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Protein Synthesis in Chloroplasts

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

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

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

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

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

Experimental

Materials

Pea seeds (*Pisum sativum* L. var. Meteor) were purchased from S. Dobie, Chester, U.K. Vermiculite (Micafil) was bought from Dupre Vermiculite Ltd., Herts., U.K. Creatine phosphokinase, ATP, UTP, GTP, CTP, dTTP, Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid], Tricine [*N*-tris(hydroxymethyl)methylglycine], phenylmethanesulphonyl fluoride, D-*threo*-chloramphenicol, cycloheximide and Triton X-100 [octylphenoxypolyethoxyethanol] were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Pronase (B grade) and carbonyl cyanide *m*-chlorophenylhydrazone were purchased from Calbiochem, London W1H 1AS, U.K., Coomassie Brilliant Blue R was from Searle Diagnostic, High Wycombe, Bucks., U.K., Bromophenol Blue and sodium dodecyl sulphate (especially pure) were from BDH Chemicals Ltd., Poole, Dorset, U.K., acrylamide was from Fluka A.G., Buchs, Switzerland, and *NN'*-methylenebisacrylamide was from Eastman Organic Chemicals, Rochester, N.Y., U.S.A.

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

Methods and procedure

Plant tissue. Pea seeds were surface sterilized in 2% (w/v) sodium hypochlorite solution for 5 min and then washed in running tap water for 24 h. The imbibed seeds were sown in moist vermiculite and grown at 22–24°C for 9 days. Mature chloroplasts were isolated from the youngest leaves of seedlings grown under a 12 h photoperiod of 2000 lx provided by white fluorescent tubes. Etioplasts were isolated from the apical buds of plants grown in darkness. Etiolated seedlings were greened under continuous white light of 2800 lx at 22–24°C. Plastids from greening seedlings were isolated from the first-formed leaflets.

Plastid isolation. Plastids were isolated by the method of Ramirez *et al.* (1968) as modified by Blair & Ellis (1973). Plant tissue was excised and 20 g homogenized for 4 s in a Polytron homogenizer (Northern Media Supply Ltd., Hull, U.K.) in 100 ml of semi-frozen sterile isolation medium [0.35 M-sucrose–25 mM-Hepes–NaOH buffer (pH 7.6)–2 mM-EDTA (disodium salt)–2 mM-sodium isoascorbate]. The homogenate was strained through two lots of eight layers of absorbant muslin and centrifuged at 2500*g* for 1 min at 4°C. The supernatant fraction was discarded and the pellet carefully resuspended in sterile KCl resuspension medium [66 mM-Tricine–KOH buffer (pH 8.3)–0.2 M-KCl–6.6 mM-$MgCl_2$]. Plastid isolations were performed in the light. Experiments were performed which showed that no differences could be found with regard to these studies between plastids isolated in the light and plastids isolated in the dark. About 50–60% of the plastids isolated by this method were highly refractive when viewed with the phase-contrast microscope, suggesting they possessed a completely limiting envelope. Studies with the electron microscope indicate that nuclei are present in these preparations to the extent of one nucleus for twelve plastids in the etioplast preparations and one nucleus for 50 plastids in the chloroplast preparations. Protein was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Assay for L-[^{35}S]*methionine incorporation into hot trichloroacetic acid-insoluble material in isolated etioplasts.* The etioplasts isolated from 20 g of tissue were resuspended in 5 ml of sterile KCl resuspension medium. As a routine, incubation mixtures with a final volume of 500 μl contained 100 μmol of KCl, 33 μmol of Tricine–KOH buffer (pH 8.3), 3.3 μmol of $MgCl_2$, 0.5 μCi of L-[^{35}S]methionine (200 Ci/mmol), 1 μmol of ATP and etioplasts containing 150–300 μg of protein. All the inhibitors tested were soluble in KCl resuspension medium at the concentrations used, and were added to the incubation mixture by replacing a portion of KCl resuspension medium with an equal portion of KCl resuspension medium plus inhibitor. Reaction components were mixed at 2°C, and the incubation was started by increasing the temperature of the incubation mixture to 20°C. This temperature was maintained during the incubation.

