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STUDIES ON THE SUBUNIT STRUCTURE OF COUPLING FACTOR 1 FROM CHLOROPLASTS

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled STUDIES ON THE SUBUNIT STRUCTURE OF COUPLING FACTOR 1 FROM CHLOROPLASTS

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in partial fulfilment of the requirements for the degree of Master of Science.

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The earth is bathed in light, and one can scarcely think of a more important single fact than that.

Isaac Asimov

ABSTRACT

A chloroplast coupling factor with latent Ca^{2+} - dependent adenosine triphosphatase activity was studied.

1. A procedure was developed which renders possible the rapid purification and concentration of coupling factor 1 from bean (*Phaseolus vulgaris*) chloroplasts to apparent electrophoretic purity.

2. Immunodiffusion of bean chloroplast and etioplast coupling factors and spinach coupling factor against an antiserum to spinach coupling factor showed at least partial identity of the bean coupling factor with that of spinach.

3. Immunoelectrophoretic analysis of extracts containing coupling factor 1 of bean leaves under dissociating conditions indicated the existence of at least two antigenically distinct subunits and at least three species of dissociated products with nonidentical electrophoretic mobilities.

4. Disc gel electrophoresis under various dissociating conditions showed the presence of two major and at least four minor species. Undissociated coupling factor showed only the two major species.

5. The apparent molecular weights of polypeptides from coupling factor 1 of bean (as determined by SDS-PAGE)

appear to be identical to those of spinach. At least six distinct species were found. The major bands had molecular weights of 42,000, 59,000 and 63,000 daltons.

6. Amino acid analysis of electrophoretically purified bean CF_1 indicates strong similarity to published values for spinach CF_1.

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LIST OF ABBREVIATIONS

The following abbreviations are used in this work:

ATP	adenosine triphosphate
ATP-ase	adenosine triphosphatase
BSA	bovine serum albumin
CAP	chloramphenicol
CF_1	coupling factor 1
CHI	cycloheximide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
IEA	immunoelectrophoretic analysis
SDS	sodium dodecyl sulphate
SDS-CF_1	CF_1 that has been treated with SDS
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
TEMED	N,N,N',N', - tetramethylethylenediamine
TES	tris(hydroxy methyl) methylaminoethane sulphonic acid
Tris	tris(hydroxy methyl) aminomethane

INTRODUCTION

The occurrence of photosynthesis is perhaps the single most important biological phenomenon known to man. Therefore, it is important to understand the role played by the various components of the chloroplast in affecting energy production through photosynthesis.

The excitation of chlorophyll pigments by light results in a series of coupled electron transfer reactions leading to the formation of two major energy products. They are:

1. NADPH - a source of reducing power for the Calvin cycle's reduction of CO_2 into sugars;
2. ATP - a source of energy for the Calvin cycle and for many other biological reactions.

One of the components believed to be involved in the terminal stages of photophosphorylation is coupling factor 1 (CF_1). It is the basis of this study. It has been shown that CF_1 is required for the light-dependent synthesis of the ATP in the chloroplast. Other properties of the CF_1 are cold-lability and latent ATP-ase activity (which can be activated by heat, trypsin, or dithiothreitol). Studies of the cold inactivation of mitochondrial F_1 showed that the native coupling factor dissociated into subunits. Since

CF_1 plays such an important role in photosynthesis it seemed desirable to obtain further information about the fine structure of this protein. Properties shared with F_1 suggested that it too might be dissociable into subunits. The purpose of this work was to determine the nature of the subunits of CF_1, if any, and supposing they existed, to determine their intracellular location of synthesis. Two approaches were used; they were immunochemistry and dissociating chemical systems. The work is divided into two sections. The first deals with experiments to establish methods of obtaining pure concentrated active ATP-ase. The second deals with experiments to determine the existence and nature of subunits.

LITERATURE REVIEW

(1) Photophosphorylation

As mentioned in the introduction light causes electron flow in chloroplasts which results in the production of energy in the form of NADPH and ATP. Oxygen, vital to life on earth, is evolved in this process.

The evolution of oxygen by chloroplasts in the presence of an artificial electron acceptor was first observed by Hill in 1937 [1]. This evolution of oxygen in the presence of chloroplasts or chloroplast fragments and an artificial electron acceptor came to be called the Hill reaction. The Hill reaction proceeds at a faster rate if the chloroplasts are supplied with ADP, orthophosphate, and Mg^{2+}. It was in 1954 that Arnon, Allen, and Whatley [2] observed this light-dependent ATP formation. Since their discovery both light-induced electron transport and the resulting phosphorylation have been studied intensively but have not yet been fully delineated.

In 1932, Emerson and Arnold [3] postulated that the photosynthetic unit consisted of some 2,500 chlorophyll molecules coupled to a chemical reaction centre where CO_2 absorbed by the plant was reduced to sugars and O_2 evolved.

The picture, in reality, has proven to be somewhat more complex.

The first inkling of this came in the 1950's when the two-light-reaction hypothesis was evolved. Emerson himself was responsible for the evolution of this theory by demonstrating what has come to be known as the "Emerson enhancement effect". He and his co-workers [4] showed that the number of oxygen molecules evolved per quantum of light absorbed decreased in the far red end of the spectrum (*ca.* 700 nm) despite the fact that the light was still being absorbed by the plant. Further, if chloroplasts illuminated at 700 nm were supplemented with light of a shorter wavelength the increase in oxygen evolved was greater than the sum of the amounts evolved at each wavelength by separate illumination. This information, combined with the cytochrome studies by Hill and Bendall [5] in 1960, lead to the formation of the two-light-reaction hypothesis which was confirmed and expanded by the spectrographic studies of Duysens [6] in 1961.

It is now generally accepted that photosynthesis in plants occurs by the action of two photosystems, called "photosystem I" and "photosystem II". They are linked by a sequential flow of electrons derived from H_2O in photosystem II, which act to reduce NADP and produce ATP. In the two-light-reaction theory photosystem II is presumed to yield a strong oxidant and a weak reductant upon absorption

of light. The oxidant is reduced by H_2O to give O_2 while the reductant, generally called substance "a" is oxidized by a chain of redox reactions involving plastoquinone, plastocyanin, cytochromes and other intermediates.

Photosystem II is the least understood of the two systems. The exact nature of the chlorophyll absorption site or of the reductant(s) involved is not known. Studies of fluorescence kinetics and oxygen evolution by Joliot [7] suggest two components may be involved. Photosystem II operating by itself has been found to be responsible for the Hill reaction and deficiencies of Mn^{2+} and Cl^- result in impaired photosynthetic activity [8]. This would suggest that they may be part of the molecule(s) of the reacting species. Ben-Hayyim and Avron [9] have recently shown that manganese ion itself can serve as an electron donor to photosystem II. The work of Kok *et al.* [10] indicates that there may be an electron storage system involved as a part of photosystem II. Cheniae has recently reviewed photosystem II reactions [11].

Photosystem I, which is activated by the longer wavelengths of light, consists of chlorophylls of various types including P 700 which acts as the collection centre for excited electrons. The electrons are probably transferred to an intermediate. As the P 700 (or the intermediate) returns to the ground state NADP is reduced and then serves to reduce CO_2 indirectly. The return to the

ground state involves electron flow in ferredoxin (see a review by San Pietro and Black [12]), ferredoxin-NADP oxidoreductase (purified and characterized by Avron and Jagendorf [13]), NADP [12, 14] and a possible multitude of cytochromes, quinones, and plastocyanins (see a capable review by Bishop [15]). In 1964 Boardman and Anderson [16] published a differential centrifugation method for resolving two distinct chlorophyll-protein complexes which they postulated may have been from photosystem I and photosystem II. Later work by Vernon *et al.* [17] using Triton X-100 and centrifugation confirmed Boardman's hypothesis. More recent work by Kung and Thornber [18] has resulted in an electrophoretic-chromatographic method for purifying the chlorophyll-protein complexes. Further study of these complexes remains to be done.

The exact flow of electrons and sites of ATP formation have not been fully traced, but it is known photophosphorylation can occur by one of two ways.

"Noncyclic photophosphorylation" involves electron flow from H_2O to NADP and involves both photosystems while "cyclic photophosphorylation" involves only photosystem I. Noncylic photophosphorylation was discovered by Arnon *et al.* [19, 20] who showed NADP reduction was accompanied by ATP formation and that there was a stoichiometric relationship.

"Cyclic photophosphorylation" was discovered by Arnon and his co-workers [21,22]. Cyclic photophosphorylation

requires an electron carrier such as phenazine methosulphate to be observable *in vitro*. *In vivo* it is assumed to be coupled to a cyclic flow of electrons from photosystem II.

The stoichiometry of photophosphorylation and the energetics of photosynthesis have not been fully developed. It is postulated that two phosphorylations per two electrons transferred occur during light-induced electron transport [23]. The coupling factors of photophosphorylation appear to be directly involved in this ATP synthesis.

(2) Coupling Factors

ATP synthesis catalyzed by illuminated chloroplasts depends on the light-driven redox reactions within the chloroplast electron chain. Components which stimulate photophosphorylation (or mitochondrial phosphorylation) without affecting the rate of electron transport have been defined as "coupling factors" [24].

Avron [25] was first to describe a coupling factor. He found that chloroplasts or chloroplast fragments pre-incubated with EDTA lose all their photophosphorylative activity but not their electron transport activity. If these chloroplasts were recombined with the EDTA extract and Mg^{2+} ions introduced, the photophosphorylative capacity could be restored to the extent of about 20 per cent. The coupling factor was found to be heat-labile, non-dialysable and required in a quantitative manner.

Two years later, in 1965, Vambutas and Racker [26] reported the presence of two soluble enzymes separated from chloroplast preparations. These enzymes would catalyze ATP hydrolysis, and one required Ca^{2+} for activity, the other Mg^{2+}. The ATP-ase activity of both enzymes was latent and could only be revealed by treatment with trypsin or heat. The Ca^{2+}-dependent ATP-ase was purified by ammonium sulphate precipitation and protamine fractionation. The ability to restore photophosphorylative capacity to chloroplast particles was irreversibly lost after trypsin treatment. They proposed that the role of the coupling factor was to catalyze the last step of transphosphorylation:

$$X\sim P + ADP \rightleftharpoons X + ATP$$

where X is an unknown phosphate donor/acceptor.

In 1966 McCarty and Racker [24] provided evidence showing that when release of latent ATP-ase was maximal, phosphorylation was minimal. This suggested that the coupling factor-ATP-ase was the same entity. They observed that 0.5M EDTA gave a maximal release of ATP-ase. The enzyme was cold-labile with the loss of activity accelerated by salts. Using antiserum to the coupling factor they were able to inhibit not only photophosphorylation, but also Ca^{2+}-activity of trypsin-treated coupling factor.

In 1968 McCarty and Racker [27] discovered that the

ATP-ase activity could be revealed by not only trypsin and heat, but also by the reducing agent dithiothreitol.

In 1969 Bennun and Racker [28] introduced a DEAE-chromatography step into the purification of coupling factor 1. Using tritium-labelled CF_1 preparations they showed, among other things, that trypsin treatment or heat treatment of coupling factor 1 abolished its ability to combine with resolved chloroplast particles, that Mg^{2+} or Ca^{2+} were required to bind the protein to the chloroplast membrane, and that coupling factor 1 when combined with the chloroplast membrane exhibited increased cold stability.

Coupling factor 1 isolated from EDTA extracts and purified by ammonium sulphate precipitation and density gradient centrifugation was studied by Howell and Moudrianakis [29]. They found properties similar to Racker's coupling factor. The purified particle with Ca^{2+}-dependent ATP-ase activity appeared as a 100 Å structure in the electron microscope. They also calculated an estimated molecular weight of 350,000 from a sedimentation coefficient of 12.7.

In 1970 Farron [30] published work showing that native coupling factor and the heat-activated ATP-ase from the coupling factor were identical in regard to electrophoretic mobility on acrylamide gel, analytical ultracentrifugation and amino acid composition. The molecular weight of both proteins was determined to be 325,000 ± 600 by equilibrium

ultracentrifugation and 358,000 ± 3,100 by low speed equilibrium centrifugation. Farron explains the disparity by the fact that stored samples tend to form aggregates. Such samples on acrylamide gel showed that some material was retarded compared to the major band. In addition to the above, Farron determined that the protein dissolved in 5M Gd·HCl/pH 7.1 had an apparent molecular weight of 62,000. Based on her amino acid analysis she found a minimum molecular weight of 28,000. Further studies on coupling factor to ATP-ase conversion by Farron and Racker [31] showed that the change was accompanied by the appearance of titratable sulphydryl groups, and that the loss of coupling activity and the activation to an ATP-ase are two independent processes. The ATP-ase form may occur through disulphide interchange. Two independent sites are affected by heating and coupling activity begins to decay at temperatures too low to elicit ATP-ase activity.

