from the formula:-

$$[\text{chlorophyll}]_{mg/l} = (20.2 \times E_{640} + 8.02 \times E_{655}).$$

M. ESTIMATION OF PROTEIN.

Two methods of protein estimation were used.

i. The method of Lowry, Rosebrough, Farr and Randall (1951).

This method was used to determine concentrations up to 1mg/ml. The following solutions were prepared.

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

Solution /

Solution B 50ml (w/v) Na_2CO_3 + 1ml Solution A.

Solution C Diluted Folin-Ciocalteau reagent. This was made 1M in acid by diluting 1:1 with distilled water. The acidity was checked by titration against standard NaOH using phenolphthalein as indicator.

The standard curve was prepared as follows. A 1mg/ml standard solution of BSA was made up in 1M NaOH. Aliquots (0.05-0.7ml) were taken and made up to 0.7ml with 1M NaOH. Solution B (7.0ml) was added, and the mixture was then left to stand for 15 minutes. Solution C (0.7ml) was added, mixed and the samples were left to stand for a further 30 minutes. The extinction of each sample was read at 750nm and the standard curve plotted. A typical standard curve is shown in fig. 6. The curve is biphasic, as noted by Campbell and Sargent (1967). Protein determinations were made on either of the straight lines.

Aliquots of unknown protein samples were precipitated with 2ml 10% TCA and left at 4°C for at least one hour. They were then centrifuged at 1 000 x g for 10 minutes on an MSE bench centrifuge. The supernatant was removed and the precipitate dissolved in 0.7ml 1M NaOH. Solution B (7.0ml) was added, mixed, and left for 15 minutes. Solution C (0.7ml) was added, and the samples left for 30 minutes. Extinctions were read at 750nm and the protein concentration determined by reference to the standard curve.

ii. The Biuret method (Gornall, Bardawill and David, 1949).

This method was used to determine protein concentration between 1 and 5mg/ml. The Biuret reagent was prepared as follows. $CuSO_4.5H_2O$ (1.50g) and sodium potassium tartrate (6.0g) were transferred to a 1 litre volumetric flask. They were then dissolved in approximately 500ml distilled water, to which 300ml of 10% (w/v) NaOH was added, and the solution was made up to the mark with distilled water. The reagent was stored in a polythene bottle.

The procedure for protein determination was as follows. To 4.0ml of reagent was added 1.0ml of sample or standard (using a stock 5mg/ml solution of BSA in 1.0N NaOH as standard). The mixture was allowed to stand for 30 minutes at room temperature and the extinction read at 540nm against a reagent blank. A typical standard curve is shown in fig. 7, showing good linearity in the range used.

Figure 6. Standard curve for the Lowry method of protein estimation.

Protein determinations were carried out on samples containing 0 to 1.0mg/ml BSA, and the extinction measured at 750nm (E_{750}). The method is described in Section II2Mi.

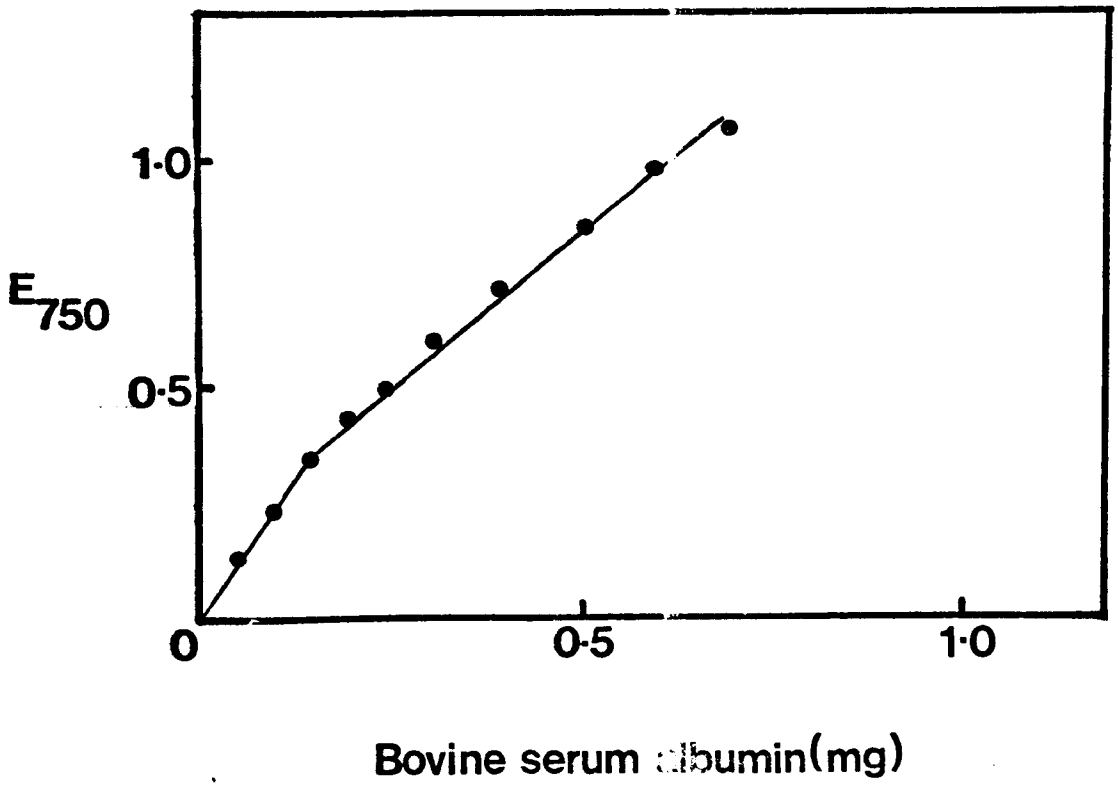
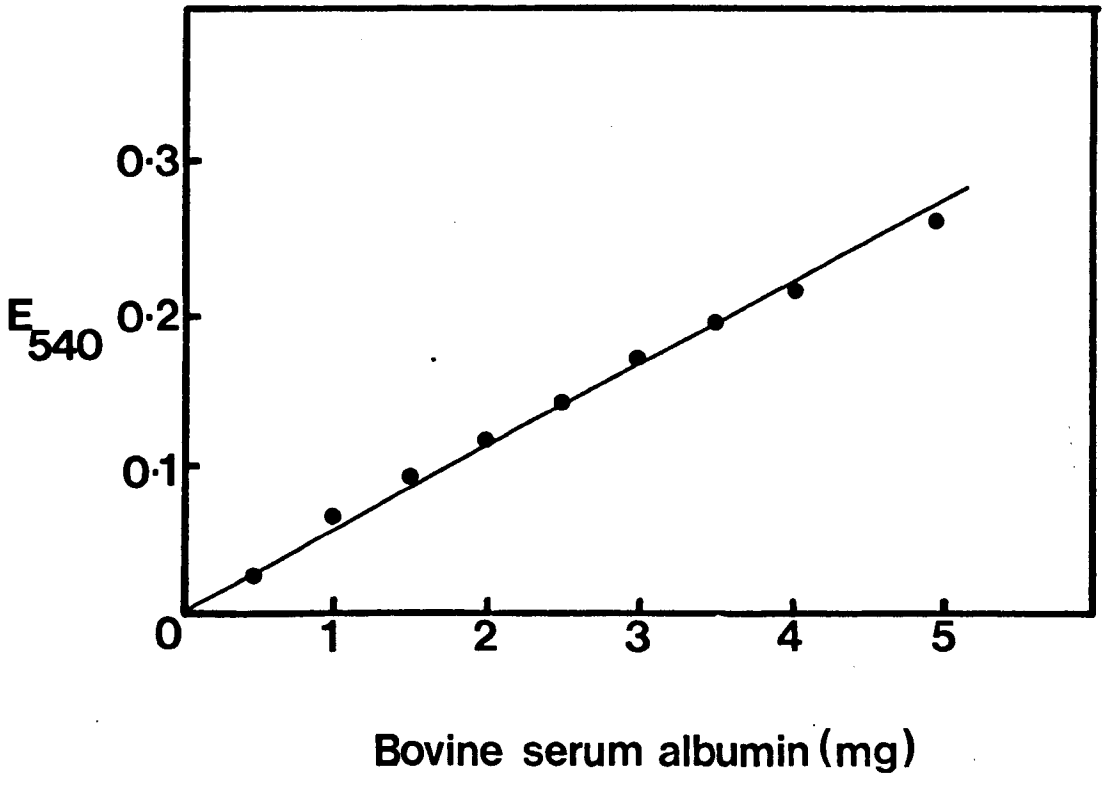

1·0
E_{750}
0·5
0
0·5
1·0
Bovine serum albumin(mg)

Figure 7. Standard curve for the Biuret method of protein estimation.

Protein determinations were carried out on samples containing 0 to 5.0mg/ml BSA, and the extinction measured at 540nm (E_{540}). The method is described in Section II2Mii.


0·3
0·2
0·1
0
E_{540}
1
2
3
4
5
Bovine serum albumin (mg)

SECTION III - RESULTS AND DISCUSSION

THE CHARACTERISTICS OF AMINO ACID INCORPORATION BY ISOLATED CHLOROPLASTS

1. INTRODUCTION.

In an attempt to identify and characterise the products of *in vitro* protein synthesis by isolated chloroplasts, it was decided initially to make a careful choice of chloroplast preparation. The aim was to obtain a chloroplast preparation which showed high rates of amino acid incorporation into protein, in order to aid the identification of newly-synthesised polypeptides. In addition, the preparation should also closely reflect the nature and pattern of protein synthesis which occurs *in vivo*. Recent advances in our knowledge of the control of carbon metabolism during photosynthesis have been achieved by the use of rapid methods of chloroplast isolation. These methods yield preparations which contain a high percentage of intact chloroplasts i.e. chloroplasts which have complete, unbroken outer double membranes. Rates of carbon dioxide fixation in such isolated chloroplasts approach those measured *in vivo* (Walker and Crofts, 1970; Jensen and Bassham, 1966; Bucke, Walker and Baldry, 1966). It might be argued that in intact chloroplasts no dilution occurs of the Calvin cycle enzymes or of the enzymes and factors associated with the photosynthetic electron transport pathway. By analogy, high rates of amino acid incorporation might be achieved by the use of intact chloroplast preparations since none of the tRNAs, enzymes or factors involved in protein synthesis would be diluted or inactivated. In addition, if chloroplast ribosomes are preserved in a biochemical environment which resembles that which exists *in vivo*, the chances are increased that the translation process will proceed with fidelity.

It is known that the outer double membrane of the chloroplast is relatively impermeable to ATP, the rate of transfer of ATP across the chloroplast envelope being of the order of 7 to 9 μmoles/mg chlorophyll/h (Heber and Santarius, 1970). By using ATP synthesised *in situ* in intact chloroplasts by photophosphorylation, higher rates of amino acid incorporation might be expected than if exogenous ATP was used as an energy source. Under the influence of light, ADP formed due to synthesis of protein will be quickly re-phosphorylated; no intermediates (such as ferredoxin) or catalysts (such as phenazine methosulphate) of photophosphorylation need to be added. Broken chloroplasts, on the other hand, will only phosphorylate ADP when such intermediates and catalysts are added (Tagawa, Tsujimoto and Arnon, 1963). With/

With these points in mind, several rapid methods of chloroplast isolation were tested for light-driven and ATP-driven [C^{14}]leucine incorporation. The ideal chloroplast preparation for *in vitro* protein synthesis studies would be one which showed a high rate of light-dependent amino acid incorporation, and which also contained a high proportion of intact chloroplasts. Rapid, crude preparations were used since it was felt that they would possess greater activity than highly purified chloroplast preparations.

Criteria of intactness of chloroplasts were then considered. The most widely used criterion is the appearance of the chloroplasts under both phase contrast and electron microscopy (Kahn and von Wettstein, 1961). In addition, more objective, biochemical criteria were sought. If amino acid incorporation is dependent on light only, this strongly suggests that protein synthesis is taking place in intact chloroplasts since, as already mentioned, broken chloroplasts will phosphorylate only in the presence of substrates and catalysts. The sensitivity of incorporation to ribonuclease was also examined. Ribonuclease cannot cross the outer membrane of the chloroplast (Margulies, Gantt and Parenti, 1968) - if amino acid incorporation in chloroplasts is insensitive to ribonuclease, this strongly suggests that protein synthesis is taking place in intact chloroplasts.

Contamination of chloroplast preparations by cytoplasmic ribosomes, mitochondria, nuclei or bacteria must be minimised. Biochemical evidence, rather than direct plating, was used to assess bacterial contamination. The extent of contamination by cytoplasmic ribosomes was judged not only by the use of specific inhibitors of ribosome function, but also by analysis of the nucleic acids present in the chloroplast preparation.

2. ISOLATION OF CHLOROPLASTS.

A. Comparison of several methods of isolation with respect to both the rate of amino acid incorporation and the yield of intact chloroplasts.

The ability of several chloroplast preparations to use either light or added ATP as an energy source for the incorporation of [C^{14}] leucine into a hot TCA-insoluble product was compared (Table 2). In addition, the fraction of intact chloroplasts in each preparation was determined/

Table 2. Incorporation of [C^{14}]leucine and yield of intact chloroplasts in several chloroplast preparations.

Method	[C^{14}]leucine incorporation (pmoles/mg chlorophyll/h) +Light	-Light	-Light+ATP	% Intact chloroplasts
1. Ramirez et al (1968) -resuspended in KCl medium	493	26	284	46%
2. Ramirez et al (1968) -resuspended in sucrose medium	60	4	57	61%
3. Jensen and Bassham (1966)	25	5	13	80%
4. Nobel (1967)	96	95	97	78%
5. Walker (1968)	42	4	21	68%

Chloroplasts were isolated from 10 day old pea leaves by methods described in Section II2A. The conditions of incubation and the determination of hot TCA-insoluble radioactivity is described in Section II2Ci.

determined by quantitative phase contrast microscopy.

The highest rates of light-stimulated amino acid incorporation were shown by chloroplasts prepared by the method of Ramirez et al (1968). These chloroplasts, resuspended in the KCl medium, showed the highest rate of incorporation achieved by any preparation under any incubation conditions. Generally a 20 fold stimulation due to light and a 10 fold stimulation due to exogenous ATP was observed, compared to controls incubated in the absence of light. When chloroplasts isolated by the same method but resuspended in sucrose medium were examined, the incorporation rate was lower than that obtained in the KCl medium. In the sucrose medium the rates of both light-driven and ATP-driven incorporation were of the same order. Chloroplasts which were resuspended in KCl medium while showing the highest rates of incorporation, contained the lowest percentage of intact chloroplasts of all methods considered, generally being around 50% intact.

Chloroplasts prepared by the methods of Walker (1968), and of Jensen and Bassham (1966) showed a 5 to 10 fold stimulation of [C^{14}] leucine incorporation when light was used as the energy source. When ATP and an ATP-generating system was used, rates of incorporation midway between the low rate obtained in the dark and the rate obtained in the light were observed. However, chloroplasts prepared by the method of Nobel (1967) show a similar rate of incorporation irrespective of the energy source.

Broken chloroplasts are often used to study chloroplast protein synthesis (Chen and Wildman, 1970). Table 3 shows the results of [C^{14}]leucine incorporation by chloroplasts isolated by the method of Ramirez et al (1968) and resuspended in TMS resuspension medium.

Table 3/

Table 3. Amino acid incorporation by a preparation of broken chloroplasts.

Incubation conditions	[C^{14}]leucine incorporation. (pmoles/mg chlorophyll/h)
Complete	35
Complete + light	36
Omitting ATP and ATP-generating system	2

Chloroplasts were isolated and resuspended as described in Section II2Av. Incubation was carried out using the ATP-generating system and cofactors for use with chloroplasts resuspended in TMS resuspension medium (Section II2Cb). [C^{14}]leucine incorporation was measured as described in Section II2Ci.

Rates of amino acid incorporation are much lower than those in light-driven protein synthesis by chloroplasts isolated using rapid techniques. In broken chloroplasts, light does not stimulate incorporation over the amount shown by the complete system with ATP as energy source.

B. Phase contrast microscopy of chloroplast preparations.

Chloroplasts isolated by the procedure of Ramirez et al (1968) and resuspended in either KCl, sucrose or TMS medium were observed in the light microscope using phase optics. Clear differences were observed between all three preparations, and the photographs of the preparations shown in Plates 1, 2 and 3 to some extent show this. Photographs rarely match the naked eye in the detail which can be observed; much depends on the depth of focus, conditions of photography and the processing of the film.

Plate 1 shows chloroplasts resuspended in sucrose resuspension medium. The most striking features are the slightly granulated appearance of the chloroplasts, due to the stacking of thylakoids into grana (Wildman et al, 1962), and the bright, refractile haloes around most of the chloroplasts. This preparation contained a high proportion of intact chloroplasts (Table 2). Plate 2, on the other hand, shows chloroplasts resuspended in TMS medium. The chloroplasts appear dark and lack the surrounding brightness of the sucrose-resuspended chloroplasts. This preparation contained only broken chloroplasts/

Plate 1. Phase contrast microscopy of pea chloroplasts isolated by the procedure of Ramirez et al (1966) and resuspended in sucrose medium (see Section II2Aiv).

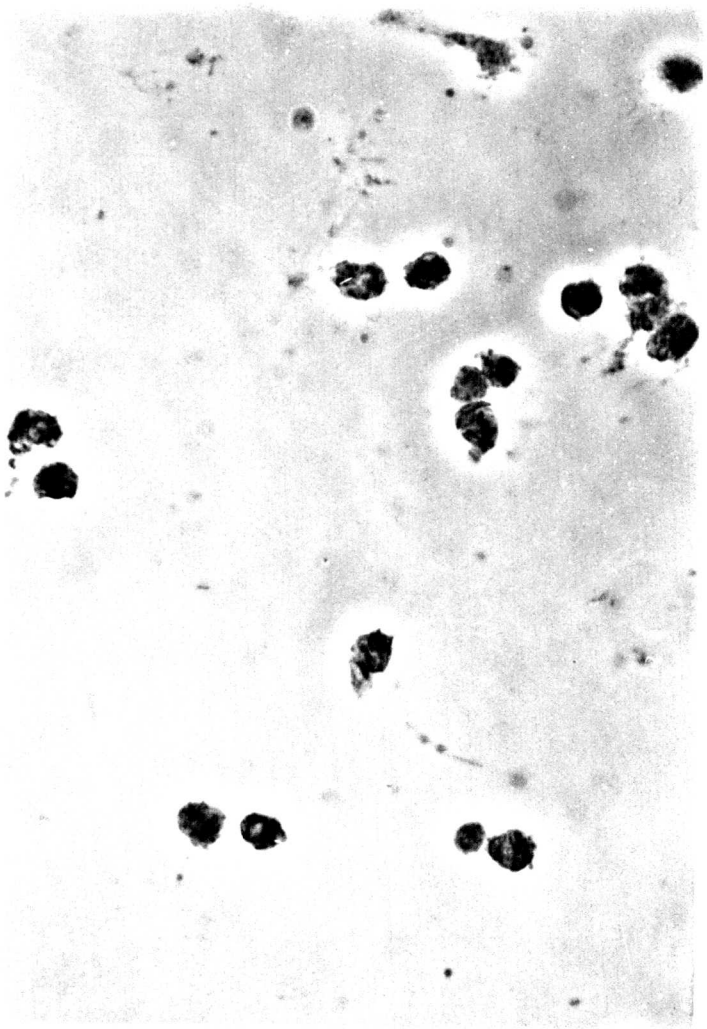


Plate 2. Phase contrast microscopy of pea chloroplasts isolated by the procedure of Ramirez et al (1968) and resuspended in TMS medium (see Section II2Av).

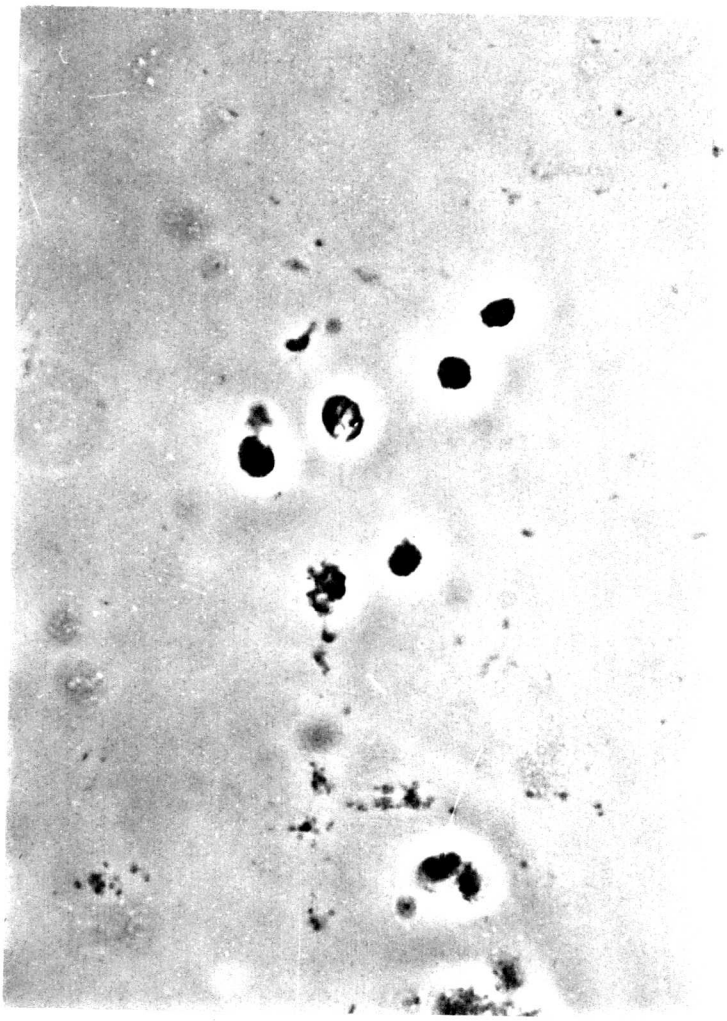


Plate 3. Phase contrast microscopy of pea chloroplasts isolated by the procedure of Ramirez et al (1966) and resuspended in KCl medium (see Section II2Aiv).

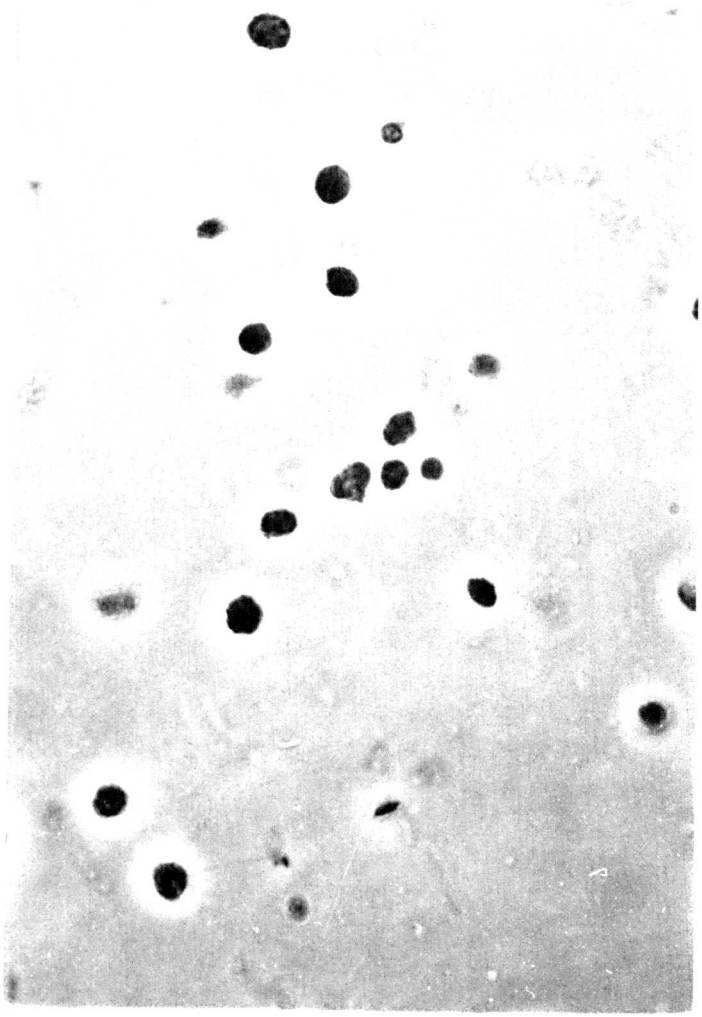


Plate 4. Electron microscopy of a section of pea chloroplasts isolated by the procedure of Ramirez et al (1966) and resuspended in KCl medium (see Section II2Aiv). I, intact chloroplast; b, broken chloroplast; ce, chloroplast envelope; gr, granum; la, lamella; st, stroma region.


B
I
ce
gr
st
la

chloroplasts. Plate 3 shows chloroplasts resuspended in KCl medium. The chloroplasts, while possessing bright haloes, lack the granulated appearance shown by sucrose-resuspended chloroplasts. The preparation of KCl-resuspended chloroplasts contained 40-50% intact chloroplasts (Table 2).

C. Electron microscopy of chloroplast preparations.

Only chloroplasts resuspended in KCl medium were examined by electron microscopy. The results are shown in Plate 4. The chloroplasts show a slightly swollen appearance, when compared with published micrographs of chloroplasts either in the intact leaf cell or resuspended in 0.35M sucrose medium (Kirk and Tilney-Bassett, 1967; Whitfeld, Spencer and Bottomley, 1972). Both broken and intact chloroplasts are present. The intact chloroplasts have complete outer envelopes, and possess recognisable lamellae, grana and a dense, granulated stroma region. The broken chloroplasts lack this type of ordered structure, and appear to consist mainly of free lamellae.

3. THE SOURCE OF ENERGY FOR AMINO ACID INCORPORATION.

Pea chloroplasts, isolated by the procedure of Ramirez et al (1968) and resuspended in KCl medium show high, light-stimulated rates of amino acid incorporation into protein. Is it certain that this incorporation uses ATP synthesised by photophosphorylation? If so, is the phosphorylation of the cyclic or non-cyclic type, or both? These questions are partially answered by the results presented in Table 4.

Table 4/

Table 4. The dependence of protein synthesis on the source of energy.

Energy source	Treatment	% Incorporation
Light	Complete	100
None	Complete	3
ATP+ATP-generating system	Complete	50
Light+ATP+ATP-generating system	Complete	125
Light	+ CCCP ($5x10^{-6}$M)	6
Light	+ DCMU ($6x10^{-7}$M)	60

Chloroplasts were isolated from pea leaves by the method of Ramirez et al (1968) and resuspended in KCl medium as described in Section II2Aiv. Incubation was at 20°C for 40 minutes in the light (see Section II2Ci). Hot TCA-insoluble [C^{14}]leucine incorporation was determined (Section II2Ci). Results are expressed as percentages of the incorporation by the complete, light-driven system.

ATP could replace light as an energy source for protein synthesis, but did not give such high rates as light alone. ATP as well as light showed little stimulation; the effect of light and ATP were not additive.

m-chlorocarbonyl cyanide phenylhydrazone,(CCCP) is an uncoupler of oxidative phosphorylation and an inhibitor of photophosphorylation (Avron and Shavit, 1965). CCCP had a strongly inhibitory effect on light-driven amino acid incorporation. 3-(3,4-dichlorophenyl)-1,1-dimethylurea,(DCMU) at low concentration specifically inhibits non-cyclic photophosphorylation (Avron and Neumann, 1968); however, chloroplasts did incorporate some [C^{14}]leucine when this inhibitor was present in the light, although at a decreased rate.

4. THE IONIC AND OSMOTIC REQUIREMENTS FOR AMINO ACID INCORPORATION.

As shown in part 2A of this Section, chloroplasts isolated by the procedure of Ramirez et al (1968) and resuspended in sucrose medium showed lower rates of light-driven amino acid incorporation than chloroplasts resuspended in KCl medium. The reason for this might lie in a difference between the osmolarities of the two resuspension media, or the explanation might lie in a requirement for potassium chloride for protein synthesis. Accordingly an experiment was/

Figure 8. The dependence of light-driven protein synthesis on KCl.

Pea chloroplasts were isolated by the procedure of Ramirez et al (1968), described in Section II2Aiv. They were then resuspended in the following media:- 66mM tricine-KOH (pH 8.3), 6.6mM $MgCl_2$, KCl varying from 0 to 0.4M (●———●); 66mM tricine-NaOH (pH 8.3), 6.6mM $MgCl_2$, NaCl varying from 0 to 0.4M (■———■); 66mM tricine-KOH (pH 8.3), 6.6mM $MgCl_2$, sucrose varying from 0 to 0.4M (▲———▲). Incubation was at 20°C for 40 minutes in the light. $[S^{35}]$methionine incorporation was measured as described in Section II2Ci.

