COPPER-PROTEINS IN PHOTOSYNTHESIS

A thesis submitted for the degree of Doctor of Philosophy

by

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ABBREVIATIONS USED

PS1, PS2 : Photosystems 1 and 2 respectively (nomenclature after Duysens et al 1961).

EPR : Electron paramagnetic resonance.

Tris buffer: Tris-(hydroxymethyl)-methylammonium chloride buffer.

$NaPO_4$, KPO_4: Buffer formed from a mixture of sodium or potassium salts, respectively, of orthophosphoric acid.

KPO_4(1:1) : Potassium phosphate buffer formed from an equimolar mixture of K_2HPO_4 and KH_2PO_4.

DEAE- : Diethylaminoethyl-.

DCMU : 3-(3', 4'-dichlorophenyl)-1, 1-dimethylurea.

NADP : Nicotinamide adenine dinucleotide phosphate.

DCPIP : Dichlorophenol-indophenol.

% digitonin: %(w/v).

mM : millimolar

nm : nanometer, equal to a millimicron.

TEMED : N,N,N',N'-tetramethylethylenediamine.

DHT : Diazonium-1-H-tetrazole.

MV : Methyl viologen.

PMS : N-methylphenazonium methosulphate.

W/m^2 : light intensity in watts per square meter.

GENERAL INTRODUCTION

The discovery of plastocyanin in 1960 confirmed the long-held belief that copper was an essential component of the electron transfer sequence in photosynthesis (Katoh 1960). Much work has appeared in the literature on the role of plastocyanin and this has been reviewed in recent articles (Vernon and Avron 1965; Hind and Olson 1968; Levine 1969).

Plastocyanin appears to act as the immediate donor of electrons to the photochemically active chlorophyll species, P700, at the reaction centre of photosystem 1 (PS1). This species has been given the notation P700, because its reversible oxidation-reduction is accompanied by an absorption change with its maximum at 700 nm (Kok 1961). The scheme of photosynthetic electron transport which is adopted in this thesis is similar to a multitude of such schemes in the literature, and is presented diagramatically in Figure 1. The locations of the various components are known with varying degrees of certainty. In the region between the two photosystems PS2 and PS1 there is still doubt about the correct sequence of the electron carriers, and also the possibility that some components have not yet been identified: such as the substance M of Levine (1969).

Although there is agreement that plastocyanin acts close to P700, there is still dispute concerning its exact relationship with the two experimentally distinguishable electron pathways to P700: the DCMU-sensitive pathway from PS2,

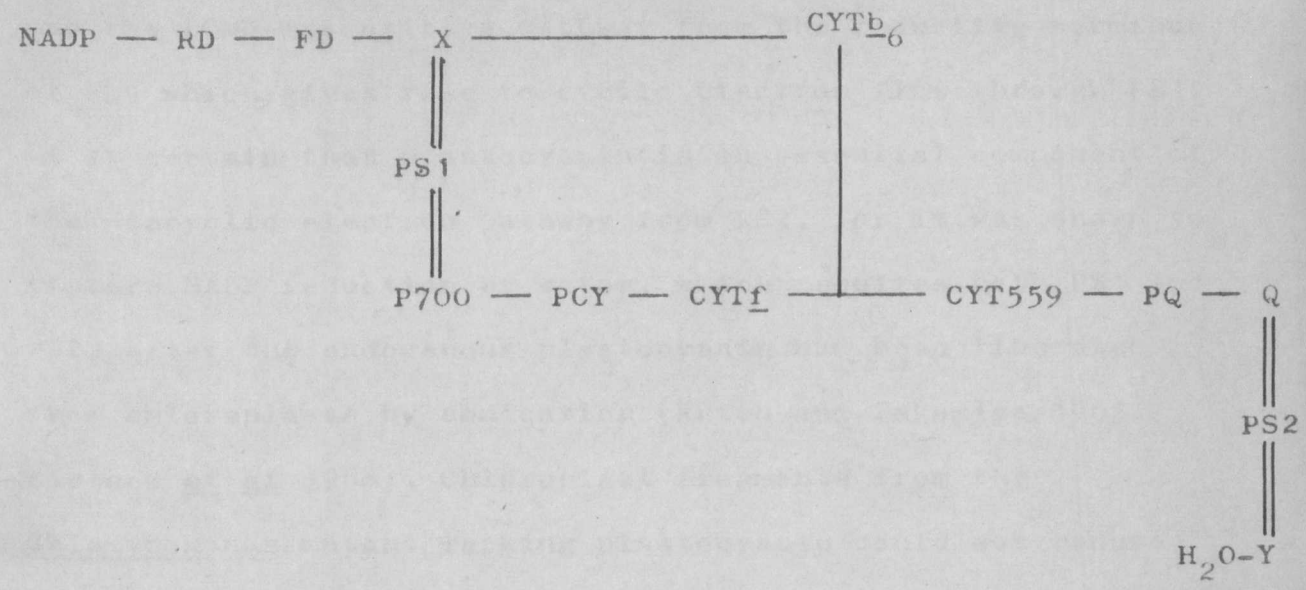


Figure 1. The scheme of electron transport in chloroplasts adopted in the present work. The abbreviations used for the electron transport components are defined here and are employed throughout:

Y : the unknown electron donor to PS2.

Q : the electron acceptor of PS2, which quenches PS2 fluorescence.

PQ : plastoquinone.

CYT559 : cytochrome 559, a b-type cytochrome.

CYTf : cytochrome f.

PCY : plastocyanin.

X : the unknown electron acceptor of PS1.

FD : ferredoxin

RD : ferredoxin-NADP oxidoreductase (E.C. 1.6.99.4).

CYTb_6 : cytochrome b_6, believed to be located on the cyclic pathway of electron transport.

and the DCMU-insensitive pathway from the reductive terminus of PS1 which gives rise to cyclic electron flow through PS1. It is certain that plastocyanin is an essential component of the noncyclic electron pathway from PS2, for it was shown to restore NADP reduction by water, which requires both PS1 and PS2, after the endogenous plastocyanin had been liberated from chloroplasts by sonication (Katoh and Takamiya 1965; Elstner et al 1968). Chloroplast fragments from the Chlamydomonas mutant lacking plastocyanin could not reduce NADP using water as electron donor unless plastocyanin was added (Gorman and Levine 1966 b). Plastocyanin added to washed granae from spinach chloroplasts could be photoreduced by PS2 (Katoh and Takamiya 1961), and plastocyanin added to detergent treated chloroplasts could be photooxidised by PS1 (Kok and Rurainski 1965). The work of Wessels with digitonin from spinach chloroplasts showed that plastocyanin was essential for the reduction of NADP by PS1 with electrons provided by the donor couple ascorbate-DCPIP (Wessels 1966). Although removal of plastocyanin from chloroplasts by sonication did not markedly affect reduction of DCPIP (dichlorophenolindophenol) by PS2 (Katoh and Takamiya 1965), chloroplast fragments from the Chlamydomonas mutant lacking plastocyanin reduced DCPIP at a rate which was ten times lower than that by chloroplasts from the wild type (Gorman and Levine 1965). It is possible that DCPIP can be reduced at two sites in chloroplasts, one site somewhere in the electron transport chain from PS2 before plastocyanin has a low activity, whilst the second site

at plastocyanin has a high activity. When the chloroplasts are disrupted by sonication the activity at the first site is greatly increased and plastocyanin is no longer required for high rates of DCPIP reduction.

The requirement for plastocyanin in cyclic electron flow around PS1 is not quite so clear. It has been demonstrated that plastocyanin is not essential for cyclic photophosphorylation catalysed by N-methylphenazonium methosulphate (PMS) in PS1 digitonin particles (called D-144) from spinach chloroplasts (Arnon et al 1968). When plastocyanin was added back to a chlorophyll-protein particle preparation from Anabaena, from which the plastocyanin had been removed and which was both deficient in PMS catalysed cyclic photophosphorylation and had a reduced ability to photoreduce NADP with ascorbate-DCPIP, phosphorylation was not affected although the electron transport reaction was restored (Lee et al 1969). However, in contrast, it has been shown that the plastocyanin-deficient mutant of Chlamydomonas has a greatly reduced rate of PMS-catalysed cyclic photophosphorylation compared to the wild type (Gorman and Levine 1965). An attempt has been made in the present work to clarify the effect of plastocyanin on cyclic electron flow by following the effect of plastocyanin on the light-induced redox changes of P700.

Hind (1968) demonstrated that adding plastocyanin gave a large enhancement of the rate of dark reduction of P700 in large Triton X-100 particles from spinach chloroplasts. The quantity of plastocyanin added was only a two-fold molar excess

over the P700 content of the chloroplast particles. This plastocyanin-P700 interaction was investigated more closely in the present work, by following the dependence of the dark reduction rate over a range of added plastocyanin concentrations below that equal to the concentration of P700, in chloroplast particles prepared using digitonin.

The work of Crane and co-workers has implicated plastoquinone C in the partial noncyclic sequence from ascorbate--DCPIP to NADP (Henninger and Crane 1967 a). It is known that plastocyanin, but not cytochrome f, is required for NADP reduction by digitonin particles with ascorbate-DCPIP as electron donor (Wessels 1966). Also, in mutants of Chlamydomonas it was found that the lack of plastocyanin resulted in the loss of NADP reduction by ascorbate-DCPIP but the lack of cytochrome 553, a cytochrome f analogue, did not inhibit this activity (Gorman and Levine 1965). Therefore if it is accepted that the site of entry of electrons from the couple ascorbate-DCPIP is at plastocyanin, then plastoquinone C must be part of the partial electron transfer sequence from plastocyanin through P700 to NADP. The redox potential of isolated plastoquinone C has been measured to be +55 mV at pH 7 (Carrier 1967), but it is possible that it could be considerably altered on interaction with other components in the chloroplast lamellae in vivo. Therefore a titration of the dark reduction of P700 by plastocyanin was made in digitonin particles of chloroplasts from which plastoquinone C had been extracted with organic solvents. The interaction of plastocyanin and P700 in the digitonin

particles from unextracted and extracted chloroplasts were compared to observe any difference which might arise from the removal of plastoquinone C. It has been shown that plastoquinones are not solubilised from chloroplasts by the action of digitonin alone (Henninger et al 1967).

To obtain chloroplasts extracted with organic solvents it was found preferable to prepare chloroplasts from spinach by the nonaqueous technique of Thalacker and Behrens (1959), rather than to prepare chloroplasts by the usual aqueous procedures and extract subsequently with organic solvents. This led to a closer investigation of the properties of non-aqueously prepared chloroplasts (referred to as nonaqueous chloroplasts), which is included in the present work in Appendix A. There have been reports in the literature of the absence of photochemical activity in nonaqueous chloroplasts (Stocking 1959; Heber and Tyszkiewicz 1962), however when the conditions of the preparation were controlled so as to prevent the loss of any chlorophyll, it was found that the chloroplasts retained many of the characteristics of aqueously prepared chloroplasts. Both PS1 and PS2 activities were detected and the usual pattern of chloroplast digitonin fragments was obtained on isodensity equilibrium centrifugation on a sucrose density gradient according to Wessels (1965).

After plastocyanin had been discovered there still remained the possibility that another copper-protein was essential for electron transport in photosynthesis, because copper analyses of chloroplasts revealed that the plastocyanin content could only account for 30-50% of the copper actually

determined (Katoh et al 1961). In the present work the concept of a basic unit of photosynthesis will be adopted and copper analyses will be referred to a unit of 400 moles of total chlorophyll a and chlorophyll b. This unit is not related to the photosynthetic unit of Gaffron (Schmid and Gaffron 1968) nor to the quantosome of Park (Park and Pon 1963), but is rather the sum of one PS1 unit and one PS2 unit, each consisting of one reaction centre and its accessory pigment molecules. The size of the basic unit can be judged from analyses of chloroplasts for P700, cytochromes, plastocyanin and ferredoxin, which lead to a value of between 400 and 500 molecules of total chlorophyll (Boardman 1968). A copper content of approximately 3 gram atoms per 400 moles chlorophyll was found by both Katoh et al (1961) and by earlier workers (Whatley et al 1951). In the present work chloroplasts were prepared by techniques, quoted in the literature as giving preparations free from contamination by other organelles, and were analysed for copper content to confirm this number of copper atoms per basic photosynthetic unit.

Apart from this analytical evidence, work on the inhibition of the Hill reaction in chloroplasts by chelating agents regarded as fairly specific for copper has suggested that there exists another copper-protein essential for photosynthetic electron transport. Therefore the site of action of the chelating agents salicylaldoxime (Trebst 1963) and cupferron (Lightbody and Krogmann 1967) were investigated in three types of experiment. Firstly, the effect of the chelating

agents on the Hill reaction with DCPIP, cytochrome c and potassium ferricyanide as Hill oxidants was measured spectrophotometrically. Secondly, their effect on the oxidation-reduction of P700 in chloroplasts on illumination and in the subsequent dark period was measured on a dual wavelength spectrophotometer. Finally, the effect of the chelating agents on the light-induced fluorescence yield increase in chloroplasts was investigated using an apparatus designed and constructed by Drs. Elgersma of the Philips Research Laboratories in Eindhoven. The fluorescence of chloroplasts at room temperature arises mainly from PS2 and the fluorescence yield increase induced by actinic light is believed to reflect the reduction of the quencher Q at the reductive terminus of PS2 (Fork and Amesz 1969). In this way the inhibition of electron transport by the chelating agents was followed at various points along the electron transfer chain from PS2 to PS1.

Pertinent to this work on the possibility of a second copper-protein in electron transport, was the discovery of a red-coloured substance, isolated as a precipitate in thawed solutions of impure plastocyanin kept in the deep freeze for several weeks. This substance could be resuspended to yield an optically-clear solution which possessed the visible spectrum of rubimedin, a protein isolated for the first time several years ago but not reported by any other group of workers (Henninger and Crane 1966 a). This red coloured substance isolated in the course of the present work will be called rubimedin, although the visible spectrum is

the only evidence to equate it with the protein of Henninger and Crane. It was found that rubimedin contained copper and therefore a study of its physical properties was made in order to decide whether it represented a genuine electron transport carrier or merely a degradation product of plastocyanin.

One of the main purposes of the present work was to characterise plastocyanin further, and to understand the manner in which it acts as an electron carrier by an analysis of its molecular properties. Since Katoh and co-workers described the properties of plastocyanin from Chlorella (Katoh 1960) and from spinach (Katoh et al 1962; Katoh and Takamiya 1964) little further work has been reported in the literature to confirm these observations. The properties of the protein from Chlamydomonas were found to agree with the data of Katoh except that the molecular weight estimated by gel filtration was lower (Gorman and Levine 1966 a). Plastocyanin from french beans has been studied by Wells (Wells 1966; Milne and Wells 1968) and his results were in agreement with Katoh for the most part, except that, again, gel filtration led to a molecular weight estimation much lower than the value found by Katoh for spinach plastocyanin. The importance of the molecular weight of plastocyanin is reflected in the recent report that plastocyanin was a two-electron acceptor of electrons in the noncyclic electron pathway from water (Chain et al 1968). Although not stated explicitly, it can be assumed that these workers obtained this result employing the molecular weight and molecular extinction coefficients of plastocyanin determined

by Katoh and co-workers. However redox titrations of plastocyanin from Chlorella (Katoh 1960), from Chlamydomonas (Gorman and Levine 1966 a), and from spinach (Katoh et al 1962) have shown that the change in redox state of plastocyanin was accompanied by the transfer of a single electron only. Therefore the molecular weight and the molecular extinction coefficients of plastocyanin were investigated in order to resolve these conflicting reports.

The properties of the plastocyanin molecule as an electron carrier in photosynthesis are determined to a large extent by the structure of the copper binding site, for this determines both the redox potential of the protein and also the rate of transfer of electrons between the copper and other oxidation-reduction species. It has been shown for Chenopodium plastocyanin that the copper is not located on the surface of the protein molecule, and electron transfer must take place between the ligand atoms of the copper and the amino acid residues on the surface of the molecule, and thence to other redox molecules (Blumberg and Peisach 1966). Other factors, such as the degree of hydrophobic character of the amino acid composition and also the isoelectric point, would also be expected to influence the interaction of plastocyanin with the other electron transport components in the chloroplast. Therefore the above properties of plastocyanin were studied and the results are presented in the first two chapters. In the third and fifth chapters the work carried out on the possibility of a second copper-protein

in photosynthesis is described. In the fourth chapter the interaction of plastocyanin with P700 and cyclic electron flow through PS1 is discussed. Appendix A contains information on the preparation and photochemical activities of nonaqueous chloroplasts; and mathematical analyses and equations are placed in Appendix B.

CHAPTER ONE. PREPARATION AND PROPERTIES OF PLASTOCYANIN

SUMMARY

1. Plastocyanin has been prepared from spinach and parsley and purified to a degree shown to be homogeneous on electrophoresis and in the ultracentrifuge. The most persistent impurity is a cytochrome b of unknown origin, which has an apparent molecular weight by gel filtration close to that of plastocyanin. The lowest value of the absorbance index obtained was about 1.6, however it is proposed that the purity of plastocyanin cannot be judged by this criterion, because of the variation of the absorption at 278 nm produced by conformational changes of the protein molecule.

2. Precipitation of plastocyanin from 0.1 M tris buffer pH 7.5 by saturated ammonium sulphate is not complete unless sodium pyrophosphate is added to raise the pH to 8.

3. The amino acid composition of plastocyanin from parsley and lettuce has been determined and compared with the published analysis for spinach plastocyanin. All three proteins are found to have similar compositions, with only minor differences, and show a high proportion of hydrophilic residues.

When parsley plastocyanin was treated with DHT, only two of the four tyrosyl residues, but both of the two histidyl residues, reacted with the reagent.

4. The isoelectric points of the plastocyanins in 50 mM sodium citrate buffer have been estimated and small differences were found. For parsley plastocyanin the isoelectric

point lay between pH 4.3 and 4.0; for lettuce plastocyanin between pH 4.0 and 3.85; and for spinach plastocyanin between pH 3.85 and 3.65. This order of isoelectric points agreed with both the net charges on the protein molecules, calculated from the amino acid composition, and also the order of electrophoretic mobility at pH 4.8 and pH 8.9.

5. The EPR spectrum of oxidised parsley plastocyanin showed that the cupric ion possessed axial symmetry and that the hyperfine splitting of the low-field signal was small, typical of native blue copper proteins. Calculations of the bonding parameters for the copper-ligand bonds indicated a considerable degree of covalency.

6. The difference spectrum between oxidized and reduced parsley plastocyanin in the ultraviolet was similar to the perturbation spectrum of tyrosyl residues in proteins produced by acidic pH. The oxidised and reduced forms of parsley plastocyanin could be separated by electrophoresis at pH 7.2 and higher pH values, but at pH 4.8 no separation occurred, suggesting that a histidyl residue was free to ionise in the reduced form but not in the oxidised form.

7. The molar absorbance coefficient of oxidised plastocyanin at 597 nm was found to be close to the published value of 4,900 with respect to copper. Spinach plastocyanin was shown to accept one electron only per molecule indicating a copper content of one atom per molecule and the absence of other electron accepting groups on the protein.

INTRODUCTION

Plastocyanin has been shown to be a typical member of a group of proteins which contain copper bound directly to the polypeptide chain without any further prosthetic group. These so-called blue copper proteins are characterised by their visible spectrum in the oxidised form which is unusually intense for copper-containing chromophores, and also by their electron paramagnetic resonance (EPR) spectrum which shows an unusually small hyperfine splitting in the low field signal (Vallee and Williams 1968). The number of copper atoms per molecule varies from one in the case of the bacterial blue proteins to eight in the case of ceruloplasmin (Brill *et al* 1964). Spinach plastocyanin was found to contain two copper atoms per molecule calculated on the basis of a molecular weight of 21,000 (Katoh *et al* 1962), and *Chenopodium* plastocyanin has been quoted as containing two copper atoms per molecule of 11,500 (Blumberg and Peisach 1966).

It is the hypothesis in the present work that plastocyanin has only a single copper atom per molecule and is analogous to the bacterial blue copper proteins, which have been shown to be active in electron transport to the cytochrome oxidase of *Pseudomonas* (Horio *et al* 1961). Plastocyanin is not capable of reacting with molecular oxygen, being different in this respect from the multi-copper proteins ascorbate oxidase, ceruloplasmin and the laccases. It is however rapidly oxidised or reduced by chemical agents (Nakamura and Ogura 1968).

The electrophoresis of plastocyanin on starch gel (Wells 1966) has shown that only two redox states of plastocyanin exist, the oxidised and the reduced forms, no intermediate semi-reduced form being noted. However, redox titration of plastocyanin has shown that it is a one-electron acceptor (Katoh 1960; Katoh et al 1962; Gorman and Levine 1966 a). Therefore if there exist two copper atoms per molecule, then these must accept electrons in separate steps, and a stable intermediate semi-reduced form might be expected to be observed. In discussing the EPR spectrum of plastocyanin (Blumberg and Peisach 1966), it was stressed that the spectrum was very similar to that of the bacterial blue copper proteins, and that although the authors believed plastocyanin to contain two divalent copper atoms in the oxidised form only a single paramagnetic species was seen by EPR.

The mechanism of oxidation-reduction of plastocyanin and the structure of the copper binding site are properties of the molecule which are important for its role in photosynthesis, and which can be investigated by standard protein chemistry techniques. In the oxidised form, the optical spectrum and the EPR spectrum can be used to probe the environment of the copper ion, however interpretation of the results is difficult (Brill et al 1964). The divalent copper ion in Chenopodium plastocyanin is present at a site showing axial symmetry from the EPR spectrum, but possessing some rhombic character from the results of the optical rotatory dispersion measurements (Blumberg and Peisach 1966).

The data on the redox potential of spinach plastocyanin (Katoh et al 1962) has been interpreted as indicating that a destabilisation of the oxidised form of the protein occurs at acid pH values owing to the dissociation of a group tentatively identified as a histidyl residue (Brill et al 1964). A detailed analysis of the binding of copper in spinach plastocyanin (Katoh and Takamiya 1964) showed that the sulphydryl group of cysteine might be implicated in copper binding, for the addition of parachloromercuribenzoate caused a decrease in the blue colour of the native oxidised plastocyanin with a corresponding release of copper from the protein. However, inspection of the time courses of the decrease in blue colour and of the increase in absorbance at 250 nm due to the formation of a sulphydryl-parachloromercuribenzoate bond (Figure 4 of this reference), indicates that the release of copper may be a secondary effect of the reaction of parachloromercuribenzoate with another group, possibly histidyl, rather than a direct displacement of copper from the sulphydryl group.

By the measurement of the proton relaxation rate for oxidised Chenopodium plastocyanin, it was shown that the copper was located inside the protein molecule rather than on the surface, because the cupric ion was not available for interaction with water molecules (Blumberg and Peisach 1966). It therefore follows that water cannot occupy one of the ligand positions of the copper atom.

Copper binding in parsley plastocyanin has been investigated in the present work by comparison of the electrophoretic mobility of the oxidised and reduced forms, and also

by observation of the difference spectrum of the two forms in the ultraviolet region of the spectrum around 280 nm. From these experiments both histidyl and tyrosyl residues could be implicated in copper binding.

Plastocyanin has been isolated from many plant sources, but the only published amino acid analysis is that for spinach plastocyanin (Katoh et al 1962). Recently a note has appeared that the amino acid composition of french bean plastocyanin is under study, but no details were given (Milne and Wells 1968). The amino acid composition of some bacterial blue copper proteins is known (Ambler and Brown 1967), and a comparison of the compositions of plastocyanins with these would be useful in judging the similarity between the electron transport blue copper proteins from chloroplasts and from bacteria. Therefore parsley plastocyanin has been analysed in the present work; the analysis of lettuce plastocyanin has already been undertaken (Searle 1966). The amino acid composition has also been used to calculate a value for the partial specific volume (Cohn and Edsall 1943), a necessary parameter for the evaluation of molecular weight by ultracentrifugation (see Chapter 2).

Electrophoresis of proteins on polyacrylamide gels was introduced by Raymond and Weintraub (1959), who employed a continuous buffer system as in classical electrophoretic techniques. Later, the disc gel electrophoresis technique employing a discontinuous buffer system was shown to give far better resolution, because the proteins could be concentrated to a very narrow starting zone (Davis 1964). However it is also

possible to obtain a narrow starting zone at the surface of the polyacrylamide gel, from the proteins in free solution, by simply using the effect of the much greater viscosity of the gel towards the protein molecules compared to the free buffer solution (Clarke 1964). A further aid to the concentration of the proteins at the gel surface is a sharp rise in conductivity going from the protein sample solution to the gel (Hjerten et al 1965). The use of gels in the form of cylindrical rods instead of flat slabs produces a symmetrical temperature distribution within the gel during electrophoresis which in turn results in sharper protein bands.

By using a continuous buffer system throughout the gel and electrode buffers, and by applying the protein sample on the gel surface in a low conductivity buffer with sucrose added to prevent convective mixing, a very simple technique results which gives the same resolution as the much more complicated discontinuous buffer system of Davis (1964). Since the completion of the work reported here an article has appeared which is in complete agreement with this statement (Brackenridge and Bachelard 1969).

Polyacrylamide gels can be formed by polymerisation of a monomer mixture either chemically with ammonium persulphate as catalyst, or with light using riboflavin as photocatalyst (Davis 1964). It was found that residual persulphate caused the oxidation of reduced cytochrome c during electrophoresis (Flatmark 1964), and, as in the present work it was required to separate the oxidised and reduced forms of plastocyanin, this method of polymerisation was avoided.

The visualisation of protein bands after electrophoresis is usually achieved with amido black. However the dye, coomassie blue, has been shown to have several advantages over amido black, the more important of which are greater sensitivity and ease of removal of excess dye (Fazekas de St.Groth et al 1963; Chrambach et al 1967).

In the present work the technique of polyacrylamide gel electrophoresis has been employed to follow the variation of the separation of oxidised and reduced plastocyanin over a range of pH values. A comparison was also made of the electrophoretic mobility and of the isoelectric points of the plastocyanins from lettuce, parsley and spinach. The isoelectric point of spinach plastocyanin had been shown to be below pH 4.7 (Katoh et al 1962), whilst that of the plastocyanin from the blue-green alga Anabaena was apparently above pH 7.5. (Lightbody and Krogmann 1967).

The preparation of plastocyanin from photosynthetically active material is a relatively straightforward procedure, depending upon an initial disruption of the chloroplast lamellae whereupon the plastocyanin is released into solution. Further purification then follows well-established protein chemistry techniques.

The disruption of the lamellae can be achieved by homogenisation in a hypotonic buffer, followed by fractionation of the filtered homogenate with acetone (San Pietro and Lang 1958), and this procedure is used in several laboratories for the preparation of plastocyanin (Hall, personal communication; West, personal communication; Akulova and Mukhin 1968).

Better yields have been found if a detergent is present during the initial homogenisation (Borchert and Wessels, personal communication). In the initial studies of Katoh and co-workers an acetone powder was first made from which the plastocyanin was extracted by deionised water or hypotonic buffer (Katoh 1960; Katoh *et al* 1961; Katoh *et al* 1962). Recently it has been shown that extraction of chloroplasts with heptane followed by resuspension in a hypotonic buffer also releases plastocyanin (Elster *et al* 1968). Fragmentation of chloroplast lamellae by digitonin has been employed as a method of obtaining chloroplast fragments free from plastocyanin (Wessels 1966), however it would be unpractical as a method of plastocyanin preparation.

Techniques employed to purify plastocyanin have included DEAE-cellulose chromatography and ammonium sulphate fractionation (Katoh 1960), gel filtration on Sephadex (Gorman and Levine 1966 a), and fractionation with calcium phosphate gel (Wells 1966). A combination of all, or some, of these four procedures is sufficient to produce purified plastocyanin. Of the impurities present in the preparation those most readily noted are the proteins with characteristic absorption spectra such as ferredoxin, ferredoxin-NADP reductase, catalase and a cytochrome *b* species, the latter being the most difficult to remove.

The purity of plastocyanin has been judged in all published work by the so-called absorbance index, which is the ratio of the absorbance of the plastocyanin at 278 nm

to the absorbance at the maximum in the visible spectrum in the oxidised form, which is at 597 nm (Katoh et al 1962). The value of 0.8 found for spinach plastocyanin has set the target for which each subsequent investigator has strived. An absorbance index of 1.9 was the lowest obtainable for Chlamydomonas plastocyanin (Gorman and Levine 1966 a), a value of 1.07 was reported for french bean plastocyanin (Wells 1966), and 1.57 was obtained for Anabaena plastocyanin (Lightbody and Krogmann 1967).

Katoh, Shiratori and Takamiya (1962) observed that although an absorbance index of 0.8 could be reached, the protein was then apparently unstable, because on subsequent handling the absorbance index rose significantly. This behaviour was confirmed by Gorman and Levine (1966 a). In the present work it was found that plastocyanin shown to be pure by electrophoresis and ultracentrifugation had an absorbance index value in the range 1.5 - 2.0, indicating that the absorbance index was not a good measure of the purity once a value of 2.0 had been attained. However in the early stages of purification the absorbance index was a valuable guide to the degree of purity.

MATERIALS AND METHODS

Materials. Spinach and parsley were obtained from a local market, washed, and after the roots and main stems were removed, stored in a deep freeze at -20° until use.

DEAE-cellulose chromatography was performed with DE22 from Whatman.

Ammonium sulphate used in salt fractionation was a grade specially low in heavy metals for enzyme work, from British Drug Houses Ltd.

Gel filtration was carried out with Sephadex dextran gels from Pharmacia. Calcium phosphate gel for differential adsorption of proteins was made according to the procedure of Tsuboi and Hudson (1957).

Concentration of protein solutions in dilute aqueous buffer was achieved using a Diaflo Model 50 ultrafiltration cell with UM1 or UM2 membranes.

All buffer solutions were made using analytical grade chemicals and double-glass-distilled water.

Buffers were made according to the compositions given by Gomori (1955) and the pH value and ionic strength of phosphate buffers was found from the data of Green (1933).

The hydrolysis of plastocyanin for aminoacid analysis was performed with M.A.K. grade hydrochloric acid from British Drug Houses, and a Beckman standard aminoacid mixture was used for calibration of the aminoacid analyser. Other chemicals used were all analytical reagent grade.

Preparation of plastocyanin. The standard procedure which is described is that for parsley plastocyanin, and it was also employed to obtain plastocyanin from spinach. Lettuce plastocyanin could not be obtained in this way, but was prepared by the method of Katoh et al (1961) from isolated chloroplasts, as described in a previous report (Searle 1966).

The whole preparation was carried out in a coldroom at a temperature near -5°.

About 5 kg parsley in 600 g lots was homogenised with twice its weight of water in a large Waring blender, the temperature remaining close to 0°. The pH of the homogenate usually fell to pH 6 which did not however denature plastocyanin. After filtering through four layers of muslin to remove large debris the pH was raised to 7.5 with tris buffer and then the green homogenate was fractionated with acetone (San Pietro and Lang 1958). To the homogenate was added half its volume of chilled acetone (-20°) and after standing 30 minutes to solubilise plastocyanin from the chloroplasts, the particulate material was centrifuged off at 1,500 g for 20 minutes at -20°. The clear pale green 33% acetone supernatant was then treated with further chilled acetone to raise the acetone concentration to 75%, whereupon the proteins formed a flocculent pale cream precipitate. After standing for 60 minutes, during which time the protein precipitate settled under gravity, most of the supernatant was siphoned off and the precipitate then recovered by centrifugation at 1,500 g for 20 minutes at -20°.

The precipitate was resuspended in about 200 ml of 10 mM tris buffer pH 7.5 at 0° with the aid of a hand homogeniser and the acetone removed by dialysis overnight at 4°, with magnetic stirring, against 10 litres of the same buffer. The dialysate was centrifuged at 2,000 g for 20 minutes at 4° to remove insoluble material, and the clear, pale, red-brown supernatant was then further treated according to the scheme presented in the Results and Discussion section.

Detection of impurities. The progress of the purification after DEAE-cellulose chromatography was followed spectrophotometrically. Impurities were noted in the visible absorption spectrum of the plastocyanin fraction and by estimation of the absorbance index (Katoh et al 1962). Catalase was easily detected by the intense absorption of its Soret band at 403 nm, which usually appeared as a shoulder in the dilute catalase solutions measured. The cytochrome b impurity was also observed by its Soret band absorption at 412 nm in the oxidised form. The presence of ferredoxin-NADP reductase could be detected by its diaphorase activity (Jagendorf 1963).

The prosthetic group of both catalase and the cytochrome b was shown to be protohaem by formation of the reduced alkaline pyridine haemochromogen (Falk 1964).

The cytochrome b contamination of a purified plastocyanin solution is expressed as % cytochrome, which is evaluated on the assumption that the cytochrome b component has a molar absorbance coefficient at 412 nm of 100,000. If the plastocyanin is estimated assuming a molar extinction coefficient of 10,000 at 278 nm, then % cytochrome = 10 x height of cytochrome Soret band/absorbance at 278 nm. As the cytochrome b has a molecular weight approximately equal to that of plastocyanin this formula expresses the fraction of cytochrome b both by weight and as a molar ratio.

Properties of plastocyanin. Amino acid analysis of parsley plastocyanin was performed on purified protein which had been treated with 1 N hydrochloric acid at room temperature

for 40 minutes, and washed with further 1 N hydrochloric acid to remove all traces of copper ions. The protein was collected by centrifugation and washed first with acetone and finally with ether to give a completely dry, white powder. The powder was dissolved in 6 N hydrochloric acid containing norleucine as an internal hydrolysis standard (Walsh and Brown 1962) and four aliquots measured out into hydrolysis tubes. These were evacuated as described by Moore and Stein (1963) to remove all oxygen, sealed, and then heated at 110° in an oven for 24, 48, 72 and 96 hours respectively. After hydrolysis all hydrochloric acid was removed by repeated evaporation on a rotary evaporator at 40°. The hydrolysate was then dissolved in sodium citrate buffer pH 2.2 and known aliquots analysed on a Beckman 120C automatic amino acid analyser, calibrated for the ninhydrin solution used with a standard amino acid mixture. Calculation of the amino acid elution peaks was by the standard half height x width method, which assumes a Gaussian form. When two peaks were too close to allow an estimation of the width at half height, then the width was calculated by a mathematical analysis of the Gaussian curves as described in Appendix B. The average of the amount of an amino acid at the four hydrolysis times was taken (except for serine and threonine), as it was found that all amino acids were fully released after 24 hours. Threonine was found to decrease linearly with time and serine in a non-linear fashion; both were extrapolated to zero time of hydrolysis (see Downs F. and Pigman W. Int.J.Prot.Chem. 1 (1969) 181.)

Titration of histidyl and tyrosyl residues in parsley plastocyanin by diazo-1-H-tetrazole was carried out as described by Horinishi et al (1964) in 0.67 M sodium bicarbonate buffer pH 8.8 without prior denaturation of the protein. The calculations were made using the extinction coefficients of Sokolovsky and Vallee (1966) and the recommended check for monoazo derivatives of histidine and tyrosine was carried out. Protein concentrations were measured by Kjeldahl digestion.

EPR spectra of plastocyanin were obtained on a Decca X-I spectrometer equipped with a magnetometer to allow field calibration and g-value calculation. The spectra are presented as the first derivative of the microwave absorption spectra. All spectra were measured at -186° in 3 mm internal diameter tubes made from spectrosil, which were free of heavy metal contamination. The copper ligand bonding parameters were calculated according to the equations given by Kivelson and Nieman (1961) for α^2, and Malmström and Vänngård (1960) for a^2 and for $(\frac{4}{7}\alpha'^2+K)$. EPR tubes were cleaned with concentrated nitric acid, washed with distilled water and dried with acetone before use. Freezing and thawing of samples was done as rapidly as possible to prevent denaturation.

Polyacrylamide gel electrophoresis. The compositions of the gel monomer mixtures were based on that originally prescribed by Davis (1964). The buffer concentration was 50 mM, at the several pH values employed, both throughout the gel and the electrode buffers, thus providing a continuous buffer system. The plastocyanin sample in 5 mM KPO_4 (1:1) buffer containing

20% sucrose was applied to the gel surface using a microsyringe, the volume of sample usually being 10 - 20 microlitres. As plastocyanin was anionic at all pH values used, the anode was always connected to the electrode of the lower buffer compartment of the Shandon gel electrophoresis apparatus.

The polymerisation mixture for the gels and the overlayering mixture were made up from three solutions, which were stored at 4° in the dark, but warmed to room temperature before use.

A. Riboflavin : (4 mg/100 ml).

B. Monomer mixture, filtered before use: (30 g acrylamide, 0.5 g bisacrylamide in 100 ml water).

C. 0.4 M buffer containing 0.75 ml TEMED per 100 ml.

The gel mix was composed of 1 part of A, 2 parts of B, 1 part of C and 4 parts of water; and the overlayering mix was composed of 1 part of A, 1 part of C and 6 parts of water. The gels were formed in glass tubes rinsed with Kodak Photoflo 200 as described by Davis (1964), and sealed at the bottom with transparent parafilm. 1 ml gel mix was pipetted into the tubes and 0.1 ml overlayering mix carefully pipetted on the surface without mixing. The use of an overlayering mix containing riboflavin and TEMED ensured the formation of a perfectly flat upper surface to the gel. The gels were then photopolymerised for at least 30 minutes. The time required is naturally dependent on the light intensity, but is also dependent upon the pH of the gel mixture. The necessary polymerisation time increases

with increasing pH for constant riboflavin, monomer and TEMED concentrations. The gels were stored overnight at 4°, and immediately before use the overlayering mixture was removed and the top of the gel washed with water, and also the parafilm removed from the bottom of the tube.

The buffers employed were: sodium acetate pH 4.8, sodium potassium phosphate pH 7.2, tris buffer pH 7.6, and sodium borate pH 8.9. All of these solutions have high buffering capacity and prevent pH changes from occurring during electrophoresis.

Electrophoresis was carried out in the cold room at 4° with apparatus and buffer at 4°. The current through each gel during electrophoresis was kept constant at a value such that the temperature of the outside of the gel rod did not rise above about 10°. After the plastocyanin sample had been applied to the gel surface, the current was kept at the low value of 1 mA per tube until a narrow blue band of plastocyanin had formed at the gel surface and migrated a few millimetres into the gel; the current could then be increased to about 5 mA per tube without the danger of convective disturbance of the protein bands.

Electrophoresis was usually continued until the band of oxidised plastocyanin had moved to about the middle of the gel. The gel was removed from the gel tube by rimming under water with a needle and forcing the gel out by pressure from a rubber teat.

Fixing and staining the gels with Coomassie Blue was performed in one of two ways; either in trichloroacetic acid as described by Chrambach et al (1967), or in methanol--acetic acid-water (5-1-5 v/v/v). The latter method is preferable for rapid staining as Coomassie Blue is more soluble in the organic solvent mixture than in trichloroacetic acid. In the organic solvent the gels are seen to shrink a little, whilst in the acid the gels increase in length. The stained gels can be kept for many months in the dark but in the light the dye fades gradually.

To estimate the isoelectric point of native oxidised plastocyanin, polyacrylamide gels were made in 50 mM sodium citrate buffer pH 4.3 and before use 50 mM sodium citrate buffer of the required pH from 3.65 to 4.30 was placed in the upper and lower electrode compartments and a current of 10 mA per tube passed for at least 45 minutes to equilibrate the gel with the required pH. Then the plastocyanin in 5 mM KPO_4 (1:1) and 20% sucrose was added to the gel surface and the direction of movement of the blue colour of the oxidised plastocyanin noted on passing a low current. With the anode connected to the lower buffer compartment electrode, the plastocyanin formed a narrow blue band at the gel surface at pH values above its isoelectric point, but when the pH of citrate buffer used was below the isoelectric point of the plastocyanin then the narrow blue band formed at the surface between the sample sucrose layer and the upper electrode buffer. In this way the isoelectric point could be judged to lie between two pH values. The iso-

electric point found was that of native oxidised plastocyanin. Below pH 4.3 the blue colour was very unstable and the plastocyanin was completely denatured within 5 - 10 minutes.

RESULTS AND DISCUSSION

Preparation of plastocyanin. The scheme for plastocyanin purification from the dialysed 33 - 75% acetone precipitate, obtained as described in the Materials and Methods section, is presented below:

1. Chromatography of extract of 33 - 75% acetone precipitate on DEAE-cellulose in tris buffer pH 7.5. Impurities with alkaline isoelectric points are removed. Ferredoxin, being tightly bound to the column, is completely absent from the plastocyanin fraction eluted from the column with 0.1 M tris buffer pH 7.5 + 0.1 M NaCl. Some catalase and ferredoxin-NADP reductase is still present.
2. Concentration of plastocyanin-containing effluent by ultrafiltration.
3. Ammonium sulphate fractionation. The plastocyanin is precipitated by ammonium sulphate containing sodium pyrophosphate. All the remaining catalase and some of the remaining reductase is removed.
4. Gel filtration in 10 mM KPO_4 buffer (1:1) on Sephadex G-75 separates plastocyanin completely from remaining reductase and partially from the cytochrome b contaminant.

5. Calcium phosphate gel adsorption. Addition of sodium acetate buffer pH 6 to the plastocyanin fraction allows adsorption onto calcium phosphate gel. Plastocyanin is eluted from the gel with 20 mM KPO_4 (1:1), and a preparation completely free from cytochrome b can be obtained.

DEAE-cellulose chromatography was performed with DE22 ion exchanger, washed as advised by the manufacturers to remove impurities. Fines were removed and the exchanger equilibrated with 10 mM tris buffer pH 7.5. Chromatography was performed in the cold room at 4°, on a gel column 20 cm x 4 cm diameter. After the extract was applied to the column, about 1.5 column volumes of 0.1 M tris buffer pH 7.5 was passed through it, before plastocyanin was eluted with 0.1 M tris buffer pH 7.5 + 0.1 M NaCl. The effluent fractions, which showed a blue colour on addition of one drop of 1 mM ferricyanide solution, were pooled and concentrated by ultrafiltration to about 50 ml volume. In agreement with previous reports, it was found that reduced plastocyanin was eluted from the column after the zone of oxidised plastocyanin. Electrophoresis experiments reported below demonstrated that the reduced form of the protein carried one more negative charge than the oxidised form at neutral pH values.

