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STUDIES ON THE STRUCTURE AND PHOTOCHEMISTRY OF CHLOROPLAST LAMELLAE

John Biggins

(Ph. D. Thesis)

January 1965

Studies on the Structure and Photochemistry of chloroplast lamellae

Contents

ABSTRACT

Fragmentation of chloroplast lamellae from Spinacia oleracea L. resulted in the formation of a sub-unit which was active in the Hill reaction. The sub-unit sedimentation coefficient was found to be 86×10^{-13}s and analysis of the boundary revealed that the preparation was polydisperse. The molecular weight was estimated as 18×10^6g-mole^{-1} and the sub-unit contained 2000 chlorophyll molecules. By density gradient sedimentation equilibrium the effective buoyant density of the sub-unit was found to be 1.175g-cc^{-1} and inhomogeneity was not detected. From the sub-unit density and the volume of a quantasome the molecular weight of a quantasome was computed as 2×10^6g-mole^{-1}.

After removal of the lipid from lamellae by the use of dodecyl sulfate and butanol, the lamellar protein fraction was found to consist of two cytochromes and a large quantity (95%) of non-heme protein. The sedimentation coefficient of the protein fraction was observed to be dependent on concentration and, at infinite dilution, was estimated to be 2.3×10^{-13}s. The concentration dependence is interpreted as indicating that the system exhibits an association-dissociation equilibrium and that the tendency for aggregation is very high. The diffusion coefficient was found to be 9.1×10^{-7}cm^2-sec^{-1} and, therefore, the molecular weight of the protein is about $22,000$g-mole^{-1}. It is suggested that the fraction, by virtue of its physical properties, is instrumental in maintaining the structure of the membrane.

A comparison of the action spectra and quantum requirements was made for NADP reduction by chloroplasts and quantasomes with water and reduced indophenol as electron donors. Between 550mu and 680mu both

systems were observed to occur with a requirement of two to three einsteins/equivalent NADP reduced. At wavelengths longer than 680mu, the reaction with water as electron donor showed an increasing requirement for quanta, whereas with reduced indophenol as donor, the reaction was observed to approach 1.5 einsteins/equivalent NADP reduced. The results reflect the participation of two pigment systems for NADP reduction and suggest that the transfer of energy between the two systems does not occur. Some characteristics of the two pigment systems are discussed.

The quantum requirements were found to increase linearly with increasing incident light intensity for both reactions. It is proposed that a photochemically generated intermediate of the photosynthetic unit with an estimated lifetime of 50msec when water is donor, and 150msec when reduced indophenol is donor, is responsible for this intensity dependence.

The action spectrum for ferricyanide reduction by chloroplasts was found to be characteristic of the short wave photosystem of photosynthesis with a region of maximum quantum efficiency at 650mu and not significantly modified by the addition of catalytic amounts of indophenol. These data support the hypothesis that oxidized indophenol is inert and that ferricyanide is the direct oxidant in this system.

Lamellar fragments consisting of about eight quantasomes are half as efficient as whole chloroplasts in the photoreduction of NADP when reduced indophenol is donor and a method is described whereby their absorption spectrum can be used to determine the true chloroplast absorption spectrum. Such a method eliminates the contribution of light scattering which seriously interferes with the calculation of absorbed light intensities in the determination of quantum requirements.

Acknowledgments

It was a privilege to be associated with Dr. K. Sauer with whom I collaborated in the investigations described in Part II of this study. I am especially grateful to him for tuition in precise physical techniques and for my introduction to photochemistry. It gives me great pleasure to thank him for his patience and for the principles he imparted throughout the investigations.

I wish to express my thanks to Dr. N. G. Pon and Dr. J. E. Hearst for guidance in aspects of physical biochemistry and to Professor F. R. Whatley and Dr. D. P. Hackett for stimulating conversations in all areas of plant biochemistry.

I particularly wish to thank Professor M. Calvin for making my residence in his laboratory a possibility and, through his inspiration, such a rewarding experience.

The immediate supervision of my graduate program was implemented by Dr. R. B. Park. I am indebted to him for his stimulation and guidance in all aspects of the work and it is a pleasure to acknowledge his continual interest and encouragement.

Abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
Chl	chlorophylls a and b
DCMU	3-(3, 4-dichlorophenyl) -1, 1- dimethylurea
DPIP	2, 6-dichlorophenolindophenol
ESR	electron spin resonance
Fd	ferredoxin
FMN	flavin mononucleotide
GTP	guanosine triphosphate
HOQNO	2-heptyl - 4 - hydroxyquinoline - N - oxide
NAD	nicotinamide adenosine dinucleotide
NADP	nicotinamide adenosine dinucleotide phosphate
$NADPH_2$	" " " " , reduced form
PMS	phenazine methosulfate
RTIC	rotor temperature indicating and control
Tris	tris (hydroxymethyl) amino methane

"We may therefore reasonably conclude that one great use of leaves is what has long been suspected by many, viz., to perform in some measure the same office for the support of vegetable life that the lungs of animals do for the support of animal life; plants very probably drawing thro' their leaves some part of their nourishment from the air.................. And may not light also, by freely entering the expanded surfaces of leaves and flowers contribute much to the ennobling of vegetables."

<div align="right">Stephen Hales, 1727</div>

General Historical Introduction

Early views on photosynthesis, based on the classical experiments of Priestley (1776), Ingen-Housz (1779), Senebier (1782) and De Saussure (1804) were that green plants, in the presence of sunlight, take up carbon dioxide, evolve oxygen and make starch. This concept prevailed through the nineteenth century with the additional finding by Engelmann (1881) that the site of oxygen evolution in the cell is the chloroplast.

Investigations by Blackman (1905, 1911) established that overall photosynthesis consists of two classes of events: photochemical events which are dependent upon light intensity but temperature independent, and chemical events, which are temperature sensitive but independent of light intensity. Emerson and Arnold (1932) elaborated earlier experiments by Brown and Escombe (1905), and Warburg and Negelein (1923) and showed that the photosynthetic yield per unit of absorbed energy of a continuously-illuminated system could be considerably improved if the light is supplied in the form of flashes. Furthermore, the dark period

can be shortened in time if the temperature is raised. This suggested that some factor is made in the light which is then used in subsequent temperature sensitive dark reactions. From these kinetic experiments they also introduced the concept of a physiological photosynthetic unit containing about 2000 chlorophyll molecules. This was later revised by Kok and Businger (1956, 1957) who made similar kinetic experiments and suggested that the physiological photosynthetic unit contains 200 to 400 chlorophyll molecules.

It was Van Niel in the early 1930's who laid the foundation upon which much of the subsequent investigations were conducted. His comparative approach to the study of photosynthetic green and purple-sulfur bacteria led him to generalize and propose that the light reaction leads to a "photolysis" of water. The reducing moiety is then associated with the mechanism for the reduction of carbon dioxide, and the oxidized moiety reacts to form oxygen which is liberated as a gas. The photosynthetic bacteria, which do not evolve oxygen and require the addition of an external reductant such as H_2S, do not have the enzymic mechanism for the rearrangement of the oxidized moiety to form oxygen but, instead, use the outside hydrogen donor to reduce the moiety and reform water and the oxidized donor. At the time, this formulation accounted for all the known facts of bacterial and higher plant photosynthesis.

Further confirmation of Van Niel's formulation (1941) came from studies by Ruben, et al. (1941) using enriched oxygen (CO_2^{18} and H_2O^{18}) as a tracer. They showed that the source of oxygen liberated during photosynthesis is water rather than carbon dioxide.

Van Niel's formulation (1941) can be represented as follows:

$$4H_2O - \text{photolysis} \begin{cases} \text{Oxidized Moiety} \quad 4[OH] \xrightarrow{\text{higher plants}} 2H_2O + O_2 \\ \\ \text{Reduced Moiety} \quad 4[H] \end{cases}$$

$$4[OH] \xrightarrow[2H_2A]{\text{bacteria}} 4H_2O + 2A$$

$$4[H] \xrightarrow[CO_2]{} \text{cellular products}$$
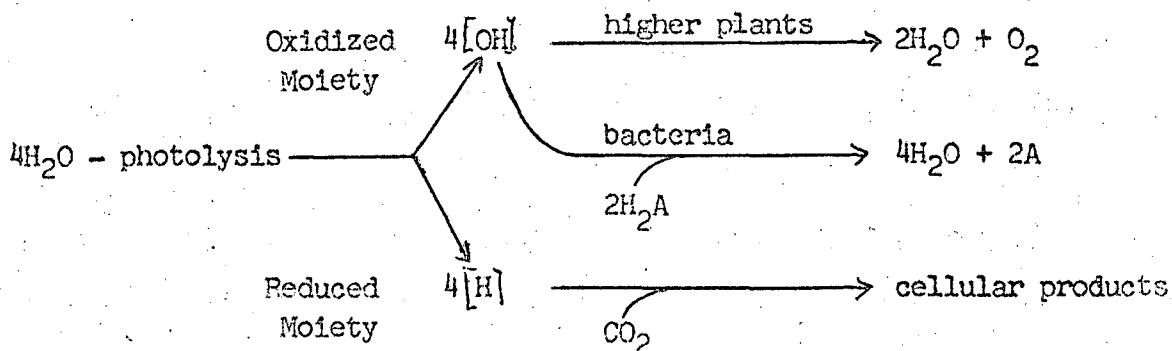
where H_2A represents an external hydrogen donor.

By the use of radioactive carbon (C^{14}) in conjunction with paper chromatography, Calvin and his collaborators completely mapped the metabolic fate of carbon dioxide during photosynthetic assimilation in green algae (see Bassham and Calvin, 1957, for review). The path was shown to be a cyclic process, the chief components being sugar phosphates. Many of the reactions in the sugar rearrangements were shown to be similar to those of the oxidative pentose cycle which was discovered shortly afterward (see Axelrod and Beevers, 1956, for review). Calvin defined the energetic and reductive requirements of the carbon reduction cycle in metabolic terms as $2NADPH_2$ and $3ATP$ per carbon dioxide assimilated.

Hill (1939) was the first to demonstrate a photochemical reaction related to photosynthesis outside the confines of the living cell. He found that isolated chloroplasts would evolve oxygen when illuminated (ie. photolysis of water) and supplied with a suitable electron acceptor such as ferric ion. However, he was unable to show that carbon dioxide could act as the electron accepter.

The demonstration of the light dependent formation of the cofactors $NADPH_2$ and ATP, together with evidence that the chloroplast is the cellular site for carbon assimilation, was achieved by Arnon et al (1954, 1957).

The production of ATP was shown to occur in isolated chloroplasts in a coupled Hill reaction with NADP as the natural physiological oxidant (non-cyclic photophosphorylation). An additional photophosphorylation occurs in the presence of an added cofactor such as vitamin K, PMS or FMN. This phosphorylation proceeds in the absence of gas evolution or uptake and is, therefore, termed cyclic photophosphorylation. Later, Trebst et al (1958) demonstrated that the production of ATP and $NADPH_2$ is associated with the chloroplast membrane fraction (the "grana" or lamellae) during the light reactions, and that the carbon dioxide assimilation occurs by means of the soluble enzymic apparatus in the chloroplast stroma.

Investigations on the structure of the chloroplast began shortly after the discovery by Engelmann (1881) that oxygen evolution occurs in the chloroplasts of algae. Pringsheim (1881) and Schmitz (1883), in a series of investigations using the light microscope, found that chloro- plasts have a granular or netlike internal structure. Meyer (1883) called such highly absorbing granular areas "grana". A complete survey of all major plant taxonomic groups was made by Heitz (1936) who showed that grana are present in many classes.

With the advent of newer techniques in electron microscopy, such as shadowing and ultra-thin sectioning, it soon became apparent that the internal structure of the chloroplast contains a bilaminate membrane sys- tem. Localized grana areas were recognized as lamellar stacks 0.5u wide and 0.8u high (Steinman, 1952; Steinman and Sjöstrand, 1955) and connected by single membranes 30 A thick to form a continuous lamellar system. Granick and Porter (1947), by means of shadowing techniques, found about 40 to 60 grana per chloroplast. Extraction of the material on the electron microscope grids with methanol showed that the residual fraction, presumed to be protein, constituted less than half the original lipo-protein mass,

and still retained some structure. No sub-units were identified in these investigations.

Additional evidence for a lamellar system in chloroplasts was provided by Frey-Wyssling and Steinman (1948), who obtained pure layer birefringence curves that they claimed arose from protein inter-weaved with oriented lipids.

Early work on the composition of the chloroplast (Menke, 1938), "chloroplastic" matter (Chibnall, 1939; Neish, 1939; Comar, 1942) and grana (Bot., 1942) showed that these preparations contained 40 to 50% protein, 20 to 40% lipid, and 5 to 10% photosynthetic pigments, and the concept of a chlorophyll-protein complex as a chloroplast sub-unit, by analogy to hemoglobin, was quite popular.

Thus, in the late 1950's the concept of structure and function in the higher plant chloroplast was that the internal lamellae contain the photosynthetic pigments and are responsible for light absorption, quantum conversion and electron transport with associated phosphorylation. The stroma fraction contained all the enzymes to catalyze the carbon reduction cycle in the presence of the ATP and $NADPH_2$ formed during electron transport.

The purpose of the work described here is twofold. Part I is a study of the molecular architecture of the chloroplast lamellae using biochemical techniques which have proved particularly useful in the study of the structure of the mitochondrion (Green, 1961). Part II is a study of some of the photochemical properties of reactions that occur during photosynthetic electron transport in the lamellae. In particular, emphasis is placed on quantum efficiencies and wavelength dependences of the reactions in order to further characterize the pigment systems responsible for quantum conversion.

Part I. Studies on the Structure of the Chloroplast Lamellae.

Introduction

Chloroplasts of higher plants are located in the mesophyll of the leaves and, as observed in the light microscope, appear as disc-shaped bodies approximately 5 u in diameter and about 2-3 u thick. The chloroplast is surrounded by a semi-permeable membrane which is osmotically active and internally appears to consist of two main phases. A number of highly absorbing areas containing the photosynthetic pigments, called "grana" are embedded in a matrix of soluble protein, the "stroma" (Weier, 1938; Weier and Stocking 1952; Rabinowitch, 1945; Granick, 1961).

By means of the electron microscope and by virtue of the recent tremendous advances made in the preparation of material for observation, the ultra-structure of the grana areas has been considerably amplified. Notable work has been accomplished by Steinman (1952), Steinman and Sjöstrand (1955), Granick and Porter (1947), Hodge, et al (1955), von Wettstein (1957 and 1959), Sager (1959), and more recently, by Park and Pon (1961) and Weier, et al (1963).

The grana are bi-laminated stacks about 0.5 u in diameter and 0.8 u high and are connected by single, larger membranes (stroma lamellae) to form a continuous lamellar system within the chloroplast (see Fig. 1).