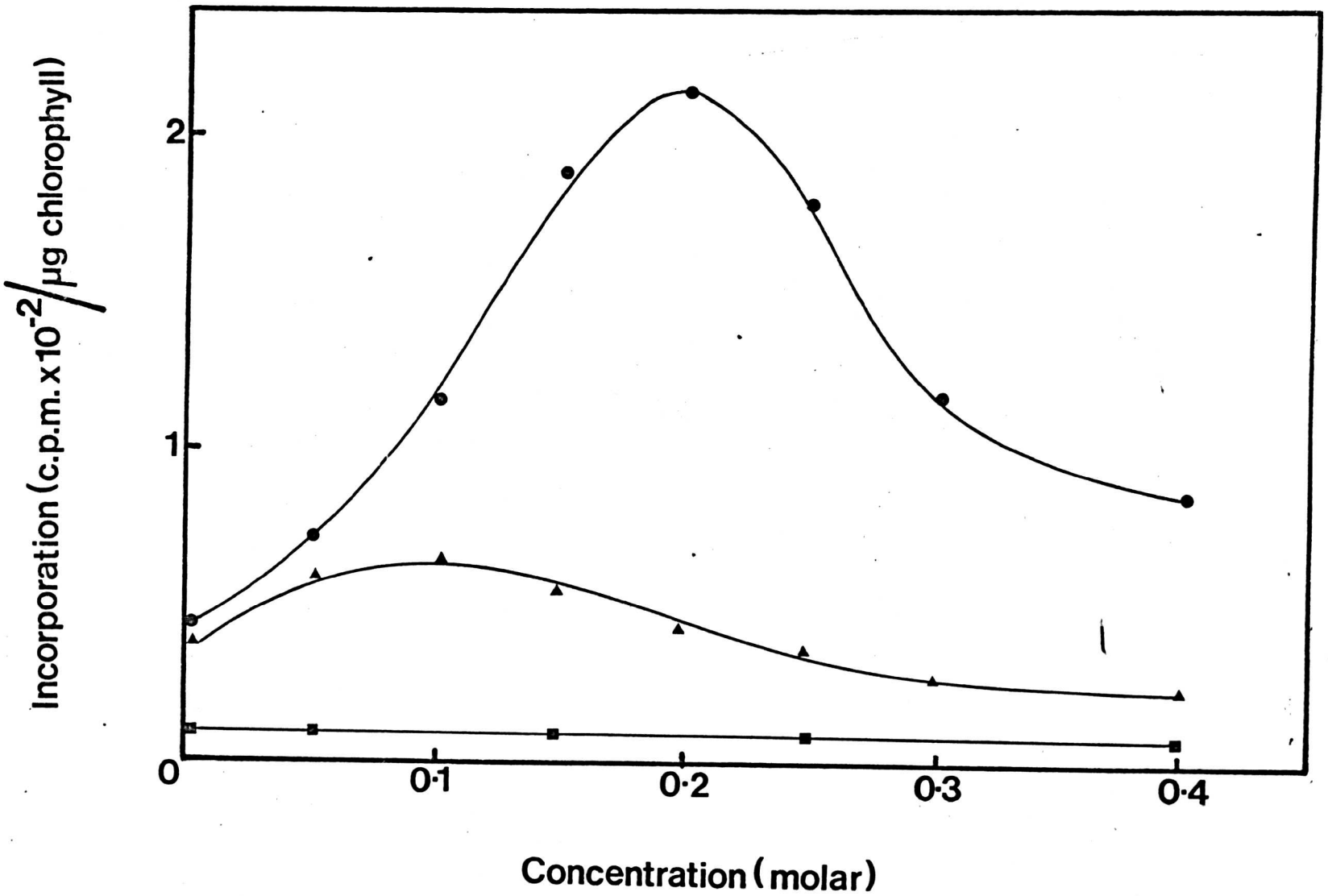
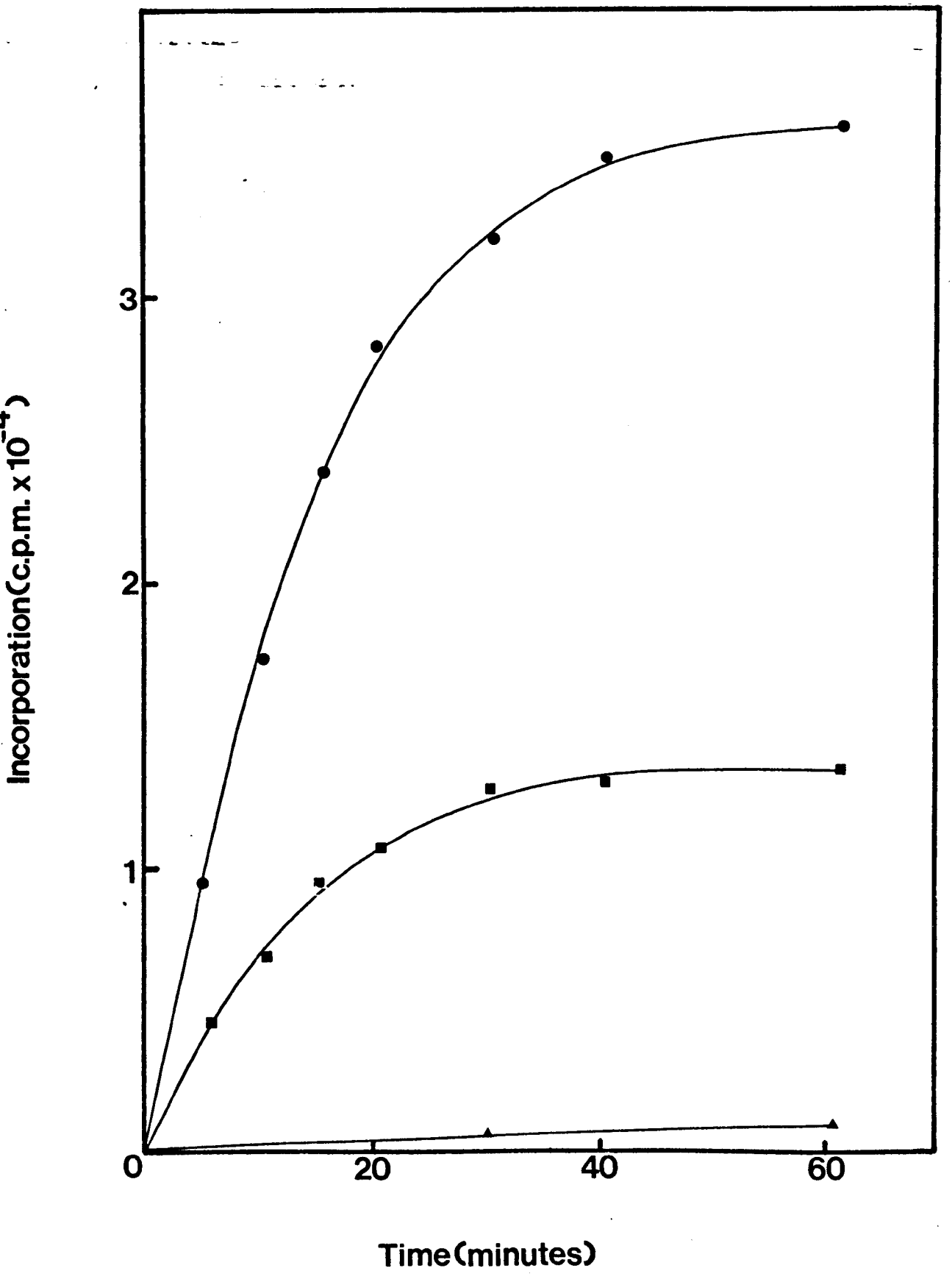

Incorporation (c.p.m. x10^{-2}/µg chlorophyll)
2
1
0
0·1
0·2
0·3
0·4
Concentration (molar)

Figure 9. The time course of light-driven protein synthesis.

Pea chloroplasts were isolated by the method of Ramirez et al (1968) and incubated in KCl medium as described in Section II2Aiv and II2Ci. At the indicated times, reactions were stopped by adding 0.5ml of a saturated solution of unlabelled L-leucine and 1.0ml of 10% (w/v) TCA to the 500µl incubation mixture. [C^{14}]leucine incorporation was measured as described in Section II2Ci. (●——●) 400µg chlorophyll per incubation mixture; (■——■) 100µg chlorophyll per incubation mixture; (▲——▲) 400µg chlorophyll per incubation mixture, but incubated in the absence of light.


Incorporation(c.p.m. x10^{-4})
3
2
1
0
20
40
60
Time(minutes)

was designed to assay amino acid incorporation at a range of sucrose and potassium chloride concentrations, keeping the buffer and magnesium chloride concentrations in the resuspension media constant. To separate the effects of potassium and chloride ions, a series of concentrations of sodium chloride was also included. The results are shown in fig. 8. Incorporation in KCl medium was greater than incorporation in sucrose medium at all concentrations measured. Optimal incorporation in KCl medium occurred at 0.2M, the concentration routinely used in the KCl resuspension medium. A concentration of 33mM potassium ions is present in the sucrose resuspension medium, since KOH is used to adjust the tricine buffer. However, this concentration is clearly not sufficient to give a high rate of protein synthesis. When NaCl replaced KCl, very low levels of incorporation resulted.

When chloroplasts were intentionally lysed by resuspending in medium lacking KCl (but containing tricine buffer and magnesium chloride), subsequent restoration of the KCl concentration to 0.2M did not restore the ability to incorporate amino acids into protein using ATP as energy source.

5. THE TIME COURSE OF THE REACTION.

A. Amino acid incorporation.

The time course of [C^{14}]leucine incorporation into protein is shown in fig. 9. The rate of incorporation begins to fall at 20 minutes and reaches zero by about 40 minutes. The final amount of incorporation (i.e. after 60 minutes of incubation) was dependent on the chlorophyll concentration of the chloroplast preparation. Incorporation in the absence of light was less than 5% of the complete, light-driven rate throughout the time of incubation.

B. Morphological changes shown by electron and phase contrast microscopy.

Changes in the structure of the chloroplasts during incubation were monitored both by phase contrast and by electron microscopy. Chloroplasts were isolated by the procedure of Ramirez et al (1968) and resuspended in KCl medium. Samples were taken at time zero (before incubation), after 15 minutes of incubation (on the linear part of the amino acid incorporation time course) and after 60 minutes of/

Plate 5. Phase contrast microscopy of pea chloroplasts isolated by the procedure of Ramirez *et al* (1968) as described in Section II2Aiv and incubated in KCl medium in the light for 15 minutes.

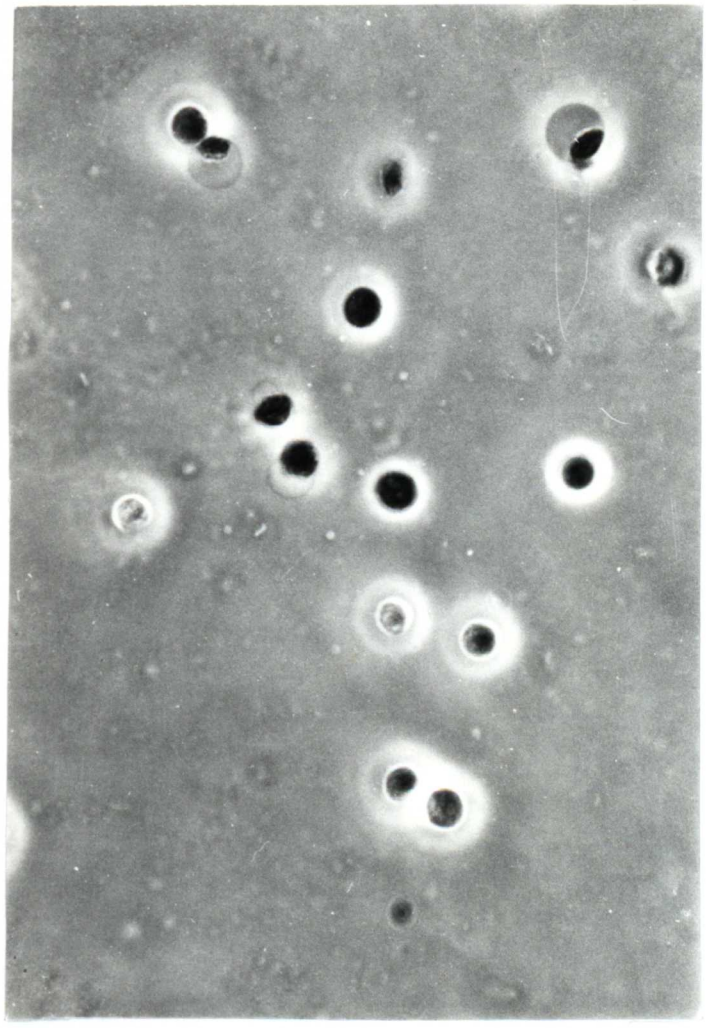


Plate 6. Phase contrast microscopy of pea chloroplasts isolated by the procedure of Ramirez et al (1968) as described in Section II2Aiv and incubated in KCl medium in the light for 60 minutes.

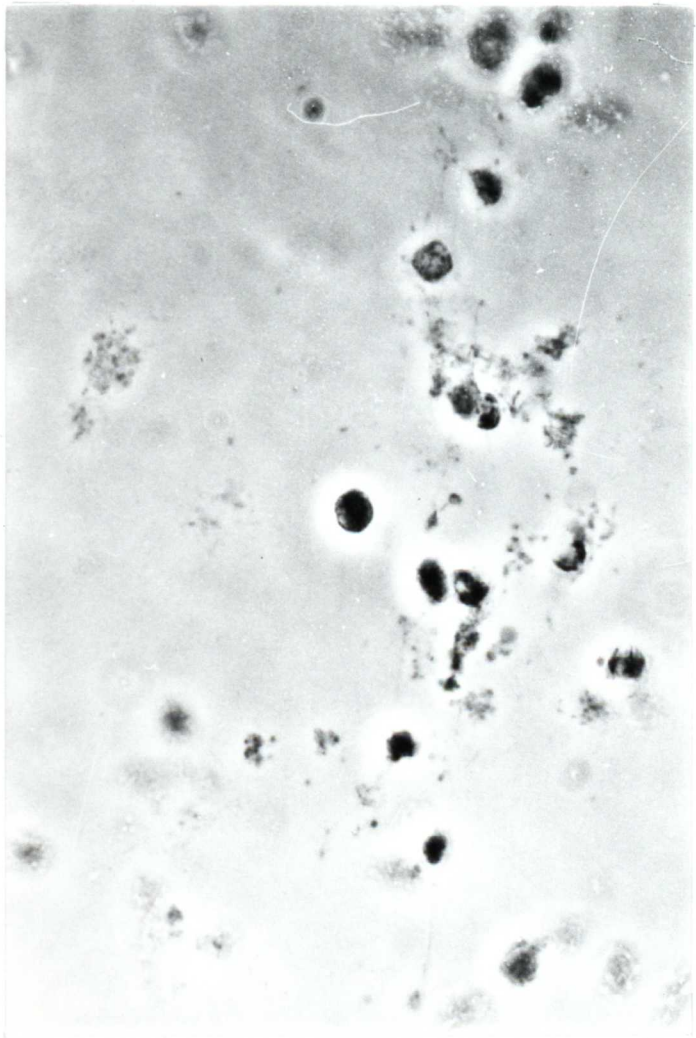
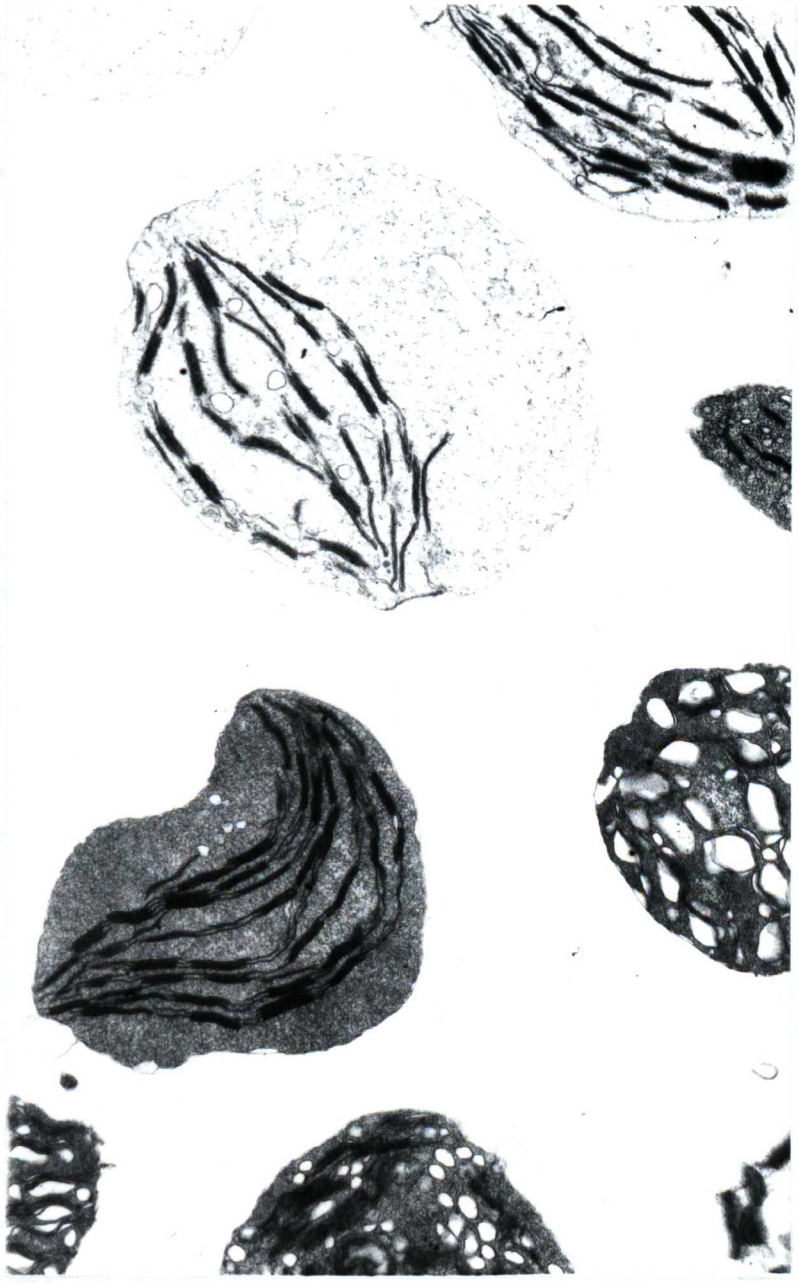


Plate 7. Electron microscopy of a section of pea chloroplasts isolated by the procedure of Ramirez *et al* (1968) as described in Section II2Aiv and incubated in KCl medium in the light for 15 minutes.

Plate 8. Electron microscopy of a section of pea chloroplasts isolated by the procedure of Ramirez et al (1968) as described in Section II2Aiv and incubated in KCl medium for 60 minutes.



of incubation at 20°C in the light (when amino acid incorporation had ceased).

Photographs of the specimens viewed by phase contrast microscopy are shown in plates 3, 5 and 6. Plate 3 has already been discussed (in Section III2B). Plate 5 shows KCl chloroplasts after 15 minutes of incubation in the light. Essentially no morphological changes can be discerned - the chloroplasts still have a dark appearance surrounded by bright haloes. Similarly after 60 minutes (plate 6), intact chloroplasts are still visible.

Examination of the same samples by electron microscopy (plates 4, 7 and 8) confirms this conclusion, that no obvious ultrastructural changes take place in the light. The outer envelopes of the chloroplasts remain intact, and the internal structure of the chloroplasts remains the same.

The photographs discussed in this section show only a small sample of the chloroplasts present in the preparation. However, when samples of chloroplasts were removed at points during the incubation and the proportion of intact chloroplasts was determined (Section II2Bia), it was found that the percentage of intact chloroplasts remained constant at about 50%, throughout the time of incubation. Thus no breakage of chloroplasts occurred during incubation.

6. THE EFFECT ON AMINO ACID INCORPORATION:-

A. of increasing [C^{14}]leucine concentration.

B. of increasing chlorophyll concentration.

In order to detect and analyse the products of <u>in vitro</u> chloroplast protein synthesis it is desirable to maximise the amount of radioactivity in newly-synthesised polypeptides. To this end, the relationship between [C^{14}]leucine incorporation and increasing chlorophyll and [C^{14}]leucine amino acid concentration was examined.

A. The effect on amino acid incorporation of increasing [C^{14}]leucine concentration.

Fig. 10 shows that a linear relationship exists between [C^{14}] leucine incorporation and the number of μCi of [C^{14}]leucine added to the incubation mixture. This shows that the rate of amino acid incorporation obtained is not limited by the concentration of [C^{14}] leucine/

Figure 10. The relationship between light-driven [C^{14}]leucine incorporation and the number of µCi of [C^{14}]leucine supplied.

Pea chloroplasts were isolated by the procedure of Ramirez *et al* and resuspended and incubated as described in figure 9. The chlorophyll concentration was 0.235mg/ml. Up to 0.625µCi [C^{14}]leucine was supplied as radioactive precursor. Incubation was at 20°C for 60 minutes in the light. [C^{14}]leucine incorporation was determined as described in Section II2Ci. A control incubation in which 0.625µCi [C^{14}]leucine was supplied, but was incubated in the absence of light, showed 7% of the incorporation of the comparable incubation in the light.

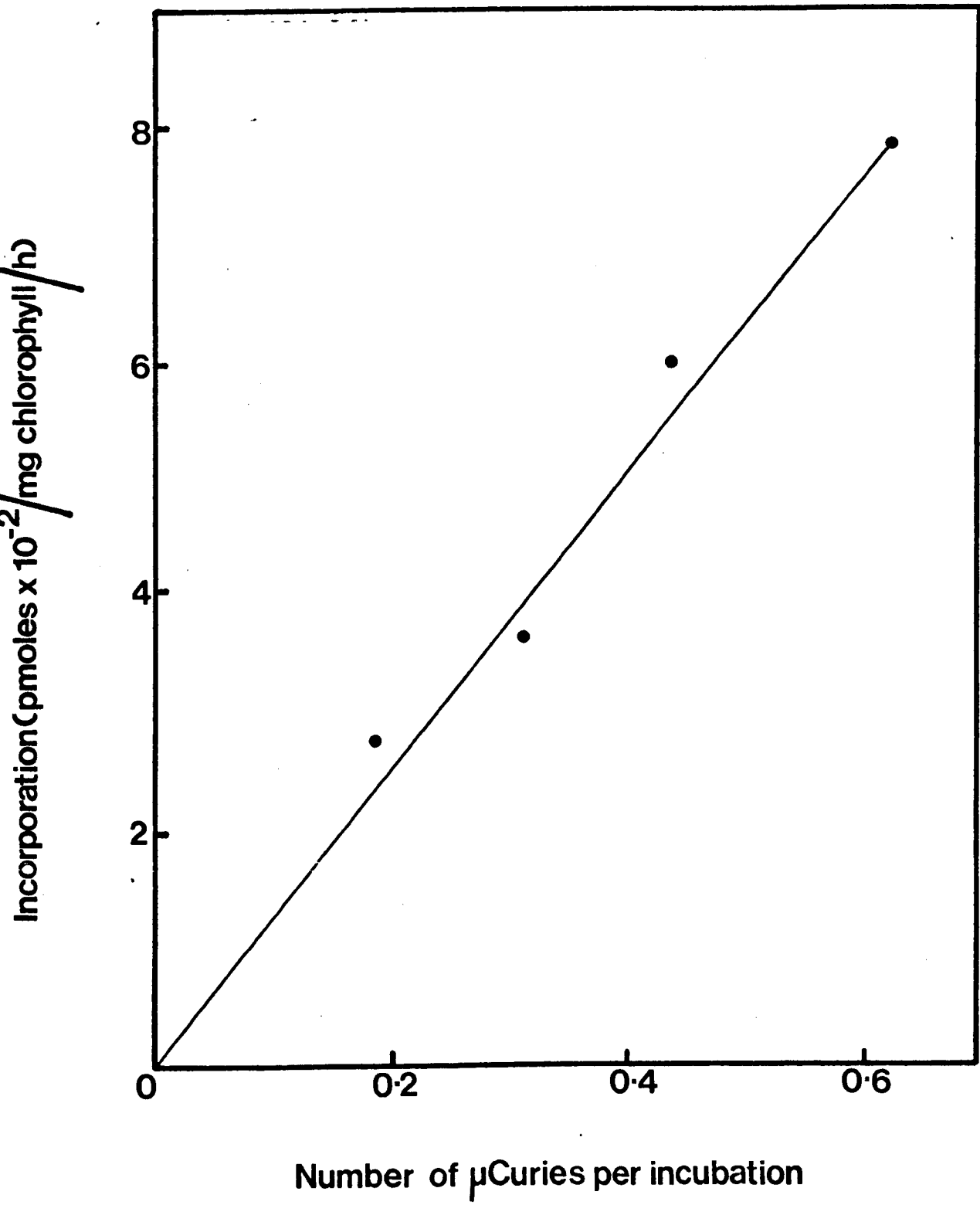
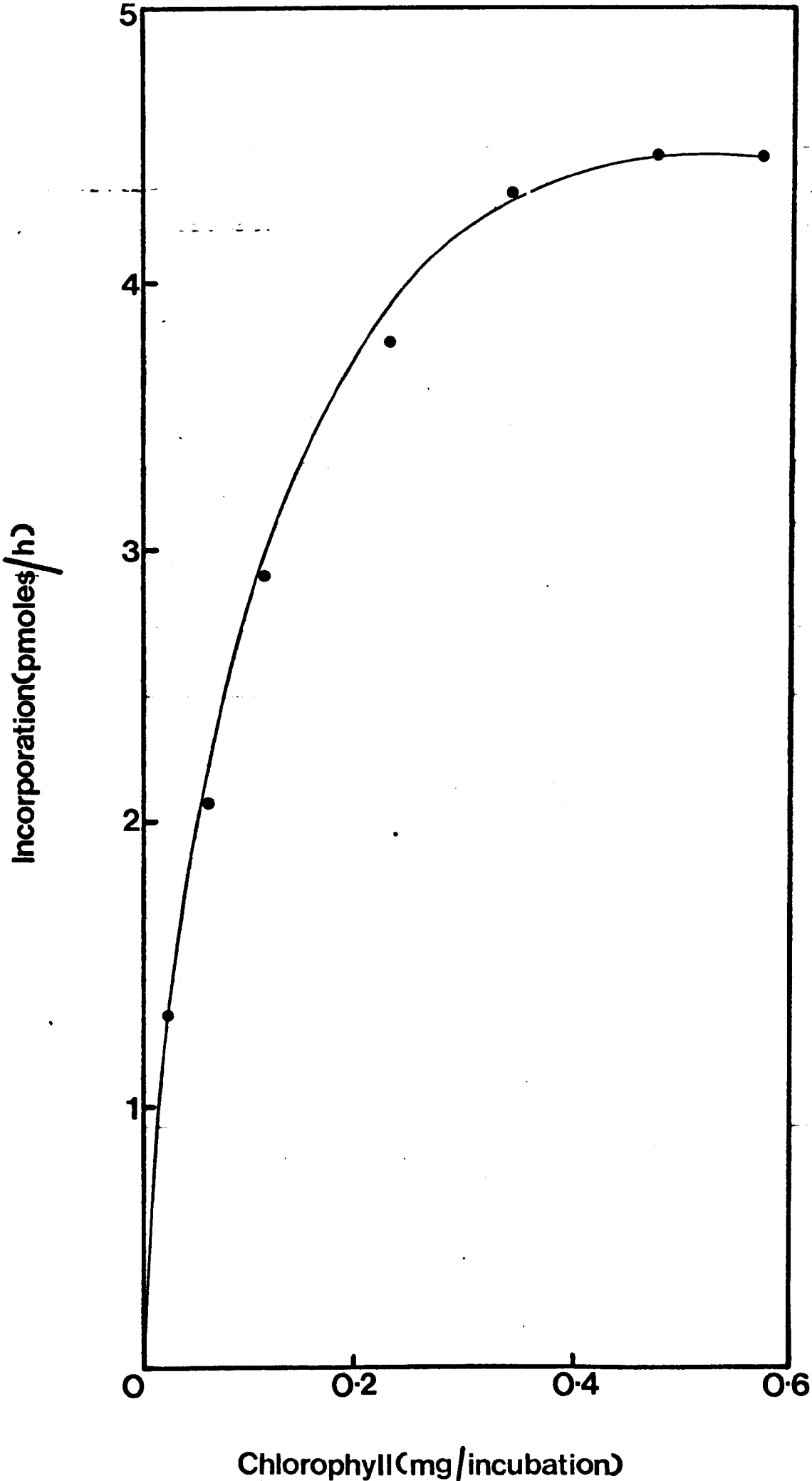

Incorporation (pmoles x 10^{-2} / mg chlorophyll / h)
8
6
4
2
0
0·2
0·4
0·6
Number of μCuries per incubation

Figure 11. The relationship between light-driven [C^{14}]leucine incorporation and the chlorophyll concentration.

The details of the experimental procedures used are given in figure 10, except that 0.625μCi of [C^{14}]leucine was supplied to each 500μl incubation, and the chlorophyll concentration varied between 0 and 1.12mg/ml.


5
4
3
2
1
0
0·2
0·4
0·6
Incorporation(pmoles/h)
Chlorophyll(mg/incubation)

leucine present in the incubation mixture. It can be calculated that over the range tested, 3% of the [C^{14}]leucine supplied is incorporated into protein.