Incubations were performed in the dark. After 1h the incubations were terminated by the addition of 100μl of a saturated aqueous solution of L-[^{32}S]methionine and 1ml of 20% (w/v) trichloroacetic acid. The suspensions were left to stand at 2°C for 30min and then heated to 90°C for 15min. The precipitate was transferred quantitatively to glass-fibre discs (Whatman GF/C, 2.5cm) and each disc was washed with 100ml of ice-cold 5% (w/v) trichloroacetic acid, 60ml of ethanol and finally with 60ml of diethyl ether. Filters were dried at 60°C for 30min and placed in scintillation vials containing 8ml of a toluene-based scintillation fluid [0.5% (w/v) 2,5-diphenyloxazole, 0.03% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene]. The radioactivity was determined in a Packard Tri-Carb model 3320 liquid-scintillation spectrometer at a counting efficiency of 70% as determined by internal standardization.

Incubation of plastids for analysis of synthesized polypeptides. The plastids isolated from 40g of tissue were resuspended in 3ml of KCl resuspension medium. Light-driven protein synthesis incubation mixtures with a final volume of 1ml contained 200μmol of KCl, 66μmol of Tricine–KOH buffer (pH8.3), 6.6μmol of $MgCl_2$, 100μCi of L-[^{35}S]methionine (300Ci/mmol) or 100μCi of L-[^{3}H]leucine (50Ci/mmol) and plastids containing 1–6mg of protein. ATP-driven protein synthesis incubation mixtures contained in addition 2μmol of ATP, 5μmol of creatine phosphate and 100μg of creatine phosphokinase. Reaction components were mixed at 2°C, and the incubations were started by raising the temperature of the incubation mixture to 20°C. This temperature was maintained during the incubation. The ATP-driven system was incubated in the dark, whereas incubations with light as the energy source were illuminated with filtered red light of 4000lx intensity as measured by the Megatron Light Meter Type E1. After 1h the incubations were terminated by the addition of 200μl of a saturated aqueous solution of L-[^{32}S]methionine and 120μl of an aqueous solution of 20% (w/v) sodium dodecyl sulphate. The incubation mixtures were boiled for 3min and then dialysed at room temperature against 5 litres of 2.5mM-Tris–glycine buffer (pH8.5), 100mM-2-mercaptoethanol and 0.2% (w/v) sodium dodecyl sulphate. At room temperature an insoluble precipitate of potassium dodecyl sulphate forms when sodium dodecyl sulphate is added to the incubation mixtures. This precipitate disappears during dialysis.

Preparation of a 150000g etioplast supernatant fraction. Etioplasts were isolated and incubated for the analysis of the products *in vitro* as described above. The incubation was terminated by the addition of L-[^{32}S]methionine and the incubation mixture was then centrifuged at 2500*g* for 2min at 2°C. The supernatant fraction and pellet were separated, and the pellet was resuspended in 1.2ml of a buffer containing 2.5mM-Tris–glycine (pH8.5) and 4mM-2-mercaptoethanol to lyse the etioplasts. The supernatant fraction was added to the lysed etioplast preparation and centrifuged at 150000*g* for 100min at 2°C. The clear supernatant was removed, 240μl of an aqueous solution of 20% (w/v) sodium dodecyl sulphate was added and the sample boiled for 3min. The 150000*g* supernatant fraction was dialysed at room temperature against 5 litres of a buffer containing 2.5mM-Tris–glycine (pH8.5), 100mM-2-mercaptoethanol and 0.2% (w/v) sodium dodecyl sulphate.

Pronase digestion of the polypeptides synthesized in isolated etioplasts. Incubation for the analysis of the products synthesized *in vitro* was performed as described. The incubation was terminated by the addition of L-[^{32}S]methionine and a lysed etioplast preparation was prepared as described above. After the supernatant fraction had been added to the lysed etioplast preparation, a 600μl sample was taken for pronase digestion. Pronase, at a concentration of 1mg/ml in a buffer containing 50mM-Tris–glycine (pH8.5) and 10mM-2-mercaptoethanol, was added to the sample to give a protein/Pronase ratio of 10:1 (w/w) and the sample was incubated at 37°C for 3h. The incubation was terminated by the addition of 65μl of an aqueous solution of 20% (w/v) sodium dodecyl sulphate and boiling for 3min. The solubilized digest was dialysed at room temperature against 5 litres of a buffer containing 2.5mM-Tris–glycine (pH8.5), 100mM-2-mercaptoethanol and 0.2% (w/v) sodium dodecyl sulphate, and the dialysed sample was then analysed by electrophoresis as described below.