Isolated chloroplasts can be physically separated into the two main phases, the stroma and the lamellae, by osmotic rupture in dilute buffer followed by ultracentrifugation. The lamellae are sedimented at about 10,000 g after 10 minutes' centrifugation, whereas the stroma proteins remain in the supernatant. The stroma protein fraction has
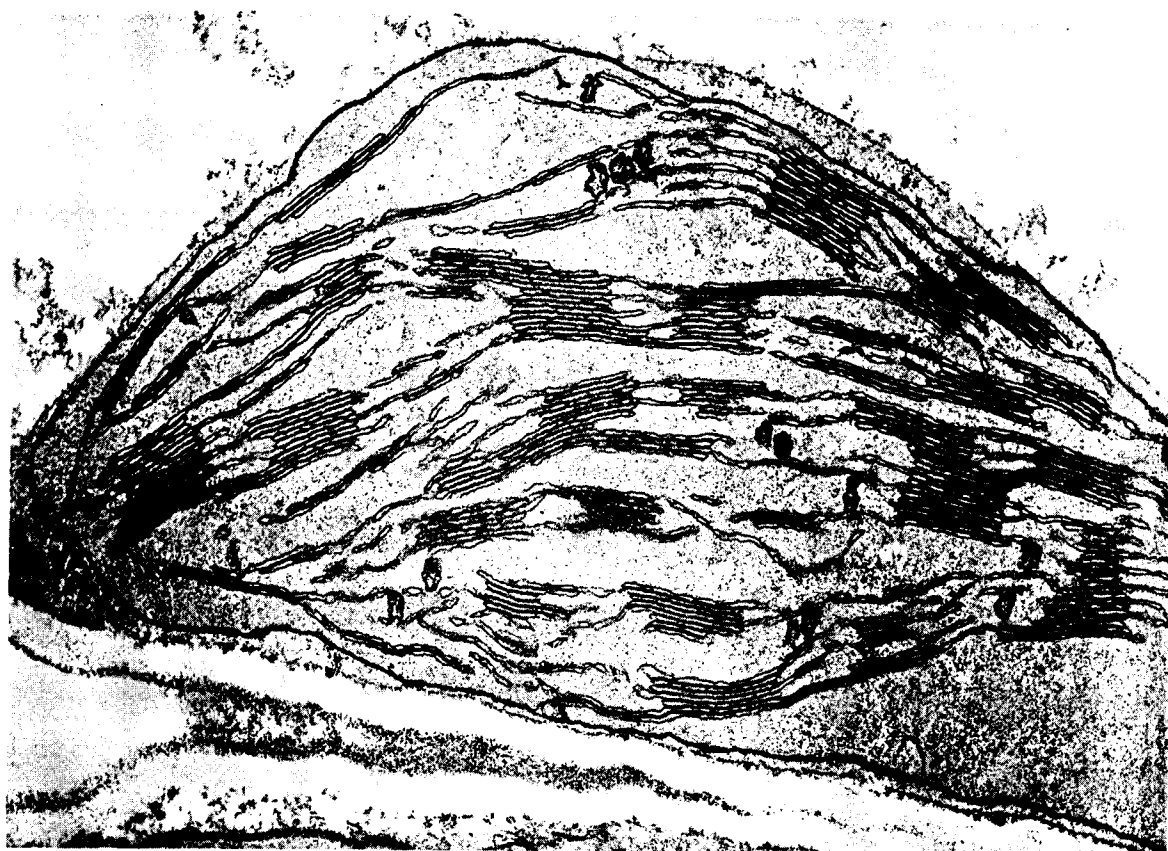
ZN-4606

Fig. 1. Ultrathin section of a chloroplast from Spinacia
oleracea L. Fixed in 2% $KMnO_4$ and embedded in
Epon. Magnification $392,000^x$. Kind courtesy Dr.
R. P. Park.

been studied from physical and enzymological viewpoints (Singer et al 1952; Lyttleton and T'so, 1958; Pon, 1960; Racker, 1955; Mayaudon, 1957; Rabin and Trown, 1964). The principal protein is a 16 Svedberg component which is also the major protein of the green leaf, and has been designated Fraction I by Wildman. Carboxydismutase (Pon, 1960; Van Noort and Wildman, 1964) activity is associated with Fraction I, but there is disagreement concerning the phosphoribulokinase and isomerase activities. It is possible that Fraction I protein is part of an organized system of multifunctional enzymes in the chloroplast concerned with CO_2 assimilation as proposed by Bassham (1963).

Fraction II protein, the minor constituent of the stroma protein, is a mixture of many enzymes and is in the order of 2 to 4 Svedbergs. Some forty enzyme activities have been identified in chloroplast material and they are presumably associated with Fraction II (see Thomas, 1960, for review). In addition to the protein, DNA and RNA have been shown to be constituents of the chloroplast stroma (see Granick, 1961, for review). Ribosomes have also been isolated from chloroplasts indicating that a fraction of the RNA is in the form of nucleoprotein (Lyttleton, 1962).

The lamellar fraction of the chloroplast contains all the components necessary for light absorption, quantum conversion and electron transport in photosynthesis. Very pure lamellae, obtained by repeated washing with dilute buffer and centrifugation, were shown to be active in quantum conversion and were analyzed by Park and Pon (1963). They showed that the lamellae are about 50% protein and 50% lipid. The lipid fraction contains all the chlorophylls, carotenoids and quinones. Park and Pon (1963) determined the distribution of the transition metals,

Cu, Mn and Fe, which are likely to be involved in oxidation-reduction reactions in the two fractions. They found that all the metals are in the protein fraction apart from Mg, which is present in the chlorophyll and Mn is present in the lowest concentration. As Mn is essential for oxygen evolution in photosynthesis (Kessler, 1957), there must be at least one atom per photosynthetic unit. Hence, they calculated a minimum molecular weight of a photosynthetic unit by calculating the material associated with one atom of manganese. Such a minimum molecular weight based on the manganese content is about 10^6 gm. $mole^{-1}$ and contains 115 chlorophylls.

Park and Pon (1961) also investigated the structure of sonicated chloroplast lamellae by electron microscopy. The lamellar fragments which were prepared for microscopy were shown to be active in quantum conversion and to support CO_2 fixation in the presence of added stroma. When shadowed, and viewed in the electron microscope, many fragments were seen to be granular in appearance and, in some cases, regular arrays of particles were seen as in Fig. 2. The particles were particularly apparent in areas where the upper layer of the bilaminated membrane had been torn by sonication, exposing the inside of the membrane. The particles were described as 200 A x 100 A oblate spheroids, and Park and Pon (1961) suggested that this particle is a repeating sub-unit of the lamellae. They later showed (1963) that by employing a suitable consistant packing arrangement in the membrane, the lamellae of all photosynthetic organelles in green plants and algae could be composed of this particle (see Fig. 3).

As the unique feature of aggregates of the particle is the ability to convert light energy to chemical potential, it was subsequently named
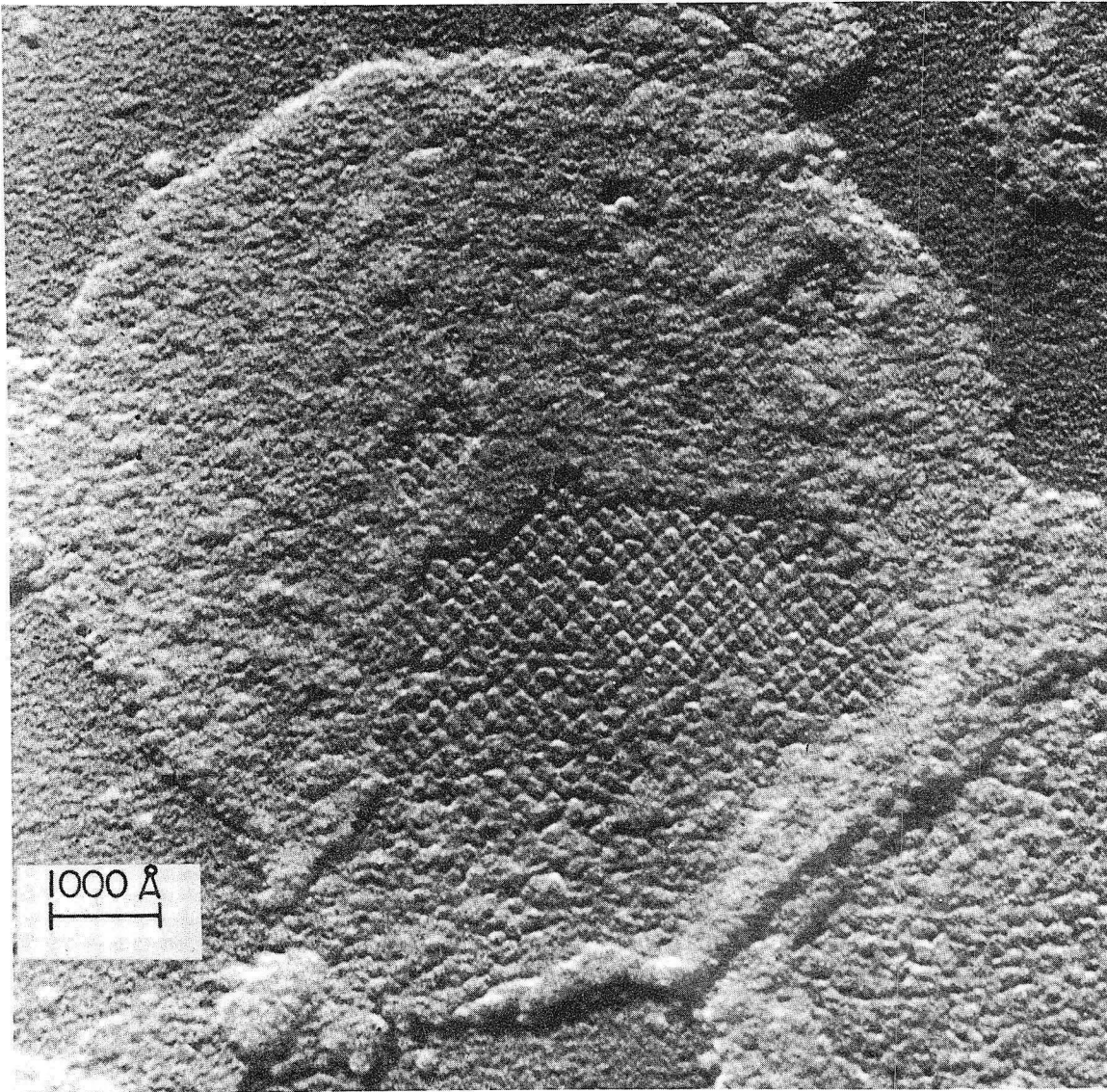
ZN-4304

Fig. 2. Spinach chloroplast lamellae washed free of chloroplast
stroma, sonicated and shadowed with chromium. Quanta-
some arrays can be seen in areas where the upper mem-
brane has been removed by the sonic treatment. Kind
courtesy Dr. R. B. Park.
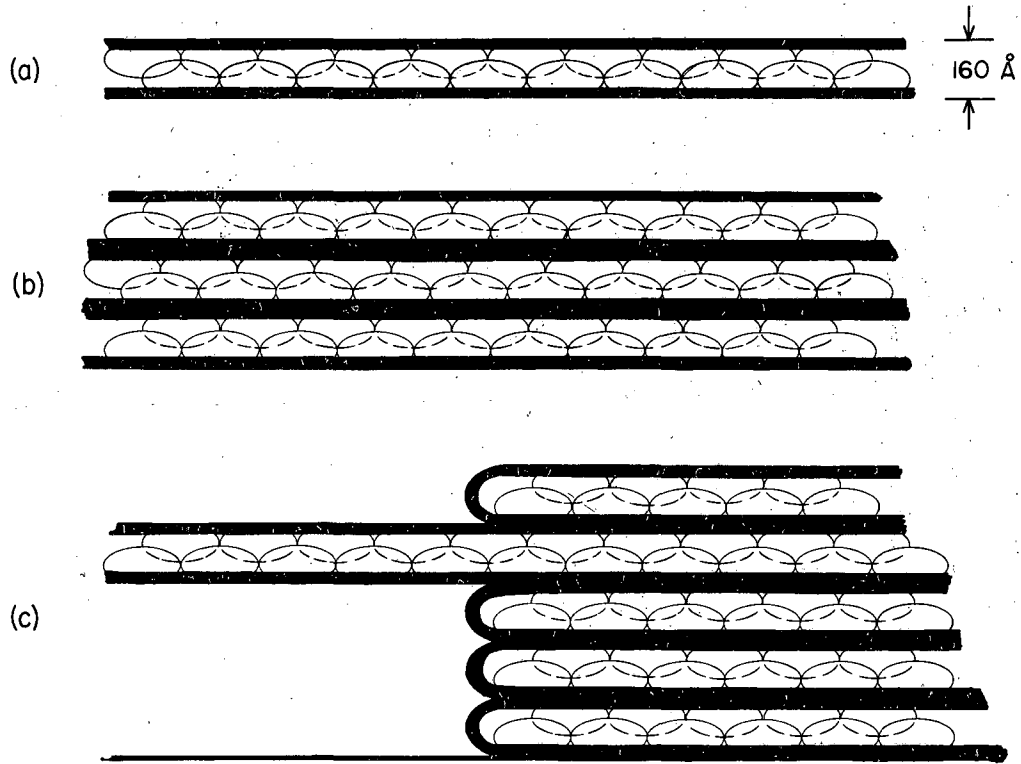
Fig. 3. Substructure of lamellar structures from photosynthetic organisms (Park and Pon, 1963). The particles, quantasomes, are granular sub-units and are osmiophilic over one surface. The model shows how the quantasome may be a fundamental repeating sub-unit of a) blue-green algae, b) Euglena and c) higher plants.

a "quantasome" (Park, 1962) and Park suggested that it may be the morphological expression of the physiological photosynthetic unit first formulated by Emerson and Arnold (1932) and more recently by Kok and Businger (1956, 1957).

Later work showed that the quantasome exists in at least three different types of packing arrangements in lamellae; a para-crystalline array, which permitted a more accurate determination of the dimensions of the quantasome, a linear array and a random array which appears most frequently (Park and Biggins, 1964). The dimensions of the quantasome as seen in the para-crystalline array are 185 A x 155 A and 100 A thick.

The lamellar fragments obtained by Park and Pon (1961) by sonication of the chloroplast lamellae are active in quantum conversion. However, the fragments are heterogenous with respect to particle size and consist of up to 8 quantasomes. Hence, the integrity of the complete lamellae is unnecessary for quantum conversion.

The lipid fraction of the chloroplast lamellae has, in the past, received a tremendous amount of attention owing to the considerable interest in the nature and state of the photosynthetic pigments. The most recent work on the composition of the lamellae, and specifically in relation to the quantasome hypothesis, is that by Lichtenthaler and Park (1963) and Lichtenthaler and Calvin (1964).

Table 1 shows the composition of a photosynthetic unit relative to one atom of manganese based on the results of the investigations at Berkeley and elsewhere.

Table 1.

Representative distribution of substances in spinach chloroplast lamellae on the basis of a minimum molecular weight of 960,000 per mole of manganese (Lichtenthaler and Park, 1963).

<u>Lipid</u> composition (moles/mole Mn)

115	chlorophylls	103,200
24	carotenoids	13,700
23	quinones	15,900
58	phospholipids	45,000
72	digalactosyldiglycerides	67,000
173	monogaloctosyldiglycerides	134,000
24	sulpholipids	20,500
	sterols	7,500
	unidentified lipid	87,800
		495,000

<u>Protein</u>

4690	N atoms as protein	464,000
1	Mn	55
6	Fe	336
3	Cu	159
		465,000
	Lipid and protein	960,000

The protein fraction has received very little direct attention other than the recognition that it contains the two cytochromes f and b_6 which appear to be restricted to photosynthetic tissues (Hill and Bonner, 1961). The fact that the protein fraction in the absence of the lipids is insoluble at physiological pH, and the absence of appropriate tools to cope with this problem are the most likely reasons for the lack of attention the protein fraction has received in the past.

However, for a complete understanding of the molecular architecture of the chloroplast lamellae, further knowledge concerning the protein fraction is required — in particular, the total number of protein species per quantasome and their molecular weights, shapes and prosthetic groups if any. As the integrity of the quantasome relies heavily upon the physical nature of the proteins, knowledge of their properties would also be advantageous.

Green and associates (1961 a,b) have made notable contributions to an understanding of the structure of the mitochondrion using bile salts and detergents to great advantage in the solubilization of the cristae. In addition, they have physically characterized all the cytochrome complexes and constituents of the mitochondrial respiratory unit or elementary particle, in the presence of detergents (see Fernandez-Moran et al, 1964). Although there are limitations concerning the validity of such measurements, it is the only approach in modern biochemistry available at this time.

The work presented here in Part I is a further study of the physical properties of chloroplast lamellae (section A) and a physical characterization of the lamellar protein fraction (section B).

IA. Studies on the Structure of Chloroplast Lamellae.

1. Preparation of chloroplasts and membrane fractions

Chloroplasts were prepared from spinach (Spinacia oleracea L.) leaves by procedures according to Arnon, et al (1956), Park and Pon (1961) or Hoch and Martin (1963). Prior to homogenization, the de-stemmed leaves were thoroughly washed and refrigerated in a sealed polyethylene bag for at least one hour. This treatment increased the turgidity of the leaves and better yields of intact chloroplasts were obtained (Whatley and Arnon, 1963). Following isolation, the chloroplasts were osmotically ruptured and the stroma fraction removed by high speed centrifugation. The membrane fraction was sonicated and, after removal of large fragments, quantasome aggregates collected as outlined in Fig. 4.