In 1972 Lien *et al.* [32] published further work on the structure of coupling factor 1. They found that cold inactivation at mildly acidic pH in the presence of neutral salts resulted in disocciation of the protein into subunits. Immunoelectrophoretic analysis showed the existence of at least two antigenically different subunits, and three electrophoretically different protein species. Recently published work by Ryrie and Jagendorf [33] has provided evidence that there may be an energy-linked conformational

change in CF_1 that is required to allow ATP synthesis. Sato *et al.* [34] have reported a *C. reinhardi* mutant which is fully ATP-ase active without prior activation by DTT or heat.

MATERIALS AND METHODS

(1) Experiments to Determine Methods for Preparing Pure Concentrated CF_1

Growing of bean plants

Seeds of *Phaseolus vulgaris* var. Kinghorn Special were rinsed in dilute hypochlorite solution (Javex; H_2O at 1:5) and grown for 12-20 days on soil in the greenhouse under 16 hour days or in total darkness if etiolated plants were desired.

Plastid isolation

Plastids were isolated by an adaptation of the method of Nobel [35]. Primary leaves were homogenized in a medium consisting of 0.4M sucrose in 40 mM TES-NaOH pH 8 at 4°C. This medium will subsequently be referred to as sucrose-TES medium. A Vir-tis 45 homogenizer was used at a rheostat setting of 10-20 for 1 minute. All preparations were made in 5 g batches with 20 ml of sucrose-TES in a 30 ml homogenizing flask. The homogenate was filtered through fine nylon cloth (15XX Flour Silk, Strong-Scott Ltd., Winnipeg) to remove debris and centrifuged at 2,000 x g for 10 minutes at 0-4°C. The pellet was then extracted with the appropriate medium.

EDTA extraction

Immediately after centrifugation at 2,000 x g the pellet obtained was resuspended in 10 mM NaCl at 0-4°C at a ratio of 1 ml NaCl per gram original leaf material (approximately 1 ml per mg chlorophyll) and immediately centrifuged at 20,000 x g for 20 minutes at 0-4°C. The pellets so obtained were then resuspended in 0.75 mM EDTA (unless otherwise indicated) at room temperature at a ratio of 10 ml EDTA per g original leaf material, and stirred for 30-60 minutes. The EDTA wash was then centrifuged at 18-20°C for 30 minutes at 35,000 x g. Pellets were discarded and the supernatant, which contains CF_1, retained. It will be referred to as "EDTA extract".

Ultrafiltration

The membranes used for ultrafiltration were "Diaflo Ultrafilters" (Amicon Corporation) Series UM-10 which have a 95 per cent exclusion limit of 10,000 MW. A large volume of EDTA extract was prepared and brought to 20 mM Tris-Cl pH 8.0 except when immunochemistry was to be done. The solution was then filtered under nitrogen at 20°C with constant stirring at 55 psig in a 350 ml Diaflo unit. When volume had been reduced to 60 ml the solution was transferred to a 60 ml Diaflo unit and ultrafiltration continued until a suitable volume was reached (generally less than 7 ml). The protein so prepared was called "CF_1 concentrate".

Pervaporation

CF_1 concentrate was put into 8/32 dialysis tubing and pervaporated at room temperature for approximately 1 hour in an open piece of glass tubing attached to a vacuum line.

Determination of ATP-ase activity

a) DTT activation

ATP-ase activity of the Ca^{2+}-dependent ATP-ase extracted from the plastids was assayed by a method adapted from McCarty and Racker [27]. An 0.7 ml aliquot of EDTA extract containing 25-100 µg of protein was combined with 0.2 ml 0.2M TES-NaOH pH 8.0 and 0.1 ml of 0.5 M DTT pH 8.0. The solution was left at room temperature for 2 hours and ATP-ase activity determined. Unless otherwise indicated DTT was used for all ATP-ase activations.

b) Trypsin activation

Trypsin activation was identical to the method of Vambutas and Racker [26] except that the optimal activation time was determined to be 12 minutes. Unless otherwise indicated the amount of protein activated was 25-75 µg.

c) Incubation and assay

The activated proteins were incubated by the method of Horak and Hill [36]. Aliquots of 0.2 or 0.4 ml were combined with 0.1 ml 50 mM $CaCl_2$, 0.25 ml 0.2 m TES-NaOH 0.1 ml 50 mM ATP and water to a final volume of 1 ml at pH 8.0. The mixture was incubated at 37°C for 20

minutes. Reaction was stopped by the addition of 0.1 ml 30% trichloroacetic acid. The released inorganic phosphate was measured as the unreduced phosphomolybdate complex according to Mozerski *et al.* [37] after DTT had been oxidized with H_2O_2.

Definition of unit and specific activity

A unit of ATP-ase is that amount of protein required to catalyze the cleavage of 1 μmole of ATP to ADP and Pi under the specified assay conditions. The specific activity is defined as units per mg dry weight protein.

Chlorophyll determination

Chlorophyll was determined spectrophotometrically in 80 per cent acetone extracts of chloroplasts using the equation of Mackinney [38].

Nitrogen and protein determination

The presence of ammonium sulphate was determined at 430 nm with "Nessler's Reagent" by the method of Williams [39]. Proteins were determined by the Lowry method [40] at 750 nm.

The ATP-ase extraction method modified from Vambutas and Racker [26] and Farron [30]

A chloroplast pellet was prepared as described above and resuspended in ice-cold sucrose-TES buffer to give a final chlorophyll concentration of 3 mg/ml. The chloroplast

suspension was then added to 16 volumes of acetone at -20°C to -10°C with rapid stirring. Acetone was decanted and the residue spread to dry on a glass plate under a stream of cold air. The residue was then extracted twice for 20 minutes with 50 mM Tris-Cl buffer pH 8.0 containing 2 mM EDTA and 4 mM ATP at a ratio of 1 ml buffer per 10 g fresh weight of leaves (combined volume). As shown later the presence of Tris may have affected the activity of the ATP-ase. The supernatants obtained after centrifugation at 20,000 x g for 15 minutes were combined. This extract was fractionated with ammonium sulphate at 0°C. Twelve grams of solid ammonium sulphate were added per 100 ml of solution with slow stirring. The solution was left overnight at 0-4°C, the precipitate was collected by centrifugation and discarded, and 13.8 g solid ammonium sulphate per 100 ml solution added at 0-4°C. The precipitate, which contained the enzyme, was collected and dissolved in a minimal volume of 20 mM Tris-SO_4 containing 2 mM EDTA pH 7.1 at room temperature. The solution was clarified by centrifugation and diluted to contain no more than 80 mM NH_4 as assayed with Nessler's Reagent. This solution is then applied to a 1.5 cm x 30 cm column of DEAE-Sephadex A-50 equilibrated with the same buffer except at 1 mM EDTA and containing 80 mM ammonium sulphate. The column was developed by a linear gradient of ammonium sulphate in the same buffer as described by Farron [30].

The Racker [41] procedure for CF_1 isolation

After chloroplasts have been isolated in the normal manner they are resuspended in 10 mM NaCl at 4°C for 10 minutes (2.5 ml NaCl solution/mg chlorophyll). The suspension is then centrifuged for 20 minutes at 20,000 x g and the pellet resuspended in the same solution at a concentration of 1 ml per 3-4 mg chlorophyll. It is then diluted to 0.1 mg chlorophyll per ml with 0.75 mM EDTA pH 7.6 and stirred with a magnetic stirrer for 10 minutes. The solution is centrifuged at 20°C for 30 minutes at 20,000 x g The supernatant contains CF_1.

Effect of addition of Tris and/or ATP to EDTA extraction solutions

An experiment was designed to test the effect of ATP and Tris-Cl on the extraction and activity of ATP-ase. The standard preparation method was used except that the 10 mM NaCl wash was excluded. Equal amounts of chlorophyll were extracted with each solution. Samples which were frozen and stored at -20°C for 30 minutes were used as controls.

(2) Electrophoretic and Immunological Methods

Micro-scale immunodiffusion

Micro-scale immunodiffusion was performed on glass slides using 0.8 per cent agarose buffered with Tris-barbital buffer as in IEA. Holes were punched with dies obtained from Gelman.

Immunoelectrophoresis

Microimmunoelectrophoresis was performed on glass slides using equipment from Gelman. Gels were 0.8 per cent Agarose (Sigma) at ionic strength 0.025 using Tris-barbital sodium barbital buffer at pH 8.8. Reservoir buffer was at 0.050 ionic strength. Gels were run for 60 minutes at 350 v and incubated for 20-24 hr at 25°C with quarter-strength antiserum, unless otherwise indicated. In most instances matched pairs of gels were run, with one stained for protein immediately after electrophoresis and the other used for immunodiffusion. After precipitin lines had developed the gels were photographed and then washed in 2 per cent NaCl for 48-72 hr to remove unprecipitated protein. The gels were then stained in 0.1 per cent Amido Black in 7 per cent acetic acid, for 1 hr, destained in 7 per cent acetic acid and photographed using a red filter and Kodak High Contrast Copy film. In some instances gels were stained with Coomassie Brilliant Blue (0.02 per cent in 12.5 per cent TCA) and destained with 12.5 per cent TCA.

Large scale double diffusion

Glass petri dishes were filled with a layer of 0.8 per cent agar containing 1 per cent NaN_3, 0.85 per cent NaCl. In one experiment a basement layer of 2 per cent agar was poured before the 0.8 per cent agar, with wells cut only to the bottom of the top layer.

Protein synthesis inhibition

Etiolated plant material was grown at 20°C in total darkness. Plant stems were cut at sufficient length to allow them to be suspended in 35 ml test tubes containing 20 ml of solution. Excised plants were placed into either chloramphenicol (10 µg/ml), cycloheximide (5 µg/ml), or distilled water, and greened at 10,000 lux for 72 hr at 21°C.

Plant tissue was then extracted by the standard extraction procedures. Post ribosomal supernatants of the buffered sucrose homogenate were prepared by centrifugation at 150,000 x g and 20°C for 1 hr. All treatments were concentrated by ultrafiltration and pervaporation and then dialyzed against 20 mM Tris-Cl pH 7.8 for 24 hr. Protein determinations were made on these final pervaporates, and IEA performed as above against full strength antiserum. ATP-ase activity was determined before ultrafiltration.

The method of Lien *et al.* [32]

IEA was performed as described above. Samples were

prepared by the above authors as follows: urea treatment was carried out by incubating freshly desalted CF_1 in the presence of the indicated amount of urea and 5 mM Tricine-NaOH/pH 7.2 at room temperature. Cold inactivation was performed in 20 mM Tris-maleate at pH 6.4 containing the desired amount of KCl and incubated for 50 minutes at 0°C. The only deviation from the method of Lien *et al.* was the use of TES instead of Tricine. Slides were stained as described above and photographed using a red filter.

Polyacrylamide gels prepared with buffers of Poulik [42]

A 7.5 per cent polyacrylamide gel was prepared using the following proportions: 4 ml acrylamide solution (as used in Davis gels below), 1.6 ml of 0.76M Tris-0.05M citrate pH 8.65, 9.6 ml distilled H_2O, 0.8 ml of 1.5 per cent ammonium persulphate, and 30 µl TEMED. Gels were 6 cm long. Reservoir buffer was 0.3M borate 0.05M NaOH pH 8.0. Electrophoresis was performed at 2 mA/gel. Protein load per gel was 250 µg. Bromphenol blue was used as a marker dye. Gels were stained as described above.

Urea-polyacrylamide gel electrophoresis as adapted from Takayama *et al.* [43]

Polyacrylamide gels (7.5 per cent) were prepared as described by the authors except that 5 times more TEMED was required to polymerize them. Gels were 5M in urea, 35 per cent in acetic acid. Reservoir buffer was 10 per cent

acetic acid. Samples were made 2M in urea and 2:1:1 phenol:acetic acid:H_2O. Sample loads per gel were kept below 100 µl, and contained approximately 100 µg of protein. Electrophoresis was performed at 1-2 mA per gel for 8 hr with water cooling. A sample of cytochrome c was run in parallel as a marker.

When immunodiffusion was to be performed the gels were cut in half longitudinally, one half stained, and the four upper protein bands excised from the unstained gels. The gel pieces were macerated, dialysed against 20 mM Tris-Cl pH 8.0 for 20 hr, and the solution used for immunodiffusion.