B. The effect on amino acid incorporation of increasing chlorophyll concentration.

The results shown in fig. 11 indicate that amino acid incorporation levels off at about 350µg of chlorophyll per incubation mixture volume of 500µl. Above this chlorophyll concentration, incorporation is presumably limited by the fact that the dense suspension of chloroplasts is not exposed to saturating levels of light under the conditions of incubation.

7. THE EFFECT OF 70S AND 80S RIBOSOMAL INHIBITORS ON CHLOROPLAST AMINO ACID INCORPORATION.

The sensitivity of amino acid incorporation by chloroplast suspensions to 70S ribosomal inhibitors would provide strong supporting evidence for the involvement of chloroplast ribosomes in protein synthesis.

Table 5. The effect of various inhibitors of 70S and 80S ribosomes on chloroplast amino acid incorporation.

Addition	% Incorporation
None	100
Cycloheximide (100µg/ml)	100
D-threo-chloramphenicol (50µg/ml)	5
L-threo-chloramphenicol (50ug/ml)	70
Lincomycin (100µg/ml)	28
Lincomycin (10µg/ml)	32
Lincomycin (1µg/ml)	40
Erythromycin (100µg/ml)	50

Chloroplasts were isolated, resuspended and incubated for 40 minutes as described in Table 4. Results are expressed as percentages of the incorporation by the complete, light-driven system.

The results presented in Table 5 show that amino acid incorporation is completely inhibited by D-threo-chloramphenicol, an inhibitor considered to be specific for 70S ribosomes, but was unaffected by cycloheximide/

cycloheximide, a frequently used inhibitor of 80S ribosomes. L-threo-chloramphenicol caused some inhibition of light-driven protein synthesis. However, when L-threo-chloramphenicol was tested on protein synthesis in the absence of light, but with ATP and an ATP-generating system as energy source, no effect was observed. The inhibition by L-threo-chloramphenicol can therefore be explained in terms of an inhibition of ATP synthesis; this isomer of chloramphenicol has been shown to inhibit oxidative phosphorylation by isolated mitochondria but not protein synthesis by isolated bacterial ribosomes (see Sections I3A and I5A). Erythromycin and lincomycin are also regarded as specific 70S ribosomal inhibitors. However, erythromycin caused less inhibition at 100µg/ml than lincomycin did at µg/ml.

8. SOLUBILISATION OF LABELLED CHLOROPLASTS BY TRITON X-100 DETERGENT.

Triton X-100 detergent has been reported to solubilise chloroplasts and mitochondria, but not nuclei, bacteria or whole leaf cells (Parenti and Margulies, 1967). This detergent could therefore be useful in assessing the contribution of bacteria, nuclei and whole cells to the total amino acid incorporation by chloroplast preparations. At the end of the incubation with [C^{14}]leucine, the labelled chloroplast suspension was solubilised with Triton X-100 at a final concentration of 2% (v/v) as described in Section II2E. A 10 000 x g pellet was prepared, and the [C^{14}]leucine incorporated into a hot TCA-insoluble product was determined.

Table 6. Solubilisation of a labelled chloroplast preparation by Triton X-100 detergent.

Treatment	[C^{14}]leucine incorporation (c.p.m.)	% Unsolubilised chloroplasts
Unsolubilised chloroplasts	37,035	100
10 000 x g pellet	226	0.61

Chloroplasts were isolated and incubated as described in Table 4. The labelled chloroplast preparation was treated with Triton X-100 as described in Section II2E. Amino acid incorporation into the 10 000 x g pellet and an unsolubilised chloroplast preparation was measured as described in Section II2Ci. Results are expressed as a percentage of/

of the incorporation by the unsolubilised chloroplast preparation.

Thus more than 99% of [C^{14}]leucine incorporated is released by Triton X-100 solubilisation of the chloroplast suspension, strongly suggesting that the amino acid incorporation is taking place in chloroplasts or mitochondria.

9. SENSITIVITY OF AMINO ACID INCORPORATION TO RIBONUCLEASE A.

Intact chloroplast envelopes exclude ribonuclease A (Margulies, Gantt and Parenti, 1968). Ribonuclease-insensitive amino acid incorporation by chloroplast preparations thus tends to imply that incorporation is taking place in intact organelles (assuming that the preparation is not heavily contaminated by bacteria). Accordingly, light-driven [C^{14}]leucine incorporation was assayed at several different ribonuclease concentrations.

Table 7. The sensitivity of light-driven amino acid incorporation by isolated chloroplasts to ribonuclease A.

Ribonuclease concentration (μg/ml)	[C^{14}]leucine incorporation (c.p.m./mg chlorophyll)	% Incorporation
0	25 200	100
0.03	24 400	97
0.30	25 400	101
3.00	22 700	90
30.00	25 900	103

Experimental details are given in Table 4 and Section II2Ci. Incubation was at 20°C for 40 minutes in the light. Results are expressed as percentages of the complete, light-driven system without added ribonuclease.

These results show quite clearly that no significant inhibition of incorporation occurred at any ribonuclease concentration tested. In addition, the time course of amino acid incorporation by these chloroplasts was quite insensitive to the presence of ribonuclease (fig. 12). This result is consistent with the microscopic evidence that the outer membranes of the chloroplasts remain intact throughout the time of incubation.

The possibility that the ribonuclease is inactivated in some manner/

Figure 12. The time course of light-driven [C^{14}]leucine incorporation in the presence and absence of ribonuclease.

Chloroplasts were isolated and incubated in KCl medium as described in figure 9. Each 500µl incubation contained 0.25µCi of [C^{14}]leucine and 95µg chlorophyll. [C^{14}]leucine incorporation was measured as described in Section II2Ci. (●——●) complete; (■——■) complete + ribonuclease (10µg/ml).

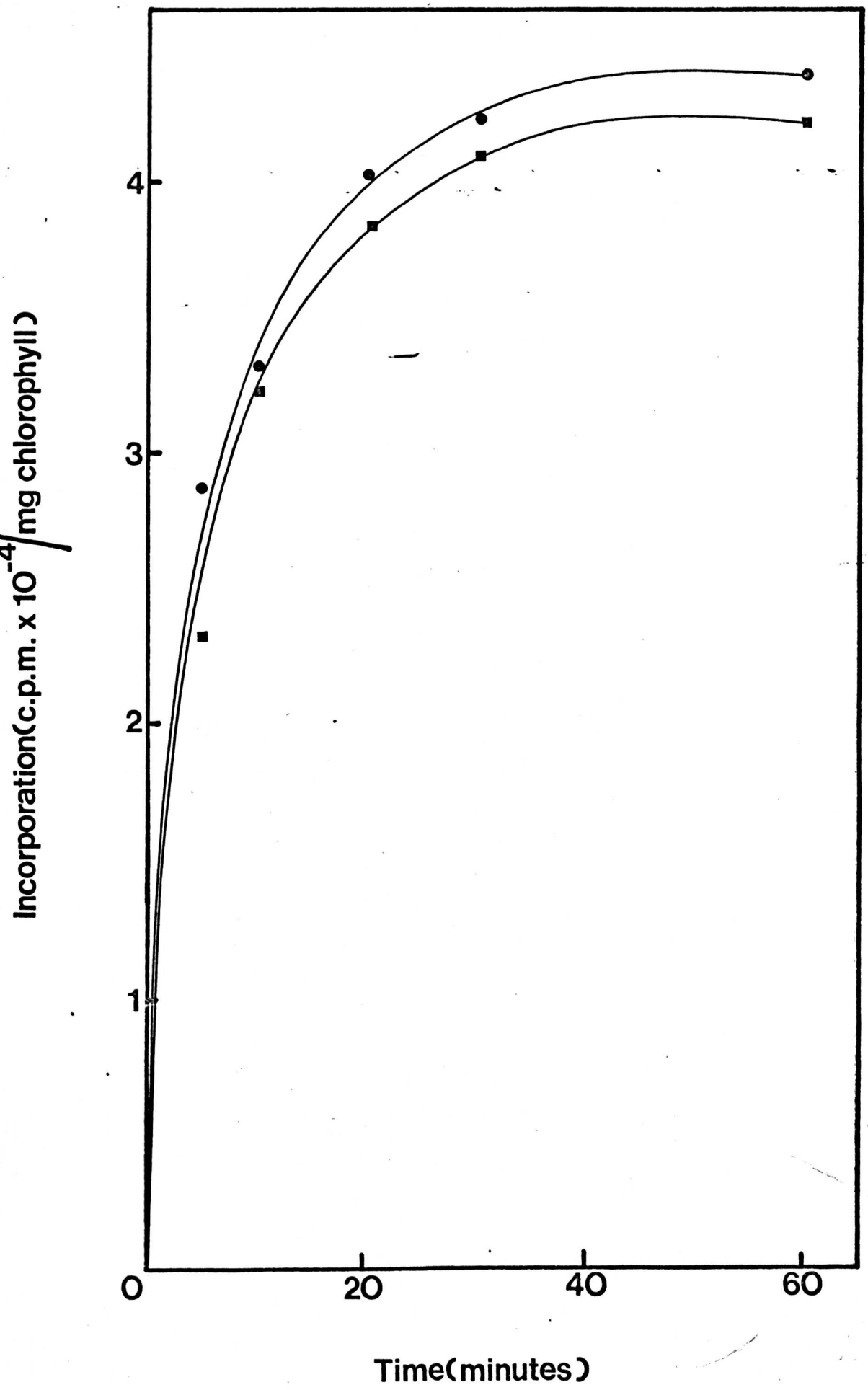
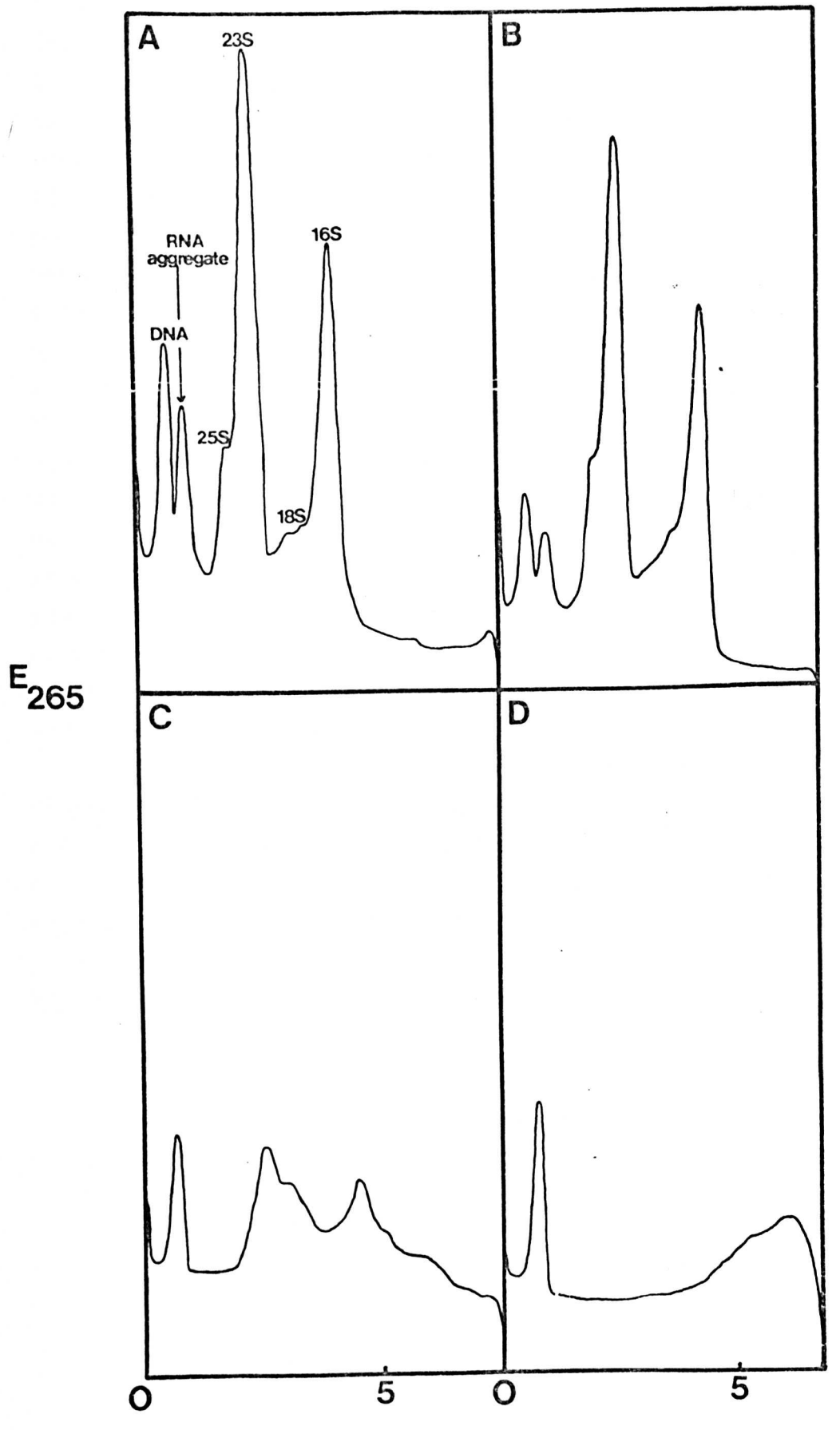

Incorporation(c.p.m. x 10^{-4}/mg chlorophyll)
4
3
2
1
0
20
40
60
Time(minutes)

Figure 13. The degradation of nucleic acids in chloroplast preparations by exogenous ribonuclease.

Total nucleic acids were extracted from chloroplast preparations (see Section II2J) A. at time zero; B. after 60 minutes of incubation in the light; C. after 60 minutes of incubation in the light in the presence of 10µg/ml ribonuclease; D. after 30 minutes of incubation in light in the presence of 2% (v/v) Triton X-100 and 10µg/ml ribonuclease. Nucleic acids were fractionated by polyacrylamide gel electrophoresis as described in Section II2K.


A
23S
B
RNA
aggregate
16S
DNA
25S
18S
C
D
E_{265}
O
5
O
5
Electrophoretic mobility(cm)

manner during the incubation is excluded by the results shown in fig. 13. Total nucleic acids were extracted from the chloroplast preparations at time zero and after 60 minutes of incubation in the light in the presence or absence of 10µg/ml ribonuclease. The nucleic acids were then fractionated by polyacrylamide gel electrophoresis as described in Section II2K. Chloroplast 23S and 16S ribosomal RNA peaks are clearly resolved. In addition, a high molecular weight peak, perhaps an aggregate of chloroplast 23S RNA was obtained. Some contaminating cytoplasmic 25S and 18S RNA was present as shoulders on the chloroplast ribosomal RNA peaks. There appears to be little endogenous nuclease activity in the chloroplast preparations since almost no degradation of the chloroplast ribosomal RNA had occurred after 60 minutes of incubation in the light (figs. 13A and 13B). However, after 60 minutes of incubation with 10µg/ml of ribonuclease, substantial hydrolysis of chloroplast ribosomal RNA occurred, although ribosomal RNA peaks were still observed (fig. 13C). In the presence of 2% (v/v) Triton X-100 (which solubilises chloroplast membranes and hence permits hydrolysis of membrane-bound ribosomes by ribonuclease), all ribosomal RNA was digested (fig. 13D). These results show that ribonuclease is not inactivated by light or any other factor present in the incubation system.

The effect of ribonuclease on [C^{14}]leucine incorporation in the absence of light, but in the presence of ATP and an ATP-generating system was then examined. The results are shown in Table 8 below. A parallel set of incubations in the light was included in the same experiment for comparison.

Table 8/

Table 8. Sensitivity of ATP-driven protein synthesis in isolated chloroplasts to ribonuclease A.

Treatment	[C^{14}] leucine incorporation (c.p.m./mg chlorophyll)	% Control
Light (control)	47 400	100
Light+RNAase (3µg/ml)	49 070	104
Light+RNAase (0.3µg/ml)	46 400	98
-Light+ATP (control)	32 300	100
-Light+ATP+RNAase (3µg/ml)	25 225	78
-Light+ATP+RNAase (0.3µg/ml)	24 015	74

Experimental details are given in Table 4 and Section II2Ci. Incubation was at 20°C for 40 minutes. Amino acid incorporation is expressed as a percentage of the incorporation of the respective control incubations.

Thus in the absence of light but with ATP as energy source, there appears to be a slightly increased level of inhibition at both concentrations of ribonuclease tested. The control series, incubated in the light, showed no inhibition due to ribonuclease.

10. DISTRIBUTION OF LABELLED PROTEIN BETWEEN THE 150 000 x g SUPERNATANT AND PELLET FRACTIONS.

Before the products of *in vitro* protein synthesis by isolated chloroplasts were characterised, the amount of radioactively-labelled protein released into the soluble phase of the chloroplast was determined. This was done in order to check that sufficient radioactivity was present in the soluble fraction to permit further analysis.

Chloroplasts were isolated, resuspended in KCl medium and incubated as previously described (Sections II2Aiv and II2Ci). [C^{14}] leucine was used as the radioactive precursor. At the end of the incubation, 0.1ml of saturated, unlabelled L-leucine was added, and the reaction mixture was transferred to a dialysis sac. The suspension was dialysed overnight at 4°C against 1 litre of 30mM tricine-KOH (pH8.3), 6.6mM MgCl, 4mM 2-mercaptoethanol (i.e. KCl medium lacking KCl, having a lowered buffer concentration and including 2-mercaptoethanol). At 150 000 x g supernatant fraction was then prepared as described in Section II2Di. The 150 000 x g pellet was dispersed in 0.5ml distilled water and transferred to a conical glass centrifuge/

centrifuge tube. Total radioactivity was determined in the supernatant, pellet and in the unfractionated reaction mixtures as described in Section II2Ci.

Table 9. The distribution of labelled protein between the 150 000 x g supernatant and pellet fractions.

Fraction	Total c.p.m.	% Unfractionated chloroplasts
Unfractionated chloroplasts	16 900	100
150 000 x g supernatant fraction	4 000	24
150 000 pellet fraction	12 000	71
(Losses)		(5)

Experimental details are given in the text and in Section II2Di. Incubation was at 20°C for 40 minutes in the light. Results are expressed as a percentage of the total radioactivity incorporated in a 500µl suspension of chloroplasts which were not subsequently fractionated by centrifugation.

Between 20 and 30% of the $[C^{14}]$leucine incorporated into protein was released into the soluble phase of the chloroplast. The remainder of the radioactivity sedimented with the 150 000 x g pellet. This fraction would be expected to include membrane proteins and both membrane-bound and free ribosomes with attached nascent polypeptides.

11. CHARACTERISTICS OF AMINO ACID INCORPORATION BY ISOLATED SPINACH CHLOROPLASTS.

Amino acid incorporation by isolated spinach chloroplasts was also examined. A brief account of the characteristics of incorporation is given here in order to illustrate its similarity to light-driven protein synthesis in isolated pea chloroplasts, and also to suggest that chloroplasts isolated from other plant species may show similar properties as regards light-driven protein synthesis.

Table 10/

Table 10. A comparison of the characteristics of amino acid incorporation by isolated pea and spinach chloroplasts.

	Spinach	Pea
1. Rates of [C^{14}]leucine incorporation (nmoles/mg chlorophyll/h)		
a. Light-driven	0.30	0.78
b. ATP-driven	0.19	0.42
c. In the absence of light or added ATP	0.01	0.05
2. Percentage inhibition of light-driven incorporation		
a. by DCMU (6×10^{-7}M)	15	60
b. by ribonuclease (0 to 30µg/ml)	0	0

Spinach and pea chloroplasts were isolated by the procedure of Ramirez et al (1968), described in Section II2Aiv, and resuspended in KCl medium. Incubation was at 20°C for 40 minutes. Amino acid incorporation was determined as described in Section II2Ci.

Spinach chloroplasts, isolated by the procedure of Ramirez et al (1968) and incubated in KCl medium showed rates of light-driven [C^{14}] leucine incorporation into protein of the order of 0.3 nmoles/mg chlorophyll/h. Rates of light-driven leucine incorporation by isolated pea chloroplasts were always greater, being in the range 0.5 to 1.0 nmoles/mg chlorophyll/h. A 30 to 40 fold stimulation of incorporation by light (as compared with incorporation in the dark) was generally observed in isolated spinach chloroplasts. When ATP was supplied as an energy source instead of light, levels of incorporation of around 50% of the complete, light-driven system were obtained with both spinach and pea chloroplasts. Resuspending and incubating spinach chloroplasts in medium with 0.35M sucrose instead of 0.2M KCl caused a marked lowering of amino acid incorporation; this effect was also noted and investigated with isolated pea chloroplasts (see fig. 8).

Light-driven amino acid incorporation by both isolated spinach and pea chloroplasts was found to be sensitive to inhibitors of photophosphorylation. DCMU (at a concentration of 6×10^{-7}M) when supplied to the complete, light-driven system caused an inhibition of 15%. This amount of inhibition due to DCMU is rather lower than that shown by pea chloroplasts. Ribonuclease, supplied to isolated spinach/

spinach chloroplasts over a range of concentrations varying from 0 to 30µg/ml, caused no inhibition of amino acid incorporation in the light; this agrees with results obtained with isolated pea chloroplasts (Table 7).

Contamination of spinach chloroplast preparations by bacteria, nuclei and whole cells appeared to be of the same order as that occurring in preparations of isolated pea chloroplasts (Table 6). Only 0.02% of the protein synthesised by the light-driven system of isolated spinach chloroplasts appeared in a 10 000 x g pellet obtained after treatment of the labelled chloroplasts with Triton X-100 detergent (see Section II2E).

12. DISCUSSION.

The results presented in this Section show that isolated chloroplasts incorporate radioactively-labelled amino acids into protein at high rates, using light as an energy source. This is not the first report of the use of light as the energy source for amino acid incorporation - the results in this Section confirm those obtained by Ramirez et al (1968) for light-driven incorporation by isolated spinach chloroplasts, and extend the observation to isolated pea chloroplasts. Acetabularia chloroplasts also appear to incorporate amino acids in vitro using light as the energy source (Apel and Schweiger, 1972). However, this light-dependence was not strict; when incubation was performed in the absence of light, rates of protein synthesis 40% of the complete, light-driven system were obtained (Apel and Schweiger, 1973). Previous descriptions of the use of light as the energy source for protein synthesis have involved the use of catalysts of photophosphorylation, and added ADP and inorganic phosphate. Spencer (1965) used pyocyanine to couple photophosphorylation to amino acid incorporation in isolated spinach chloroplasts. Similarly, Griffiths and Lozano (1970) used phenazine methosulphate, which is chemically related to pyocyanine, to obtain light-dependent incorporation in isolated pea chloroplasts. In the work presented in this Section, however, no added catalysts or cofactors of photophosphorylation were required for light-dependent protein synthesis since the incorporation proceeded in intact chloroplasts.

The rate of light-driven incorporation of labelled amino acids by isolated pea chloroplasts is higher than that obtained with isolated spinach/

spinach chloroplasts (Section III 11). The rates reported in this Section are not so high as those originally reported by Ramirez et al (1968). They obtained rates of almost 3 nmoles [C^{14}]leucine incorporated/mg chlorophyll/h, whereas the rates reported in this Section for similarly prepared spinach chloroplasts are of the order of 0.3 nmoles/mg chlorophyll/h (Table 10). Comparison of other published rates is difficult, since authors tend either not to publish vital information (such as specific activities of radio-isotopes or counting efficiencies) or express rates in different forms, e.g. c.p.m./mg protein/h. Parenti and Margulies (1967), working with chloroplasts isolated from Phaseolus vulgaris, obtained a rate of [C^{14}]leucine incorporation of 1 nmole/mg chlorophyll/h. The rates reported in this Section, while not being the highest reported, appear sufficiently high to permit identification of the products of in vitro chloroplast protein synthesis.

Much effort was devoted to establishing the optimal conditions for protein synthesis by isolated pea and spinach chloroplasts. A vital component of the incubation medium appears to be the high concentration of KCl present in the KCl resuspension medium (fig. 8). The concentration of 0.2M KCl routinely used gave both the highest rate of amino acid incorporation observed (fig. 8 and Table 2) and also yielded a chloroplast preparation in which at least 50% of the chloroplasts were intact. Incubation in sucrose resuspension medium reduced incorporation in both isolated pea (fig. 8) and spinach chloroplasts (Section III 11), although at least 60% of the chloroplasts were intact (Table 2). These results suggest that KCl functions both as an osmoticum and as a cofactor for protein synthesis. The low concentration of potassium ions present in the sucrose resuspension medium (Section III4) was not sufficient to give a high rate of protein synthesis; sucrose therefore appears to function only as an osmoticum. It is interesting to note that Ibrahim et al (1973) have obtained similar results with rat liver mitochondria where the use of 200mM KCl gave a higher rate of protein synthesis in isolated mitochondria than when a conventional sucrose medium was used.

The concentration of potassium ions inside the chloroplast may well be different from that present in the KCl resuspension medium. Larkum (1968) estimated the concentrations of sodium, potassium and chloride ions in non-aqueously isolated chloroplasts of the alga Tolypella/

Tolypella intricata. He found high concentrations of 340mM potassium and 340mM chloride ions in the chloroplasts. However, low concentrations of sodium ions were found (about 36mM). These figures are consistent with the observations in this Section that protein synthesis requires high potassium ion concentrations. The inhibition of protein synthesis by sodium ions found in isolated chloroplasts (fig. 8) is in good agreement with the observation that, in vivo, sodium ion concentrations are low. It is also interesting to note that chloroplast polysomes isolated from Vicia faba are stable at higher (i.e. 0.2M), rather than lower KCl concentrations (T.A. Dyer, personal communication).

Is it certain that the amino acid incorporation is taking place on chloroplast ribosomes? Some alternative sources of possible incorporation can be considered and excluded. Helmsing (1970) has shown that isolated nuclei from Drosophila melanogaster incorporate amino acids into proteins associated with the polytene chromosomes. The incorporation was sensitive to chloramphenicol but not cycloheximide, showing a certain similarity to amino acid incorporation by chloroplasts and mitochondria. Nuclear incorporation by preparations of isolated pea or spinach chloroplasts can be excluded for two reasons. Firstly, less than 1% of incorporated radioactivity was present in a 10 000 x g pellet prepared after labelled chloroplasts were treated with Triton X-100 detergent. This pellet would be expected to include nuclei, bacteria and whole leaf cells (Parenti and Margulies, 1967). Secondly, although many preparations were examined by phase contrast microscopy, nuclei were rarely observed.

Bacterial contamination has been a great problem in studying protein synthesis in both isolated chloroplasts (Gnanam, Jagendorf and Ranalletti, 1969; Margulies et al, 1968) and isolated mitochondria (Beattie, Basford and Koritz, 1967). Characteristics of amino acid incorporation by bacterial contaminants in preparations of organelles include a lack of dependence on added ATP or other energy source; linear time courses of incorporation extending over several hours; and insensitivity of incorporation to ribonuclease. Incorporation in chloroplast preparations showed a normal hyperbolic time course (fig. 9) and was strictly dependent on added energy, either as ATP or light. No compounds which could act as fermentable substrates such as sucrose or mannitol, were present in the KCl resuspension medium routinely/

routinely used. In addition, all media, glassware etc. were sterilised before use to minimise bacterial contamination. For these reasons, amino acid incorporation by contaminating bacteria can be excluded. Incorporation was however insensitive to ribonuclease; this point will be considered later.

Incorporation by contaminating cytoplasmic ribosomes also appears unlikely. Very little cytoplasmic ribosomal RNA was detected when the nucleic acids of the chloroplast preparations were extracted and fractionated (fig. 13A). Incorporation was also completely insensitive to cycloheximide (Table 5), an inhibitor considered to be specific for 80S ribosomes.

The contribution of mitochondria to the amino acid incorporation observed is rather more difficult to assess due to the great similarity between the protein-synthesising systems of the two types of organelle. However, the great stimulation of protein synthesis produced by light argues strongly for synthesis on chloroplast ribosomes only, irrespective of the evidence provided by the use of selective inhibitors of 70S ribosomes (Table 5). Incorporation on the ribosomes of any contaminant would require the export of ATP synthesised by photophosphorylation from the chloroplast, and the uptake and utilisation of the ATP by the contaminating organelle, bacterium or ribosome. As already mentioned, the permeability of the outer membrane of the chloroplast to ATP is low (Heber and Santarius, 1970) and a considerable dilution of any 'leaked' ATP would occur in the external medium. Such an argument would preclude incorporation by any organism, ribosome or organelle other than the chloroplast.

The source of energy for light-driven protein synthesis was shown to be ATP synthesised by photophosphorylation on the basis of the complete inhibition of amino acid incorporation by CCCP (Table 4), an inhibitor of photophosphorylation. However, further identification, i.e. whether photophosphorylation was of the cyclic or non-cyclic type or both, proved difficult. Ramirez et al (1968) showed that light-driven amino acid incorporation was completely insensitive to DCMU (an inhibitor of non-cyclic photophosphorylation) but was inhibited by desaspidin (an inhibitor of cyclic photophosphorylation), and concluded that all the energy for protein synthesis in chloroplasts was provided by cyclic photophosphorylation. The results presented in Table 10 show 40% inhibition of incorporation due to DCMU in isolated pea chloroplasts/

chloroplasts and rather less inhibition (15%) in isolated spinach chloroplasts. These results disagree with those of Ramirez et al (1968), and indicate that some ATP used for protein synthesis may be synthesised by non-cyclic photophosphorylation.