For some of the studies (see Sec. B) large quantities of lamellae were required and, for these purposes, chloroplasts were isolated by a continuous flow procedure. Ten kg of spinach leaves were homogenized in 25 l 0.35 M-NaCl, 0.02 M tris-Cl pH 8.0 in 500 g, 1 l portions, and strained through 8 layers of cheesecloth. The brei was centrifuged in a Servall RC-2 Automatic Superspeed Refrigerated Centrifuge equipped with a Szent-Györgyi and Blum 8-tube continuous flow system. The centrifugation conditions found to best compromise between yield and purity of chloroplasts were a flow rate of 60 ml/min and 4000 rpm. The sediment from 10 kg leaves (about 300 ml wet-packed volume) was suspended in 10 l 0.035 M-NaCl, 2 x 10^{-3} M-tris-Cl pH 8, stirred for one hour and then centrifuged at 17,000 rpm (34,000 g) at a flow rate of aproximately 10 ml/min. The sedimented green membrane fraction was then

```
                        - → chloroplasts (isolated in isotonic buffer)
                                │  homogenize in 0.035 M NaCl,
                                │     2 x 10⁻³ M tris-Cl, pH 8
   repeat twice                 │
                                │  30,000 g, 10 min
                                ▼
          ┌─────────────────────────────────────────────┐
   sediment P₁                                  supernatant S₁

   (membrane fraction) and starch            (stroma fraction)

   semi-pure lamellae
          │
          │   sonicate, 9 kc, 90 sec Raytheon 100w magnetostriction oscillator
          │   30,000 g, 10 min.
          │
          ▼
          ┌─────────────────────────────────────────────┐
   sediment                                     supernatant S

   (unbroken lamellae, large
    fragments and starch)
   fra
                                       small lamellar fragments

                                          make 0.1 u with NaCl
                                          30,000 g, 10 min.
                                                │
                                                ▼
          ┌─────────────────────────────────────────────┐
   sediment P₂                                  supernatant

   (purified chloroplast lamellae)        (residual stroma fraction
                                           detached by sonication)

   Quantasome aggregates
```

Homogenize in 0.035 M NaCl, 2×10^{-3} M tris-Cl, pH 8, $30,000$ g, 10 min.

sediment P_1 supernatant S_1

(membrane fraction) and starch   (stroma fraction)

semi-pure lamellae

sonicate, 9 kc, 90 sec Raytheon 100w magnetostriction oscillator $30,000$ g, 10 min.

sediment   supernatant S

(unbroken lamellae, large fragments and starch) fra

small lamellar fragments

make 0.1 u with NaCl $30,000$ g, 10 min.

sediment P_2 supernatant

(purified chloroplast lamellae)   (residual stroma fraction detached by sonication)
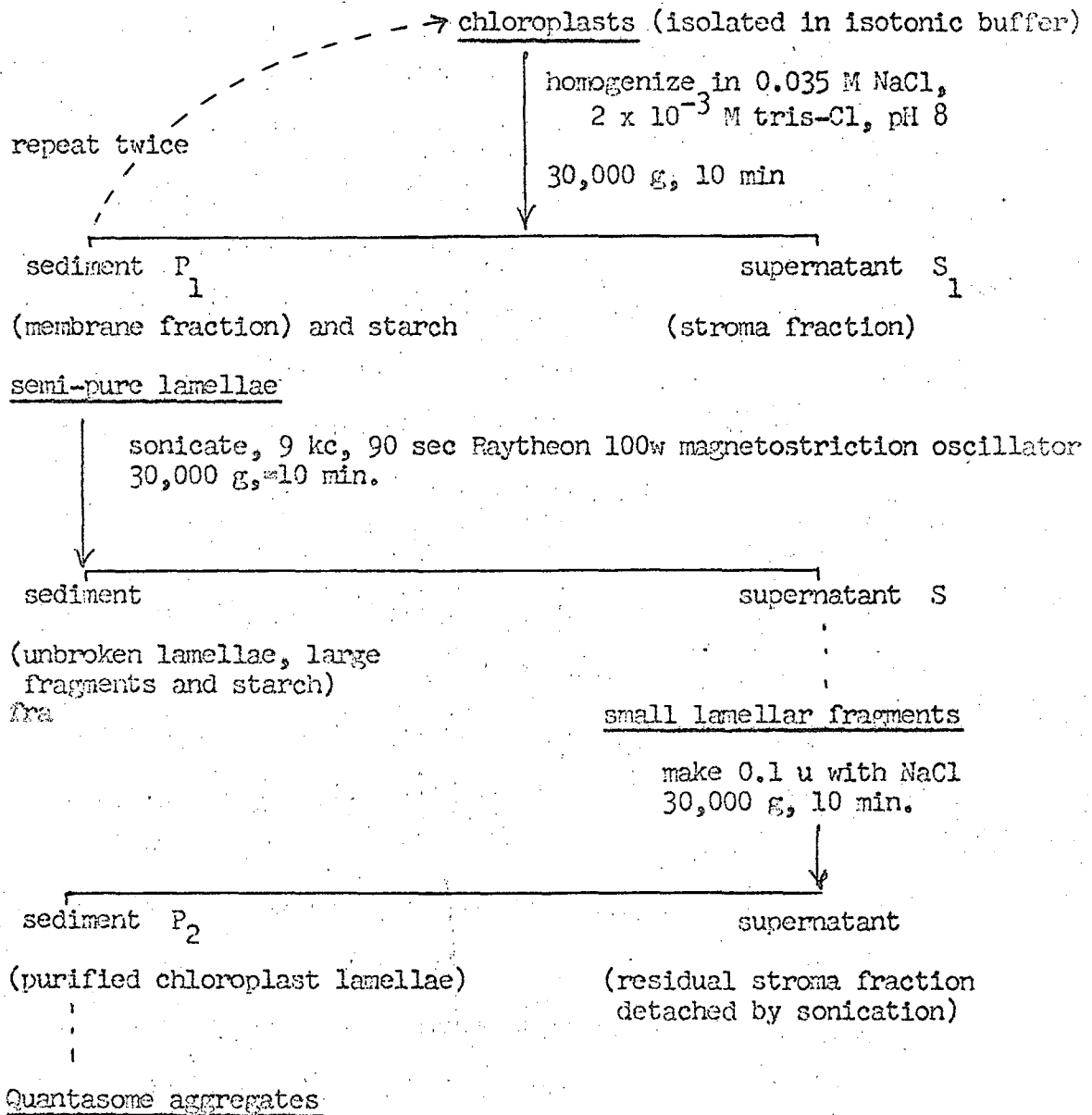
Quantasome aggregates

Fig. 4. Scheme for the Isolation of Chloroplast Fragments

resuspended in 200 ml dilute buffer and the preparation continued in the usual fashion. The supernatant from the final continuous-flow centrifugation is an excellent source of carboxydismutase.

Preparation of lamellar sub-unit

The preparation of quantasome aggregates as shown in Fig. 4 yields a heterogeneous system with respect to particle size. The preparation can be partially resolved by differential ultracentrifugation by a procedure according to Park and Pon (1961), yielding fractions of up to 8 quantasomes. These preparations are satisfactory for metabolic and spectroscopic studies, but their poly-dispersity impedes certain physical studies.

A survey of membrane fragmentation techniques was made in an attempt to prepare monomeric quantasomes or a monodisperse sub-unit of the membrane. It was found that detergents such as cholate, deoxycholate, Na-dodecyl sulfate and Tween were ineffective in the preparation of active sub-units, but were very effective in removing lipid from the membrane. Finally, a technique based on principles previously employed by Smith (1960) for the preparation of proto-chlorophyll holochrome from etiolated bean seedlings was used. This method, outlined below, utilizes a low concentration of cysteine at high pH and, in combination with sonic rupture, an active membrane preparation was obtained which, when partially characterized, appeared ultracentrifugally homogeneous. Rigorous analysis of the sedimentation velocity data, however, revealed that the preparation is polydisperse but not to a considerable extent.

Procedure

Quantasome aggregates were prepared and suspended in 0.1 M-glycine, 0.05 M-KOH and 0.02 M-cysteine-HCL (gly-KOH-cys-SH) and homogenized

gently by means of a Potter-Elvehjem homogenizer with a Teflon pestle. This treatment fragmented the membrane. The treated membranes were then ultracentrifuged at 105,000 g for 15 minutes to remove large fragments; the supernatant was retained as the preparation for study. Preparations were stored for periods of up to one week under nitrogen, but determinations of activity in the Hill reaction were carried out immediately after preparation.
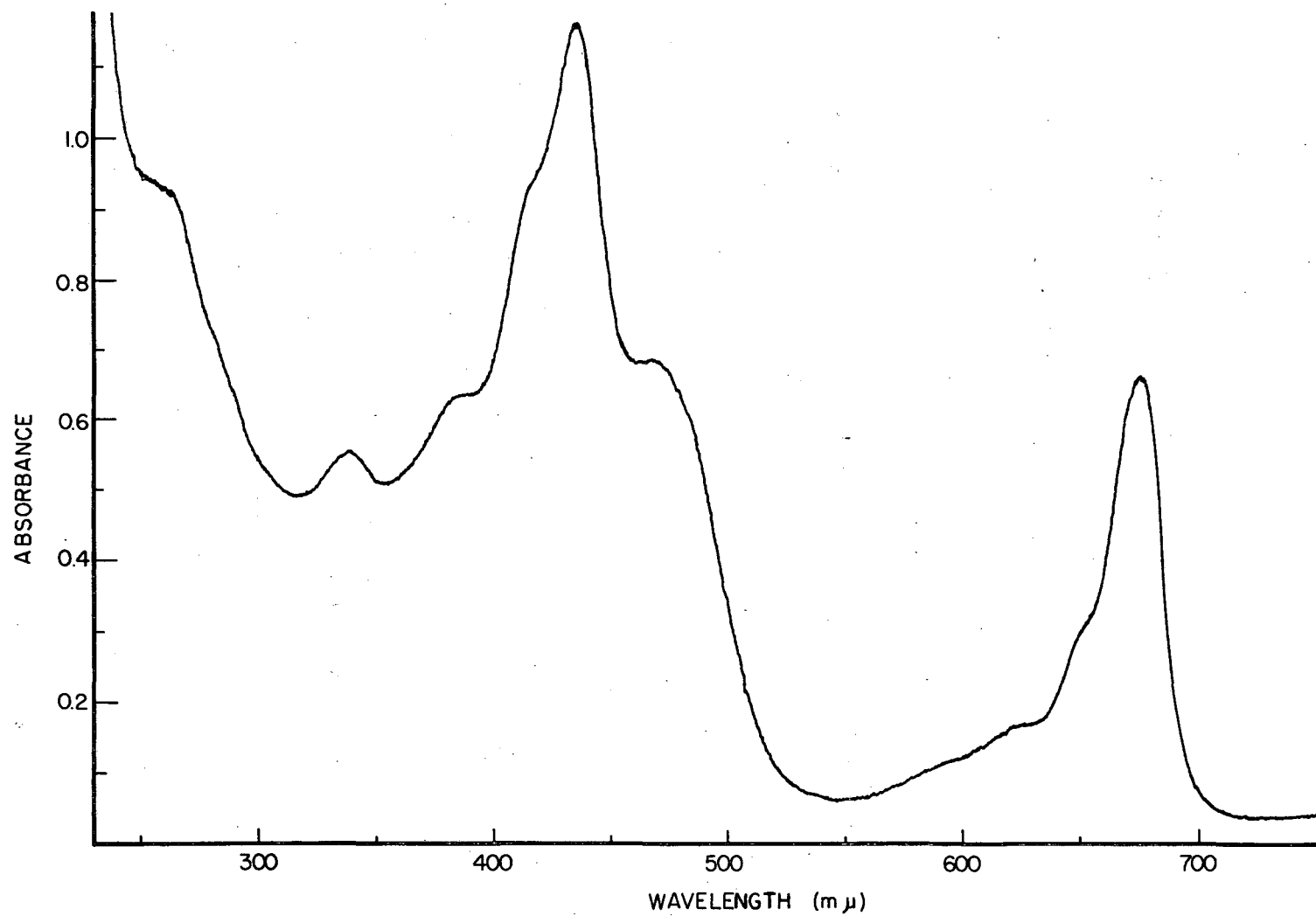
2. Absorption spectrum

If the preparation is a representative sub-unit of the photosynthetic membrane, then the entire pigment complement should be present and the absorption spectrum should be identical to that of intact lamellae.

Many investigators have reported the absorption spectra of photosynthetic systems (see French, 1960), and the most recent work for higher plants is that by Sauer and Park (1963). They measured the absorption spectrum of sonicated spinach lamellae by means of a scattered transmission technique. They found that the absorption maximum in the red region of the spectrum is not shifted from 678.5 mu in the preparation of chloroplasts, by sonication or by the separation of the small lamellar fragments by differential ultracentrifugation.

Fig. 5 shows the absorption spectrum of the sub-unit preparation prepared by treatment with the gly-KOH-cys-SH buffer pH 9. The absorption maximum in the red region of the spectrum is 677 mu. Thus, there is a shift of 1.5 mu from the value _in vivo_ and for untreated lamellae. It is highly probable that this shift is due to treatment of the lamellae with the gly-KOH-cys-SH buffer.

Treatment of photosynthetic organelles with organic solvents and detergents lead to similar shifts of this absorption maximum to shorter

MUB-1117

Fig. 5. Absorption spectrum of chloroplast lamellar sub-units
prepared by sonication and treatment with gly-KCH-cys-SH
buffer. The absorption maximum in the red is 677 mu.

wavelengths. The magnitude of the shift depends upon the concentration and type of additive and, eventually, extraction of the pigments occurs. Smith (1941) found that 2.5% digitonin, 3% bile salts such as Na-taurocholate and Na-glycocholate and 0.5 to 0.25% deoxycholate lead to a shift of the red maximum to 675 mu with a concomitant increase in fluorescence. Sodium dodecyl sulfate (0.25%_ led to a much greater shift to 670 mu. α-Picoline and dioxane (Takashima, 1952), 1% Dupanol and 1% Span (Chiba, 1960) and dodecylbenzene sulfone (Itoh, et al, 1963) also lead to similar shifts.

A thorough study of such absorption maximum shifts induced by organic solvents and detergents was made by Sauer and Park (1963) in an investigation of the possibility that such shifts be directly correlated with the loss of photochemical activity and, thus, permitting use of the position of the absorption maximum as a sensitive measure of the structural integrity of the photosynthetic apparatus. Although the results were complex, in general they found that there was a loss in photochemical activity at solvent concentrations lower than was required to produce a measurable spectral shift. From this work, then, one would anticipate a loss of photochemical activity if a shift is detectable.

3. Activity of the lamellar sub-unit

A further prerequisite that the sub-unit truly represents a fragment of the photosynthetic membrane is that it be active in quantum conversion and, for this purpose, the Hill reaction was used as a criterion. Ferricyanide was used as an oxidant and oxygen was measured manometrically.

Procedure

The sub-unit preparation was subjected to exclusion chroma-tography on Sephadex G-75 (coarse) in order to remove excess gly-KOH-cys-SH buffer. Three ml preparation (1 mg chlorophyll/ml) in gly-KOH-cys-SH was placed on a Sephadex/2.0 x 50 cm column equilibrated with 10^{-2} M-phosphate buffer pH 7.0, 10^{-3} M KCL. Elution was carried out with the same buffer and, in this way, the preparation was rapidly transferred to the reaction buffer. Control quantasome aggregates were treated similarly except that they were not exposed to the gly-KOH-cys-SH buffer.

The Warburg vessels contained 2 ml of the eluted preparations (0.8 mg chlorophyll) with 0.5 ml potassium ferricyanide (30 umoles) in the side arms. The reaction was carried out in an Aminco Warburg Apparatus (American Instr. Co., Maryland) adapted for illumination from below with white Tungsten light and at 15° C.

After an equilibration period of 5 minutes the ferricyanide was added from the side arms and the samples illuminated (10,000 ft candles, white light).

Results

	Treatment	uMoles O_2 evolved/mg chlorophyll/hr
1	None	39.8
2	"	35.8
3	gly-KOH-cys-SH, excess removed	14.5
4	"	15.75

Table 2. Hill reaction of quantasome aggregates (#1 ¢ #2) and aggre-gates treated with gly-KOH-cys-SH buffers for 30 min and then subjected to exclusion chromatography on Sephadex to remove excess buffer (#3 ¢ #4).