Method for the determination of molecular weight by SDS-PAGE

a) Protein standards

Bovine γ-globulins (Cohn II), pyruvate kinase (rabbit skeletal muscle), and trypsin (bovine pancreas) were obtained from Sigma; cytochrome c (equine heart) and bovine serum albumin (fatty acid poor) from Calbiochem. The lactic dehydrogenase was a contaminant in the pyruvate kinase preparation. Extensive reduction with β-mercapto-ethanol would have reduced the number of oligomers of the various standards, but it was considered advantageous to have them since they provided extra data for the standard curve.

b) Preparation of electrophoretically pure CF_1-disc electrophoresis according to Davis [44]

CF_1-concentrate, prepared as described elsewhere, was applied to polyacrylamide gels prepared according to Davis [44] at approximately 400 µg protein per gel. Upper gel was 3 per cent at pH 6.9, running gel was 7 per cent at pH 8.9 (Tris-Cl buffer). Reservoir buffer was Tris-glycine pH 8.3. Electrophoresis was run at 2 mA per gel for 4 hr and the gels stained for ATP-ase activity by the method of Horak and Hill [36]. The ATP-ase bands were then excised from the gels and placed into 6 mm I.D. destaining tubes in the bottoms of which was a plug of 7 per cent polyacrylamide (as above). Dialysis tubing was tied to the ends of each tube and electrophoresed in the same buffer as above at 2 mA per tube for 3.5 hr to ensure that all protein had migrated into the dialysis tubing. This was confirmed by staining the excised gel slices for protein. The aliquots in the dialysis tubing were combined into one large tube (less than 5 ml final volume) and dialysed overnight against 2 l of 10 mM Tris-Cl pH 8.0. The sample was then pervaporated for several hours, dialysed for another 3-4 hr against 10 mM Tris-Cl pH 8.0 and then prepared for SDS-gels.

c) Preparation of samples for SDS-electrophoresis

The protein standards were dissolved in 0.75 mM EDTA, 20 mM Tris-Cl pH 8.0, allowed to stand at room

temperature for 1 hr and then prepared (as was the CF_1) by an adaptation of the method of Tzagaloff [45] where all proteins were brought to 1 per cent SDS, 1 per cent mercaptoethanol, 0.01M sodium phosphate pH 7.1, 0.003 per cent bromphenol blue, and incubated for 30 minutes at 70°C. Protein concentration was not allowed to exceed 2 mg/ml. Glycerol was added either before or after incubation to increase sample density.

d) SDS-polyacrylamide gels

Polyacrylamide gels (7.5 per cent) were prepared by the method of Weber and Osborn [46] except that solutions were not deaerated, and less TEMED was required to induce polymerization. Gels were 6 cm long. Tank buffer was as described by Weber and Osborn.

e) Electrophoresis

Samples were layered onto gels and then overlayed with tank buffer. Standards were generally applied at 50 µg (in 100 µl) per gel, though as little as 10 µg would give visible bands in most cases. CF_1 was applied in 100 µl and 200 µl amounts and contained 150-175 µg protein per 100 µl except in the case of purified CF_1 where the concentration was 40 µg/100 µl. Electrophoresis at room temperature was performed at 6-8 mA per gel for 3-4 hr.

f) Staining

Gels were stained for 1 hr in 0.1 per cent Amido

Black in 7 per cent acetic acid and destained overnight with 7 per cent acetic acid. Some preliminary work using 0.02 per cent Coomassie Brilliant Blue in 12.5 per cent TCA showed it to be not as effective as Amido Black.

g) Measurement of relative mobilities

Because gels swell upon staining-destaining, it was necessary to measure them twice. Assuming that all gels are equally affected the relative mobility was calculated by the formula:

$$\text{MOBILITY} = \frac{\text{distance of protein migration}}{\text{gel length after staining}} \times \frac{\text{gel length before staining}}{\text{distance of bromphenol blue migration}}$$

RESULTS AND DISCUSSION

(1) Preparation of CF_1

Several methods that have been successful in the preparation of spinach CF_1 were tested for the extraction of bean CF_1. Most of these methods were not completely suited to this study and a procedure was developed that produces CF_1 that is homogeneous by disc gel electrophoresis.

The cold lability of the ATP-ase presents problems as far as storage of extracts is concerned. Therefore, it was desirable to have a procedure that took as little time as possible so that an active ATP-ase could be used in the work to follow. A high degree of purity was also required since the subunit structure was to be studied. The level of ATP-ase in etiolated tissue is very low, and this, combined with the naturally low leaf weight of etiolated plants, meant that the method had to show high recovery over a broad range of tissue weight extracted. A low salt concentration in extraction media was desirable so as to limit the amount of dialysis required for pervaporated concentrates.

A comparison of trypsin and DTT activation of bean and spinach ATP-ase

The papers consulted in the development of a preparative procedure for bean CF_1 dealt with spinach CF_1, and the method of ATP-ase activation varied, being either by DTT or trypsin. An experiment was performed to determine the best method of ATP-ase activation for this study, and to determine whether the bean and spinach ATP-ases were differently affected by the activation procedures.

Results are indicated in Table I. A 20 minute trypsin activation was used instead of the 5 minute activation used by Vambutas and Racker [26]. The Farron [30] method was used for extraction, and a water wash of the acetone powders preceeded the EDTA wash.

The source of the ATP-ase does not appear to be a factor in determining the method of activation. The reasons for lower ATP-ase activity with trypsin could be several. The length of time, amount of protein activated, temperature, etc., are critical in trypsin activation. The use of trypsin activation entails a day-to-day analysis of optimal time of activation and protein load and is therefore much more inconvenient to use than the DTT activation.

In addition to this experiment a second experiment was performed in which trypsin and DTT activation were performed over a range of protein concentrations. The NaCl-EDTA extraction of chloroplasts was used. Trypsin

Table I A comparison of DTT activation and trypsin activation of bean and spinach ATP-ase.

Tissue	Activity µmoles Pi released/mg protein/hr	
	DTT	Trypsin
Bean	15	10
Spinach	27	20

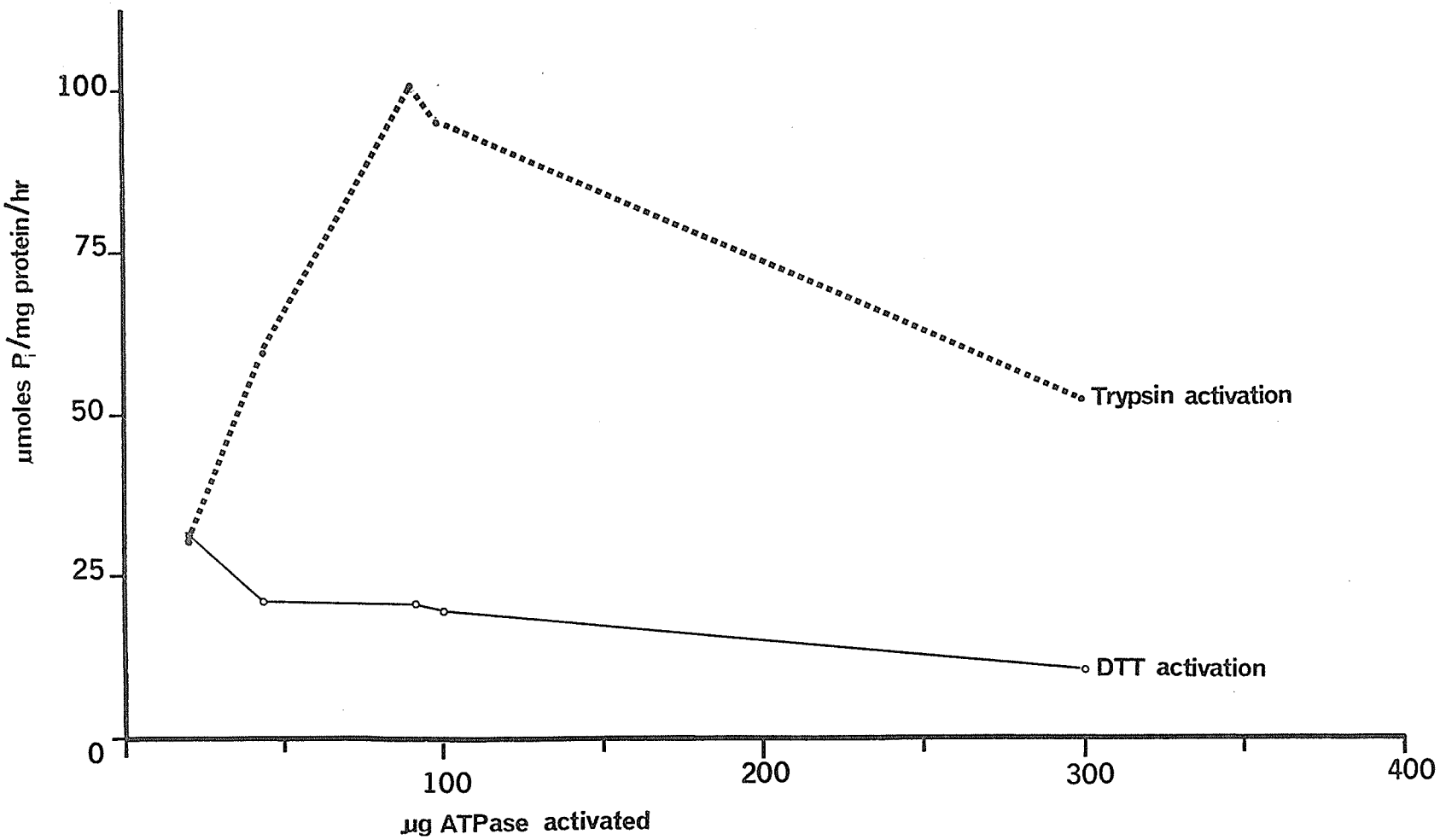
activation was for 12 minutes and 50 mM DTT was used for DTT activation. Proteins were concentrated by Diaflo ultrafiltration and then diluted with EDTA extraction solution. Figure 1 shows that the behaviour of the two types of activation is markedly different as determined by ATP-ase assay.

Trypsin activation shows a maximum activation concentration. This is probably due to the opposing processes of protein digestion (at low ATP-ase concentrations) versus protein activation (at higher protein concentrations). In the region of ATP-ase excess the trypsin is incapable of fully activating all of the protein, in the region of low ATP-ase concentration the trypsin not only activates, but digests the ATP-ase, leading to a loss of activity. In the case of DTT activation it is believed that the DTT acts by stimulating disulphide interchange [26]. Increasing concentration of ATP-ase results in a slight decrease in activation, but the DTT concentration is high [27] and the limiting factor becomes length of exposure to DTT. Since DTT activation is not as dependent on protein concentration, nor does exposure to DTT for excessive periods destroy ATP-ase activity it was decided to use this method for ATP-ase activation.

Effect of BSA in homogenization medium on DTT activation and ATP-ase activity

The plastid isolation medium normally used was 0.4M

Figure 1. Effect of protein concentration on ATP-ase activation by trypsin or DTT.


100
75
50
25
0
µmoles P_i/mg protein/hr
100
200
300
400
µg ATPase activated
Trypsin activation
DTT activation

sucrose 0.04M TES-NaOH and 0.2 per cent fatty-acid-poor bovine serum albumin at pH 8. The inclusion of fatty acid poor BSA was shown to increase CF_1 stability as determined by the photophosphorylation assay [48]. Since it was desirable to purify CF_1 for future experiments and the BSA was known to be carried through the EDTA extraction an experiment was performed to determine whether the exclusion of BSA from the homogenization medium had any effect on ATP-ase activity or DTT activation. Two gram amounts of bean leaves were homogenized in 20 ml of sucrose-TES medium with DTT, and assayed for ATP-ase activity as described in Methods except that the 10 mM NaCl wash was excluded.

Table II shows that the exclusion of BSA from the homogenization medium had no significant effect on either recovery of ATP-ase from the chloroplast or the DTT activation of the ATP-ase. It was therefore decided to exclude BSA in future homogenization media to decrease the amount of extraneous protein present in CF_1 preparations.

CF_1 preparation procedure for electrophoretic and immunological studies

One of the first methods attempted in the purification of bean CF_1 was a modification of methods used successfully for spinach CF_1 by Vambutas and Racker [26] and Farron [30]. Several experiments were carried out up to the point of ammonium sulphate precipitation.

Table II A comparison of DTT activations of bean ATP-ase isolated with and without 0.2 per cent BSA in the leaf homogenisation medium.

Additions to homogenisation medium	Total chlorophyll in extracted pellet (mg)	Total protein extracted (mg)	ATP-ase activity - DTT µmoles Pi released/mg protein/hr	ATP-ase activity + DTT µmoles Pi released/mg protein/hr	Recovery units ATP-ase/mg chlorophyll
none	2.24	2.20	31.5	107	1.7
0.2% BSA	2.19	2.00	29.1	105	1.6

Approximately 120 g of leaves were used. The expected yields (based on Farron's results [30] for spinach) versus the observed yields are shown on Table III. Bean leaves gave a lower yield per given weight of tissue than did spinach and the percentage recovery from the ammonium sulphate precipitation was lower. In addition, the specific activity of the bean ATP-ase is considerably lower than that of spinach. It is possible that bean leaves have a naturally lower level of CF_1 or the coupling factor may have a slight structural difference that results in lower specific activity. Alternatively, the extraction procedure used may not be favorable for removing CF_1 from bean leaves even though it is suitable for spinach.

Experiments in which the pellet was washed several times in EDTA extraction solution failed to give a significant change in yield on a μg protein basis.

When locally purchased spinach was prepared in a manner as above the yield obtained from the EDTA wash was 176 mg protein (from 73 mg chlorophyll) representing 54 units of ATP-ase activity. This is approximately twice the expected protein yield and about one-tenth of the expected unit yield of ATP-ase (cf. Table II). The reason for the excess protein released may be that the time used for the buffered EDTA washes was about twice as long as that recommended by Farron as a result of difficulties encountered in resuspending the acetone powder. The low

Table III A comparison of observed ATP-ase yields for bean leaves versus reported [30] ATP-ase yields for spinach leaves.