About 50% of the chloroplasts isolated and resuspended in KCl medium were intact (Table 2). Does light-driven amino acid incorporation take place in these intact chloroplasts, or in broken chloroplasts, or in both? The most conclusive evidence on this point comes from the studies on the sensitivity of amino acid incorporation to ribonuclease (Table 7). Light-driven incorporation was found to be insensitive to ribonuclease at all concentrations tested and throughout the time course of incorporation (fig. 12). This strongly argues for incorporation taking place inside intact chloroplasts into which ribonuclease cannot penetrate (Margulies et al, 1968). Exogenous ribonuclease will hydrolyse the RNA only of broken chloroplasts and contaminating cytoplasmic ribosomes (fig. 13C).

In ATP-driven protein synthesis in the absence of light, a slightly increased amount of inhibition of incorporation by ribonuclease was observed (Table 9). This may indicate that broken chloroplasts incorporate amino acids into protein in small amounts, but only when exogenous ATP is supplied. In the absence of added ADP, phosphate, phenazine methosulphate etc., the rate of photophosphorylation is low (Tagawa et al, 1963). However, when ATP was supplied as well as light, little stimulation of incorporation resulted (Table 4). If broken chloroplasts incorporated amino acids actively in the presence of exogenous ATP a much greater stimulation might have been expected when both light and ATP were used as the energy source. The results obtained in ATP-driven protein synthesis in the absence of light can be explained by assuming that incorporation is taking place mainly in intact chloroplasts. Although the outer envelope of the chloroplast is permeable to ATP only to the extent of around 7 to 9 μmoles/mg chlorophyll/h (Heber and Santarius, 1970), the rates of leucine incorporation obtained were around three orders of magnitude less than this figure, being about 0.5 to 1.0 nmoles/mg chlorophyll/h. Therefore the influx of ATP can easily account for the rates of amino acid incorporation obtained in ATP-driven synthesis. The generation of ATP in situ in intact chloroplasts may be much more efficient/

efficient as a source of energy for protein synthesis, leading to a higher rate of amino acid incorporation (Table 4).

The time course of light-driven amino acid incorporation was shown to be hyperbolic, with the rate of incorporation reaching zero by about 40 minutes of incubation (fig. 9). Why does incorporation stop after this time? The chloroplasts do not lose their outer membranes; this was shown by direct observation (Section III5B) and inferred from the time course in the presence of ribonuclease (fig. 12). The chloroplast ribosomal RNAs are unaffected by 60 minutes of incubation in the light (figs. 13A and 13B); this suggests that the chloroplast ribosomes are not inactivated by endogenous nuclease action. Protein synthesis may cease because the photochemical systems or the amino acid carrier system (Nobel and Cheung, 1972) is inactivated. Another reason may be that initiation of protein synthesis does not take place for some reason, e.g. loss or inactivation of an initiation factor.

The results presented in this Section fulfill the main aims set out in the Introduction to the Section. Protein synthesis, dependent on light, taking place mainly in intact chloroplasts at a high rate was obtained. These results provide a firm basis for the analysis of the products of <u>in vitro</u> chloroplast synthesis.

SECTION IV - RESULTS AND DISCUSSION

ANALYSIS OF THE SOLUBLE PRODUCTS OF *IN VITRO* CHLOROPLAST PROTEIN SYNTHESIS

1. INTRODUCTION.

The results discussed in Section III showed that high, light-driven rates of amino acid incorporation into protein occur in isolated pea and spinach chloroplasts. The characteristics of incorporation were similar for the chloroplasts of both species. In this Section it was decided to restrict the analysis of the products of *in vitro* protein synthesis to isolated pea chloroplasts. Rates of light-driven protein synthesis were higher in isolated pea chloroplasts (Table 10); also peas could be grown more readily than spinach and in larger quantity.

It was also decided to restrict the analysis to the proteins of the soluble phase of the chloroplast. As already described in Section I, the protein chemistry of the chloroplast membrane is poorly understood. Some of the soluble proteins, such as Fraction I protein and ferredoxin, have been better characterised and can be easily identified. Identification is also aided if only completed polypeptide chains are examined; peptides at various stages of elongation would be difficult to characterise. Chloroplast ribosomes have been reported to bind nascent polypeptides very strongly (Chen and Wildman, 1970). By examining supernatant proteins, only released and therefore completed polypeptides will be studied. For these reasons, a 150 000 x g supernatant fraction was prepared from a radioactively-labelled chloroplast preparation (Section II2Di). Under these conditions of centrifugation chloroplast lamellae and ribosomes are pelleted, leaving a clear supernatant containing only soluble proteins and tRNAs (Francki *et al*, 1965).

What methods can be used to establish the identity of a newly-synthesised protein? Ultimately, the only method is to show that labelled amino acids are incorporated into a sequence identical to that of the authentic protein. Rather than sequence a complete protein, the labelling of tryptic peptides is frequently used to compare *in vitro*-synthesised proteins with the authentic protein (Campbell and Kernot, 1962) and thus establish identity. All other methods are at best only suggestive. In this Section, polyacrylamide gel electrophoresis and gel chromatography were used to initially characterise the products of *in vitro* chloroplast protein synthesis, and then tryptic peptide analysis was used to confirm the identity of the soluble products of chloroplast protein synthesis.

2./

2. ELECTROPHORETIC ANALYSIS ON NON-DENATURING POLYACRYLAMIDE GELS.

A. Analysis on 5.0% and 4.0% acrylamide gels.

The in vitro-synthesised proteins present in the 150 000 x g supernatant fraction were analysed firstly by polyacrylamide gel electrophoresis in the absence of a denaturant. Fig. 14 shows the results of two separations on gels of different acrylamide concentration. In both electrophoreses only one symmetrical peak of radioactivity can be distinguished, and in each case this radioactive peak is associated with an amidoblack-staining (i.e. protein) band. However, the radioactive peak is associated with different protein bands on gels of different acrylamide concentration.

In fig. 14A, the labelled peak is coincident with a protein band which can be identified as Fraction I protein by comparison with a purified marker. In fig. 14B, however, the labelled peak migrates exactly with a band of lower mobility than Fraction I protein. Analyses of labelled supernatants on non-denaturing gels were routinely performed on 4.0% and 5.0% gels. The difference in electrophoretic mobility of the labelled peak was always observed; its coincidence with Fraction I protein on 5.0% gels and with the protein band of lower mobility on 4.0% gels was consistently found.

B. Analysis of in vivo, radioactively-labelled purified pea Fraction I protein on 5.0% and 4.0% acrylamide gels.

The mobility of in vivo-labelled Fraction I protein contrasts with that shown by the in vitro-labelled peak described above and in fig. 14. On both 4.0% and 5.0% gels both radioactive peaks and Fraction I protein bands were found to be exactly coincident. No labelled proteins of lower mobility than Fraction I were seen (figs. 15A and 15B). These results also indicate the purity of the in vivo-labelled Fraction I protein. Proteins can be detected on gels with greater sensitivity if they are radioactively-labelled than by their dye-binding properties. Since not only one protein band but also one radioactive peak was detected on non-denaturing gels, this testifies to the purity of the in vivo-labelled Fraction I protein used in subsequent work.

C. Analysis of in vitro products synthesised

i. at time zero.

ii. in the absence of light.

iii./

Figure 14. Analysis of the products of light-driven protein synthesis by isolated pea chloroplasts on non-denaturing polyacrylamide gels.

A suspension of pea chloroplasts (500µl) was incubated with 1µCi [C^{14}]leucine in KCl medium for 60 minutes at 20°C in the light. The chlorophyll concentration was 0.76mg/ml. A 150 000 x g supernatant fraction was prepared as described in Section II2Di. Aliquots (100µl) of supernatant extract were analysed by electrophoresis on A. 5.0% acrylamide gels, and B. 4.0% acrylamide gels. The details of electrophoresis and the subsequent staining, scanning and slicing of gels are given in Section II2Dii. The solid line represents the absorbance at 620nm and the histogram shows the radioactivity in each 1mm gel fraction.

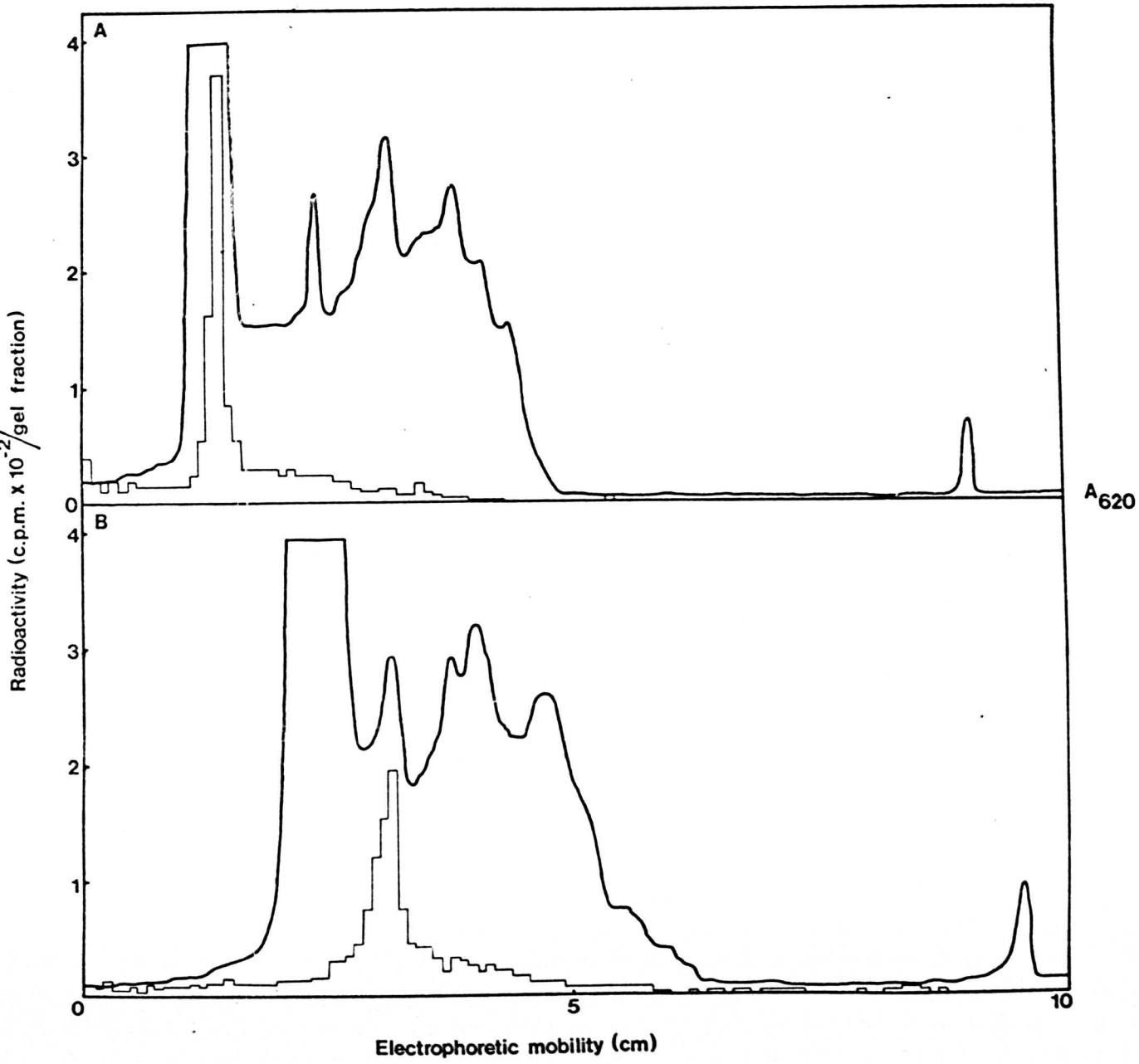

A
B
4
3
2
1
0
5
10
Radioactivity (c.p.m. x 10^{-2}/gel fraction)
Electrophoretic mobility (cm)
A_{620}

Figure 15. Polyacrylamide gel electrophoresis of *in vivo*, radioactively-labelled purified pea Fraction I protein.

Fraction I protein, labelled *in vivo* with [S^{35}] methionine, was prepared as described in Section II2G. Electrophoresis was performed A. on a 5.0% acrylamide gel and B. on a 4.0% acrylamide gel. A total of 70µg protein was loaded on each gel. All other details are given in figure 14.

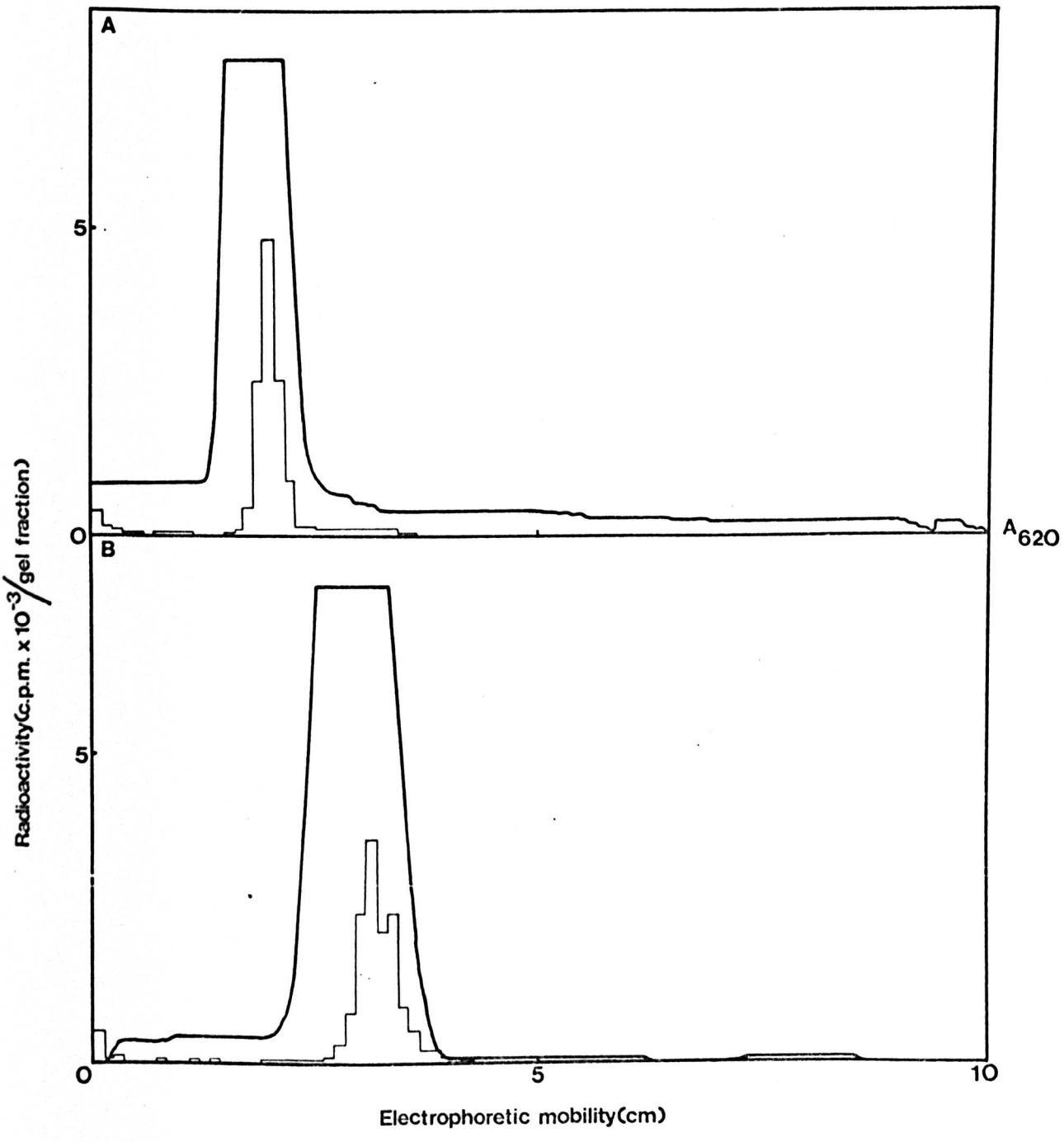
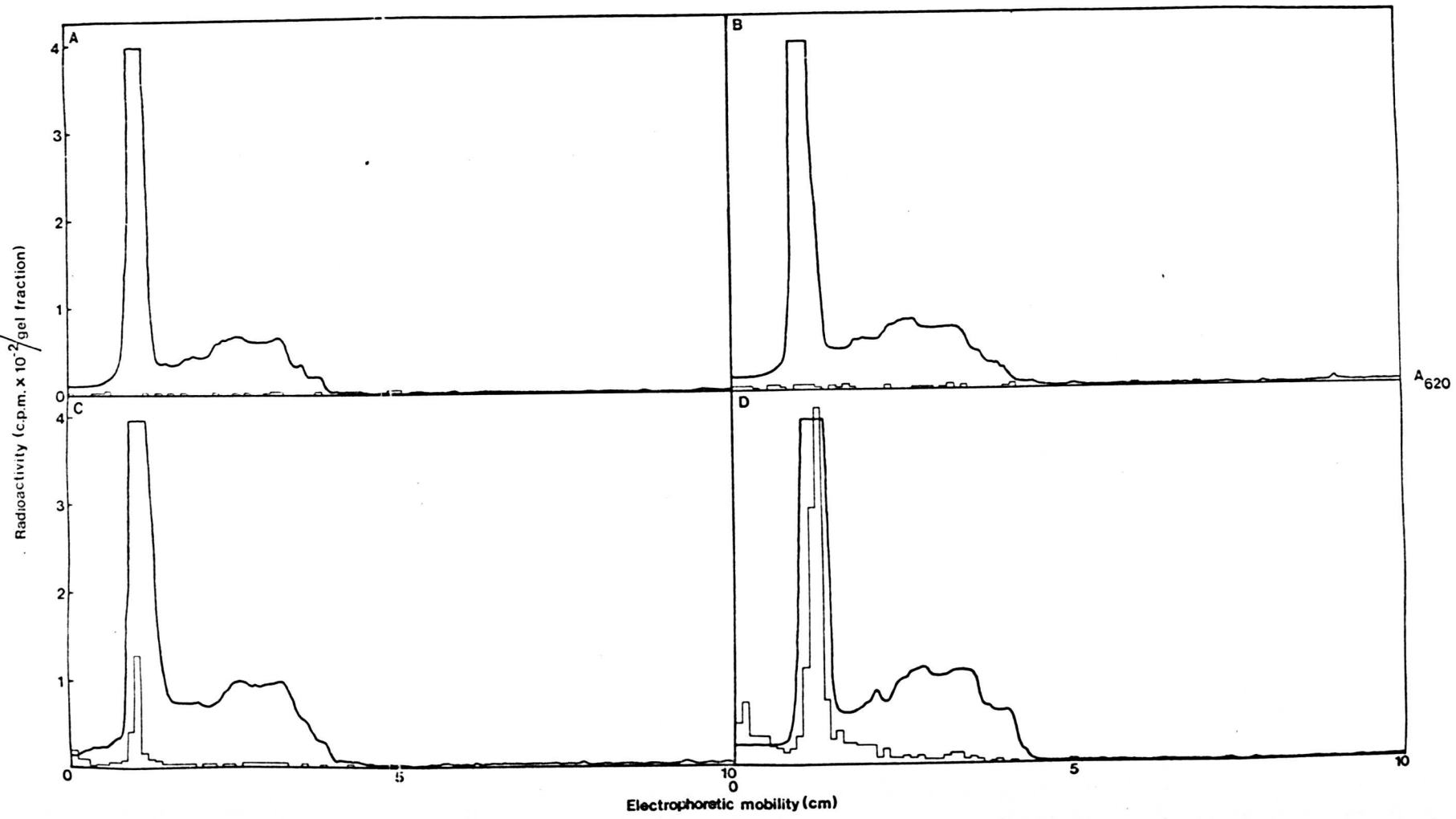

A
5
0
A620
B
5
0
5
10
Radioactivity(c.p.m. x 10-3/gel fraction)
Electrophoretic mobility(cm)

Figure 16. Analysis of the soluble products of *in vitro* chloroplast protein synthesis on non-denaturing polyacrylamide gels : the dependence of protein synthesis on the source of energy.

Aliquots of chloroplasts (400µl, containing 120µg chlorophyll) were incubated at 20°C with 1µCi of [C^{14}]leucine in KCl medium A. for zero minutes; B. for 60 minutes in the absence of light or added ATP; C. for 60 minutes in the absence of light, but with ATP and an ATP-generating system supplied; D. for 60 minutes in the light. A 150 000 x g supernatant fraction was prepared from each sample, and 100ul was analysed by electrophoresis on 5.0% acrylamide gels. All other details are given in figure 14.


A
B
C
D
Radioactivity (c.p.m. x 10^{-2}/gel fraction)
A_{620}
Electrophoretic mobility (cm)
0
1
2
3
4
5
10

iii. in the absence of light, but in the presence of exogenous ATP.

The results of these experiments are shown in fig. 16. When [C^{14}]leucine incorporation was stopped at time zero no labelled proteins were present in the 150 000 x g supernatant fraction (fig. 16A). This shows that the labelled peak observed in the light-driven system is not due to the binding of [C^{14}]leucine to a pre-existing supernatant protein. When the chloroplasts were incubated in the absence of light, no labelled peaks were obtained (fig. 16B). This shows that no synthesis occurs due to endogenous ATP in chloroplasts, and also indicates that the labelled peak obtained in the light is not due to contamination by micro-organisms. When ATP was supplied exogenously in the absence of light some synthesis of a supernatant protein occurred (fig. 16C). This protein has the same mobility as that which is synthesised in the light (fig. 16D). Both migrate with Fraction I protein on 5.0% gels. However, the labelling of the peak is greatly reduced when ATP is used as the energy source for protein synthesis instead of light. This result parallels that obtained for total amino acid incorporation in light and ATP-driven protein synthesis (Section III3).

In all experiments, controls incubated in the absence of light were always inserted. No counts were found on the gels of such control supernatant proteins.

3. ELECTROPHORETIC ANALYSIS ON SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GELS.

A. Analysis on 8.0%, 10.0% and 12.0% acrylamide gels.

When the proteins of the 150 000 x g supernatant fraction were denatured with SDS in the presence of 2-mercaptoethanol and analysed by polyacrylamide gel electrophoresis, the results shown in fig. 17 were obtained. Although many protein bands appeared after staining, one band stained very intensely giving a full scale deflection on scanning in the Chromoscan densitometer at high sensitivity. This band was identified as the large subunit of Fraction I protein by comparison with a purified marker. When the distribution of labelled proteins on the gel was examined, it was found that only one discrete radioactively-labelled peak was present. This peak migrated exactly with the large subunit of Fraction I protein on 8.0%, 10.0% and 12.0% gels. Neither the small subunit of Fraction I protein nor any other protein/

Figure 17. Polyacrylamide gel electrophoresis of the soluble, *in vitro* products of chloroplast protein synthesis on sodium dodecyl sulphate-polyacrylamide gels of varying acrylamide concentration.

Pea chloroplasts (500µl, containing 150µg chlorophyll) were incubated with 1µCi [C^{14}]leucine in KCl medium for 60 minutes at 20°C in the light. A 150 000 supernatant fraction was prepared and denatured with SDS as described in Section II2Di. Aliquots (100µl) of this mixture were fractionated by electrophoresis on A. an 8.0% SDS-gel; B. a 10.0% SDS-gel; C. a 12.0% SDS-gel. All other details are given in figure 14. LSU, large subunit; SSU, small subunit.

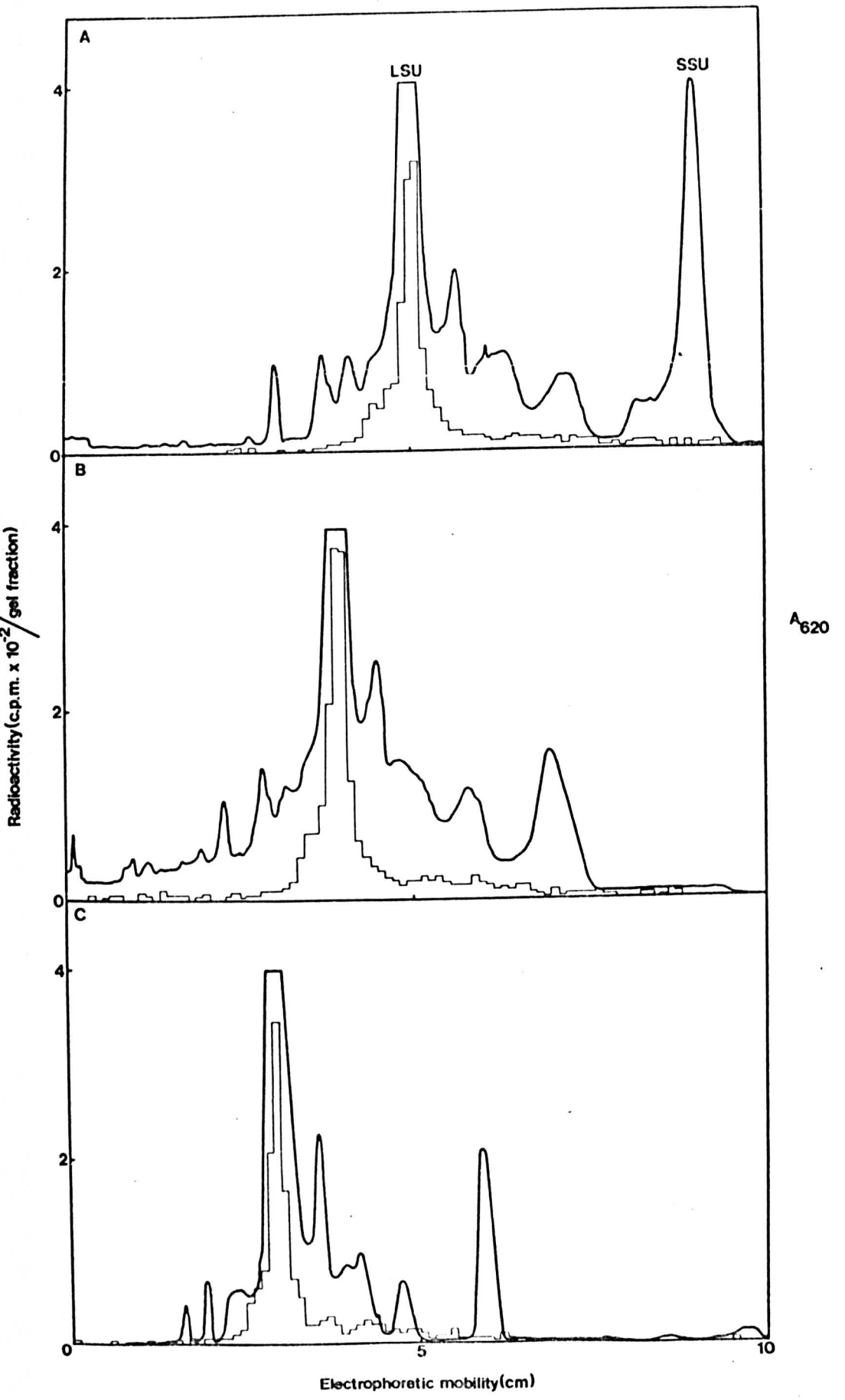
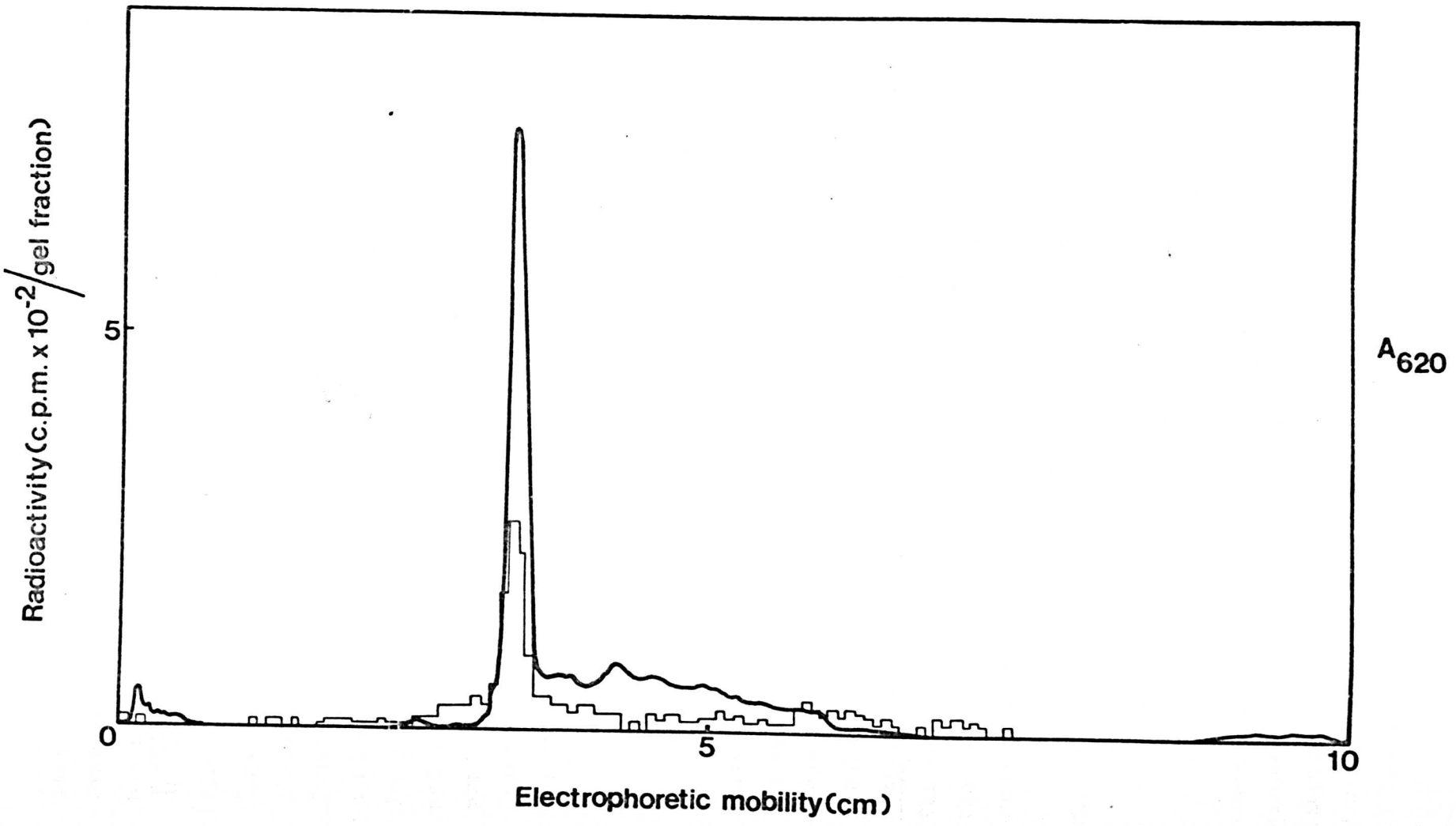

A
LSU
SSU
B
C
Radioactivity (c.p.m. x 10⁻²/gel fraction)
A620
Electrophoretic mobility (cm)
0
2
4
5
10

Figure 18. Comparison of the mobility of the large subunit of Fraction I protein and the soluble, *in vitro* product of chloroplast protein synthesis.