Analytical sodium dodecyl sulphate–polyacrylamide gel electrophoresis. The polyacrylamide-gel electrophoresis system used contained sodium dodecyl sulphate and urea in the buffers. Gels were polymerized in glass tubes (6mm×100mm) and consisted of 15% (w/v) acrylamide–0.3% bisacrylamide made up in a buffer containing 0.25M-Tris–HCl (pH8.5), 0.1% (w/v) sodium dodecyl sulphate and 3% (w/v) urea. The running buffer contained 50mM-Tris–glycine (pH8.5), 0.1% (w/v) sodium dodecyl sulphate and 10mM-2-mercaptoethanol or 8mM-cysteine. Gels were prerun at 10mA/gel for 3h. Samples (100μl), containing 100–400μg of protein, were layered directly on the gels with sucrose (10%, w/v) and Bromophenol Blue (0.005%, w/v). The samples were concentrated by applying a current of 0.5mA/gel for 30min. Electrophoresis was then carried out at 5mA/gel at room temperature; to obtain maximum separation of the polypeptides the electrophoresis was continued 30% longer than the time taken for the marker dye to reach the bottom of the gel. Experiments were performed to ensure that this did not cause protein bands or radioactive peaks to be lost from the bottom of the gel. Gels were stained in Coomassie Brilliant Blue R [0.1% (w/v) in 50% (v/v) methanol and 7.5% (v/v) acetic acid] and the excess

of stain was removed by washing in 50% (v/v) methanol containing 7.5% (v/v) acetic acid. The gels were calibrated for molecular weight as described (Eaglesham & Ellis, 1974). The gels were scanned at 620nm in a Joyce–Loebl Chromoscan, swollen in 7.5% (v/v) acetic acid to remove the methanol, frozen at −90°C for 30min and then sliced into 1mm slices with a Mickle gel slicer. The slices were solubilized in 200μl of H_2O_2 (100vol.) for 3h at 80°C in capped scintillation vials and, after cooling the vials, 8ml of Triton X-100–toluene scintillation fluid [0.4% (w/v) 2,5-diphenyloxazole, 0.05% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene–Triton X-100 (2:1, v/v)] was added. Samples were counted for radioactivity in a Packard Tri-Carb model 3320 liquid-scintillation spectrometer. L-[^{35}S]Methionine alone was determined at a counting efficiency of 88% as determined by internal standardization. In gel slices containing both L-[^{35}S]methionine and L-[^{3}H]leucine the radioactivity due to each isotope was estimated by the channels-ratio method of Hendler (1967).

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

Preparation of the large subunit from pea Fraction I protein labelled in vivo with L-[^{35}S]methionine. Labelled Fraction I protein was prepared by the method of Blair & Ellis (1973) from the leaves of excised pea seedlings that had been fed with L-[^{35}S]-methionine during greening. Purified labelled Fraction I protein was dissociated into its constituent subunits by solubilization in 50mM-Na_2HPO_4–NaOH buffer (pH11.2). The subunits were separated by chromatography on Sephadex G-100 (superfine grade) with 50mM-Na_2HPO_4–NaOH buffer (pH11.2) as the eluting buffer. The three peak fractions containing the large subunit from Fraction I protein were pooled, and the protein was precipitated with 20% (w/v) trichloroacetic acid at 4°C for 30min. The protein was washed by resuspension and centrifugation in ethanol–ether (3:1, v/v) (twice) and ether (once). This material was stored at 4°C and used for tryptic peptide analysis.

Tryptic peptide analysis. The samples for analysis were resuspended in a mixture of formic acid and methanol (4:1, v/v). Performic acid oxidation was performed as described by Bray & Brownlee (1973). The oxidized samples were dried with N_2, resuspended in water, and freeze-dried twice to remove all traces of performic acid. Tryptic digestion was performed by dissolving the samples (250μg of protein) in 50mM-NH_4HCO_3 (pH8.0) containing 1mM-$CaCl_2$ and by adding 12.5μg of TPCK (1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one)-inactivated trypsin dissolved in 50mM-NH_4HCO_3 (pH8.0)–1mM-$CaCl_2$ (protein/trypsin, 20:1, w/w). Incubation was at 37°C for 7h. After digestion, samples were freeze-dried and then dissolved in 150μl of water. The samples were centrifuged at 1500*g* for 5min to remove particulate matter. Finally, the samples were freeze-dried again and dissolved in 15μl of water. Samples contained 50×10^3–100×10^3c.p.m. The tryptic peptides were separated by the two-dimensional 'mapping' procedure of Sargent & Vadlamudi (1968). This technique allows two peptide mixtures to be 'fingerprinted' simultaneously and under identical conditions. Separation occurs on thin layers of silica gel with acetic acid–formic acid–water (4:1:45, by vol.) as the electrophoresis vector, and butan-1-ol–acetic acid–water (3:1:1, by vol.) for the chromatography vector. After drying, the thin-layer plates were exposed to Kodirex X-ray film for 8–12 days.