Treatment	Total protein (mg)	Specific activity	Total units	Per cent yield
Spinach				
EDTA wash	85	5.2	442	100
$(NH_4)_2SO_4$ precipitation	28.6	14.2	405	92
Bean				
EDTA wash	36	0.72	26	100
$(NH_4)_2SO_4$ precipitation	13.3	1.25[1]	17.8	68

[1] Determined with 0.33 mM NH_4^+ present. Inhibition by NH_4^+ is 45 per cent at 10 mM NH_4^+ [26].

yield in terms of units of ATP-ase activity was perhaps due to the low quality of the spinach used.

The preparation of acetone powder as described by Farron [30] requires an additional step not encountered in the EDTA wash of chloroplasts. To determine whether this approach provided any advantages other than storage of the acetone powders, 60 g of leaves were homogenized in 16.7 ml buffered sucrose and equal aliquots of homogenate were treated by either the Farron method or by extraction of the chloroplast pellet with 1 mM EDTA containing 4 mM ATP at pH 8.0. Both pellets were extracted with their respective EDTA solutions for 1 hr. As Table IV indicates there was no significant difference (less than 1 per cent) between these two methods on a per mg chlorophyll basis. For this reason it was decided that all future preparations of CF_1 would be made without using the acetone powder step of Farron [19].

The inclusion of a 10 mM NaCl wash of the chloroplast material prior to the EDTA extraction was used by Racker [41] in his preparation of spinach ATP-ase. Bean ATP-ase was prepared using his method except that the adjustment of the final chlorophyll concentration was to 0.4 mg chlorophyll/ml versus 0.1 mg/ml. The important fact learned was that the 10 mM NaCl wash contained 44 per cent of the total protein recovered in both the NaCl and EDTA washes, but only 18 per cent of the total ATP-ase activity.

Table IV A comparison of two methods of CF_1 preparation.

Treatment	Chlorophyll (mg)	Protein (µg/ml EDTA extract)	ATP-ase activity (µmoles Pi released/mg protein/hr)	Total units ATP-ase recovered	Units/mg Chlorophyll
Farron method	15.8	620	25.3	13.08	0.828
1 mM EDTA method	14.5	560	26.0	12.12	0.836

Thus, the 10 mM NaCl wash became a part of the standard extraction procedure. The shortest possible exposure of the chloroplast pellet to 10 mM NaCl at 0-4°C was used. Sufficient data was obtained to show that the NaCl wash contained 40-50 per cent of the protein released in both washes, but often less than 5 per cent of the ATP-ase units. Allowing the NaCl wash to warm up or stand for even a couple of minutes resulted in release of large amounts of ATP-ase into the NaCl wash. If NaCl was included in the EDTA solution the release of ATP-ase was prevented, as is demonstrated in Figure 2, where equal amounts of chlorophyll were extracted with 0.75 mM EDTA* and 1.0 mM EDTA containing either 0, 1, 2, or 5 mM NaCl at pH 8.0. That the concentration of NaCl in the assay mixtures was not a significant factor in the specific activity determinations, was shown by Vambutas and Racker [26] who found only 5 per cent inhibition of ATP-ase activity at 10 mM NaCl. The highest concentration of NaCl in an assay mixture in this experiment was 0.14 mM.

One millimolar EDTA washes extracted about 20 per cent more protein than did their respective 0.75 mM EDTA washes but they had lower specific activity and therefore lower yields in terms of ATP-ase.

The volume of EDTA solution used to extract CF_1 from chloroplast pellets was found to have an effect on both

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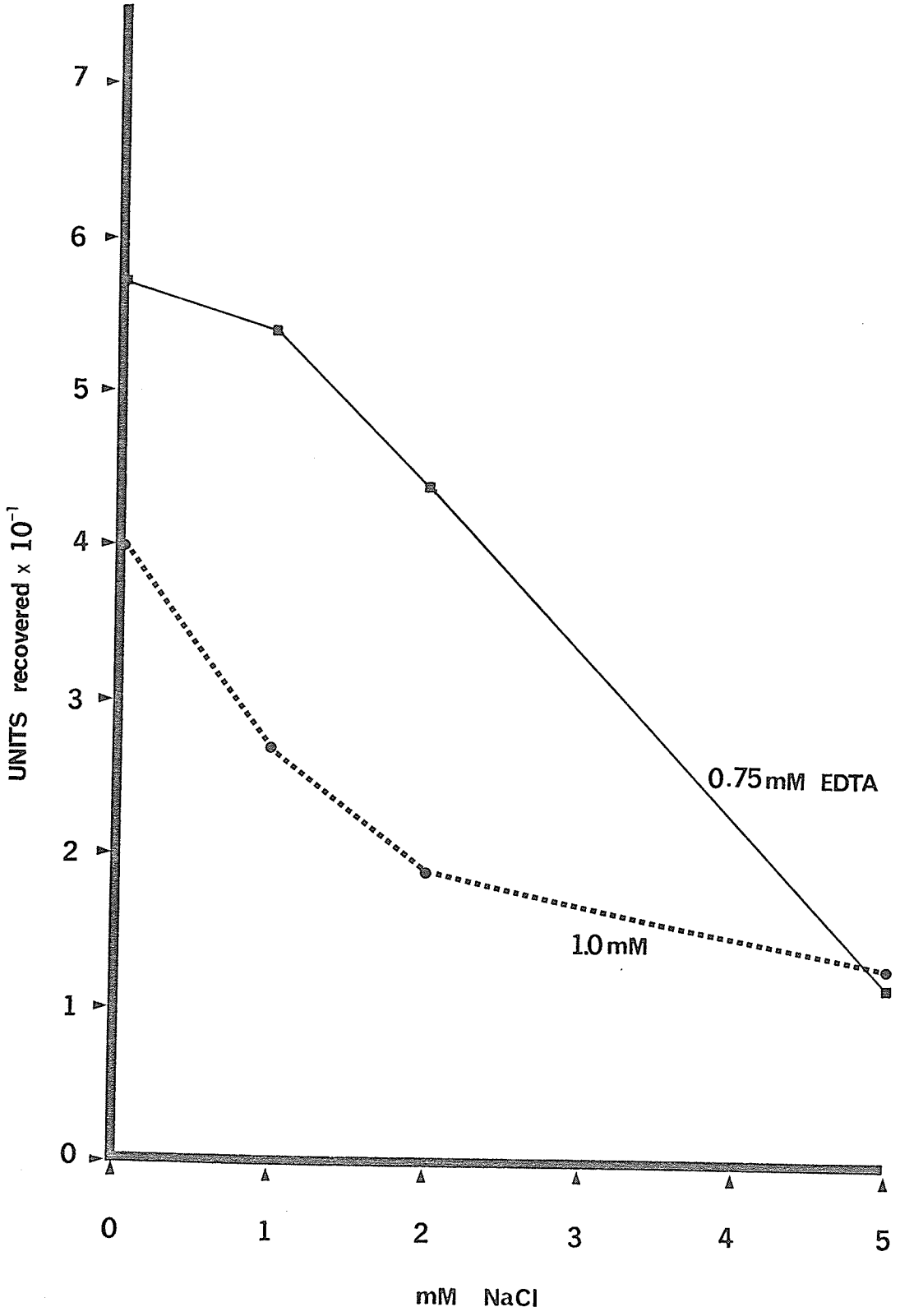
* used by Racker [41]

Figure 2. A plot of units ATP-ase recovered versus mM NaCl at two different concentrations of EDTA.


UNITS recovered x 10^{-1}
7
6
5
4
3
2
1
0
0.75mM EDTA
1.0mM
0
1
2
3
4
5
mM NaCl

activity and yield of the ATP-ase (Table V). A chloroplast pellet washed with 10 mM NaCl was suspended in 125 ml of 0.75 mM EDTA 4 mM ATP at pH 7.6 and allowed to stand for 25 minutes. The suspension was centrifuged at 35,000 x g for 20 minutes and the pellet resuspended in a second 125 ml aliquot of EDTA. After 40 minutes the suspension was centrifuged and the two supernatants combined. This data was compared to an experiment in which two 15 ml aliquots were prepared in the same manner. The use of a larger volume of EDTA (Table V) resulted in a higher specific activity of the released ATP-ase and a higher unit yield. The amount of protein released was not significantly affected.

ATP had been routinely included in the extraction procedure at either 2 mM or 4 mM concentration as it had been reported it stabilized the activity of the ATP-ase [26]. Because some procedures covered a span of several days it was desirable to exclude the ATP, as its breakdown lead to very high blank values in the ATP-ase assay and to inhibition of the ATP-ase activity by the ADP released. Storage in the cold would prevent ATP breakdown but unfortunately the ATP-ase becomes inactivated [24].

In addition to this it had been observed on several occasions that the resuspension of the chloroplast pellet in 0.75 mM EDTA at pH 8 led to a pH drop of approximately 0.8 pH units, while a similar resuspension in 0.75 mM EDTA

Table V A comparison of two different EDTA volumes in their ability to extract ATP-ase from a chloroplast pellet.

Volume EDTA (ml)	Chlorophyll (mg)	Protein (mg)	Units ATP-ase
30	35	29.3	9.08
250	34	31.2	15.97

2 mM ATP at pH 8 caused a drop of 1.1 pH units. Since ATP-ase is especially unstable on the acid side of neutrality [24] it was feared that a loss of activity might be occurring as the EDTA was normally unbuffered. Table VI shows a comparison of ATP-ase activity for CF_1 extracted from equal amounts of chloroplast pellet using 4 different CF_1 extraction media varying in buffer and ATP content. The inclusion of ATP had no significant effect on ATP-ase activity and the presence of buffer had disastrous results on activity as well as releasing additional protein. ATP-free 0.75 mM EDTA at pH 8 seems the best extraction medium for our purposes. The fact that ATP seemed to have little effect on maintaining activity may have been due to the short storage period (approximately 24 hr). The effect of Tris may be due to the ionic strength of the solution or to Tris-EDTA interaction which may have resulted in release of large amounts of non-ATP-ase protein.

Table VI A comparison of four EDTA extraction media in their effects on protein yield and enzymic activity of ATP-ase.

Additions to 0.75 mM EDTA pH 8.0	Protein (µg/ml EDTA)	ATP-ase activity (µmoles Pi/mg protein/hr)
A. none	100	21
B. 2 mM ATP	100	22
C. 2 mM ATP 20 mM Tris-Cl	130	1.8
D. 20 mM Tris-Cl	136	-*

* Blank value though consistent with other blank values was slightly higher (~10 per cent) than the "active" enzyme incubation.

(2) Electrophoretic and Immunological Experiments

In the second part of this work CF_1 extract concentrated by ultrafiltration and pervaporation was used for a series of experiments on subunit structure of the coupling factor. They consist of two basic types of experiments: (i) immunochemical experiments using an antiserum to CF_1 and its subunits, and (ii) electrophoretic experiments in which various dissociating treatments of CF_1 were made and their effect analysed by electrophoresis.

Immunological activity of spinach CF_1, bean CF_1, and bean etioplast CF_1

Many of the properties of the latent Ca^{2+}-dependent ATP-ase of bean chloroplasts and etioplasts are consistent with its being identical to the coupling factor isolated from spinach [48]. The results of immunodiffusion experiments shown in Figures 3, 4, and 5 indicate that there are antigenically similar sites on bean chloroplast and etioplast ATP-ase when reacted with an antibody to spinach CF_1. The plate in Figure 3 was prepared using two layers of agar; all other plates had only one layer. In Figure 3 a U-shaped line of immunological identity exists between all three samples. Figures 4 and 5 show lines between spinach CF_1 and both bean CF_1 (Figure 4) and bean etioplast CF_1 (Figure 5). The lack of a line for bean CF_1 (Figure 5) is probably due to the relatively low protein concentration used.

Figure 3. Immunodiffusion of anti-CF_1 and normal rabbit serum versus bean, spinach, and bean etioplast ATP-ase.