Pea chloroplasts (500µl, containing 210µg chlorophyll) were incubated with 50µCi of [S^{35}]methionine (specific activity 90Ci/mmole) in KCl medium for 60 minutes at 20°C in the light. An SDS-denatured 150 000 x g supernatant fraction was prepared as described in Section II2Di. A 10µl aliquot of this fraction (containing 36µg protein) was analysed by electrophoresis on a 10.0% SDS-gel. All other details are given in figure 14, except that the gel was sliced in 0.75mm fractions.


Radioactivity (c.p.m. x 10^{-2}/gel fraction)
5
0
5
10
Electrophoretic mobility (cm)
A_{620}

protein on the gel was labelled to any significant extent.

The results shown in fig. 17 do not prove that the labelled peak has exactly the same molecular weight as the large subunit of Fraction I protein, since the exact peak height of the large subunit was not determined. A suspension of chloroplasts was incubated with [S^{35}]methionine of high specific activity, and small volumes of the 150 000 x g supernatant fraction were analysed on 10.0% gels. As fig. 18 shows, this reduced the loading of the large subunit so that it could be scanned as a peak, and not as a full scale deflection. However, sufficient radioactivity was present in the in vitro-synthesised peak (due to the high specific activity of the [S^{35}]methionine) to make analysis of the radioactivity in the gel both feasible and significant. Fig. 18 shows that both the soluble in vitro product of chloroplast protein synthesis and the large subunit of Fraction I protein have exactly the same mobility and hence molecular weight (Shapiro, Vinuela and Maizel, 1967) on 10.0% SDS-gels.

B. Analysis of in vivo radioactively-labelled purified pea Fraction I protein.

When a sample of pure, in vivo -labelled Fraction I protein was analysed by electrophoresis on 10.0% SDS-gels, distinct labelling of the small subunit was observed (fig. 19). This labelling pattern was never observed on gels of in vitro-labelled supernatant proteins. The labelling of the large subunit shows similarity to that obtained in vitro. In addition, a small peak of low mobility is labelled in vivo. This may be an aggregate of large or smallsubunits - the staining of the band appeared to decrease on increasing the 2-mercaptoethanol concentration.

C. Analysis of in vitro products synthesised

i. in the absence of light.

ii. in the absence of light, but in the presence of exogenous ATP.

The results from these experiments agree with those performed on non-denaturing gels, described in Section IV2C. As shown in fig. 20A, reduced synthesis of the in vitro-synthesised peak occurs when exogenous ATP is used as the energy source instead of light (fig. 20B). However, the radioactive peak migrates exactly with the large subunit of Fraction I protein when ATP is used to stimulate its synthesis. In the/

Figure 19. Analysis of *in vivo*, radioactively-labelled purified pea Fraction I protein by sodium dodecyl sulphate-gel electrophoresis.

Fraction I protein, labelled *in vivo* with [S^{35}] methionine, was prepared as described in Section II2G, and denatured with SDS (Section II2Di). Electrophoresis was performed on a 10.0% SDS-gel, loading 70µg protein on the gel. All other details are given in figure 14.

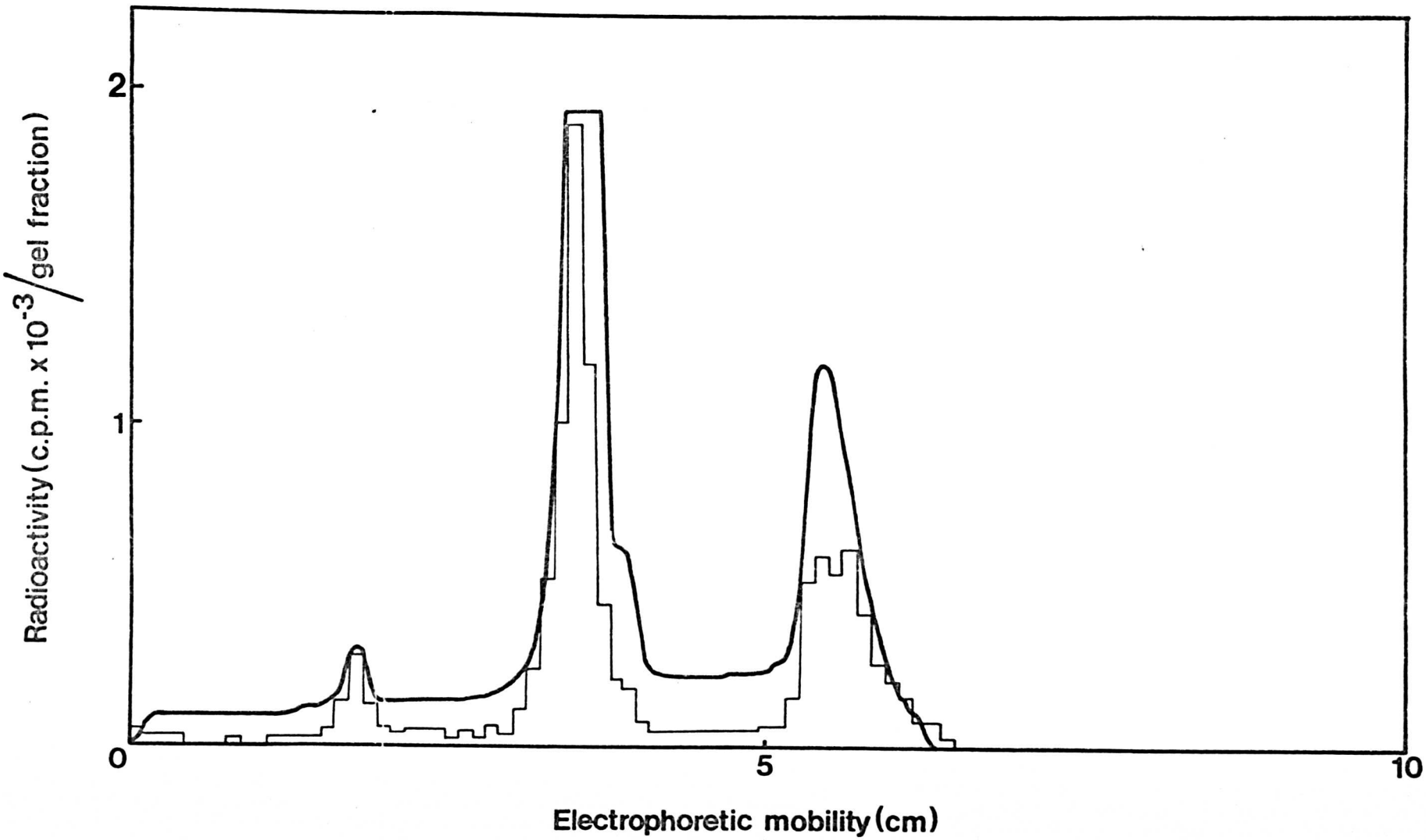

Radioactivity (c.p.m. x 10^{-3}/gel fraction)
2
1
0
5
10
Electrophoretic mobility (cm)

Figure 20. Analysis of the soluble, in vitro products of chloroplast protein synthesis by electrophoresis on sodium dodecyl sulphate-gels : the dependence of protein synthesis on the energy source.

Pea chloroplasts (400µl, containing 230µg chlorophyll) were incubated with 1µCi [C^{14}]leucine for 60 minutes at 20°C A. in the absence of light, with ATP and an ATP-generating system supplied; B. in the light; C. in the absence of light or ATP. A 150 000 x g supernatant fraction was prepared from each incubation, denatured with SDS, and 100µl analysed by electrophoresis on 10.0% SDS-gels as described in figure 14.

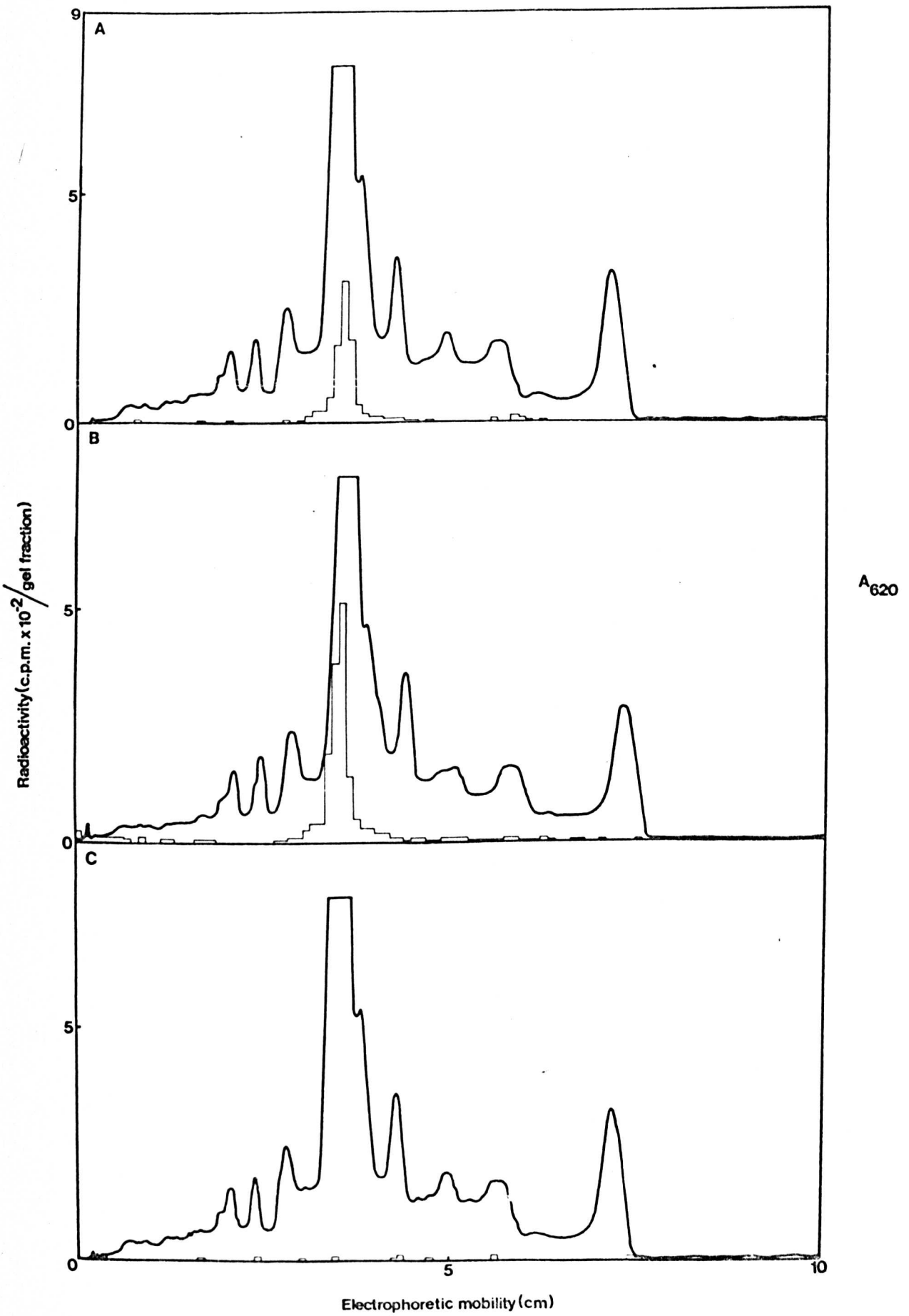
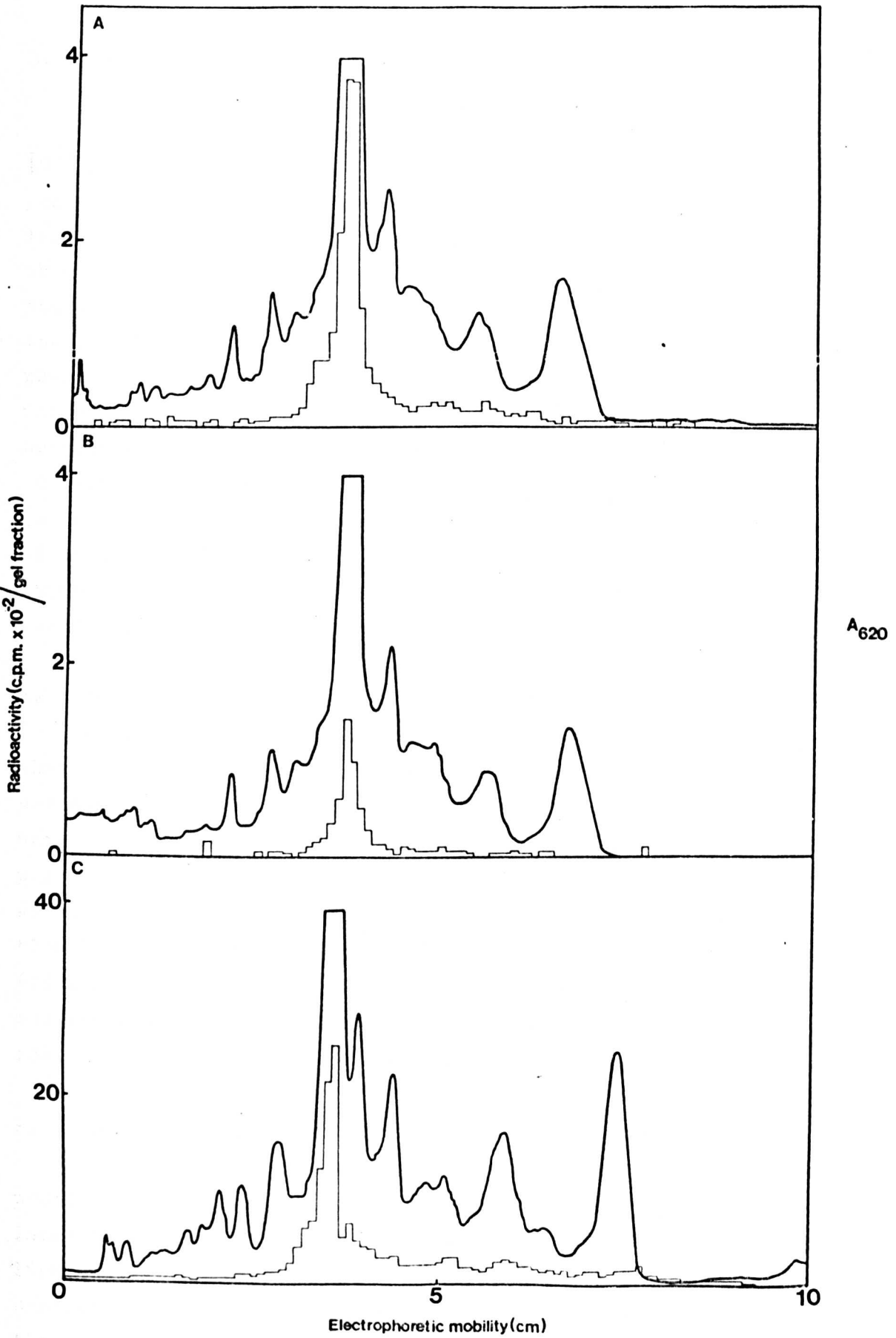

A
B
C
9
5
0
5
0
5
0
5
10
Radioactivity (c.p.m. x 10⁻²/gel fraction)
A620
Electrophoretic mobility (cm)

Figure 21. Polyacrylamide gel electrophoresis of soluble, in vitro products of chloroplast protein synthesis using three different labelled amino acids as precursor.

A volume of 500µl of chloroplasts (containing 150µg chlorophyll) was incubated in KCl medium in the light for 60 minutes at 20°C A. with 1µCi [C^{14}]leucine (specific activity 331mCi/mmole); B. with 1µCi [C^{14}] phenylalanine (specific activity 513mCi/mmole); C. in a separate experiment with 20µCi [S^{35}]methionine (specific activity 20Ci/mmole). Aliquots (100µl) of the 150 000 x g supernatant fraction prepared from each incubation were analysed by electrophoresis on 10.0% SDS-gels. All other details are given in figure 14.


A
B
C
4
2
0
4
2
0
40
20
0
5
10
Radioactivity (c.p.m. x 10⁻²/gel fraction)
A620
Electrophoretic mobility (cm)

the absence of light or exogenous ATP, no synthesis took place (fig. 20C).

D. Analysis of products synthesised using [C^{14}]phenylalanine or [S^{35}]methionine as protein precursor.

When chloroplasts were incubated in KCl medium in the light with [C^{14}]phenylalanine or [S^{35}]methionine as protein precursor, a single peak of radioactivity was obtained which migrated exactly with the large subunit of Fraction I protein. No qualitative difference was observed in the results obtained with [C^{14}]leucine (fig. 21A), [C^{14}] phenylalanine (fig. 21B) or [S^{35}]methionine (fig. 21C). However, the radioactive peak was labelled to a lesser extent with [C^{14}] phenylalanine than with [C^{14}]leucine, although the [C^{14}]phenylalanine was of higher specific activity than the [C^{14}]leucine. The [S^{35}] methionine had an even greater specific activity than either of the C^{14}-labelled amino acids. When [S^{35}]methionine at high concentration (at 20μCi per incubation) was used as a precursor, no additional peaks of incorporated radioactivity were observed (fig. 21B); only the peak which co-electrophoresed with the large subunit of Fraction I protein was labelled.

E. Analysis of products in the presence of 70S ribosomal inhibitors.

Fig. 22 shows the labelling of the 150 000 x g supernatant proteins when chloroplasts were incubated in the light in the presence of various selective inhibitors of 70S ribosomes. When chloroplasts were incubated with 50μg/ml D-*threo*-chloramphenicol, no labelled peaks were observed (fig. 22A). In the presence of spectinomycin (fig. 22B) and lincomycin (fig. 22C) synthesis of the single labelled peak was clearly reduced, when compared to the complete, light-driven system (fig. 22D). Lincomycin appears to be a better inhibitor of pea chloroplast ribosomes than spectinomycin; greater inhibition was achieved with lincomycin at 2μg/ml than with spectinomycin at 50μg/ml.

F. Analysis of products synthesised in TMS resuspension medium.

Broken chloroplasts are often used to study *in vitro* chloroplast protein synthesis (Chen and Wildman, 1970). It was therefore of interest to apply the same analytical methods used in the study of light-driven protein synthesis to identify any products of protein synthesis in preparations of broken chloroplasts. Fig. 23A shows the results of ATP-driven protein synthesis in pea chloroplasts incubated/

Figure 22. The effect of inhibitors of 70S ribosomes on the *in vitro* synthesis of chloroplast soluble proteins.

Pea chloroplasts (400µl, containing 170µg chlorophyll) were incubated in KCl medium with 1µCi [C^{14}]leucine at 20°C for 60 minutes in the light A. with 50µg/ml D-*threo*-chloramphenicol; B. with 50µg/ml spectinomycin; C. with 2µg/ml lincomycin; D. with no addition (control). A 150 000 x g supernatant fraction was prepared from each incubation, and 100µl were then analysed on 10.0% SDS-gels, as described in figure 14.

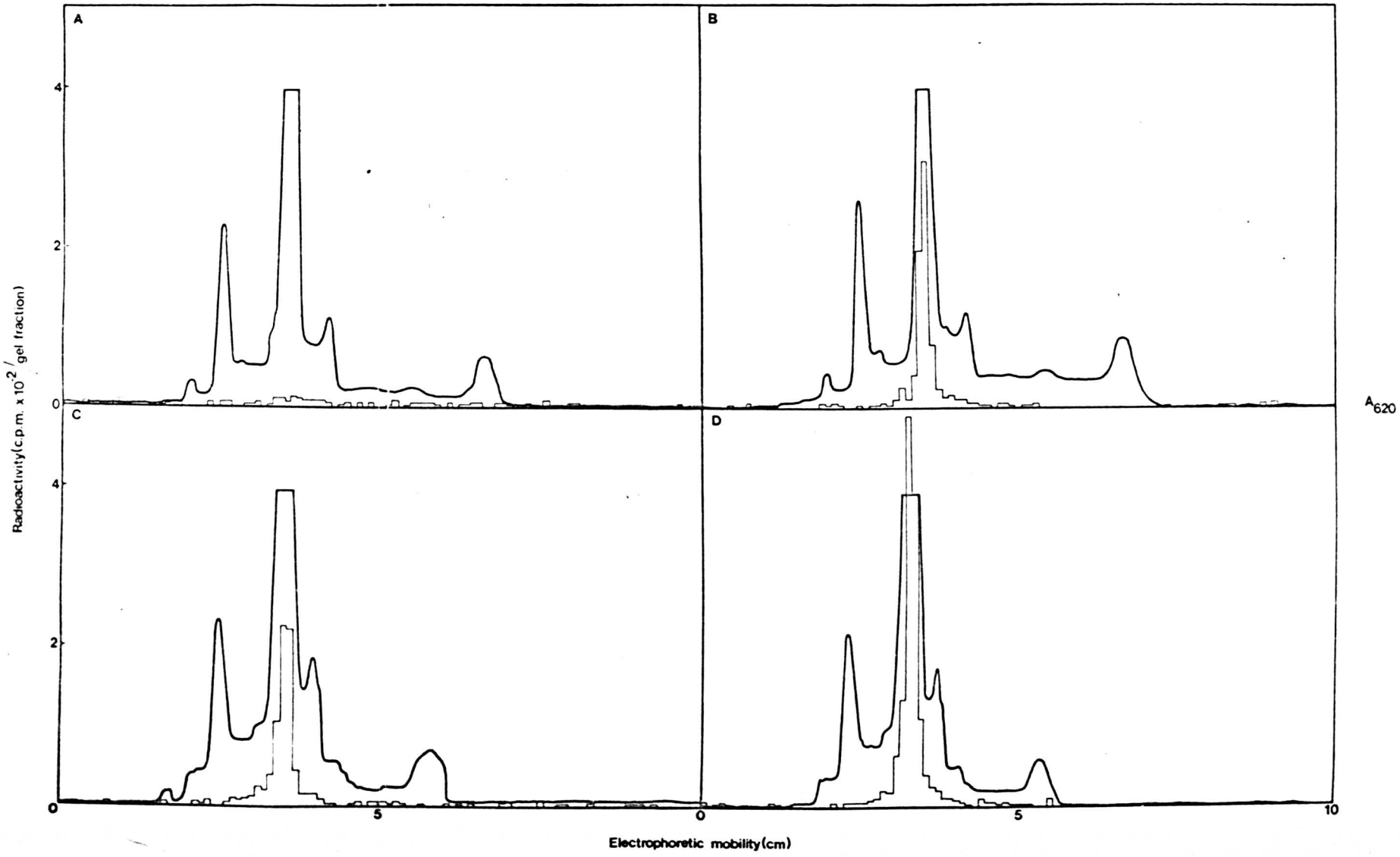

A
B
C
D
4
2
0
5
10
Radioactivity(c.p.m. x10^{-2}/gel fraction)
A_{620}
Electrophoretic mobility(cm)

Figure 23. A comparison of the products of *in vitro* protein synthesis by chloroplasts resuspended in TMS and KCl media.

A. Chloroplasts were isolated by the procedure of Ramirez *et al* (1968), and resuspended and incubated in TMS medium with 1μCi [C^{14}]leucine (Section II2Av) for 60 minutes at 20°C, using the ATP and ATP-generating system described in Section II2Ci for chloroplasts incubated in TMS medium.
B. Chloroplasts were isolated and incubated in TMS medium as described in A. above, but without ATP or an ATP-generating system.
C. Chloroplasts were isolated by the procedure of Ramirez *et al* (1968) and resuspended and incubated in KCl medium for 60 minutes at 20°C in the light with 1μCi [C^{14}]leucine.
The chlorophyll concentration in A., B. and C. was 410μg/ml. A 150 000 x g supernatant fraction was prepared from each sample, and 100μl analysed by electrophoresis on 10.0% SDS-gels, as described in figure 14.