The calculation of the quantity of solid ammonium sulphate required (W grams) to increase the concentration of ammonium sulphate in a solution of V ml, by N g/100 ml was found from the relationship: $W = NV/100-0.406\ N$. The concentrat-

ion of ammonium sulphate will always be expressed as N grams per 100 ml of final solution, or N % w/v. To the ferricyanide-oxidised plastocyanin fraction at 4° in 0.1 M tris buffer pH 7.5 + 0.1 M NaCl, was added solid ammonium sulphate to give a concentration of 38% w/v, and the brown precipitate obtained on centrifugation at 10,000 *g* for 10 minutes at 4° was discarded. The blue supernatant was treated further with solid ammonium sulphate at 4° to raise the concentration to 58% w/v, and 20 grams solid tetra-sodium pyrophosphate added per 100 ml of the saturated ammonium sulphate solution to raise the pH to about 8. The plastocyanin was precipitated quantitatively, as seen by the lack of blue colour in the supernatant, and collected by centrifugation at 20,000 *g* for 20 minutes at 4°. The ammonium sulphate did not dissolve completely at pH 7.5 when added to obtain a saturated solution, but when the pH was raised by addition of pyrophosphate it then dissolved completely. The presence of 0.1 M tris buffer was sufficient to safeguard against large pH changes when the slightly acid ammonium sulphate, and the very alkaline pyrophosphate, were added to the plastocyanin solution. It was sometimes found that the plastocyanin precipitate floated on centrifugation in saturated ammonium sulphate, and the protein was then collected by filtration through a glasswool pad.

The plastocyanin precipitate was redissolved in the minimum volume of 10 mM KPO_4 (1:1) and after a short-term dialysis at 4° against the same buffer to remove most of the salt, it was applied to a Sephadex G-75 column (55 cm x 1.6 cm diameter), which was eluted at 1° with 10 mM KPO_4 (1:1). The

plastocyanin fraction could be followed by its blue colour on the column and was clearly separated from a faster-eluting yellow zone of the reductase, of apparent molecular weight 35,000. The plastocyanin contained a considerable amount of the cytochrome b contaminant still (about 1%, see Figure 1-2 (a)), which was eluted from Sephadex G-75 just prior to the plastocyanin (see Figure 1-1 (B)).

Completion of the purification was then achieved by calcium phosphate gel adsorption. The plastocyanin-containing fractions from gel filtration were combined and 0.1 M sodium acetate buffer pH 6 and also a few drops of ferricyanide added. This solution at 0° was then treated with sufficient 45 mM calcium phosphate gel suspension in water to adsorb all the blue colour of the oxidised plastocyanin from solution, as seen when the gel was spun down by centrifugation in a bench centrifuge in the cold room at 4° for a few minutes. The gel was washed twice by resuspension in distilled water, to remove unadsorbed proteins and also acetate buffer. Plastocyanin was then eluted from the gel by resuspension in water and addition of KPO_4 (1:1) to a concentration of 20 mM. In calculations of the final phosphate concentration allowance was made for the packed volume of the gel, which was one-third of the volume of the gel suspension added. The gel, containing most of the impurities was then discarded. To the plastocyanin solution was added fresh gel and also acetate buffer. The plastocyanin was now adsorbed more readily by the gel because of the absence of competition by impurities. The elution was carried out as before but first with 10 mM then with 20 mM

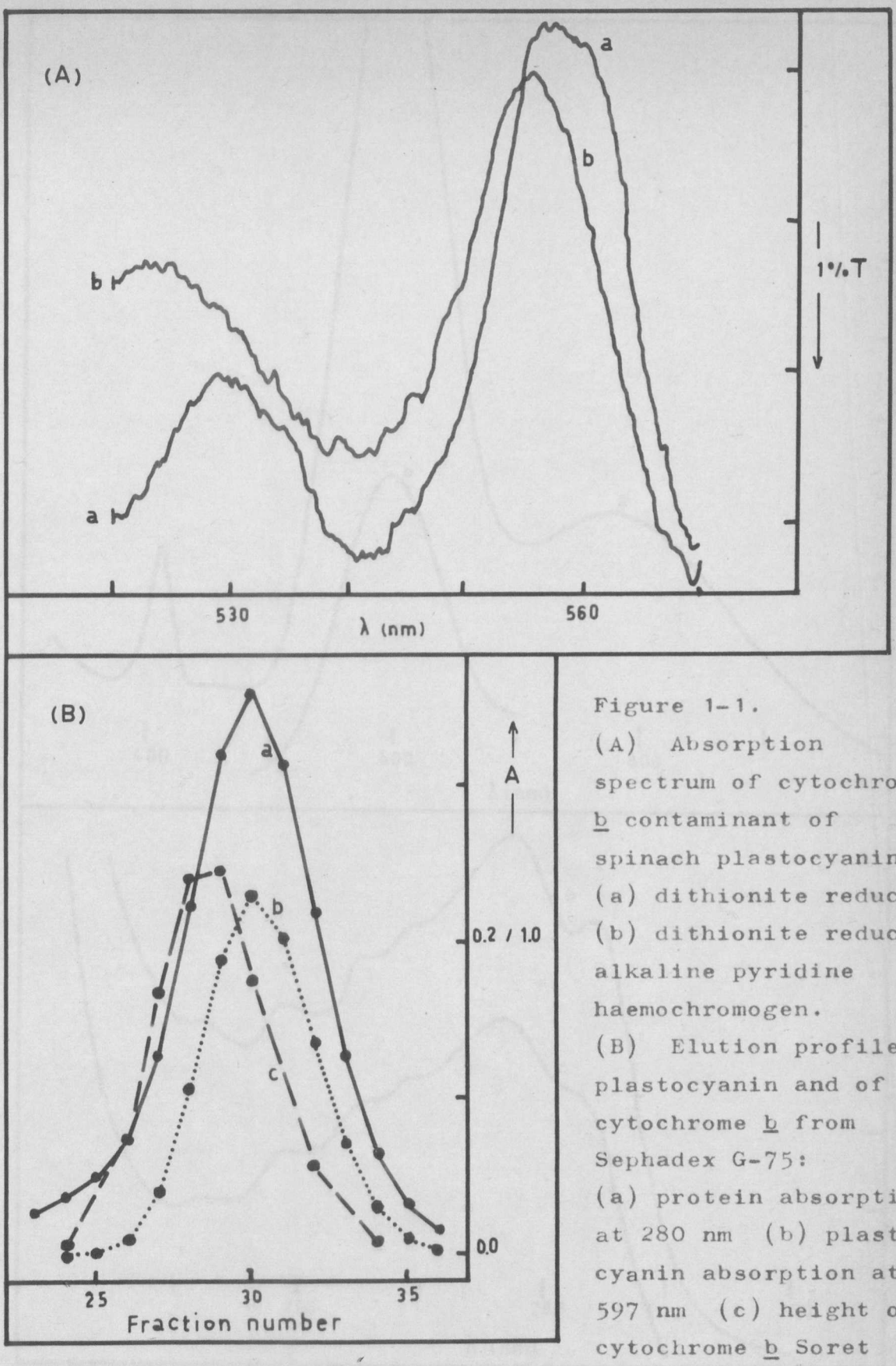


Figure 1-1.
(A) Absorption spectrum of cytochrome b contaminant of spinach plastocyanin: (a) dithionite reduced (b) dithionite reduced alkaline pyridine haemochromogen.
(B) Elution profile of plastocyanin and of cytochrome b from Sephadex G-75: (a) protein absorption at 280 nm (b) plastocyanin absorption at 597 nm (c) height of cytochrome b Soret band at 412 nm. As shown, the absorbance scale for (c) is magnified 5x compared to that for (a) and (b).

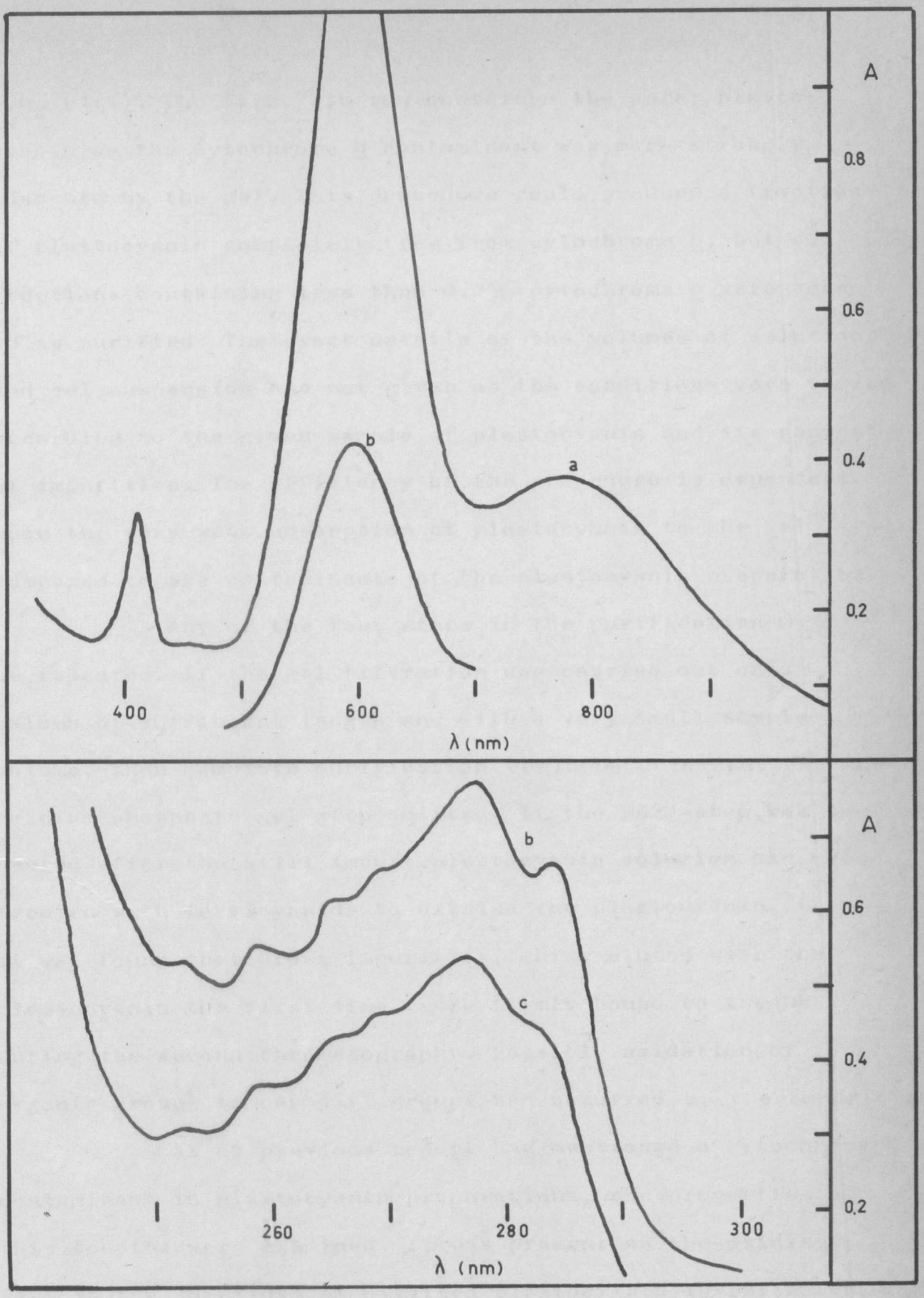


Figure 1-2. (a) Absorption spectrum of oxidised spinach plastocyanin showing some cytochrome b contamination. (b) Diluted 2.76 times to show 597 nm and ultraviolet peaks. (c) Absorption spectrum of a reduced sample of spinach plastocyanin to compare 284 nm shoulder.

KPO_4 (1:1). The first elution contained the purer plastocyanin as the cytochrome b contaminant was more strongly adsorbed by the gel. This procedure could produce a fraction of plastocyanin completely free from cytochrome b, but all fractions containing less than 0.25% cytochrome b were accepted as purified. The exact details of the volumes of solutions and gel suspension are not given as the conditions were varied according to the given sample of plastocyanin and its content of impurities. The efficiency of the procedure is dependent upon the very weak adsorption of plastocyanin to the gel compared to the contaminants of the plastocyanin preparation.

Any of the four steps in the purification may be repeated. If the gel filtration was carried out on a column of sufficient length and with a very small sample volume, then complete purification could be obtained, and the calcium phosphate gel step omitted. If the DE22-step was repeated after the still impure plastocyanin solution had been treated with ferricyanide to oxidise the plastocyanin, then it was found that brown impurities, which eluted with the plastocyanin the first time, were firmly bound to the DE22 during the second chromatography. Possibly oxidation of organic groups to carboxyl groups had occurred on the impurities.

As no previous report had mentioned a cytochrome b contaminant in plastocyanin preparations, the properties of this species were examined. It was present as the oxidised form in the solutions of oxidised plastocyanin investigated, with the Soret band at 412 nm. On addition of ascorbate the cytochrome remained oxidised, for the Soret band was

found to remain at 412 nm. However, when dithionite was then added, reduction occurred, the reduced form showing the Soret band at 422 nm and an assymmetrical α-band at 557 - 558 nm. The prosthetic group of the cytochrome was shown to be protohaem by formation of the dithionite-reduced alkaline pyridine haemochromogen in 1% NaOH and 20% pyridine at room temperature. The symmetrical α-band of the haemochromogen was found to be at 556 nm (Figure 1-1 (A)).

The purity of the plastocyanin preparation was judged by four criteria: (i) an absorbance index below 2 and a cytochrome b content below 0.25%; (ii) the presence of only one protein band on polyacrylamide gel electrophoresis in 50 mM sodium borate buffer pH 8.9 containing 1 mM sodium isoascorbate; (iii) the linearity of the sedimentation equilibrium ultracentrifugation plots (see Chapter 2); and (iv) the absence of arginine on amino acid analysis of the plastocyanin (see below for details).

Properties of plastocyanin. The amino acid analysis of spinach plastocyanin has been published (Katoh et al 1962), however it was based on only one hydrolysis for 30 hours, no correction being made for loss of serine and threonine. The data are presented here, with a 10% correction added for serine, and calculated for a molecular weight of 10,500. The amino acid composition of lettuce plastocyanin was investigated previously (Searle 1966), but subsequently an error in the calibration of the amino acid analyser was detected and the results have been recalculated, a recovery of 88.6% after hydrolysis replacing the value of 117% obtained previously.

The recalculated data are presented in Table 1-1. The amino acid composition of parsley plastocyanin, showing an absorbance index of 1.97 and no detectable cytochrome b contamination, was determined as described in the Materials and Methods section and the results are presented in Table 1-2. No tryptophan or arginine was found, and the cysteine content was not measured.

Native parsley plastocyanin was also examined for histidine and tyrosine content by titration with DHT, at pH 8.8 and room temperature, as described by Horinishi et al (1964). A solution of oxidised parsley plastocyanin having a concentration of 1.10 mg/ml (as shown by Kjeldahl nitrogen analysis using a factor of 6.10 to convert nitrogen to protein content) when diluted 29.2 times was found to contain 2.00 bisazotyrosyl residues and 2.22 bisazohistidyl residues per molecule of 10,400, from the absorbance values A_{480} = 0.215 and A_{550} = 0.100, measured on the Cary 14 spectrophotometer. The check on the absence of monoazotyrosyl residues was made and a difference of only 2% was seen when the bisazohystidyl content was calculated from the absorbance values at 480 nm and 600 nm (A_{600} = 0.047). A reduced solution of parsley plastocyanin (concentration 0.634 mg/ml) was also examined after dilution 29.2 times: 1.98 bisazotyrosyl residues and 1.96 bisazohistidyl residues were found per molecule. The check for monoazotyrosyl residues was again carried out, and a difference of only 2.5% was found between the bisazohistidyl contents calculated from the absorbances at 550 nm and at 600 nm (A_{480} = 0.112, A_{550} = 0.057 and A_{600} = 0.028). Therefore in neither solution of

TABLE 1-1. AMINO ACID ANALYSIS OF LETTUCE PLASTOCYANIN.

The data are recalculated from the results presented previously (Searle 1966). The first column gives the values of HxW used in the recalculations, as those utilised previously were in error. The quantities of amino acids are given for 1 ml of the lettuce plastocyanin solution in 6 N HCl before hydrolysis. The numbers of residues per mole are calculated assuming 5 residues of lysine in a molecular weight of 10,800.

Amino acid	HxW/micromole	HxW calculated	Micromoles	Residues per mole
Lys	62.3	4.36	0.070	5
His	55.2	1.53	0.028	2
NH_3	48.5	6.49	0.134	9
Asp	45.4	8.57	0.189	12
Thr	45.5	4.01	0.088	6
Ser	47.9	7.40	0.154	10
Glu	46.1	7.36	0.160	10
Pro	10.6	0.74	0.070	4
Gly	45.6	9.42	0.206	13
Ala	46.8	6.47	0.138	9
Val	44.6	10.15	0.228	14
Met SO_2	47.5	1.14	0.024	2
Ile	47.4	1.47	0.031	2
Leu	47.4	4.48	0.095	6
Tyr	48.9	2.43	0.050	3
Phe	49.5	4.50	0.091	6
Cys	47.5	0.71	0.015	1

TABLE 1-2. AMINO ACID ANALYSIS OF PARSLEY PLASTOCYANIN

The experimental procedures are described in the text. The quantities of amino acids in micromoles per ml of the original plastocyanin solution in 6 N HCl is shown for the several times of hydrolysis at 110°. For all amino acids except threonine and serine the average value was used; for threonine and serine the value found by extrapolation to zero time was adopted. The number of amino acid residues per mole were calculated assuming two moles of histidine per mole of protein.

Amino Acid	Time of hydrolysis (hours) 24	48	72	96	Calculated	Residues per mole
Lys	0.177	0.175	0.220	0.163	0.181	7
His	--	0.046	0.043	0.046	0.045	2
NH_3	--	0.326	--	0.304	0.315	13
Asp	0.285	0.285	0.309	0.262	0.285	11
Thr	0.140	0.130	0.137	0.113	0.152	6
Ser	0.122	0.102	0.098	0.081	0.150	6
Glu	0.320	0.320	0.356	0.307	0.323	12
Pro	0.116	0.120	--	0.118	0.118	5
Gly	0.335	0.319	0.353	0.300	0.325	12
Ala	0.257	0.237	0.257	0.229	0.240	9
Val	0.232	0.228	0.259	0.239	0.234	9
Met	--	0.019	--	0.020	0.020	1
Ile	0.065	0.070	0.075	0.072	0.072	3
Leu	0.099	0.098	0.104	0.099	0.100	4
Tyr	0.088	0.086	0.085	0.085	0.086	4
Phe	0.148	0.146	0.157	0.149	0.150	6

TABLE 1-3. COMPARISON OF THE AMINO ACID COMPOSITIONS OF PLASTOCYANINS AND OF PSEUDOMONAS BLUE COPPER-PROTEINS.

The amino acid composition of parsley plastocyanin (P) was determined in the present work; that of lettuce plastocyanin (L) was recalculated from analysis carried out previously (Searle 1966); and that for spinach plastocyanin (S) was recalculated from the results of Katoh *et al* (1962) for a molecular weight of 10,500. The composition of the blue copper-protein from *P.fluorescens* (PF) was described by Ambler and Brown (1967), and that from *P.aeruginosa* (PA) by Coval *et al* (1961). The data are given in residues per mole protein.

Amino acid	P	L	S	PF	PA
Lys	7	5	7	11	12
His	2	2	2	4	4
NH_3	13	9	8	13	12
Arg	0	0	0	1	1
Asp	11	12	12	18	19
Thr	6	6	6	10	11
Ser	6	10	9	10	11
Glu	12	10	12	10	11
Pro	5	4	5	4	5
Gly	12	13	12	11	14
Ala	9	9	7	7	8
Val	9	14	8	10	10
Met	1	2	3	6	6
Ile	3	2	2	4	4
Leu	4	6	6	10	10
Tyr	4	3	2	2	2
Phe	6	6	5	6	1
Trp	0	0	0	1	-
Cys	-	1	1	3	2
TOTAL($-NH_3$)	98	105	99	128	131

parsley plastocyanin did monoazotyrosine interfere with the estimation of the bisazo-derivatives. Although in both solutions, the histidine content was titrated quantitatively, only two out of the four tyrosyl residues (see Table 1-2) were detectable. Therefore two tyrosyl residues must be buried within the protein molecule and were not available for reaction with DHT.

The amino acid compositions of the plastocyanins are compared with those of the blue copper-proteins from Pseudomonas in Table 1-3. The molecular weight of Pseudomonas fluorescens blue protein is 14,100 from the amino acid analysis, compared to 10,000 - 11,000 for the plastocyanins. The proteins are similar in amino acid composition, showing a preponderance of hydrophobic residues; and a genetic relationship between them, although not revealed by this simple comparison, might be seen in amino acid sequence studies. The possession of an excess of hydrophilic residues over hydrophobic residues in the copper-proteins active in electron transport, may well be an important factor for the interaction of these proteins with other redox carriers.

The active centre of plastocyanin was also studied in the present work. In Figure 1-2 it is seen that spinach plastocyanin showed a considerable difference in the structure of the shoulder at 284 nm, in the ultraviolet region, between its oxidised and reduced forms. This suggested that the microenvironment of one of more tyrosyl residues was altered when the redox state of the protein changed. The ultraviolet difference spectrum of parsley plastocyanin in 10 mM KPO_4 pH 7 was

investigated more precisely, by measuring the absorbance difference between the reduced and oxidised forms, on a Cary 14 spectrophotometer with both the A, 0.0-1.0 and the A, 0.0-0.1 slidewires, at room temperature (see Figure 1-3). The absorbance of the two solutions at 278 nm was equalised (A_{278} = 0.776) by dilution of the oxidised form with 10 mM KPO_4 pH7. The difference spectrum could not be ascribed to a greater concentration of the oxidised reference solution, because further considerable dilution did not remove the negative peaks seen at 285 nm and 280 nm. The close similarity of the structure of the difference spectrum at 285 - 280 nm to that seen for the acid-induced difference spectrum of ovomucoid, or the perturbation of tyrosine produced by 20% ethylene-glycol (see Donovan 1969) confirmed that one or more tyrosyl residues changed environment with alteration of the redox state of plastocyanin. It is proposed that a tyrosyl residue is part of the copper binding site, or at least closely linked with it in the three-dimensional structure of the protein molecule.

The EPR spectrum of oxidised parsley plastocyanin is shown in Figure 1-4. The hyperfine splitting in the low field signal is not constant for all of the four peaks, but is 64, 64 and 44 gauss, respectively, for the separation of lines 1 and 2, 2 and 3, 3 and 4 starting from the lowest field. A similar distribution of hyperfine splittings was found for Pseudomonas fluorescens blue copper-protein (Brill et al 1968). The position of $g_{//}$, taken to be at the minimum between lines 2 and 3 was 2,955 gauss ($g_{//}$ = 2.241). The microwave absorption

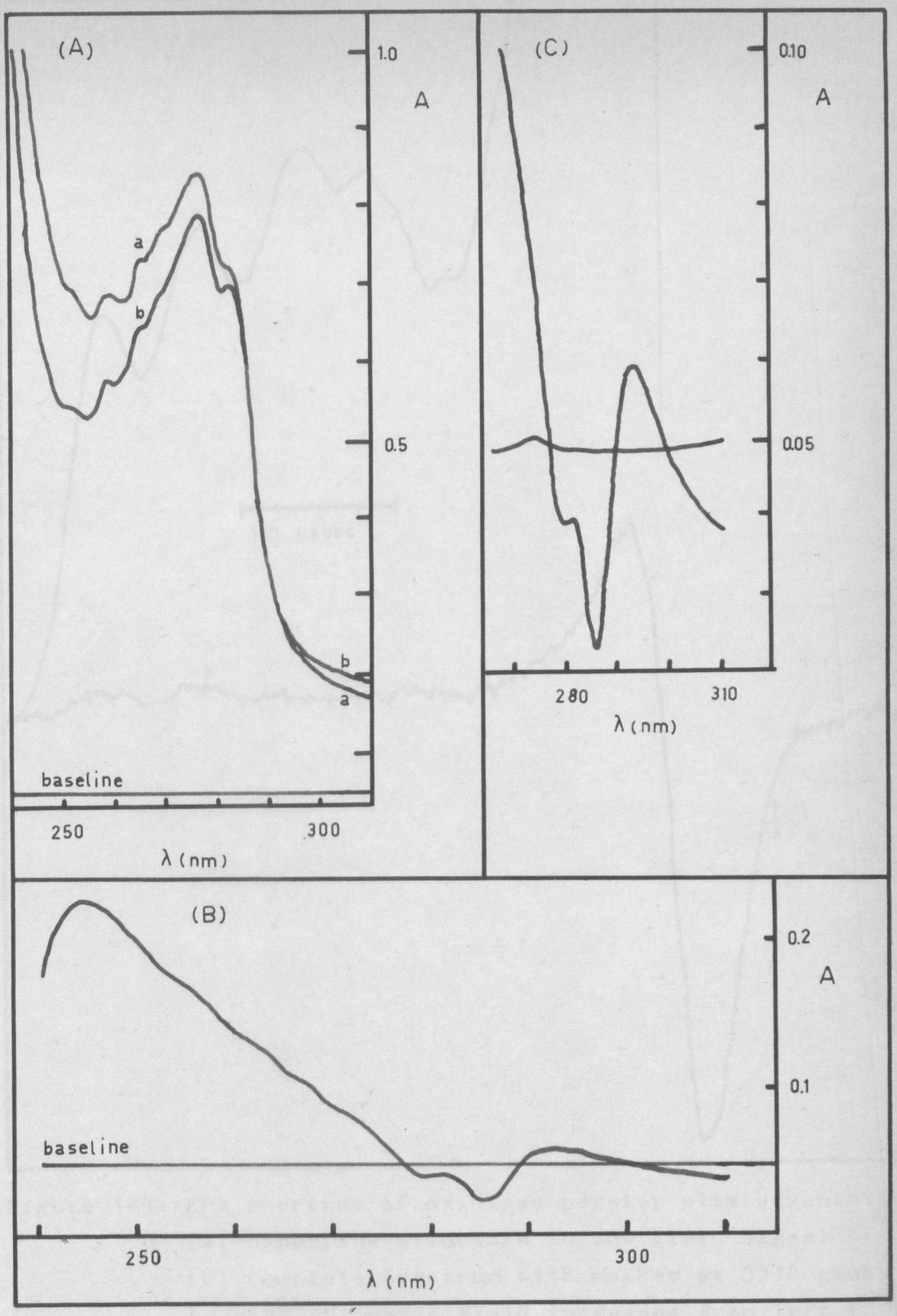


Figure 1-3. (A) Fine structure of UV absorption of (a) reduced and (b) oxidised parsley plastocyanin of different concentrations. (B) UV difference spectrum of reduced minus oxidised parsley plastocyanin solutions having equal absorbance at 278 nm. (C) As for (B) but on more sensitive absorbance scale.

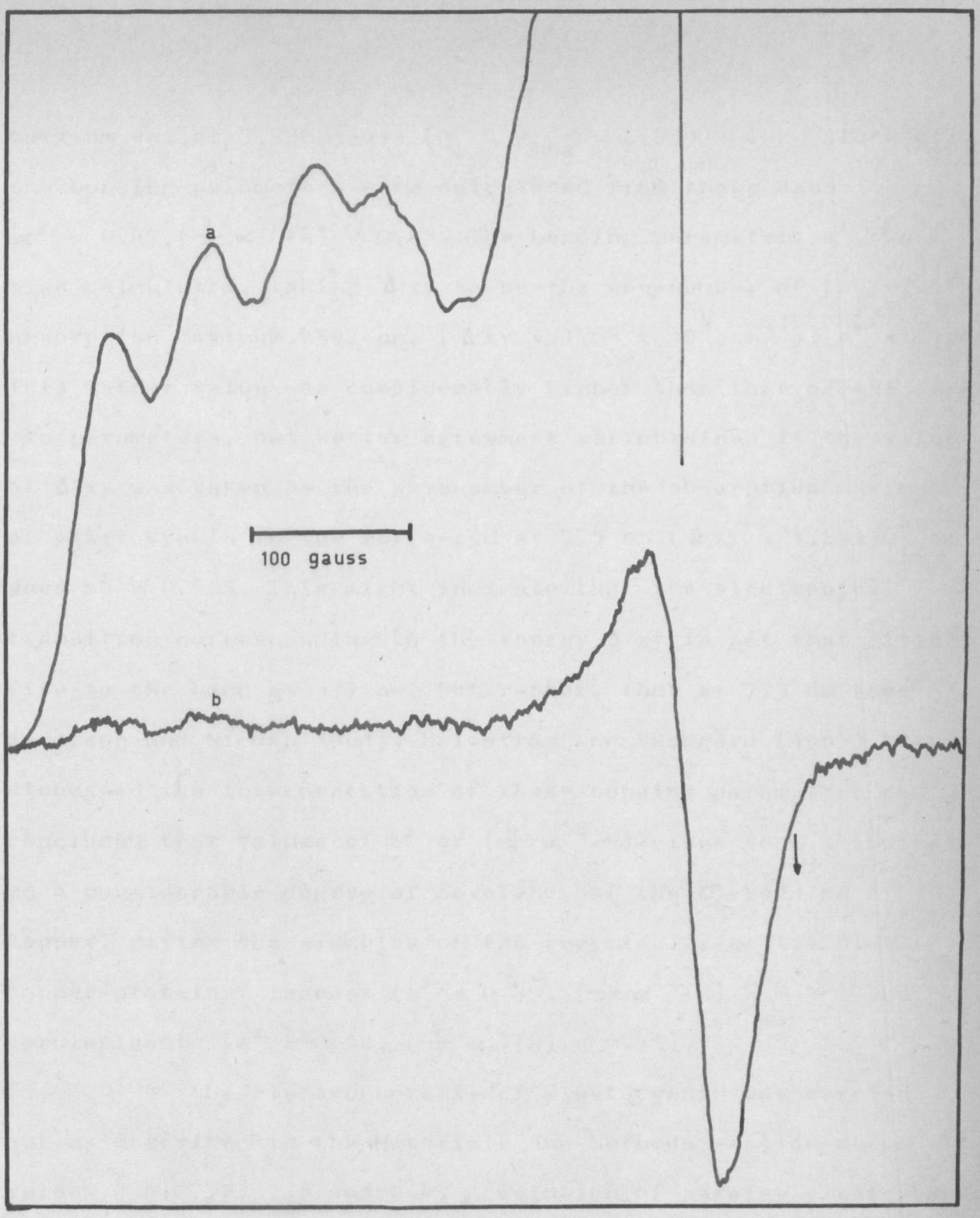


Figure 1-4. EPR spectrum of oxidised parsley plastocyanin:
(a) hyperfine structure in low field signal
(b) complete spectrum with marker at 3312 gauss ($g = 2$). Magnetic field increases from left to right. Temperature -186°. The control spectrum of the spectrosil tube was featureless except for a small cavity signal close to $g = 2$.

maximum was at 3,230 gauss ($g_{\perp} = g_{max} = 2,050$). The values of the bonding parameters were calculated from these data: $\alpha^2 = 0.47, (\frac{4}{7}\alpha'^2 + k) = 0.43$. The bonding parameter, a^2, was also calculated, taking Δ xy to be the wavenumber of the visible absorption maximum, 597 nm, (Δ xy = 1.68×10^4 cm^{-1}): $a^2 = 0.606$. This latter value was considerably higher than that of the other two parameters, but better agreement was obtained if the value of Δ xy was taken as the wavenumber of the absorption maximum of plastocyanin in the infra-red at 775 nm (Δ xy = 1.29×10^4 cm^{-1}), when $a^2 = 0.465$. This might indicate that the electronic transition corresponding to the energy Δ xy is not that giving rise to the band at 597 nm, but rather, that at 775 nm (see Kivelson and Nieman 1961). Malmström and Vänngård (1960) have discussed the interpretation of these bonding parameters and concluded that values of a^2 or $(\frac{4}{7}\alpha'^2 + k)$ close to 0.5 indicated a considerable degree of covalency of the σ-bonding of the copper, giving the examples of the enzymically-active blue copper-proteins, laccase ($a^2 = 0.49$, $(\frac{4}{7}\alpha'^2 + k) = 0.48$) and ceruloplasmin ($a^2 = 0.52$, $(\frac{4}{7}\alpha'^2 + k) = 0.45$).

The electrophoresis of plastocyanin was carried out as described in the Materials and Methods section at pH values 4.8, 7.2, 7.6 and 8.9. A solution of parsley plastocyanin in 5 mM KPO_4 (1:1) containing 20% sucrose, having a concentration of 0.5 mg/ml and being 50% oxidised, was used in most experiments. Samples of 10 microlitres were normally used on each electrophoresis gel rod. At pH 4.8, only one band was visualised on staining with Coomassie Blue, but at pH 7.2, 7.6 and 8.9 two bands could be seen, after staining, of approximate-

ly equal intensity. The separation between the bands was 10 - 15% of the distance of migration for all three pH values. The identity of the two protein bands was demonstrated in an experiment at pH 7.6: 15 micrograms of spinach plastocyanin (again 50% oxidised) were applied to a gel rod, and after electrophoresis for 60 minutes at 5 mA, one blue band could be seen in the gel corresponding to the native oxidised plastocyanin. A small volume of 1 mM ferricyanide solution, in electrophoresis buffer and containing 10% sucrose, was layered on the gel surface and the ferricyanide zone passed down the gel rod by continued electrophoresis at 5 mA. When the ferricyanide reached the blue band of oxidised plastocyanin, still only one blue band was visible. However as the ferricyanide migrated further, a second blue band appeared, ahead of the first, and separated from it by 10% of the migration distance. This second band was native reduced plastocyanin. On staining in Coomassie Blue, the same gel rod showed two protein bands at the positions of the blue bands of plastocyanin. Therefore it may be concluded that reduced plastocyanin migrated faster, at pH 7.2 and above, than did oxidised plastocyanin, but no separation occurred at pH 4.8. The net charge on a molecule of spinach plastocyanin at pH 7.6 can be deduced from its amino acid composition to be -9, and a variation of its charge by one unit should give a mobility change of about 10%. It is proposed therefore that one histidyl residue is free to ionise in reduced plastocyanin but not in oxidised plastocyanin, implicating imidazole in copper binding in the oxidised form, as also

suggested by Brill et al (1964).

Reduced, pH 4.8 : Cu^{+} His^{+} pH 7.2 : Cu^{+} His

Oxidised, pH 4.8 : Cu^{2+}——His pH 7.2 : Cu^{2+}——His

Figure 1-5 shows the titration of a solution of reduced spinach plastocyanin by potassium ferricyanide. The beginning of the oxidation of plastocyanin showed no lag, indicating the absence of a species with a redox potential lower than native plastocyanin. The initial phase of the titration, corresponding to quantitative transfer of electrons from plastocyanin to ferricyanide had a slope of ΔA_{597}/ml ferricyanide = 1.296. Thus the absorbance at 597 nm increased by 4,650 for the transfer of one mole of electrons, for ferricyanide is known to be a one-electron acceptor. When the initial slope was extrapolated to the final value of A_{597} reached (A_{597} = 0.257), this was found to correspond to 1.66×10^{-7} moles of ferricyanide added. By Kjeldahl nitrogen estimation (taking the nitrogen to protein factor as 6.33) the plastocyanin concentration was found to be 0.564 mg/ml, which corresponded to 1.61×10^{-7} moles of plastocyanin in the 3 ml sample, assuming a molecular weight of 10,500. Therefore to completely convert the reduced spinach plastocyanin into oxidised plastocyanin, one mole of electrons was required per mole of plastocyanin, indicating the presence of one gram atom of copper in one molecule of 10,500. The molar absorbance coefficient was therefore estimated to be 4,650.

The molar absorbance coefficients of the plastocyanins were also found by measurement of the absorbance of solutions of known protein concentration. For a parsley plasto-

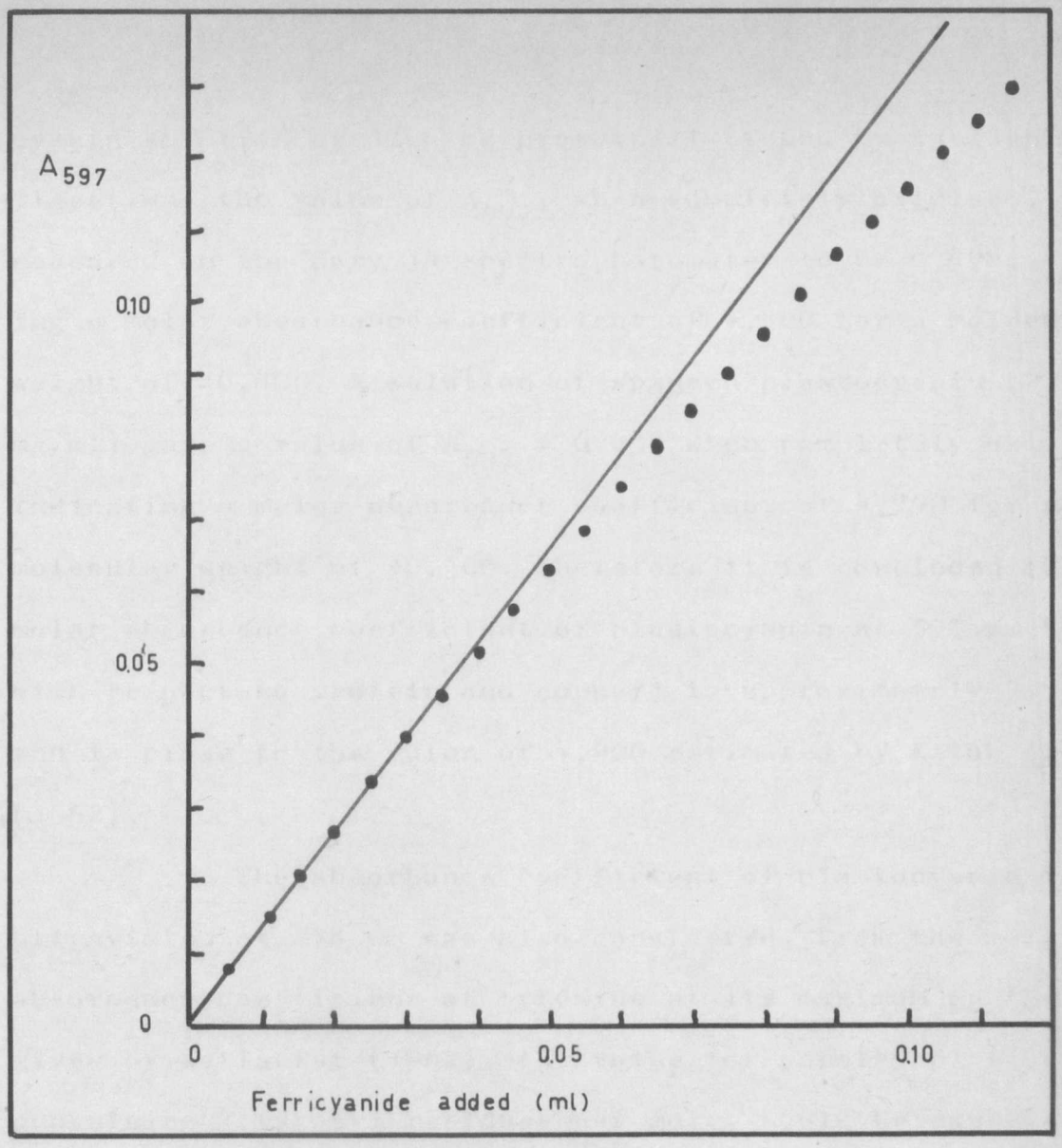


Figure 1-5. Ferricyanide oxidation of reduced spinach plastocyanin. 3 ml plastocyanin solution in 10 mM KPO_4 pH 7 was treated with 5 microlitre aliquots of 0.837 mM ferricyanide and the increase in A_{597} followed on a Cary 14 spectrophotometer with the A,0.0-0.1 slidewire. Absorbance readings were corrected for dilution. The solution was stirred magnetically. Temperature 20°. When excess ferricyanide was added A_{597} rose to 0.257. At the beginning of the titration, the redox potential was sufficiently low for stoichiometric transfer of electrons from plastocyanin to ferricyanide.

cyanin solution of 1.08 mg protein/ml (found by Kjeldahl digestion) the value of A_{597}, when completely oxidised, was measured on the Cary 14 spectrophotometer to be 0.498; yielding a molar absorbance coefficient of 4,800 for a molecular weight of 10,400. A solution of spinach plastocyanin (0.564 mg/ml) gave a value of A_{597} = 0.257 when completely oxidised, indicating a molar absorbance coefficient of 4,790 for a molecular weight of 10,500. Therefore it is concluded that the molar absorbance coefficient of plastocyanin at 597 nm (both with respect to protein and copper) is approximately 4,800, and is close to the value of 4,900 estimated by Katoh et al (1962).

The absorbance coefficient of plastocyanin in the ultraviolet at 278 nm was also considered. From the molar absorbance coefficient of tyrosine at its maximum in the UV given by Wetlaufer (1962), the value for parsley plastocyanin, containing 4 tyrosyl residues per mole, would be expected to be about 4,800. Further contribution to the absorbance in this region might be expected from copper-ligand bonds but not from other amino acid side-chains. Therefore the theoretical minimum of the absorbance index of parsley plastocyanin is about 1.0. As the tyrosine content of spinach and lettuce plastocyanins is lower, the theoretical minimum of the absorbance index would also be lower. However values of the absorbance index were usually found to be from 1.5 to 2.0 for both spinach and parsley plastocyanins. It is proposed that these higher values arose not from contamination by other proteins, nor by loss of

copper from the active centre, but rather from an increase in absorbance at 278 nm produced by a contribution from the tail of the far-UV absorbance band. The amount of absorbance at 278 nm contributed by this band, which arises from peptide bond absorption, might be expected to vary with a change in the conformation of the plastocyanin. The conformation giving rise to the very low value of the absorbance index found by Katoh et al (1962) for spinach plastocyanin (0.8) might be unstable, reverting readily to another conformation possessing a larger value of A_{278}. As the absorbance index of most plastocyanin solutions handled in the present work was close to 2.0, a value of the molar absorbance coefficient of 10,000 was used to estimate protein concentrations approximately at 278 nm.