The results in Table 2 show that the sub-unit is active in quantum conversion but 60% of the activity is lost by treatment with the gly-KOH-cys-SH buffer. The loss in activity in the preparation suggests that either the photosynthetic process is inhibited in the enzymological sense or, more likely, that the unit for quantum conversion is structurally modified. The fact that the preparation of the sub-unit also leads to a spectral shift of the red absorption maximum (see IA2) very strongly suggests a change in the structural integrity of the photosynthetic unit.

Several active chloroplast sub-units or chlorophyll-proteins have been described previously. Wolken (1963) showed that "chloroplastin" photoreduces dichlorobenzenone indophenol and forms "labile phosphate" in the presence of a mixture of many cofactors (α-ketoglutarate, ascorbate, cytochrome c, riboflavin 5-phosphate, Mg^{++}). Chloroplastin is prepared from Euglena by a 2% digitonin extraction of the cells (Wolken and Schwertz, 1956) which leads to the formation of a pigment-protein of MW=290,000. This is in good agreement with Smith and Pickles (1941) who showed that 2.5% digitonin extracts of spinach leaves yield a pigment-protein complex of MW=265,000. However, Smith (1941) showed that a spectral shift of 3.5mu to 675 mu was associated with the addition of digitonin.

Kahn (1963) treated spinach chloroplast fragments with Triton X-100 and separated a chlorophyll protein of unspecified molecular dimensions but containing 0.8 to 1.2 mg protein/mole chlorophyll. The chlorophyll was chlorophyll a only. The particle photoreduced ferricyanide but oxygen was not evolved and boiled preparations behaved similarly.

Allen, et al (1963) prepared a particle from Chlorella pyrenoidosa
by treatment of the cells by freezing in 10% methanol, grinding,
sonication and differential centrifugation. The particles are from
70 Å to 300 Å dia. and have a chlorophyll a/b ratio of one, and, the
red absorption maximum is 673 mu (ie. a chlorophyll b enrichment).
The particle generates a single line ESR signal upon illumination
which is similar to that induced by long red light in normal photosyn-
thetic systems. However, it does not decay in the dark and can only
be discharged when ferricyanide is added. The particle photoreduces
DPIP but not quinone.

Both Wessels (1962) and Boardman and Anderson (1964) showed that
the treatment of spinach chloroplasts with low concentrations of
digitonin (0.2 to 0.5%) does not impair the photoreduction of NADP
when DPIP and ascorbate are present. However, when water is electron
donor, NADP reduction does not occur. Wessels also showed that
photosynthetic phosphorylation of the cyclic type (Arnon, et al,
1955) occurs and, hence, low concentrations of digitonin appear to
act like DCMU, as observed experimentally. No reports on particle
size were given, but Boardman and Anderson indicate that the treatment
results in a shift of the absorption maximum of 2 mu to shorter
wavelengths.

Other preparations of chlorophyll-proteins or particles from
photosynthetic tissues reported by Chiba (1960), Takamiya et al (1963),
Takashima (1952), Itoh, et al (1963) and Nishimura and Takamatsu (1957)
were either photochemically inactive or the measurements were not
attempted.

4. Sedimentation coefficient and analysis for polydispersity

Quantasome aggregates which have been clarified by differential ultracentrifugation are polydisperse on the basis of measurements made in the electron microscope, and the particles consist of up to eight quantasomes. These preparations do not give a discrete boundary in the analytical ultracentrifuge even after extensive differential preparative ultracentrifugation (Pon, 1962). The preparation described here was investigated by analytical ultracentrifugation and was found to sediment as a single component with a broad boundary. The boundary was analyzed for polydispersity.

Procedure

Analytical ultracentrifugation was carried out on a Spinco Model E ultracentrifuge equipped with a rotor temperature indicating and control system. The ultraviolet absorption optical system was modified for the purpose of measuring pigment absorption of the subunit preparation in the blue region of the spectrum. This was accomplished by removing the chlorine-bromine filter from below the centrifuge chamber and replacing the housing. A cut-off filter absorbing all wavelengths below 300 mu was then attached to the bottom of the housing with masking tape. The focus of the optical system did not appear to be radically modified as very sharp meniscii were obtained on the absorption photographs. Exposure times of 2.5 sec were found to be satisfactory when the absorbance of the preparations under study were in the order of unity and blue sensitive, medium speed Kodak commercial safety film was used.

By virtue of the very high extinction of the photosynthetic pigments and use of the absorption technique, it was possible to

analyze very dilute solutions of the preparations, i.e., about 10^{-6} M-quantasomes. Experiments were carried out in 0.1 M-glycine, 0.05 N-KOH and 0.02 M-cysteine-HCL. An AN-D rotor and 12 mm, Kel-F, 4° single sector cell were used. Absorption photographs were scanned by means of a recording microdensitometer.

The densitometer tracings of the sedimentation velocity experiments show the concentration of sub-units in the centrifugal field. The log radii of the 50% concentration points were plotted against time and a straight line was obtained, the slope of which was related to the sedimentation coefficient by the expression:

$$s = \frac{1}{\omega^2} \cdot \frac{dx}{dt},$$

where, s = sedimentation coefficient, cm/sec/dyne/g, ie. sec,

ω^2 = angular velocity, radians/sec,

x = distance of the 50% concentration points in cm from the

axis of rotation and

t = time in sec.

Hence,

$$s = \frac{2.303}{\omega^2} \cdot \log \frac{x}{\Delta \Delta t} .$$

Results

Fig. 6 shows an example of an absorption photograph taken during a sedimentation velocity experiment, and it can be seen that the boundary spreads quite cons'derably. Fig. 7 shows the superimposed densitometer tracings of such an absorption photograph and Table 3 shows the sedimentation coefficient of the lamellar sub-units at three concentrations. The preparations were sufficiently dilute to be in

the linear range of film sensitivity and the sedimentation coeffi-
cient is independent of concentration.

Table 3. Sedimentation coefficient of the lamellar sub-units at
three concentrations.

sub-unit concentration A_{675}	rotor speed rpm	sedimentation coefficient Svedbergs
0.8	24,630	87.1
0.71	39,460	86.0
0.33	39,460	86.8

Full use of the sedimentation velocity data was used by analysis
of the boundary for polydispersity. The function

$$g(s) = \frac{dc/dx}{c_o} \cdot (\frac{x^2}{x_{min}}) \cdot x\omega^2 t$$

was computed for several values of s for a boundary position during
a sedimentation velocity experiment by a procedure according to
Schumaker and Schachman (1957). Fig. 8 shows the weight distribution
of sedimentation coefficients in a sample of lamellar sub-units, and
it can be seen that the preparation is polydisperse. However, the
bulk fraction lies within 15% of the mean. Account of the contribution
to boundary spreading due to diffusion would lessen the extent of this
apparent polydispersity but this would be small for such a large sub-unit.

These results show that the sub-unit preparation is polydisperse
and the mean sedimentation coefficient is 86 Svedbergs. The analysis
for polydispersity used by analysis of the boundary during a sedimentation
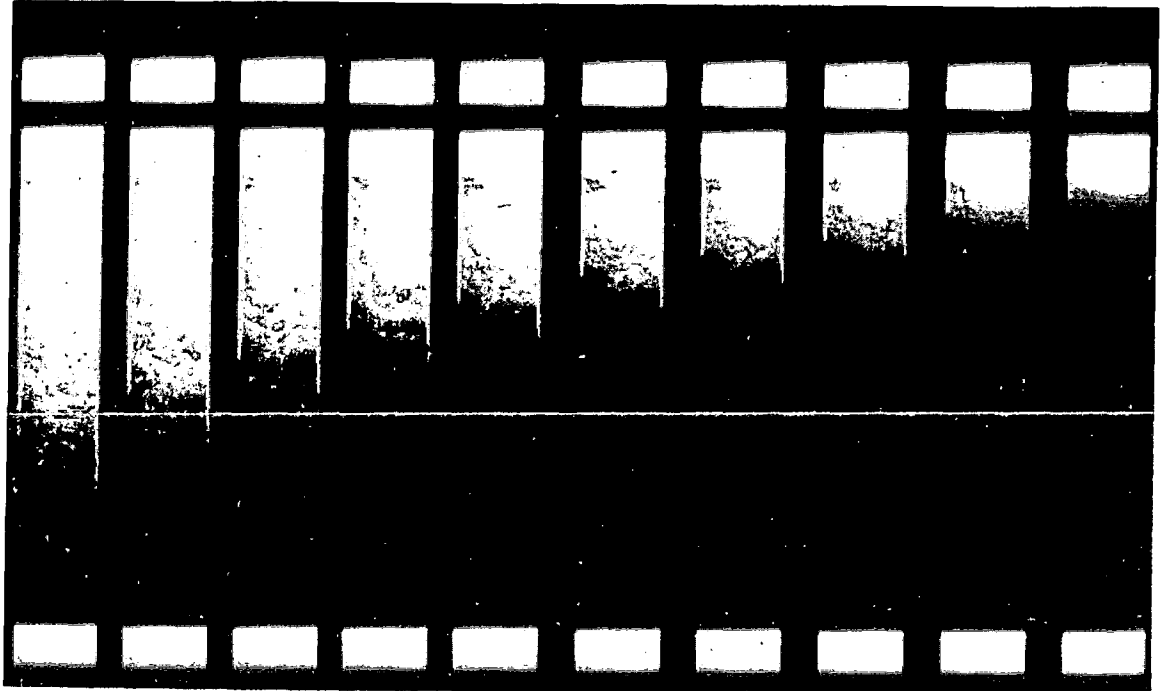
ZN-3545

Fig. 6. Sedimentation velocity of lamellar sub-units. Absorption
photograph taken at two-minute intervals at 439 mu. Rotor
speed 39, 460 rpm, 0. 1 μNaCl, 20° C, sub-unit absorbancy at
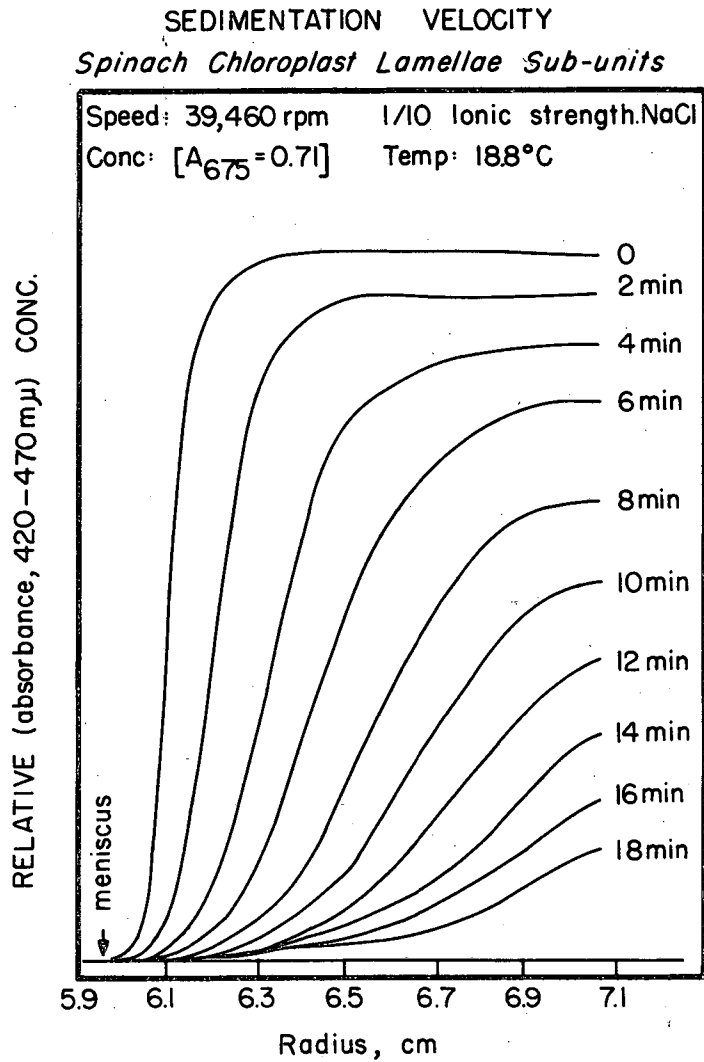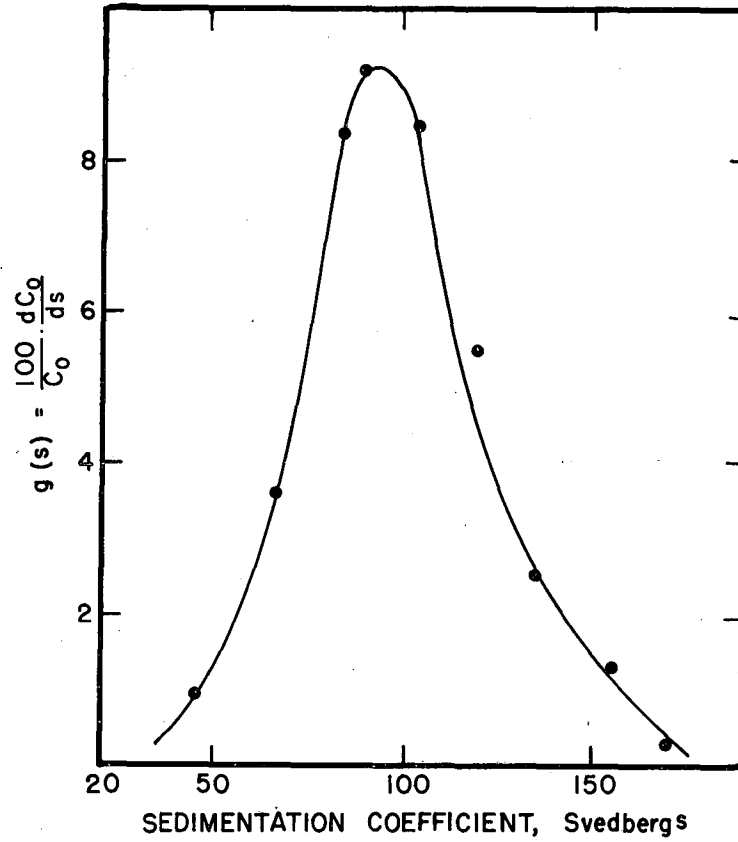675 mu is 0.71.

Fig. 7. Sedimentation velocity of spinach chloroplast lamellar sub-unit. Superimposed densitometer tracings of absorption photographs taken during an experiment at 39,460 rpm.

MU-33778

Fig. 8. Weight distribution of sedimentation coefficients in a
preparation of lamellar sub-units. Analysis of a boundary
during a sedimentation velocity experiment.

velocity experiment, is very sensitive and was used by Shumaker and Schachman (1957) in the analysis of DNA. The criterion of purity previously employed was the appearance of a single peak in the ultracentrifuge. However, treatment of sedimentation data of very dilute solutions of DNA, in the manner described, showed that DNA was quite polydisperse. Hence, casual inspection of a boundary in a sedimentation velocity experiment is insufficient in deciding the purity of a single macromolecular species. For a multimacromolecular complex the problem is even more acute. It is anticipated that if other large cellular particles such as electron transport particles (Green, 1961; Fernandez-Moran et al, 1964), ribosomes (see Arnstein, 1963 for review), chromatophores (Schachman et al, 1952) are investigated similarly, then a comparable distribution of sizes would be seen.

5. Density gradient sedimentation equilibrium

The sub-units were investigated by density gradient sedimentation equilibrium in order to determine the density of the material and to ascertain the extent of possible heterogeneity by an additional technique.

Procedure

Sucrose was chosen as the low molecular weight solute for centrifugal redistribution and formation of the density gradient as high concentrations of electrolytes such as $CsCl_2$ led to aggregation of the lamellar sub-units.

Twenty microliters of a sub-unit preparation ($A_{677} = 0.7$) was added to 1 ml of 0.45 g sucrose/ml and centrifuged at 50,740 rpm at 3°C. A single 4° sector, 12 mm, aluminum centerpiece cell was used.

The approach to sedimentation equilibrium for sucrose was observed by the standard schlieren optical system of the ultracentrifuge. This took 72 hours, and during this time the sub-units migrated centrifugally from the meniscus and centripetally from the cell bottom until, at equilibrium, they formed a discrete band close to the cell center where the net buoyancy term was zero.

A 439 mu interference filter (Corning) was placed below the lower collimating lens of the schlieren optical system in the ultra-centrifuge, and photographs were taken using Kodak spectroscopic plates, type 1-D. Thus it was possible to simultaneously record the density gradient by schlieren optics and the sub-unit band by 439 mu absorption on the same plate. Fig. 9 shows an example of such a photograph at equilibrium after 72 hr.