Legend: A = spinach CF_1 antiserum
N = normal rabbit serum
S = spinach extract
B = bean extract
E = etiolated bean leaf extract

Fig.3

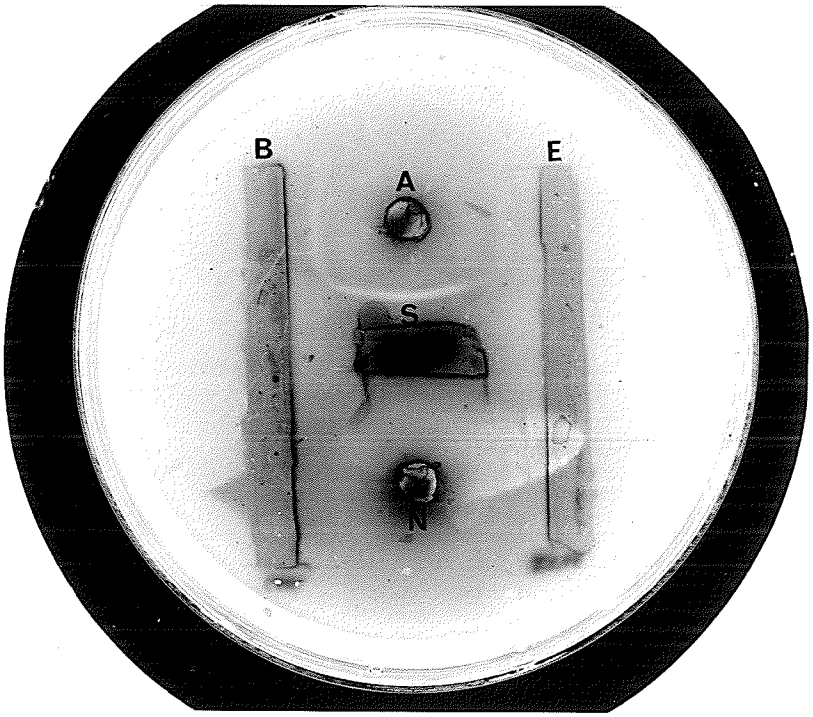


Figure 4. Immunodiffusion of spinach and bean CF_1 versus CF_1 antiserum and normal rabbit serum.
Legend as in Figure 3.

Fig.4

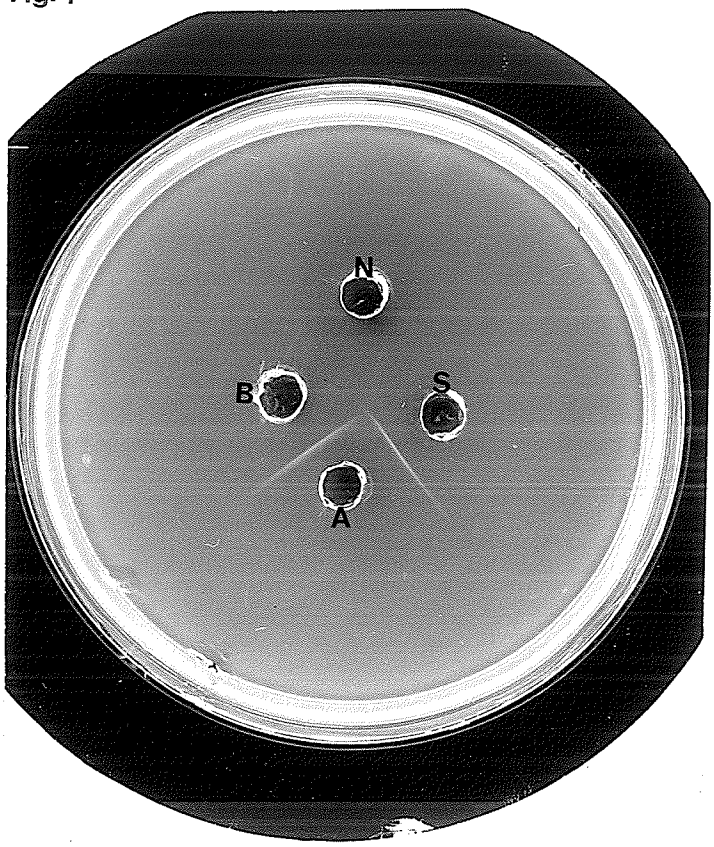
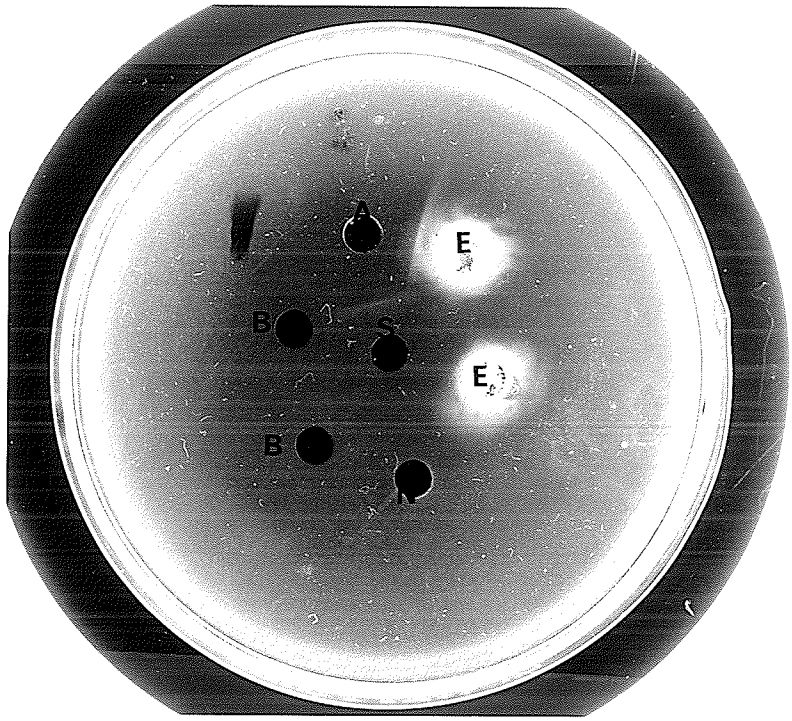


Figure 5. Immunodiffusion of spinach, bean, and bean etioplast CF_1 versus spinach CF_1 antiserum and normal rabbit serum. Legend as in Figure 3.

Fig.5



The partial identity (i.e. extension of part of the spinach precipitin line beyond the bean precipitin line) suggests that there is an antigenic factor present in spinach CF_1 which is not present in bean CF_1. As a consequence, some of the antibodies will not react with the bean CF_1 (since it lacks the antigenic factor) but will react with spinach CF_1. This could mean one of two things, either spinach CF_1 is structurally different from bean CF_1 or, the preparative procedures used affect the proteins in different manners, exposing an antigenic site in spinach CF_1, but not in bean CF_1. As will be shown later, on the basis of subunit studies, the spinach CF_1 appears to be electrophoretically identical to bean CF_1.

Immunoelectrophoretic analysis of CF_1

To determine the most effective dilution of antiserum to use against CF_1 a series of micro-scale double diffusions was performed. CF_1 was serially diluted from 1/2 to 1/32 as was anti-CF_1. The undiluted CF_1 load was 12 µg/well. Table VII lists intensity of precipitin lines as a function of the ratio of the antiserum dilution to the antigen dilution.

The 1:1 ratio (i.e. full strength antiserum versus 12 mg/ml CF_1) gave best results, but since such high loads of protein (12 mg/ml) were seldom used it was decided that a 1/4 dilution of antiserum would be sufficient to produce

Table VII Relative intensities of precipitin arcs at various ratios of antiserum versus CF_1 (volume to weight).

\+ visible precipitate
++ moderate precipitate
+++ heavy precipitate

Dilution of CF_1 antiserum adjusted to CF_1 concentration of unity	Intensity of precipitin line
8	-
4	-
2	-
1	+++
1/2	++
1/4	+
1/8	+
1/16	-
1/32	-

precipitin arcs.

A similar experiment was performed using immuno-electrophoretic analysis (IEA). Three microlitre aliquots of CF_1 (60, 130, 240, 450, 650, 950 µg/ml) were electrophoresed and reacted with full-strength antiserum. In a second set of experiments, a series of dilutions of antiserum (1/2 to 1/32) were reacted with 3 µl of CF_1 (950 µg/ml) after electrophoresis. After 24 hr of incubation at 37°C single precipitin arcs were observed at the 1/32 and 1/4 dilutions of antiserum. After washing with 2 per cent NaCl for 32 hr the gels were stained with Coomassie Brilliant Blue and arcs were seen with all dilutions of antiserum. The arcs at 1/4 and 1/8 dilution were the sharpest, and the 1/4 dilution of antiserum was routinely used in later experiments of subunit structure.

In an attempt to dissociate CF_1 into subunits an aliquot was treated with 0.1 per cent SDS and IEA performed either with or without 0.1 per cent SDS in the gels and buffer. No precipitin arcs were detected at a protein load of 22 µg/well. IEA of SDS-treated BSA against anti-BSA gave precipitin arcs in a gel containing SDS. Thus, SDS-treated BSA could still elicit an immune response from its antiserum under conditions where SDS treated CF_1 would not.

Solubilizing the coupling factor in Tris-Cl buffer containing urea and Triton X-100 and performing IEA using the methods of Demus and Mehl [49] proved disappointing.

6c

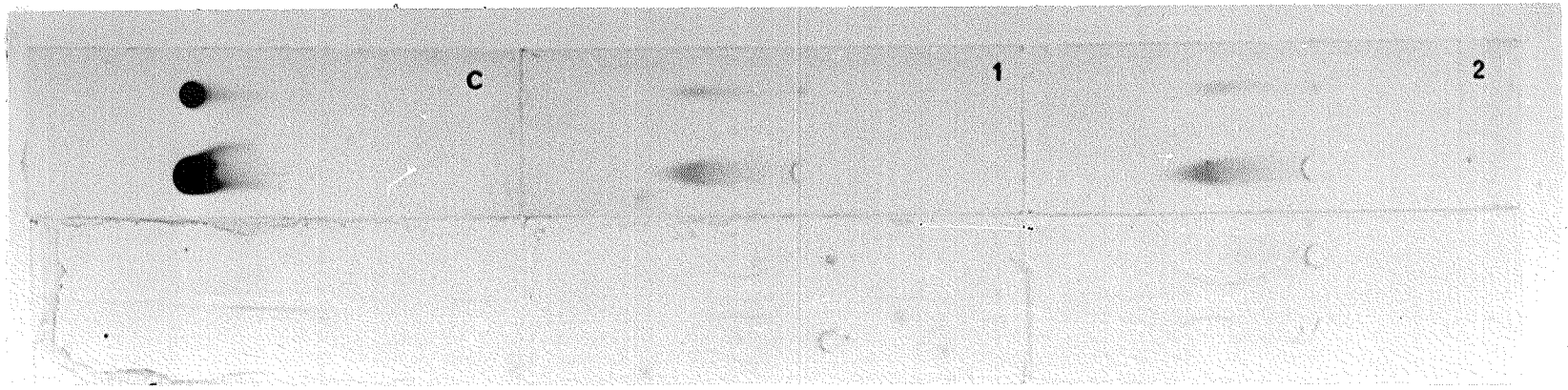
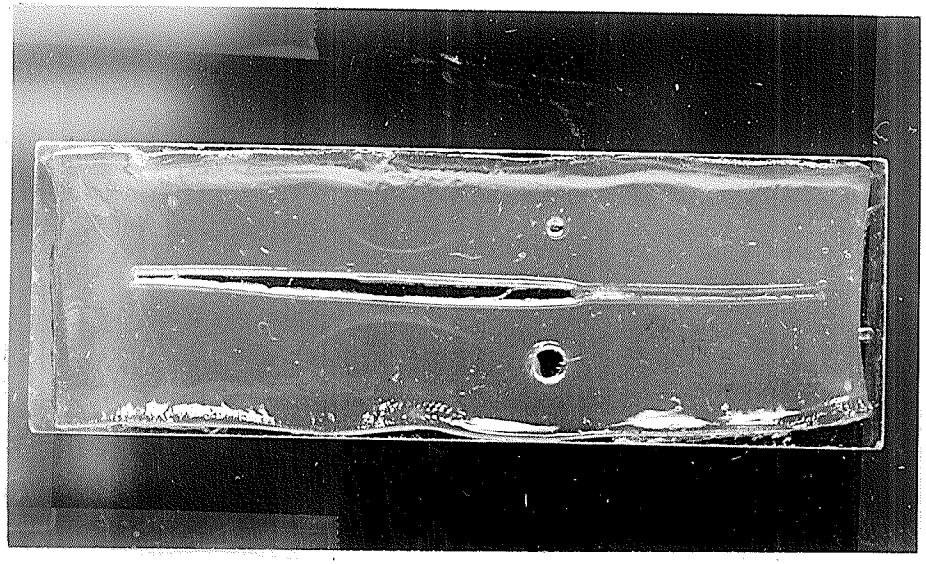


Fig. 6a



6b

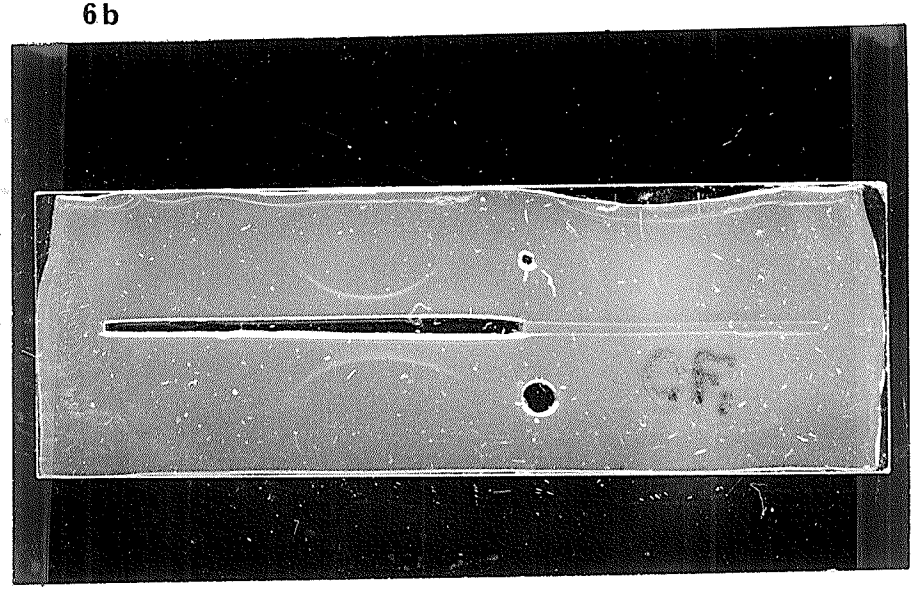


Figure 6. IEA precipitin arcs after cold treatment: a) bean CF_1, b) spinach CF_1, c) stained gels of above compared to electrophoretic patterns of samples run in parallel.
Legend: C = BSA control
1 = bean CF_1
2 = spinach CF_1

The protein stain of gels not reacted with antiserum showed that the BSA control migrated as a spot, but CF_1 was highly concentrated to the anodic side of the well with a diffuse leading edge, suggesting that the CF_1 would not migrate into the gel. No further IEA was attempted with this system.