Finally, the electrophoretic mobilities of the plastocyanins from lettuce, parsley and spinach were compared at both pH 4.8 and pH 8.9. Comparison was made by electrophoresis of mixtures of the proteins on the same gel, as comparison of mobilities between different gel rods was not possible. It was found that at both pH values, lettuce and spinach plastocyanins ran together, ahead of parsley plastocyanin. The isoelectric point of plastocyanin in 50 mM sodium citrate buffer was found for parsley to be between pH 4.3 and 4.0; for lettuce to be between pH 4.0 and 3.85; and for spinach to be between pH 3.85 and 3.65. This order of isoelectric points correlates both with the electrophoretic mobilities and with the net charges of the protein molecules calculated from the amino acid compositions (see Table 1-3). The isoelectric points were only estimated in citrate buffer, and the effect of the binding of buffer ions to the proteins was not investigated.

Concluding remarks. The amino acid analysis of the plastocyanins shows that they are very comparable in composition, and sequence analysis might reveal lengths of the polypeptide chain which are analogous in the three proteins. The lower tyrosine content in spinach and lettuce plastocyanins compared to parsley plastocyanin may have arisen from tyrosine degradation during hydrolysis. In the analysis of spinach plastocyanin, the hydrolysis tube was apparently not evacuated, and oxygen present would destroy part of the tyrosine content (Katoh et al 1962).

The involvement of tyrosine in copper-binding might be examined further by comparison of tyrosine fluorescence in the oxidised and reduced forms (Teale 1960). However initial studies revealed that fluorescence was not detectable from either form with the equipment available. There was no difference in tyrosine or histidine detected, between the oxidised and reduced forms of parsley plastocyanin on DHT titration, but as nitrite was present in the reagent solution this might be expected to reduce the oxidised plastocyanin.

Plastocyanin shows a great similarity to bacterial blue copper-proteins in amino acid composition, EPR spectrum and in containing one copper atom per molecule. The isoelectric point of all three plastocyanins was highly acidic, which might point to its interaction in vivo with a basic protein.

The data of Chain et al (1968) which indicated that plastocyanin was a two-electron acceptor, might be expected to show that it was in fact a one-electron acceptor, if the molar extinction coefficient of 4,800 found in the present work was employed to estimate plastocyanin, in place of the value of 9,800 determined by Katoh et al (1962).

CHAPTER TWO. THE MOLECULAR WEIGHT OF PLASTOCYANIN

SUMMARY

1. The molecular weights of plastocyanins from spinach, parsley and lettuce were estimated from measurements of the internal volume of the gel matrix available to the proteins on gel filtration over a Sephadex G-75 column calibrated with globular proteins of known molecular weight. In the elution buffer used, 0.01 M $NaPO_4$ + 0.5 M NaCl pH 6.1, cytochrome *c* was not adsorbed to the gel and pepsin was stable; however ribonuclease behaved anomalously. Spinach plastocyanin had an apparent molecular weight of 11,800 which remained constant for both the oxidised and reduced forms, and also over the concentration range 0.003 to 0.13 mg/ml measured at the elution profile maximum. Plastocyanin from parsley had an apparent molecular weight of 10,600 in both oxidised and reduced forms and that from lettuce had an apparent molecular weight of 12,200.

2. The fraction of the internal volume of the gel matrix σ available to parsley plastocyanin on gel filtration on Sephadex G-75 was measured in various elution buffers made up from sodium and potassium phosphates and sodium and potassium chlorides. The value of σ was found to vary by up to 20% depending on the ions present, but did not vary with change in ionic strength.

3. The partial specific volumes of the three plastocyanins were measured in 0.1 M KPO_4 (1:1) at 20° and the values found were, for spinach (2.75 mg/ml) 0.702, for

parsley (3.28 mg/ml) 0.705, and for lettuce (7.71 mg/ml) 0.705. However when the partial specific volumes were calculated from the amino acid composition neglecting the copper content, higher values were obtained: spinach 0.724, parsley 0.726 and lettuce 0.729. The lower values found by experiment were used in the calculations of molecular weight by sedimentation equilibrium.

4. Sedimentation equilibrium centrifugation was used to obtain another estimate of plastocyanin molecular weight. The values found in 0.1 M KPO_4 (1:1) at 20° employing Rayleigh interference optics were for spinach 8,600, for parsley 9,800, and for lettuce plastocyanin 10,400. As the value for spinach plastocyanin appeared to be in error, it was re-examined, in a different ultracentrifuge with a different preparation of plastocyanin and with Schlieren optics instead of interference optics. In 0.1 M KPO_4 (1:1) at 20° the value found was 10,250 at a protein concentration of 2.77 mg/ml. However the same preparation at a higher concentration (6.62 mg/ml) gave the unexpectedly low value of 7,650.

5. The molecular weights of the plastocyanins estimated from amino acid analysis were for spinach 10,500, for parsley 10,400, and for lettuce 10,800. Therefore there is agreement between the three methods that the molecular weight of plastocyanin is close to 11,000.

6. The diffusion coefficients and Stokes radii of the plastocyanins were estimated from the gel filtration data, using Acker's equation to relate the parameter σ to the Stokes

radius, and the Stokes-Einstein equation to obtain the diffusion coefficient from the Stokes radius. The values found, corrected to 20° and to water, were, for the Stokes radius in angstroms, for spinach plastocyanin 17.1, for parsley 16.4 and for lettuce 17.3. The corresponding diffusion coefficients were for spinach plastocyanin 12.5×10^{-7}, for parsley 13.0×10^{-7}, and for lettuce 12.4×10^{-7} sq cm per second. As the concentration of plastocyanin in gel flitration was low, these values were taken as corresponding to zero protein concentration.

7. The diffusion coefficient of spinach plastocyanin was also measured in the ultracentrifuge using a synthetic boundary cell, with two protein concentrations. The values found were extrapolated to zero protein concentration to yield a corrected value of 14.2×10^{-7} sq cm per second at 20° and in water.

INTRODUCTION

A study of spinach plastocyanin purified to a stage with absorbance index of about 1.0 gave a value of the molecular weight of the protein of 21,000 (Katoh *et al* 1962). This value was calculated from the values of the sedimentation coefficient and diffusion coefficient, determined in separate experiments, and the value of the partial specific volume obtained by calculation from the known amino acid composition. Analysis of the protein yielded an estimate of one gram atom of copper in 10,800 grams of protein, or two copper atoms per molecule.

However later work with plastocyanins from other sources appeared to indicate that the molecular weight of spinach plastocyanin might in fact be much lower. Gel filtration on Sephadex yielded 13,000 as an estimate of the molecular weight of Chlamydomonas plastocyanin, although the Sephadex column used was not calibrated directly with standard proteins of known molecular weight giving rise to some uncertainty in the precision of the estimate (Gorman and Levine 1966 a). In a recent note (Milne and Wells 1968), the preliminary results were given of a study of the molecular weight of french bean plastocyanin. Amino acid analysis indicated a minimum molecular weight of 11,000, although in contrast, peptide mapping studies apparently indicated a minimum molecular weight of 22,000. Gel filtration estimates of the molecular weight varied from 11,000 to 16,000 depending upon the composition of the eluting buffer. The confusion existing in the literature over the correct molecular weight of plastocyanin in turn renders the number of copper atoms per molecule uncertain. It was felt that the molecular weight of plastocyanin was sufficiently important to warrant a detailed study.

During earlier work on lettuce plastocyanin it was found that the apparent molecular weight of the protein on gel filtration was about 16,000 (Searle 1966), the Sephadex column being calibrated with standard proteins according to the procedure of Andrews (1964). This work has now been extended to plastocyanin from parsley and spinach as well as lettuce, and both gel filtration and ultracentrifuge techniques have been employed.

Since Andrews introduced the use of dextran gels to estimate molecular weights of globular proteins many investigators have successfully employed the technique. However the theoretical interpretation of the relationship between elution volume from the Sephadex column of a protein and the molecular parameters of the protein has remained a matter of dispute. Various theoretical models have been proposed to describe the degree to which a protein molecule can penetrate into the dextran gel matrix, but the treatment of Ackers (1967) is preferred here, because it does not assume any definite geometrical structure for the pores of the gel particles and yet can explain all the experimental results. For a series of proteins of similar shape and density a good correlation of molecular weight with elution volume is observed. However, as discussed by Ackers, it has been shown that elution volumes are best correlated with the molecular diameter or the Stokes radius of the proteins. The calibration procedure of Ackers has been verified by later work (De Vincenzi and Hedrick 1967; Yoza and Ohashi 1969).

Gel filtration on a Sephadex column calibrated with standard proteins of known molecular weight and known Stokes radius allows an estimation to be made of the molecular weight and Stokes radius of plastocyanin. The diffusion coefficient of plastocyanin can then be calculated from the Stokes radius by use of the Stokes-Einstein equation (see Appendix B).

The choice of standard proteins for calibration of the Sephadex column for the study of plastocyanin is restricted to globular proteins of accurately known molecular weight in the range 10,000 - 60,000, and known diffusion coefficient. These proteins must be completely stable during the several hours required for them to be eluted from the Sephadex column, and therefore active proteolytic enzymes cannot be used because of autodigestion. Pepsin is stable within a restricted pH range near pH 6, showing autodigestion below about pH 5 (Bovey and Yanari 1960) and instability at pH 7 and above (Edelhoch 1957; Porcelli 1968). It has been shown that cytochrome c behaves anomalously on Sephadex gels in dilute buffers near neutrality, being retarded by electrostatic interaction with the few carboxyl groups on the gel matrix. However this interference may be overcome by increasing the ionic strength of the elution buffer (Siegel and Monty 1966; Andrews 1962).

Although gel filtration is useful in providing an estimate of the molecular weight of plastocyanin, the method lacks precision, as is demonstrated by the variation of apparent molecular weight with buffer composition for french bean plastocyanin (Milne and Wells 1968). Nevertheless gel filtration studies were considered to be valuable in allowing comparison with previous work.

As a check on the gel filtration results it was considered desirable to investigate plastocyanin by ultracentrifuge techniques as well. The molecular weight was

determined accurately by sedimentation equilibrium, employing a short column of sample solution to minimize the time required to reach equilibrium (Van Holde and Baldwin 1958). Sedimentation equilibrium centrifugation is considered a better technique than sedimentation velocity in the case of plastocyanin, because it is a small protein with a low sedimentation coefficient but high diffusion coefficient. In sedimentation velocity experiments the protein boundary would therefore move very slowly and would not be sharp because of the high rate of diffusion. However in sedimentation equilibrium experiments, the high diffusion coefficient is beneficial in shortening the time required to reach the equilibrium state.

The diffusion coefficient of spinach plastocyanin determined previously (Katoh et al 1962) must be regarded as unusually low for a protein of molecular weight 21,000 as determined by these workers. The approximate relationship derived by Longsworth (1955) would indicate a value of 11×10^{-7} sq cm per second in contrast to the value of 6.6×10^{-7} sq cm per second quoted. Therefore the diffusion coefficient of spinach plastocyanin was determined in the ultracentrifuge to check the estimate obtained by gel filtration and to compare with the previously published value.

The monochromatic light of wavelength 546 nm used in the optical system of the Spinco ultracentrifuge is absorbed appreciably by oxidised plastocyanin and use of the oxidised form would require very long exposure times of the photographic plates. Therefore in all ultracentrifuge work the plastocyanin

was in the reduced form. A gross difference in molecular weight between the oxidised and reduced forms was discounted by gel filtration experiments.

In order to calculate molecular weights from sedimentation equilibrium studies it is necessary to know the partial specific volume of plastocyanin. A small error in the value of the partial specific volume results in a large error in the calculated molecular weight (see Appendix B), and therefore a good precision is required in its measurement. To evaluate the partial specific volume the density of a protein solution of accurately known concentration must be measured. Of the methods available to measure the density of a protein solution the most sensitive is the density gradient column method of Linderstrom-Lang (Schachman 1957). This technique has the further advantage that only a very small volume of sample solution is required, in contrast to the large volumes needed in pycnometry.

In addition to the density of the plastocyanin solution the protein concentration was also required. It was essential to avoid any assumptions about the molar extinction coefficients and molecular weight, and therefore estimation by spectrophotometry was not possible. Dry weight measurements are susceptible to large errors, and nitrogen determinations by Kjeldahl digestion, which would have consumed large quantities of purified material, were also avoided. However it has been shown that the specific refractive increment for proteins is quite constant for a wide variety of proteins

(Perlmann and Longsworth 1948), and it was found convenient to measure plastocyanin concentrations by refractometry, assuming a value for the specific refractive increment of plastocyanin of that of an average protein. This assumed value was the same as that employed in a recent publication (Blethen et al 1968).

The partial specific volume of a protein can also be calculated by addition of the specific volumes of the constituent amino acid residues (Cohn and Edsall 1943). The assumptions involved in such a calculation appear to be justified by the many examples in the literature for which the calculated value and the experimentally-determined value are found to agree. Schachman (1957) quotes the case of ribonuclease to illustrate the calculation. However the effect of the copper content of plastocyanin on the validity of the assumptions is unknown. It has been found for the copper-protein ascorbate oxidase, that the experimentally-determined value was significantly lower than the value calculated from the amino acid composition (Nakamura et al 1968), indicating that the presence of copper in the protein caused a tightening of the protein structure and a consequent increase in density.

MATERIALS AND METHODS

Materials. Plastocyanin prepared from spinach, parsley and lettuce and purified as described in Chapter 1, was used in all experiments except those in the ultracentrifuge with schlieren optics, when purified spinach plastocyanin prepared according to Borchert and Wessels (personal communication) was employed.

For ultracentrifuge work plastocyanin was reduced by ascorbate, put into 0.1 M KPO_4 (1:1) using a Sephadex G-25 column, and concentrated either by ultrafiltration in a Diaflo Model 50 cell or by dialysis against dry Sephadex powder. The plastocyanin was then dialysed to equilibrium for 4 hours at 4° against 0.1 M KPO_4 (1:1), with the dialysis vessel closed to reduce evaporation. The dialysis medium was used as the plastocyanin solvent solution for the measurement of partial specific volume and for the reference solution in the double sector ultracentrifuge cells used throughout this work.

For the gel filtration experiments to estimate molecular weight and Stokes radius, the plastocyanin was applied in gel filtration buffer: 0.01 M $NaPO_4$ (1:1) with 0.5 M sodiumchloride added, pH 6.1, measured at the operating temperature of the column. In experiments in which the elution volume of parsley plastocyanin was investigated for varying elution buffers, the plastocyanin sample was applied in 5 mM KPO_4 (1:1) to the Sephadex G-75 column. The standards used to calibrate the column were: ribonuclease (Sigma, Bovine pancreas); cytochrome c (Sigma, Horse heart, Type III); trypsinogen (Sigma, Type I 1x crystallised); chymotrypsinogen (Sigma, Bovine pancreas 6x crystallised Type II); haemoglobin (Sigma, Bovine Type I); pepsin (Sigma, 2x crystallised); Bovine serum albumin; Blue dextran 2000 (Pharmacia); Glucose. All chemicals used in making the buffer solutions were analytical reagent grade.

Gel filtration. The Sephadex G-75 column, used throughout the gel filtration studies, was made with deaerated gel suspension in a jacketed, fixed-capacity column of 1.6 cm

internal diameter and 60 cm height, with cross-section area 2 sq cm. The height of the gel bed was about 55 cm. The elution buffer was fed by gravity, employing a constant hydrostatic head of 30 cm provided by a Marriott flask and giving a buffer flow-rate of 12 ml/hour. The effluent was collected with a Beckman fraction collector in fractions of 2.53 ml, both the fractions collected and also the column were cooled to 1° by the refrigeration system of the fraction collector. The elution profiles of the proteins were measured spectrophotometrically at 230 nm for dilute solutions and at 280 nm for more concentrated solutions; Blue Dextran 2000 was also measured spectrophotometrically, at its absorption maximum in the visible at 625 nm. When the elution volume of bovine serum albumin was investigated, the elution profiles of the Blue Dextran and the protein were found to overlap and corrections had to be made for the absorption of the Blue Dextran at 230 nm to obtain the bovine serum albumin elution profile. The elution volume of the Blue Dextran was taken as the volume corresponding to the position of the half height of the leading edge of the Blue Dextran peak, as the elution peak is not symmetrical (Granath and Kvist 1967). The position of the protein peaks were found by extrapolating the slopes of the leading and trailing edges of the elution profile to the apex (Andrews 1964).

The internal volume of the gel matrix available to glucose was taken as an estimate of the total internal volume of the gel. Glucose was detected in the column effluent using the glucostat reagent (Worthington Biochemical Corp., Freehold, N.J. U.S.A.). When the elution buffer was changed, a decrease in

gel bed height and flow rate was prevented by flowing the buffer upwards for a short time, before allowing the new buffer to flow normally through the column for 12 hours prior to use of the column.

Elution volumes from the column are expressed in fractions of 2.53 ml. The internal volume of the gel matrix available to a substance (ΔV) is the difference between the elution volume of the substance and that of Blue Dextran 2000. Each sample applied to the column contained Blue Dextran, and ΔV was read directly each time. The parameter σ is defined as the fraction of the internal volume of the gel matrix available to a protein, $\sigma = \Delta V$ (protein)/ΔV (glucose).

Ultracentrifugation. The sedimentation equilibrium studies using Rayleigh interference optics were performed on a Spinco Model E ultracentrifuge at the University of Oxford. The temperature was 20° and the rotor speed 15,220 r.p.m. Prior to insertion in the cell, the refractive index of the solvent was balanced with that of the protein solution in a Bryce-Phoenix differential refractometer by addition of 1,3-butanediol. The protein concentration was measured refractometrically assuming a specific refractive increment of 1.84×10^{-4} per g/litre (Perlmann and Longsworth 1948). Each run lasted for 16 hours overnight, after which time equilibrium had been reached and photographs of the interference patterns were taken. The hinge point, the radial distance at which the protein concentration was equal to the original concentration, was found by the white-light fringe technique (Richards et al

1968). The concentration distribution, at equilibrium, through the protein solution was then calculated from the interference fringe displacements measured with a microcomparator. A run was made with lettuce, parsley and spinach plastocyanins in the multicell rotor An-G, and lettuce plastocyanin was also investigated in run number 1767-1768 in the single cell An-D rotor.

Sedimentation equilibrium studies on spinach plastocyanin using schlieren optics were carried out on a Spinco Model E ultracentrifuge in Eindhoven. The temperature was 20° and the An-D rotor used at 21,740 r.p.m. Photographs of the equilibrium schlieren pattern were taken after 16 - 20 hours and measured on a microcomparator. Straight baselines were invariably found at the low speed used. As the data was analysed using the Lamm plot (Creeth and Pain 1967) a knowledge of the original concentration was not required to calculate the molecular weight.

The diffusion coefficient of spinach plastocyanin was measured in a capillary-type synthetic boundary cell, with a rotor speed of 6,995 r.p.m. and a temperature of 24°. A silicone oil layer on the surface of the protein solution facilitated smooth overlayering. The time of overlayering was taken as: the time when the rotor was accelerated rapidly from 2,000 r.p.m. plus one third of the time taken to accelerate from 2,000 r.p.m. to 6,995 r.p.m. Photographs were taken of the protein solution-solvent boundary, with schlieren optics, at short time intervals after overlayering had occurred, until the boundary was so broad that it spread to the solution

boundaries. The photographs were measured on a microcomparator and the boundary profiles shown to be Gaussian. Therefore the plastocyanin was homogeneous. The diffusion coefficient could be calculated by the increase in the parameter σ^2 of the Gaussian curves with increasing time after overlayering. Runs were made at two protein concentrations and numbered runs 87 and 88. The protein concentrations were measured from the absorbance at 278 nm.

Partial specific volume. The densities of the plastocyanin solutions were measured with a density gradient column, placed in a constant temperature room at 20°. The gradient was made from paraffin-bromobenzene, and was calibrated with 1.5%, 2.0% and 2.5% potassium chloride solutions whose densities were found in the International Critical Tables. The same pipette was used for all solutions to produce drops of equal size. The height of the centre of the drops of plastocyanins, solvent, and potassium chloride standards were read after settling for a fixed time of 20 minutes.

The concentrations of the plastocyanin solutions were measured refractometrically at room temperature in a Bryce-Phoenix differential refractometer.

RESULTS AND DISCUSSION

Gel filtration. The estimation of the molecular weight of the three plastocyanins is shown in Figure 2-1. The values of the elution volumes shown are the averages of several experiments (see Table 2-1 for the individual results). The

TABLE 2-1. THE ELUTION VOLUMES OF STANDARD PROTEINS * AND PLASTOCYANIN FROM SEPHADEX G-75.

Details of the experiment and methods employed are given in the legend to Figure 2-1.

Protein	ΔV						
	Individual experiments						Average
Bovine serum albumin	3.3	3.0	3.2	3.2			3.18
Pepsin	7.4	7.2	7.1	6.9	7.2	7.0	7.13
Haemoglobin	7.9						7.90
Chymotrypsinogen	10.1	10.2	10.1				10.13
Trypsinogen	10.4	10.1	10.1				10.20
Ribonuclease	13.9	13.9	13.7				13.83
Cytochrome c	13.4	13.5	13.3	13.5			13.43
Lettuce pcy **	14.3	14.6	14.2	14.2			14.33
Spinach pcy **	14.5	14.5	14.5	14.5	14.4	14.6	14.50
Parsley pcy **	15.2	15.4	15.2	15.1			15.23

** pcy is the abbreviation for plastocyanin. All plastocyanins were oxidised.

* The references for the molecular weights of the standard proteins used are as follows:

Bovine serum albumin: Creeth (1958).
Pepsin : Rajagopalan et al (1966).
Haemoglobin : Andrews (1964).
Chymotrypsinogen : Zmrhal (1962).
Trypsinogen : Tietze (1953).
Ribonuclease : Scheraga and Rupley (1962).
Cytochrome c : Margoliash (1962).

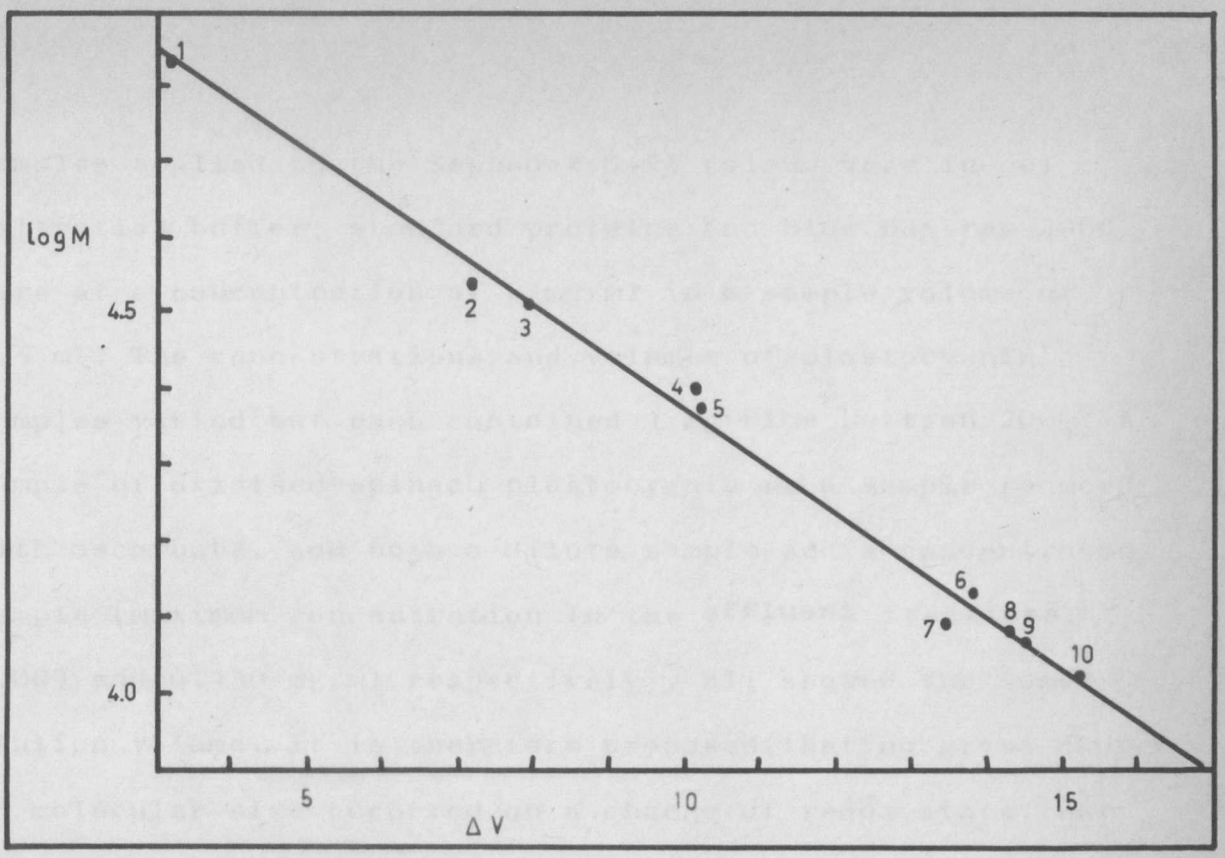


Figure 2-1. The relationship between molecular weight and the elution volume from the Sephadex G-75 column. The details of the gel filtration procedure are given in the text; log M is: logarithm$_{10}$ (molecular weight); and Δ V is the difference between the elution volume of the protein and the elution volume of Blue Dextran 2000, expressed in numbers of fraction tubes (2.53 ml each). The standard proteins used were:

1. Bovine serum albumm, M = 67,000.
2. Pepsin, M = 34,160.
3. Haemoglobin, M = 32,500 (dissociated).
4. Chymotrypsinogen, M = 25,000.
5. Trypsinogen, M = 23,700.
6. Ribonuclease, M = 13,680.
7. Cytochrome c, M = 12,380.

The molecular weights found for the plastocyanins were:

8. Lettuce plastocyanin, M = 12,200.
9. Spinach plastocyanin, M = 11,800.
10. Parsley plastocyanin, M = 10,600.

samples applied to the Sephadex G-75 column were in gel filtration buffer; standard proteins and Blue Dextran 2000 were at a concentration of 2 mg/ml in a sample volume of 0.5 ml. The concentrations and volumes of plastocyanin samples varied but each contained 1 mg Blue Dextran 2000. A sample of oxidised spinach plastocyanin and a sample reduced with ascorbate, and both a dilute sample and a concentrated sample (maximum concentration in the effluent fractions, 0.003 and 0.130 mg/ml respectively), all showed the same elution volume. It is therefore proposed that no gross change in molecular size occurred on a change of redox state, nor did spinach plastocyanin dissociate at low concentrations. When parsley plastocyanin was run on the same column, with 0.1 mM sodium isoascorbate added to the gel filtration buffer, to be certain that the plastocyanin remained in the reduced form during gel filtration, elution volumes of 14.7 and 14.9 were measured in contrast to an average of 15.2 seen before. It is possible that a small change in shape accompanied the redox change in parsley plastocyanin, but the effect was small and confirmation of the difference in elution volume of oxidised and reduced forms is required.

The elution volume of ribonuclease from the column was anomalous, as it was eluted after cytochrome c although its molecular weight is greater than the latter protein. The nativeness of the cytochrome c used, was shown by an estimation of the absorbance at 695 nm of a 0.332 mM solution of the protein: $A_{695} = 0.210$. Taking the molar absorbance coefficient at 695 nm to be 800 (Schejter et al 1963), this indicated that

79% of the protein was native monomeric ferricytochrome c, the remainder probably being accounted for by the reduced cytochrome c present in the sample. In addition the data for cytochrome c, but not for ribonuclease, agreed with the equation given by Determann and Michel (1966) for Sephadex G-75:

$$\log M = 5.624 - 0.752 (V_e/V_o).$$

The value of V_o (the elution volume of Blue Dextran) for the column was found to be 12.9 fraction tubes, and V_e was 26.7 for ribonuclease and 26.3 for cytochrome c, giving values of V_e/V_o of 2.07 and 2.04 respectively. However the values of V_e/V_o calculated with the above equation from the known molecular weights were 1.98 and 2.04 respectively. The reason for the anomalous behaviour of ribonuclease may be connected with the high salt concentration (0.5 M).

The equation of the line drawn through the points of the standard proteins in Figure 2-1, which was obtained by the method of least squares (see Appendix B) is given by:

$$\log M = 5.037 - 0.06667 \Delta V.$$ (ΔV expressed as fractions of 2.53 ml each).

When the elution buffer for the Sephadex G-75 column was varied, the elution volume of parsley plastocyanin was found to change, although the total internal volume of the gel matrix remained constant (Table 2-2). This might be explained by a loose conformation of the parsley plastocyanin, which can be altered by binding of ions to the polypeptide chain. The effect was apparently not dependent on pH or ionic strength but was specific for the ions composing the buffer: both Na^+

TABLE 2-2. VARIATION OF THE ELUTION VOLUME OF PARSLEY PLASTOCYANIN FROM SEPHADEX G-75 WITH CHANGE IN BUFFER COMPOSITION.

The same Sephadex G-75 column was used throughout after equilibration with each buffer in turn. Temperature 1°. The elution volumes (ΔV) are expressed as the number of fractions (each 2.53 ml) between the elution peak of plastocyanin or glucose and the void volume found with Blue Dextran 2000 *. The parameter σ is the fraction of the internal volume of the gel matrix available to plastocyanin. I is the ionic strength of the buffer.

Elution buffer	ΔV		σ	pH	I
	Plastocyanin	Glucose			
0.01 M KPO_4 (1:1)+0.19 M KCl	16.7	24.5	0.681	6.5	0.21
0.01 M KPO_4 (1:1)+0.19 M KCl	16.8	24.6	0.682	6.5	0.21
0.2 M KPO_4 (1:1)	15.1	24.8	0.609	6.7	0.40
0.01 M KPO_4 (1:1)+0.5 M KCl	16.9	25.0	0.676	6.1	0.51
0.01 M $NaPO_4$ (1:1)+0.5 M NaCl	15.7	25.0	0.628	6.1	0.51
0.01 M $NaPO_4$ (1:1)+0.5 M NaCl	15.8	25.2	0.626	6.1	0.51
0.1 M $NaPO_4$ (1:1)	14.2	24.5	0.580	6.8	0.20
0.1 M KPO_4 (1:1)	15.0	24.6	0.610	6.8	0.20
0.1 M K_2PO_4	14.2	24.2	0.580	8.2	0.30
0.1 M KPO_4 pH 5.8	16.3	24.4	0.668	5.8	0.12

* Each sample contained 2 mg Blue Dextran 2000, 0.5 mg glucose and 0.1 mg oxidised parsley plastocyanin (cytochrome b content 0.2%) in 0.5 ml of 5 mM KPO_4 (1:1) buffer.

and HPO_4^{2-} lowered the elution volume, whereas K^+ and $H_2PO_4^-$ raised it. This variation rendered the estimation of the molecular weight of plastocyanin by gel filtration uncertain by up to 20%.

Partial specific volume. From the amino acid compositions given in Table 1-3, the partial specific volumes were calculated by the method of Cohn and Edsall (1943), assuming a random distribution of the amide groups between the aspartic and glutamic acid residues, and one cysteinyl residue per molecule of all three plastocyanins. The values calculated were 0.724 for spinach, 0.726 for parsley and 0.729 for lettuce plastocyanin.

The partial specific volumes of the plastocyanins were also measured, as described in the Materials and Methods section, to be 0.702 for spinach, and 0.705 for both lettuce and parsley plastocyanins. The considerably lower values measured compared to those calculated, indicated that the calculation was probably in error, possibly by the assumption that the copper content of the plastocyanins had no effect on the specific volumes of the constituent amino acids. The sample of lettuce plastocyanin used was slightly oxidised and it was possible to observe that the protein remained native throughout the measurements, showing no denaturation at the aqueous-organic solvent interface. The concentrations were determined at room temperature to be 2.75 mg/ml for the spinach plastocyanin solution, 3.28 mg/ml for the parsley, and 7.71 mg/ml for the lettuce plastocyanin. The error arising from not making the

measurements at 20° is negligible, for Perlmann and Longsworth (1948) stated that the specific refractive increment varied only 0.5% for a 10° change around 20°. The densities of the solutions were 1.01158 for the spinach plastocyanin, 1.01172 for parsley and 1.01300 for lettuce plastocyanin. The density of the solvent, 0.1 M KPO_4 (1:1) was measured to be 1.01078. These values were calculated taking the densities of the standard solutions as 1.01103 for 2% KCl and 1.01423 for 2.5% KCl, as given in the International Critical Tables, Volume III, page 87.

Sedimentation equilibrium ultracentrifugation. In Figure 2-2 the results are shown for the sedimentation equilibrium runs on lettuce plastocyanin using Rayleigh interference optics. The plot of log J against r^2 in Figure 2-2 (A) is seen to have a slight upward curvature indicating a small degree of heterogeneity. The protein concentration was 6.40 mg/ml and the slope of 0.0680 led to a value of the molecular weight of 10,400, taking $(1-\bar{v}\rho)$ equal to 0.288. Another sample of lettuce plastocyanin was investigated in run number 1767-1768 shown in Figure 2-2 (B): protein concentration 7.71 mg/ml, and this sample gave a perfectly straight plot, with a slope of 0.0763 leading to an estimation of the molecular weight of 11,700.

In Figure 2-3 (A) a solution of spinach plastocyanin (concentration 3.41 mg/ml) gave a straight line also, showing it to be homogeneous; but the slope of 0.0568 led to a low value of the molecular weight, namely 8,600, when $(1-\bar{v}\rho)$ was taken equal to 0.291. In Figure 2-3 (B) a sample of parsley

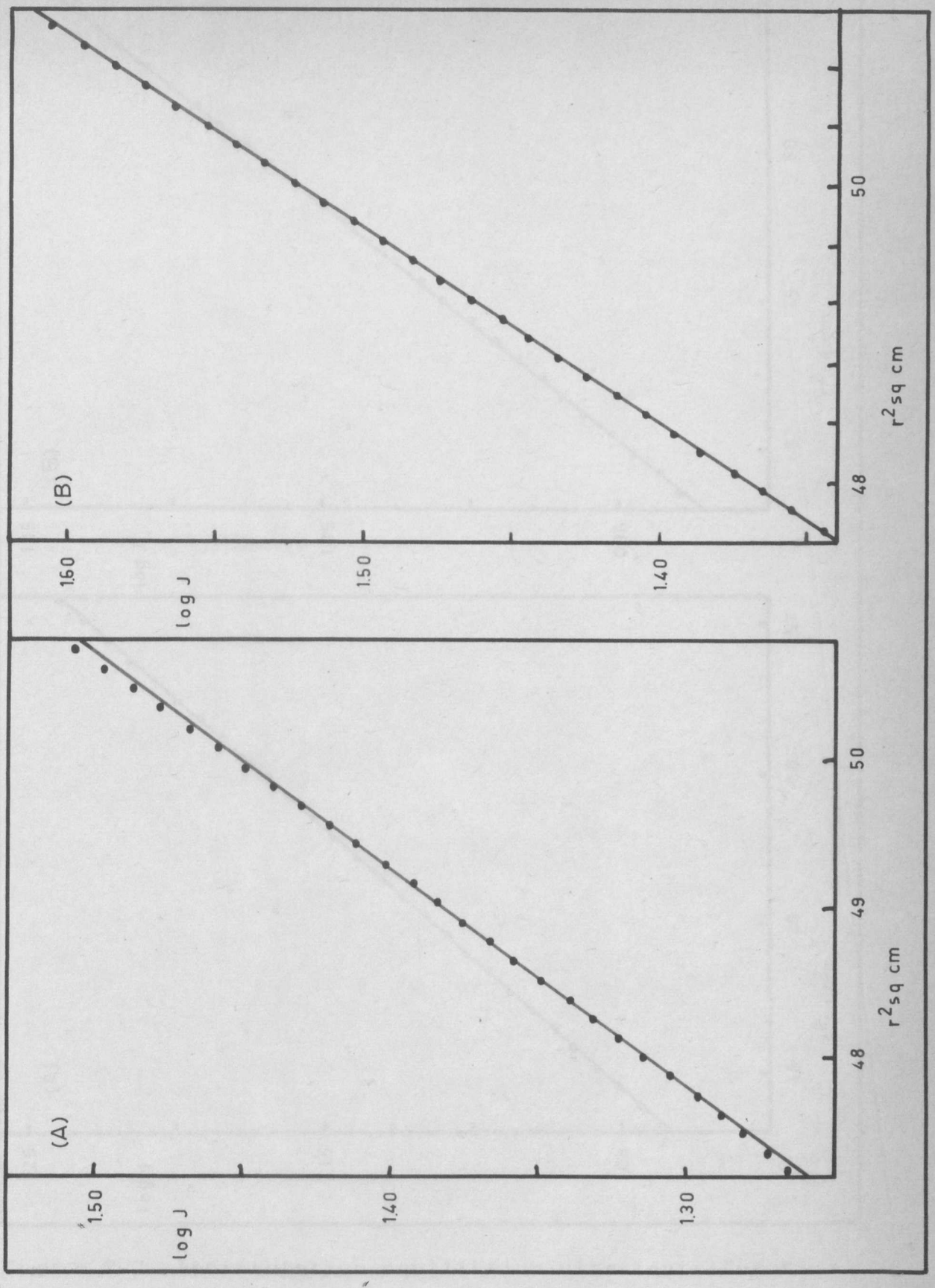


Figure 2-2. Sedimentation equilibrium ultracentrifugation of lettuce plastocyanin using Rayleigh optics. J is the fringe displacement and r the radial distance in cm. (A) An-G rotor run. (B) Run number 1767-1768. Speed 15,220 r.p.m. Temperature 20°.

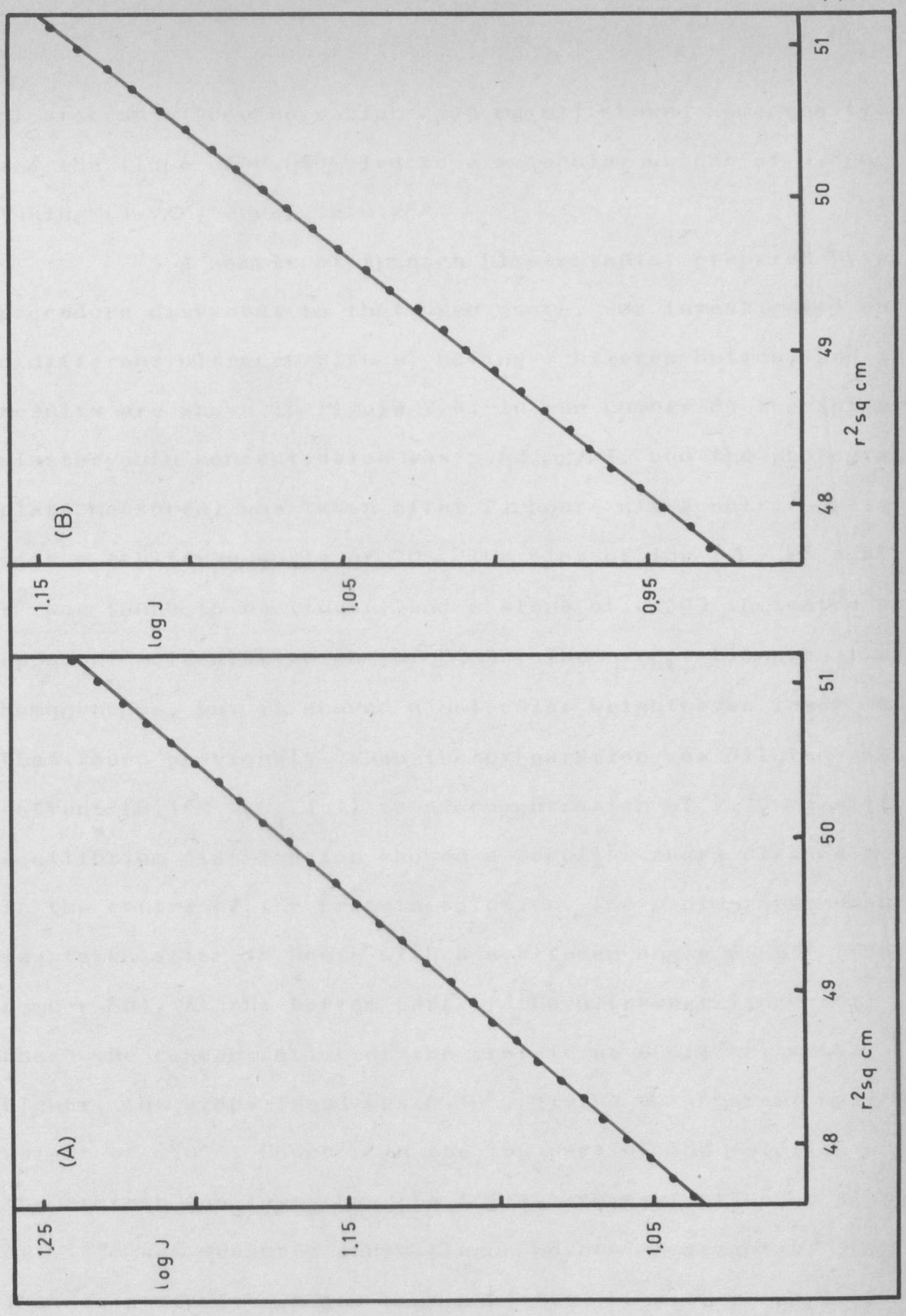


Figure 2-3. Sedimentation equilibrium ultracentrifugation of (A) spinach, and (B) parsley plastocyanin in the An-G rotor using Rayleigh optics. Other details as for Figure 2-2.

plastocyanin (concentration 2.73 mg/ml) showed homogeneity, and the slope of 0.0640 led to a molecular weight of 9,800, taking $(1-\bar{v}\rho)$ equal to 0.288.