Results and Discussion

Characteristics of protein synthesis in isolated etioplasts

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

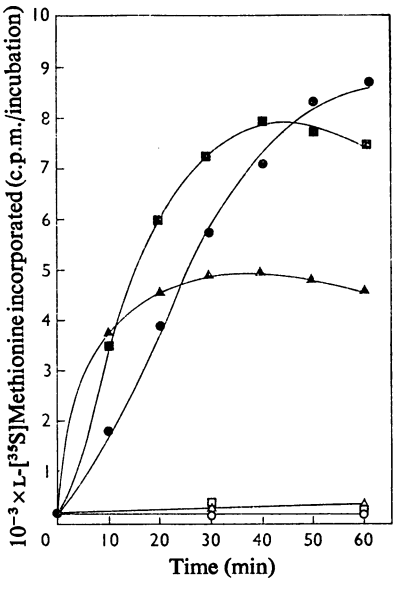


Fig. 1. *Effect of ATP and temperature on the incorporation of* L-[^{35}S]*methionine into a hot-trichloroacetic acid-insoluble product in isolated etioplasts*

Etioplasts were prepared as described under 'Methods and procedure'. Incubation mixtures (final vol. 500μl) contained 300μl of etioplast suspension (190μg of protein), 100μl of KCl resuspension medium (pH8.3) containing 0.5μCi of L-[^{35}S]methionine (200Ci/mmol) and either 100μl of KCl resuspension medium containing 10mM-ATP (■, ●, ▲) or 100μl of KCl resuspension medium alone (□, ○, △). Incubations were terminated and assayed for incorporation as described under 'Methods and procedure'. ●, 10°C, +ATP; ○, 10°C, −ATP; ■, 20°C, +ATP; □, 20°C, −ATP; ▲, 30°C, +ATP; △, 30°C, −ATP.

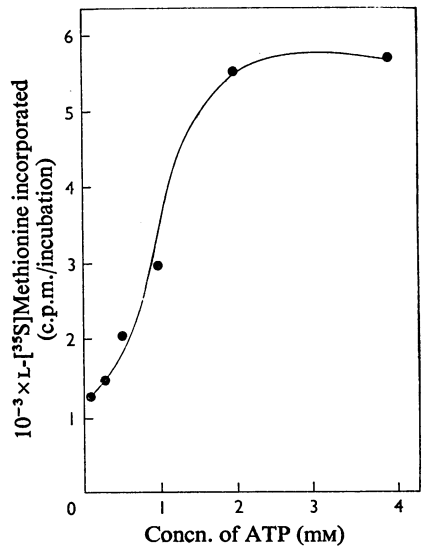


Fig. 2. *Effect of ATP concentration on the incorporation of* L-[^{35}S]*methionine into a hot-trichloroacetic acid-insoluble product in isolated etioplasts*

Etioplasts were prepared as described under 'Methods and procedure'. Incubation mixtures (final vol. 500μl) contained 300μl of etioplast suspension (300μg of protein), 100μl of KCl resuspension medium (pH8.3) containing 0.5μCi of L-[^{35}S]methionine (200Ci/mmol) and 100μl of ATP in KCl resuspension medium (rebuffered to pH8.3). Incubations were terminated after 1h and assayed for incorporation as described under 'Methods and procedure'.

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

To find optimum incubation conditions, the effect of changing some parameters of the incubation were investigated. The effect of changing the ATP concentration is shown in Fig. 2. L-[^{35}S]Methionine incorporation is stimulated by increasing the ATP concentration up to a value of 2mM, but no further stimulation occurs up to 4mM-ATP. Under our conditions the addition of an ATP-generating system did not alter the total incorporation in etioplasts. The effect of altering the incubation pH is shown in Fig. 3. Maximum incorporation is obtained at a pH of 8.3. This pH is also the optimum value for protein synthesis in isolated chloroplasts. Although L-[^{35}S]-methionine incorporation in isolated etioplasts is stimulated by a high K^+ ion concentration in the incubation medium (Fig. 4), the degree of stimulation is not as great as in isolated chloroplasts. The K^+ ion stimulation of incorporation in etioplasts confirms the suggested role of this ion as a cofactor for protein synthesis in isolated chloroplasts rather than as a factor that stimulates incorporation by an involvement in, for example, photophosphorylation (Blair & Ellis, 1973).