Results

Fig. 10 shows the sedimentation equilibrium distribution of a the sub-units and it can be seen that there is one band. Fig. 11 shows a plot of the log relative sub-unit concentration versus square of the band width, and the relationship is linear, indicating that the band is a Gaussian distribution. Hence, the preparation is likely to be homogeneous with respect to density. The density of sucrose at the radius corresponding to the peak of the sub-unit band is 1.175 g-cc^{-1}. This is the point where the sum of the forces acting on the sub-units is zero and is the effective density of the macro-molecular solute (Meselson et al, 1957).

The real density of the lamellar sub-units is likely to be less than the effective density as measured in the sucrose gradient as the experiment was conducted in a sucrose solution of high osmotic
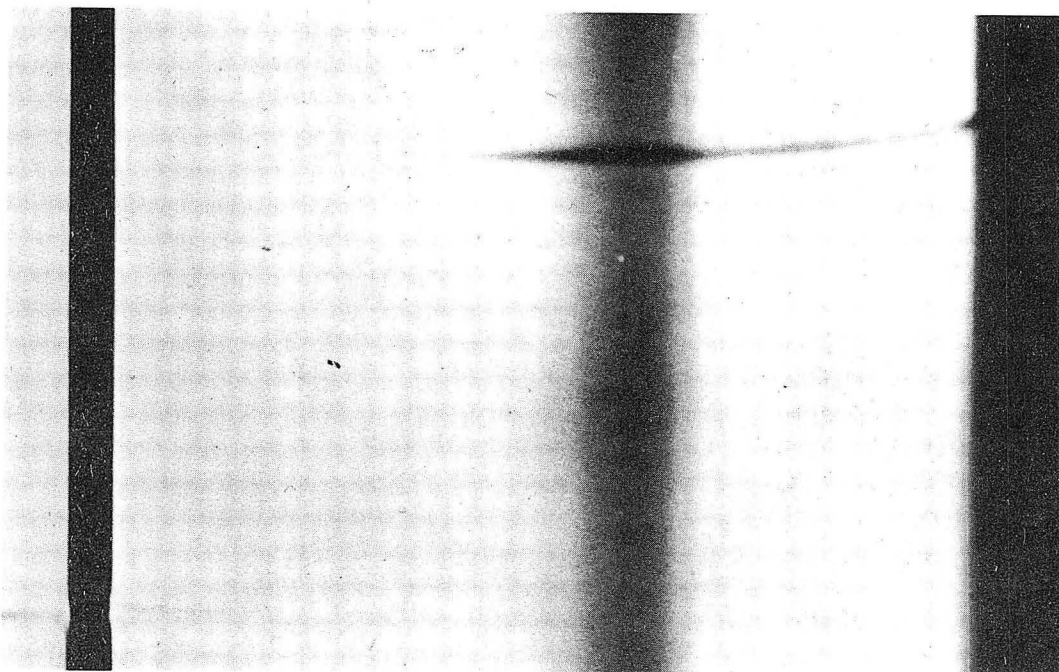
ZN-4610

Fig. 9. Density gradient sedimentation equilibrium of lamellar
   sub-units. The sub-units are banded near the center of the
   cell at a point in the sucrose density gradient where their
   effective buoyant density is equivalent. The sucrose is at
   sedimentation equilibrium. Rotor speed 50,740 rpm, time
   72 hr, sucrose 1.315 M and 4°C.

- 37 -

Fig. 10. Densitometer tracing of an absorption photograph taken
at density gradient sedimentation equilibrium of lamellar
sub-units.
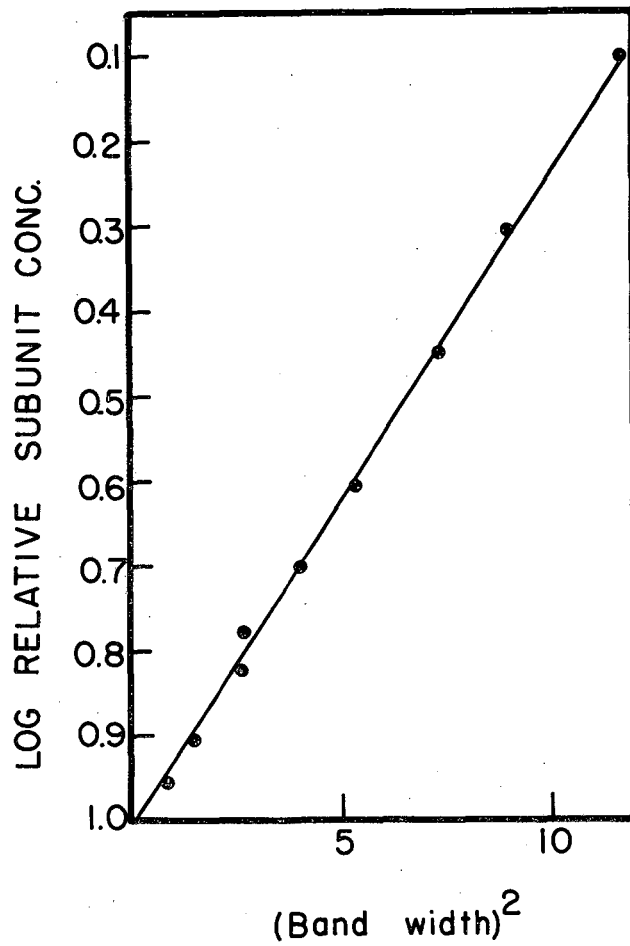
MU-27712

Fig. 11. Plot of log relative sub-unit concentration versus band
width of the lamellar sub-units at density gradient sedimen-
tation equilibrium. The linear plot is indicative of a Gaus-
sian distribution and highly suggestive of density homogeneity
in the preparation.

pressure and the degree of dehydration of the sub-units is unknown. However, in spite of this possible error, the measurement is probably good to 1% accuracy.

The value obtained here for the density of the sub-units is expected for material of composition 50% lipid and 50% protein, assuming the density of a pure protein to be about 1.4 g/cc and that of pure lipid to be about 0.8 g/cc. Bergeron (1959) measured the density of a preparation of chromatophores from Chromatium and obtained a vlue of 1.2486 g/cc. The chromatophore he prepared contained 66% protein and therefore one would expect a slightly higher density.

6. Electron microscopy

The sub-units were investigated by electron microscopy in collaboration with Dr. R. B. Park to determine the molecular size, shape and polydispersity.

Procedure

Single drops (50ul) of a very dilute solution of a preparation of sub-units were placed on 200 mesh copper grids with formvar films, the excess liquid drawn off with an absorbant tissue and air dried. One per cent phosphotungstate pH 7 was then applied to the grids similarly. Grids prepared in this way were then viewed by means of an Akashi Tronscope.

Results

Fig. 12 is an example of a photograph taken at high magnification. The particles appear spherical but of a large range of diameters. The range is from 300 $\overset{\circ}{A}$ to 700 $\overset{\circ}{A}$, but there appear to be a majority of particles with a diameter of about 500 $\overset{\circ}{A}$. In some cases the

ZN-4605

Fig. 12. Electron micrograph of spinach lamellae-sub-units
negatively stained with 1% phosphotungstate. The white
line is 0.1 u.

material appears to be broken, giving a "C"-shaped fragment, and, in general, the particles seem hollow.

7. Summary

Chloroplast lamellar fragments were treated with gly-KOH-cys-SH buffer pH 9 and a sub-unit was isolated by differential ultracentrifugation which is active in the Hill reaction. The absorption spectrum is similar to that of unfragmented lamellae but the red absorption maximum has shifted 1.5 mu.

The sedimentation coefficient of the preparation was found to be 86 Svedbergs and analysis of the boundary showed the preparation to be polydisperse. As determined in a sucrose density gradient, the effective density of the sub-unit is 1.175 g cc^{-1}. The sub-units are largely spherical in shape and appear hollow. The size range is from 300 $\overset{o}{A}$ to 700 $\overset{o}{A}$, the majority in the order of 500 $\overset{o}{A}$.

8. Discussion

From the sedimentation coefficient, density and diameter of the sub-unit it is possible to compute the molecular weight of the particles using the expressions:

$$f = 6\pi r \eta$$

and $$M = \frac{(1-\bar{v}p)}{Nfs},$$

where f = frictional coefficient,

s = sedimentation coefficient,

η = viscosity,

r = radius,

p = solution density,

$\bar{v}$ = partial specific volume,

N = Avogadros number,

and M = molecular weight.

Using s = 86 x 10^{-13}, $\bar{v}$ = 0.85 cc-g^{-1} and r = 300 $\overset{o}{A}$, then the mole-
cular weight of the sub-unit is in the order of 18 x 10^{6}.g-mole^{-1}.
Such a unit contains 2000 chlorophylls and 9 quantasomes. The partial
specific volume, $\bar{v}$, used here is estimated from the reciprocal of
the measured effective buoyant density and, therefore, the calculations
based upon this are considered with caution.

On the basis of electron microscope measurements, the quantasome
aggregates described by Park and Pon (1961) are particles containing
up to about 8 quantasomes (Park, personal commun.). Hence, the sub-
unit preparation described here and the quantasome aggregates described
by Park and Pon are in the same size range but the two preparations
have quite different hydrodynamic properties. Pon (personal commun.)
showed that the quantasome aggregates do not exhibit a discrete
boundary in/analytical ultracentrifuge, whereas the sub-unit described
the
here does. A possible explanation of the fact that the two prepara-
tions are hydrodynamically different is that they are of quite different
shape. This would affect the frictional coefficient which would result
in different sedimentation characteristics.

That the two preparations are different in shape has been confirmed
by observations in the electron microscope. The quantasome aggregates
are elongated (Park, personal commun.) whereas the sub-units here are
more spherical. Such a difference in the shape of the two types of
particles is likely to be due to either the methods of preparation or
the condition of the starting material. It is possible that sonication
at a certain fixed frequency leads to fragmentation of the lamellae
along linear arrays of quantasomes in the horizontal plane of the mem-
brane. These arrays could then be further fragmented until a critical
size is attained (which could well be a function of the oscillator

frequency.

An additional factor that could influence the pattern of fragmentation of the lamellae is the physiological condition of the plant from which the chloroplasts are prepared. Park and Biggins (1964) observed varying degrees of quantasome crystallinity in lamellae prepared from spinach grown during summer and winter conditions. The extent of crystallinity could possibly be an important factor in the final size and shape of the lamellar fragments. It would appear that greater control over the condition of the plant material would be profitable in future analytical investigations.

The difference in pH or presence of the sulphydryl in the case of the sub-unit preparation could lead to excessive charge repulsion on the protein. This, together with the hydrophobic properties of the lipid on the other surface, could lead to a curling of the fragmented array in order to distribute the charges and, ultimately, form a sphere with lipid in the center and protein on the outside.

This mechanism for membrane fragmentation described above could also explain the formation of spherical chromatophores by sonication of the bacterial photosynthetic membrane. Bergeron (1959) proposed a model for the substructure of a preparation of chromatophore from Chromatium. He obtained 300 $\overset{o}{A}$ diameter hollow particles by sonication and suggested that they were spheres with an inner layer of lipid and an outer layer of protein. However, such particles have not been observed in sections of whole cells and Cohen-Bazire suggests that chromatophores are fragments of an initially continuous membrane (Cohen-Bazire & Kunisawa, 1961; Cohen-Bazire, 1963). The dimensions of the preparation of lamellar sub-units described here are larger than those of Bergeron's chromatophore but similar to those of chromato-

phores isolated by Schachman et al (1952). It is possible that the final form of both particles in vitro is strictly a function of the mode of preparation.

Use of the value obtained in this study for the density of the lamellar sub-units, in conjunction with the molecular size of a quantasome as determined from electron microscope measurements of shadowed preparations of sonicated lamellae, show that the molecular weight of the quantasome is about two million (Park and Biggins, 1964). This is twice the minimum molecular weight based on the weight of material associated with one atom of manganese. Hence, a quantasome contains 225 molecules of chlorophyll, 1 cytochrome f and 1 cytochrome b_6 (see Part I, sec. B). The total composition is given in Table 4.

This chlorophyll content per quantasome is surprisingly close to that of the physiological photosynthetic unit as determined by Kok and Businger (1956). However, only the isolation of a homogeneous active particle containing 200 chlorophylls would represent unequivocal evidence that the quantasome is the photosynthetic unit. A further restriction would be that such a particle is indivisible in terms of photochemical activity.

Table 4

Composition of the Quantasome (Park & Biggins, 1964)

Lipid (composition in moles/quantasome[1])

230	chlorophylls[2,3]		206,400
160	chlorophyll a	143,000	
40	chlorophyll b	63,000	
48	carotenoids[2]		27,400
14	ß-carotene	7,600	
22	lutein	12,600	
6	violaxanthin	3,600	
6	neoxanthin	3,600	
46	quinone compounds[22]		31,800
16	plastoquinone A	12,000	
8	plastoquinone B	9,000	
4	plastoquinone C[4]	3,000	
8-10	α-tocopherol	3,800	
4	α-tocopherylquinone	2,000	
4	vitamin K_1	2,000	
116	phospholipids[5]		90,800
144	digalactosyldiglyceride[6]		134,000
346	monogalactosyldiglyceride[6]		268,000
48	sulpholipid[7]		41,000
?	sterols[8]		15,000
	unidentified lipids		145,000
		Total	990,000

(Cont.)

Table 4 (Cont.)

Protein

9,380 N atoms protein	928,000
10 non-heme iron	560
2 cytochrome-iron	112
2 manganese	110
6 copper	218
Total	930,000
Total lipid plus protein	1,920,000

1. Wolf, et al. (1962); Debuch (1962).

2. Lichtenthaler & Calvin (1963).

3. Park & Pon (1963).

4. Henninger, et al. (1963).

5. Wintermans (1960).

6. Wintermans (1962).

7. Benson, et al. (1959).

8. Menke & Jacob (1942).

Part I. B. Studies on the Lamellar Protein Fraction.

Introduction

The lamellae from the chloroplasts of higher plants have received considerable attention as far as composition and enzymic activity is concerned (see Granick, 1961, for review). The most recent work on composition by Park and Pon (1961) shows that the membrane is 50% protein and 50% lipid. This is in agreement with many other workers although the lipid content is slightly higher. They attribute this difference to the fact that the lamellae they analyzed were absolutely free of the soluble proteins of the stroma. The presence of the stroma protein leads to an exceptionally high protein content.

Of the two fractions, the lipid fraction has been studied the most extensively due to the considerable interest in the photosynthetic pigments. This lipid fraction contains the chlorophylls, carotenoids, quinones, phospholipids, di- and mono-galactosyldiglycerides and sulpholipids (Lichtenthaler and Park, 1963).

The protein fraction contains the two photosynthetic cytochromes f and b_6 and the transition metals iron (non-heme), manganese and copper. The protein fraction has received very little direct attention, presumably owing to the extreme insolubility of the material at physiological pH and the lack of appropriate enzymological tools to cope with the problem.

Davenport and Hill (1952) purified cytochrome f from an ammoniacal ethanolic extract of the fresh leaves of parsley by ammonium sulfate fractionation and calcium phosphate gel adsorption. The pure protein was shown to be MW = 110,000 and contained two hemes. The heme-

protein linkage was found to be similar to that of cytochrome c and the redox potential was estimated as $E_8^1 = 0.365v.$

Indirect evidence concerning the properties of the protein fraction comes from work primarily designed to investigate the nature of the chlorophyll-protein link in attempts to elucidate the state of chlorophyll in vivo (Smith, 1940, 1941). For this purpose a variety of detergents and organic solvents were used to solubilize the membranes and led to the formation of a "clear" solution. It was decided that a similar use of detergents for the removal of lipid from the lamellae and solubilization of the protein would be a fruitful approach to the study of the chloroplast protein fraction, as these methods had also been shown to be of great value in the study of the mitochondrion, where similar problems of protein insolubility prevail (Green, 1961).

This section describes the preparation and properties of the protein fraction in an attempt to derive information pertinent to the molecular architecture of chloroplast lamellae.

1. Preparation of the chloroplast lamellar protein fraction

Quantasome aggregates were prepared in large quantities by the continuous flow procedure, solubilized in 0.2% sodium dodecyl sulfate pH 7.1 and allowed to stand at 4°C for one hour with gentle stirring. During this process, the membranes dispersed and a clear "solution" was obtained.