A sample of spinach plastocyanin, prepared by a procedure different to that used above, was investigated on a different ultracentrifuge, having schlieren optics, and the results are shown in Figure 2-4. In run number 84 the spinach plastocyanin concentration was 6.62 mg/ml, and the photographic plate measured, was taken after 21 hours ultracentrifugation with a schlieren angle of 70°. The plot of log $(\Delta y/r)$ against r^2 was found to be linear, and a slope of 0.103 indicated an apparent molecular weight of 7,650. The preparation was thus homogeneous, but it showed a molecular weight even lower than that found previously. When the preparation was diluted with solvent (0.1 M KPO_4 1:1) to a concentration of 2.77 mg/ml the equilibrium distribution showed a peculiar sharp discontinuity in the centre of the protein solution. The photograph measured was taken after 18 hours with a schlieren angle of 55° (run number 86). At the bottom part of the ultracentrifuge cell where the concentration of the protein at equilibrium was higher, the slope found was 0.108, giving an apparent molecular weight of 8,050. However in the top part of the solution where the protein was less than the initial concentration, a slope of 0.138 was measured indicating a molecular weight of 10,250. The discontinuity in the Lamm plot for the dilute sample is difficult to interpret. The protein sample solution was homogeneous, as seen in run number 84, and the cytochrome contaminant

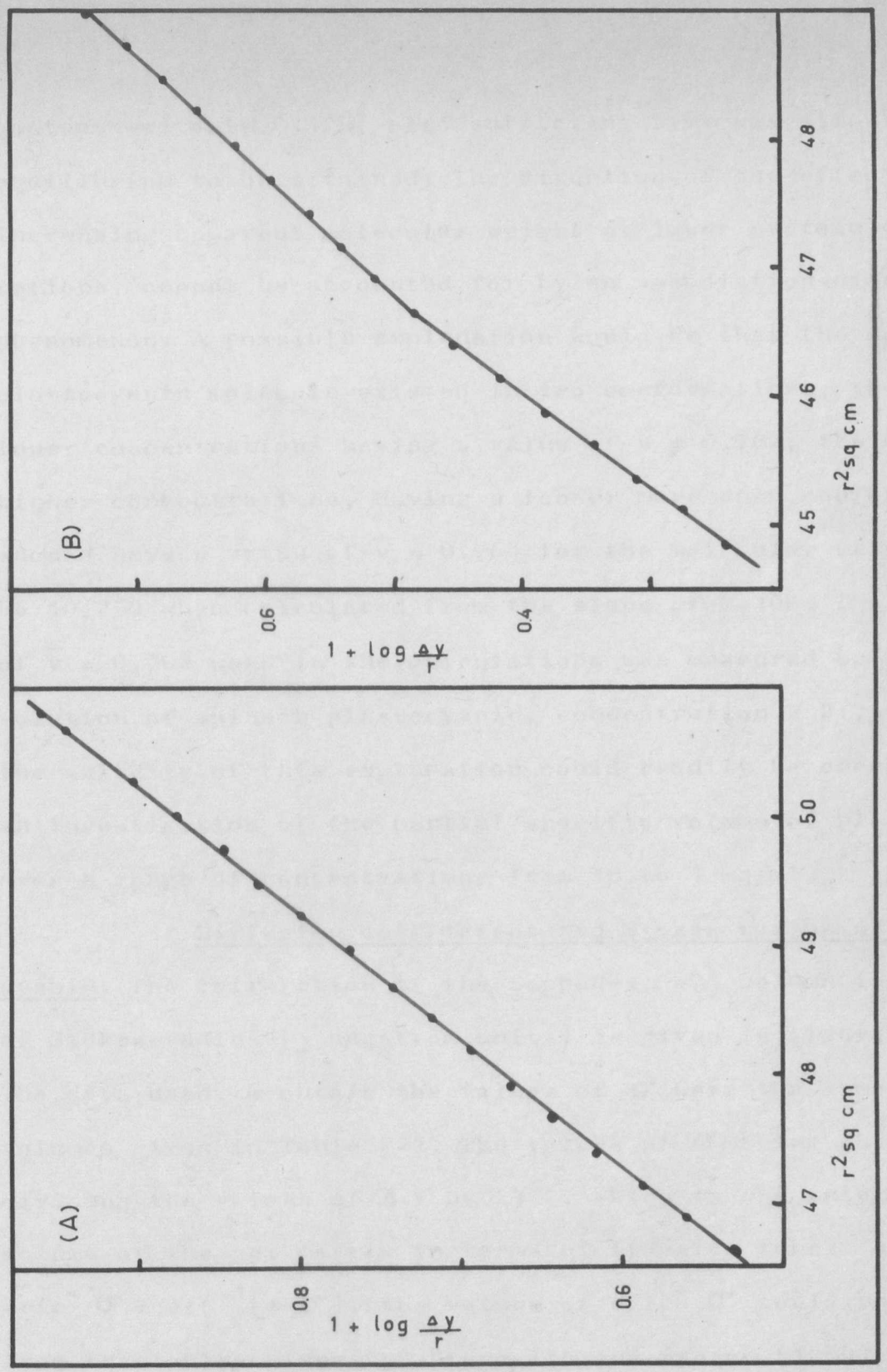


Figure 2-4. Sedimentation equilibrium ultracentrifugation of spinach plastocyanin using Schlieren optics. Analysis of the data is presented as Lamm plots: r is the radial distance and Δ y is the vertical displacement of the Schlieren pattern from the baseline. Speed 21,740 r.p.m. Temperature 20°. The two runs are identical except for concentration of plastocyanin: (A) run number 84 , 6.62 mg/ml; (B) run number 86 , 2.77 mg/ml.

content was only 0.12%; also sufficient time was allowed for equilibrium to be attained. The direction of the effect, an increasing apparent molecular weight at lower protein concentrations, cannot be accounted for by an association-dissociation phenomenon. A possible explanation would be that the spinach plastocyanin molecule existed in two conformations, the one at lower concentrations having a value of $\bar{v}$ = 0.702; the one at higher concentrations, having a looser more open conformation, should have a value of $\bar{v}$ = 0.764 for the molecular weight to be 10,250 when calculated from the slope of 0.108. The value of $\bar{v}$ = 0.702 used in the calculations was measured on a dilute solution of spinach plastocyanin, concentration = 2.75 mg/ml. The validity of this explanation could readily be checked by an investigation of the partial specific volume of plastocyanin over a range of concentrations from 10 to 1 mg/ml.

Diffusion coefficient and Stokes radius of plastocyanin. The calibration of the Sephadex G-75 column in terms of Stokes radius (a angstrom units) is given in Figure 2-5. The data used to obtain the values of σ were the same elution volumes given in Table 2-1. The values of σ being obtained by dividing the values of ΔV by 24.2, which is the internal volume of the gel matrix in terms of fraction tubes. As $\text{erfc}^{-1}\sigma = \text{erf}^{-1}(1-\sigma)$, the values of $\text{erfc}^{-1}\sigma$ could be found from the tables given by Abramowitz and Stegun (1964) for erf x (see Appendix B). The diffusion coefficients of the standard proteins, at zero protein concentration, were found in the literature and the values for $D_{20,w}(cm^2/sec)$ used, were

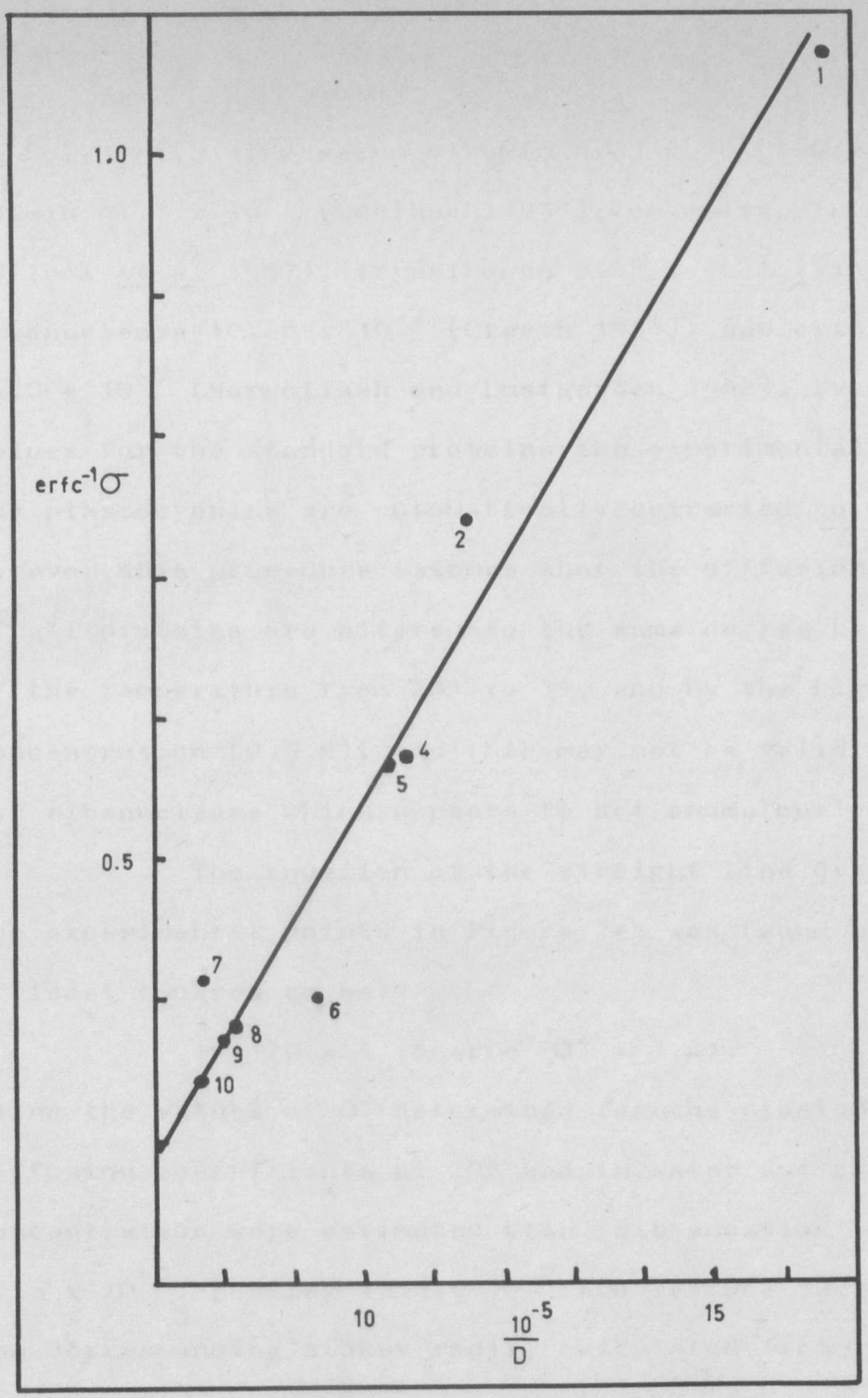


Figure 2-5. The relationship between Stokes radius and the elution volume from the Sephadex G-75 column. The numbers refer to proteins which are listed in the legend to Figure 2-1. The details of the gel filtration procedure are given in the text; $\text{erfc}^{-1}\sigma$ is a function of the elution volume, defined in Appendix B; $10^{-5}/D$ is related to the Stokes radius (a): $a = 2.14\ (10^{-5}/D)$, where D is the diffusion coefficient of the protein at 20° and in water.

as follows: bovine serum albumin 6.03×10^{-7} (Creeth 1958); pepsin 8.71×10^{-7} (Edelhoch 1957); chymotrypsinogen 9.48×10^{-7} (Wilcox et al 1957); trypsinogen 9.68×10^{-7} (Tietze 1953); ribonuclease 10.68×10^{-7} (Creeth 1958); and cytochrome c 13.0×10^{-7} (Margoliash and Lustgarten 1962). By using $D_{20,w}$ values for the standard proteins the experimental values for the plastocyanins are automatically corrected to 20° and to water. However this procedure assumes that the diffusion coefficients of all proteins are altered to the same degree by the lowering of the temperature from 20° to 1°, and by the high salt concentration (0.5 M), and this may not be valid, particularly for ribonuclease which appears to act anomalously.

The equation of the straight line drawn through the experimental points in Figure 2-5 was found by the method of least squares to be:

$$10^{-5}/D = 11.8 \text{ erfc}^{-1}\sigma + 3.61.$$

Using the values of σ determined for the plastocyanins, their diffusion coefficients at 20° and in water and at zero protein concentration were estimated with this equation to be: spinach 12.5×10^{-7}, parsley 13.0×10^{-7} and lettuce 12.4×10^{-7} cm^2/sec. The corresponding Stokes radii, calculated from the Stokes--Einstein equation (taking the viscosity of water at 20° to be 0.01002 g/sec.cm: $a(cm) = 2.14 \times 10^{-13}/D$) were found to be: spinach 17.1, parsley 16.4 and lettuce 17.3 angstrom units.

The diffusion coefficient of spinach plastocyanin was also determined in a capillary-type synthetic boundary cell at 24° in the Spinco Model E ultracentrifuge equipped with schlieren optics. Two runs were made. Run number 87 at a protein

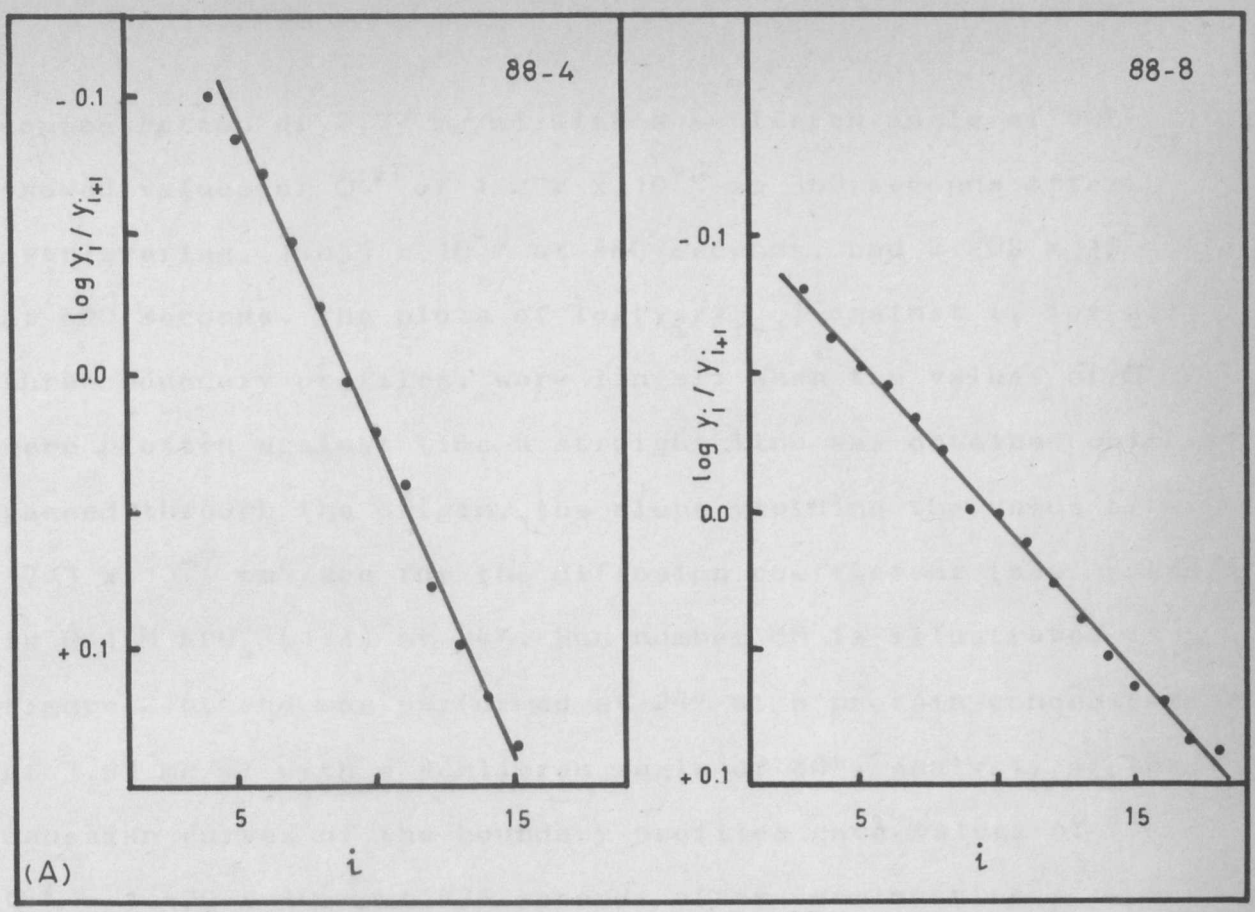
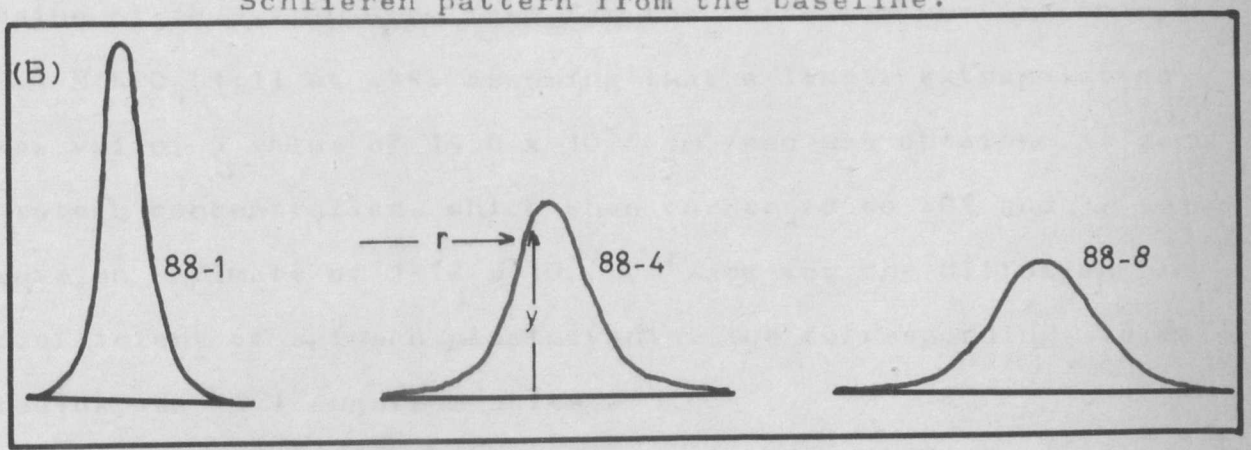


Figure 2-6. Ultracentrifuge run number 88. Determination of the diffusion coefficient of spinach plastocyanin in a synthetic boundary cell using Schlieren optics. (A) i is an arbitrary, but constant, interval of radial distance across the protein solution-solvent boundary and y is the vertical displacement of the Schlieren pattern from the baseline.



(B) Profiles of the solution-solvent boundary at increasing times after overlayering: 88-1 at 1.5 min, 88-4 at 7.25 min, and 88-8 at 15.5 min. Diffusion is rapid. Schlieren angle 80°. Temperature 24°. The diffusion coefficient is evaluated from the profiles by the analysis illustrated in (A).

concentration of 2.77 mg/ml with a schlieren angle of 60° showed values of σ^2 of 1.272×10^{-3} at 360 seconds after overlayering, 1.655×10^{-3} at 480 seconds, and 2.202×10^{-3} at 600 seconds. The plots of $\log(y_i/y_{i+1})$ against i, for all three boundary profiles, were linear. When the values of σ^2 were plotted against time, a straight line was obtained which passed through the origin, the slope yielding the value of 17.3×10^{-7} cm^2/sec for the diffusion coefficient (see Appendix B) in 0.1 M KPO_4 (1:1) at 24°. Run number 88 is illustrated in Figure 2-6, and was performed at 24° at a protein concentration of 3.97 mg/ml with a schlieren angle of 80°. Analysis of the Gaussian curves of the boundary profiles gave values of $\sigma^2 = 1.670 \times 10^{-3}$ at 435 seconds after overlayering, 2.382×10^{-3} at 660 seconds, and 3.387×10^{-3} at 930 seconds. As shown for the profiles 88-4 and 88-8 in Figure 2-6, the plots of $\log(y_i/y_{i+1})$ against i cm were linear. The plot of σ^2 against time passed through the origin, and the slope gave a value of 18.3×10^{-7} cm^2/sec for the diffusion coefficient in 0.1 M KPO_4 (1:1) at 24°. Assuming that a linear extrapolation was valid, a value of 15.0×10^{-7} cm^2/sec was obtained at zero protein concentration, which when corrected to 20° and to water gave an estimate of 14.2×10^{-7} cm^2/sec for the diffusion coefficient of spinach plastocyanin. The corresponding Stokes radius was 15.1 angstrom units.

Conclusions. It is proposed that the value of the diffusion coefficient determined by Katoh *et al* (1962) was in error. The value of 6.6×10^{-7} cm^2/sec found would be a likely estimate of the diffusion coefficient of a globular protein of

molecular weight close to 60,000 rather than 20,000 or 10,000. The equation of Longsworth (see Appendix B), which is applicable to typical globular proteins, leads to an estimate of 11.2×10^{-7} cm^2/sec for the diffusion coefficient of a protein with molecular weight equal to 20,000 and $\bar{v} = 0.70$; and 14.1×10^{-7} cm^2/sec for a protein of molecular weight 10,000 and $\bar{v} = 0.70$. This latter value is in close agreement with those determined in the present work. Moreover if the diffusion coefficient of spinach plastocyanin is taken as 12.5×10^{-7} cm^2/sec and used with the value of $S_{20,w} = 1.72$ determined by Katoh *et al* (1962), then a molecular weight of 11,200 can be calculated, again in agreement with estimations of the molecular weight reported in the present work. It is not proposed that Katoh and co-workers were dealing with a dimer, but rather that a two-fold error was made in the diffusion coefficient measurements reported by them.

Evidence that plastocyanin can be regarded as a globular protein is provided by calculation of the Stokes radius from the equation: $f/f_o = a \left(\frac{3 \bar{v} M}{4 \pi N}\right)^{-1/3}$. From the data given by Siegel and Monty (1966), an average value of $f/f_o = 1.25$ can be assumed for a typical globular protein. For spinach plastocyanin M = 10,500 and $\bar{v} = 0.702$, and taking f/f_o equal to 1.25 yields an estimate of 17.9 angstrom units for the Stokes radius, close to that obtained by gel filtration.

Amino acid analysis of the proteins (see Table 1-3) gave estimates of the molecular weight for spinach plastocyanin 10,500, for parsley 10,400 and for lettuce 10,800.

It is possible for both gel filtration and sedimentation equilibrium studies to give imprecise estimates of the molecular weight. In gel filtration, errors can arise from the assumption that all proteins studied have the same molecular shape; and the effect of the buffer ions on particular proteins may differ as seen by the anomalous behaviour of ribonuclease, and also by the variation of elution volume of plastocyanin with elution buffer composition. In ultracentrifugation, the molecular weight estimate depends upon the accuracy of the value of the partial specific volume used. An error in $\bar{v}$ is magnified 3 times in the molecular weight value calculated.

CHAPTER THREE. THE COPPER CONTENT OF CHLOROPLASTS, AND RUBIMEDIN

SUMMARY

1. The copper content of preparations of purified chloroplasts was found to be between 0.97 and 2.07 gram atoms per basic photosynthetic unit with an average value of 1.48.

2. Rubimedin was isolated on ammonium sulphate fractionation of a thawed solution of impure plastocyanin stored in the deep freeze for several weeks. It could be homogenised to give a clear solution but it was sedimented by centrifugation at 100,000 g. The method of isolation was not reproducible.

3. Rubimedin, as isolated, was coloured red with a single maximum in its absorption spectrum at 475 - 478 nm. It was only partially reduced by ascorbate, but was completely bleached by dithionite. Immediate bleaching was also found on addition of sodium hydroxide and the difference spectrum of untreated against alkali-treated rubimedin showed a maximum at 320 nm in addition to that at 475 nm. The untreated rubimedin did not show an absorption maximum in the ultraviolet region of the spectrum.

4. Rubimedin was found to possess an EPR spectrum, and it is proposed that divalent copper is the paramagnetic species. The spectrum shows no hyperfine structure and has the parameters, $g_{//} = 2.260$ and $g_{\perp} = 2.097$. Calculation of the copper-ligand bonding parameter indicated considerable ionic

character. The EPR signal was lost on addition of dithionite, whilst addition of ascorbate only reduced the signal intensity by 30% but changed the shape of the spectrum slightly.

5. Both copper and iron were detected in rubimedin after digestion of organic material, the ratio Cu:Fe being 1.06. The copper content could be chelated by diethyldithiocarbamate, the copper-diethyldithiocarbamate complex being characterised after extraction into carbon tetrachloride.

INTRODUCTION

The copper content of chloroplast material was first investigated by Neish (1939), who found that 75% of the copper content of whole leaves was contained in a crude chloroplast fraction. A copper analysis of a chloroplast fragment fraction from sugar beet (Whatley *et al* 1951) revealed that it contained 64% of the copper content of the whole leaf, and that there were 3 gram atoms of copper per 410 moles of chlorophyll. The chloroplast fragment fraction analysed by these workers did not contain intact chloroplasts as these were discarded after a preliminary low speed centrifugation, the fragments being subsequently centrifuged down at 20,000 *g* for 2 hours. It is probable that the fragment fraction also contained mitochondria, which possess the copper containing enzyme, cytochrome oxidase.

The copper content of spinach chloroplasts has been investigated (Katoh *et al* 1961), and the results obtained agreed with those of the previous workers, indicating approx-

imately 3 gram atoms of copper per basic photosynthetic unit of 400 moles of chlorophyll. The chloroplasts employed for analysis were centrifuged down at low speed, and so were probably intact rather than fragmented, and also probably free from gross mitochondrial contamination. When these chloroplasts were analysed for plastocyanin it was found that they contained an average of one gram atom of plastocyanin copper per 360 moles of chlorophyll. The plastocyanin was estimated spectrophotometrically using a molar extinction coefficient with respect to copper of 4900 at 597 nm which was subsequently re-confirmed (Katoh et al 1962) and is supported by the work described in Chapter 1.

The plastocyanin content of wild type Chlamydomonas was found to be equal to one gram atom of plastocyanin copper per 430 moles of chlorophyll (Gorman and Levine 1966 a). A recent analysis of spinach chloroplasts (Arnon et al 1968) was in agreement with the results of Katoh and co-workers, one gram atom of plastocyanin copper being found per 345 moles of chlorophyll. It is therefore apparent that one gram atom of plastocyanin copper is present per basic photosynthetic unit in chloroplasts.

Further analyses of chloroplasts for copper content have been published, but gross contamination by extraneous copper is indicated by the high values found. The total copper content of quantosomes was 20 gram atoms per 400 moles of chlorophyll (Park and Pon 1963). Anderson, Boardman and David (1964) found 7.5 gram atoms of copper per

400 moles of chlorophyll in chloroplasts and 6.5 gram atoms of copper per 400 moles of chlorophyll in digitonin particles sedimented at 144,000 g from which the plastocyanin had been solubilised (Arnon et al 1968).

Therefore the problem arose of the nature of the remaining two gram atoms of copper per basic photosynthetic unit, as no other copper-protein besides plastocyanin had been identified as a chloroplast constituent.

It was decided to carry out further analyses of chloroplasts for copper content to attempt to establish the excess of copper found over plastocyanin content. The isolation of spinach chloroplasts free from contaminating organelles was considered essential if copper analyses were to be meaningful. Because of the difficulties involved in avoiding contamination completely, it was decided to prepare chloroplasts by several different methods and note any consistency in the ratios of copper to chlorophyll found in the several preparations.

The normal procedure for chloroplast isolation from homogenates of spinach leaves is by differential centrifugation, the chloroplasts being sedimented rapidly at low centrifugal fields because of their large size. Contamination by nuclei is possible but mitochondria would be expected to be absent. However, recent work by Tolbert and co-workers has shown that such preparations of spinach chloroplasts do contain small amounts of mitochondria, as well as peroxisomes (Yamazaki and Tolbert 1969). Purification of broken chloroplasts could be achieved by isopycnic centrifugation on

a sucrose density gradient, however intact chloroplasts were not separated from mitochondria by this procedure.

A method has been described for Vicia faba which produced chloroplasts, having a broken bounding membrane, free from both mitochondrial and nuclear contamination (Kung and Williams 1968). The procedure employed was a combination of two methods previously published for purification of spinach chloroplasts: those of Jagendorf (1955) and of James and Das (1957). As discussed by Jagendorf, the separation of chloroplasts from other organelles by equilibrium density centrifugation is critically dependent upon the density of the chloroplasts themselves, which varies from one plant to another and even with age for the same plant. The densities chosen for the separation media should be such that the greatest possible yield of chloroplasts is obtained without contamination from other organelles. Many methods have appeared in the literature claiming to isolate pure chloroplasts with intact bounding membranes by the use of sucrose density gradients, however yields are low and the integrity of the chloroplasts is invariably not 100% (Leech 1964; Still and Price 1967; Harvey and Brown 1969).

The preparation of spinach chloroplasts by the nonaqueous technique on a density gradient has been shown to give a light fraction free from nuclei and cell debris (Thalacker and Behrens 1959). Electron microscopy has demonstrated that the nonaqueous chloroplasts of lowest density seen on isodensity centrifugation contained very little mitochondrial or cytoplasmic contamination (Stocking

et al (1968). The density of nonaqueous chloroplasts prepared in the usual hexane-carbon tetrachloride medium is greater than that of chloroplasts prepared by aqueous techniques because of the removal of lipids of low density (Thalacker and Behrens 1960). A good yield of pure nonaqueous chloroplasts is only obtained if the spinach plants are kept in the dark prior to harvesting in order to reduce the starch content. If this is not done the presence of the high density starch granules increases the chloroplast density to that of nuclei and other contaminants (Thalacker and Behrens 1959; Stocking 1959).

Whilst selecting methods of chloroplast preparation which avoided contamination it had also to be borne in mind that genuine copper-containing components of the chloroplast lamellae active in photosynthesis might be extracted. The bounding membrane of the chloroplast is very liable to damage and can be ruptured simply by pellet formation at low centrifugal force (Harvey and Brown 1969). The procedures of Tolbert and of Kung and Williams actually produce pure broken chloroplasts which have lost both bounding membrane and stroma. However in the isotonic or hypertonic solutions used, the chloroplast lamellae remain unswollen so that the components of the photosynthetic electron transport chain remain in situ, although soluble proteins of the stroma are washed out (Ridley and Leech 1968). Because of the difficulty of maintaining the integrity of intact chloroplasts during long purification procedures, it was decided to analyse both chloroplasts prepared

with minimum damage by a rapid differential centrifugation method and also broken chloroplasts purified on density gradients, rather than attempt to isolate pure intact chloroplasts in sufficient quantity for copper analysis.

The preparative method for nonaqueous chloroplasts ensures that no water-soluble component of the chloroplast lamellae is lost, and it must be regarded as unlikely that any copper containing component would be soluble in organic solvents, although it was claimed that a fraction of the copper content of quantosomes was soluble in organic solvents (Park and Pon 1963).

The isolation of rubimedin by Henninger and Crane (1966 a) raised the possibility of a new electron transport component of photosynthesis. During the preparation of plastocyanin from parsley and from spinach, described in Chapter 1, a red-coloured substance was found, of which the method of isolation is described later in this chapter. Analysis of this substance showed that it possessed the same absorption spectrum as rubimedin, and significantly for this work, that it contained copper.

The effect of rubimedin on the photochemical activities of chloroplasts has been described in three similar publications (Henninger and Crane 1966 a; Henninger and Crane 1967 a; Henninger and Crane 1967 b). Since these papers appeared no further work on rubimedin has been reported, and before rubimedin can be accepted as an electron transport component confirmation of the published isolation procedure

and of the activity of the protein would have to be presented by an independent group of workers. There is a possibility that the preparation described by Henninger and Crane contained both the substance rubimedin, an artefact inactive in photosynthetic electron transport, and another yet unidentified protein which has the ability to stimulate NADP reduction as described. The aqueous extract of heptane-treated chloroplasts contains many proteins and the purification procedure used was not rigorous (Henninger and Crane 1966 a).

In this chapter questions are raised concerning rubimedin and the possibility of a second copper-protein in chloroplasts, but no definite answers are given. However it is proposed that plastocyanin is the only copper-protein involved in photosynthetic electron transport, and that the substance rubimedin, isolated apparently only a few times by Henninger and Crane and only under peculiar circumstances described in the present work, is an artefact. It is however an artefact with rather specific properties.

MATERIALS AND METHODS

Copper content of chloroplasts. Chloroplasts were prepared from spinach grown in a greenhouse at 24° under a 16 hours light and 8 hours dark regime, normally for a period of 3-4 weeks. However in some work, leaves from more mature plants were required and the leaves were harvested after 5-6 weeks of growth. All centrifugations with chloroplasts in aqueous media were performed at 2° - 4°.

200 g spinach leaf was blended with twice its weight of 0.5 M sucrose + 0.05 M tris buffer pH 7.8 in a Sorvall Omnimixer at 0° for 2 x 20 sec. The homogenate was filtered through nylon net, centrifuged at 5,000 g for 25 minutes and the supernatant discarded. The chloroplast precipitate was resuspended in 0.5 M sucrose + 0.05 M tris buffer pH 7.8, centrifuged at 500 g for 3 minutes and the precipitate, containing large debris, was discarded. Whole chloroplasts were then precipitated at 1,200 g for 7 minutes, and resuspended in 15 ml 0.05 M tris buffer pH 7.8 to give preparation C_1.

The 1,200 g supernatant was spun further at 5,000 g for 25 minutes to give a broken chloroplast fraction, the supernatant then being discarded. The precipitate was resuspended in 0.5 M sucrose + 0.05 M tris buffer pH 7.8 to a volume of 15 ml, and 2.5 ml layered on each of six discontinuous sucrose gradients made following Yamazaki and Tolbert (1969). The gradients were made at 4° by pipetting into tubes of the Spinco SW 25·1 swinging bucket rotor, successively 6 ml 2.5 M, 6 ml 1.8 M, 8 ml 1.5 M and 8 ml 1.3 M sucrose solutions all containing 0.05 M tris buffer pH 7.8. The gradients were then centrifuged in a Spinco Model L centrifuge at 40,000 g for 2 hours. The broken chloroplast fragment zones in the 1.3 M sucrose layer were collected with a pipette and after diluting two fold with 0.05 M tris buffer pH 7.8, the fragments were centrifuged down at 5,000 g for 15 min. The pellet was resuspended in 6 ml 0.05 M tris buffer pH 7.8 to give the pure chloroplast fragment preparation T.

60 g leaves from mature spinach plants was blended with 0.5 M sucrose + 0.05 M tris buffer pH 7.8, and filtered. The filtrate was centrifuged at 500 g for 3 minutes, the precipitate discarded and the supernatant centrifuged further at 1,200 g for 7 minutes. The 1,200 g pellet of whole chloroplasts was resuspended in 10 ml 0.05 M tris buffer pH 7.8 to give preparation C_2.

100 g leaves from mature spinach plants was blended in 0.3 M sucrose + 0.05 M tris buffer pH 7.8, and the pellet of whole chloroplasts obtained as above. The chloroplasts were resuspended in 0.3 M sucrose + 0.05 M tris buffer pH 7.8 and placed on a two-layer discontinuous gradient made according to James and Das (1957). This was formed from glycerol and 0.3 M sucrose + 0.05 M tris buffer pH 7.8 respectively, in the proportions 60:40 and 25:75. After centrifuging in a swinging bucket rotor at 1,300 g for 10 minutes, the dark green chloroplast band at the interface was collected with a pipette. The chloroplasts were then precipitated at 1,200 g for 10 min. after diluting the suspension 4-fold with 0.3 M sucrose + 0.05 M tris buffer pH 7.8. The pellet was resuspended in 30 ml of medium of density 1.17 (Jagendorf 1955), and spun in a tube of the Spinco SW 25·1 swinging bucket rotor in a Spinco Model L centrifuge for 2 hours at 40,000 g. The dark-green top pellet was collected, diluted with 0.3 M sucrose + 0.05 M tris buffer pH 7.8 and the chloroplast fragments precipitated by centrifugation at 12,000 g for 15 min. The pellet was resuspended in 5 ml 0.05 M tris buffer pH 7.8 to give preparation J.

Nonaqueous chloroplasts were prepared from freeze dried spinach leaves as described by Thalacker and Behrens (1959). To prevent loss of chlorophyll from the chloroplasts, the freeze-dried material was blended in a Sorvall Omnimixer at -15° in a salt-ice bath and all centrifugations were performed at -5°. Chloroplasts of density between 1.25 and 1.30 were collected. The purified chloroplasts, after evaporation of organic solvent in vacuo, were resuspended in 5 ml 0.05 M tris buffer pH 7.8 to give preparation N.

Chlorophyll was measured according to Bruinsma (1963). The absorbance at 652 nm of a filtered 80% acetone solution of the chloroplast pigments was measured on a Cary 14 spectrophotometer, and the chlorophyll concentration in mg/ml calculated as $A_{652}/36$. An average chlorophyll molecular weight of 900 was assumed in calculations of moles of chlorophyll present. The Cary wavelength scale was shown to be accurate to one angstrom unit.

Copper in the chloroplast preparations was measured by atomic absorption spectrophotometry, after total destruction of the organic material by digestion with nitric and sulphuric acids, to an accuracy of 0.05 micrograms and with correction for reagent blanks. All glassware was cleaned with concentrated nitric acid, but polythene containers were used during the chloroplast preparations wherever possible.

Rubimedin. The isolation of rubimedin was found not to be reproducible, The experiments described in this chapter were performed on rubimedin obtained from one preparation from parsley. 6 kg parsley leaves was homogenised in

hypotonic buffer, the homogenate fractionated with acetone, and the 33 - 75% acetone precipitate dialysed and fractionated on a DEAE-cellulose column. The fraction eluting off the column with 0.1 M tris buffer pH 7.5 + 0.1 M NaCl was collected and stored at -20° for 3 weeks. Thus far the procedure was exactly as for a plastocyanin preparation described in Chapter 1. The solution after thawing had a more red-brown colour than before freezing but did not contain a precipitate. Solid ammonium sulphate was added to give a concentration of 35% w/v, the final pH being 6.85, and the red-brown precipitate collected by centrifugation at 17,000 g for 15 min at 4°. The precipitate was homogenised with 0.01 M $NaPO_4$ pH 7.0 containing 0.1 M NaCl, recentrifuged at 5,000 g for 10 min at 4°, and the brown precipitate discarded. The pink-yellow supernatant was centrifuged at 100,000 g for 1 hour at 4°, to give a red pellet and a yellow supernatant, the latter being discarded. The red pellet was resuspended in 3 ml buffer to give a clear solution, and was used in the investigations described later.

Rubimedin was found during some other plastocyanin preparations from parsley and spinach, but not consistently. Attempts to repeat the method of rubimedin preparation described by Henninger and Crane (1966 a) have failed.

Measurements of the EPR spectrum of rubimedin and calculation of the bonding parameter, a^2, were carried out as described in Chapter 1 for parsley plastocyanin.

Copper and iron analysis of the purified rubimedin was carried out, after complete destruction of organic material with H_2SO_4 and H_2O_2, using Boehringer test kit solutions

(Boehringer, Mannheim, Germany) with correction for reagent blanks. The standard copper and iron solutions provided with the kits were used for calibration of the spectrophotometric estimation. All glassware was cleaned with conc. HNO_3 to remove heavy metal contamination. To 0.1 ml rubimedin solution was added 0.05 ml M.A.R. conc. H_2SO_4 (36 N) and the solution heated. After cooling, 0.05 ml H_2O_2 (100 volumes) was added and the solution was re-heated to complete the oxidation, and destroy all traces of peroxide. The clear, colourless solution was diluted with water to 2 ml, and then 1 ml of either the copper or the iron reagent solution was added. The copper-bathocuproine disulphonate complex was measured at 480 nm and the iron-bathophenanthroline disulphonate complex was measured at 535 nm.

Amino-acid analysis of rubimedin was performed on material which had been dialysed against 1 mM sodium diethyldithiocarbamate in tris buffer pH 7.5 followed by extraction of the copper-diethyldithiocarbamate complex with carbon tetrachloride. The protein material was then dialysed extensively against 0.01 M KCl to remove any diethylamine produced by diethyldithiocarbamate decomposition. Hydrochloric acid was added to a final concentration of 6 N and the amino acid composition of this solution, after 24 hours hydrolysis at 108°, determined as described in Chapter 1, but omitting norleucine. To convert amounts of ammonia nitrogen to protein, a factor of 6.25 was used.

Rubimedin was also analysed for inorganic sulphur content using the methylene blue method described by Brumby et al (1965).

RESULTS AND DISCUSSION

Copper content of chloroplasts. The results of the analysis of purified chloroplasts for copper are shown in Table 3-1. The average value for the eight determinations was 1.48 gram atoms copper per basic photosynthetic unit, which is two times lower than that reported by Katoh *et al* (1961). No special precautions were taken against contamination during chloroplast preparations except that analytical grade chemicals and deionised water were used throughout, and all glassware was rinsed with conc. HNO_3, and plastic centrifuge tubes rinsed with an EDTA solution. On comparing preparations C_1 and C_2 it is seen that although duplicate samples corresponded well, the two preparations did not. It is possible the difference lay in the age of the leaves from which the chloroplasts were made. Preparation J from mature leaves also showed a higher copper content than preparation T, made from young expanding leaves. It is apparent also that the preparation of purified broken chloroplast fragments from whole chloroplasts did not result in the loss of a copper-containing component. The small quantity of nonaqueous chloroplasts available rendered the result with sample N liable to a greater error than for the other samples.

Properties of rubimedin. The absorption spectrum of parsley rubimedin is shown in Figure 3-1 (A). The only peak in the spectrum is at 478 nm, no peak being seen in the protein absorption region around 280 nm. On addition of ascorbate the spectrum was only partially bleached, and the difference spectrum of untreated rubimedin against ascorbate-reduced rubimedin showed a maximum at 475 - 478 nm. Dithionite completely bleached

TABLE 3-1. THE COPPER CONTENT OF PURIFIED SPINACH CHLOROPLASTS.