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

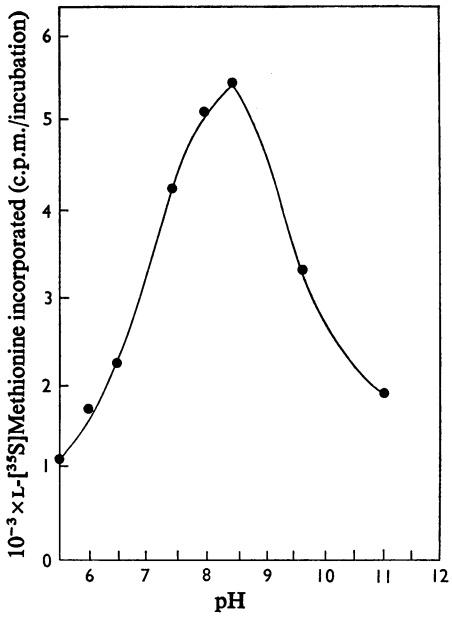


Fig. 3. *Effect of pH on the incorporation of* L-[^{35}S]*methionine into a hot-trichloroacetic acid-insoluble product in isolated etioplasts*

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

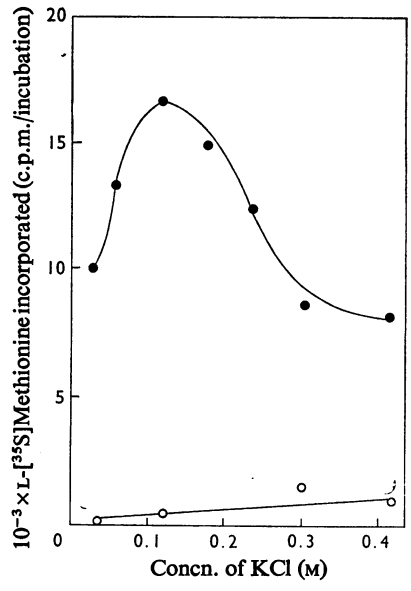


Fig. 4. *Effect of K^+ ion concentration on the incorporation of* L-[^{35}S]*methionine into a hot-trichloroacetic acid-insoluble product in isolated etioplasts*

Etioplasts were prepared as described under 'Methods and procedure', except that 5ml portions of the homogenized tissue were centrifuged individually. The etioplast pellets (900μg of protein) were resuspended in 500μl of modified KCl resuspension medium in which the total K^+ ion concentration varied from 33 to 453mM (pH8.3), containing either 0.5μCi of L-[^{35}S]methionine (200Ci/mmol) and 2mM-ATP (●) or 0.5μCi of L-[^{35}S]methionine (200Ci/mmol) alone (○). Incubations were terminated and assayed as described under 'Methods and procedure'.

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

Etioplasts were isolated and incubated as described under 'Methods and procedure'. The Triton X-100 solubility of the material containing the incorporated L-[^{35}S]methionine was determined by addition of an equal volume of 4% (v/v) Triton X-100 to the incubation after 1h, centrifuging the solubilized preparation at 12500*g* for 10min at 4°C, separating the supernatant fraction and pellet and precipitating the protein in these fractions with 10% (w/v) trichloroacetic acid. Incorporation was assayed as described under 'Methods and procedure'. Incorporation by the complete ATP-driven system is called 100.

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

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

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

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

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

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

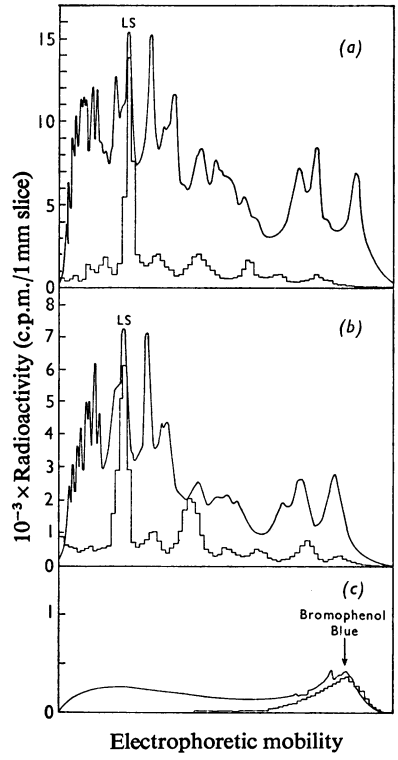


Fig. 5. *Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of the polypeptides synthesized in vitro in etioplasts and the sensitivity of these polypeptides to Pronase*