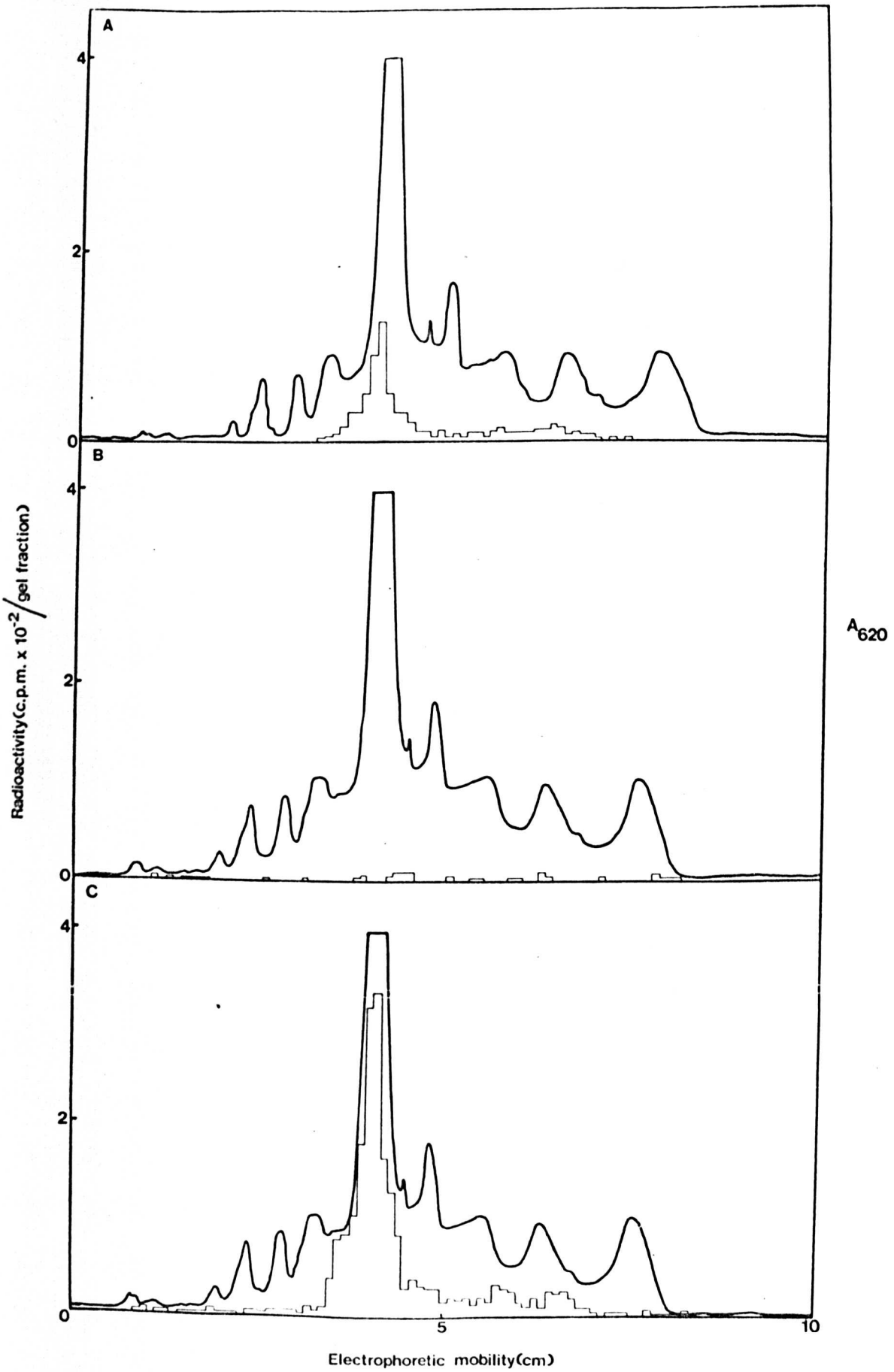
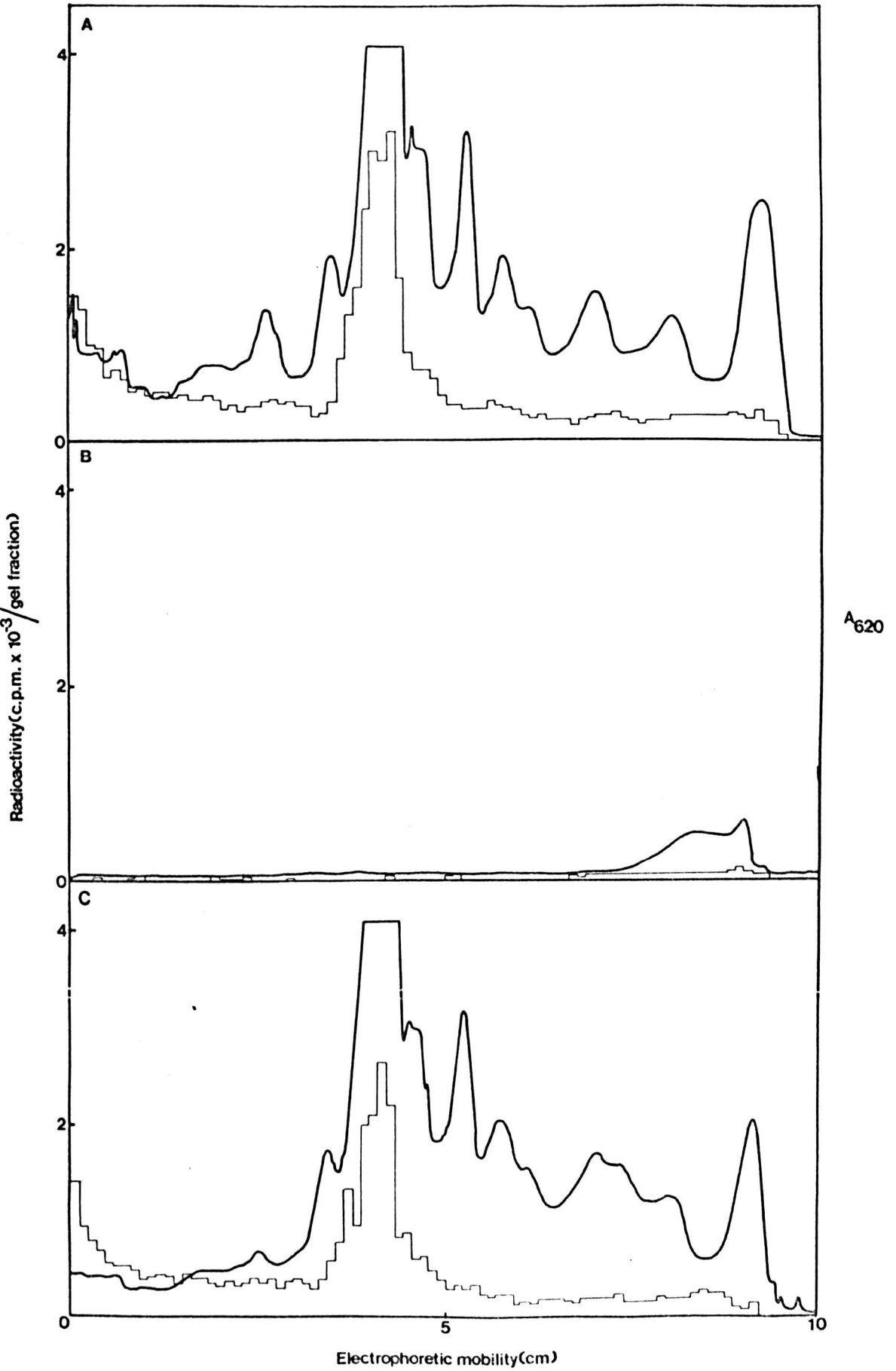

A
B
C
4
2
0
5
10
Radioactivity(c.p.m. x 10^{-2}/gel fraction)
A_{620}
Electrophoretic mobility(cm)

Figure 24. The stability of the soluble, radioactively-labelled product of *in vitro* chloroplast protein synthesis to digestion by pronase and ribonuclease A. Details of the procedure are given in Section II2Diii. A. control incubation; B. pronase-treated; C. ribonuclease-treated.


A
B
C
4
2
0
5
10
Radioactivity(c.p.m. x 10⁻³/gel fraction)
A620
Electrophoretic mobility(cm)

incubated in TMS resuspension medium. Synthesis of a single labelled peak, which was coincident with the large subunit of Fraction I protein, was found in broken chloroplasts. The amount of the labelled peak which was synthesised was greatly reduced when compared to the complete, light-driven control (fig. 23C). No synthesis took place in the absence of ATP and an ATP-generating system (fig. 23B).

G. Analysis of products digested by pronase and ribonuclease A.

The sensitivity of the soluble, in vitro-labelled product to digestion by pronase and ribonuclease was investigated. The results are shown in fig. 24. When the 150 000 x g supernatant fraction was incubated without either enzyme, no digestion of the labelled peak occurred (fig. 24A). However, when the supernatant fraction was incubated with pronase (fig. 24B), both the radioactive peak and all amidoblack-staining bands on the gel were hydrolysed. In contrast, the labelled peak was completely stable to the action of ribonuclease (fig. 24C), as were all the stained bands.

4. ANALYSIS OF PRODUCTS BY SEPHADEX G100 CHROMATOGRAPHY.

The technique of gel chromatography was used as an additional method of fractioning the products of in vitro chloroplast protein synthesis, and also as a method of purifying the labelled products. The details of the procedure are given in Section II2H. A 150 000 x g chloroplast supernatant fraction labelled in vitro with [S^{35}] methionine, was co-chromatographed on Sephadex G100 with purified pea Fraction I protein in an SDS-containing buffer (fig. 25A). Similarly, purified pea Fraction I protein labelled in vivo with [S^{35}] methionine was analysed by SDS-Sephadex G100 chromatography (fig. 25B). The in vivo-labelled Fraction I protein showed distinct labelling of both large and small subunits, in agreement with the analysis on SDS-gels (fig. 19). The in vitro-labelled supernatant fraction, while showing clear labelling of the large subunit, also appeared to have incorporated [S^{35}] methionine into the small subunit. This result is in direct contradiction to that obtained by analysis on SDS-gels (fig. 17).

This problem was further investigated by precipitation of the large and small subunit fractions of figs. 25A and 25B, and re-running aliquots/

Figure 25. Elution of *in vivo*, radioactively-labelled pea Fraction I protein, and the products of *in vitro* chloroplast protein synthesis on Sephadex G100, in a sodium dodecyl sulphate-containing buffer.

A. Pea chloroplasts (500µl, containing 180µg chlorophyll) were incubated in KCl medium with 50µCi [S^{35}]methionine at 20°C for 60 minutes in the light. A 150 000 x g supernatant fraction was prepared (Section II2Di). A volume of 1.5ml of supernatant fraction (whose protein concentration was 3.85mg/ml) was mixed with 7mg of purified pea Fraction I protein and denatured by incubating with 0.2ml 10% (w/v) SDS and 20µl 2-mercaptoethanol for 60 minutes at 37°C. The mixture (which contained approximately 1.25×10^6 c.p.m.) was applied to a Sephadex G100 column (2.5 x 45cm) and eluted at a flow rate of 10ml/h with an SDS-containing buffer (Section II2H). Fractions (1.8ml) were collected and a 100µl aliquot removed from each fraction, dissolved in 8ml Triton-toluene scintillant and counted in a Packard Tricarb spectrometer at 15% gain, open window. The extinction at 280nm of each fraction was determined in a Unicam SP500 spectrophotometer.
B. Fraction I protein, labelled *in vivo* with [S^{35}] methionine, was purified as described in Section II2G. A total of 12mg of labelled protein was mixed with 7mg of unlabelled pea Fraction I protein and denatured by adding 0.5ml 10% (w/v) SDS and 20µl 2-mercapto-ethanol. Incubation was at 37°C for 60 minutes. The details of the conditions of chromatography are given in A. above. E_{280} (■———■); radioactivity (▲———▲).

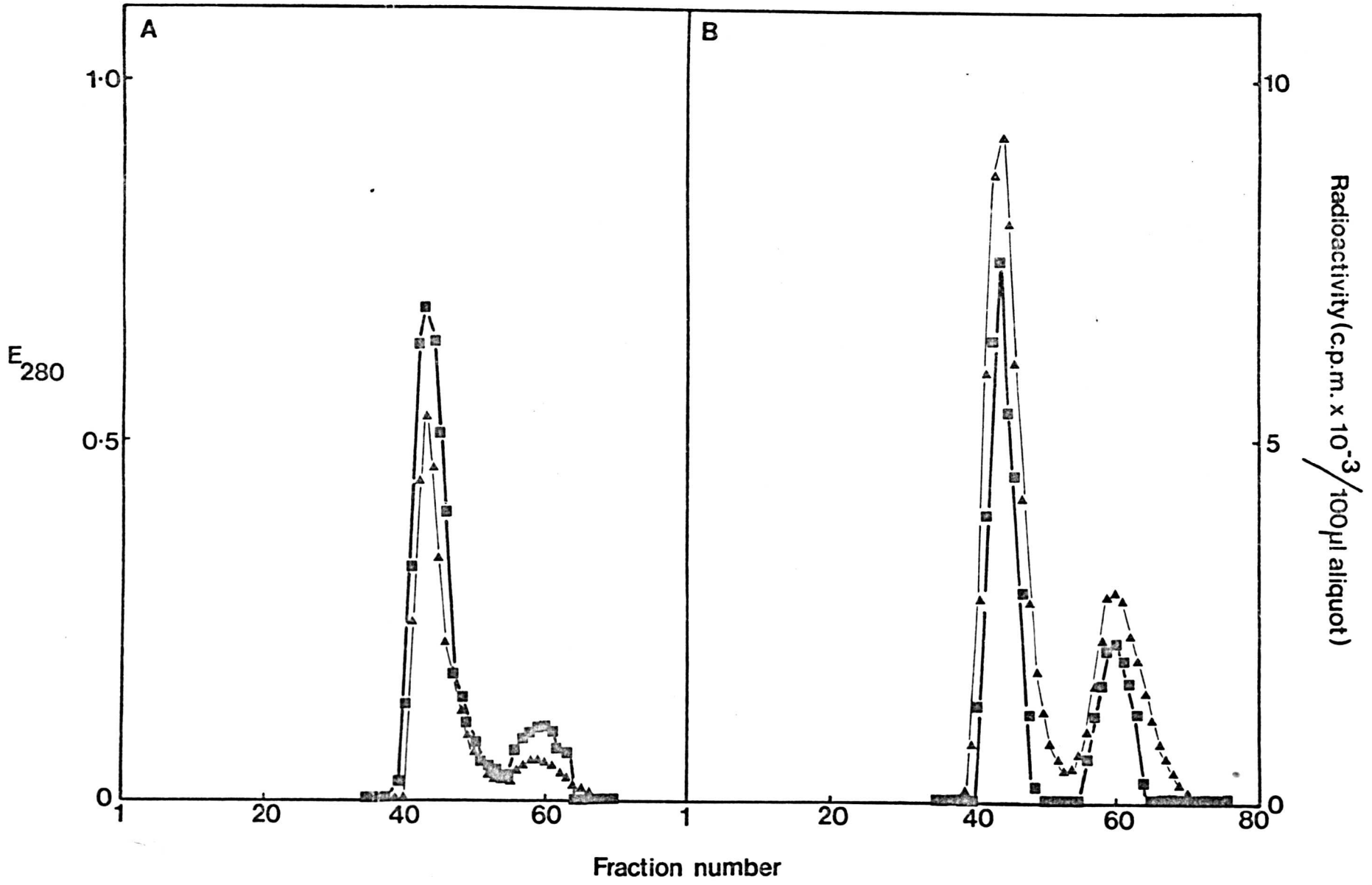

A
B
1·0
0·5
0
E_{280}
10
5
0
Radioactivity (c.p.m. x 10^{-3} / 100µl aliquot)
1
20
40
60
80
Fraction number

Figure 26. Purification of radioactively-labelled large and small subunits of Fraction I protein.

Peak fractions of [S^{35}]methionine-labelled large and small subunit proteins obtained by chromatography on Sephadex G100 (figs. 25A and 25B) were precipitated in 90% (v/v) acetone. The precititates were centrifuged at 1 000 x g for 15 minutes at 4°C (r_{av} 21.6cm) in an MSE 6L. Each pellet was dissolved in 100µl 2.5mM tris-19mM glycine (pH 8.5), 20µl 2% (w/v) SDS and 10µl 2-mercaptoethanol, and incubated at 37°C for 60 minutes. Aliquots of each protein solution were analysed by electrophoresis on 10.0% SDS-gels. A. 20µl large subunit, obtained by co-chromatography with labelled 150 000 x g chloroplast supernatant fraction. B. 30µl large subunit, labelled *in vivo*. C. 50µl small subunit, labelled *in vivo*. D. 50µl small subunit, obtained by co-chromatography with labelled 150 000 x g chloroplast supernatant fraction. All other details are given in figure 14.

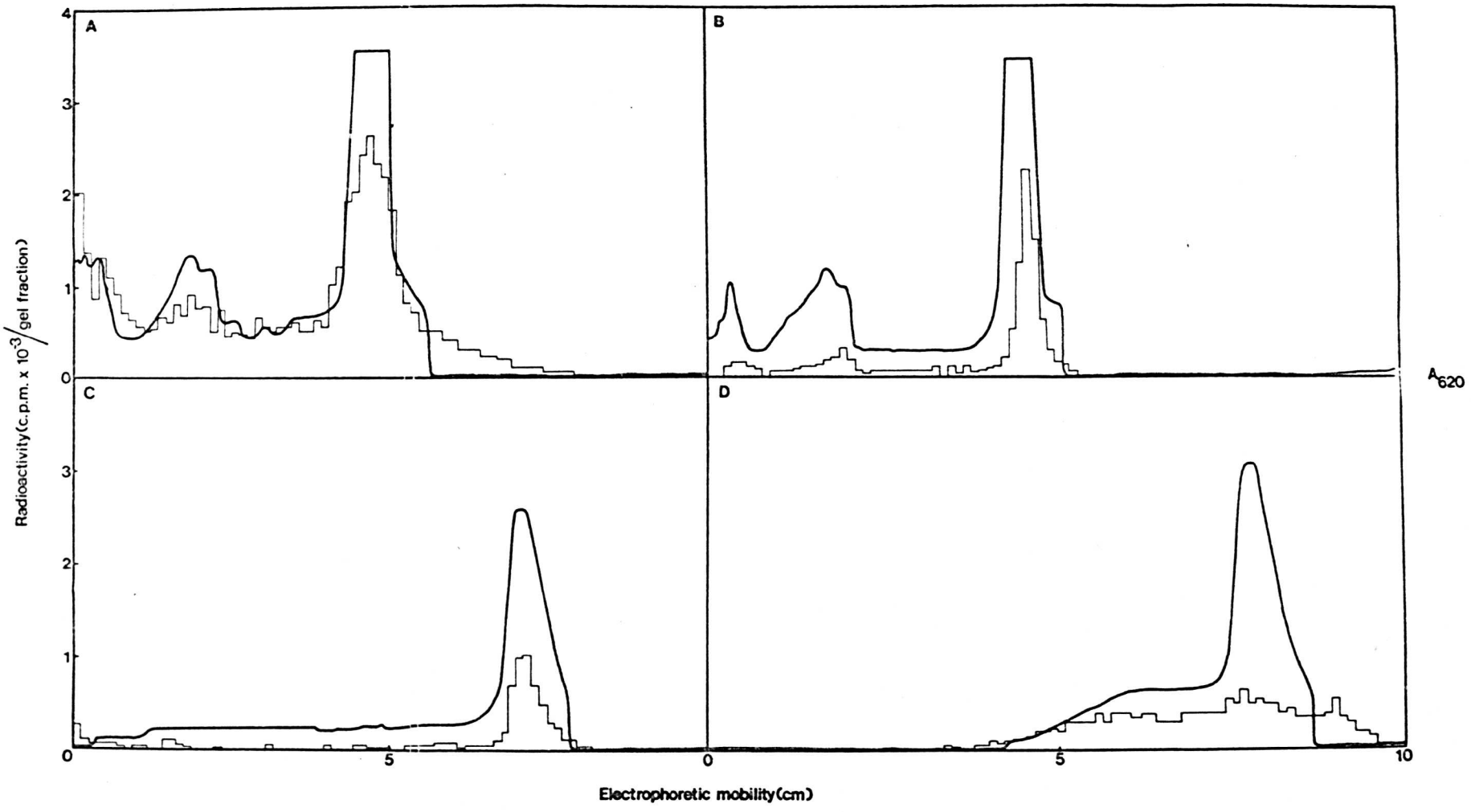
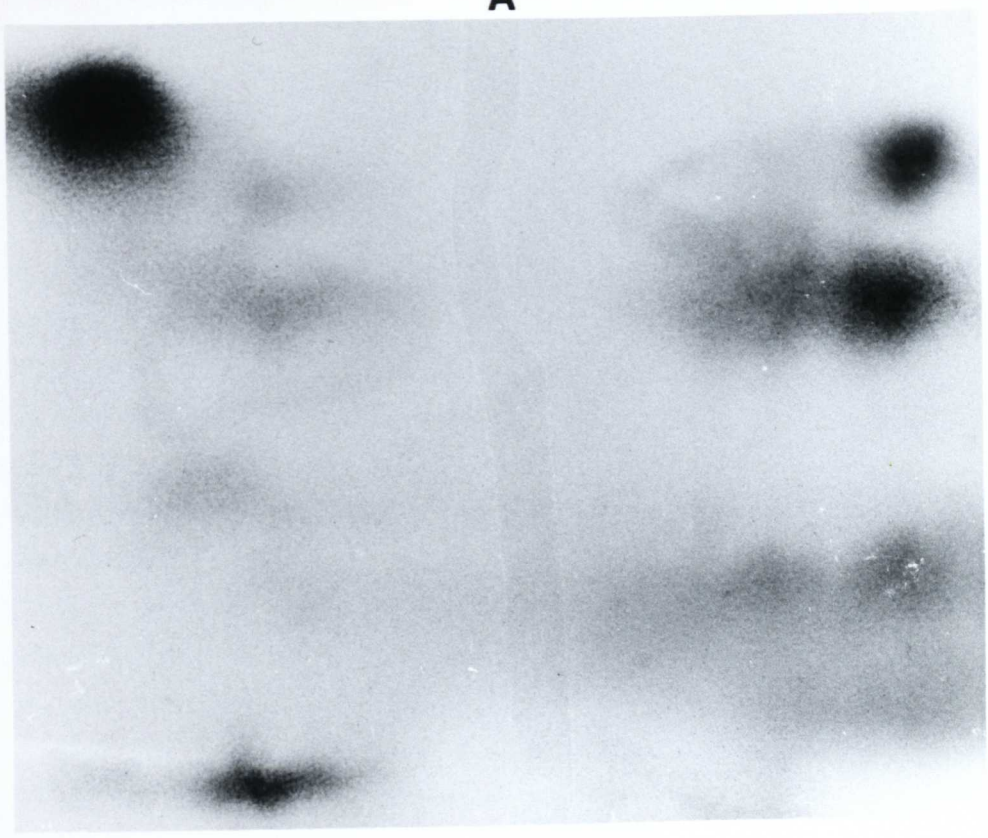
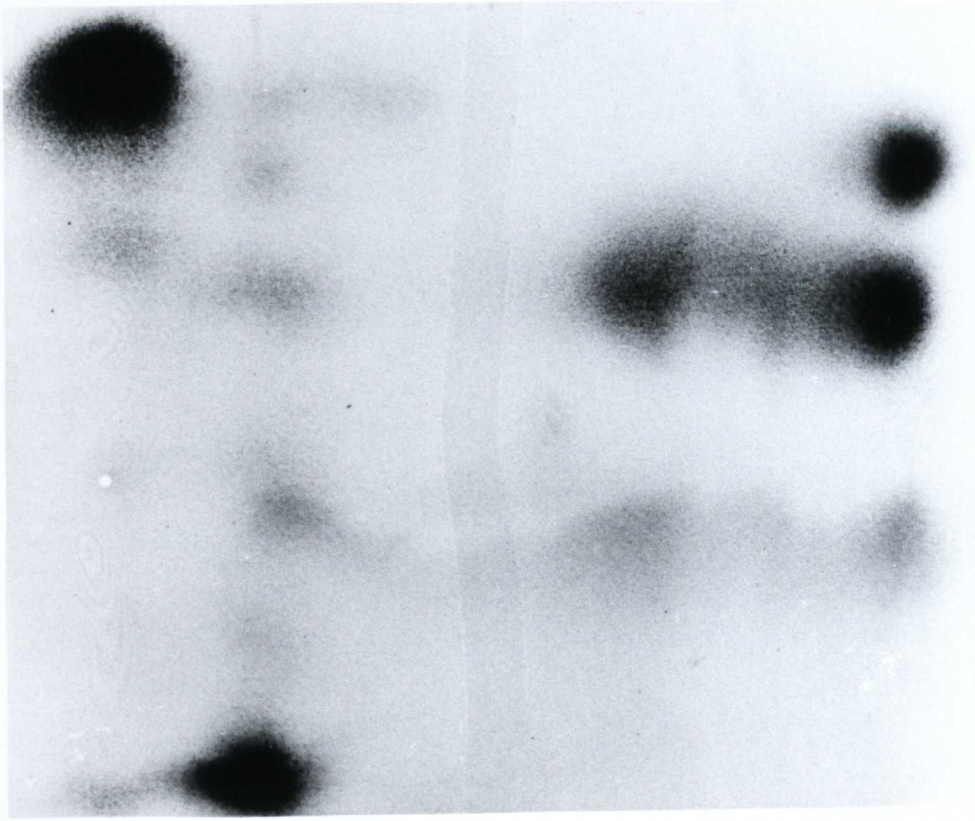

A
B
C
D
Radioactivity(c.p.m. x 10-3/gel fraction)
A620
Electrophoretic mobility(cm)
0
1
2
3
4
5
10

Plate 9. Autoradiographs of tryptic peptide maps of A. the soluble, *in vitro* product of light-driven chloroplast protein synthesis and B. *in vivo*-labelled large subunit of Fraction I protein.

A



B



aliquots of the purified subunit proteins on 10.0% gels (Section II2H). In fig. 26A, a gel of purified large subunit protein labelled *in vitro* with [S^{35}]methionine is shown. Although the gel was overloaded, the large subunit was discretely labelled; the high molecular weight protein already noted (Section IV3B) was also labelled. Similarly, large subunit obtained from purified, *in vivo*-labelled Fraction I protein was labelled by a discrete, coincident peak of radioactivity (fig. 26B).

The small subunit protein prepared from *in vivo*-labelled Fraction I showed the expected discrete labelling profile (fig. 26C). However, no discrete peak of radioactivity was observed on SDS-gels of small subunit protein prepared by Sephadex G100 chromatography of an *in vitro*-labelled chloroplast supernatant fraction (fig. 26D). The radioactivity obtained on this gel, and in the small subunit fractions of fig. 25A, may be low molecular weight oligopeptides or free [S^{35}] methionine. The apparent *in vitro*-labelling of the small subunit shown when the supernatant fraction was analysed by gel chromatography could not be removed by adding unlabelled L-methionine at 10mM to the buffer against which the chloroplasts were dialysed at the end of the incubation (Section II2Di).

5. TRYPTIC PEPTIDE ANALYSIS OF THE LARGE SUBUNIT OF PEA FRACTION I PROTEIN.

Tryptic peptide analysis was performed as the final stage in the identification of the soluble, *in vitro* product of chloroplast protein synthesis. A two-dimensional map of the [S^{35}]methionine-labelled tryptic peptides of the *in vitro*-synthesised product was compared with a map of the [S^{35}]methionine-labelled tryptic peptides of *in vivo*-labelled large subunit of Fraction I protein (Section II2I). As shown in plates 9A and 9B, both *in vivo* and *in vitro*-labelled proteins share five major [S^{35}]methionine-labelled tryptic peptides. Some minor peptides may also be common to both proteins.

6. DISCUSSION.

The results presented in this Section show that isolated pea chloroplasts synthesise a single soluble protein. Fractionation of the soluble products of *in vitro*, light-driven protein synthesis on non-denaturing polyacrylamide gels showed a difference in mobility of the/

the labelled peak depending on the concentration of acrylamide used. Although Fraction I protein appeared to be labelled *in vitro* when the 150 000 x g supernatant fraction was analysed on 5.0% acrylamide gels (fig. 14A), on 4.0% gels the same supernatant fraction showed incorporation of [C^{14}]leucine into a peak of lower mobility than Fraction I protein (fig. 14B). It must be concluded that complete Fraction I protein is not synthesised by isolated pea chloroplasts, especially since purified, *in vivo*-labelled Fraction I protein showed exact coincidence of radioactivity and protein on both 5.0% (fig. 15A) and 4.0% (fig. 15B) gels. However, the results obtained by fractionation on non-denaturing gels do show that the synthesis of the *in vitro* product is energy-dependent - in the absence of light no synthesis took place (fig. 16B). ATP could substitute for light as an energy source although incorporation into the labelled peak was reduced (fig. 16C), closely reflecting the results shown in Table 4 for total amino acid incorporation by the chloroplast preparation. The existence of the labelled peak could not be explained by the binding of [C^{14}]leucine to pre-existing protein since no discrete, labelled peak was obtained at time zero (fig. 16A); this result is important since gel slices were not treated with hot trichloroacetic acid.

Labelling of the subunits of the supernatant proteins was investigated by electrophoresis on SDS-gels. Again, only one discrete, radioactively-labelled peak was obtained. This peak migrated with the large subunit of Fraction I protein when the same supernatant fraction was analysed on 8.0%, 10.0% and 12.0% SDS-gels (fig. 17). The labelled peak had exactly the same mobility and hence molecular weight (Shapiro *et al*, 1967) as the large subunit of Fraction I protein (fig. 18). Neither the small subunit of Fraction I protein, nor any other protein on the gel was labelled to an appreciable extent when either [C^{14}]leucine (fig. 21A), [C^{14}]phenylalanine (fig. 21B) or [S^{35}] methionine at high concentration (fig. 21C) was used as protein precursor. The use of these three amino acids makes it unlikely that an additional protein would fail to be labelled due to the lack of, for example, methionine in its sequence. In addition, the large amounts, and the higher specific activity of the [S^{35}]methionine used might have been expected to label other proteins if they were being synthesised in low amounts. In conclusion, no other protein on the gel was labelled under any labelling conditions used *in vitro*, within the limits of detection/

detection of the analytical techniques used. Labelling of the small subunit was detected, but only when in vivo-labelled Fraction I protein was analysed by electrophoresis on SDS-gels (fig. 19) and by elution on Sephadex G100 using an SDS-containing buffer (fig. 25B). It must be added, however, that some apparent labelling of the small subunit was detected in vitro when analysis of the supernatant fraction was performed on Sephadex G100 (fig. 25A); this point will be returned to later.

The soluble product of in vitro protein synthesis by isolated chloroplasts shows the characteristics of a protein. It was always coincident with an amidoblack-staining (i.e. protein) band on both denaturing and non-denaturing gels; it was digested by pronase (fig. 24B) but not by ribonuclease (fig. 24C), and it could be labelled by several protein precursors : $[C^{14}]$leucine, $[C^{14}]$phenylalanine and $[S^{35}]$methionine (fig. 21). The reduced incorporation shown with $[C^{14}]$ phenylalanine compared to $[C^{14}]$leucine (Section IV3D) might reflect either the amino acid composition of the large subunit, the relative pool sizes of phenylalanine and leucine in the chloroplast, or a difference in the permeability of the outer envelope of the chloroplast to phenylalanine and leucine.

Since no additional labelled peaks were observed when the supernatant fraction was analysed by SDS-gel electrophoresis, it must be concluded that the labelled peaks found on denaturing and non-denaturing gels are the same proteins. On the basis of gel electrophoresis this labelled protein can tentatively be identified as the large subunit of Fraction I protein. The rather confusing results obtained on non-denaturing gels (fig.14) can be explained by assuming that the labelled large subunits are aggregated and show differing mobilities on different percentage acrylamide gels. On 5.0% gels, both Fraction I protein and the aggregated labelled protein have the same mobility. This phenomenon of altering relative mobilities has been described for several different proteins (Gordon, 1969). Only on a 4.0% gel can Fraction I protein and the labelled protein be resolved.