The methods of preparation of the chloroplasts are described in the Materials and Methods section. Total chlorophyll was determined according to Bruinsma (1963). Copper was determined by atomic absorption spectrophotometry after digestion with HNO_3/H_2SO_4.

Preparation*	Chlorophyll concentration mg/ml	Volume analysed ml	Chlorophyll content mg	Copper content microgr.	Copper per 400 moles chlorophyll** (g.atoms)
C_1	3.94	4.0	15.8	2.9	1.04
C_1	3.94	4.0	15.8	2.5	0.90
C_2	1.79	4.5	8.0	2.6	1.83
C_2	1.79	4.5	8.0	2.5	1.76
T	3.51	2.3	8.1	1.3	1.00
T	3.51	3.0	10.5	3.2	1.72
J	1.30	4.0	5.2	1.9	2.07
N	1.20	3.06	3.7	1.0	1.54

* The preparations are,

C_1 and C_2 : differential centrifugation at low centrifugal field.

T : density gradient method of Yamazaki and Tolbert (1969).

J : density gradient technique of James and Das (1957) combined with the method of Jagendorf (1955).

N : nonaqueous chloroplasts, the 1.25-1.30 density fraction of Thalacker and Behrens (1959).

** Copper content is expressed as the number of gramatoms found in one basic photosynthetic unit of 400 moles of total chlorophyll a and chlorophyll b, assuming an average molecular weight of 900 for chlorophyll.

the red colour of the rubimedin, a clear colourless solution being obtained. Addition of sodium hydroxide (final concentration 0.4% w/v) to rubimedin also resulted in the loss of the visible absorption, the difference spectrum of untreated against alkali-treated rubimedin showing a second peak at 320 nm (Figure 3-1 (B)). Rubimedin was precipitated when treated with 1.2 N HCl, but the red colour was not discharged; however 6 N HCl caused the rubimedin solution to change colour from red to yellow and then to colourless. The stability of the red chromophore to 1.2 N HCl distinguishes rubimedin from the transferrin-metal complexes which have similar absorption spectra (Feeney and Komatsu 1967).

The EPR spectrum of rubimedin is given in Figure 3-2. The spectra shown were obtained at -186°, but the spectrum was also seen at both -100° and -22°. The signal was attributed to divalent copper. The four spectra shown in Figure 3-2 were all obtained with the same sample of rubimedin, at first untreated, then ascorbate added and finally dithionite added. To add a reducing agent, the frozen rubimedin was thawed rapidly, ascorbate or dithionite added, the solution mixed and then refrozen. This procedure did not denature the rubimedin. When excess ascorbate was added the signal intensity was reduced by 30% and furthermore the relative heights of the two maxima in the spectrum appeared to change. When dithionite was then added, no EPR signal was observed, the spectrum being identical to the spectrum of the EPR sample cavity itself. The parameters of the parsley rubimedin EPR spectrum at -186° were

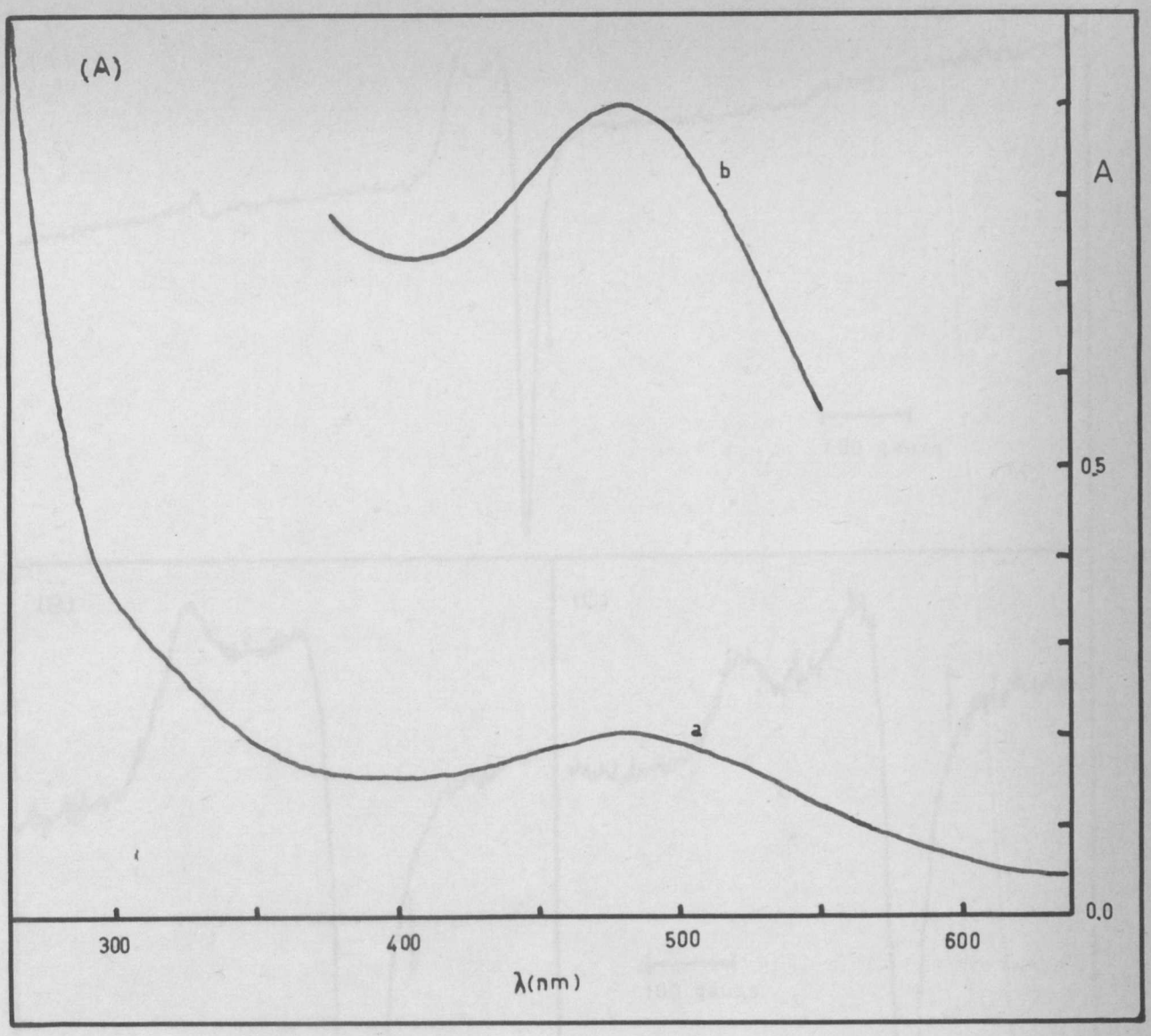
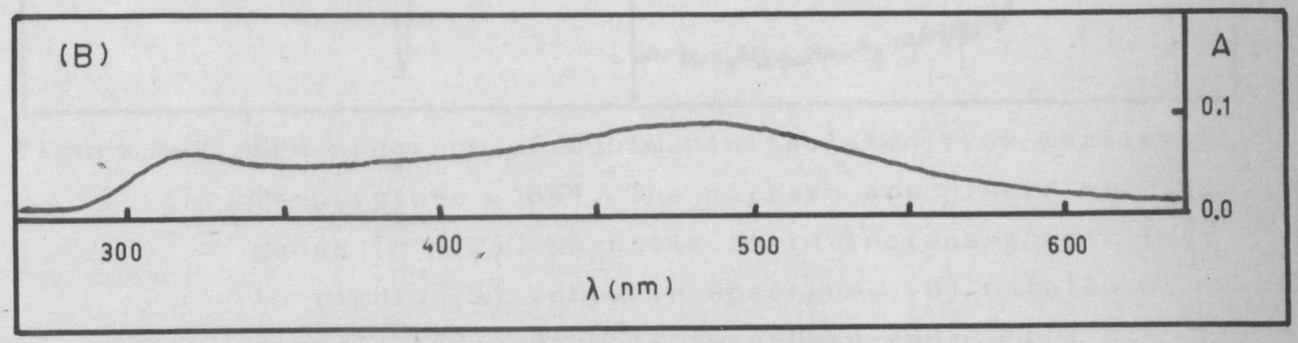


Figure 3-1. (A) Absorption spectrum of rubimedin isolated from parsley. Baseline is at zero absorbance; (a) the complete visible and ultraviolet spectrum; (b) a more concentrated solution clearly showing the absorption maximum at 478 nm.



(B) Difference spectrum of untreated rubimedin minus alkali-treated rubimedin. A second absorption maximum is revealed at 320 nm.

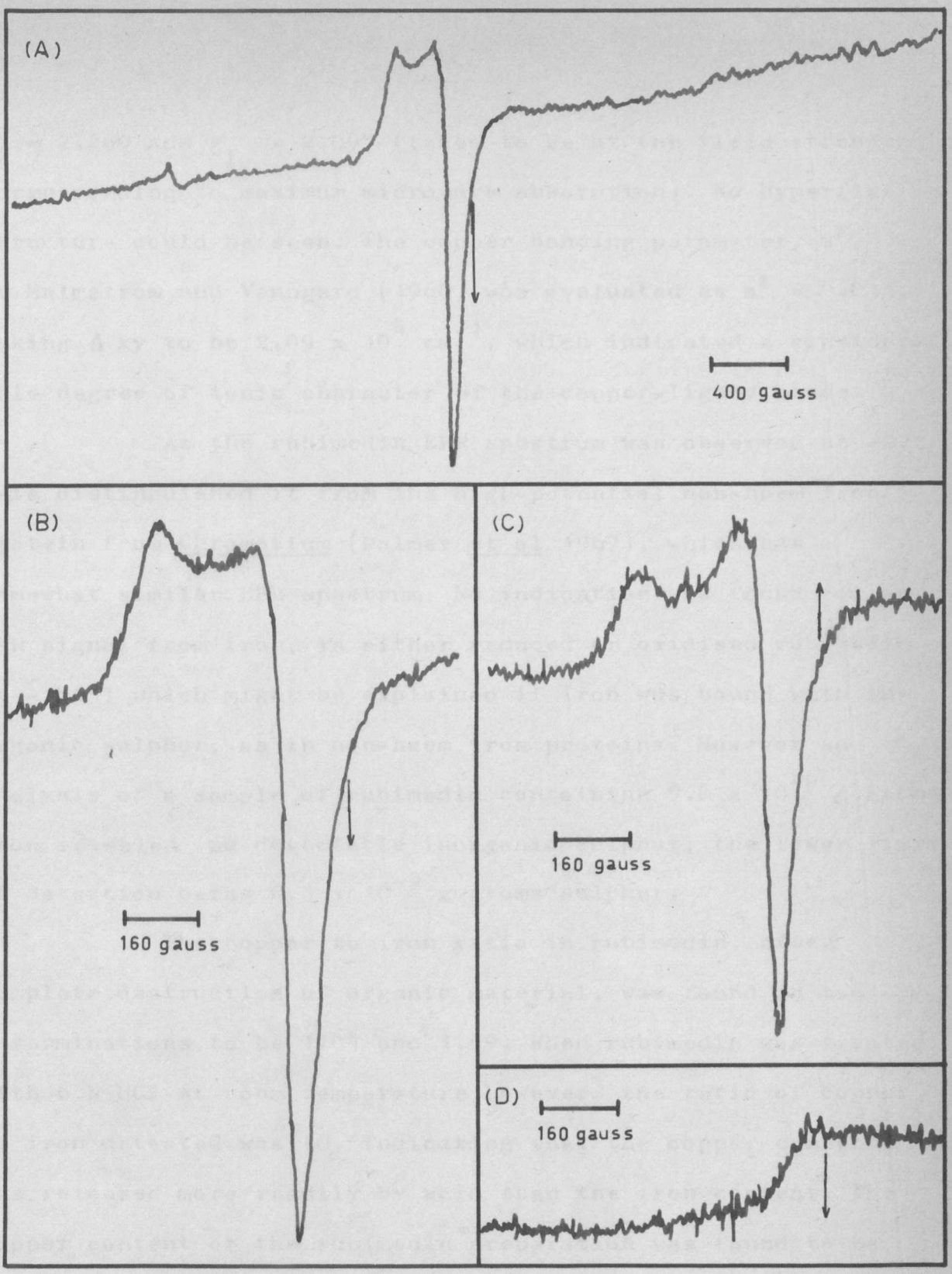


Figure 3-2. EPR spectrum of rubimedin isolated from parsley. Temperature -186°. The markers are placed at 3312 gauss (g = 2). Magnetic field increases from left to right. (A) complete spectrum, (B) details of the signal, (C) sodium isoascorbate added, (D) dithionite added.

$g_{//}$ = 2.260 and $g_{\perp}$ = 2.097 (taken to be at the field strength corresponding to maximum microwave absorption). No hyperfine structure could be seen. The copper bonding parameter, a^2, of Malmstrom and Vanngard (1960) was evaluated as $a^2 = 0.815$, taking Δ xy to be 2.09×10^4 cm^{-1}, which indicated a considerable degree of ionic character of the copper-ligand bonds.

As the rubimedin EPR spectrum was observed at -22° this distinguished it from the high-potential non-haem iron protein from Chromatium (Palmer et al 1967), which has a somewhat similar EPR spectrum. No indication was found for an EPR signal from iron, in either reduced or oxidised rubimedin at -186°; which might be explained if iron was bound with inorganic sulphur, as in non-haem iron proteins. However an analysis of a sample of rubimedin containing 9.8×10^{-9} g.atoms iron revealed no detectable inorganic sulphur, the lower limit of detection being 0.3×10^{-9} g.atoms sulphur.

The copper to iron ratio in rubimedin, after complete destruction of organic material, was found in two determinations to be 1.04 and 1.09. When rubimedin was treated with 6 N HCl at room temperature however, the ratio of copper to iron detected was 50, indicating that the copper content was released more readily by acid than the iron content. The copper content of the rubimedin preparation was found to be 0.407 microgram atoms/ml, thus the total copper content was 1.22 microgram atoms. From the same batch of parsley 4.46 microgram atoms of plastocyanin copper was obtained, and therefore the quantities of rubimedin and plastocyanin were quite

comparable. The molar absorbance coefficient of rubimedin at 478 nm was estimated to be 2,300 with respect to copper. On dialysis of rubimedin against bathocuproine disulphonate at pH 7.5 for 16 hours the red colour of the rubimedin remained unaffected, but when dithionite was added the orange-brown colour of cuprous-bathocuproine disulphonate appeared. When rubimedin was dialysed against 1 mM sodium diethyldithiocarbamate at pH 7.6 for 18 hours the pink colour was discharged and the absorption maximum at 465 nm of the rubimedin-copper-diethyldithiocarbamate complex appeared. The brown complex could be sedimented at 5,000 *g* for 5 minutes at 4° to leave a colourless supernatant. It is therefore proposed that the red colour of rubimedin arises from divalent copper bound to the protein.

A sample of rubimedin, after removal of copper as described in the Materials and Methods section, was analysed for amino acid composition. A total of amino acids equivalent to 0.45 mg protein/ml rubimedin preparation was found, which indicated 1100 grams of protein per gram atom of copper. However ammonia equivalent to 0.20 mg protein/ml was also detected, which may have arisen from amino acid degradation during hydrolysis, catalysed by traces of heavy metals remaining in the rubimedin.

0.8 ml diluted rubimedin preparation was homogenised with 2 ml methanol and 2 ml chloroform, when the rubimedin formed a pink layer at the interface of the two organic phases. As the chromophore was not extracted into organic solvent, rubimedin was distinguished from the red protein (a beta-carotene/protein complex) of Ji *et al* (1968).

Conclusions. It was the purpose of the work described in this chapter to decide whether or not a second copper protein was involved in photosynthetic electron transport. The analysis of chloroplasts for copper content was equivocal, and further work must be done to make a definite decision whether there is one or two gram atoms of copper per basic photosynthetic unit. Rubimedin has been demonstrated to contain copper, bound in a manner shown by its EPR spectrum to be distinct from that in plastocyanin (see Chapter 1), or in nonspecific copper-proteins formed on denaturation of blue copper-proteins. The EPR spectrum of copper bound nonspecifically is similar to that of copper in the Cu^{2+}-transferrin complex (Aasa and Aisen 1968) with a large hyperfine splitting in the low-field signal of about 150 gauss. The EPR spectrum of rubimedin shows, in contrast, no hyperfine splitting. Taken together with the observed copper to iron ratio close to unity, it suggests that rubimedin could be a genuine copper-containing component.

However the lack of corroborative reports of rubimedin isolation from groups of workers other than its discoverers, and the inconsistency with which rubimedin was isolated by the procedure given in the present work, must be regarded as obstacles to the acceptance of rubimedin as a chloroplast protein. At the present moment, there is no evidence to show that plastocyanin is not the only copper-protein involved in photosynthetic electron transport.

CHAPTER FOUR. INTERACTION OF PLASTOCYANIN AND P700

SUMMARY

1. A digitonin particle preparation was obtained from both chloroplasts and nonaqueous chloroplasts by incubation with 0.5% digitonin at 0° for 75 minutes at a digitonin to chlorophyll ratio of 11:1 (w/w), followed by removal of large fragments by centrifugation at 10,000 g.

2. In the dark the P700 content was kept reduced by an internal, endogenous pool of reductant. On illumination with blue light P700 was photo-oxidised, and in a subsequent dark period it was re-reduced. The spectrum of the measured changes corresponded with published oxidation-reduction spectra of P700. Addition of 0.016 mM DCMU did not affect the dark reduction rate, indicating lack of PS2 reducing activity.

3. Addition of small amounts of plastocyanin, in the reduced form, prior to illumination, caused an increase in the subsequent dark reduction rate of P700. The increase of dark reduction rate in chloroplast particles was proportional to the square of the plastocyanin concentration, but in nonaqueous chloroplast particles it was linearly proportional to plastocyanin. Methylviologen did not inhibit the plastocyanin-induced increase. To obtain a 50% decrease in the half-time of dark reduction of P700 in chloroplast particles, an amount of added plastocyanin was required equal to only 5.4% of the P700 content. A similar decrease in half-time in nonaqueous chloroplast particles was obtained with plastocyanin equal to 6.9% of the P700 content.

4. DCPIP could also increase the dark reduction rate of P700 but amounts in excess of the P700 content were required, and furthermore methylviologen could inhibit the DCPIP-induced increase.

5. The degree of photo-oxidation of P700 was found to increase as illumination was continued up to 60 seconds, when the steady state level of oxidation was almost attained. As the illumination period was increased from 5 seconds to 60 seconds the half-time for P700 dark reduction in the subsequent dark period increased correspondingly from 9 seconds to 40 seconds.

6. Addition of a many-fold excess of plastocyanin over P700 content resulted in the P700 remaining in the reduced state on illumination, because of rapid donation of electrons through plastocyanin to oxidised P700.

INTRODUCTION

The type of experiment to be described in this chapter is similar to that employed by Hind, in which plastocyanin was added back to detergent-treated chloroplasts, deficient in plastocyanin, and the effect on the light-induced oxidation-reduction changes of P700 was followed with a dual wavelength spectrophotometer (Hind 1968). It may be argued that in this type of experiment there can be no certainty that the added plastocyanin will return to the site occupied in vivo, however it will be shown that extremely small amounts of plastocyanin, in relation to P700, are capable of causing

large increases in the rate of dark reduction of P700, implying a highly specific interaction. A further uncertainty in this technique is that alterations may occur in the elctron transport pathways on fragmenting the chloroplasts in order to release the endogenous plastocyanin. It is possible that artefactual cyclic electron transport pathways from the reductive terminus to P700 are produced, for it is known that 0.5% digitonin solubilises some of the cytochrome *f* and cytochrome b_6 content, and all of the ferredoxin and ferredoxin-NADP reductase in addition to plastocyanin (Arnon *et al* 1968). For the present work to be meaningful these artefacts had to be minimised.

The chloroplast digitonin particles used in this work were prepared according to the procedure of Anderson and Boardman (1966), with a low concentration of digitonin (0.5%) to minimise the disruption of electron transport pathways and the solubilisation of the cytochromes *f* and b_6 whilst completely extracting plastocyanin (Arnon *et al* 1968). In order that solubilised cytochromes should not be lost from the system it was decided to employ in these experiments the supernatant after centrifugation of the digitonin-treated chloroplasts at 10,000 g. This supernatant being perfectly clear avoids the interference in spectrophotometric work associated with light-scattering heavy particles. It is also enriched in PS1 content compared to untreated chloroplasts. The digitonin particles remaining in the supernatant after centrifugation have only a small fraction of their original plastocyanin still

bound to the particles. The endogenous plastocyanin released from the chloroplasts and present in the 10,000 g supernatant was found in the present work to have a negligible activity compared to plastocyanin added subsequently. It is known that digitonin does not denature plastocyanin even at a concentration of 1.3%, for the protein released from chloroplasts on treatment with 1.3% digitonin showed electron transport activity when added back to PS1 particles (Wessels 1965). It is possible that the endogenous plastocyanin released by 0.5% digitonin treatment is enclosed within, or is on the surface of detergent micelles, where its ability to interact directly with P700 is greatly diminished, whereas plastocyanin added subsequently will be in a freely soluble form.

It has been shown (Arnon et al 1968) that the small chloroplast particles, present in the 10,000 g supernatant after incubation of chloroplasts with 0.5% digitonin, can reduce NADP when ascorbate-DCPIP is used as the electron source and the proteins ferredoxin, ferredoxin-NADP reductase and plastocyanin are added back. Furthermore they are capable of cyclic photophosphorylation with PMS as cofactor. This is in complete agreement with many reports of the stability of PS1 activity towards digitonin treatment. It has been demonstrated that the particles present in the 10,000 g supernatant are virtually inactive in oxygen evolution and the transfer of electrons from water to P700 may be neglected (Anderson and Boardman 1966; Henninger et al 1967). Therefore, with no artificial electron source added to the 10,000 g supernatant, P700 can only be

reduced by the cyclic backflow of electrons from the reductive terminus of PS1 along a pathway which may represent the cyclic pathway in vivo.

The electron transport pathway of cyclic photophosphorylation is a subject of considerable uncertainty. Studies with algal mutants, shown by analysis to be lacking certain electron transport carriers, have provided useful results in identifying the necessary components of the cyclic pathway. In Chlamydomonas, chloroplasts from the mutant lacking plastocyanin did not carry out PMS catalysed cyclic photophosphorylation, whilst the lack of cytochrome 553, a cytochrome f analogue, only reduced the phosphorylation rate slightly compared to the wild type rate (Gorman and Levine 1965). The conclusion was that plastocyanin, but not cytochrome 553, was required for cyclic photophosphorylation. However, a Scenedesmus mutant lacking cytochrome f was found not to possess cyclic photophosphorylation in vivo as measured by anaerobic glucose assimilation, although it did carry out PMS catalysed cyclic photophosphorylation (Powls et al 1969), demonstrating the difference between the cyclic electron pathway in vivo and that when a cofactor is added.

Arnon et al (1968) found that digitonin particles, sedimented at 144,000 g, could carry out cyclic photophosphorylation with PMS as cofactor but not with ferredoxin or menadione as cofactor. It was apparent that different cyclic electron pathways were involved with different cofactors. Plastocyanin, when added back to the particles, did not alter the phosphorylation

rate with PMS nor restore cyclic photophosphorylation with ferredoxin or menadione, and so it is probable that in digitonin particles PMS is able to interact directly with P700 and does not require plastocyanin as an intermediate. It is the hypothesis in the present work that plastocyanin is the only natural electron donor to P700 (Hind and Olson 1968; Levine 1969) and as cyclic electron flow through PS1 depends on P700 then plastocyanin must be regarded as an essential component of the cyclic electron transport pathway in vivo, as well as the noncyclic electron transport pathway. The cyclic pathway seen in the digitonin particles used in the present work is taken to be the same as that present in vivo, involving cytochromes *f* and b_6 and plastocyanin (see Figure 1 in the general introduction), as in the schemes of cyclic electron flow proposed by Levine (1969) and also by Hildreth (1968). The scheme adopted requires X, but does not involve ferredoxin in cyclic flow. It has been claimed that cyclic photophosphorylation induced by addition of ferredoxin to chloroplasts represents the in vivo cyclic photophosphorylation as it was found to be inhibited by antimycin A (Tagawa *et al* 1963), however it was recently shown that antimycin A inhibited PMS catalysed phosphorylation as well (Drechsler *et al* 1969), making this evidence indecisive.

Although cytochromes *f* and b_6 are still present in the chloroplast particles prepared with 0.5% digitonin they are removed more completely by 1.3% digitonin. It has been found that in purified PS1 particles prepared from chloroplasts with 1.3% digitonin (Wessels 1966) plastocyanin also catalyses

an increased rate of dark reduction of P700 as seen in the present work. However, ascorbate is required to be present and the reduction of P700 probably does not take place by cyclic electron flow but rather by donation of electrons from ascorbate via plastocyanin as intermediate (Voorn and Wessels, personal communication).

The involvement of plastoquinone in PS1 reactions is also discussed in this chapter because of the report that plastoquinone C was an essential component for the noncyclic transport of electrons from ascorbate-DCPIP to NADP via PS1, implying a close association with plastocyanin and P700 (Henninger and Crane 1967 a). The extraction of plastoquinones A and C caused a large decrease in the above activity which was restored by re-addition of plastoquinone C. However the assay system employed (Keister et al 1962) did not include plastocyanin, and it is apparent that the authors regarded plastocyanin as an alternative for DCPIP and did not use them together (Henninger and Crane 1966 a). After extraction of chloroplasts with heptane, plastocyanin is released by subsequent aqueous treatment (Elstner et al 1968). It is known that plastocyanin is an essential intermediate between DCPIP and P700 (Wessels 1966), and that any PS1 activity seen without addition of plastocyanin to plastocyanin deficient chloroplasts can be ascribed to remaining endogenous plastocyanin (Davenport 1965). Therefore the restoration of electron flow to P700 in the absence of plastocyanin must be an artefact, the addition of plastoquinone C allowing direct donation of electrons from reduced DCPIP to P700 without plastocyanin.

It is established that plastoquinone A is required for cyclic photophosphorylation with either PMS, menadione or flavinmononucleotide as cofactor (Whatley and Horton 1963). However it is accepted that the location of plastoquinone A is at a site near the reductive terminus of PS2 and not close to P700 (see Figure 1 in the general introduction).

An analysis of digitonin particle fractions from spinach chloroplasts to locate the site of action of plastoquinones A and C did not yield clear-cut results (Henninger et al 1967) because of the complications arising from the presence of osmiophilic globules or plastoglobuli in chloroplasts. These are lipid storage particles which contain a large fraction of the plastoquinone content of the chloroplast (Hind and Olson 1968). Therefore the distribution of plastoquinones between PS1 and PS2 could not be decided in this manner.

In the present work the role of plastoquinone in the cyclic electron pathway around PS1 was investigated by following the light-induced oxidation-reduction changes of P700 as described above, but with extracted chloroplasts. The chloroplasts were prepared by the nonaqueous technique, and extracted with heptane for four hours at room temperature to remove both plastoquinones A and C (Henninger and Crane 1966 b), before resuspension in aqueous buffer and treatment with digitonin. The results obtained were compared to the results with unextracted chloroplast digitonin particles to note any effects of the removal of plastoquinone C.

In order to follow the redox state of P700 in the chloroplast particles the difference in absorption at 700 nm and at 740 nm is followed on a dual wavelength spectrophotometer. No other electron carrier shows light-induced absorbance changes in this spectral region except plastocyanin whose contribution to the absorption difference would be negligible. In its reduced form P700 has an absorption maximum at 700 nm, but in the oxidised form this absorption band is bleached; no significant absorption change occurs at 740 nm and this is employed as the reference wavelength (Kok 1961). The molar absorbance coefficient of the reduced form at 700 nm is not known, but a value of 100,000 is usually assumed and is employed in this work (Beinert and Kok 1964). Recently a value of 42,000 has been estimated (Schliephake *et al* 1968), but this would appear to be small for the molar extinction coefficient of a chlorophyll *a* species at the red peak maximum. In chloroplasts there is one P700 per basic photosynthetic unit of 400 moles of chlorophyll, and in purified PS1 particles the ratio chlorophyll:P700 is found to be approximately 200 as would be expected for a molecule which is the unique reaction centre for PS1 (Wessels 1966). As discussed in the review of Levine (1969), Kok has advanced the hypothesis that P700 may not be a distinct molecular entity, but rather one of the molecules of C705 (a chlorophyll *a* species absorbing maximally at 705 nm) which becomes unique by virtue of excitation by light and transfer of an electron from plastocyanin to X. It has been recently found that in spinach

chloroplasts the reduction of P700 measured spectrophotometrically could only account for approximately one-fifth of the electron transfer shown to occur during the time taken to complete P700 reduction (Malkin 1968). Of the three explanations proposed by Malkin, the presence of a pool of electron acceptors at the redox potential of P700 is preferred, as the redox potentials of plastocyanin, cytochrome f and possibly also a fraction of the cytochrome 559 content (Bendall 1968) are close to that of P700.

The experimental procedure used in the present work is to follow the reduction of P700 in the dark, as the dark equilibrium redox state is re-established, immediately after an extended period of actinic light during which time the P700 redox state had been shifted towards almost complete oxidation. The electrons to reduce P700 are present as a pool of reduced components at the reductive terminus of PS1 which is built up during the light period by the action of PS1. This reducing pool can be built up in the absence of ferredoxin but is dependent on X, the electron acceptor species of PS1.

Electrons from X may be transferred in a noncyclic pathway either to ferredoxin and thence to NADP via ferredoxin--NADP reductase, or to oxygen either directly or via the catalyst methyl viologen the reduced form of which is a semiquinone rapidly oxidised by molecular oxygen. Oxidised DCPIP can also accept electrons from the reductive terminus of PS1, being rapidly reoxidised by the electron carriers at the oxidative terminus of PS1 in an external cyclic pathway (Ke 1967).

The electrons may also be transferred internally from X to P700 and it is this cyclic electron flow which is involved in the present work. The scheme of electron transport from X is presented in Figure 4-1, and is similar to that described in a recent article on the back flow of electrons in PS1 (Fujita and Murano 1968). The redox potential of X has been estimated to be -700 mV (Kok et al 1965) whilst that of P700 has been found to be about +430 mV at pH 7 (Kok 1961).

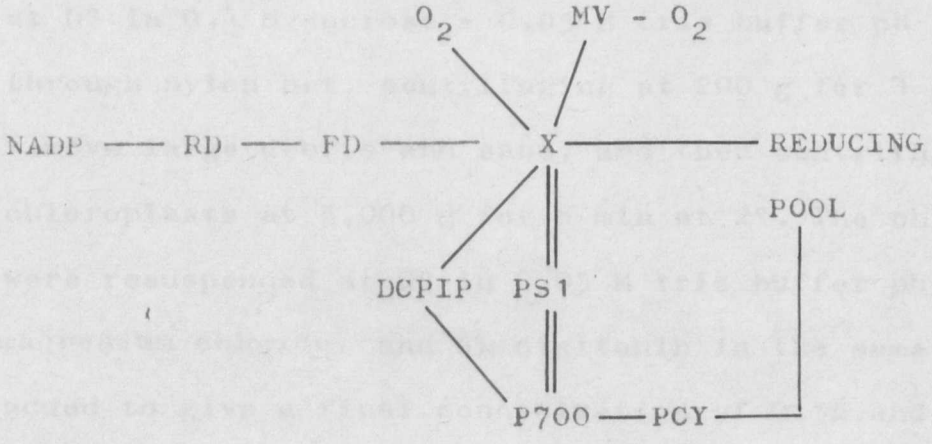


Figure 4-1. Paths of electron transport from X, the electron acceptor of PS1. RD is the ferredoxin-NADP reductase, and PCY is plastocyanin.

MATERIALS AND METHODS

Materials. Purified plastocyanin was prepared from spinach by the method of Borchert and Wessels (personal communication), and was in the reduced form in 10 mM KPO_4 pH 7 at a concentration of 1.0 mg/ml. For use in the plastocyanin

titration experiments the solution was diluted and 5 microlitre aliquots were added. Methyl viologen (1.5 mM) and DCPIP (0.15 mM) were freshly made solutions, the DCPIP being filtered before use. DCMU was added as a 4 mM solution in 50% ethanol. All the chloroplast digitonin particles used in this work were prepared from spinach leaves harvested from 3-4 weeks old plants grown in a greenhouse as described in Chapter 3.

Aqueously-prepared chloroplasts were made by grinding freshly-picked spinach leaves with acid-washed sand at 0° in 0.4 M sucrose + 0.05 M tris buffer pH 7.8, filtering through nylon net, centrifuging at 200 g for 1 min at 2° to remove large debris and sand, and then centrifuging down the chloroplasts at 5,000 g for 8 min at 2°. The chloroplasts were resuspended at 0° in 0.05 M tris buffer pH 7.0 + 0.01 M magnesium chloride, and 4% digitonin in the same buffer was added to give a final concentration of 0.5% and a digitonin to chlorophyll ratio of 11:1. After incubation at 0° for 75 min, the suspension was centrifuged in the Spinco SW 39 swinging bucket rotor at 4° for 30 minutes at 10,000 g. The 10,000 g supernatant was used as the aqueous chloroplast digitonin particle preparation after dilution to a chlorophyll concentration of 73 mg/litre with 0.05 M tris buffer pH 7.0 + 0.01 M $MgCl_2$.

Nonaqueous chloroplasts were prepared, as described in Appendix A, in hexane-carbon tetrachloride at -5°. The chloroplasts, equivalent to 10 mg of chlorophyll, were sedimented, resuspended in 200 ml heptane and placed on a

rotary shaker at 24° for two hours. A connection was maintained between the chloroplasts and the air to further oxidation of plastoquinols to plastoquinones, as the latter are more readily extracted. During the extraction the chloroplasts were protected from light. After two hours shaking, the chloroplasts were centrifuged down, 200 ml fresh heptane added, and shaking continued for a further two hours. The chloroplasts were centrifuged again, the sediment transferred with a little fresh heptane to a beaker which was placed in a desiccator containing P_2O_5, and the heptane removed on evacuation. The chloroplasts were resuspended in 0.05 M tris buffer pH 7.0 + 0.01 M $MgCl_2$, digitonin was added as for the aqueous chloroplasts, and the 10,000 g supernatant so obtained, after dilution to 73 mgs chlorophyll/litre, was employed as the nonaqueous chloroplast digitonin particle preparation.

In one experiment only, shown in Figure 4-4 (B), a preparation of digitonin particles was made from nonaqueous chloroplasts prepared as described in Appendix A, without further extraction by heptane. The chloroplasts free of organic solvent were resuspended as for the other chloroplasts in 0.05 M tris buffer pH 7.0 + 0.01 M $MgCl_2$, digitonin was added to a final concentration of 0.5%, but the digitonin to chlorophyll ratio was 4:1 only. The details of incubation and centrifugation were as for the preparation described above. When mentioned elsewhere in the text this digitonin particle preparation is referred to specifically, and is differentiated from the nonaqueous chloroplast digitonin particle preparation.

Methods. All experiments were performed on an Aminco-Chance dual-wavelength spectrophotometer equipped with a flat-bed recorder whose maximum sensitivity corresponded to a full scale deflection equal to an absorbance change of only 0.005. Actinic side illumination was provided by a tungsten lamp whose intensity remained constant. A spring-loaded shutter allowed an accurate timing of the period for which the sample was illuminated. Two filters were placed between the lamp and the sample: a blue perspex filter showing no transmission in the visible above 570 nm, and an infrared filter (Balzer IR 1256/283), which cut off infra-red radiation. The lamp filament was focussed in the plane of the sample cuvette, and an intensity of 90 W/m^2 was measured in the sample space with a YSI-Kettering Model 65 Radiometer. The absorbance measured was the difference in absorbance of the sample at 700 nm and 740 nm, $A = A_{700} - A_{740}$. An oxidation of P700 resulted in a bleaching of its absorption band at 700 nm, which was seen as a decrease in absorbance, and recorded as a downward movement of the pen on the recorder. The photo-multiplier was protected from the actinic side illumination by placing a red filter (Schott RG 5) directly in front of it. The voltage supplied to the lamp providing the measuring light-beam of wavelengths 700 and 740 nm, could be continuously varied, allowing a variation in the measuring beam intensity. The measuring beam slit width was kept constant at 0.5 mm, equivalent to a bandpass of 3 nm. The sample was placed in an Aminco cuvette, clear on all four sides, with pathlength

10 mm and width 5 mm. All of the measuring beam passed through solution which was being illuminated by blue light. The size of the hole in the cuvette holder allowing side illumination had to be enlarged to achieve this condition, and allow illumination of the whole of the sample in the cuvette. This modification was made after the experiment with nonaqueous chloroplasts shown in Figure 4-4 (B), and the poor configuration of side illumination during that experiment might account for the apparently low activity of plastocyanin found.

The redox state of P700 in the 10,000 *g* supernatants was very sensitive to light. Additions were made to a sample in a darkened room, the sample was placed in the cuvette compartment with a light-tight cover, and left for at least five minutes to reach the dark steady state before commencing measurements. The measuring beam was found to affect the redox state of P700 appreciably, causing an oxidation, and therefore the measuring beam was continuously passed through the sample during the dark equilibration period. The effect recorded on illuminating with blue light was therefore the transition between two light regimes: very low intensity far red light; and a combination of intense blue light with the far red light.

The dark reduction rate of P700 was measured by following the value of ΔA with time; ΔA being the difference between A at time t and the final steady level of A reached in the dark. The plot of the logarithm of ΔA against time was linear up to a point corresponding to completion of about

80% of the dark reduction, and it is this slope $(\frac{d \log \Delta A}{dt})$ which is referred to as the dark reduction rate of P700.

Chlorophyll concentrations were measured as described in Chapter 3.

To estimate P700 concentration, the following procedure was adopted. The solution of chloroplast digitonin particles at a chlorophyll concentration of about 10 mg/litre was placed in the sample cuvette and the value of A was noted whilst the blue light illuminated the sample, giving what is assumed to be complete P700 oxidation (A_o). The side illumination was cut off, a trace of sodium isoascorbate was added and the measuring beam was also cut off from the sample. After 1 minute the measuring beam was allowed to pass through the sample and the value of A recorded. The measuring beam was once more cut off and the sample once again left in total darkness for 1 minute. The value of A was then remeasured and usually found to have remained steady. This value corresponded to complete P700 reduction (A_R). Assuming a molar absorbance coefficient of 10^5, the molar concentration of P700 was calculated as $A_R - A_o/10^5$. The value of $A_R - A_o$ was about 0.005 and was easily measurable.

RESULTS AND DISCUSSION

When a solution of chlorophyll a in acetone was observed in the Aminco-Chance, under the conditions used in the experiments described in this chapter, no effect of the

fluorescence induced by the blue actinic light was seen on the recorder. Therefore chloroplast fluorescence would not be expected to interfere with the absorbance measurements made. Scattered actinic light (maximum emission at 455 nm) was prevented from reaching the photomultiplier by the red filter. The principle of operation of the spectrophotometer rules out interference of delayed light emission in the measurement of the absorbance difference, $A_{700} - A_{740}$. The absorbance changes ascribed to P700 oxidation-reduction on illumination with blue light were shown to have the spectrum of P700 (Kok 1961) and to be reproducible in successive light periods.

When 16 micromolar DCMU was added to aqueous chloroplast particles containing 0.46 n moles P700 and with 0.6 micrograms of plastocyanin present, no inhibition was seen of the dark reduction rate. PS2 did not therefore supply the electrons which reduced P700 in the dark.

Figure 4-2 shows the effect of adding small amounts of plastocyanin to a preparation of chloroplast digitonin particles. The photo-oxidation of P700 was unaffected at the low concentration sufficient to increase the dark reduction rate significantly (B); but in (C) the amount of photo-oxidation observed was decreased; and when in (D) a 15-fold molar excess of plastocyanin was present, no photo-oxidation was seen. It was shown in a control experiment that excess plastocyanin caused the P700 to remain reduced upon illumination; and the lack of absorbance change was not due to the P700 being oxidised in the dark. Note that in (D) the absorbance and time scales are magnified compared to those in (A), (B) and (C).