Lipid was most effectively removed from the detergent-dispersed solution by the slow addition of sufficient butanol at -5°C to give a final volume 20% greater than the original detergent solution. After rapid stirring for about 30 min, the solution was centrifuged at 1000 g for 15 min and the upper organic layer removed by careful aspiration. Complete removal of lipid was achieved by one or more

repetitions of this procedure. The aqueous phase was then dialyzed exhaustively against 10^{-2} M-tris-Cl pH 8.0 to remove as much butanol and detergent as possible.

An additional method of extracting lipid from the detergent-treated quantasome aggregates was by $-10°C$ acetone precipitation. The protein was precipitated at 80-85% acetone and was shown to be lipid-free after several washes with cold $-15°C$ acetone. After removal of the acetone by evacuation, it was possible to dissolve 75% of the protein in 0.02% sodium dodecyl sulfate 0.05 M-tris Cl pH 8.0 by extraction overnight. During this procedure the washing and removal of acetone was conducted as rapidly as possible and at $-10°C$ to minimize protein denaturation.

The protein was concentrated by ammonium sulfate precipitation or lyophilization after dialysis against 10^{-4} M tris-Cl pH 8. The protein fraction was yellow-brown in color and was stored at 4°C.

2. Absorption spectrum and oxidation-reduction difference spectra

Procedure

A Cary Model 14 Automatic Recording Spectrophotometer with a Scatter Transmission Attachment was used. Oxidation and reduction difference spectra were obtained using the 0 to 0.2 optical density slide wire on the spectrophotometer. Difference spectra of the protein fraction in sodium dodecyl sulfate were compared with those of 80% acetone - extracted lamellae and an acetone powder solubilized by a large quantity of sodium cholate.

i) 80% acetone extraction. Quantasome aggregates (ie. 500 mg dry weight) were prepared and suspended in 40 ml 10^{-2} M tris-Cl pH 8. This suspension was poured into 160 ml $-10°C$ acetone and stirred vigorously. After about 5 min stirring the suspension was rapidly

filtered by means of a medium glass sinter and Büchner attachment with the aid of a water pump. The brown-yellow residue was washed with cold acetone (80%) and never allowed to dry (this avoided cracking the pad of protein on the sinter funnel, prevented channelling and also made the final aqueous suspension considerably easier). After washing with cold 80% acetone until the washings were colorless, the pad was covered with 5 ml ice-cold 10^{-1} M-tris-Cl pH 8 and, as soon as the acetone was displaced from the pad (ca 1 ml), the filter was disconnected from the pump. The wet residue was then scraped from the filter and suspended in 10 ml ice-cold 10^{-1} M-tris-Cl pH 8. The suspension was then sonicated using a Bronwell Biosonik Sonicator (Blackstone Ultrasonics, Bronwell Scientific, Rochester, N. Y.), operated at full power for 5 min. The sonicate was then centrifuged to remove particles large enough to settle and cause anomalies during the spectrophotometric measurements.

ii) Cholate solubilization. Quantasome aggregates were prepared (ie. 500mg dry weight) and suspended in 10^{-2} M-tris-Cl pH 8. The suspension was then poured into a volume of -20°C acetone one hundred times greater than the volume of the suspension, vigorously stirred for 2 min and then filtered on a large Büchner funnel through Whatman #4 filter paper. The filtering was accelerated by the use of a water pump and was conducted as rapidly as possible. The residue was washed with -20°C acetone and the filter paper plus residue placed in a large desiccator and evacuated to remove acetone.

The dry acetone powder was then suspended in 10^{-1} M tris-Cl pH 8 by means of a glass pestle and homogenizer and 2 mg cholic acid per mg powder added. The pH was carefully controlled and maintained at 8 by the addition of base (0.1 N NaOH). The suspension was stirred

gently overnight at about 4°C and then ultracentrifuged at 105,000 g
for 30 min in a Model L Spinco preparative ultracentrifuge with a #40
rotor to remove insoluble matter. About 70% of the material was
solubilized by this treatment.

iii) Difference spectrophotometry. Cytochrome contents of the
protein fraction and other lamellar preparations were measured by
difference spectrophotometry. Identical samples were introduced into
both reference and sample beams of the Cary Model 14 Automatic Recording
Spectrophotometer equipped with a Scatter Transmission Accessory and
full-scale expansion. A base line was run of "untreated" material
versus "untreated" and then the samples were either oxidized or reduced
by the addition of very small quantities of solid potassium ferricyanide
or sodium dithionite. The spectral region of the α and β bands of
the ferrocytochromes was studied (ie. 500mu to 600mu). In later work,
the split compartment cuvettes described by Yankeelov (1963) obtained
from Pyrocell (Pyrocell Man. Co., New York, N. Y.) were used. Ferri-
cyanide and dithionite both absorb in the far ultraviolet, but, the
use of these cuvettes permitted accurate, unambiguous measurement of
the Soret region of the cytochrome as the concentration of oxidant or
reductant in both analyzing beams was identical.

The underlying assumptions during the determination of the cyto-
chrome content of the lamellar protein fraction were that the autoxidi-
zable cytochrome b_6 was present in aerated solution as the ferricyto-
chrome, and that cytochrome f was present as the ferrocytochrome
(Hill and Bonner, 1961). Owing to this difference in oxidation states
in air at pH 8, the measurement of the two cytochromes in the presence
of each other was considerably simplified.

All the samples were finally adjusted to 0.1 M-tris-Cl pH 8, and, when the split compartment cells were used, ferricyanide and dithionite solutions were 10^{-5} M and in the same buffer.

Results

Fig. 13 shows the absorption spectrum of the lamellar protein fraction in 0.002% Na dodecyl sulfate, 0.05 M-tris pH 8. There is a large absorption band at 279mu and a shoulder at 290mu. The difference spectrum, oxidized minus reduced, in the visible region shows the α, β and Soret absorption bands of the ferrocytochromes f and b_6. The density of the absorption band in the ultraviolet is seven times greater than that of the combined Soret bands, indicating that the greater proportion of the protein is not hemoprotein.

Fig. 14 is a difference spectrum, oxidized minus untreated lamellar protein. This shows the absorption spectrum of ferrocytochrome f. The absorption bands are $\alpha = 554$, $\beta^a = 530$, $\beta = 524$ and $\gamma = 421$. This is in very good agreement with the absorption maxima reported by Hill and Bonner (1961). However, in this work, the β band is split whereas Hill and Bonner only found the β^1 band at low temperature. The absorption maxima ratios α/β and γ/α are also in good agreement and with the data on highly purified ferrocytochrome f (Davenport and Hill, 1952).

Fig. 15 is a difference spectrum of untreated lamellar protein minus reduced protein and shows the absorption spectrum of ferrocytochrome b_6. The absorption maxima are $\alpha = 561$, $\beta = 530$ and $\gamma = 431$. This result is in agreement with the values given by Hill and Bonner (1961) for 80% acetone-extracted lamellae although it appears that in the isolated lamellar protein the bands are shifted slightly to

shorter wavelengths. The ratio α/β is very similar.

Fig. 16 shows difference spectra of the region of the α and β bands of an 80% acetone powder of pure chloroplast lamellae for comparison, and in an attempt to repeat the data of Hill and Bonner (1961). The agreement is good in both cases, showing that the low detergent concentration does not further modify the spectra.

Fig. 17 shows difference spectra of the lipid-free lamellae solubilized in cholate (2 mg/mg protein). The absorption maxima of the ferrocytochromes do not appear to be shifted, but the density of the band of the ferrocytochrome b_6 is less.

Table 5 summarizes the spectrophotometric data in this study, together with that of Hill and Bonner, Davenport and Hill, Sironval and Engelert-Dujardin and Lundegårdh.

The cytochrome content of the chloroplast lamellar protein was determined from these spectrophotometric measurements and knowledge of the weight of protein in solution. Using the α band extinction coefficients of 2.5×10^4 for cytochrome f and 2.0×10^4 for cytochrome b_6, then the cytochrome b_6/f ratio is 0.92. 1.1×10^6 g of the protein would contain molar amounts of the two cytochromes, and, hence, there is one of each cytochrome per quantasome or approximately 230 chlorophylls.

These values for the cytochrome concentration are lower than the concentrations measured by Davenport and Hill for cytochrome f in parsley and elder (1952), and Lundegårdh (1962) for spinach. However, the agreement with Sironval and Engelert-Dujardin (1963) and Hill and Bonner (1961) is fairly good. Sironval and Engelert-Dujardin reported the b_6/f ratio as 1 but Hill and Bonner did not state the extinction coefficients they used in their calculations.

Table 6 summarizes the data in this study and the literature values for comparison. The variation in values is quite high and it is suggested that variation in the physiological condition of the starting materials could contribute to such discrepancies.

These results show that the lamellar protein fraction contains the two photosynthetic cytochromes f and b_6 in approximately equal concentrations and about 9 uM. This is the same concentration as determined in intact lamellae suggesting that all the cytochrome is recovered in the protein fraction when solubilized by detergent. The absorption maxima of the ferrocytochromes are very similar to those described previously for lamellae extracted with acetone. It appears that very low concentrations of Na-dodecyl sulfate and proportionately high concentrations of Na-cholate do not radically modify the absorption spectra.

The majority of the protein fraction is non-heme protein and the physical properties described in the next section suggest that it contributes to maintaining the structure of the membrane.
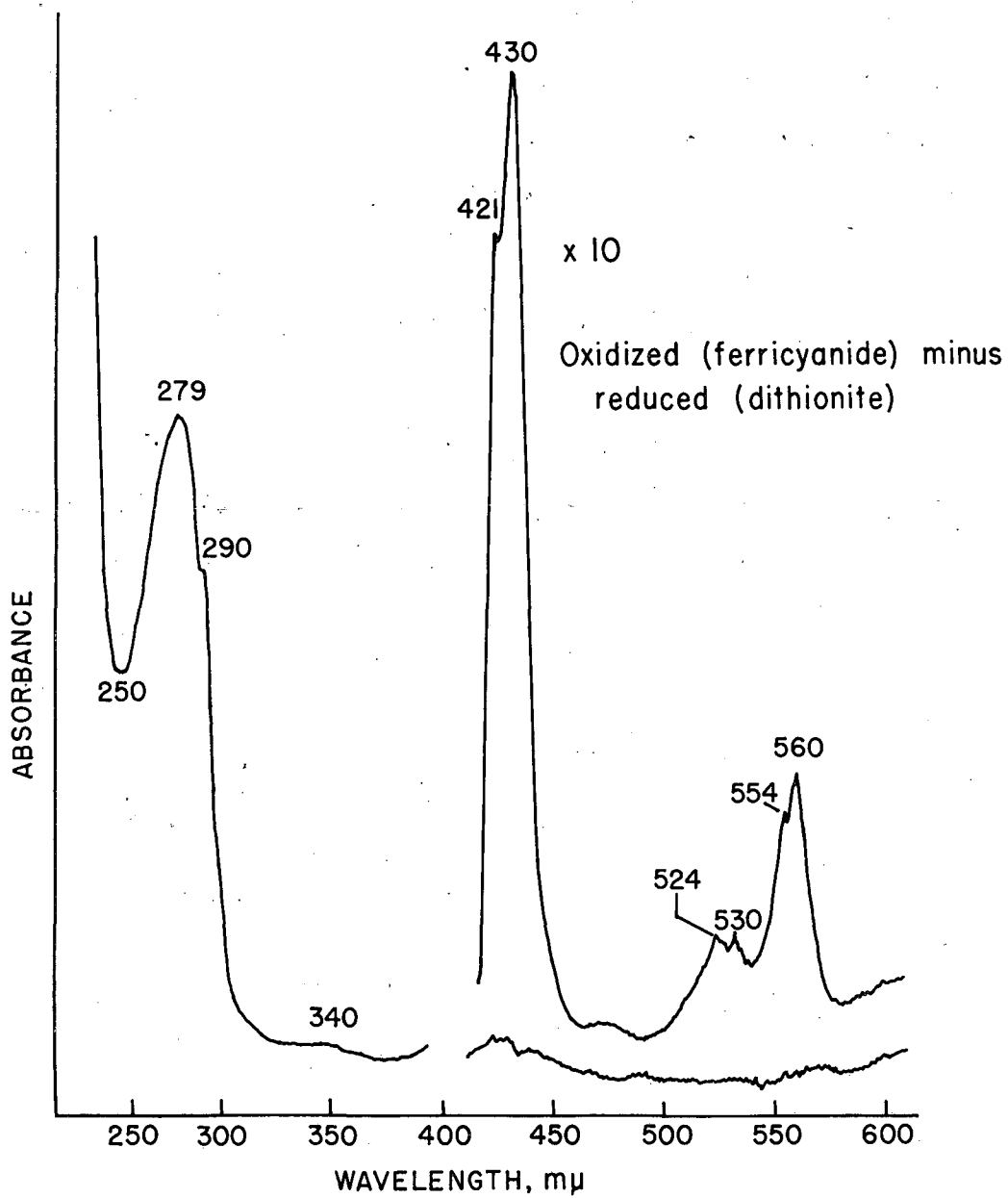
Fig. 13. Absorption spectrum of the chloroplast lamellar protein
fraction. The spectrum in the visible region is a difference
spectrum, oxidized minus reduced.

CHLOROPLAST LAMELLAR PROTEIN
FRACTION
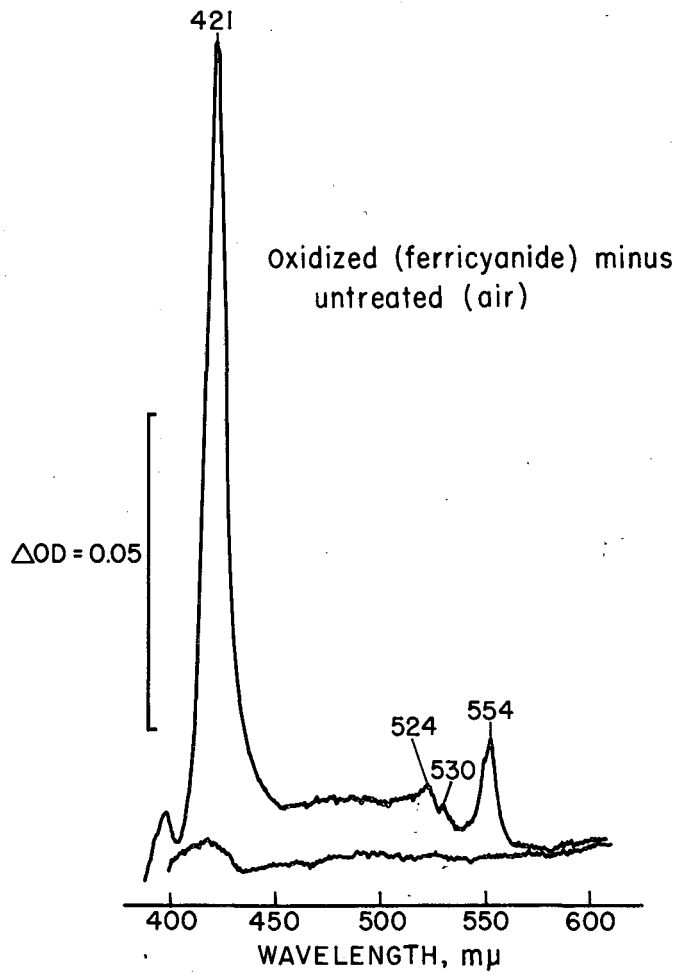
Fig. 14. Chloroplast lamellar protein fraction. Oxidized (ferri-
cyanide) minus untreated. This shows the absorption spec-
trum of cytochrome f.