Etioplasts were isolated from 20–30g of tissue and incubated with L-[^{35}S]methionine (300Ci/mmol) (*a*) and (*c*), or L-[^{3}H]leucine (50Ci/mmol) (*b*). Plastid isolation and incubation, and Pronase digestion of the products synthesized *in vitro*, was performed as described under 'Methods and procedure'. Gels were loaded with 100μl of solubilized incubation mixture: (*a*), 130μg of protein; (*b*), 160μg of protein; (*c*) 100μl of the Pronase digest. Electrophoresis and related procedures were performed as described under 'Methods and procedure', except for (*c*) in which the electrophoresis was stopped when the marker dye reached the bottom of the gel. ——, E_{620}; ⁻⊔⁻ (histogram), radioactivity; LS, large subunit of Fraction I protein.

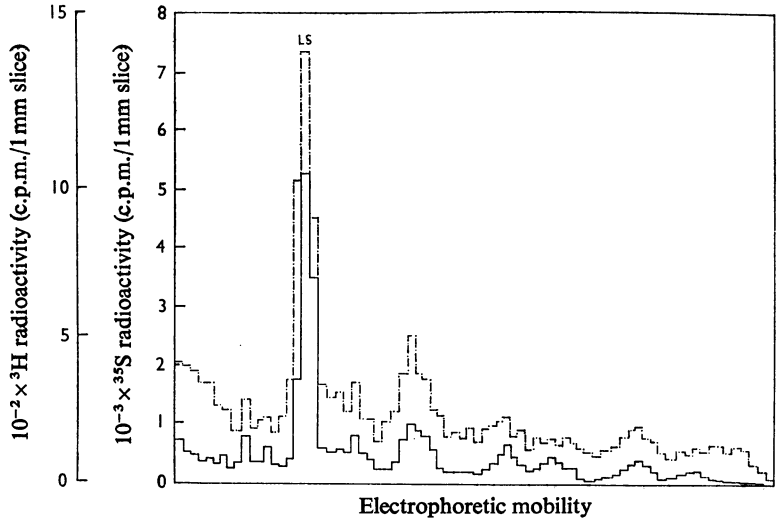
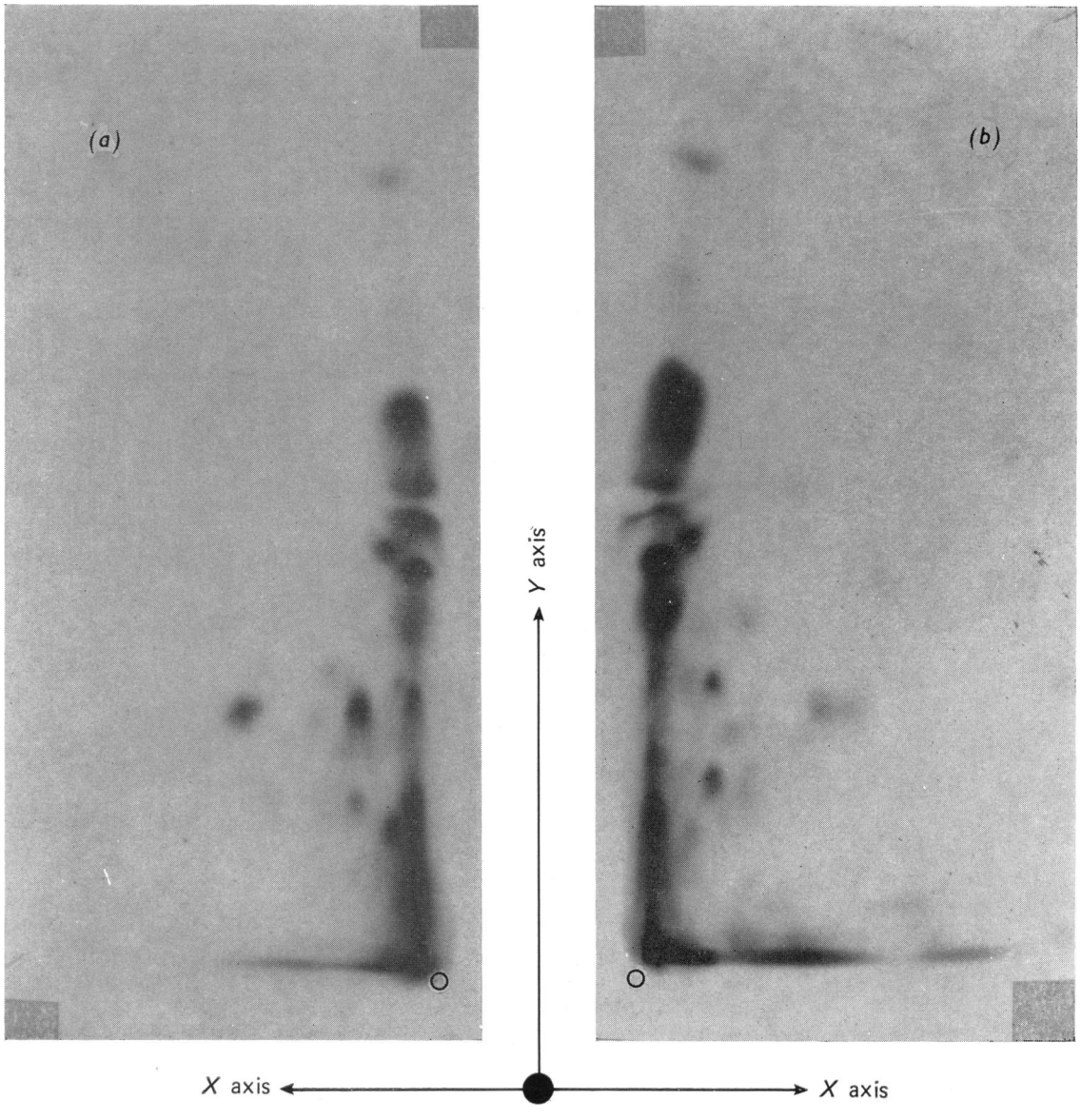