In an effort to find a dissociating system in which anti-CF_1 - CF_1 would react, a microscale immunodiffusion was performed using CF_1 which had been brought to 0, 2, and 5M urea and 15 per cent acetic acid. Immunodiffusion performed at 25°C for 36 hr gave a line only in the absence of urea. When 3.5 per cent BSA was similarly tested it gave a precipitin line for all the wells, but the 2M and 5M urea samples required 96 hr of incubation for lines to develop. Once again a BSA model system reacted where a similarly treated CF_1 failed to do so.

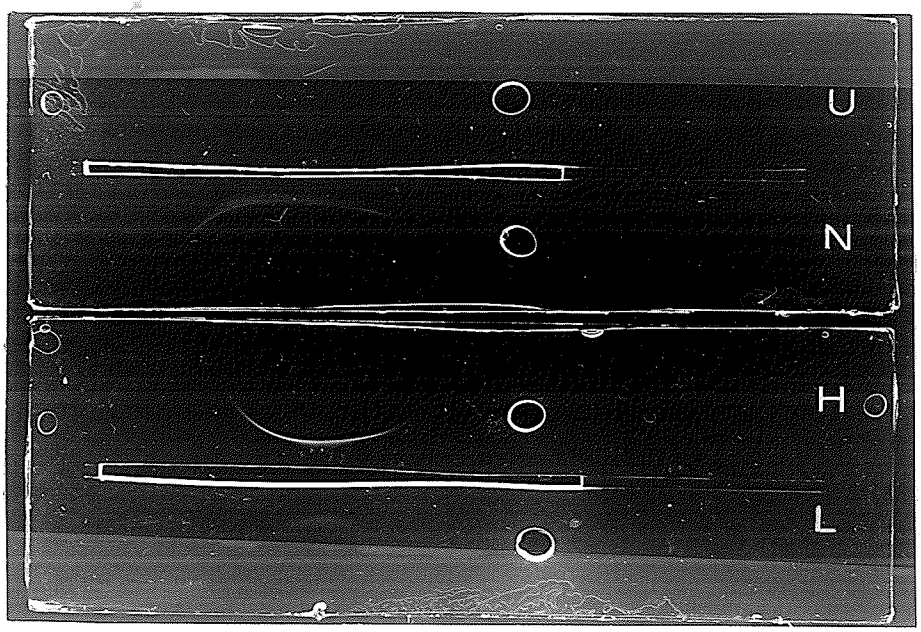
Since none of the above CF_1 systems would react with its antiserum, a less harsh dissociation method was needed. Cold treatment was chosen. A CF_1 concentrate at 2,300 µg/ml was left at 0-4°C for 16 hr. IEA was performed using 1/4 strength antiserum. Figure 6(a) shows that only one precipitin line was visible (with a 23 µg load). A repeat of the experiment using spinach CF_1 at 1,700 µg/ml gave similar results (Figure 6(b)). Staining with Amido Black of both immunodiffused and non-immunodiffused gels run in parallel is shown in Figure 6(c). As can be seen the protein of both bean and spinach CF_1 gave multiple spots

Figure 7. Precipitin arcs of CF_1 after treatment with urea or KCl-cold: a) bean CF_1, b) spinach CF_1.

Legend: U = urea treated (4 ug in well)
N = native (70ug)
H = KCl-cold treated high protein concentration (27 ug)
L = KCl-cold treated low protein concentration (4 ug)

See text for details.

7a



7b

but only one precipitin arc. It would seem from this that the antiserum was either too dilute or that the titer against subunits was too low to cause additional precipitin lines to form.

The method that proved successful for IEA of CF_1 was taken from Lien *et al.* [32]. The differences from above mentioned methods are: (i) full strength antiserum was used; (ii) incubation was performed at 20°C (vs. 25°C); and (iii) a different dissociating method was used.

Lien *et al.*, using IEA of either urea or KCl dissociated spinach CF_1 showed "... the existence of at least two antigenically distinct subunits" [32], and three other products with non-identical electrophoretic mobilities. Two dissociating systems were tested on bean and spinach CF_1 in this work. They were: (i) 3M urea in 5 mM TES-NaOH pH 7.2 at room temperature for 1 hr, and (ii) 0.3M KCl in 20 mM Tris-maleate (pH 6.4) at 0-4°C for 50 minutes. All loads on gels were 4 µg/well excepting a 27 µg load used in the KCl - cold denaturation, and the non-denatured control which was 70 µg.

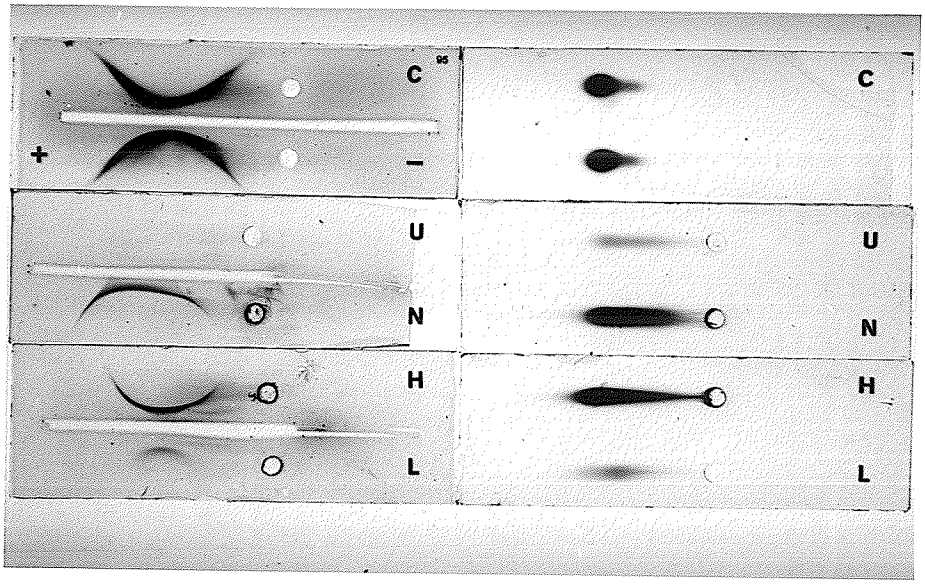
Figure 7(a) shows precipitin arcs obtained with bean CF_1. The urea treatment gave no line (U), and the KCl - cold treatment gave only a smudge at low concentration (L). Both native (N) and KCl - cold treated CF_1 at high concentration (H) showed two arcs having identical immunological properties but different electrophoretic mobilities.

Figure 7(b) shows results for the same experiment as above but using spinach CF_1. The two main arcs are especially distinct (N) and one shows a spur. This spur could be due to a species with identical electrophoretic mobility as the main arc, but with an extra antigenic determinant (if we call the arc farthest from the trough the spur). Formation of subunit(s) in the native sample may have been due to the fact that the protein was stored for at least 24 hr before use. The greater intensity of the native CF_1 arcs is likely because of the high protein load - about three times higher than the "high" KCl - cold load and about twenty times higher than the other loads.

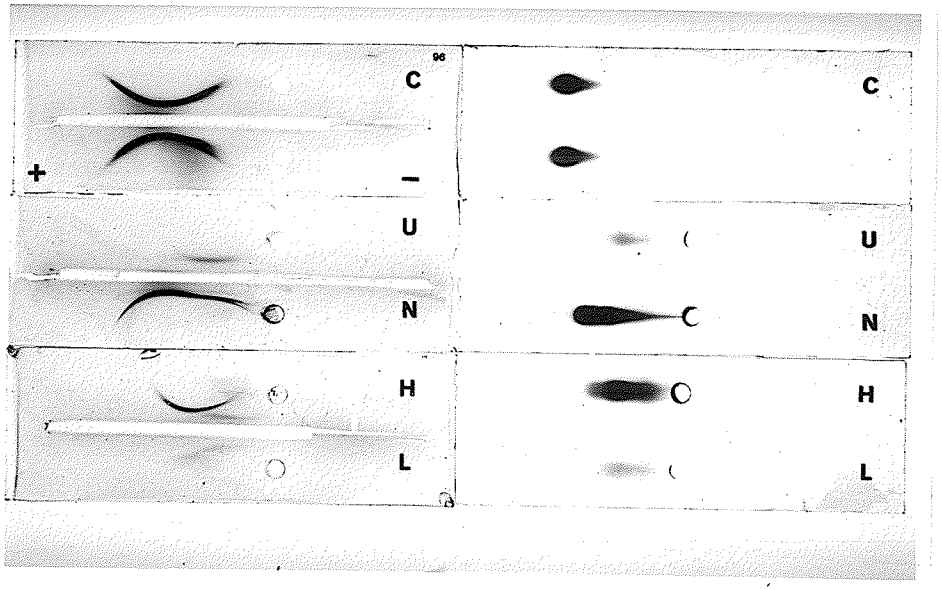
In Figure 8(a) and 8(b) Amido Black staining shows the location of proteins before immunodiffusion. In addition to enhancing the arcs shown in Figure 7, the staining reveals additional precipitin lines. A third arc becomes apparent in the KCl - cold treated samples (H and L) giving therefore at least two antigenically distinct species. No additional arc is revealed in the native sample implying that the newly revealed arc is due to a subunit. It is not possible to say which of the two arcs in the native CF_1 is intact CF_1 and which is due to a subunit. Perhaps in future experiments the IEA slides could be stained for ATP-ase activity. The major arcs of undenatured bean and spinach CF_1 can be superimposed exactly except for the spur of spinach CF_1. It is of interest to recall that anti-spinach CF_1 gave only

Figure 8. Immunoelectrophoretic and electrophoretic patterns of: a) bean, and b) spinach CF_1 under various dissociating conditions.
Legend as in Figure 7.
C = BSA-anti-BSA control
Gels on the left hand side are after electrophoresis and immunodiffusion. Those on the right hand side are after electrophoresis only.

8a



8b



partial identity between bean and spinach CF_1 (Figures 4 and 5). This would imply that the spur is not an artifact, but that there is a difference in the nature of antigenic determinants to be found in spinach and bean CF_1, with bean CF_1 lacking a determinant that spinach has. Because the extra arcs revealed by staining are of approximately equal intensity for both high and low protein concentrations (H and L), it would seem that the reason subunits may not have been detected in earlier gels was the low titer of the antiserum to the subunits relative to the titer for intact CF_1. For each precipitin arc there is a point where additional CF_1 protein will not increase the intensity of the arc as all the antibodies have been bound, but for the arc revealed by Amido Black staining this point is much lower than for those arcs visible without staining. Thus, at low CF_1 concentrations there may be more than enough of a particular subunit to precipitate all of its respective (low titer) antibody, yet at the same concentration of CF_1 the large arcs become less intense because there is insufficient CF_1 protein to react with all of the antibodies present.

Figure 8(b) (H) shows particularly well that at least three distinct protein species exist after KCl - cold treatment as shown by the slides stained after electrophoresis only. It can be tentatively concluded that at least two immunoelectrophoretically distinct protein species were observed in this experiment, and that there may be a

slight immunological difference between bean CF_1 and spinach CF_1 when anti-spinach CF_1 is used.

Effect of protein synthesis inhibitors on ATP-ase formation in greening chloroplasts

Cycloheximide inhibits the cytoplasmic ribosomal system while chloramphenicol inhibits the chloroplastic ribosomal systems. It is known that the ATP-ase activity of EDTA-washed etioplasts is extremely low and increases upon greening [50]. It could be that the enzyme subunits are synthesized in each subcellular location and combined in the chloroplast, or that the enzyme is synthesized in the chloroplast and needs to be stimulated by a cytoplasmic component. Inhibitor studies indicate that both ribosomal systems may be required for CF_1 synthesis [50]. It was hoped that if subunits were synthesized in either system they would elicit an immune response.