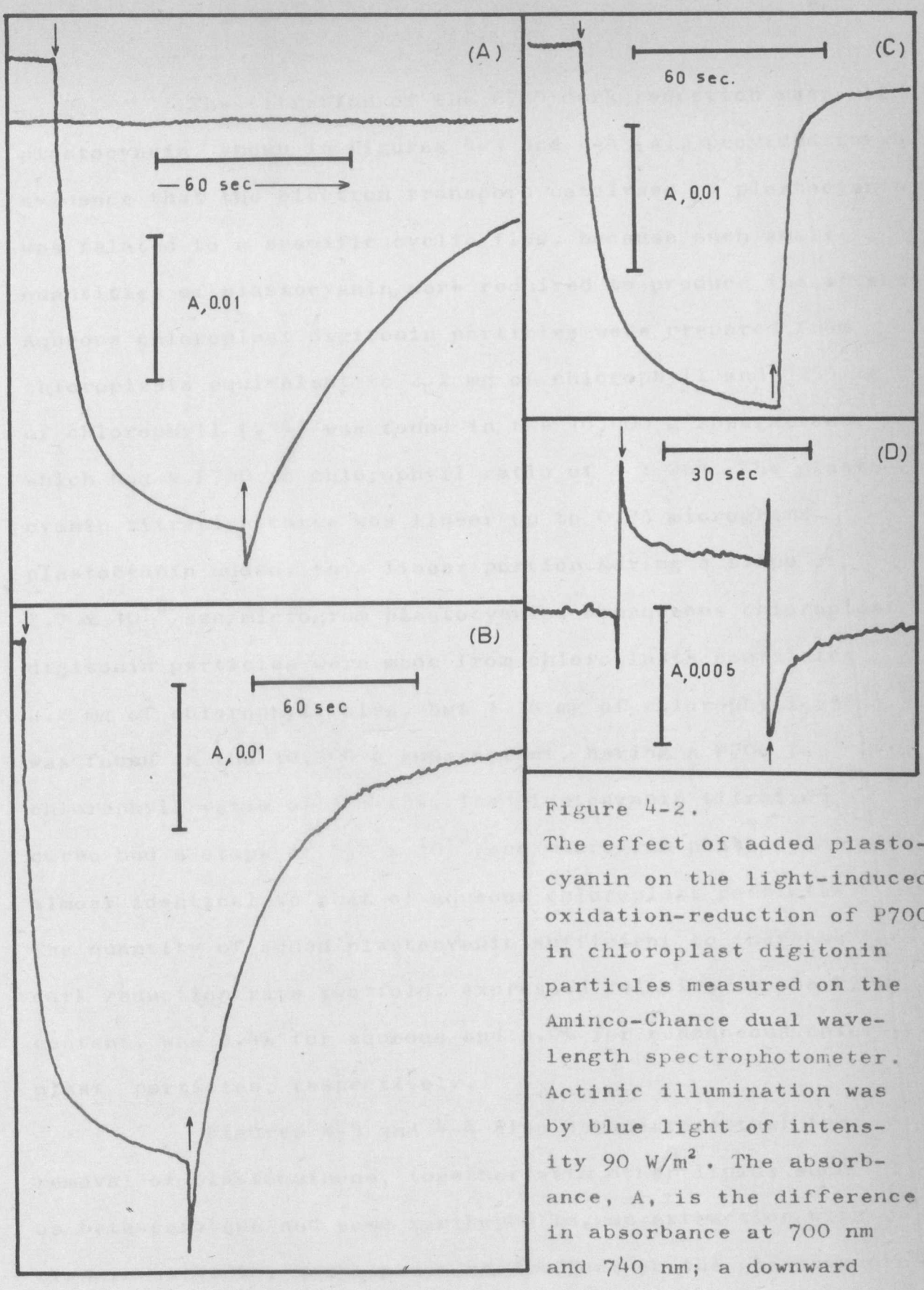


Figure 4-2.
The effect of added plastocyanin on the light-induced oxidation-reduction of P700 in chloroplast digitonin particles measured on the Aminco-Chance dual wavelength spectrophotometer. Actinic illumination was by blue light of intensity 90 W/m^2. The absorbance, A, is the difference in absorbance at 700 nm and 740 nm; a downward movement corresponds to a decrease in this absorbance and an oxidation of P700. The amount of plastocyanin added to 1.5 ml chloroplast particles containing 0.46 n moles P700 was (A) none, (B) 0.03 n moles, (C) 0.2 n moles, (D) 7.5 n moles. In (A) the final dark steady state level is shown.

The titration of the P700 dark reduction rate with plastocyanin, shown in Figures 4-3 and 4-4 (A), provided further evidence that the electron transport catalysed by plastocyanin was related to a specific cyclic flow, because such small quantities of plastocyanin were required to produce the effects. Aqueous chloroplast digitonin particles were prepared from chloroplasts equivalent to 2.2 mg of chlorophyll and 0.55 mg of chlorophyll (25%) was found in the 10,000 g supernatant, which had a P700 to chlorophyll ratio of 1 : 264. The plastocyanin titration curve was linear up to 0.25 micrograms plastocyanin added, this linear portion having a slope of 2.7×10^{-2}/sec/microgram plastocyanin. Nonaqueous chloroplast digitonin particles were made from chloroplasts containing 2.2 mg of chlorophyll also, but 1.16 mg of chlorophyll (53%) was found in the 10,000 g supernatant, having a P700 to chlorophyll ratio of 1 : 238. The plastocyanin titration curve had a slope of 3.0×10^{-2}/sec/microgram plastocyanin, almost identical to that of aqueous chloroplast particles. The quantity of added plastocyanin sufficient to increase the dark reduction rate two fold, expressed relative to the P700 content, was 5.4% for aqueous and 6.9% for nonaqueous chloroplast particles, respectively.

Figures 4-3 and 4-4 also demonstrate that the removal of plastoquinone, together with other lipids such as beta-carotene and some xanthophylls, on extraction with organic solvents, has a pronounced effect on the plastocyanin catalysed dark reduction of P700. Whereas in aqueous chloroplast particles, the dark reduction rate becomes proportional

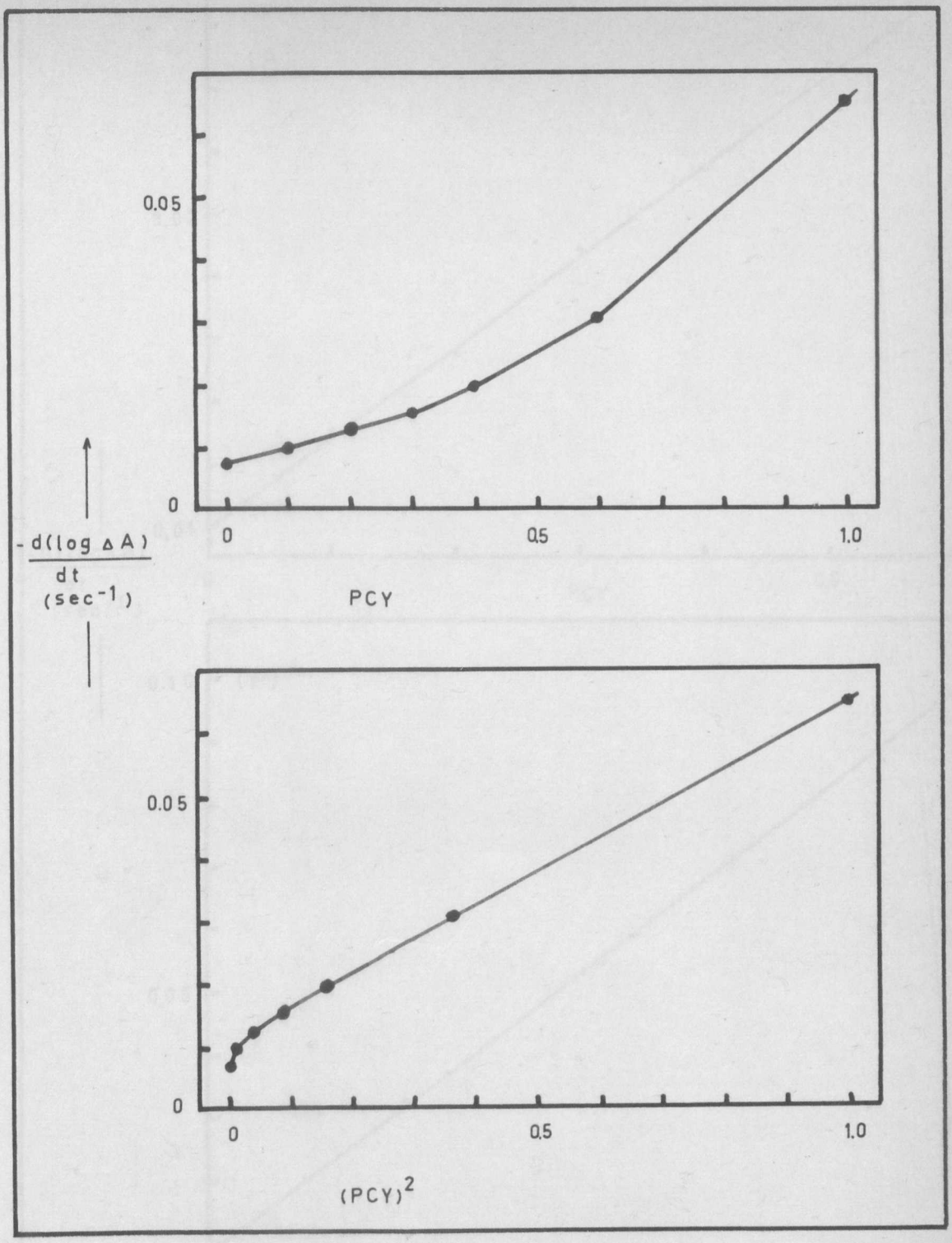


Figure 4-3. Titration by plastocyanin of the dark reduction rate of P700 in aqueously-prepared chloroplast digitonin particles. See legend to Figure 4-2 for experimental details. PCY is the amount of plastocyanin added, in micrograms of protein, to 1.5 mls of particles containing 0.46 n moles P700. ΔA is the difference between A at time t, and the final dark steady state level of A. The dark reduction rates found were plotted against both plastocyanin added and the square of the plastocyanin added.

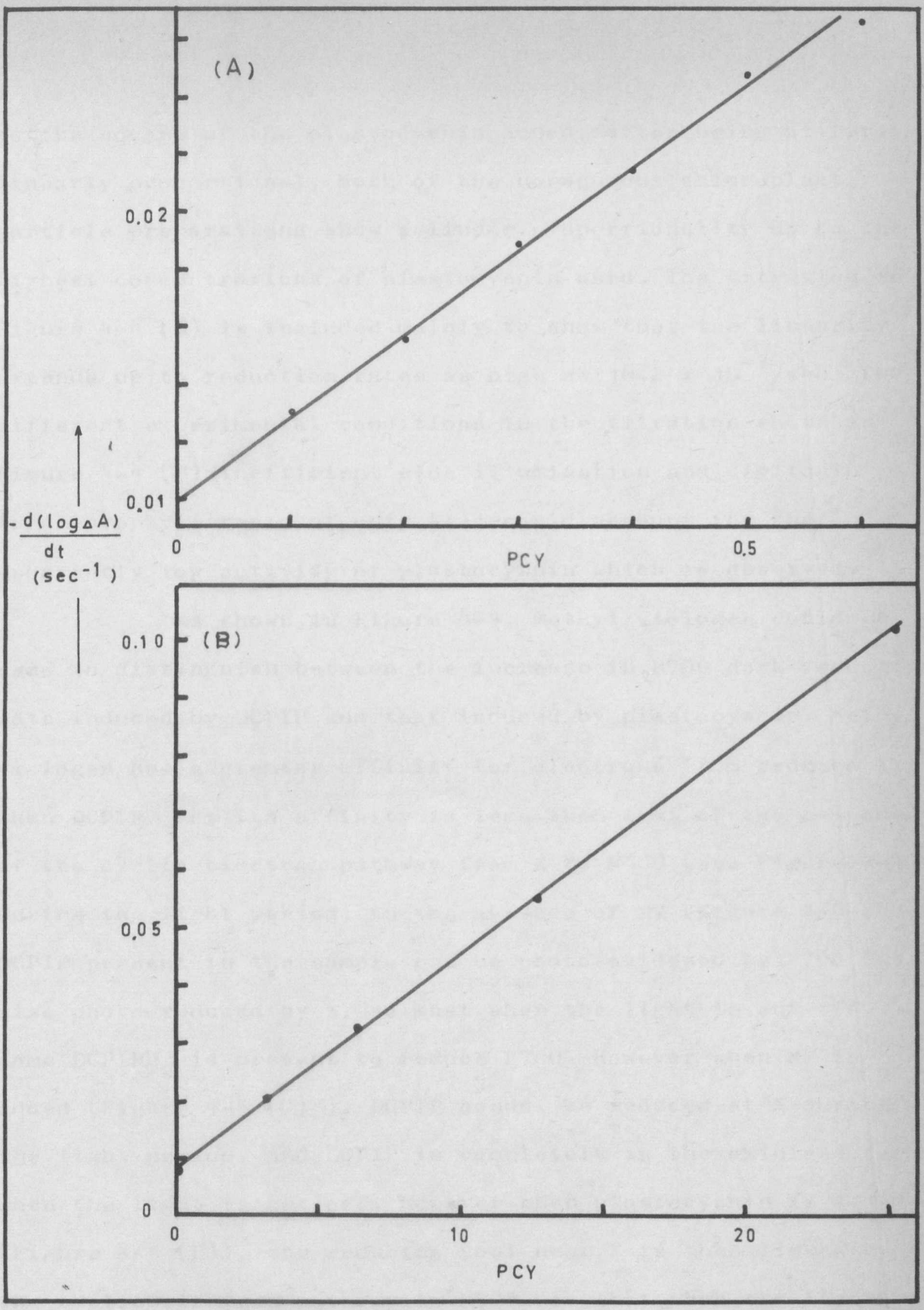


Figure 4-4. Plastocyanin titration of the P700 dark reduction rate in nonaqueous chloroplast digitonin particles. (A) 1.5 ml containing 0.51 n moles P700, plastoquinones A and C extracted. (B) particles from which only plastoquinone A was extracted, experimental conditions were different from the titrations shown in Figure 4-3 and in (A): see text for details.

to the square of the plastocyanin added, after being at first linearly proportional, both of the nonaqueous chloroplast particle preparations show a linear proportionality up to the highest concentrations of plastocyanin used. The titration in Figure 4-4 (B) is included mainly to show that the linearity extends up to reduction rates as high as 10.2×10^{-2}/sec. The different experimental conditions in the titration shown in Figure 4-4 (B) (inefficient side illumination and digitonin to chlorophyll ratio of only 4:1) could account for the apparently low activity of plastocyanin which is observed.

As shown in Figure 4-5, methyl viologen could be used to distinguish between the increase in P700 dark reduction rate induced by DCPIP and that induced by plastocyanin. Methyl viologen has a greater affinity for electrons from reduced X than DCPIP, but its affinity is less than that of the components of the cyclic electron pathway from X to P700 (see Figure 4-1). During the light period, in the absence of MV (Figure 4-5 (B)), DCPIP present in the sample can be photo-oxidised by P700 but also photo-reduced by X, so that when the light is cut off, some $DCPIPH_2$ is present to reduce P700. However when MV is added (Figure 4-5 (C)), DCPIP cannot be reduced at X during the light period, and DCPIP is completely in the oxidised form when the light is cut off. However when plastocyanin is added (Figure 4-5 (D)), the reducing pool near X is then linked by the cyclic electron pathway to P700, so that when the light is now cut off, electrons are available to reduce P700 from the reducing pool even though methyl viologen is present. In agreement with these observations, no inhibition of P700 dark

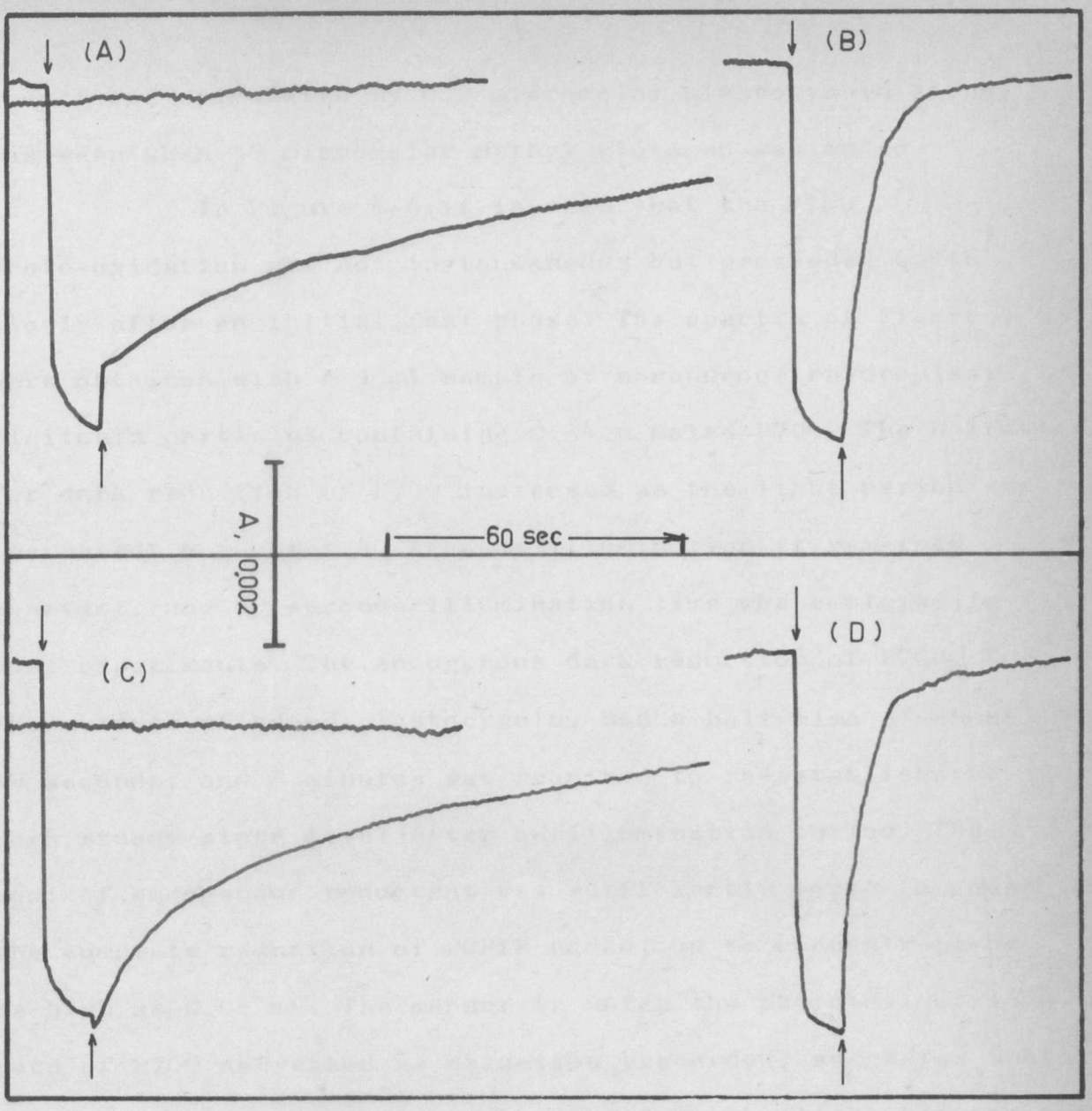


Figure 4-5. P700 oxidation-reduction changes in chloroplast digitonin particles: the effect of methyl viologen. (A) particles with no addition; (B) 5 micromolar DCPIP added; (C) then 50 micromolar methyl viologen added; and finally (D) 0.3 micromolar plastocyanin added also. The sample volume of 1.5 ml contained 0.13 micromolar P700. In (A) and (C) the final dark steady state level is shown. For details of abbreviations and methods see the legend to Figure 4-2.

reduction, stimulated by 0.2 micromolar plastocyanin alone, was seen when 50 micromolar methyl viologen was added.

In Figure 4-6 it is seen that the P700 photo-oxidation was not instantaneous but proceeded quite slowly after an initial fast phase. The spectra of Figure 4-6 were obtained with a 1 ml sample of nonaqueous chloroplast digitonin particles containing 0.34 n moles P700. The half-time for dark reduction of P700 increased as the light period was increased, but after 40 seconds illumination it remained constant, and 60 seconds illumination time was employed in most experiments. The endogenous dark reduction of P700, in the absence of added plastocyanin, had a half-time of about 40 seconds, and 5 minutes was required to re-establish the dark steady state level after an illumination period. The pool of endogenous reductant was sufficiently large to cause the complete reduction of DCPIP added, up to concentrations as high as 0.08 mM. The manner in which the photo-oxidation rate of P700 decreased as oxidation proceeded, suggested that an equilibrium state was being reached in the light between a photo-oxidation, and a photo-reduction by a direct leakage of electrons from reduced X. When illumination ceased, dark reduction by endogenous reductant then occurred. The half-time for P700 dark reduction increased as the illumination period was extended, indicating that the pool of electron donors constituting the endogenous reductant could be gradually photo-oxidised by PS1 through P700.

A peculiar effect was seen in Figure 4-6, curve E, when the voltage of the lamp providing the measuring light

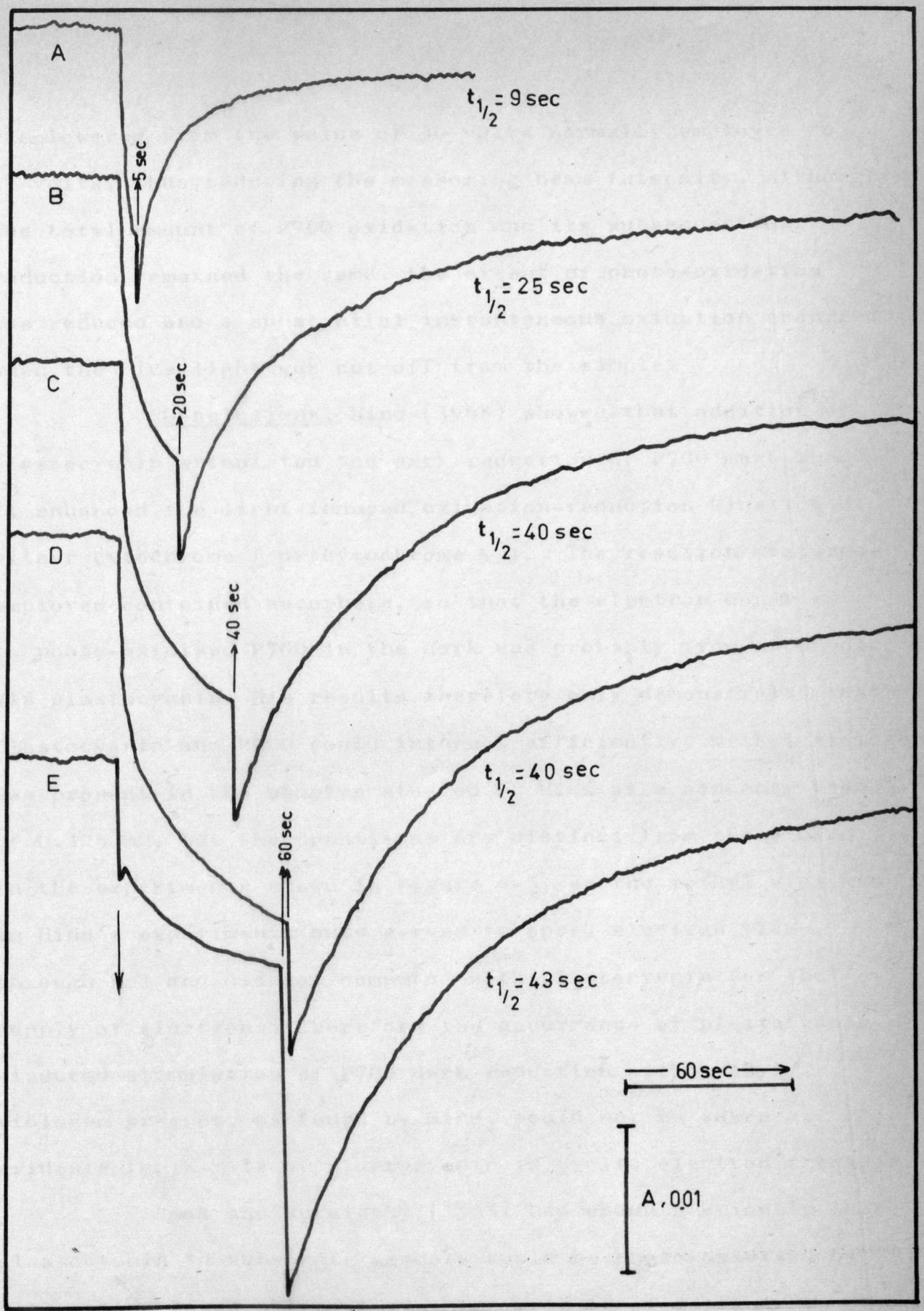


Figure 4-6. Effect on P700 oxidation-reduction changes in chloroplast digitonin particles of increasing the light period. The measuring lamp of the Aminco-Chance was used at 30 volts for A-D but at 17 volts for E, otherwise D and E are identical. The half times shown are for the dark reduction rate. See Figure 4-2 for other details.

was lowered from the value of 30 volts normally employed to 17 volts, thus reducing the measuring beam intensity. Although the total amount of P700 oxidation and its subsequent dark reduction remained the same, the extent of photo-oxidation was reduced and a substantial instantaneous oxidation occurred when the blue light was cut off from the sample.

Conclusions. Hind (1968) showed that addition of plastocyanin stimulated the dark reduction of P700 more than it enhanced the light-induced oxidation-reduction kinetics of either cytochrome f or cytochrome 559. The reaction mixtures employed contained ascorbate, so that the electron donation to photo-oxidised P700 in the dark was probably from ascorbate via plastocyanin. His results therefore only demonstrated that plastocyanin and P700 could interact efficiently. Methyl viologen was present in the samples studied by Hind at a concentration of 0.125 mM, but the conditions are distinct from those used in the experiments shown in Figure 4-5, as the methyl viologen in Hind's experiments only served to speed electron flow through PS1 and did not compete with plastocyanin for the supply of electrons. Therefore the occurrence of plastocyanin--induced stimulation of P700 dark reduction with methyl viologen present, as found by Hind, could not be taken as evidence for a role of plastocyanin in cyclic electron transport.

Kok and Kurainski (1965) had shown previously that plastocyanin in substrate amounts could be photo-oxidised by detergent-treated chloroplasts. The rate of oxidation was proportional to the concentration of reduced plastocyanin, as

expected for a reaction in which one molecule of reduced plastocyanin transferred an electron to one molecule of oxidised P700. The concentrations of plastocyanin used were greater than 80 times the P700 concentration of the chloroplasts.

In the present work, the results illustrated in Figure 4-5 show that plastocyanin can stimulate the dark reduction rate of plastocyanin by a pathway from X which is resistant to methyl viologen inhibition. This pathway is proposed to be the cyclic electron transport pathway around PS1, which in vivo allows cyclic photophosphorylation. The extremely high efficiency of the added plastocyanin in catalysing the P700 dark reduction, supports the hypothesis that the plastocyanin returns to its specific site in the 10,000 g chloroplast digitonin particles, as a complex with P700.

The titration of the dark reduction in chloroplast particles shown in Figure 4-3 can be interpreted to imply that two molecules of plastocyanin are involved in the rate-limiting step of cyclic electron flow, which in these plastocyanin-deficient particles is presumably at the point of donation of electrons from plastocyanin to P700. This result is unexpected as it has been found that chloroplasts contain plastocyanin and P700 in a 1:1 ratio (Arnon et al 1968), and both plastocyanin and P700 are one-electron acceptors.

When chloroplasts are extracted with organic solvents, carotenoids and plastoquinones are removed. However as shown in Figure 4-4 the stimulation of the dark reduction of P700 by plastocyanin still occurred. The role of beta-carotene in

photosynthesis has been proposed to be light energy collection for supply to the photochemically active chlorophyll (Goedheer 1969), and it cannot be regarded as essential for electron transport (see Appendix A). The plastoquinones are redox carriers active in electron transport; however, their absence from the chloroplast particles did not prevent the reduction of photo-oxidised P700 by plastocyanin, which is in disagreement with the finding of Henninger and Crane (1967 a) that plastoquinone C was required for PS1 reactions. Although electron donation to P700 was not prevented by extraction with hexane-carbon tetrachloride (Figure 4-4 (B)), the dark reduction rate was found to be linearly proportional to the plastocyanin concentration, rather than to the square of the plastocyanin concentration. It is proposed that the interaction of P700 with plastocyanin was modified by a configurational change of the PS1 reaction centre on removal of beta-carotene. The electron transfer reaction giving rise to the proportionality of dark reduction rate to the square of plastocyanin added above 0.25 micrograms (Figure 4.3), was prevented from occurring by this configurational change.

The instantaneous oxidation, seen when the blue actinic light was cut off in curve E of Figure 4-6, was also noted in experiments with chloroplasts treated with electron transport inhibitors (see Chapter 5).

CHAPTER FIVE. THE SITES OF INHIBITION OF SALICYLALDOXIME, CUPFERRON AND ORTHOPHENANTHROLINE

SUMMARY

1. The inhibition of electron transport was studied in spinach chloroplasts prepared in 0.4 M sucrose containing 0.05 M KPO_4 pH 6.5 and 0.01 M NaCl, and resuspended in the hypotonic buffer, 0.05 M KPO_4 pH 6.5 + 0.01 M NaCl. These chloroplasts did not photoreduce ferricyanide unless 2 mM ammonium chloride was added to uncouple photophosphorylation.

2. 10 mM salicylaldoxime completely inhibited photoreduction of both 1 mM ferricyanide and 0.1 mM DCPIP, but photoreduction of 0.033 mM cytochrome c was not inhibited.

1.1 mM cupferron inhibited ferricyanide reduction by 52%, but 10 mM cupferron was required to inhibit DCPIP photoreduction by 37%. Cytochrome c photoreduction was inhibited 37% by 10 mM cupferron, but the degree of inhibition was unstable and increased during illumination of the chloroplasts. Orthophenanthroline, like DCMU, inhibited all photoreductions in the chloroplasts.

3. The degree of inhibition of DCPIP reduction by 5 mM salicylaldoxime was found to be dependent on the light intensity, increasing with increasing actinic light intensity.

4. The photoreduction of DCPIP by heat-inactivated chloroplasts, which had been restored by addition of 11 mM hydroxylamine, was inhibited by 10 mM salicylaldoxime.

5. The effect of the inhibitors on the fluorescence yield induction in the chloroplasts was investigated. Orthophenanthroline produced an effect identical to that seen with DCMU. Cupferron appeared to reduce the fluorescence yield by acting as an electron acceptor similar to ferricyanide. The effect of salicylaldoxime was again different, and appeared to give an effect similar to that produced by heating chloroplasts.

6. The effect of the inhibitors on the light-induced oxidation-reduction changes of P700 in the chloroplasts appeared to indicate that they inhibited electron transport in different ways. On illumination with blue light, chloroplasts showed a small degree of P700 oxidation which was rapidly reversed in the dark. The photosystem 2 inhibitor, DCMU, did not change the degree of photo-oxidation, but caused a rapid, very large further oxidation of P700 to occur when illumination was ceased, followed by a slow dark reduction phase. Addition of 10 mM cupferron produced very little effect. Addition of 10 mM salicylaldoxime, like heating the chloroplasts at 50° for 5 minutes, caused a much greater degree of P700 oxidation to appear on illumination, compared to untreated chloroplasts; further oxidation was seen on ceasing illumination, followed by a dark reduction phase, as noted for DCMU. Addition of 0.1 mM orthophenanthroline gave an effect intermediate between that of heating and of addition of DCMU.

INTRODUCTION

The inhibition of photosynthetic oxygen evolution by salicylaldoxime was first noted in whole Chlorella cells (Green et al 1939). Inhibition by a low concentration of salicylaldoxime could be reversed by dialysis. Salicylaldoxime is known to be able to form a complex with cupric ions - $Cu(C_7H_6NO_2)_2$ - and it was therefore proposed that the inhibition was caused by chelation of essential copper by salicylaldoxime. Studies of the effect of copper deficiency on the ability of chloroplasts to reduce DCPIP in the Hill reaction suggested that a copper-containing component of the electron transport pathway from water to DCPIP existed on the reducing side of PS2 (Spencer and Possingham 1960). The reduction of DCPIP by chloroplasts from copper deficient plants was 60% of the control rate.

When Trebst (1963) investigated the inhibition by salicylaldoxime of chloroplast photoreactions he assumed that plastocyanin was the site of inhibition of the chelating agent. He found that 10 mM salicylaldoxime inhibited oxygen evolution, ferricyanide reduction and coupled noncyclic photophosphorylation, and cyclic photophosphorylation with menadione as cofactor, in hypotonically broken chloroplasts. Reduction of NADP by water was inhibited but the partial noncyclic pathway from the donor couple ascorbate-DCPIP to NADP was not. Trebst concluded that salicylaldoxime inhibited electron transport at a site between the two light reactions and not on the oxygen evolution side of PS2, as shown in Figure 5-1.

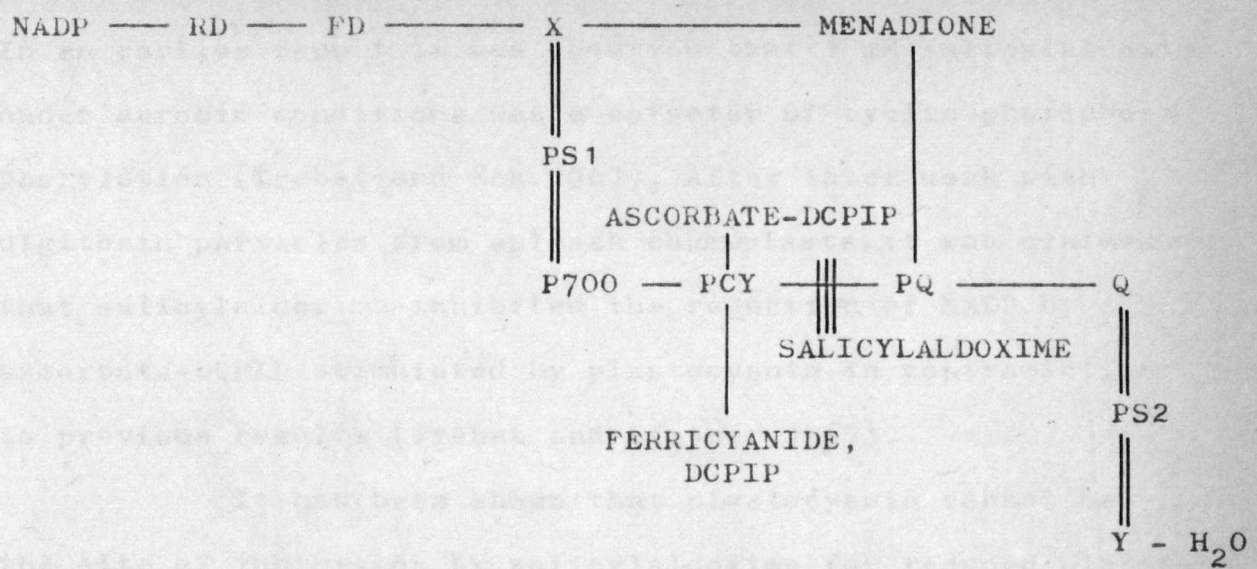


Figure 5-1. The site of salicylaldoxime inhibition indicated by the results of Trebst (1963). The abbreviations for the electron transport components are defined in the legend to Figure 1 in the general introduction.

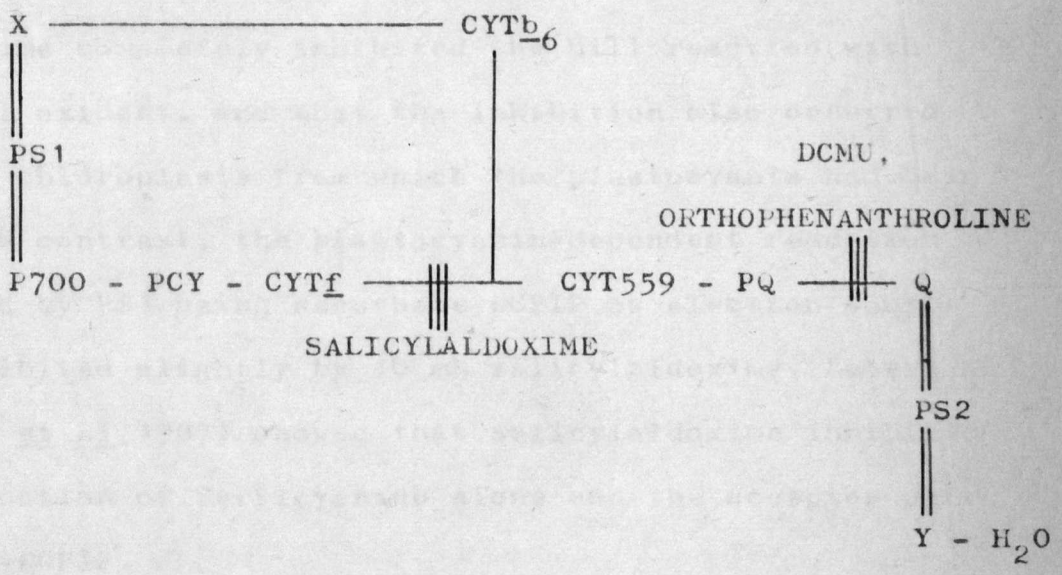


Figure 5-2. The site of salicylaldoxime inhibition indicated by the results of Hildreth (1968). The site of inhibition of orthophenanthroline and DCMU is also shown. The abbreviations used are defined in the legend to Figure 1 in the general introduction.

In an earlier report it was observed that 1 mM salicylaldoxime under aerobic conditions was a cofactor of cyclic photophosphorylation (Trebst and Eck 1963). After later work with digitonin particles from spinach chloroplasts, it was claimed that salicylaldoxime inhibited the reduction of NADP by ascorbate-DCPIP stimulated by plastocyanin, in contradiction to previous results (Trebst and Elstner 1967).

It has been shown that plastocyanin cannot be the site of inhibition by salicylaldoxime, for reduced plastocyanin was not affected by incubation with 10 mM salicylaldoxime for 30 minutes, and although oxidised plastocyanin was 50% denatured in two hours, this rate was too slow to be able to account for the inhibition of electron transport in chloroplasts, which is apparent immediately after addition of salicylaldoxime (Katoh and San Pietro 1966). These authors observed that 10 mM salicylaldoxime completely inhibited the Hill reaction with DCPIP as Hill oxidant, and that the inhibition also occurred in sonicated chloroplasts from which the plastocyanin had been liberated. In contrast, the plastocyanin-dependent reduction of methyl red by PS1 using ascorbate-DCPIP as electron source was only inhibited slightly by 10 mM salicylaldoxime. Later work (Renger et al 1967) showed that salicylaldoxime inhibited both the reduction of ferricyanide alone and the acceptor pair ferricyanide-DCPIP.

From the above experimental results it became apparent that salicylaldoxime inhibited electron transport between PS2 and PS1 at a site distinct from plastocyanin and

probably close to the reductive terminus of PS2. There was the possibility that this site was a second copper containing component of the photosynthetic electron transport chain.

Working with the marine green alga Ulva lobata, Fork and Urbach (1965) attempted to locate the inhibition site by following the effect of salicylaldoxime on light-induced absorbance changes in vivo. There was some doubt over their assumption that the absorbance change measured at 591 nm represented redox changes of plastocyanin, however, later work appeared to justify this, because the spectrum of the absorbance change around 591 nm could be fitted to the oxidation--reduction difference spectrum of plastocyanin (Fork and de Kouchkovsky 1968). Further doubt about this work arose from the length of time required to achieve inhibition. Complete inhibition of the absorbance change at 591 nm required a preincubation with 10 mM salicylaldoxime for 20 minutes, which might however be explained by the requirement of the inhibitor to be transported across the cell membrane to the site of inhibition. However results on cytochrome f redox changes obtained by preincubation with salicylaldoxime for periods of up to one hour must be regarded with circumspection as no effect on these changes was found for incubation periods less than 15 minutes. From these studies it may be concluded that short-term incubation with 10 mM salicylaldoxime inhibits the transfer of electrons from PS2 to plastocyanin, whereas long-term incubation denatures the plastocyanin.

The inhibition of cytochrome oxidation-reduction changes has been investigated by Hildreth (1968), and it was found that 4 mM salicylaldoxime inhibited electron transport at a site between cytochrome *f* and cytochrome b_6, for the kinetics of photo-oxidation of cytochrome *f* were unaffected by the inhibitor whereas cytochrome b_6 photo-oxidation was inhibited. This confirmed a previous report that 10 mM salicylaldoxime inhibited the photo-oxidation of cytochrome b_6 (Chance *et al* 1966). As cytochrome b_6 is believed to lie on the cyclic electron pathway (Boardman and Anderson 1967), it may be concluded that salicylaldoxime can inhibit both non-cyclic and cyclic electron transport, which requires that the site of inhibition be at a point common to both pathways and prior to cytochrome *f*. This is illustrated in Figure 5-2.

A report has appeared recently on the inhibition of photophosphorylation in algae by low concentrations of salicylaldoxime (Tanner *et al* 1969). It was found that 1 mM salicylaldoxime could strongly inhibit glucose assimilation in *Chlorella*, whilst the oxygen evolution and carbon dioxide fixation reactions were not affected. The effect of 1 mM salicylaldoxime was apparently an uncoupling of phosphorylation from electron transport, as assimilation of glucose in the dark, dependent on oxidative phosphorylation, was also inhibited. The inhibition of cyclic photophosphorylation was also noted by Urbach and Simonis (1964) and contrasts with the lack of inhibition reported by Trebst and Eck (1963) for 1 mM salicylaldoxime.

Cupferron was employed by Lightbody and Krogmann (1967) as a specific copper chelating agent which could inhibit photosynthetic electron transport in apparently the same manner as salicylaldoxime. Reduction of methyl red at the reductive terminus of PS1 by electrons from water was inhibited by 1 mM cupferron, but the partial noncyclic pathway from ascorbate--DCPIP to methyl red requiring only PS1 was not inhibited. The possibility of a second copper containing component between PS2 and PS1 would therefore be increased if it could be shown that the two copper chelating agents, salicylaldoxime and cupferron, inhibited electron transport at the same site.

Since it was first reported that orthophenanthroline could strongly inhibit the Hill reaction, it has been assumed that it inhibited by virtue of its metal-chelating properties (Arnon 1950). Krogmann and Jagendorf (1959) found that the inhibition could be reversed readily by dialysis, which would not be expected if inhibition was caused by chelation of a metal ion. The effect of orthophenanthroline on the fluorescence induction of algae was shown to be similar to that of phenylurethane (DCMU) suggesting an identical site of inhibition (Kautsky et al 1960). This was confirmed by studies on the fluorescence induction of isolated spinach chloroplasts (Murata et al 1966). The site of inhibition of DCMU is known to be immediately after the quencher Q of PS2 fluorescence and before plastoquinone in the electron transfer sequence from PS2 to PS1 (Kok and Cheniae 1966). It is probable therefore that orthophenanthroline acts at this site also, rather than

inhibiting a metal-containing enzyme, so that orthophenanthroline provides an example of an electron transport inhibitor which, although a potent metal chelator, does not inhibit electron transport by virtue of its metal chelating ability.

The three chelating agents have been studied in the present work in three types of experiment with spinach chloroplasts: spectrophotometric measurement of the Hill reaction, fluorescence induction measurements and a study of the light induced redox changes of P700.

The PS2 activity of chloroplasts has been measured with three Hill oxidants: DCPIP, cytochrome c and potassium ferricyanide.