Fig. 6. *Co-electrophoresis of etioplast and chloroplast products synthesized in vitro in sodium dodecyl sulphate–polyacrylamide gels*

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

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

These results suggest that not only the characteristics and requirements, but also the products of protein synthesis *in vitro* in both pea etioplasts and chloroplasts are qualitatively the same. One of the peaks of activity common to the fractionation pattern of both the etioplast and chloroplast products synthesized *in vitro* is more highly labelled than the others, and runs with an apparent molecular weight of about 6×10^{4}. The identity of this polypeptide has been established in the light-driven chloroplast system as the large subunit of Fraction I protein (Blair & Ellis, 1973). This polypeptide is the only product present *in vitro* in the 150000*g* supernatant fraction of chloroplasts, and Fig. 7 shows that this polypeptide is also the only product of etioplasts *in vitro* that is present in this fraction. By inference from the chloroplast situation, these results suggest that the 6×10^{4}-mol. wt. polypeptide synthesized in isolated etioplasts is the large subunit of Fraction I protein; this suggestion has been confirmed by comparing the L-[^{35}S]methionine-labelled tryptic peptides of the product labelled *in vitro* with those of the large subunit from Fraction I protein labelled *in vivo* with L-[^{35}S]methionine. Plate 1(*a*) shows that the etioplast product *in vitro* shares seven major L-[^{35}S]methionine-labelled tryptic peptides with the large subunit of Fraction I protein labelled with L-[^{35}S]methionine *in vivo* (Plate 1*b*). Some minor peptides may also be common to both proteins. This number of methionine tryptic peptides for the large subunit of Fraction I



EXPLANATION OF PLATE 1

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

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

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

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

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

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

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

Several conclusions can be drawn from the results shown in Fig. 8. First, light cannot be used as the source of energy for protein synthesis in isolated etioplasts; this confirms the picture suggested by Table 1. Under our conditions light-driven protein synthesis can first be detected in plastids isolated 3h after the onset of illumination. Secondly, in the first 48h of greening there appears to be an increase of about threefold in the protein-synthesizing capacities of the isolated plastids. Drumm & Margulies (1970) reported a similar increase for plastids from *Phaseolus vulgaris* L., and have concluded that this represents a real increase in the protein-synthesizing ability rather than alterations in the amino acid pool sizes or concentrations of inhibitors. Thirdly, it is clear that whereas light-driven protein synthesis occurs in plastids isolated after 96h of greening (Fig. 8*h*), added ATP will no longer stimulate protein synthesis in these plastids (Fig. 8*d*). This observation has not been further investigated, but may represent an alteration in the permeability of the plastid envelope to ATP. Fourthly, the fractionation of the polypeptides synthesized in plastids isolated after 96h of illumination shows a radical change in the major product. Whereas the major product in isolated etioplasts is the large subunit of Fraction I protein, almost the only polypeptide synthesized in plastids isolated after 96h of greening is an unidentified membrane-bound protein with a molecular weight of about 3.2×10^4 (Fig. 8*h*). Whether this alteration in the major product synthesized *in vitro* of plastid ribosomes reflects a similar alteration *in vivo* is not known. However, there are clearly controls exerted on the production of proteins in isolated plastids, and the mechanisms of these controls deserve further study.