CHLOROPLAST LAMELLAR PROTEIN
FRACTION
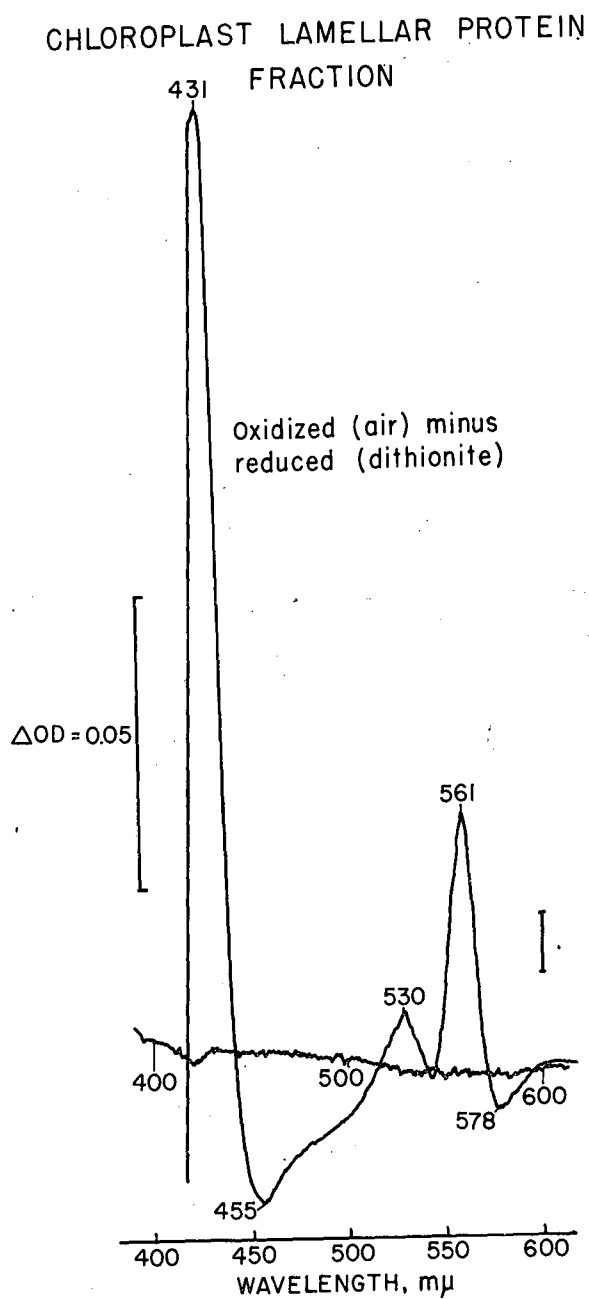
Fig. 15. Chloroplast lamellar protein fraction. Untreated (ie. air oxidized) minus reduced (dithionite). This shows the absorption spectrum of cytochrome b_6.

SPINACH CHLOROPLAST LAMELLAE

80% ACETONE EXTRACTED

A- Base line

B- Untreated minus dithionite - reduced

C- Ferricyanide - oxidized minus untreated

D- Oxidized minus reduced

560

560

554

524

530

530

531

B

C

D

A

A

$\Delta A = 0.01$

500   600 mμ
WAVELENGTH

500   600 mμ
WAVELENGTH
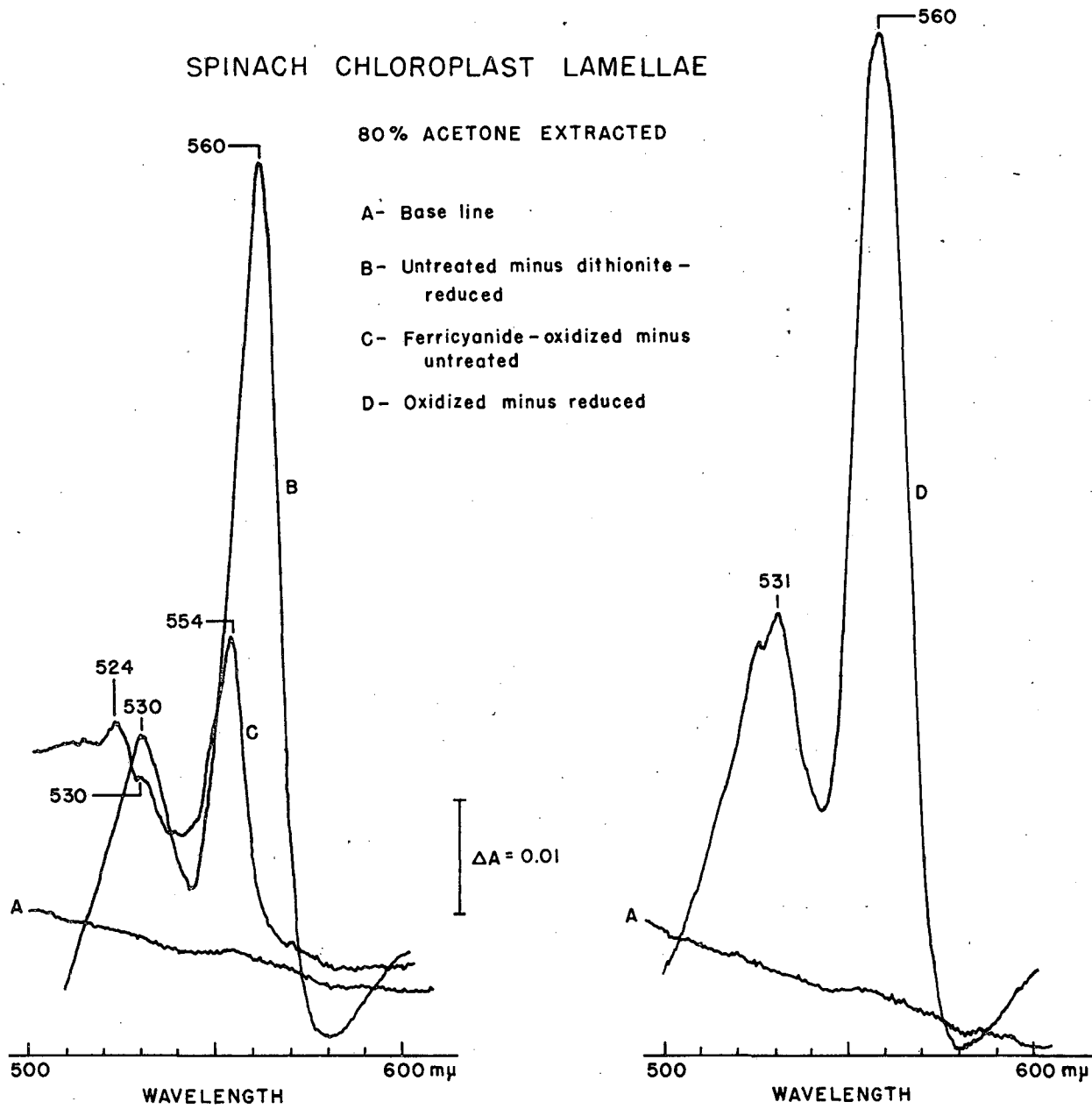
Fig. 16. Difference spectra of an 80% acetone powder of pure chloroplast lamellae. Powder suspended in 10^{-1} M tris-Cl pH 8.0.

SPINACH CHLOROPLAST LAMELLAR PROTEIN

A. FERRICYANIDE-OXIDIZED MINUS UNTREATED
B. UNTREATED MINUS DITHIONITE-REDUCED
C. OXIDIZED MINUS REDUCED

Fig. 17. Difference spectra of lipid free chloroplast lamellae
solubilized in cholate (2 mg/mg protein).

MUB-4234

Table 5

Spectral Data of Chloroplast Cytochromes[*]

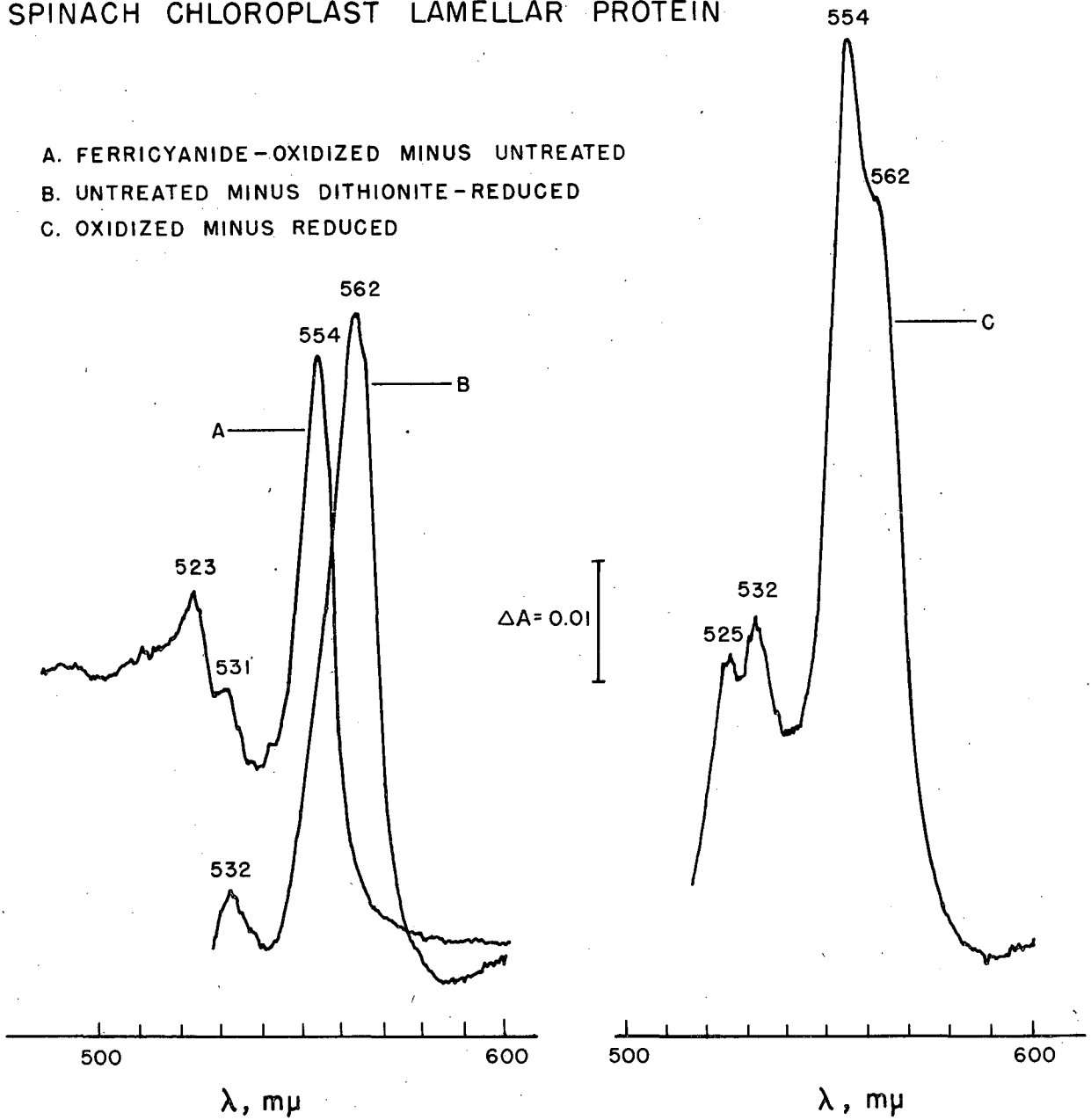| material	Cytochrome	Absorption Maxima			Ratios		Reference
		α	β	Soret	α/β	$\frac{\text{Soret}}{\alpha}$	
lamellar protein	b_6	561	530	431	4.4	4	This work
	f	554	530,524	421	2.1	7.1	
80% acetone extracted lamellae	b_6	560	530	——	3.9	——	" "
	f	554	530,523	——	1.9	——	
cholate treated	b_6	562	532	——	5	——	" "
	f	554	531,523	——	2.2	——	" "
80% acetone extracted lamellae 20°C −190°C	b_6 f b_6 f	563 554.5 557 552,548	534 524 530 529,524	429 422 427 420.8	4 2 —— ——	—— 7 —— ——	Hill and Bonner (1961)
80% acetone extracted lamellae	b_6 f	560 554	530 530	—— ——	—— ——	—— ——	Sironval & Engelert-Dujardin (1963)
pure cytochrome from parsley	f	554.5	524	421	2	7	Davenport & Hill (1952)

[*]In the computation of these ratios the absorption minima between the

α and β bands was taken as a "base line". Many spectra reported in

the literature were not absolute and did not show a true base line.

Hence, these values were computed for comparison only and do not

reflect the absolute absorption maxima densities.

Table 6

Cytochrome content of chloroplasts –
this study and literature values

Plant	cytochrome ratio b_6/f	molar ratio $\dfrac{\text{chlorophyll}}{\text{cytochromes } f \text{ and } b_6}$	Author
parsley	—	380 (f only)	Davenport and Hill (1952)
elder	—	430 (f only)	
spinach	1	200 to 300	Sironval and Engelert-Dujardin (1963)
spinach	—	400	Lundegardh (1962)
spinach	1.3	—	Hill and Bonner (1961)
spinach	0.92	about 230*	this work

*calculated on the basis of the composition of a quantasome of 50% protein and containing 230 chlorophyll molecules (Park and Biggins, 1964).

3. Sedimentation coefficient

Procedure

A Spinco Model E analytical ultracentrifuge was used with an RTIC system. An AN-D rotor with thermistor assembly and a standard single $4°$ section cell with Al centerpiece were used. Schlieren optics and Kodak spectroscopic plates type 1-D were employed.

The protein solution in 0.02% sodium dodecyl sulfate was exhaustively dialyzed against 10^{-2} M tris-Cl pH 8.0 for 48 hours and centrifuged at 110,000 g for 30 min in a Spinco Model L ultracentrifuge, #40 rotor. The supernatant was then dialyzed to equilibrium against 500 ml 0.002% sodium dodecyl sulfate, 5×10^{-3} M tris-Cl pH 8 and 0.1 M NaCl. The dialysate was subsequently used for dilution of the protein solution in order to produce varying protein concentrations. Sedimentation velocity studies were carried out at 59,480 rpm at $20°C$ as a function of 6 concentrations over a tenfold range of dilution. Plots of log dc/dx_{max} versus time were made and the slope related to the sedimentation coefficient as described in Section IA4.

Results

Fig. 18 shows a schlieren photograph of the protein fraction during such a sedimentation velocity determination. The material appears as a single boundary and the color, due to the two cytochromes f and b_6, is clearly associated with this boundary. Furthermore, the color remained associated with the boundary throughout the sedimentation. Fig. 19 shows a plot of the boundary position with time during a sedimentation velocity determination. The positions are measured from the schlieren photograph directly and as measured by scanning the photograph with a densitometer. The points are virtually coincident showing that the heme proteins are of very similar sedimentation

coefficient to that of the major component in the boundary. Fig. 20 (FV 3077) shows the dependence of sedimentation coefficient of the lamellar protein on concentrations. At high concentrations (3 mg/ml to 10 mg/ml) there appears to be a linear dependence of sedimentation coefficient on concentration, i.e., the higher the concentration, the lower the sedimentation coefficient. This behaviour is typical of many proteins and is largely due to the increase in solution viscosity at higher concentrations and centripetal flow of solvent. At lower concentration, the reverse behaviour is apparent. Schwert (1949) noted this type of anomaly for α-chymotrypsin and suggested that the system was undergoing a rapid association-dissociation equilibrium. It is likely that this interpretation could apply here in the lamellar protein system, for the bulk of the material is structural in nature and, as evidenced by its insolubility at physiological pH, the tendency for aggregation is very high.