Etiolated bean plants were cut near the base and greened in either water, cycloheximide, or chloramphenicol. The primary leaves were extracted with EDTA, and the EDTA wash, water wash, and post-ribosomal supernatant of the sucrose homogenization medium were all analyzed immuno-electrophoretically and enzymically.

It is known that etiolated bean plants have low levels of ATP-ase activity [48]. Therefore, it was expected that the EDTA washes would contain small amounts of ATP-ase and that the formation of subunits would be indicated by

the relative intensity of precipitin arcs.

Table VIII shows the results of these experiments. Precipitin arcs were visible in the case of EDTA washes of H_2O-greened and CHI-greened leaves. The precipitin line for CHI-greened leaves was extremely faint despite the high protein load which is probably indicative of the low level of ATP-ase in etiolated material before greening.

The location of the two precipitin arcs indicated that they were likely the intact ATP-ase. Thus, under the conditions employed in these experiments there does not appear to be any appreciable synthesis of CF_1 subunits in the presence of chloramphenicol or cycloheximide that are precipitable by the spinach antiserum.

Dissociation experiments

In addition to immunoelectrophoresis, several electrophoretic methods were used to look at the number and kinds of subunits of CF_1. Since CF_1 is cold-labile [24] and most stable in the range pH 7 - pH 8, it was decided to test the effects of cold, salts, and extremes of pH on CF_1 structure.

In one experiment CF_1 concentrate in 20 mM Tris Cl pH 8 containing 0.75 mM EDTA was exposed to various pH conditions (pH 3 - pH 11), or NaCl (0.1M and 0.2M), or $NaNO_3$ (0.2M) for 24 hr at either 20°C or 0-4°C. The pH of samples was attained by adding NaOH solution or HCl directly to the sample. Salt concentrations were achieved by addi-

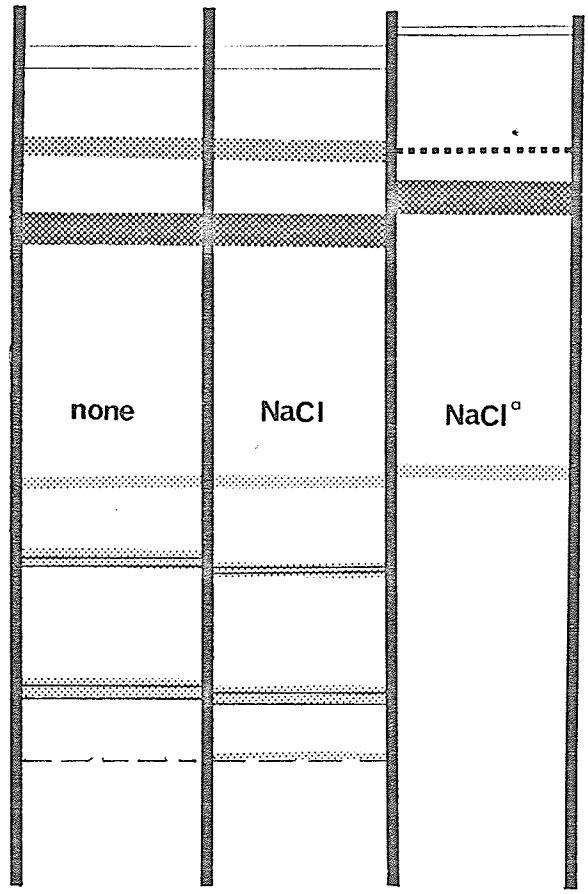
Table VIII The effects of protein synthesis inhibitors on enzymic and immunological ATP-ase activity extracted from greening chloroplasts.
(- = no arc observed; + = arc observed)

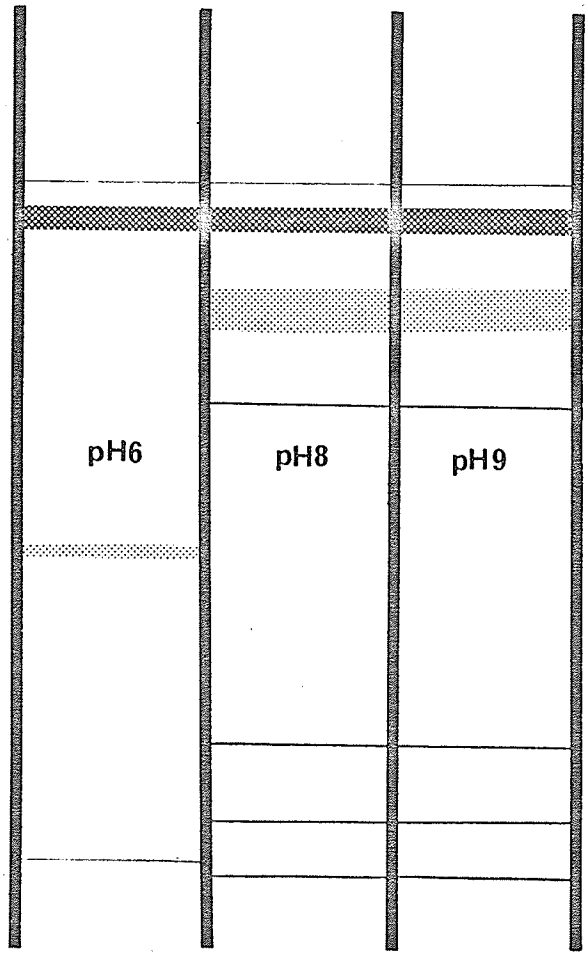
Sample	Greening regime	ATP-ase activity (μmoles Pi released/mg protein/hr)	Immuno-precipitin arc	Protein load of gel (μg)
10 mM NaCl Wash	H_2O	0.88	-	14
	CHI	0.	-	15
	CAP	0.	-	26
EDTA Wash	H_2O	11	+	5.4
	CHI	2.3	+	20
	CAP	0.	-	6.3
Post-ribosomal Super-natants	H_2O	0.31	-	37
	CHI	0.	-	33
	CAP	0.05	-	49

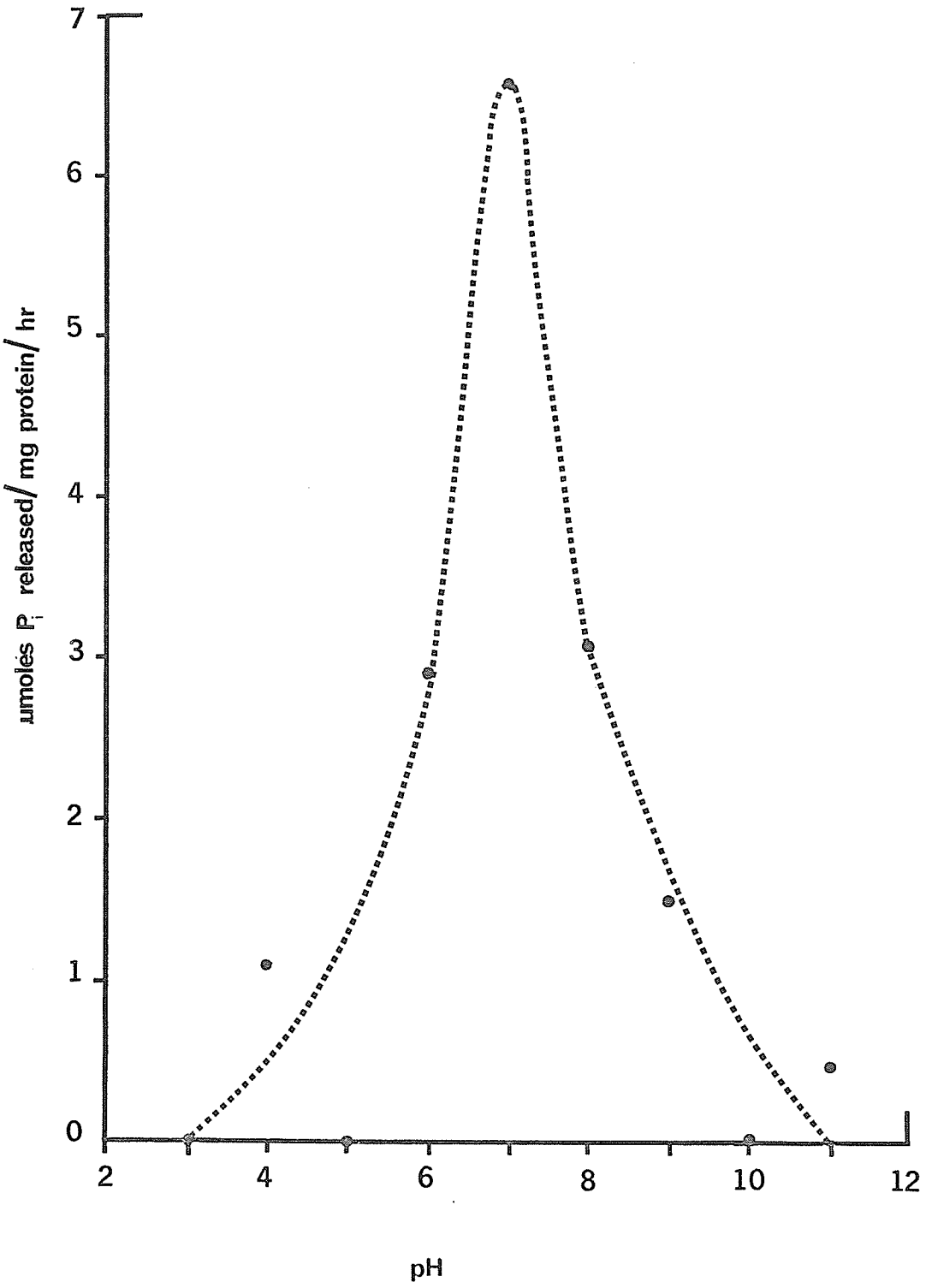
tion of solid salt. The samples were then examined by disc gel electrophoresis, according to Davis [44]. Figure 9 diagramatically illustrates the bands found in some representative gels. The major conclusions drawn from this experiment were: (i) cold was the major factor in subunit formation as the gel samples treated at 20°C failed to give more than two major bands; (ii) basic pH in the cold encouraged CF_1 dissociation; and (iii) acid pH below 5 precipitated the protein at both 0°C and 20°C. Gels of samples exposed to cold only and stained for ATP-ase activity, showed a single ATP-ase band located in the same position as gels run with a non-treated control. The effect of $NaNO_3$ was the same as that of NaCl and cold treatment, except that the upper major band was denser in the $NaNO_3$ treated samples. This may simply be a salt effect as samples were not dialysed before electrophoresis. The fact that the location of the bands of gels exposed to extremes of pH at 0-4°C differ from those exposed to NaCl at 0-4°C is more than likely due to a pH effect as samples were applied to the gels without adjusting their pH.

In addition to the electrophoretic data, the aliquots treated in the cold to various pH values were analysed for ATP-ase activity. The data in Figure 10 show that pH 7.0 is the optimum pH for stability of the enzyme when stored at 0-4°C. Values at either side of neutrality lead to a rapid loss of activity. Electro-

Figure 9. Diagram of representative polyacrylamide gels of CF_1 extract exposed to various dissociating conditions. All treatments at 0-4 degrees except $NaCl^a$ which is a sample at 20 degrees.


none
NaCl
NaCl°


pH6
pH8
pH9


7
6
5
4
3
2
1
0
μmoles P_i released/ mg protein/ hr
2
4
6
8
10
12
pH

phoretically, the cold-treated pH 7 sample showed only one band in addition to the two "major bands", whereas samples to the basic side showed increased band intensity of "minor bands" and lower ATP-ase activity. Those to the acid side of pH 7 showed extra bands (pH 6) or precipitation.

Polyacrylamide gel electrophoresis by other methods

To observe the effect of a different buffer system from that of Davis on cold-denatured CF_1 bands, a buffer system used by Poulik [42] for starch gel electrophoresis was adapted to polyacrylamide gels.

The results were much the same as those found by the Davis system, that is, two major bands near the top of the gel (about 1.5 cm from the cathode end of a 6 cm gel) and four minor bands beneath them. The only difference is that the Tris-citrate-borate system of Poulik gave very sharp thin bands, and the two major bands were so close together (less than 1 mm) that they appeared to be a single band until viewed at 25 power by microscope. In comparison, the bands of the Davis disc gels could be as much as 4 mm apart.

In another experiment, a method modified from Takayama *et al.* [43] was used to prepare CF_1 and electrophoretic gels. Both sample and gel contained urea.

Samples electrophoresed on these gels showed six bands, four bands in the upper half of the gel (anode) and

two lighter bands in the lower half. Thus, the same number of species had been found by three different electrophoretic procedures (excluding bands which appeared from time to time in some preparations near the very tops of gels).

Immunodiffusion of protein bands isolated from the four upper bands failed to show precipitin lines. This may have been due to the low protein concentration (about 1 µg/well) or it may have been due to protein damage from the urea, phenol, acetic acid solvent used for these systems.