The points along the electron transport pathway from the reductive terminus of PS2 at which DCPIP and ferricyanide can accept electrons has been discussed in a recent article (Fork and Amesz 1969). It has been shown that both plastoquinone A (Bishop 1959) and cytochrome 559 (Levine 1968) are required for DCPIP and ferricyanide reduction. Gorman and Levine (1965) found that both DCPIP and ferricyanide photoreductions were greatly diminished in Chlamydomonas mutants lacking either plastocyanin or cytochrome 553, indicating that the site of reduction was at or beyond these components. The claim that plastoquinone C was the site of DCPIP reduction (Henninger and Crane 1967 a) is not supported by other evidence and is probably the result of an artefact as discussed in Chapter 4. Chloroplasts from Scenedesmus mutants lacking cytochrome f were found to have a negligible DCPIP photoreduction rate compared to chloroplasts from the wild type (Powls et al 1969).

It has been reported in a preliminary note that the detergent Tween 20 inhibited the Hill reaction in chloroplasts with ferricyanide or NADP as Hill oxidant, but not that with DCPIP as Hill oxidant (Krogmann et al 1968). The reduction of NADP by PS1 with ascorbate-DCPIP was unaffected by Tween 20 treatment, and so the inhibition by Tween 20 occurred between the two light reactions, possibly by solubilisation of plastocyanin. The lack of inhibition of DCPIP photoreduction could be explained if the detergent opened up a second site for DCPIP reduction, unavailable to ferricyanide, on the PS2 side of the site of Tween 20 inhibition.

Pratt and Bishop (1968) found that a mutant of Scenedesmus lacking an active P700 could still carry out the photoreduction of ferricyanide and DCPIP, although at a reduced rate compared to the wild type. Another group of workers found for the same mutant that ferricyanide could not elicit the electron paramagnetic resonance signal or absorbance change at 700 nm typical of P700, although ferricyanide could accept electrons from PS2 for it was able to catalyse noncyclic photophosphorylation (Gee et al 1969). It is therefore apparent that P700 cannot be the site of ferricyanide or DCPIP photoreduction. The probable site of interaction of these electron acceptors in chloroplasts is at plastocyanin, as shown in Figure 5-1.

The photoreduction of cytochrome c in chloroplasts by electrons from water has been shown to be of two types (Keister and San Pietro 1963). A low rate of endogenous photo-

reduction occurred without addition of catalyst, which was stimulated ten-fold by saturating amounts of ferredoxin. The endogenous reduction was not coupled to photophosphorylation, it was partially inhibited by increasing the concentration of tris buffer pH 7.8 from 0.02 M to 0.15 M, and it was not affected by removal of endogenous ferredoxin on washing the chloroplasts. In contrast the ferredoxin dependent reduction was not inhibited, but stimulated by the higher concentration of tris buffer, and was also coupled to photophosphorylation. The effect of tris buffer can now be understood as a combination of a partial inhibition of the oxygen evolution reaction (Yamashita and Butler 1968), and a large stimulation of the electron transfer from the reductive terminus of PS1 to cytochrome c. Apparently therefore in endogenous photoreduction cytochrome c accepts electrons at a site distinct from that in ferredoxin catalysed photoreduction.

Chloroplasts treated for a few minutes with a detergent, 1% saponin, were found to be able to photoreduce cytochrome c with 650 nm light and to photo-oxidise cytochrome c with 710 nm light, showing that in this preparation cytochrome c could interact with the electron transport chain between the two photosystems (Kok *et al* 1963). Pratt and Bishop (1968) found that a mutant of *Scenedesmus* lacking PS1 activity could photoreduce cytochrome c, although at a much lower rate than the wild type, suggesting that PS2 alone can reduce cytochrome c.

The photoreduction of cytochrome c by PS1 alone, seen with hydroquinones such as reduced trimethyl-p-benzoquinone (Kelly and Sauer 1965), is not related to the present work. The photoreduction observed here is the endogenous photoreduction of the chloroplasts, occurring without addition of ferredoxin or any of the many other catalysts which may be employed to stimulate the reduction rate.

The fluorescence of isolated spinach chloroplasts measured at room temperature is mainly emitted from PS2. The fluorescence yield is enhanced by activation of PS2 and diminished by activation of PS1, and the variation of the fluorescence yield is believed to reflect the redox state of the quencher Q (see Figure 1 in the general introduction), which only quenches fluorescence when it is in its reduced form (Fork and Amesz 1969). The anaerobic redox titration of the fluorescence yield of spinach chloroplasts has been recently reported, clearly demonstrating this concept (Cramer and Butler 1969). The degree of reduction of Q achieved by illumination of chloroplasts aerobically, as is done in the present work, is small compared to that seen anaerobically in the presence of dithionite.

Fluorescence induction is the rise in the fluorescence yield of the chloroplasts from the low value obtaining at the start of actinic illumination (F_o) to the final steady state value reached in the light (F_{ss}). The profile of the fluorescence induction curve is affected by alteration of the electron transport pathways in the chloroplasts; for example

by the addition of inhibitors of electron transport, or by chemical oxidising or reducing agents. From an analysis of the effects of the chelating agents, salicylaldoxime and cupferron, on the fluorescence induction of chloroplasts, the manner in which they modify electron transport through the reductive terminus of PS2 can be deduced.

The redox state of P700 in chloroplasts can similarly be used as an indicator of electron flow through the oxidative terminus of PS1, the high molar extinction coefficient of P700 allowing a very sensitive measurement of its redox state by dual wavelength spectrophotometry. Therefore the effects of the chelating agents on the light-induced oxidation-reduction changes of P700 in chloroplasts were observed.

MATERIALS AND METHODS

Materials. Chloroplasts employed in the study of the effect of chelating agents on electron transport were prepared from spinach 3-4 weeks old, grown as described in Chapter 3. The leaves were picked early in the morning, a few hours after the 16 hours light period was started. About 10 g leaf was ground with sand in 30 ml 0.4 M sucrose + 0.05 M KPO_4 pH 6.5 + 0.01 M NaCl at 0°, filtered through nylon net and the filtrate centrifuged at 200 g for 1 minute at 2° to remove debris and sand. The chloroplasts were sedimented at 5,000 g for 8 minutes at 2°, and resuspended at 0° in 0.05 M KPO_4 pH 6.5 + 0.01 M NaCl.

The chloroplasts were prepared freshly each day, stored at 0° in the dark as a concentrated suspension (chlorophyll concentration $\geq$ 0.5 mg/ml), and used within 4 hours. The chlorophyll content of the chloroplasts was measured as described in Chapter 3.

1 mM DCPIP was a freshly prepared and filtered solution. A 10 mM ferricyanide solution was prepared and stored at 4° in the dark. Horse heart cytochrome c Type III was obtained from Sigma and was stated to be salt-free. The 1.5 mM methyl viologen solution was stored at 4°. A 4 mM DCMU solution in 50% ethanol was used. Both orthophenanthroline and cupferron were in aqueous solution. Salicylaldoxime was first dissolved in methanol and then diluted with 9 volumes of water to give a 0.1 M solution in 10% methanol. Heated chloroplasts were obtained by placing a small volume of chloroplast suspension in a tube, immersed in a water bath at 50° and rotated rapidly to obtain an immediate rise in temperature and accurate time of heating.

Hill reactions. The photoreduction of added Hill oxidants by water, in 0.05 M KPO_4 pH 6.5 + 0.01 M NaCl buffer, was followed in a Cary 14 recording spectrophotometer modified as described by Massini and Voorn (1967). DCPIP reduction was observed by the decrease in absorption at 620 nm, employing a molar absorbance coefficient at pH 6.5 of 18,000. The absorbance at this wavelength is 0.986 times that at 610 nm, and the molar absorbance coefficient at 610 nm was found by Punnett (1959) to be 18,200 at pH 6.5. At pH 7.8 the molar absorbance coefficient at 620 nm was taken to be 22,000.

Photoreduction of ferricyanide in the presence of 2 mM ammonium chloride was followed by the decrease in absorbance at 420 nm using a molar absorbance coefficient at this wavelength of 1,070. Cytochrome c photoreduction was observed as the increase in absorbance at 550 nm, assuming the difference in molar absorbance coefficient of oxidised and reduced forms at this wavelength to be 19,000 (Kelly and Sauer 1965). The reduced cytochrome c was reoxidised in the dark periods unless 1 mM potassium cyanide was present. The temperature of the sample cuvette was kept constant at 21° by circulating cooled water directly around it. The sample solution was kept stirred with a small magnetic bead at the bottom of the cuvette and out of the light path. Side illumination was by red light obtained from a tungsten lamp with a red glass filter (Schott RG 2) combined with an infrared filter. The voltage provided to the lamp was adjustable and allowed a variation in the intensity of actinic light. No filters were used in front of the photomultiplier.

The final sample volume was 3 ml. When inhibitors were added, the control rate of the uninhibited chloroplasts was measured at first, then the inhibitor was added to give a total volume of 3 ml. This was found necessary as the Hill activity of the chloroplasts was not stable and was not consistent between chloroplast preparations. The degree of inhibition found was always expressed as a percentage of the uninhibited rate of the same sample of chloroplasts. The photoreactions were usually followed for a period of 1-2 minutes.

Fluorescence induction. The fluorescence of chloroplasts was measured at room temperature in an apparatus constructed by Drs. Elgersma. The design of the apparatus was based on that described by Malkin and Kok (1966), but modified in one important respect: the fluorescence yield data were recorded on magnetic tape, which was played back at reduced speed, the fluorescence curve being presented on chart paper. Illumination was with light of wavelength 520 nm and intensity 1 W/m^2. The fluorescence emitted by the chloroplasts at wavelengths greater than about 670 nm was transmitted by a red filter placed in front of the photomultiplier (9558 EMI), but scattered green actinic light was not transmitted. 3 ml chloroplast suspension, in 0.05 M KPO_4 pH 6.5 + 0.01 M NaCl, having a chlorophyll concentration of 2.7 mg/litre, and containing various additions of electron transport modifiers, was placed in the sample cuvette. The sample was kept in the dark for a period longer than 5 minutes before the green actinic light was passed through the cuvette, and the fluorescence which was emitted at right angles to the actinic light beam, measured by the photomultiplier. At the low chloroplast concentrations used, the fluorescence measured was found to be proportional to chlorophyll concentration, but at higher concentrations errors would arise from non-uniform illumination of the sample and also from re-absorption of fluorescent emission. The induction curve of the fluorescence samples was found to be unstable over a period of several hours, even though they were kept at 0° in the dark. However the fluor-

escence induction of chloroplasts kept as a concentrated suspension was found to be reproducible even after 5 hours at 0°. Therefore fluorescence induction samples were made up from the concentrated chloroplast suspension shortly before use.

P700 redox changes. The oxidation-reduction changes of P700 in chloroplasts were measured on an Aminco-Chance dual-wavelength spectrophotometer, as described in Chapter 4 for chloroplast digitonin particles. The chloroplast concentration used was 45 mg chlorophyll/litre, which gave absorbance changes in the range, 0.004 - 0.020. 0.3 M sucrose was present in the sample suspension to minimise the settling of the chloroplasts, which gave rise to a downward-sloping baseline. In all experiments 25 micromolar MV was added to increase noncyclic electron flow of electrons from PS2 to PS1 through P700, and so emphasise any effect of added electron transport inhibitors on this electron pathway.

RESULTS AND DISCUSSION

Fluorescence induction. The induction of the fluorescence yield of chloroplasts, after various treatments to modify the electron transport pathways through PS2, are shown in Figures 5-3 and 5-4. The biphasic increase typical of untreated chloroplasts (see for example, Malkin and Kok 1966) was shown by the chloroplasts prepared as described in the Materials and Methods section. The ratio of F_{ss} to F_o was 1.92 and the steady state was reached after 25 seconds (Figure 5-3 (A)). However after the chloroplasts had been heated to 50° for 5 minutes the biphasic character of the curve was lost and the value of F_o increased (Figure 5-3 (B)). A similar effect was seen when 10 mM salicylaldoxime was added to chloroplasts (the final concentration of methanol being 1%). When 1% methanol was added alone to chloroplasts, to act as a methanol control, the value of F_o was the same as for untreated chloroplasts, and a biphasic increase in fluorescence yield was still seen (Figure 5-3 (C)). The final steady state level of fluorescence yield was constant for all samples shown in Figure 5-3.

In Figure 5-4 the value of F_{ss} seen for the samples varied. When either 8 micromolar DCMU or 0.05 mM orthophenanthroline were added to a chloroplast suspension, the effect was to remove the biphasic rise and to increase the value of both F_o and of F_{ss}. A characteristic feature of the fluorescence yield curve in the presence of either of these inhibitors was its linear increase from F_o (Figure 5-4 (B)). When 1 mM potassium ferricyanide was present, F_o remained un-

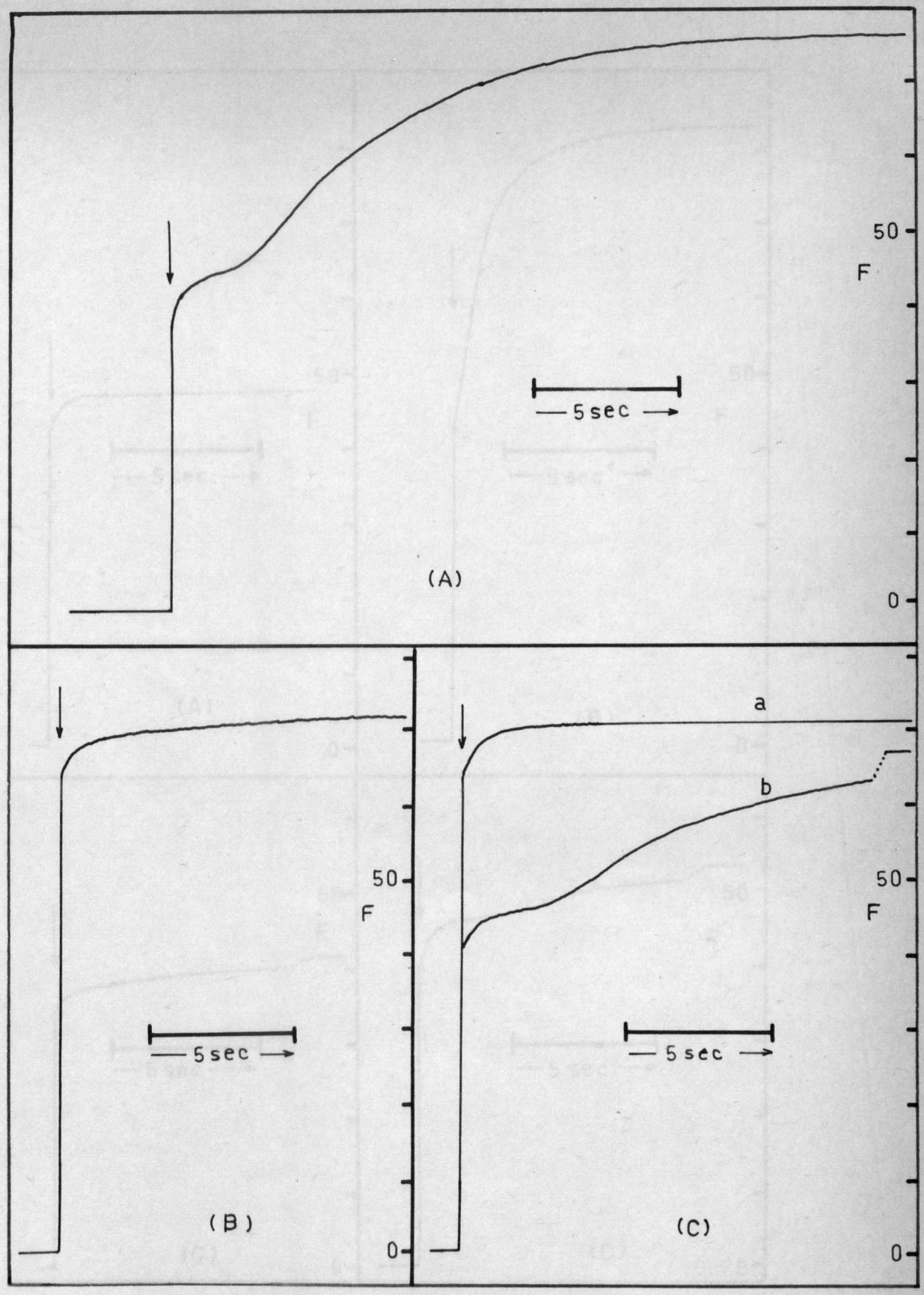


Figure 5-3. Induction of fluorescence yield (F) of chloroplasts. (A) untreated; (B) chloroplasts heated to 50° for 5 min; (C) (a) 10 mM salicylaldoxime added, (b) methanol control. Chlorophyll concentration 2.7 mg/litre. The steady state fluorescence yield after 60 sec is shown for curve (C,b).

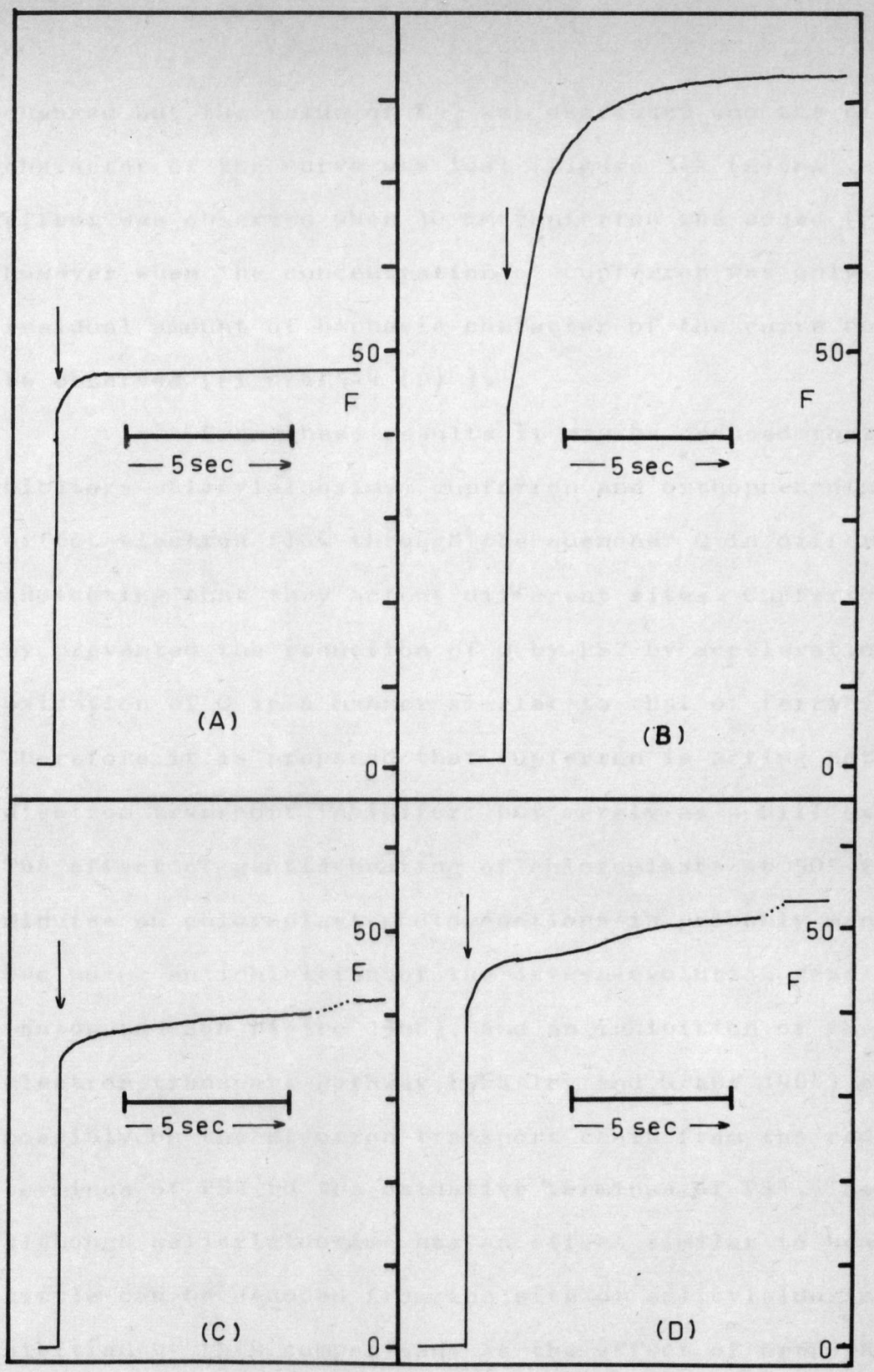


Figure 5-4. Induction of fluorescence yield (F) of chloroplasts, with addition of (A) 1 mM ferricyanide, (B) 0.05 mM orthophenanthroline, (C) 10 mM cupferron, (D) 1 mM cupferron. Chlorophyll concentration 2.7 mg/litre. The steady state fluorescence yield after 60 sec is shown for curves C and D.

changed but the value of F_{ss} was decreased and the biphasic character of the curve was lost (Figure 5-4 (A)). A very similar effect was observed when 10 mM cupferron was added (Figure 5-4(C)); however when the concentration of cupferron was only 1 mM a residual amount of biphasic character of the curve could still be observed (Figure 5-4 (D)).

From these results it may be deduced that the inhibitors salicylaldoxime, cupferron and orthophenanthroline affect electron flow through the quencher Q in different ways, indicating that they act at different sites. Cupferron apparently prevented the reduction of Q by PS2 by accelerating the oxidation of Q in a manner similar to that of ferricyanide. Therefore it is proposed that cupferron is acting not as an electron transport inhibitor, but merely as a Hill oxidant. The effect of gentle heating of chloroplasts at 50° for 5 minutes on chloroplast photoreactions is probably many-fold, including an inhibition of the oxygen-evolution reaction (Katoh and San Pietro 1968), and an inhibition of the cyclic electron transport pathway (Whatley and Grant 1964) at a site possibly on the electron transport chain from the reductive terminus of PS2 to the oxidative terminus of PS1. Therefore although salicylaldoxime has an effect similar to heating, little can be deduced from the site of salicylaldoxime inhibition by this comparison. As the effect of orthophenanthroline was so similar to that of DCMU it appears that orthophenanthroline inhibits electron transport from the reductive terminus of PS2 immediately after Q (see also Murata et al 1966).

P700 oxidation-reduction changes. Each sample investigated had a volume of 3.05 ml and was placed in a 10 mm x 10 mm Aminco cuvette, clear on all four sides. The chloroplast concentration corresponded to 45 mg chlorophyll/litre, and by assuming that the P700 content of the chloroplasts was one molecule per 400 molecules of total chlorophyll and the molar absorbance coefficient for the P700 absorbance change was 10^5, then an absorbance change of 0.0125 was expected for complete conversion of reduced P700 to oxidised P700. All absorbance changes measured will be referred to this value as 100%. The chloroplast suspension contained 0.3 M sucrose to reduce settling of the chloroplasts, but some settling still occurred.

In Fig. 5-5 (A) the light-induced redox changes of P700 in untreated chloroplasts are shown. The absorbance change seen in the light represented photo-oxidation of only 32% of the P700 content, a steady state being reached between photo-oxidation by PS1 and reduction by cyclic electron flow from the reductive terminus together with photoreduction by PS2. The light and dark steady states were reached very rapidly. When the light was cut off from the sample, the reducing power in the electron transport chain between PS2 and PS1, which was provided by the supply of electrons from water, caused the fast dark reduction of P700 (see also Malkin 1968). Addition of 10 mM cupferron caused a decrease in the photo-oxidation seen, but the total amount of oxidation was the same as for untreated chloroplasts because a small amount of instantaneous oxidation occurred when the actinic light was cut off. The dark reduction was inhibited and a half-time of 8 seconds was measured.

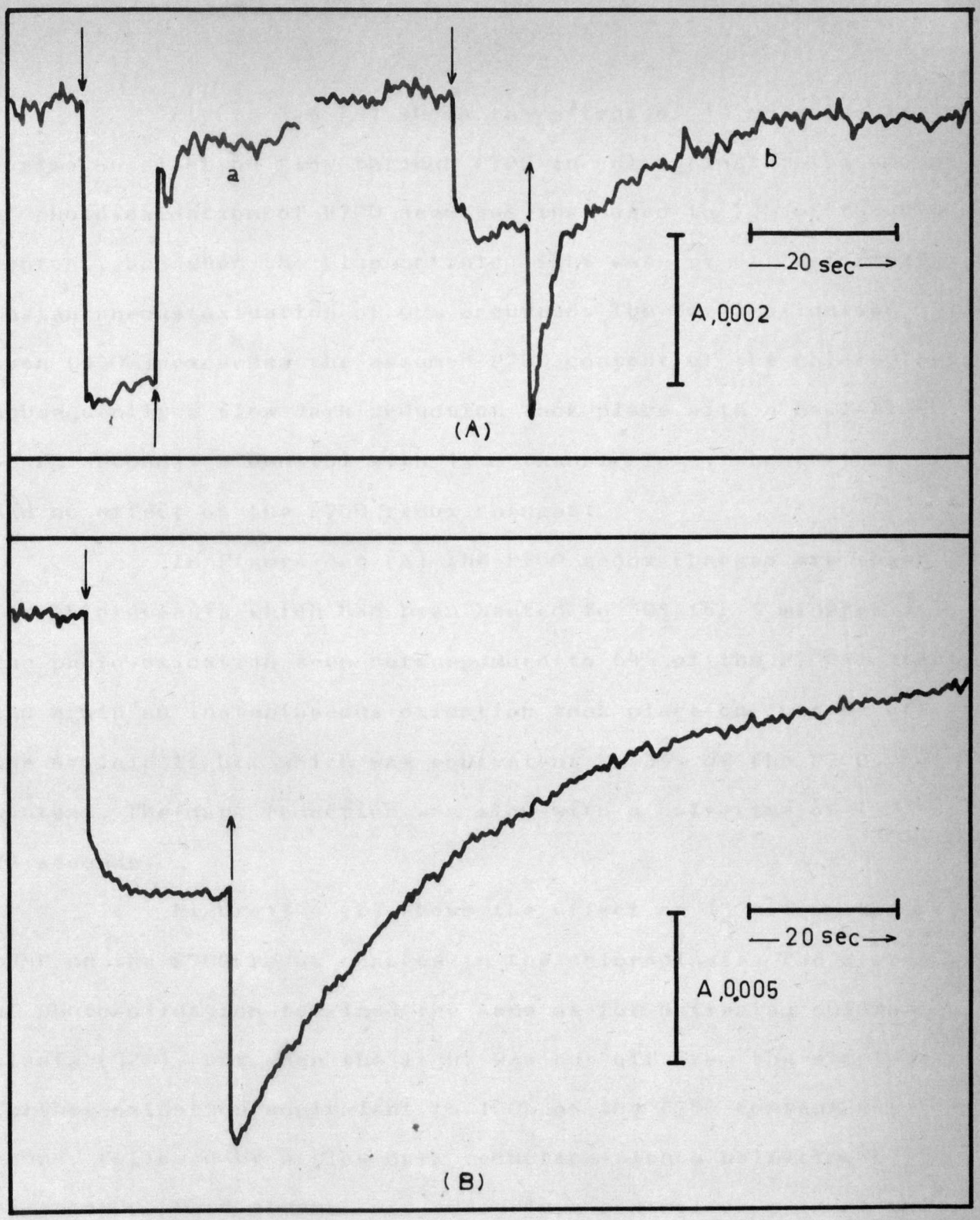


Figure 5-5. Effect of the chelating agents on the light-induced oxidation-reduction changes of P700 in chloroplasts with 25 micromolar MV present to speed electron flow through PS1. Chlorophyll concentration was 45 mg/ltr. Illumination and other experimental conditions as for Figure 4-2. (A), (a) untreated chlorplasts, (b) 10 mM cupferron added; (B) 10 mM salicylaldoxime added.

Figure 5-5 (B) shows the effect of 10 mM salicylaldoxime on electron flow through P700 in chloroplasts: the amount of photo-oxidation of P700 seen was increased to 72% of the P700 content, and when the blue actinic light was cut off a further instantaneous oxidation of 68% occurred. The total oxidation seen (150%) exceeded the assumed P700 content of the chloroplasts. Subsequently a slow dark reduction took place with a half-time of 23 seconds. A control with 1% methanol alone, showed that it had no effect on the P700 redox changes.

In Figure 5-6 (A) the P700 redox changes are shown for chloroplasts which had been heated to 50° for 5 minutes. The photo-oxidation seen corresponded to 64% of the P700 content, and again an instantaneous oxidation took place on cutting off the actinic light, which was equivalent to 35% of the P700 content. The dark reduction was slow with a half-time of 27 seconds.

Figure 5-6 (B) shows the effect of 13 micromolar DCMU on the P700 redox changes in the chloroplasts. The degree of photo-oxidation remained the same as for untreated chloroplasts (32%), but when the light was cut off from the sample a further oxidation equivalent to 100% of the P700 content was found, followed by a slow dark reduction with a half-time of 38 seconds. When ethanol was added in a control experiment, no alteration in the redox changes of the chloroplasts was seen for a time of incubation equal to that used in the measurement of DCMU-treated chloroplasts.

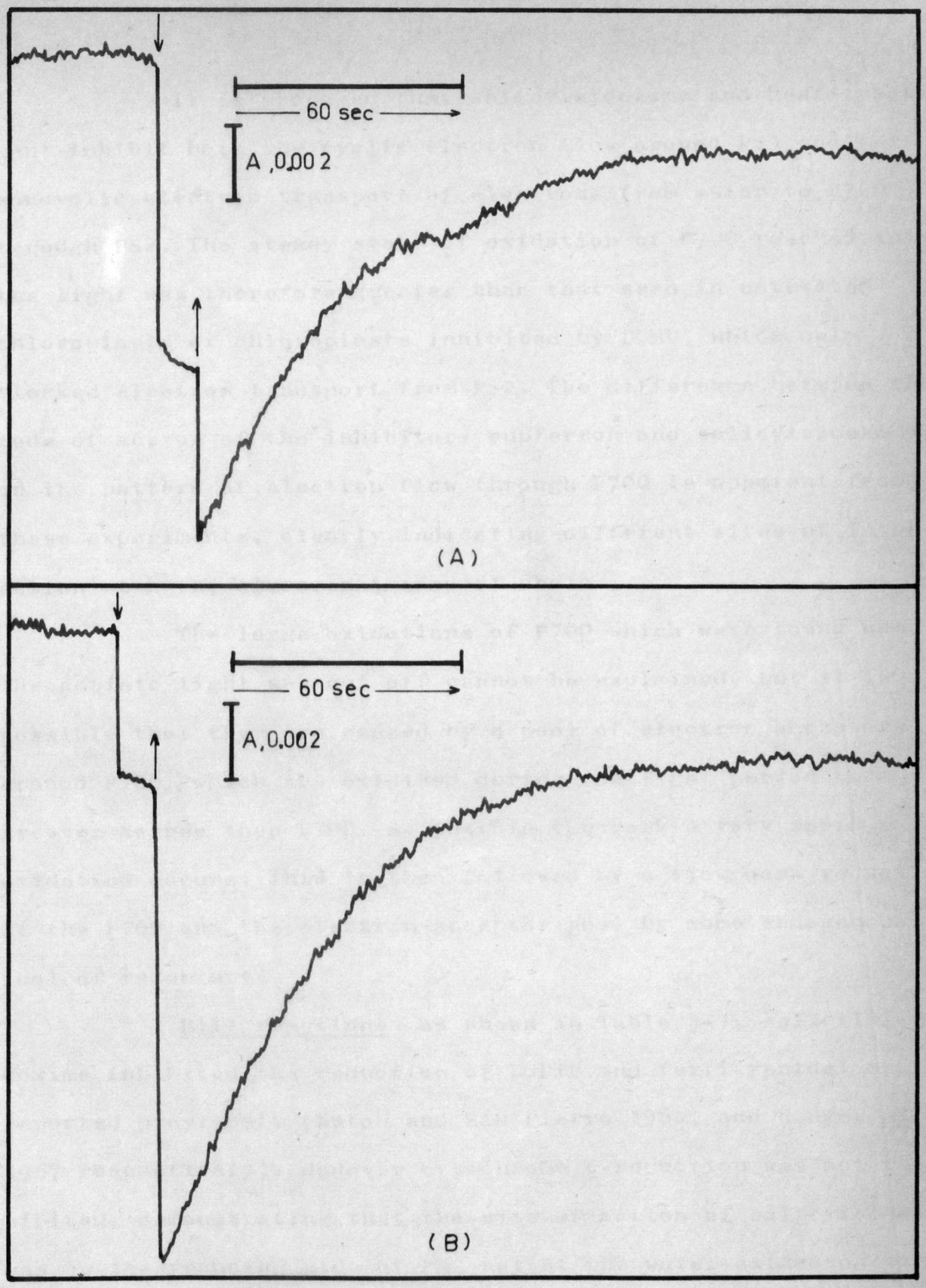


Figure 5-6. Effect on the light-induced oxidation-reduction of P700 in chloroplasts, with 25 micromolar MV present, by (A) heating to 50° for 5 minutes and (B) adding 13 micromolar DCMU. Chlorophyll concentration 45 mg/litre. Other conditions as for Figure 4-2.

It is proposed that salicylaldoxime and heat-treatment inhibit both the cyclic electron flow around PS1 and the noncyclic electron transport of electrons from water to P700 through PS2. The steady state of oxidation of P700 reached in the light was therefore greater than that seen in untreated chloroplasts or chloroplasts inhibited by DCMU, which only blocked electron transport from PS2. The difference between the mode of action of the inhibitors cupferron and salicylaldoxime on the pattern of electron flow through P700 is apparent from these experiments, clearly indicating different sites of interaction with the electron transport chain.

The large oxidations of P700 which were found when the actinic light was cut off cannot be explained, but it is possible that they are caused by a pool of electron acceptors around P700, which are oxidised during the light period to a greater degree than P700, so that in the dark a very rapid oxidation occurs. This is then followed by a slow dark reduction of the P700 and the electron acceptor pool by some endogenous pool of reductant.

Hill reactions. As shown in Table 5-1, salicylaldoxime inhibited the reduction of DCPIP and ferricyanide, as reported previously (Katoh and San Pietro 1966, and Renger *et al* 1967 respectively). However cytochrome *c* reduction was not inhibited, demonstrating that the site of action of salicylaldoxime was on the reducing side of PS2 not at the water-oxidation step. The small amount of inhibition observed with 10 mM salicylaldoxime could be attributed to the effect of methanol, for in

TABLE 5-1. INHIBITION OF HILL REACTIONS BY SALICYLALDOXIME.

The photoreduction of Hill oxidants was measured on a Cary 14 spectrophotometer as described in the Materials and Methods section. The red actinic light had a maximum intensity at 660 nm and a total intensity of 20 W/m^2 (lamp voltage 31). The gas phase was air. The components of the sample (3 ml) were: 0.05 M KPO_4 buffer pH 6.5, 0.01 M NaCl, chloroplasts equivalent to 0.015 - 0.050 mg chlorophyll depending on the Hill oxidant used, and either 0.1 mM DCPIP or 1 mM ferricyanide or 0.033 mM cytochrome c. When ferricyanide reduction was measured, 2 mM ammonium chloride was also added. Salicylaldoxime was added as a 0.1 M solution in 10% methanol. Results are given as % inhibition of the uninhibited rates, which were variable for different chloroplast preparations, but typical values were (in micromoles reduced/mg chlorophyll/hour) for ferricyanide 230, for DCPIP 142 and for cytochrome c 52.

Hill oxidant	% inhibition by salicylaldoxime (final concentration)	
	5.25 mM	10 mM
Ferricyanide	56	88
DCPIP	58	97
Cytochrome c	0	6

a control experiment methanol alone gave an inhibition, which was not constant but increased with time. The lack of inhibition of cytochrome c reduction is illustrated in Figure 5-7 (B), where it is also seen that 13 micromolar DCMU could inhibit the cytochrome c reduction completely. In the dark period following photoreduction of cytochrome c, the reduced cytochrome c was slowly reoxidised. This reoxidation was not prevented by anaerobic conditions, but could be completely stopped by addition of 1 mM KCN, which did not inhibit the water-splitting reaction (see also Trebst 1963). The restoration of electron donation to PS2 in inactivated chloroplasts by hydroxylamine has been described by Izawa et al (1969), and it was found in the present work that 10 mM salicylaldoxime could inhibit this restored electron transport (a photoreduction rate of 11 micromoles DCPIP/mg chlorophyll/hour) in heat-treated chloroplasts (Figure 5-7 (A)). The lack of inhibition of cytochrome c photoreduction by salicylaldoxime cannot be ascribed to the low electron flow rate through the electron transport chain with cytochrome c as Hill oxidant, because when DCPIP was used as Hill oxidant with the light intensity reduced to obtain identical rates to those seen with cytochrome c, the inhibition of DCPIP photoreduction was still apparent.

Lightbody and Krogmann (1967) followed methyl red photoreduction by sonicated lamellae fragments at pH 7.8 and under a nitrogen atmosphere and found an inhibition of electron transport from water by cupferron. However when DCPIP photoreduction at pH 7.8 or pH 6.5 was measured anaerobically in the present work, the degree of inhibition obtained was much

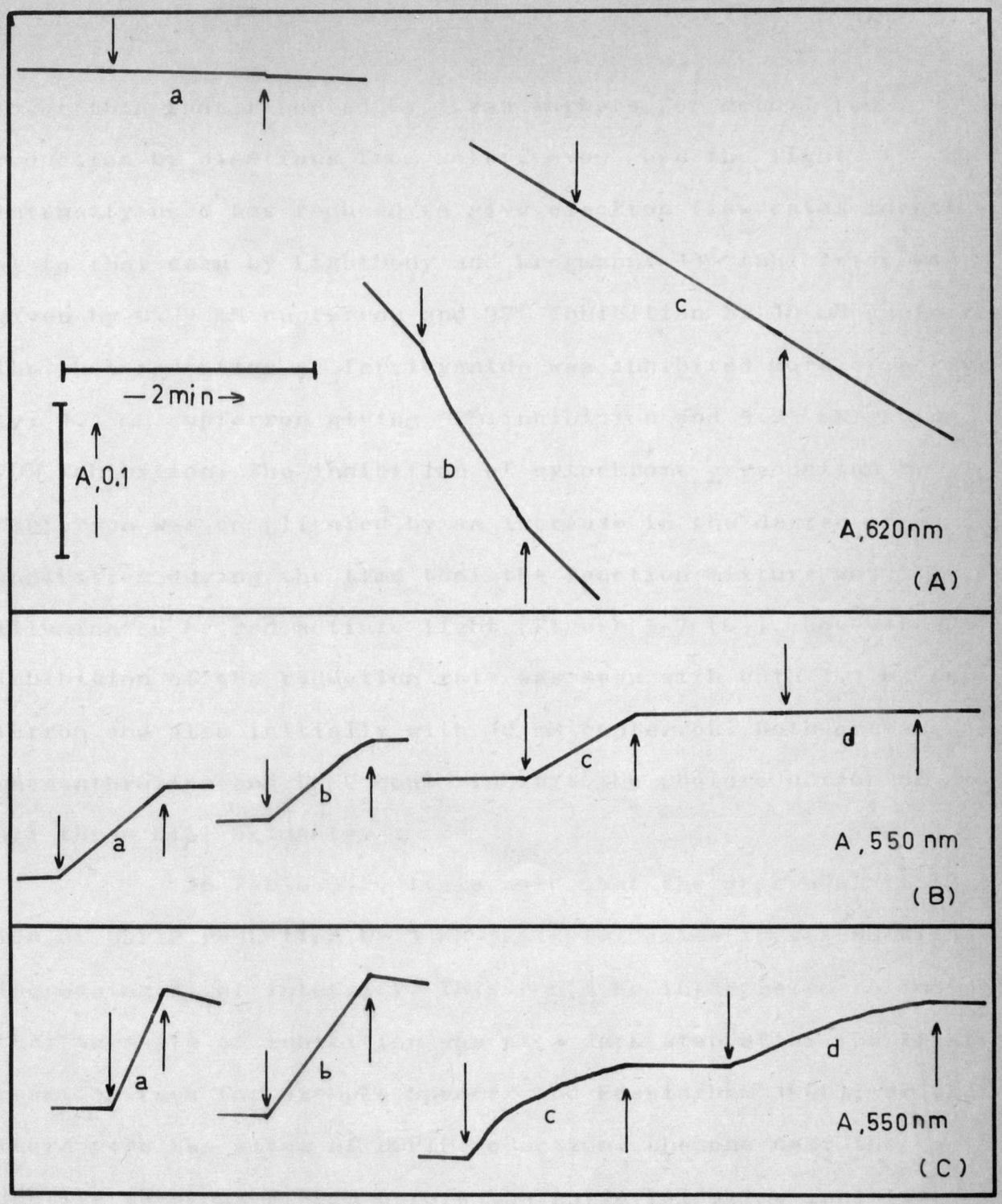


Figure 5-7. The inhibition of PS2 photoreductions by chelating agents. The time and absorbance scales apply to all curves shown. (A) DCPIP reduction by, (a) chloroplasts heated to 50° for 5 minutes, (b) 11 mM NH_2OH added, and (c) 10 mM salicylaldoxime added. (B) cytochrome c reduction, (a) control rate, (b) with 5.25 mM salicylaldoxime, (c) with 10 mM salicylaldoxime, (d) 13 micromolar DCMU added. (C) cytochrome c reduction, (a) control rate, (b) 1.1 mM cupferron added, (c) 10 mM cupferron added, and (d) with 10 mM present inhibition increases during the light period.

lower than that reported by these workers for methyl red reduction by electrons from water, even when the light intensity used was reduced to give electron flow rates identical to that seen by Lightbody and Krogmann. 13% inhibition was given by 0.99 mM cupferron and 37% inhibition by 10 mM cupferron. The photoreduction of ferricyanide was inhibited more effectively: 1.1 mM cupferron giving 52% inhibition and 5.25 mM giving 70% inhibition. The inhibition of cytochrome c reduction by cupferron was complicated by an increase in the degree of inhibition during the time that the reaction mixture was illuminated by red actinic light (Figure 5-7 (C)). However 37% inhibition of the reduction rate was seen with both 1.1 mM cupferron and also initially with 10 mM cupferron. Both orthophenanthroline and DCMU could inhibit the photoreduction of all three Hill oxidants.