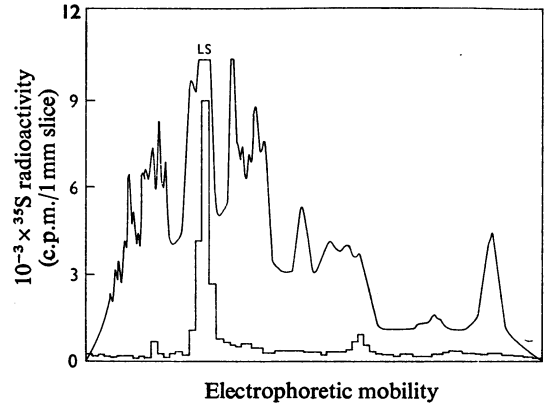


Fig. 7. *Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of labelled* 150000*g etioplast supernatant fraction*

Etioplasts were isolated and incubated as described under 'Methods and procedure'. The products labelled *in vitro* were fractionated by centrifugation at 150000*g*, the supernatant fraction was electrophoresed and the radioactivity determined as described under 'Methods and procedure'. The gel was loaded with 100μl of dialysed sample (220μg of protein). ——, E_{620}; ⁻⊔⁻ (histogram), radioactivity; LS, large subunit of Fraction I protein.

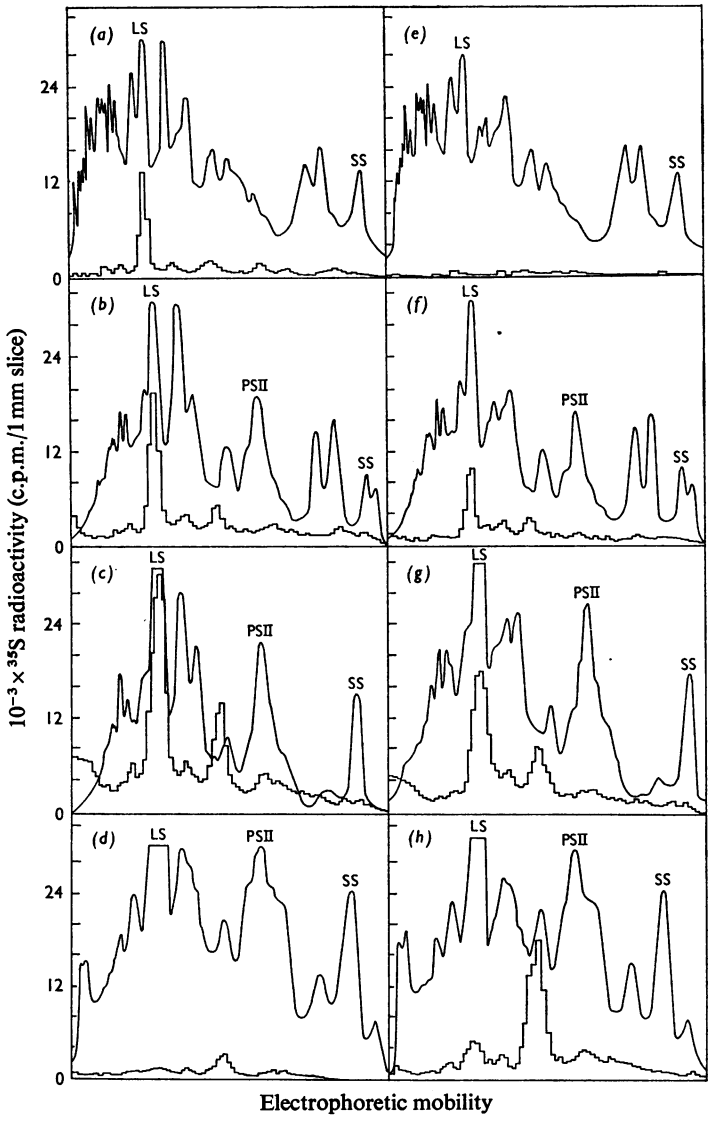


Fig. 8. *Sodium dodecyl sulphate–polyacrylamide gel electrophoresis of the polypeptides synthesized in vitro in plastids isolated during greening*

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

The results presented in this paper lead to the conclusion that, irrespective of the energy source used, plastids isolated during greening synthesize the same polypeptides that are synthesized in both isolated etioplasts and chloroplasts. This conclusion provides support for the idea that only a very limited spectrum

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

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

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