Complete, labelled Fraction I protein was not detected on non-denaturing gels (fig. 14B). This raises a question as to why native Fraction I protein could not be synthesised using pre-existing unlabelled small subunit present in the chloroplast. A possible inference is that there is no pool of small subunits within the chloroplast/

chloroplast, or that such a pool does exist but assembly of native Fraction I protein from large and small subunits is defective in some way in the isolated chloroplast. The latter possibility seems unlikely since subunits appear to associated readily into native enzymes according to principles of symmetry and thermodynamics (Klotz, Langerman and Darnall, 1970). If no pool of small subunits is present in the chloroplast this must also indicate that there is no rapid, free equilibration in the chloroplast of large and small subunits between both pre-existing enzyme molecules and newly-synthesised large subunits.

Is the labelled protein produced by cleavage or proteolysis of a higher molecular weight species, or is the labelled protein a precursor for lower molecular weight species? No evidence was found in favour of these ideas. When chloroplast supernatant extract, labelled *in vitro* with [S^{35}] methionine, was incubated for 4 hours at 37°C, no protein bands of lower molecular weight than the large subunit of Fraction I became labelled, or was there any heterogeneity produced in the labelled peak, which again ran coincident with Fraction I protein large subunit (fig. 24A). This implies that no specific or non-specific proteases are present in the supernatant fraction.

Does synthesis take place on chloroplast ribosomes? Incorporation of [C^{14}] leucine into the labelled peak was completely inhibited by D-*threo*-chloramphenicol (fig. 22A) at low concentration, an inhibitor specific for 70S ribosomes. Similarly other selective inhibitors of 70S ribosomes, spectinomycin (fig. 22B) and lincomycin (fig. 22C) reduced the synthesis of the labelled protein. These results strongly suggest that synthesis does take place on chloroplast ribosomes. The great stimulation of synthesis produced by light also argues for synthesis on chloroplast ribosomes, as already discussed in Section III12. When the *in vitro*-labelled supernatant fraction was analysed by SDS-gel electrophoresis, synthesis of the labelled peak was again shown to be energy-dependent (fig. 20), agreeing with the results obtained on non-denaturing gels (fig. 16). No synthesis occurred in the absence of light (fig. 20C) and synthesis was reduced when ATP was used as the energy source (fig. 20A).

When chloroplasts were incubated in TMS medium, which produces broken chloroplasts only, synthesis of Fraction I protein large subunit was again obtained when ATP and an ATP-generating system were present instead of light (fig. 23A). However, the extent of [C^{14}] leucine/

leucine incorporation into the labelled peak was very much less than in the control system which uses light as energy source (fig. 23C). This shows that synthesis of a specific protein can be detected in a conventional preparation of broken chloroplasts. In Section III12, it was inferred that some amino acid incorporation might take place in broken chloroplasts (which represent around 50% of the chloroplasts present in the KCl preparation) in the presence of exogenous ATP. The results described in Section IV3F lend support to this inference, and indicate that ATP-driven protein synthesis may proceed at a low rate in broken chloroplasts present in the routine KCl preparation.

The previous failure to demonstrate synthesis of specific proteins in isolated chloroplasts may be due in part to insufficient care in the choice of analytical methods. Polyacrylamide gel electrophoresis has rarely been used to analyse the products of *in vitro* chloroplast protein synthesis, with inconclusive results (Chen and Wildman, 1970; Margulies, 1970). Sucrose gradients have frequently been used (Margulies and Parenti, 1968; Chen and Wildman, 1970) but do not provide the sensitivity and resolution of gel electrophoresis.

Both chromatography on Sephadex in the presence of a denaturant such as SDS, and SDS-gel electrophoresis provide a fractionation based on molecular weight (Fish, Reynolds and Tanford, 1970; Shapiro *et al*, 1967). However, when the labelled supernatant fraction was analysed by both techniques a contradictory result was obtained. No labelling of the small subunit of Fraction I protein was observed when the supernatant proteins were fractionated by SDS-gel electrophoresis (figs. 17 and 21). When the same supernatant extract was analysed by chromatography in an SDS-containing buffer on Sephadex G100, label appeared in fractions containing the small subunit of Fraction I protein (fig. 25A). Was this labelling of the small subunit real or apparent? The question was resolved by analysing by gel electrophoresis, small subunit obtained from elutions of *in vitro*-labelled supernatant extract and *in vivo*-labelled Fraction I protein. A discrete peak, coincident with the small subunit was obtained only with the *in vivo*-labelled protein (fig. 26C). The labelling of the small subunit *in vitro* was therefore an artefact which could not be reproduced upon subsequent analysis by SDS-gel electrophoresis (fig. 26D). The basis of this artefact was not discovered, or even fully investigated, but may consist of free [S^{35}]methionine bound to some component of the chloroplast/

chloroplast which elutes with the small subunit of Fraction I protein. Fractionation of macromolecules of molecular weight less than 15 000 daltons by gel chromatography or electrophoresis in the presence of SDS is unreliable (Fish et al, 1970) and so the precise nature of the binding or state of the [S^{35}]methionine cannot be easily identified. The artefact could not be removed (Section IV4) and might perhaps be borne in mind when radioactively-labelled extracts are analysed by gel chromatography.

The fact that both SDS-gel electrophoresis and SDS-gel chromatography fractionate proteins on the basis of their molecular weight is an essential weakness in the use of these techniques to identify unknown proteins. If two proteins co-electrophorese on an SDS-gel, this does not mean that they are the same but merely that they both have the same molecular weight. Some additional criteria must be sought if identification is to be conclusive. Two-dimensional peptide mapping provides a direct comparison of the primary structures of proteins. This technique was used to identify conclusively the soluble product of protein synthesis by isolated chloroplasts.

The purity of the samples used for peptide mapping was checked by gel electrophoresis and the results are shown in figs. 26A and 26B. This shows that most of the radioactivity present on the gels was coincident with the large subunit of Fraction I protein when both in vivo-labelled large subunit (fig. 26B) and the in vitro product (fig. 26A) were analysed. Both gels were overloaded, each showing an additional labelled band of higher molecular weight. This band is believed to be an aggregate of large subunit since it appears to increase in intensity when the 2-mercaptoethanol concentration is lowered. It was concluded that the two protein samples were sufficiently pure for peptide analysis.

The tryptic peptide maps shown in plate 9 confirm the identity of the soluble protein synthesised in isolated pea chloroplasts. A high degree of similarity was shown between the peptide map of the soluble protein synthesised in vitro, and a similar map of in vivo-labelled large subunit of Fraction I protein. At least five major, and possibly more minor radioactively-labelled peptides were shared by both in vivo and in vitro-labelled proteins. Absolute comparison between the two maps is difficult since it was found to be impossible to label the large subunit in vitro to the specific activity obtained in in vivo-labelled large subunit. However, the high degree of similarity between the two peptide/

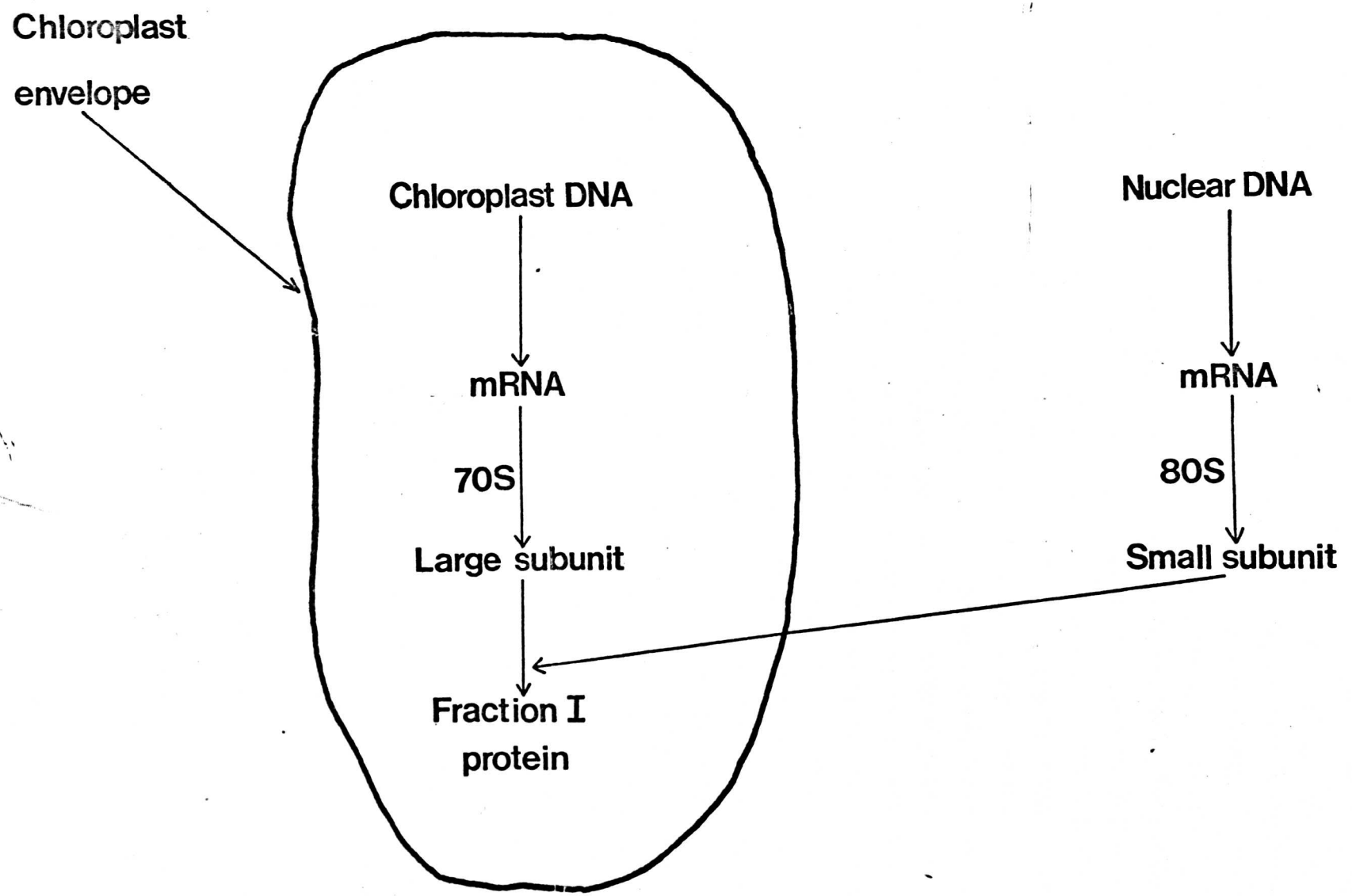
peptide maps conclusively demonstrates that the large subunit of Fraction I protein is synthesised as the single soluble product of light-driven protein synthesis in isolated intact chloroplasts.

SECTION V - GENERAL DISCUSSION

The results presented in this thesis show that only one subunit of one of the many soluble chloroplast proteins, namely the large subunit of Fraction I protein, is synthesised on chloroplast ribosomes. In addition, these results constitute the first unequivocal demonstration of the synthesis of a specific protein by isolated chloroplasts (Blair and Ellis, 1972, 1973; Ellis, Blair and Hartley, 1973). The results of the study of *in vitro* chloroplast protein synthesis described in this thesis are in good agreement with results obtained *in vivo* using selective inhibitors of 70S ribosomes. Fraction I protein is one of the few proteins which the many *in vivo* studies suggest is synthesised on chloroplast ribosomes. Moreover, the *in vitro* study agrees with the results obtained *in vivo* by Criddle *et al* (1970), which show that the synthesis of the large subunit of barley Fraction I protein is preferentially inhibited by chloramphenicol whereas that of the small subunit is preferentially inhibited by cycloheximide (Section I5A). Genetic analysis of inter-specific hybrids of tobacco suggest that the large subunit of Fraction I protein is coded in the chloroplast DNA, while the small subunit is coded in the nuclear DNA (Section I4Ci). Combining all these results, a model which describes our current view of the co-operation between nucleus, chloroplast and cytoplasm in the biosynthesis of Fraction I protein is shown in fig. 27. The large subunit is thus both encoded and synthesised within the chloroplast whereas the small subunit is both encoded and synthesised outside the chloroplast. This model therefore requires protein, but not nucleic acid to cross the chloroplast envelope. This raises again the possibility of the existence in the chloroplast envelope of a protein translocase which might recognise the small subunit and all the other chloroplast proteins which are synthesised in the cytoplasm and are transported into the chloroplast (Section I5A).

This model also requires that a messenger RNA for the large subunit is transcribed from chloroplast DNA and translated on chloroplast ribosomes. Such messenger RNA is probably synthesised in very small amounts during the time of incubation of isolated pea chloroplasts in the light, since actinomycin D at 10µg/ml had no effect on light-driven chloroplast protein synthesis (Blair and Ellis, 1973). However, the same concentration of actinomycin D inhibited light-driven incorporation of [H^3]uridine into RNA by the same chloroplast preparation/

Figure 27. Model of co-operation between chloroplast and nuclear genomes, and chloroplast and cytoplasmic ribosomes in the biosynthesis of Fraction I protein (modified after Kawashima and Wildman, 1972).


Chloroplast
envelope
Chloroplast DNA
mRNA
70S
Large subunit
Fraction I
protein
Nuclear DNA
mRNA
80S
Small subunit

preparation by 85% (M.R. Hartley, personal communication). It must be concluded therefore that protein synthesis in isolated chloroplasts probably uses messenger RNA synthesised before the chloroplasts are isolated. Messenger RNA for the large subunit of Fraction I protein is present in a preparation of RNA extracted from the chloroplast fraction of spinach leaves, since the large subunit is synthesised by a cell-free protein-synthesising system from E. coli, using spinach chloroplast RNA as template (A.M. Wheeler and M.R. Hartley, personal communication).

The nature of the mechanism which regulates the relative rates of synthesis of the two subunits of Fraction I protein in the two cellular compartments is unknown. It was inferred that there is little or no pool of small subunits in the isolated chloroplasts (Section IV6). This may mean that small subunits are assembled, with large subunits, into complete Fraction I protein as soon as they enter the chloroplast. Thus the availability of small subunits within the chloroplast may control the biosynthesis of Fraction I protein. Possible points of control might therefore include the synthesis of small subunits on cytoplasmic ribosomes, and the transfer of small subunits from the cytoplasm into the chloroplast. It might also be argued that in isolated chloroplasts no initiation of protein synthesis takes place; synthesis of Fraction I protein large subunit might therefore occur merely by the completion of pre-existing polypeptide chains. Small subunit might therefore act as an initiation factor. This possibility can be excluded since small subunit is not required for the synthesis of large subunit in the E. coli cell-free system using spinach chloroplast RNA as template (A.M. Wheeler and M.R. Hartley, personal communication). The remaining possibility, namely that small subunit acts as a transcriptional cofactor in the synthesis of large subunit messenger RNA, can be tested when a pure preparation of chloroplast DNA-dependent RNA polymerase becomes available.

The 150 000 x g supernatant fraction contains only 25% of the labelled amino acids incorporated into protein by the chloroplast preparation (Table 9). The remaining 75% sediments with the pellet, containing lamellae and ribosomes. The membrane-bound products of light-driven protein synthesis by isolated, intact pea chloroplasts have been analysed (Eaglesham and Ellis, 1973). The chloroplast lamellae were shown to contain six radioactively-labelled peaks, ranging/

ranging in molecular weight between 1.8×10^4 and 8.5×10^4 daltons, with a major peak of 3.2×10^4 daltons. Unfortunately, none of these membrane-bound proteins could be identified, although cytochrome f, the chloroplast coupling factor, and the proteins associated with photosystems I and II could be excluded from possible consideration. It is not known whether these labelled proteins account for all the radioactivity incorporated into the 150 000 x g pellet. Labelled amino acids might also be incorporated into nascent polypeptides and ribosomal proteins.

It would appear therefore that chloroplasts are capable of synthesising very few of their many proteins. On the other hand, chloroplast ribosomes can account for up to 50% of total leaf ribosomes (Boardman, Francki and Wildman, 1966). Why are so many ribosomes required to synthesise so few proteins? One explanation may be that one of their products, the large subunit of Fraction I protein, occurs in much larger quantities than any other protein in the leaf.

The co-operation of cytoplasmic and organelle ribosomes which exists in the biosynthesis of Fraction I protein in chloroplasts also takes place in the biosynthesis of several mitochondrial proteins. Both mitochondrial and cytoplasmic ribosomes are involved in the synthesis of the subunits of the rutamycin-sensitive ATPase of yeast mitochondria (Tzagoloff and Meagher, 1972), the cytochrome oxidase (Sebald et al, 1973) and the cytochrome b-containing membrane protein (Weiss, 1972) of Neurospora crassa mitochondria. However, work on Fraction I protein is aided by the fact that the protein is soluble, can be easily purified, and consists of only two non-identical subunits whereas all three mitochondrial proteins mentioned are membrane-bound, with consequent difficulties in purification, and consist of many non-identical subunits. The products of protein synthesis in isolated mitochondria are membrane-bound (Ashwell and Work, 1970; Tzagoloff and Akai, 1972) whereas 25% of the amino acids incorporated into protein by isolated chloroplasts are present in the soluble phase of the chloroplast, thus aiding identification. Chloroplasts therefore possess some advantages over mitochondria as a system for studying protein synthesis in isolated organelles.

In principle, the methods described in this thesis used to study protein synthesis is isolated pea chloroplasts could be applied to the study/

study of *in vitro* protein synthesis in chloroplasts from a range of higher plants and algae. It was noted in Table 10 that similar characteristics of amino acid incorporation are shown by both isolated pea and spinach chloroplasts. The results presented in this thesis on the identification of the soluble products of *in vitro* chloroplast protein synthesis have been confirmed for isolated spinach chloroplasts (Whitfeld *et al*, 1972). Thus the large subunit of Fraction I protein is synthesised as the sole soluble product of protein synthesis in both isolated pea and spinach chloroplasts. Harris *et al* (1973) have shown that in chloroplasts isolated from *Euglena gracilis* by conventional methods, labelled amino acids are incorporated into a soluble fraction which co-migrates with Fraction I protein on agarose column chromatography. The labelling of the subunits of *Euglena* Fraction I protein was not investigated. No discrete, membrane-bound proteins were synthesised in isolated *Euglena* chloroplasts. Apel and Schweiger (1973), however, have shown that in isolated *Acetabularia* chloroplasts two membrane proteins are synthesised. The synthesis of one of these membrane proteins was inhibited by cycloheximide. The preparations of *Acetabularia* chloroplasts were shown to contain cytoplasmic 26S RNA, again emphasising the need for caution in interpreting results obtained with isolated *Acetabularia* chloroplasts (Bidwell, 1972). It would appear, therefore, that there is a need to study light-driven protein synthesis in intactchloroplasts isolated from some species of algae. The products of *in vitro* chloroplast protein synthesis in higher plants and algae could then be directly compared. This might give some indications as to the possible functions of chloroplast ribosomes in species widely separated in evolution.

The most useful extension of the work described in this thesis would be the purification of the messenger RNA for the large subunit of Fraction I protein. Apart from being the first demonstration of a messenger RNA from a plant source, important studies on the mechanism of chloroplast protein synthesis and the expression of the large subunit gene during chloroplast development might then be possible. Eventually, with the availability of purified chloroplast RNA-polymerase, a protein-synthesising system dependent on chloroplast DNA might be developed. This would provide a direct method of studying the function of chloroplast DNA.

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APPENDIX - PUBLISHED PAPERS

1. Blair, G.E. & Ellis, R.J.(1972) Biochem.J. 127,42P.
2. Blair, G.E. & Ellis, R.J.(1973) Biochem.Biophys.Acta. 319,223-234.

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Light-Driven Synthesis of the Large Subunit of Fraction I Protein by Isolated Chloroplasts

By G. E. BLAIR and R. J. ELLIS (*Division of Biological Sciences, University of Warwick, Coventry CV4 7AL, U.K.*)

Treatment of greening cells with 70S ribosomal inhibitors such as lincomycin results in the inhibition of the synthesis of fraction I protein, but not that of other photosynthetic enzymes or chloroplast RNA polymerase (Ellis & Hartley, 1971). Fraction I protein is the major protein found in chloroplasts, and consists of large and small subunits (Rutner & Lane, 1967). The differential labelling of the large and small subunits *in vivo* (Kawashima, 1970) and the sensitivity of such labelling to chloramphenicol and cycloheximide suggests that only the large subunit is made by chloroplast ribosomes (Criddle *et al.*, 1970) However, conclusive evidence can only come from studies with isolated chloroplasts.

Isolated chloroplasts are known to carry out the incorporation of labelled amino acids into protein by means of a 70S ribosomal system, but there has been no convincing identification of any specific protein that these ribosomes synthesize *in vitro* (Kirk, 1970). We believe that the reason for this is that precautions were not taken to ensure that incorporation takes place only in intact chloroplasts in which conditions for correct termination and release of polypeptide chains are likely to be optimal. We now report that isolated intact pea chloroplasts synthesize the large subunit of fraction I protein but not the small subunit.

Chloroplasts were isolated by the rapid method of Ramirez *et al.* (1967) from 7–10-day-old pea plants (*Pisum sativum*) grown at 2000lx on a 12h photoperoid. Incorporation of [^{14}C]leucine into protein is stimulated 20-fold by red light in the absence of either added ATP or catalysts of photophosphorylation; rates of incorporation are in the range 0.5–1.0nmol of [^{14}C]leucine/h per mg of chlorophyll at 20°C. Incorporation is inhibited by chloramphenicol, by lincomycin and by lysis of the chloroplasts, but not by ribonuclease. Lysed chloroplasts supplemented with ATP and GTP show very low incorporation. We believe therefore that protein synthesis is proceeding only in intact chloroplasts.

Analysis of the chloroplast soluble protein by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis and gel filtration revealed only one labelled product, which migrates exactly with the large subunit of fraction I protein. This product is not found at zero time, in chloroplasts incubated in the dark or in the presence of chloramphenicol.

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PROTEIN SYNTHESIS IN CHLOROPLASTS

I. LIGHT-DRIVEN SYNTHESIS OF THE LARGE SUBUNIT OF FRACTION I PROTEIN BY ISOLATED PEA CHLOROPLASTS

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(Received April 16th, 1973)

SUMMARY

Intact isolated pea chloroplasts use light energy to incorporate labelled amino acids into protein. 25 % of this incorporation is present in a 150 000 × *g* chloroplast supernatant fraction. When this supernatant is analysed on sodium dodecyl sulphate polyacrylamide gels only one polypeptide is labelled. This polypeptide is the large subunit of Fraction I protein, a major protein constituent of the chloroplast. Identity of the soluble *in vitro* product with the large subunit of Fraction I protein was established by comparing a tryptic map of its [^{35}S]methionine-labelled peptides with a tryptic map of the large subunit of Fraction I protein labelled *in vivo* with [^{35}S]methionine. We conclude that only one of the many chloroplast soluble proteins, namely the large subunit of Fraction I protein, is synthesised on chloroplast ribosomes.

INTRODUCTION

Chloroplasts contain 70-S ribosomes which can represent up to 50 % of the total ribosomes in a plant cell[1], and a question arises as to the function of these ribosomes. The problem of identifying those proteins which are made by chloroplast ribosomes has been approached in two ways: (a) by supplying 70-S ribosomal inhibitors to cells making chloroplasts and determining which proteins are no longer synthesised, and (b) by identifying the products of *in vitro* protein synthesis by isolated chloroplasts. The results of inhibitor experiments suggest that many of the chloroplast proteins are synthesised by cytoplasmic ribosomes and only relatively few by the chloroplast ribosomes, but the uncertainties of *in vivo* inhibitor experiments do not allow definite conclusions to be drawn[2]. The *in vitro* approach is free from these uncertainties but previous work has failed to provide convincing identification of any of the proteins synthesized by isolated chloroplasts[3,4]. We now report a system in which isolated chloroplasts synthesise identifiable proteins using light as the source of energy[5].

Abbreviations: CCCP, *m*-chlorocarbonyl cyanide phenylhydrazone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; Tricine, *N*-tris(hydroxyethyl)methylglycine.

MATERIALS AND METHODS

Plant material and the isolation of chloroplasts

Pea seeds (*Pisum sativum* var. Feltham First) were grown in compost for 7–10 days under a 12-h photoperiod of 2000 lux provided by white fluorescent tubes. Chloroplasts were isolated from the youngest leaves essentially according to the method of Ramirez *et al.*[6]. 15 g of young leaves were homogenised for 4 s in a Polytron homogeniser in 100 ml of ice-cold isolation medium, containing 0.35 M sucrose, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES)–NaOH, 2 mM EDTA, 2 mM sodium isoascorbate (pH 7.6). The homogenate was immediately strained through eight layers of muslin and centrifuged at 2500 × *g* for 1 min at 4 °C. The supernatant was decanted and the pellet resuspended in either (a) 'KCl resuspension medium' containing 0.2 M KCl, 66 mM *N*-tris(hydroxyethyl)methylglycine (Tricine)–KOH, 6.6 mM $MgCl_2$ (pH 8.3), or (b) 'sucrose resuspension medium' containing 0.35 M sucrose, 66 mM Tricine–KOH, 6.6 mM $MgCl_2$ (pH 8.3), or (c) 'mercaptoethanol resuspension medium' containing 25 mM Tricine–KOH, 10 mM $MgSO_4$, 5 mM 2-mercaptoethanol (pH 8.0).

Lysed chloroplasts were prepared by resuspending in KCl resuspension medium lacking KCl, and then restoring the KCl to 0.2 M. Chlorophyll was determined[7], and the 2500 × *g* pellets were resuspended to a chlorophyll concentration of 300–400 μg/ml.

All media and glassware were sterilised to minimise bacterial contamination.

Incubation of chloroplasts and assay for amino acid incorporation

300 μl of chloroplasts were incubated in a final volume of 500 μl with 0.5 μCi of either [^{14}C]leucine (3 μM) or [^{35}S]methionine (36 nM). For tryptic peptide analysis only, chloroplasts, resuspended in 1.5 ml of KCl resuspension medium, were incubated with 150 μCi [^{35}S]methionine (0.9 μM). In light-driven protein synthesis, tubes were illuminated at 20 °C with filtered red light at 4000 lux as measured by a Megatron light meter Type E1. In ATP-driven protein synthesis an ATP and ATP-generating system was used, containing 2 mM ATP, 5 mM creatine phosphate and 100 μg/ml creatine phosphokinase. Chloroplasts resuspended in mercaptoethanol resuspension medium were incubated at 20 °C with 100 mM KCl, 1.25 mM ATP, 0.125 mM GTP, 5 mM creatine phosphate and 200 μg/ml creatine phosphokinase.

Radioactively labelled protein was extracted as previously described[8], and counted by liquid scintillation spectrometry in toluene–0.5 % PPO scintillant at 70 % counting efficiency.

Preparation of 150 000 × g chloroplast supernatant

150 000 × *g* chloroplast supernatants were prepared as follows. After incubation the chloroplast preparation was dialysed against 1 litre of 2.5 mM Tris–glycine, 10 mM 2-mercaptoethanol (pH 8.5) at 4 °C. When preparing samples for denaturation with sodium dodecyl sulphate, the 2-mercaptoethanol concentration was increased to 100 mM. The dialysed preparations were centrifuged at 150 000 × *g* for 1 h at 4 °C, and the clear supernatant was removed. The protein concentration was determined[9]. Supernatants were denatured by adding sodium dodecyl sulphate such that sodium dodecyl sulphate:protein was at least 2 : 1 (w/w)[10], and 2-mercaptoethanol

was added to a final concentration of 100 mM. This mixture was incubated for 1 h at 37 °C.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed using 6 mm × 100 mm gels. Supernatants without sodium dodecyl sulphate were fractionated on 4.0 % acrylamide –0.2 % bisacrylamide gels in 50 mM Tris–glycine electrophoresis buffer (pH 8.5) with 10 mM 2-mercaptoethanol. 100 μl (100–200 μg protein) of sample was layered directly on to the gel with sucrose, and electrophoresis was performed at 100 V (5 mA/gel) for 2 h at room temperature. Running conditions were the same for sodium dodecyl sulphate gels, except that 8.0 %, 10.0 %, and 12.0 % acrylamide gels were used, and sodium dodecyl sulphate was added to the electrophoresis buffer to 0.03 %. Sodium dodecyl sulphate was also present in the gels at a concentration of 0.03 %. Bromophenol blue was used as a marker dye.

After electrophoresis, gels were fixed in 7 % acetic acid for at least 30 min, and then stained in 0.5 % amido black dye in 7 % acetic acid for 1 h. Gels were destained electrophoretically for 30 min at a current of 1 A. Each gel was scanned at 620 nm in a Joyce–Loebl Chromoscan, frozen on powdered dry ice and then sliced into 1-mm fractions using a Mickle gel slicer. The fractions were solubilized in 0.1 ml H_2O_2 (100 vol.) for 1 to 2 h at 70 °C. 8 ml of Triton–toluene scintillant[11] was added and radioactivity was measured by liquid scintillation spectrometry at 90 % counting efficiency.