In Table 5-2, it is seen that the degree of inhibition of DCPIP reduction by 5 mM salicylaldoxime increased with increasing light intensity. This could be interpreted to indicate that the site of inhibition was at a dark step after the light reaction (see for example Spencer and Possingham 1960); or that there were two sites of DCPIP reduction, the one near the reductive terminus of PS2 before the salicylaldoxime-inhibited step transferring electrons to DCPIP at low light intensities, and the second near PS1 past the salicylaldoxime-inhibited step in the electron transport chain could reduce DCPIP when the light intensity was increased.

TABLE 5-2. DEPENDENCE OF SALICYLALDOXIME INHIBITION OF DCPIP REDUCTION ON THE LIGHT INTENSITY.

The photoreduction of DCPIP was measured as described in the legend to Table 5-1, except that the DCPIP concentration was 0.167 mM, chloroplasts equivalent to 0.027 mg chlorophyll were present, and the light intensity was varied by changing the lamp voltage. The uninhibited rate was measured at each light intensity, then the inhibited rate was measured first with the lamp voltage increasing and subsequently with it decreasing, in order to check the stability of the reduction rate. At the end of the experiment the DCPIP was still not completely reduced. Reduction rates are expressed as micromoles DCPIP reduced/mg chlorophyll/hour).

Lamp voltage	Uninhibited rate	Salicylaldoxime uninhibited rate			% inhibition
		Light increasing	Light decreasing	Average	
16	5.2	5.6	5.4	5.5	-5
21	26.2	20.6	21.4	21.0	20
26	66.1	40.8	39.3	40.0	39
31	124.1	45.8	45.8	45.8	63

Conclusions. The results reported here support the location of the site of inhibition of salicylaldoxime shown in Figure 5-2: 10 mM salicylaldoxime inhibits electron transport between cytochrome f and cytochrome 559 at a point common to both the cyclic electron transport pathway and the noncyclic electron transport pathway from PS2.

The copper chelating agent cupferron, introduced by Lightbody and Krogmann (1967), does not effectively inhibit electron transport in chloroplasts. The inhibition of the Hill reaction with ferricyanide could be simply a competition for electrons from PS2.

Therefore salicylaldoxime and cupferron do not inhibit at the same site and it is probable that neither chelating agent inhibits by virtue of binding the active copper of a hypothetical second copper-containing chloroplast component. Orthophenanthroline inhibits electron transport, but its site of action at the DCMU-inhibition locus strongly suggests that it does not inhibit by virtue of its chelating ability.

APPENDIX A NONAQUEOUS CHLOROPLASTS

Preparation. The method of preparation of non-aqueous chloroplasts is based upon that described by Thalacker and Behrens (1959). Freeze-dried spinach leaves are homogenised in a nonpolar organic solvent and the chloroplasts released by this treatment separated from large debris, nuclei and mitochondria by centrifugation over a density gradient prepared from hexane and carbon tetrachloride. The maintenance of the integrity of the photoreaction centres in the chloroplasts is dependent upon the prevention of the loss of chlorophyll. This is achieved by using temperatures below 0° when the chloroplasts are in contact with hexane/carbon tetrachloride mixtures. When hexane or heptane alone is used, then the temperature can be safely raised to 20 - 25° without extraction of chlorophyll.

A typical preparation proceeded as follows: Spinach grown for 3 - 4 weeks in a greenhouse, under a 16 hours light regime, was given an extended dark period of 20 hours to reduce the starch content, and then harvested. 60 g leaf material was stripped from the stems and roots, placed in a chilled mortar, and liquid nitrogen added in excess to freeze the material completely. This was then placed in a freeze-drier, consisting of a closed metal vessel containing silica gel placed in a deep freeze, the vessel being evacuated with an oil pump. Freeze-drying took about 2-3 days. The material was then broken up into a fine powder using a pestle and stored in vacuo over P_2O_5 until use.

The leaf powder (about 7 g) was homogenised at -15 to -20° in a salt-ice bath in a Sorvall Omnimixer, having teflon end bearings and a teflon sealing ring, with 200 ml hexane-carbon tetrachloride mixture 50:50 (v/v) for 2 x 2 minutes. The green homogenate was layered over an equal volume of hexane-carbon tetrachloride 25:75 (v/v) at -20° in glass bottles and centrifuged at -5° in a swinging bucket rotor at 2,000 g for 10 minutes. The chloroplasts formed a layer at the interface of the two mixtures (densities 1.13 and 1.36 at +20°). The upper yellow layer containing extracted carotenoids was removed by pipette, and the remaining supernatant decanted from the green sediment of large debris into hexane at -20° to reduce the density to 1.13. The chloroplasts could then be precipitated by centrifugation at 2,000 g for 10 minutes at -5°, resuspended in a little hexane, and transferred to a beaker, which was placed in a desiccator containing P_2O_5 and evacuated to remove all traces of organic solvents. The chloroplasts could be stored dry, in vacuo, for at least a week without loss of activity, when kept at -20° in the dark. The yield of chloroplasts was equivalent to 20 mg of chlorophyll from 60 g fresh spinach leaf material.

An important consideration in the preparation is the complete absence of water during the treatment of leaf material with the organic solvents. The freeze-drying procedure must remove all water content, and the organic solvents must be water-free, which is achieved by filtering the solvents at -20° through fast-flow filter paper.

The preparation given is that for impure chloroplasts in the greatest yield. Further purification can be obtained by using a continuous gradient, or a discontinuous gradient with more gradient steps, between densities 1.25 and 1.36.

The degree of extraction of lipids can be varied as required by further treatment of the chloroplast preparation with organic solvents under strictly controlled conditions, particularly with regard to temperature.

Properties. The chloroplasts can be resuspended in aqueous buffer at 0° with a Potter hand-homogeniser. The maximum of the extinction spectrum of the resuspended chloroplasts at the red absorption band of chlorophyll was found to be 680-681 nm, when measured on the Cary 14 spectrophotometer. However when the same suspension was measured on the Cary, with a scattered light transmission attachment, the maximum was at a wavelength 4 nm lower: 676-677 nm. This rather large effect of scattering on the extinction maximum is due to the high refractive index of the nonaqueous chloroplasts (Butler 1964).

When the resuspended chloroplasts were treated with 1.3% digitonin at a digitonin to chlorophyll ratio of 10:1, the pattern of the digitonin particles of the 80,000 g supernatant on a 10 - 30% sucrose gradient was similar to that of ordinary aqueously-prepared chloroplasts (see Wessels 1966). A small difference between them was in the colours of the various bands, which were dark green, pink, apple-green and green-yellow, for the bands in order from the bottom of the

centrifuge tube. Cytochromes *f* and b_6 were present in the pink band and plastocyanin was detected in the colourless top zone. The lower dark green band (F_I) was the fraction active in PS1 electron transport.

PS1 activity. NADP reduction by digitonin particles prepared from nonaqueous chloroplasts was measured as described by Wessels (1966). If the F_I fraction was prepared as described above, from nonaqueous chloroplasts extracted further with hexane-carbon tetrachloride 50:50 (v/v) at 5°, then it was possible to completely remove beta-carotene. Chloroplasts equivalent to 2.5 mg chlorophyll were treated with 1.3% digitonin: 1.11 mg chlorophyll was found in the 80,000 g precipitate, and a total of 1.11 mg chlorophyll was found in fractions collected from the sucrose gradient. The F_I fraction gave a NADP reduction rate, with ascorbate-DCPIP and the enzymes added, of 10.8 micromoles/mg chlorophyll/hour; the activity of the F_{III} fraction was zero as expected. The beta-carotene content of the active F_I fraction was less than one-tenth of a mole per 250 moles total chlorophyll, when the pigment content of the fraction was analysed by thin layer chromatography on Kieselguhr as described by Wessels (1968).

Although the NADP reducing activity is low, it is sufficient to demonstrate that beta-carotene cannot be regarded as an essential electron transport component of PS1 as suggested by Lundegårdh (1969). In more recent work in co-operation with Dr. Wessels, rates of NADP reduction as high as 160 micromoles/mg chlorophyll/ hour have been seen with an

F_I fraction from nonaqueous chloroplasts. The NADP reduction cannot be ascribed to a nonenzymatic reaction of solubilised chlorophyll as plastocyanin is found to be required absolutely (see Massini and Voorn 1968).

PS2 activity. It was found possible to demonstrate PS2 activity in nonaqueous chloroplasts. The ability of the chloroplasts to oxidise water is lost during their preparation, either in the freeze-drying stage or during the isolation in organic solvents. However when manganese chloride is added as artificial electron source, electron transport can occur from the oxidative terminus of PS2 to the reductive terminus of PS2. During the isolation of the chloroplasts, plastoquinone A is extracted and the reduction of DCPIP or ferricyanide cannot be observed (Bishop 1959). However added parabenzoquinone can substitute for the extracted plastoquinone at the reductive terminus of PS2, and its reduction to hydroquinone can be followed spectrophotometrically at 290 nm.

The experimental conditions were as follows. The reduction was measured anaerobically on the Cary 14 spectrophotometer modified as described in Chapter 5 for side illumination with red light. An ultraviolet filter showing 60% transmission at 290 nm (Schott UG11) was placed in front of the photomultiplier to prevent interference from scattered actinic light; neutral density filters were placed in the reference beam to obtain the necessary balance between sample and reference light beams. The 3 ml sample solution was placed in a Thunberg cuvette, cooled by circulating water at a temperature

of 21°, and the sample stirred magnetically. 10 mM parabenzoquinone solution was freshly made up in water each day from freshly-sublimed parabenzoquinone, and the solution was kept in the dark. The molar absorbance coefficient difference at 290 nm for parabenzoquinone and hydroquinone was determined to be 2,250. 0.1 M $MnCl_2$ in water was made freshly each day. A 4 mM DCMU solution in 50% ethanol was used.

The results are presented in Table A-1 for non-aqueous chloroplasts resuspended in 0.1 M sodium acetate buffer pH 6.5. In control experiments it was shown that parabenzoquinone and also hydroquinone were quite stable in sodium acetate buffer pH 6.5 under anaerobic conditions, with and without manganese chloride present, and either in the dark or under illumination with red light. The stability was checked by a steady value of A_{290}. Under aerobic conditions parabenzoquinone was stable in the presence of manganous ions but hydroquinone was gradually oxidised. It was found that the presence of tris buffer caused an instability of parabenzoquinone, seen as an increase in A_{290}. Under the experimental conditions used in the present work, the value of A_{290} was constant in the dark periods both before illumination started and after illumination had ceased. For a standard sample, made up as described in the legend to Table A-1, a total absorbance change at 290 nm of 0.14 was found after a light period of 8 minutes, corresponding to a hydroquinone concentration of 0.062 mM.

TABLE A-1. THE PHOTO-REDUCTION OF PARABENZOQUINONE BY NON-AQUEOUS CHLOROPLASTS WITH MANGANESE AS ELECTRON DONOR.

The reduction of parabenzoquinone was measured in the Cary 14 spectrophotometer as the increase in absorbance at 290 nm on illumination by red light of intensity 100 W/m^2. Temperature 21°. The standard sample volume (3 ml) contained: 1 mM parabenzoquinone; 0.1 M sodium acetate buffer pH 6.5; chloroplasts equivalent to 0.05 mg chlorophyll; and 3.3 mM manganese chloride. Omissions and additions are noted under remarks. All samples were anaerobic, gasphase: nitrogen.

Parabenzoquinone reduction rate (micromoles/mg chlorophyll/hour)	Remarks
49	Standard system.
0	$MnCl_2$ omitted.
0	Parabenzoquinone omitted.
0	13 micromolar DCMU added.
40	Ethanol control for DCMU addition.
21	0.05 mM hydroquinone added.
13	0.25 mM hydroquinone added.

The reduction rates quoted in Table A-1 are initial rates, for the rate of reduction of parabenzoquinone decreased progressively as hydroquinone was formed. The initial rate in one experiment was 49, which declined to 26 after 4 minutes light; after a 4 minutes dark period, the rate found was the same: 26. This again decreased in the light, until after a further 5 minutes, the rate was only 11. All rates are given as micromoles parabenzoquinone reduced/mg chlorophyll/hour.

The inhibition of parabenzoquinone photo-reduction by hydroquinone is also seen when hydroquinone is added prior to illumination (Table A-1). The standard redox potential of the quinone/quinol couple at pH 6.5 is +325 mV, and addition of hydroquinone to give a ratio of parabenzoquinone: hydroquinone = 4 would not lower the redox potential sufficiently to account for the inhibition of the reduction rate. It is proposed that hydroquinone inhibits by setting-up a cyclic electron flow around PS2, as it has been shown recently that hydroquinone can donate electrons to the oxidative terminus of PS2 (Yamashita and Butler 1969).

The restoration of electron donation to PS2 by manganese chloride has been recently described for chloroplasts inhibited by washing with 0.8 M tris buffer pH 8 (Yamashita et al 1969).

APPENDIX B. MATHEMATICAL RELATIONSHIPS

EPR. The equations used to evaluate the bonding parameters of the copper-ligand bonds in plastocyanin and rubimedin are:

(i) $$g_{//} = 2.0023 - \frac{8 \lambda a^2}{\Delta xy}$$

Where $g_{//}$ is the g-factor of the low field signal; λ is the spin-orbit coupling of the free Cu^{2+} ion ($= -828\ cm^{-1}$); Δxy is the wave number of the absorbance maximum in the visible.

(ii) $$(\tfrac{4}{7} \alpha'^2 + K) = (g_{//} - 2) + \frac{3}{7} (g_{\perp} - 2) - \frac{A_{//}}{P} .$$

Where $g_{//}$ is the g-factor of the low field signal; $g_{\perp}$ is the g-factor of the high field signal; $A_{//}$ is the hyperfine splitting constant of the low field signal; P is a constant, for Cu^{2+}: $P = 0.035\ cm^{-1}$.

(iii) $$\alpha^2 = \frac{A_{//}}{0.036} + (g_{//} - 2) + \frac{3}{7} (g_{\perp} - 2) + 0.04.$$

This is the equation of Kivelson and Nieman (1961); symbols as for (ii).

Gaussian curves. The equation of a Gaussian curve is: $y = N \exp - \frac{(x-x_o)^2}{2\sigma^2}$. When i cm is a constant interval along the x-axis, then

$$y_i / y_{i+1} = \exp \frac{(i+1-x_o)^2 - (i-x_o)^2}{2\sigma^2} .$$

Taking logarithms and expanding the brackets:

$$\ln(y_i/y_{i+1}) = \frac{2i+1-2x_o}{2\sigma^2} = \frac{1}{\sigma^2}.i + \frac{(1-2x_o)}{2\sigma^2}$$

Therefore, $2.3026 \log_{10}(y_i/y_{i+1}) = \frac{1}{\sigma^2}.i$ + constant, and the reciprocal of the slope of the plot of $\log_{10}(y_i/y_{i+1})$ against i will give the value of σ^2 for the Gaussian curve.

For the estimation of diffusion coefficient (D cm^2/sec) from profiles of the protein solution-solvent boundary, in a synthetic boundary cell at various times (t sec), the value of σ^2 is used directly: $\sigma^2 = 2$ Dt.

The same analysis can be used to estimate the width at half height (W) of partially overlapping Gaussian curves when visual estimation is not possible during amino acid analysis. When σ^2 is found for each curve as described above, then $W = 2\ (2 \ln 0.5\sigma^2)^{\frac{1}{2}}$.

Partial specific volume. The relationship given by Schachman (1957) was used to evaluate the partial specific volume ($\bar{v}$) from the measured concentration of the protein solution, x (g/ml), and the densities of the protein solution, d (g/ml) and of the solvent, d_o (g/ml):

$$\bar{v}\ (ml/g) = \frac{1}{d_o} - \frac{1}{x}.\frac{(d-d_o)}{d_o}.$$

Sedimentation equilibrium. (i) The basic equation for the distribution at equilibrium of the concentration (c) of a protein of molecular weight (M) and partial specific

volume $\bar{v}$ (ml/g) with radial distance r (cm) in a solution of density ρ(g/ml) at a temperature T°K and angular velocity ω (radians/sec) is:

$$\frac{d \ln c}{d r^2} = \frac{M(1-\bar{v}\rho)\omega^2}{2 RT} \text{ , where } R = 8.315 \times 10^7 \text{ erg/degree.mole.}$$

When Rayleigh interference optics are used, the concentration is measured in terms of absolute fringe number (J).

(ii) An alternative form of the basic equation is the Lamm plot (Creeth and Pain 1967):

$$\frac{d \ln(\frac{1}{r} \cdot \frac{dn}{dr})}{dr^2} = \frac{M(1-\bar{v}\rho)\omega^2}{2 RT}$$

dn/dr is the refractive index gradient at the radial distance r(cm), and is measured with a microcomparator as the displacement of the equilibrium schlieren pattern from the baseline (Δ y mm). The left hand side of the equation then becomes: $\frac{d \ln(\Delta y/r)}{dr^2}$, and as $\ln(\Delta y/r)$ is negative, the data are presented in Chapter 2 as $(1 + \log_{10} \Delta y/r)$.

(iii) When the plastocyanin samples are homogeneous both these plots yields straight lines and measurement of the slope gives the value of $M(1-\bar{v}\rho)\omega^2/2 RT$.

Diffusion and gel filtration. (i) The treatment of Ackers (1967) is used in the present work to estimate diffusion coefficient and Stokes radius on gel filtration, the basic equation being:

$a = a_o + b_o \, \text{erfc}^{-1}\sigma$, where a_o and b_o are constants.

The Stokes radius of a protein (a cm) being related to σ, the fraction of the internal volume of the gel matrix available to that protein:

$$\sigma = V_p/V_i \text{ , also } V_e = V_o + \sigma V_i \text{ ,}$$

where V_p = penetrable volume; V_i = total internal volume of the gel matrix; V_e = the elution volume of the protein; V_o = void volume of the gel bed.

The function $\text{erfc}^{-1}\sigma$ is the error function complement of the Gaussian distribution of the fraction of the internal volume of the gel matrix penetrable by a molecule, and can be obtained from published tables of erf x (Abramowitz and Stegun 1964); for $\text{erfc}^{-1}\sigma = \text{erf}^{-1}(1-\sigma)$.

(ii) The Stokes-Einstein equation is used to relate the diffusion coefficient of a protein (D) to its Stokes radius (a cm) at a temperature T°k and in a medium of viscosity (η g/sec.cm):

$$D(\text{cm}^2/\text{sec}) = kT/6\pi\eta a, \text{ where } k = 1.3804 \times 10^{-16} \text{ erg/degree.}$$

(iii) The shape of a protein molecule is related to its Stokes radius by the relationship:

$$f/f_o = a.\left(\frac{3\,\bar{v}\,M}{4\,\pi\,N}\right)^{-\frac{1}{3}}$$.(See for example Siegel and Monty 1966).

N is Avogadro's number (6.023×10^{23} atoms/mole) and other symbols have been defined above.

(iv) The measured values of the diffusion coefficient at temperature t° and in a medium of viscosity η (g/sec.cm) is corrected to the standard conditions of 20° and water using the equation given by Schachman (1957):

$$D_{20,w} = D_{meas.} \cdot \frac{293}{(273+t)} \cdot \frac{\eta}{\eta_w} \cdot \frac{\eta_{t,w}}{\eta_{20,w}} .$$

(v) The approximate relationship of Longsworth (1955), allows an estimation of the diffusion coefficient (D cm^2/sec) of a protein:

$$D = 2.7 \times 10^{-5} (M \bar{v})^{-\frac{1}{3}} .$$

Method of least squares. To obtain the best fit for a straight line through a set of experimental points the method of least squares is used. The straight line obtained is given by, $y = m x + c$. If there are k observations of x and y, then the best values for m and c are:

$$c = \frac{\left[\Sigma(x_n^2).\Sigma y_n - \Sigma x_n . \Sigma(x_n y_n)\right]}{\left[k.\Sigma(x_n^2) - (\Sigma x_n)^2\right]}$$

$$m = \frac{\left[k.\Sigma(x_n y_n) - \Sigma x_n . \Sigma y_n\right]}{\left[k.\Sigma(x_n^2) - (\Sigma x_n)^2\right]}$$

ABSTRACT

Plastocyanin has been isolated from lettuce, parsley and spinach, purified using DEAE-cellulose, ammonium sulphate fractionation, gel filtration and calcium phosphate gel adsorption and shown to be homogeneous by gel electrophoresis, ultracentrifugation and amino acid analysis. Plastocyanin, like bacterial blue copper proteins, contains one copper atom per molecule and has a predominantly hydrophilic amino acid composition. The EPR spectra are also similar, however, plastocyanin has a lower molecular weight than bacterial blue copper proteins. The copper binding site of parsley plastocyanin probably involves histidyl and tyrosyl residues of the protein. The isoelectric points of plastocyanin from the three sources were all found to be near pH 4; but the protein from spinach was somewhat more acidic than that from lettuce, which was in turn more acidic than that from parsley, in agreement with the electrophoretic mobility and the amino acid composition. An acidic isoelectric point is apparently characteristic of higher plant plastocyanin.

The molecular weights of the three plastocyanins were estimated to be near 11,000 both by gel filtration and by sedimentation equilibrium studies. The diffusion coefficient and the Stokes radius of spinach plastocyanin have been estimated both from the gel filtration data and by synthetic boundary experiments in the ultracentrige, and the values found to disagree with published results. It is proposed that the source of error in the determination of the literature

value of the molecular weight of spinach plastocyanin (21,000) lies in a two-fold underestimation of the diffusion coefficient. The value of the Stokes radius observed in the present work agrees with that expected for a typical globular protein of 11,000 molecular weight. The plastocyanin molecule could apparently have a loose conformation for a variation of 20% was seen in the elution volume of parsley plastocyanin from Sephadex G-75 depending on the buffer composition.

Analysis of copper by atomic absorption spectrophotometry in preparations of purified chloroplasts gave between one and two gram atoms per 400 moles of total chlorophyll, not allowing a decision to be reached concerning the possibility of a second copper-containing component in addition to the one molecule of plastocyanin per basic photosynthetic unit.

The effect of plastocyanin on cyclic electron flow in chloroplast digitonin particles, deficient in PS2 activity, was investigated by observation of the light-induced redox changes of P700 by dual wavelength spectrophotometry as plastocyanin was added back to the particles. It is proposed that plastocyanin is a component of both cyclic and noncyclic electron transport pathways. The increase in the dark reduction rate of photo-oxidised P700, which was found when plastocyanin was added in small quantities, was at first linearly proportional, and then proportional to the square of the plastocyanin concentration. When plastoquinones A and C were removed by extraction with non-polar solvents the dark reduction rate remained linearly proportional to plastocyanin concentration,

at all concentrations studied. Whereas the dark reduction by electrons from the reductive terminus of PS1 through plastocyanin was unaffected by the presence of methyl viologen, the similar increase in dark reduction rate induced by DCPIP was inhibited by methyl viologen.

Rubimedin has been isolated and found to contain equal amounts of copper and iron. It possessed a well-defined EPR spectrum, believed to arise from copper bound in an axially symmetrical site, and distinct from the spectrum of copper bound nonspecifically to protein. The visible spectrum was bleached on reduction with dithionite. It is however proposed that rubimedin is an artefact, and that plastocyanin is the only copper-containing component active in photosynthetic electron transport.

Inhibition of electron transport by the copper chelating agent salicylaldoxime is between cytochrome 559 and cytochrome f at a site common to both cyclic and noncyclic electron pathways and distinct from plastocyanin. The mechanism of inhibition probably does not involve chelation of copper. Cupferron, also a copper chelating agent, inhibits electron transport, but at a site distinct from the salicylaldoxime inhibition site. Its mechanism of inhibition is probably simply by competition for electrons from PS2.

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Laboratory of the Philips Research Laboratories. The investigation of the molecular weight of plastocyanin by interference optics, and the measurement of the partial specific volume of plastocyanin, were carried out in the Department of Clinical Biochemistry of the University of Oxford, and the co-operation of Dr. A.R. Peacocke is acknowledged. The few days spent in the Department of Botany of King's College, University of London, at the invitation of Professor F.R. Whatley and Dr. D.O. Hall are remembered with gratitude.

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REFERENCES

Aasa R. and Aisen P. J.Biol.Chem. 243 (1968) 2399.

Abramowitz M. and Stegun I.A. "Handbook of Mathematical Functions with formulas, graphs and mathematical tables". Superintendent of Documents, Washington D.C. (1964).

Ackers G.K. J.Biol.Chem. 242 (1967) 3237.

Akulova E.A. and Mukhin E.N. Dokl.Akad.Nauk.SSSR 180 (1968) 734.

Ambler R.P. and Brown L.H. Biochem.J. 104 (1967) 784.

Anderson J.M. and Boardman N.K. Biochim.Biophys.Acta 112 (1966) 403.

Anderson J.M., Boardman N.K. and David D.J. Biochem.Biophys.Res. Communs. 17 (1964) 685.

Andrews P. Nature 196 (1962) 36.

Andrews P. Biochem.J. 91 (1964) 222.

Arnon D.I. in "A symposium on Copper Metabolism" Eds. W.D. McElroy and B. Glass. John Hopkins Press (1950) 89.

Arnon D.I., Tsujimoto H.Y., McSwain B.D. and Chain R.K. in "Comparative Biochemistry and Biophysics of Photosynthesis". Eds. K.Shibata et al. University of Tokyo Press (1968) 113.

Beinert H. and Kok B. Biochim.Biophys.Acta 88 (1964) 278.

Bendall D.S. Biochem.J. 109 (1968) 46P.

Bishop N.I. Proc.Natl.Acad.Sci. U.S. 45 (1959) 1696.

Blethen S.L., Boeker E.A. and Snell E.E. J.Biol.Chem. 243 (1968) 1671.

Blumberg W.E. and Peisach J. Biochim.Biophys.Acta 126 (1966) 269.

Boardman N.K. Adv.in Enzymology 30 (1968) 1.

Boardman N.K. and Anderson J.M. Biochim.Biophys.Acta 143 (1967) 187.

Borchert M.T. and Wessels J.S.C. in press* (personal communication).

Bovey F.A. and Yanari S.S. in "The Enzymes" Eds. P.D.Boyer, H.Lardy and M.Myrback. Academic Press. 4 (1960) 63.

Brackenridge C.J. and Bachelard H.S. J.Chromatog. 41 (1969) 242.

Brill A.S., Martin R.B. and Williams R.J.P. in "Electronic Aspects of Biochemistry".Ed. B.Pullmann. Academic Press. (1964) 519.

Brill A.S., Bryce G.F. and Maria H.J. Biochim.Biophys.Acta 154 (1968) 342.

Bruinsma J. Photochem.Photobiol. 2 (1963) 241.

Brumby P.E., Miller R.W. and Massey V. J.Biol.Chem. 240 (1965) 2222.

Butler W. Ann.Rev.Plant Physiol. 15 (1964) 451.

Carrier J-M. in "Biochemistry of Chloroplasts" Ed. T.W.Goodwin. Academic Press II. (1967) 551.

Chain R.K., Tsujimoto H.Y., McSwain B.D. and Arnon D.I. Plant Physiol. 43 (Supplement) (1968) S13.

Chance B., De Vault D., Hildreth W.W., Parson W.W. and Nishimura M. in "Energy Conversion by the Photochemical Apparatus". Brookhaven Symp.Biol. No.19 (1966) 115.

Chrambach A., Reisfeld R.A., Wyckoff M. and Zaccari J. Anal.Biochem. 20 (1967) 150.

Clarke J.T. Ann.N.Y.Acad.Sci. 121 (1964) 428.

Cohn E.J. and Edsall J.T. in "Proteins, amino acids, and peptides as ions and dipolar ions". Reinhold Publishing Corporation. New York. (1943) 370.

---

* Biochim.Biophys.Acta 197 (1970) 78.

Coval M.L., Horio T. and Kamen M.D. Biochim.Biophys.Acta 51 (1961) 246.

Cramer W.A. and Butler W.L. Biochim.Biophys.Acta 172 (1969) 503.

Creeth J.M. J.Phys.Chem. 62 (1958) 66.

Creeth J.M. and Pain R.H. in "Progress in Biophysics and Molecular Biology". Eds. J.A.V.Butler and H.E.Huxley, Pergamon Press. 17 (1967) 219.

Davenport H.E. in "Non heme iron proteins: Role in Energy Conversion". Ed. A.San Pietro. Antioch.Press. Yellow Springs. Ohio. (1965) 115.

Davis B.J. Ann.N.Y.Acad.Sci. 121 (1964) 404.

Determann H. and Michel W. J.Chromatog. 25 (1966) 303.

De Vincenzi D.L. and Hedrick J.L. Biochemistry 6 (1967) 3489.

Donovan J.W. J.Biol.Chem. 244 (1969) 1961.

Drechsler Z., Nelson N. and Neumann J. Biochim.Biophys.Acta 189 (1969) 65.

Duysens L.N.M., Amesz J. and Kamp B.M. Nature 190 (1961) 510.

Edelhoch H. J.Amer.Chem.Soc. 79 (1957) 6100.

Elstner E., Pistorius E., Böger P. and Trebst A. Planta 79 (1968) 146.

Falk J.E. "Porphyrins and Metalloporphyrins. BBA Library, Volume 2" Elsevier Publishing Company. (1964) 181.

Fazekas de St.Groth S., Webster R.G. and Datyner A. Biochim.Biophys.Acta 71 (1963) 377.

Feeney R.E. and Komatsu St.K. Structure and Bonding 1 (1967) 149.

Flatmark T. Acta Chem.Scand. 18 (1964) 1656.

Fork D.C. and Urbach W. Proc.Natl.Acad.Sci.U.S. 53 (1965) 1307.

Fork D.C. and de Kouchkovsky Y. Biochim.Biophys.Acta 153 (1968) 891.

Fork D.C. and Amesz J. Ann.Rev.Plant Physiol. 20 (1969) 305.

Fujita Y. and Murano F. in "Comparative Biochemistry and Biophysics of Photosynthesis". Eds. K.Shibata et al. University of Tokyo Press. (1968) 161.

Gee R., Saltman P. and Weaver E. Biochim.Biphys.Acta 189 (1969) 106.

Goedheer J.C. Biochim.Biophys.Acta 172 (1969) 252.

Gomori G. in "Methods in Enzymology" Eds. S.P.Colowick and N.O.Kaplan. Academic Press. I (1955) 138.

Gorman D.S. and Levine R.P. Proc.Natl.Acad.Sci.U.S. 54 (1965)1665.

Gorman D.S. and Levine R.P. Plant Physiol. 41 (1966 a) 1637.

Gorman D.S. and Levine R.P. Plant Physiol. 41 (1966 b) 1648.

Granath K.A. and Kvist B.E. J.Chromatog. 28 (1967) 69.

Green A.A. J.Amer.Chem.Soc. 55 (1933) 2331.

Green L.F., McCarthy J.F. and King C.G. J.Biol.Chem. 128 (1939)447

Hall D.O.(personal communication).

Harvey M.J. and Brown A.P. Biochim.Biophys.Acta 172 (1969) 116.

Heber U. and Tyszkiewicz E. J.Expt.Bot. 13 (1962) 185.

Henninger M.D. and Crane F.L. Biochem.Biophys.Res.Communs. 24 (1966 a) 386.

Henninger M.D. and Crane F.L. J.Biol.Chem. 241 (1966 b) 5190.

Henninger M.D. and Crane F.L. J.Biol.Chem. 242 (1967 a) 1155.

Henninger M.D. and Crane F.L. Nature 214 (1967 b) 921.

Henninger M.D., Magree L. and Crane F.L. Biochim.Biophys.Acta 131 (1967) 119.

Hildreth W.W. Plant Physiol. 43 (1968) 303.

Hind G. Biochim.Biophys.Acta 153 (1968) 235.

Hind G. and Olson J.M. Ann.Rev.Plant Physiol. 19 (1968) 249.

Hjerten S., Jerstedt S. and Tiselius A. Anal.Biochem. 11 (1965) 219.

Horinishi H., Hachimori Y., Kurihara K. and Shibata K. Biochim. Biophys.Acta 86 (1964) 477.

Horio T., Sekuzu I., Higashi T. and Okunuki K. in "The Haematin Enzymes". Eds. J.E.Falk, R.Lemberg and R.K.Morton. Pergamon Press. (1961) 302.

Izawa S., Heath R.L. and Hind G. Biochim.Biophys. Acta 180 (1969) 388.

Jagendorf A.T. Plant Physiol. 30 (1955) 138.

Jagendorf A.T. in "Methods in Enzymology" Eds. S.P.Colowick and N.O.Kaplan, Academic Press. VI (1963) 430.

James W.O. and Das V.S.R. New Phytologist 56 (1957) 325.

Ji T.H., Hess J.L. and Benson A.A. in "Comparative Biochemistry and Biophysics of Photosynthesis" Eds. K.Shibata et al. University of Tokyo Press. (1968) 36.

Katoh S. Nature 186 (1960) 533.

Katoh S. and Takamiya A. Nature 189 (1961) 665.

Katoh S. and Takamiya A. J.Biochem. 55 (1964) 378.

Katoh S. and Takamiya A. Biochim.Biophys.Acta 99 (1965) 156.

Katoh S. and San Pietro A. Biochem.Biophys.Res.Communs. 24 (1966) 903.

Katoh S. and San Pietro A. in "Comparative Biochemistry and Biophysics of Photosynthesis". Eds. K. Shibata et al. University of Tokyo Press. (1968) 148.

Katoh S., Suga I., Shiratori I. and Takamiya A. Arch.Biochem. Biophys. 94 (1961) 136.

Katoh S., Shiratori I. and Takamiya A. J.Biochem. 51 (1962) 32.

Kautsky H., Appel W. and Amann H. Biochem.Z. 332 (1960) 277.

Ke B. Plant Physiol. 42 (1967) 1310.

Keister D.L. and San Pietro A. Arch.Biochem.Biophys. 103 (1963)45.

Keister D.L., San Pietro A. and Stolzenbach F.E. Arch.Biochem. Biophys. 98 (1962) 235.

Kelly J. and Sauer K. Biochemistry 4 (1965) 2798.

Kivelson D. and Nieman R. J.Chem.Phys. 35 (1961) 149.

Kok B. Biochim.Biophys.Acta 48 (1961) 527.

Kok B. and Rurainski H.J. Biochim.Biophys.Acta 94 (1965) 588.

Kok B. and Cheniae G.M. in "Current Topics in Bioenergetics" Ed. D.R.Sanadi. Academic Press. Volume 1 (1966) 1.

Kok B., Hoch G. and Cooper B. Plant Physiol. 38 (1963) 274.

Kok B., Rurainski H.J. and Owens O.V.H. Biochim.Biophys.Acta 109 (1965) 347.

Krogmann D.W. and Jagendorf A.T. Arch.Biochem.Biophys. 80 (1959) 421.

Krogmann D.W., Mendelin E.A. and Young A.M. Fed-Proc. 27 (1968) 827. Abstract No. 3458.

Kung S.D. and Williams J.P. Biochim.Biophys.Acta 169 (1968) 265.

Lee S.S., Young A.M. and Krogmann D.W. Biochim.Biophys.Acta 180 (1969) 130.

Leech R.M. Biochim.Biophys.Acta 79 (1964) 637.

Levine R.P. Science 162 (1968) 768.

Levine R.P. Ann.Rev.Plant Physiol. 20 (1969) 523.

Lightbody J.J. and Krogmann D.W. Biochim.Biophys.Acta 131 (1967) 508.

Longsworth L.G. in "Electrochemistry in Biology and Medecine". Ed. T.Shedlovsky. John Wiley. (1955).

Lundegårdh H. Nature 221 (1969) 280.

Malkin S. Biochim.Biophys.Acta 162 (1968) 392.

Malkin S. and Kok B. Biochim.Biophys.Acta 126 (1966) 413.

Malmström Bo.G. and Vänngård T. J.Mol.Biol. 2 (1960) 118.

Massini P. and Voorn G. Photochem.Photobiol. 6 (1967) 851.

Massini P. and Voorn G. Biochim.Biophys.Acta 153 (1968) 589.

Margoliash E. J.Biol.Chem. 237 (1962) 2161.

Margoliash E. and Lustgarten J. J.Biol.Chem. 237 (1962) 3397.

Milne P.R. and Wells J.R.E. Proceedings of the Australian Biochemical Society (1968) 12.

Moore S. and Stein W.H. in "Methods in Enzymology". Eds. S.P. Colowick and N.O.Kaplan. Academic Press. VI (1963)819.

Murata N., Nishimura M. and Takamiya A. Biochim.Biophys.Acta 120 (1966) 23.

Nakamura T. and Ogura Y. J.Biochem. 64 (1968) 267.

Nakamura T., Makino N. and Ogura Y. J.Biochem. 64 (1968) 189.

Neish A.C. Biochem.J. 33 (1939) 300.

Palmer G., Brintzinger H., Estabrook R.W. and Sands R.H. in "Magnetic Resonance in Biological Systems" Eds. A.Ehrenberg, B.G. Malmström, T.Vänngård. Pergamon Press. (1967) 159.

Park R.B. and Pon N.G. J.Mol.Biol. 6 (1963) 105.

Perlmann G.E. and Longsworth L.G. J.Amer.Chem.Soc. 70 (1948) 2719.

Porcelli G. Experientia 24 (1968) 211.

Powls R., Wong J. and Bishop N.I. Biochim.Biophys.Acta 180 (1969) 490.

Pratt L.H. and Bishop N.I. Biochim.Biophys.Acta 153 (1968) 664.

Punnett T. Plant Physiol. 34 (1959) 283.

Rajagopalan T.G., Moore S. and Stein W.H. J.Biol.Chem. 241 (1966) 4940.

Raymond S. and Weintraub L.S. Science 130 (1959) 711.

Renger G., Vater J. and Witt H.T. Biochem.Biophys.Res.Communs. 26 (1967) 477.

Richards E.G., Teller D.C. and Schachman H.K. Biochemistry 7 (1968) 1054.

Ridley S.M. and Leech R.M. Planta 83 (1968) 20.

San Pietro A. and Lang H.M. J.Biol.Chem. 231 (1958) 211.

Schachman H.K. in "Methods in Enzymology" Eds. S.P.Colowick and N.O.Kaplan, Academic Press. IV (1957) 32.

Schejter A., Glauser S.C., George P. and Margoliash E. Biochim. Biophys.Acta 73 (1963) 641.

Scheraga H.A. and Rupley J.A. Adv.in Enzymology 24 (1962) 161.

Schliephake W., Junge W. and Witt H.T. Z.Naturforsch. 23b (1968) 1571.

Schmid G.H. and Gaffron H. J.Gen.Physiol. 52 (1968) 212.

Searle G.F.W. M.Sc.Thesis, University of Warwick 1966.

Siegel L.M. and Monty K.J. Biochim.Biophys.Acta 112 (1966) 346.

Sokolovsky M. and Vallee B.L. Biochemistry 5 (1966) 3574.

Spencer D. and Possingham J.V. Aust.J.Biol.Sci. 13 (1960) 441.

Still C.C. and Price C.A. Biochim.Biophys.Acta 141 (1967) 176.

Stocking C.R. Plant Physiol. 34 (1959) 56.

Stocking C.R., Shumway L.K., Weier T.E. and Greenwood D. J.Cell.Biol. 36 (1968) 270.

Tagawa K., Tsujimoto H.Y. and Arnon D.I. Proc.Natl.Acad.Sci.U.S. 49 (1963) 567.

Tanner W., Loffler M. and Kandler O. Plant Physiol. 44 (1969) 422.

Teale F.W.J. Biochem.J. 76 (1960) 381.

Thalacker H. and Behrens M. Z.Naturforsch. 14b (1959) 443.

Thalacker H. and Behrens M. Experientia 16 (1960) 165.

Tietze F. J.Biol.Chem. 204 (1953) 1.

Trebst A. Z.Naturforsch. 18b (1963) 817.

Trebst A. and Eck H. Z.Naturforsch. 18b (1963) 105.

Trebst A. and Elstner E. in "Biochemistry of Chloroplasts" Ed. T.W.Goodwin. Academic Press. II (1967) 531.

Tsuboi K.K. and Hudson P.B. J.Biol.Chem. 224 (1957) 879.

Urbach W. and Simonis W. Biochem.Biophys.Res.Communs. 17 (1964)39.

Vallee B.L. and Williams R.J.P. Proc.Natl.Acad.Sci.U.S. 59 (1968) 498.

Van Holde K.E. and Baldwin R.L. J.Phys.Chem. 62 (1958) 734.

Vernon L.P. and Avron M. Ann.Rev.Biochem. 34 (1965) 269.

Vernon L.P., Shaw E.R. and Ke B. J.Biol.Chem. 241 (1966) 4101.

Voorn G. and Wessels J.S.C. (personal communication).

Walsh K.A. and Brown J.R. Biochim.Biophys.Acta 58 (1962) 596.

Wells J.R.E. Nature 212 (1966) 896.

Wessels J.S.C. Biochim.Biophys.Acta 109 (1965) 614.

Wessels J.S.C. Biochim.Biophys.Acta 126 (1966) 581.

Wessels J.S.C. Biochim.Biophys.Acta 153 (1968) 497.

West J. (personal communication).

Wetlaufer D.B. Adv.Protein Chem. 17 (1962) 303.

Whatley F.R. and Horton A.A. Acta.Chem.Scand. 17 (1963) Supplement 1 p.140.

Whatley F.R. and Grant B.R. Fed.Proc. 23 (1964) 227.

Whatley F.R., Ordin L. and Arnon D.I. Plant Physiol. 26 (1951)414.

Wilcox P.E., Kraut J., Wade R.D. and Neurath H. Biochim.Biophys. Acta 24 (1957) 72.

Yamashita T. and Butler W.L. Plant Physiol. 43 (1968) 1978.

Yamashita T. and Butler W.L. Plant Physiol. 44 (1969) 435.

Yamashita K., Itoh M. and Shibata K. Biochim.Biophys.Acta 189 (1969) 133.

Yamazaki R.K. and Tolbert N.E. Biochim.Biophys.Acta 178 (1969) 11.

Yoza N. and Ohashi S. J.Chromatog. 41 (1969) 429.

Zmrhal Z. Coll.Cz.Chem.Communs. 27 (1962) 2934.