Amino acid analysis of bean CF_1

Using a Beckman Model 121 amino acid analyzer, an amino acid analysis of CF_1 was made directly from polyacrylamide gels according to Houston [51]. The protein analysed was obtained by excising ATP-ase active bands from three Davis [44] gels whose total load had been 1,000 µl of CF_1 concentrate (2,600 µg protein/ml). Table IX shows the values obtained for 15 residues analysed and compares them to those obtained for spinach CF_1 by Farron [30]. It would appear that bean and spinach CF_1 are very similar in amino acid composition, if not the same. The agreement between values is quite good in view of the small sample analysed and the variation of published values [52]. Glycine determination was not accurate due to the presence of glycine in the gels from the Tris-glycine buffer. The use of sarcosine

Table IX Amino acid composition of bean CF_1 compared to a reported value for spinach CF_1 [30].

Amino acid	Spinach CF_1 Number of residues per cysteic acid	Bean CF_1 Alanine = 23
lysine	11	15
histidine	2	2
arginine	16	11
cysteic acid	1	- a
aspartic acid	19	22
threonine	18	16
serine	15	17
glutamic acid	35	31
proline	10	9
glycine	21	- a
alanine	23	23
valine	18	15
methionine	7	4
isoleucine	17	14
leucine	24	26
tyrosine	7	7
phenylalanine	7	7

a = not determined

should eliminate this problem.

Determination of molecular weight of some CF_1 subunits by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Weber and Osborn [46] and proteins prepared according to Tzagaloff [45]. Three different protein samples were analysed; they were: (i) spinach CF_1 concentrate; (ii) bean CF_1 concentrate; and (iii) electrophoretically purified bean CF_1.

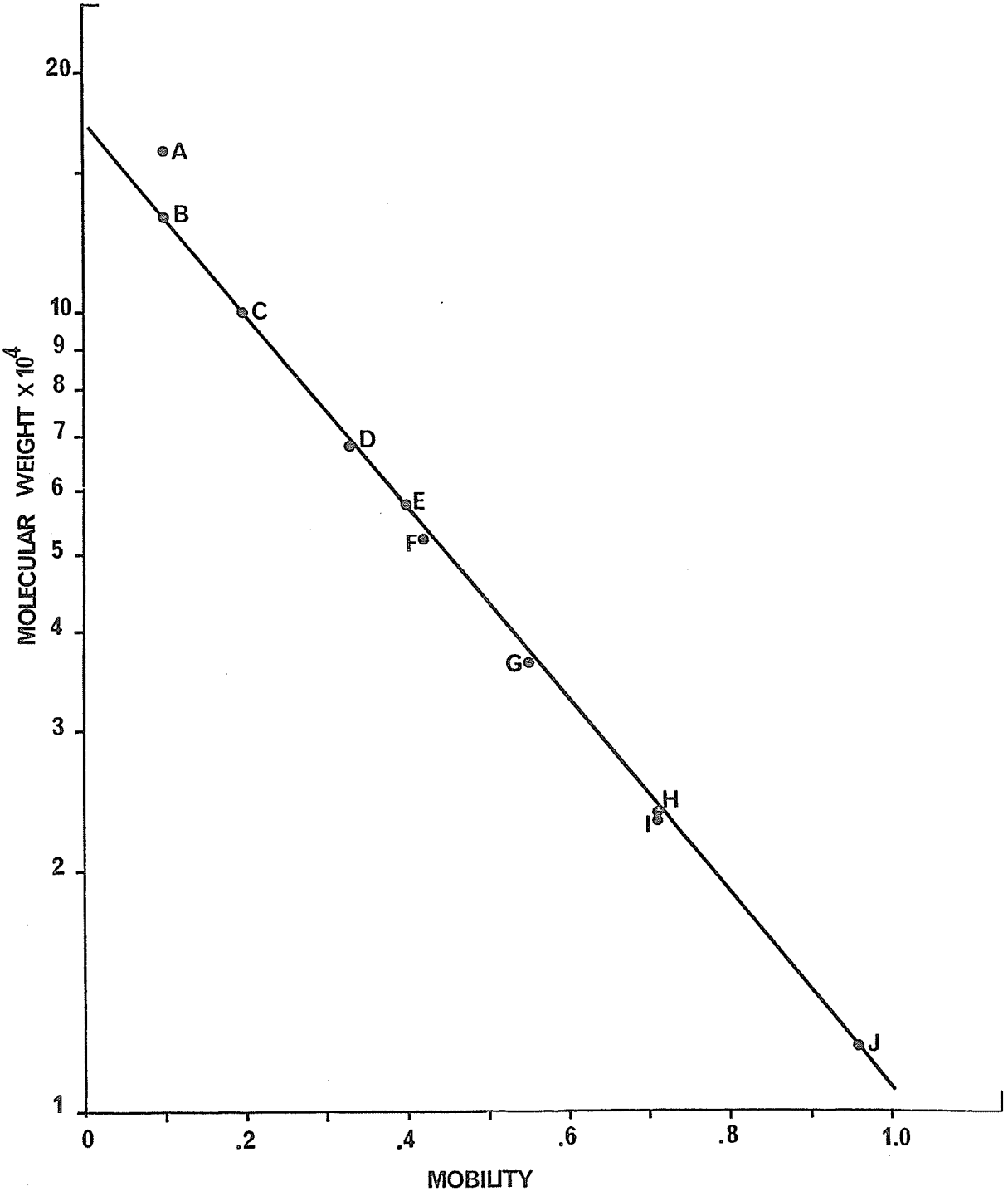
Two methods were employed to determine the molecular weight of the polypeptides obtained by SDS denaturation of CF_1. In one, the unknown and standards were run on different gels in parallel; in the other, the standard and unknown were run in the same gel. The external standard method was preferred since most standards were not sufficiently pure and when combined with the unknown led to superimposition of bands, thus making location and identification of bands difficult. Cytochrome c and BSA were generally used as internal standards. The average values obtained by either method were not significantly different, and the values shown on Table X are an average of values obtained by both methods. Several experiments were performed and in each case a curve of logarithm of the molecular weight of standards versus relative mobility was prepared as shown in Figure 11. Values of the unknown proteins were interpolated from such graphs.

Dunker and Rueckert [53] have shown that an inflec-

Table X Apparent molecular weight of CF_1 subunits.

This table lists the average molecular weights and the standard error of the mean of polypeptides found in SDS-gels of protein extracts prepared as described in the text. Those marked by an asterisk were found with electrophoretically pure CF_1.

Molecular weight x 1,000	Standard Error of the mean x 1,000	Number of values
135*	9	13
124*	4	13
114*	4	12
63*	2	14
59*	2	16
42*	6	16
34	2	10
26	1	10
22	1	5
14	1	6
11.3	0.8	4


MOLECULAR WEIGHT x 10^4
20
10
9
8
7
6
5
4
3
2
1
A
B
C
D
E
F
G
H
I
J
0
.2
.4
.6
.8
1.0
MOBILITY

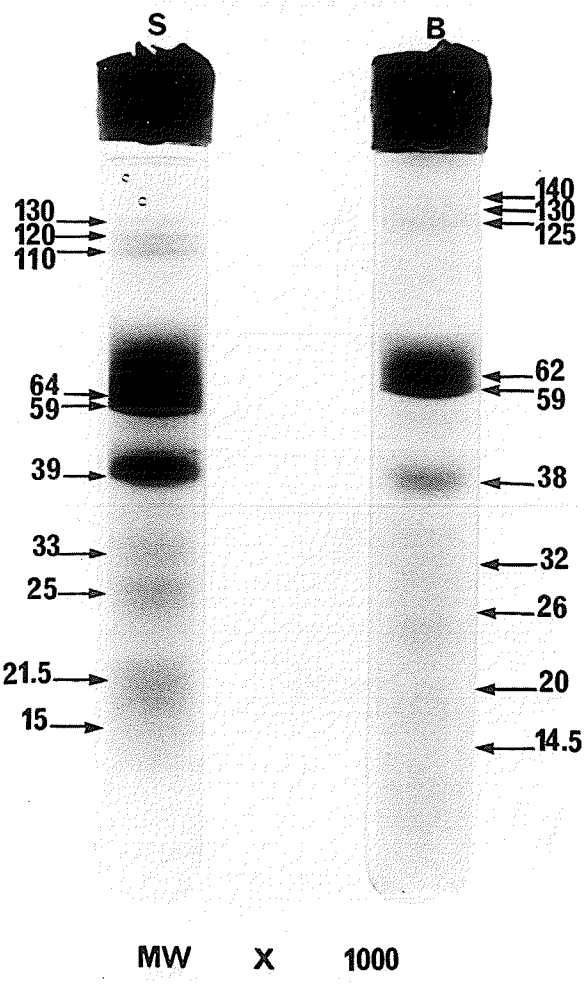
were run. The most intense bands were in the 59,000 - 63,000 molecular weight range. This doublet was not visible at high protein loads due to the heavy tailing of the lighter molecular weight band. The intensity of these bands would suggest that they may be the basic subunit(s) of CF_1.

Judging from the data of Table X it would appear that the ATP-ase molecule may consist of an even lower molecular weight subunit - a basic unit of 14,000 - 16,000 molecular weight (assuming of course, that the lighter weight subunits are not contaminants).

It is of value to know that Farron [30] has calculated a minimum molecular weight of 28,000 based on amino acid analysis and obtained protein with an apparent molecular weight of 62,000 based on the sedimentation equilibrium value in 5M guanadine·HCl pH 7.1. Published molecular weights for CF_1 have been: 325,000; 358,000 (Farron[30]); and 350,000 (Howell and Moudrianakis [29]). If the higher values are correct and we assume a possible hexameric structure for CF_1, then the 59,000 molecular weight subunit would give a hexamer of 354,000 molecular weight, and the 63,000 molecular weight subunit a hexamer of 378,000 molecular weight.

Figure 12. Typical separation of polypeptide chains of SDS-treated CF_1 concentrates. Gel "S" is spinach CF_1. Gel "B" is bean CF_1. Gels are from two different experiments. MW= molecular weight. Gel B contains approximately 280 ug protein; gel S 580 ug protein.

12


S
B
130
120
110
64
59
39
33
25
21.5
15
140
130
125
62
59
38
32
26
20
14.5
MW X 1000

SDS GELS

GENERAL DISCUSSION

Coupling factor 1 has been found in several higher plants such as spinach [25, 26], bean [54], maize [55], pea [50]; in algae such as *Anabaena variabilis* [56]; and in photosynthetic bacteria such as *Rhodopseudomonas capsulata* [57]. The presence of a coupling factor of identical function and with similar properties in a variety of photosynthetic organisms suggests that the coupling factor may have a universal presence.

The procedure of purification and concentration developed in this work has the advantage that concentrates of CF_1 can be rapidly obtained in comparison to published chromatographic methods. The fact that an electrophoretically pure coupling factor gave the same types of bands on sodium dodecyl sulphate gels as the CF_1 concentrate from which it was derived (for molecular weights above 40,000) suggests that the purity of the CF_1 concentrate is quite high. The low ATP-ase activity of the coupling factor is difficult to explain. A paper by Ranalletti *et al.* [54] in which the unit yields of bean ATP-ase were the same as, or lower than, those obtained during the course of this work suggests that this may be a property of the bean factor. The fact that spinach CF_1 isolated at the same

time as bean CF_1 always had higher unit yield suggests that either there is some unknown preparative factor omitted in this work, or if not, then that there is a naturally low level of ATP-ase activity associated with bean CF_1. The fact that partial identity with spinach CF_1 was observed and that there was no "spur" observed with bean CF_1 after immunoelectrophoresis suggests that there may be some slight structural difference between bean and spinach CF_1. Further immunochemical work was not possible due to the extremely limited supply of antiserum.

The data obtained by electrophoresis and immunochemistry suggests that there must be at least two distinct subunits of CF_1.

Some suggestions for future work on this topic would be to use the method of Cashman and Pitot [58] to characterize the molecular weights of the immunoprecipitate species against appropriate controls using SDS polyacrylamide gels.

Another way SDS-gel electrophoresis might prove useful would be for isolating the various bands. The SDS can be removed (e.g. by the method of Weber and Kuter [59]) and antiserum to each species prepared for use in further experiments. These same bands could also be analysed for amino acid composition. By using the method of Houston [51] it should be possible to characterize the amino acid compositions of the individual bands directly from polyacryl-

amide gels with little difficulty. This in turn might lead to a better understanding of the subunit structure of CF_1 by providing information such as minimum molecular weight and frequency of important amino acid residues in the various protein species. This procedure might provide some very interesting data about a *Chlamydomonas reinhardi* mutant recently reported by Sato *et al.* [34] which is unable to fix CO_2 and has a coupling factor that is fully ATP-ase active without prior stimulation by DTT or heat.

The preparation of antisera might be simplified if vaccines were prepared directly from acrylamide gel slices by the method of Sussman *et al.* [60] or Hartman *et al.* [61].

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