Purification of Fraction I protein

Fraction I protein was purified from leaves of 10–15-day-old pea seedlings following a procedure modified from that of Kawashima and Wildman[12]. All operations were performed at 4 °C. 200 g of leaves were blended with 300 ml of ice-cold 0.025 M Tris–HCl, 0.05 M NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.04 M 2-mercaptoethanol and 2 mM phenylmethyl sulphonyl fluoride (obtained from Sigma and used to inhibit serine proteases), pH 7.4 (Buffer A) in an Atomix blender for 60 s. The homogenate was strained through eight layers of muslin and centrifuged at 10 000 × *g* for 30 min. The supernatant was removed and centrifuged at 105 000 × *g* for 60 min. The yellowish 105 000 × *g* supernatant was passed through a column of coarse grade Sephadex G-25 (6.5 cm × 50 cm) in order to remove low molecular weight contaminants. Protein was eluted in the void volume with 0.025 M Tris–HCl, 0.05 M NaCl, 0.5 mM EDTA, and 10 mM 2-mercaptoethanol, pH 7.4 (Buffer B) at a flow rate of 20 ml/min. Approximately 400 ml of protein solution was collected. Solid $(NH_4)_2SO_4$ was added, and the precipitate which appeared between 35 and 45 % saturation was collected by centrifugation at 10 000 × *g* for 10 min, and resuspended in 2 ml of 0.05 M Tris–HCl, 0.5 mM EDTA, 10 mM 2-mercaptoethanol, pH 8.0 (Buffer C). The protein solution was dialysed overnight against 1 litre of Buffer C.

The protein was adsorbed on a column (1.5 cm × 15 cm) of DEAE-cellulose (Whatman DE52) previously equilibrated with Buffer C. The column was thoroughly washed with more Buffer C, and Fraction I protein was eluted with Buffer C to which NaCl had been added to 100 mM. Protein was precipitated by adding solid $(NH_4)_2SO_4$ to 50 % saturation, and the precipitate was spun down at 10 000 × *g* for 10 min and resuspended in 5 ml of Buffer B. Finally, the protein was applied to a Sephadex G-200 column. (2.5 cm × 90 cm) and eluted with Buffer B at a flow rate of 2.5 ml/h.

Fraction I protein elutes just after the void volume, and fractions from this part of the elution profile (with A_{280}/A_{260} of 1.8 or greater) were pooled, precipitated with 50 % satd $(NH_4)_2SO_4$ and resuspended in 2.5 mM Tris–glycine, 10 mM 2-mercaptoethanol (pH 8.5), and stored at 4 °C. When analysed by polyacrylamide gel electrophoresis on 4.0 % gels only one band of low mobility, characteristic of Fraction I protein, could be seen even at high loadings (*i.e.* greater than 200 μg protein per gel).

Purification of in vivo radioactively-labelled Fraction I protein

Peas (Feltham First var.) were grown in the dark for 9 days. 40 shoots were excised about 5 cm below the apex and the cut ends placed in small vials, each containing four shoots and 0.5 ml sterile distilled water with 8 μCi [^{35}S]methionine (0.9 μM). The shoots were illuminated for 3 days with 12 000 lux from white fluorescent tubes. The vials were regularly topped up with sterile distilled water. The green apices (3.1 g) were ground in a chilled pestle and mortar in 15 ml ice-cold Buffer A and then Fraction I protein was purified as above. On 4.0 % gels, only one low mobility band stained with amido black dye, and all the radioactivity on the gel was coincident with this band when the gel was sliced, solubilised, and counted.

Separation of large and small subunit of Fraction I protein

Large and small subunits of Fraction I protein were purified on Sephadex G-100 in an sodium dodecyl sulphate-containing buffer[13]. Chloroplast supernatant was prepared, mixed with 5–10 mg purified pea Fraction I protein and denatured with sodium dodecyl sulphate as described. The protein was applied to a Sephadex G-100 column (2.5 cm × 45 cm) and eluted with 50 mM Tris–HCl, 0.1 mM EDTA, 0.5 % sodium dodecyl sulphate, 10 mM 2-mercaptoethanol (pH 8.6) at a flow rate of 20 ml/h at room temperature. 2-ml fractions were collected, the absorbance read at 280 nm and 100-μl aliquots analysed for radioactivity in Triton–toluene scintillant. Peak fractions corresponding to large and small subunits were precipitated and washed twice in 90 % acetone and then dried *in vacuo*. The purity of each subunit was checked by re-running on 10.0 % sodium dodecyl sulphate gels.

Tryptic peptide mapping and autoradiography

The protein (2 mg) was resuspended in 500 μl of 0.2 M ammonium acetate buffer (pH 8.5) and 100 μg of L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone-inactivated trypsin (Worthington) was added. Incubation was at 37 °C for 4 h. The digest was acidified with 5 % formic acid and dried *in vacuo*. Peptides were resuspended in 50 μl of 0.1 M ammonia and the whole digest was spotted on Whatman 3MM paper The peptides were separated in the first dimension by chromatography in *n*-butanol–acetic acid–water (3:1:1, by vol.) for 15 h and in the second dimension in pyridine–acetic acid–water (1:10:89, by vol.) at pH 3.5 at 2 kV for 1.5 h. The resulting map was sprayed with 0.2 % ninhydrin in acetone and developed at 110 °C for 30 min. It was then placed in contact with Kodak Blue-Brand X-ray film for 2 to 4 weeks.

Pronase and ribonuclease digestion of chloroplast [50 000 × g supernatant

Pronase (B grade Calbiochem) and ribonuclease A (Type IA Sigma) were freshly made up to a concentration of 1 mg/ml in 50 mM Tris–glycine, 10 mM 2-mercaptoethanol (pH 8.5). 10 μg of each enzyme (or an equivalent volume of buffer to act as a control) was added to 700 μg of chloroplast supernatant protein, and incu-

bated at 37 °C for 4 h. The digests were then denatured with sodium dodecyl sulphate and the products were separated by electrophoresis on 10.0 % sodium dodecyl sulphate gels as described.

Nucleic acid extraction and fractionation

Total nucleic acids were extracted from chloroplast preparations as described previously[11]. Nucleic acid was fractionated according to the method of Loening[14]. The degree of degradation of chloroplast ribosomal RNA by exogenous ribonuclease was assessed by calculating the area under each peak of ribosomal RNA and relating this to the amount of DNA present, as an RNA:DNA ratio (deoxyribonuclease-free ribonuclease was used).

Sources of chemical and biochemicals

Creatine phosphokinase, pancreatic ribonuclease A (Type IA), creatine phosphate, dATP (Grade I), dGTP (Type II-S), 3-phosphoglycerate, HEPES, Tricine, poly(U), D-*threo*-chloramphenicol, cycloheximide, indole-3-acetic acid, gibberellic acid (Grade III), phenazine methosulphate, and Triton X-100 were purchased from Sigma.

Pronase (B grade) and *m*-chlorocarbonyl cyanide phenylhydrazone (CCCP) (A grade) was from Calbiochem; trypsin (TPCK inactivated) from Worthington Corp.; 3′,5′-cyclic AMP from Koch Light Labs; amido black from G. T. Gurr; bromophenol blue from Hopkin and Williams, Ltd.; acrylamide from Fluka AG; and bisacrylamide from Eastman Kodak Co.

[^{14}C]Leucine and [^{14}C]phenylalanine were purchased from the Radiochemical Centre, Amersham, and [^{35}S]methionine both from the Radiochemical Centre and from New England Nuclear.

3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) and actinomycin D were kindly supplied by Fisons, Ltd, and Merck Sharp and Dohme, respectively; lincomycin was a gift from Upjohn, Ltd.

RESULTS AND DISCUSSION

The rationale of our approach is that in order to obtain an identifiable product, we must use conditions in which correct elongation, termination and release of polypeptide chains occur in isolated chloroplasts. Such conditions seemed to us to be more likely met in intact chloroplasts than in the broken preparations which are commonly used[15]. We therefore used a technique which was developed to prepare intact chloroplasts capable of high rates of CO_2 fixation[6]. By using light as the source of energy for protein synthesis it is therefore possible to ensure that amino acid incorporation is taking place solely in intact chloroplasts, since broken chloroplasts cannot synthesise ATP in the absence of added substrates and catalysts[24].

Characteristics of amino acid incorporation by isolated chloroplasts

The preparations of chloroplasts contain between 40 and 50 % intact chloroplasts as judged by phase contrast microscopy and incorporate radioactively labelled amino acid into protein when illuminated (Fig. 1). The rates of incorporation are between 0.5 and 1.0 nmole [^{14}C]leucine per mg chlorophyll per hour. The rate of incorporation falls to zero after approximately 20 min. A vital component of the

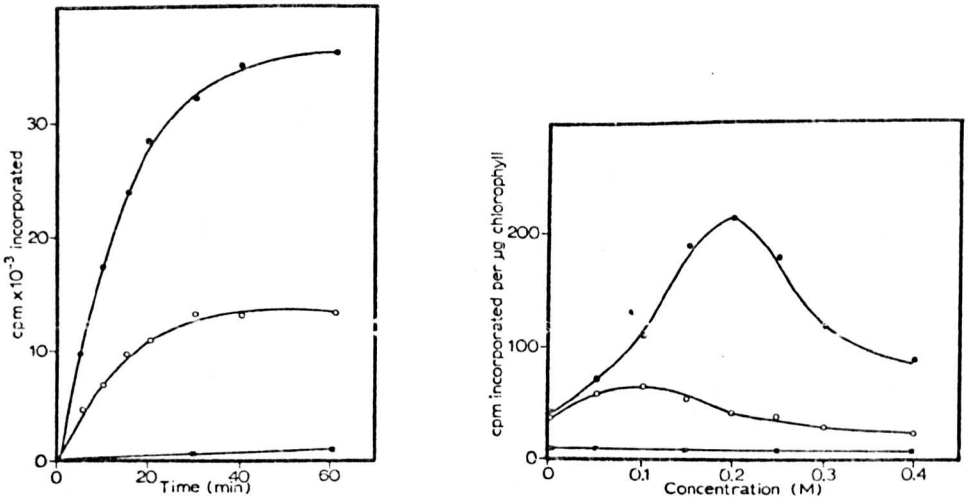

Fig. 1. Time course of light-driven chloroplast protein synthesis. Chloroplasts were isolated and incubated in KCl resuspension medium as described in Materials and Methods. At the indicated times, reactions were stopped by adding 0.5 ml of a saturated solution of [^{12}C]leucine and 1.0 ml 10 % (w/v) trichloroacetic acid to the 500 μl incubation mixture. Radioactively labelled protein was extracted and measured as described previously[8]. ●–●, 400 μg chlorophyll per incubation mixture; ○–○, 100 μg chlorophyll per incubation mixture; ■–■, 400 μg chlorophyll per incubation mixture, but incubated in the dark.

Fig. 2. Dependence of light-driven chloroplast protein synthesis on KCl. Chloroplasts were isolated as described in Materials and Methods and resuspended in the following media: 66 mM Tricine–KOH (pH 8.3), 6.6 mM $MgCl_2$, KCl varying from 0 to 0.4 M (●–●); 66 mM Tricine–NaOH (pH 8.3), 6.6 mM $MgCl_2$, NaCl varying from 0 to 0.4 M (■–■); 66 mM Tricine–KOH (pH 8.3), 6.6 mM $MgCl_2$, sucrose varying from 0 to 0.4 M (○–○). Incubation was at 20 °C for 40 min in the light, as described in Materials and Methods. Incorporation of [^{35}S]methionine was measured as in Fig. 1, except that satd [^{32}S]methionine was used to stop the reaction.

TABLE I

CHARACTERISTICS OF LIGHT-DRIVEN PROTEIN SYNTHESIS BY ISOLATED PEA CHLOROPLASTS

Pea chloroplasts were isolated and incubated in KCl resuspension medium as described in Materials and Methods. Incorporation by the complete, light-driven system in 40 min is called 100.

Energy source	*Treatment*	*Incorporation*
Light	Complete	100
None	Zero time	0.5
None	Complete	3.0
ATP+ATP-generating system	Complete	50
Light+ATP+ATP-generating system	Complete	125
Light	+Ribonuclease (30 μg/ml)	95
None	Lysed	5
ATP+ATP-generating system	Lysed	7.5
Light	+CCCP (5 μM)	6
Light	+DCMU (1 μM)	38
Light	+D-*threo*-Chloramphenicol (50 μg/ml)	5
Light	+Lincomycin (5 μM)	25
Light	+Cycloheximide (100 μg/ml)	100
Light	+Actinomycin D (10 μg/ml)	100

incubation medium is the high concentration of KCl present in the KCl resuspension medium. When chloroplasts are incubated in sucrose resuspension medium, protein synthesis is greatly reduced (Fig. 2). KCl is present at low concentration (33 mM) in the sucrose resuspension medium, in order to adjust the Tricine buffer; however, this concentration is not sufficient to give a high rate of protein synthesis. Replacement of KCl by NaCl prevents all light-dependent incorporation (Fig. 2). We have therefore routinely incubated chloroplasts in the KCl resuspension medium. If the chloroplasts are lysed in medium lacking KCl, subsequent restoration of the KCl does not restore the ability to incorporate amino acids into protein (Table I). We suggest that KCl is acting both as an osmoticum and as a cofactor for protein synthesis.

Some characteristics of this chloroplast system are shown in Table I. Light can only be partially replaced as an energy source by added ATP and an ATP-generating system, while addition of ATP as well as light gives a only slight stimulation. Lysed chloroplasts show very low incorporation even when supplied with ATP. Inhibitors of photophosphorylation such as CCCP and DCMU inhibit protein synthesis, as do antibiotics specific for 70-S ribosomes, such as D-*threo*-chloramphenicol and lincomycin. Ribonuclease is not an inhibitor in this system. Analysis of the ribosomal RNA by polyacrylamide gel electrophoresis shows that addition of ribonuclease causes RNA to be hydrolysed to a percentage equal to the percentage of broken chloroplasts; this confirms a previous report that ribonuclease cannot penetrate intact chloroplasts[16]. Actinomycin D does not inhibit protein synthesis at 10 μg/ml; this concentration inhibits light-driven incorporation of [^{3}H]uridine into RNA by the same chloroplast preparation by 85 % (Hartley, M. R., personal communication). Incorporation is not stimulated by the addition of the plant hormones indole-3-acetic acid or gibberellic acid, or by inorganic phosphate, cyclic AMP, $NADP^+$, or phenazine methosulphate; addition of 3-phosphoglycerate causes inhibition of protein synthesis. Addition of poly(U), which stimulates phenylalanine incorporation by intact mitochondria[17], does not have this effect in this chloroplast system. We conclude from these characteristics that protein synthesis is proceeding in intact chloroplasts only, is being driven by photophosphorylation and is probably using messenger RNA synthesised before the chloroplasts were isolated.

Incorporation of labelled amino acids by other components of the incubation medium can be excluded. Bacterial contamination of the chloroplast preparation was minimised by using sterile glassware and media. If the preparations were solubilised at the end of the incubation in 2 % (v/v) Triton X-100 detergent (which is known to solubilise chloroplasts and mitochondria, but not bacteria, nuclei and whole leaf cells[18]), less than 0.1 % of total radioactivity incorporated into protein was present in a 10 000 $\times$ *g* Triton pellet. Amino acid incorporation by contaminating pea nuclei can also be excluded; nuclei were rarely observed when preparations were examined by phase contrast microscopy. The great enhancement of protein synthetic activity by light, and its sensitivity to inhibitors of photophosphorylation strongly argues for chloroplast, rather than mitochondrial, protein synthesis.

Analysis of the products of chloroplast protein synthesis by polyacrylamide gel electrophoresis

The identification of an *in vitro* synthesised protein is aided if soluble, and therefore complete, polypeptide chains only are examined. This was done by prepa-

ring a 150 000 × *g* supernatant from radioactively labelled chloroplast preparations. This supernatant contains no ribosomes or chloroplast lamellae.

When chloroplast supernatant proteins are fractionated on sodium dodecyl sulphate gels, many protein bands can be stained. However, one band stains with amido black dye to a much greater extent that the others; this band is the large subunit of Fraction I protein (Fig. 3). Fraction I protein is the major protein found in plants and is present exclusively in the chloroplast[19]; it has a molecular weight of just over 500 000 and when dissociated with sodium dodecyl sulphate and fractionated on sodium dodecyl sulphate gels, it separates into a large and a small subunit of widely differing molecular weight[13]. The purified enzyme until recently had been shown to possess only one enzymic activity, namely that of ribulose diphosphate carboxylase[19] (3-phospho-D-glycerate carboxylase, EC 4.1.1.39). However, an additional oxygenase activity has recently been demonstrated[20] in protein purified from both spinach and

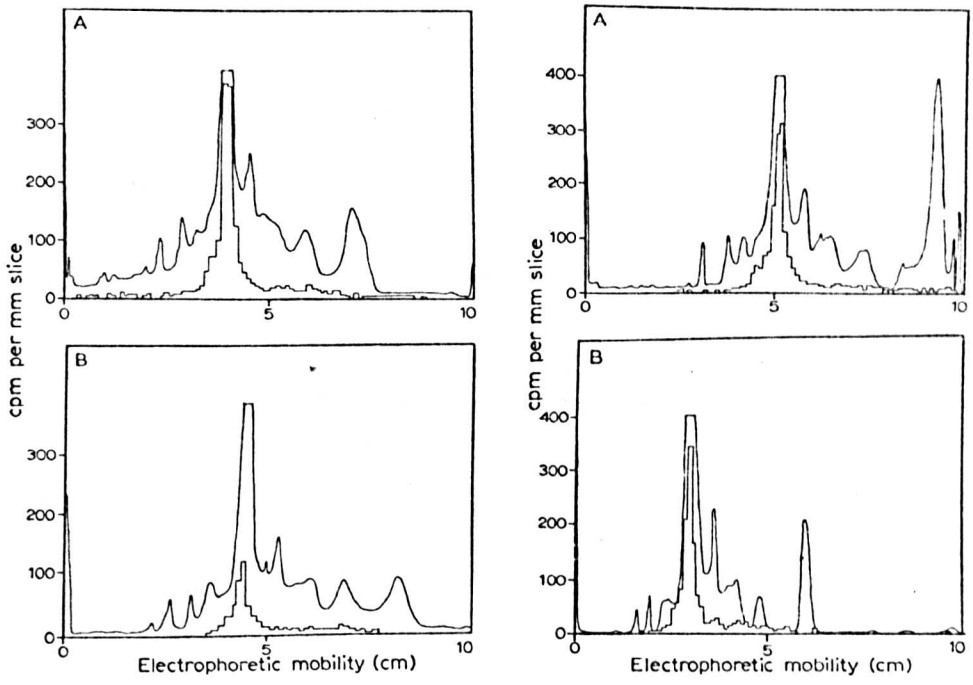

Fig. 3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of labelled chloroplast supernatant from both light-driven and broken chloroplast preparations. (A) Chloroplasts were incubated in KCl resuspension medium with light as energy source. (B) Chloroplasts were incubated in mercaptoethanol resuspension medium with the ATP, ATP-generating system and cofactors described in Materials and Methods. In both A and B, chlorophyll concentration was 300 μg/ml and 1 μCi [^{14}C]leucine (3 nmoles) was used. 150 000 × *g* supernatants were prepared and denatured as described. 100 μl of each supernatant was fractionated on 10.0 % sodium dodecyl sulphate gels. The smooth line represents the absorbance at 620 nm and the histogram shows the radioactivity in each 1-mm gel fraction.

Fig. 4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of labelled chloroplast supernatant fractionated on 8.0 % and 12.0 % acrylamide gels. Details of preparation of the labelled chloroplast supernatant as in Fig. 3A. (A) Fractionation on 8.0 % acrylamide gel. (B) Fractionation on 12.0 % acrylamide gel.

soybean leaves, implying a role for this protein in both photosynthesis and photorespiration.

The results shown in Fig. 3A show that only one discrete, radioactively labelled peak is obtained in isolated chloroplasts; this peak migrates exactly with the large subunit of Fraction I protein. Neither the small subunit nor any other protein on the gel is labelled to an appreciable extent. This result has also been obtained using [^{35}S]methionine, or [^{14}C]phenylalanine as the labelled precursor. When the same supernatant is fractionated on 8.0 % and 12.0 % acrylamide gels (Fig. 4), the single radioactive peak again runs coincidently with the large subunit of Fraction I protein.

If chloroplasts are incubated in mercaptoethanol resuspension medium which produces broken chloroplasts of a type often used for studying protein synthesis[1,2], synthesis of Fraction I protein large subunit is again observed when ATP and an ATP-generating system are present instead of light (Fig. 3B). However, the extent of incorporation of [^{14}C]leucine into the labelled peak is very much less than in the system which uses light as energy source (Fig. 3A). ATP can replace light as the energy source for the synthesis of Fraction I protein large subunit in the KCl resuspension medium, although incorporation is reduced, as would be expected from the data presented in Table I. The radioactive peak is not found when chloroplasts are incubated in the dark or in the presence of 50 μg/ml D-*threo*-chloramphenicol.

The radioactively labelled peak is completely sensitive to digestion by pronase (Fig. 5B) but not ribonuclease (Fig. 5C). In the control incubation (Fig. 5A) no degradation of the labelled peak is evident, implying that the supernatant is not markedly contaminated by proteases. The labelled peak obtained is not likely to be an enzymic degradation product of a higher molecular weight species.

When the supernatant is fractionated on 4.0 % acrylamide gels in the absence of sodium dodecyl sulphate (Fig. 6), the single radioactive peak which is found migrates separately from native Fraction I protein. This shows that newly synthesised large subunits do not equilibrate with pre-existing Fraction I protein.

Tryptic peptide analysis of the large subunit of Fraction I protein

Tryptic peptide analysis of the soluble *in vitro* product shows that it shares five major [^{35}S]methionine-labelled peptides with a tryptic digest of Fraction I protein large subunit labelled *in vivo* with [^{35}S]methionine. In addition, some minor peptides may also be common to both the *in vitro* and *in vivo* synthesised proteins (Fig. 7). This evidence confirms the indication from polyacrylamide gels that the labelled product is the large subunit of Fraction I protein.

Fraction I protein is one of the few proteins which the many *in vivo* studies with 70-S ribosomal inhibitors suggest is synthesised by chloroplast ribosomes[2]. Moreover, it has been shown by a double-labelling method with barley leaves that the synthesis of the large subunit of Fraction I protein is preferentially inhibited by chloramphenicol whereas that of the small subunit is preferentially inhibited by cycloheximide[21]. The results described here show that only one subunit of the many chloroplast soluble proteins is synthesized on chloroplast ribosomes. However, the 150 000 × *g* supernatan fraction contains only 25 % of the labelled amino acids incorporated into protein by the chloroplast preparation. The remainder sediments with the pellet, containing lamellae and ribosomes. The chloroplast lamellae have been shown to contain one major radioactive peak of molecular weight 32 000 and several minor peaks (Eagles-

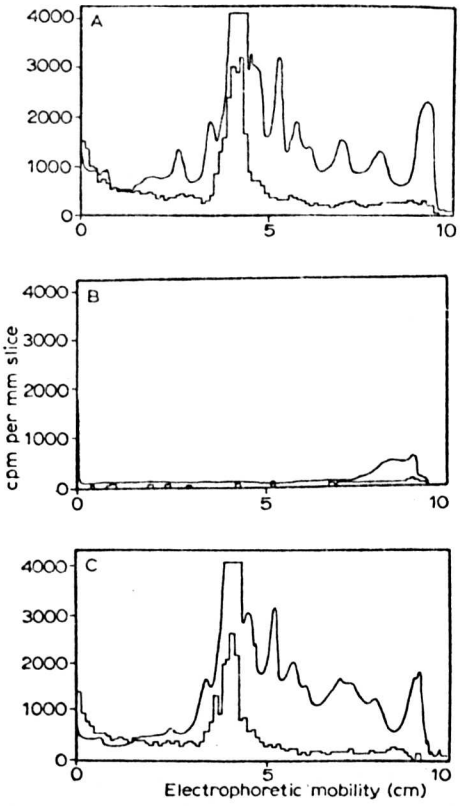

Fig. 5. Stability of the soluble radioactive product of chloroplast protein synthesis to digestion by pronase and ribonuclease. Details of the procedure used are given in Materials and Methods. (A) Control incubation. (B) Pronase treated. (C) Ribonuclease treated. Other details in Fig. 3A.

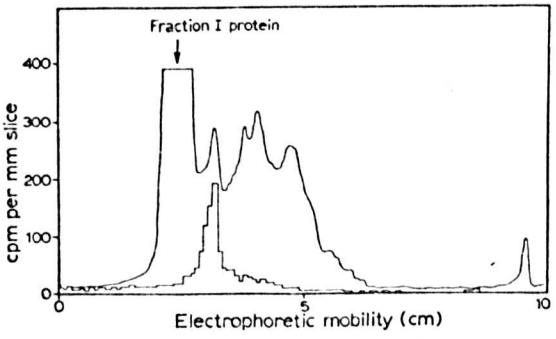

Fig. 6. Polyacrylamide gel electrophoresis of labelled chloroplast supernatant in the absence of sodium dodecyl sulphate. 150 000 × *g* supernatant was prepared as described in Materials and Methods. 100 μl of supernatant was fractionated on 4.0 % acrylamide gel in the absence of sodium dodecyl sulphate. Other details as in Fig. 3.

ham, A. R. J. and Ellis, R. J., unpublished). Therefore, chloroplasts appear to be capable of synthesising only very few of their many proteins. On the other hand,

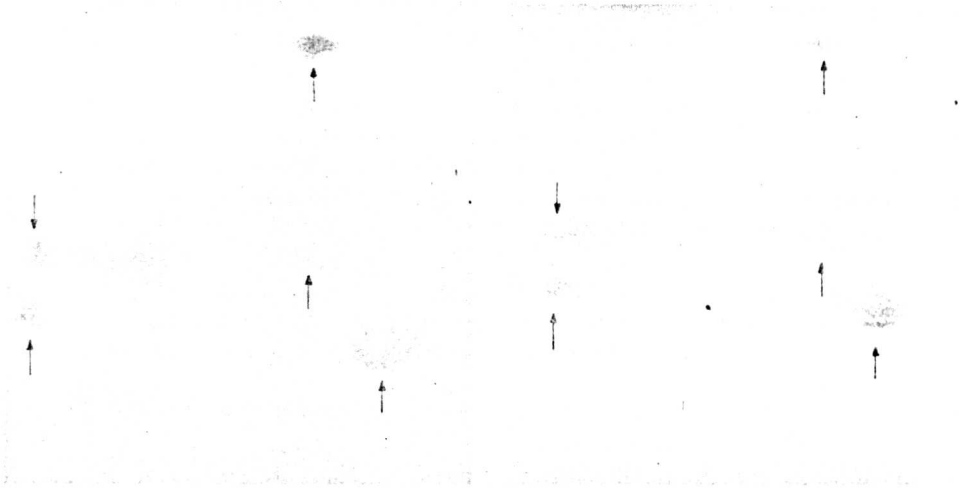

Fig. 7. Autoradiographs of tryptic peptide maps of (A) soluble *in vitro* labelled product and (B) *in vivo* labelled large subunit of Fraction I protein.

chloroplast ribosomes can account for up to 50 % of total leaf ribosomes. Why are so many ribosomes required to synthesise so few proteins? One explanation may be that one of their products, the large subunit of Fraction I protein, occurs in much greater quantities than any other protein in the leaf. Most of the soluble proteins of the chloroplast, including the small subunit of Fraction I protein, appear to be synthesised on cytoplasmic ribosomes. An inference must therefore be drawn that there exist specific mechanisms to transport into the chloroplast all those chloroplast proteins which are made on cytoplasmic ribosomes. One possibility is that a membrane protein exists in the outer envelope which recognises a site common to those proteins destined for the plastid.

A model which describes our current view of the cooperation between plastid and nuclear genomes in the synthesis of Fraction I protein is shown in Fig. 8. Genetic

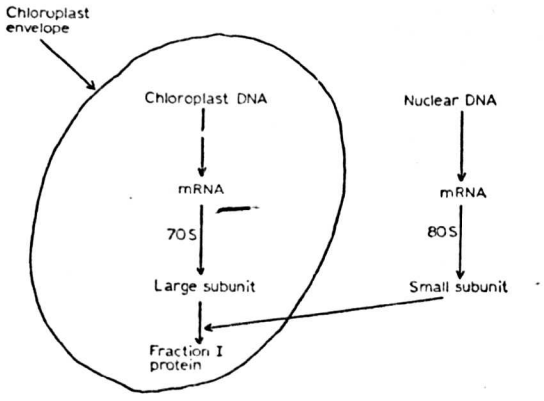

Fig. 8. Model of cooperation between plastid and nuclear genomes in the synthesis of Fraction I protein (modified from Kawashima and Wildman[22]).

analyses of Fraction I protein mutants in tobacco suggest that the small subunit is coded in the nuclear DNA[22], but that the large subunit is coded in the chloroplast DNA[23]. The large subunit is thus both encoded and synthesised within the chloroplast while the small subunit is both encoded and synthesised outside the chloroplast. This model therefore requires protein, but not nucleic acid to cross the chloroplast envelope. The data shown in Fig. 6 suggest that there is little or no pool of small subunits in the isolated chloroplasts. The nature of the mechanism which regulates the relative rates of synthesis of the subunit in the two cellular compartments is unknown.

The demonstration that isolated chloroplasts synthesise the large subunit of Fraction I protein as the sole detectable soluble product points the way to the first isolation of a messenger RNA from a plant source.

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