By extrapolation of the linear portion of the data to infinite dilution, the sedimentation coefficient of the "dimer" here is 4.5×10^{-13} s. Extrapolation of the lower concentration points to infinite dilution permits an estimate of the sedimentation coefficient of the "monomeric" form as 2.3×10^{-13} s. Owing to this anomalous behaviour of the protein fraction, both these extrapolations are hazardous and the results are considered with caution.
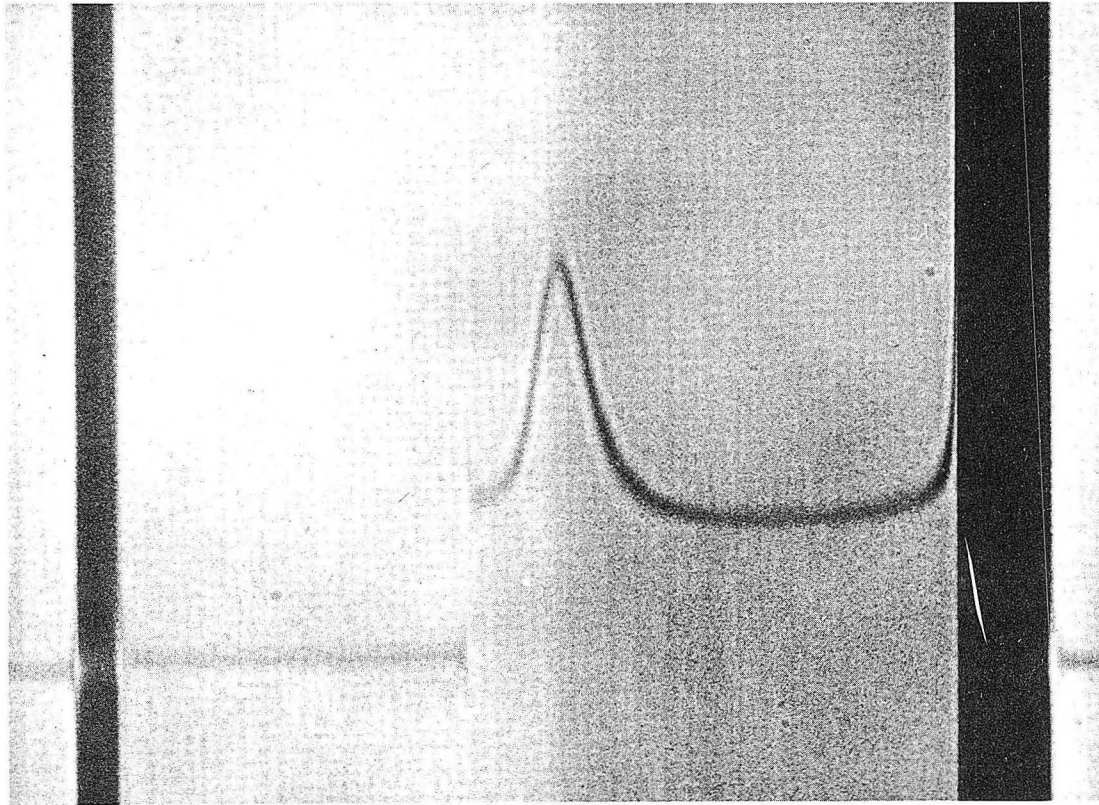
ZN-4609

Fig. 18. Schlieren photograph of the chloroplast lamellar protein
fraction during a sedimentation velocity determination. The
color due to the cytochromes is clearly associated with the
boundary. Protein 7 mg/ml in 0. 1 M-NaCl, 0.002% Na-dodecyl
sulfate, 5×10^{-2} M tris pH 8. Rotor speed 59,780 rpm, 4°C.
Phase plate 60°. Photograph taken 12 min after reaching speed.

SEDIMENTATION VELOCITY OF CHLOROPLAST LAMELLAR PROTEIN.
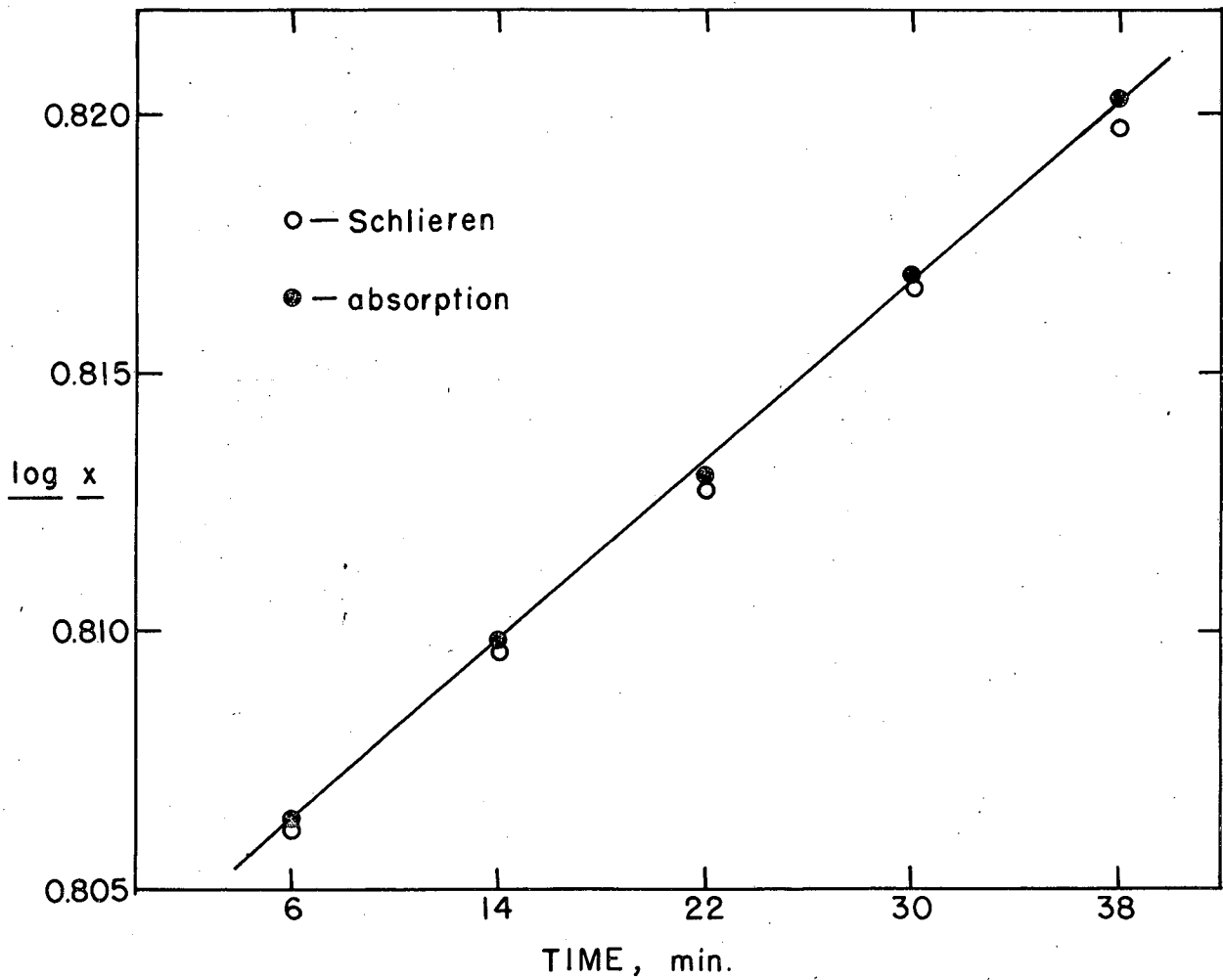
Fig. 19. Boundary position of the chloroplast lamellar pro-
tein fraction during a sedimentation velocity experiment.
The 50% concentration points were obtained from the
schlieren photograph directly (—o—) and by scanning
with a densitometer (—●—).
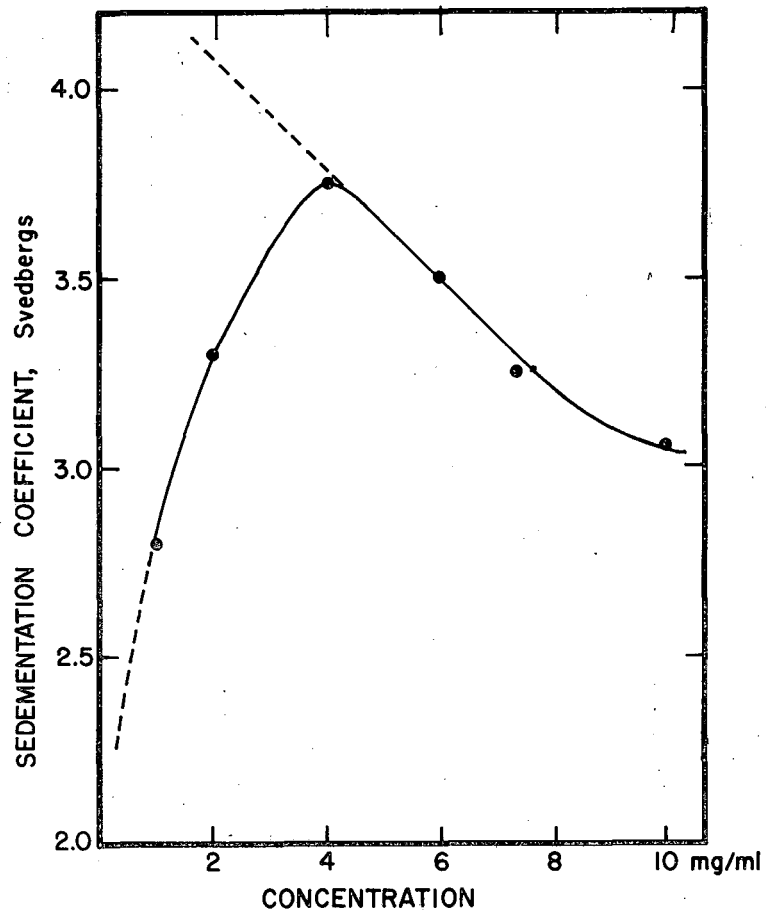
Fig. 20. Dependence of sedimentation coefficient on concentration of the lamellar protein fraction. All experiments conducted in 0.1 M-NaCl, 0.002% Na-dodecyl sulfate and 5×10^{-2} M-tris pH 8.

4. Diffusion coefficient

Procedure

The apparent diffusion coefficient of the protein fraction was determined in the analytical ultracentrifuge in a layering experiment by a procedure according to Ehrenberg (1957).

The protein was dialyzed to equilibrium against 0.1 M NaCl, 0.002% sodium dodecyl sulfate and 10^{-2} M tris-Cl pH 8.0. A capillary-type, double sector, synthetic boundary cell (Spinco No. 306075, filled-Epon) was used with an AN-D rotor. After careful layering of the buffer at low speed, the centrifuge was adjusted to 10,580 rpm to stabilize the boundary. The boundary then spread due to diffusion and the experiment was concluded after one hour. Photographs were taken during the experiment by means of the schlieren optical system and Kodak spectroscopic plates type 1-D. Figs. 21 and 22 show photographs of the boundary shortly after layering and after 48 min diffusion.

The data were evaluated by the "maximum ordinate" method, where the relation

$$d^2 = 16 \ k^2 \ D(t - t_o)$$

was used. Here d is the distance along the baseline (rotational radius) between the points on the gradient curve having

$$dc/dx = dc/dx_{max}/e \ ,$$

k is the magnification factor and t, time. The diffusion coefficient, D, was obtained from the slope of a plot of d^2 versus t as shown in Fig. 23. The diffusion coefficient of the protein fraction is 9.1×10^{-7} cm sec^{-1}. It was also noted that during the experiment the area under the gradient curve remained constant, indicating that
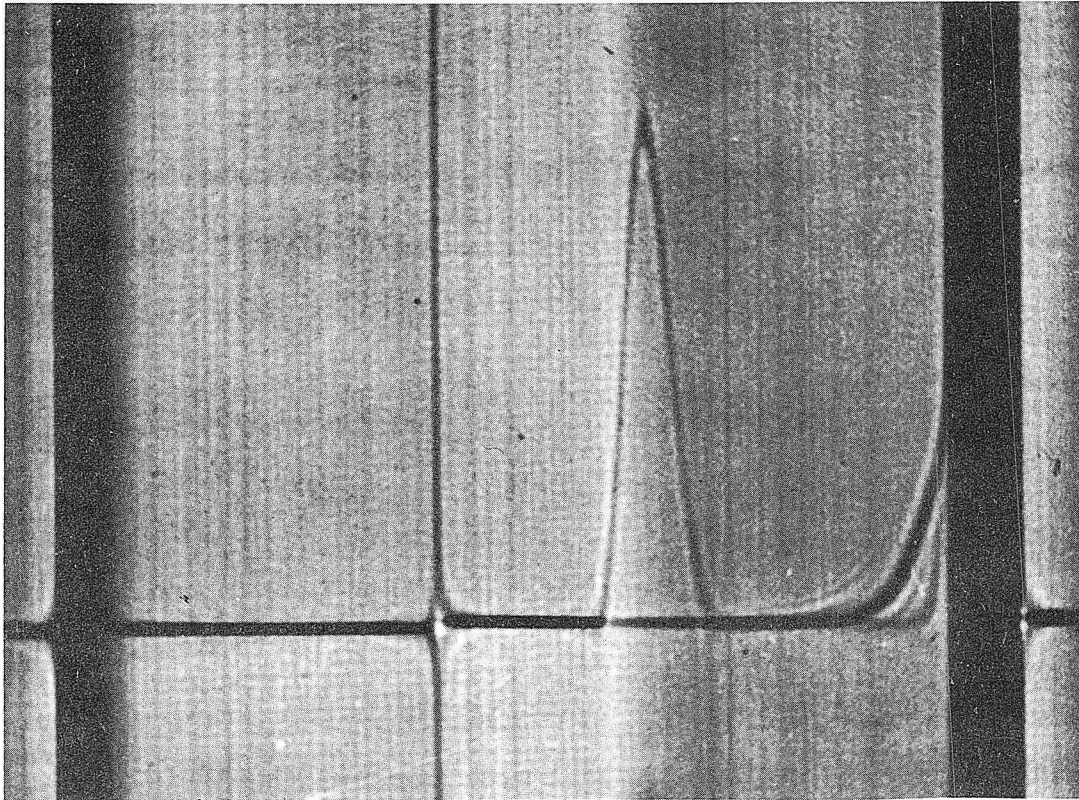
ZN-4608

Fig. 21. Synthetic boundary of chloroplast lamellar protein
fraction during a determination of the diffusion coefficient
in the analytical ultracentrifuge. The buffer, 0.1 M-NaCl,
0.002% Na-dodecyl sulfate, 0.05 M-tris-Cl pH 8, was
layered over the protein solution (6 mg/ml) at low speed.
The rotor speed was then increased to 10,580 rpm and main-
tained to stabilize the boundary. Photograph taken 30 min
after layering, phase plate 70°.
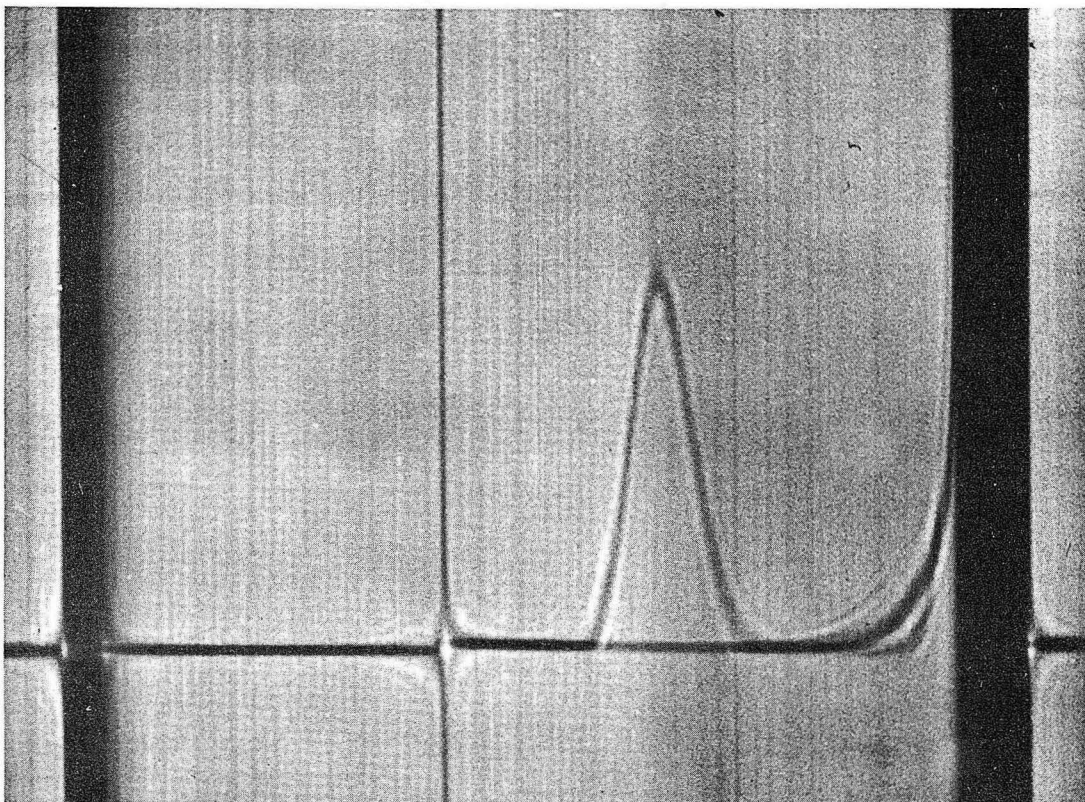
ZN-4607

Fig. 22. As in Fig. 21 but 48 min after formation of the synthetic boundary.
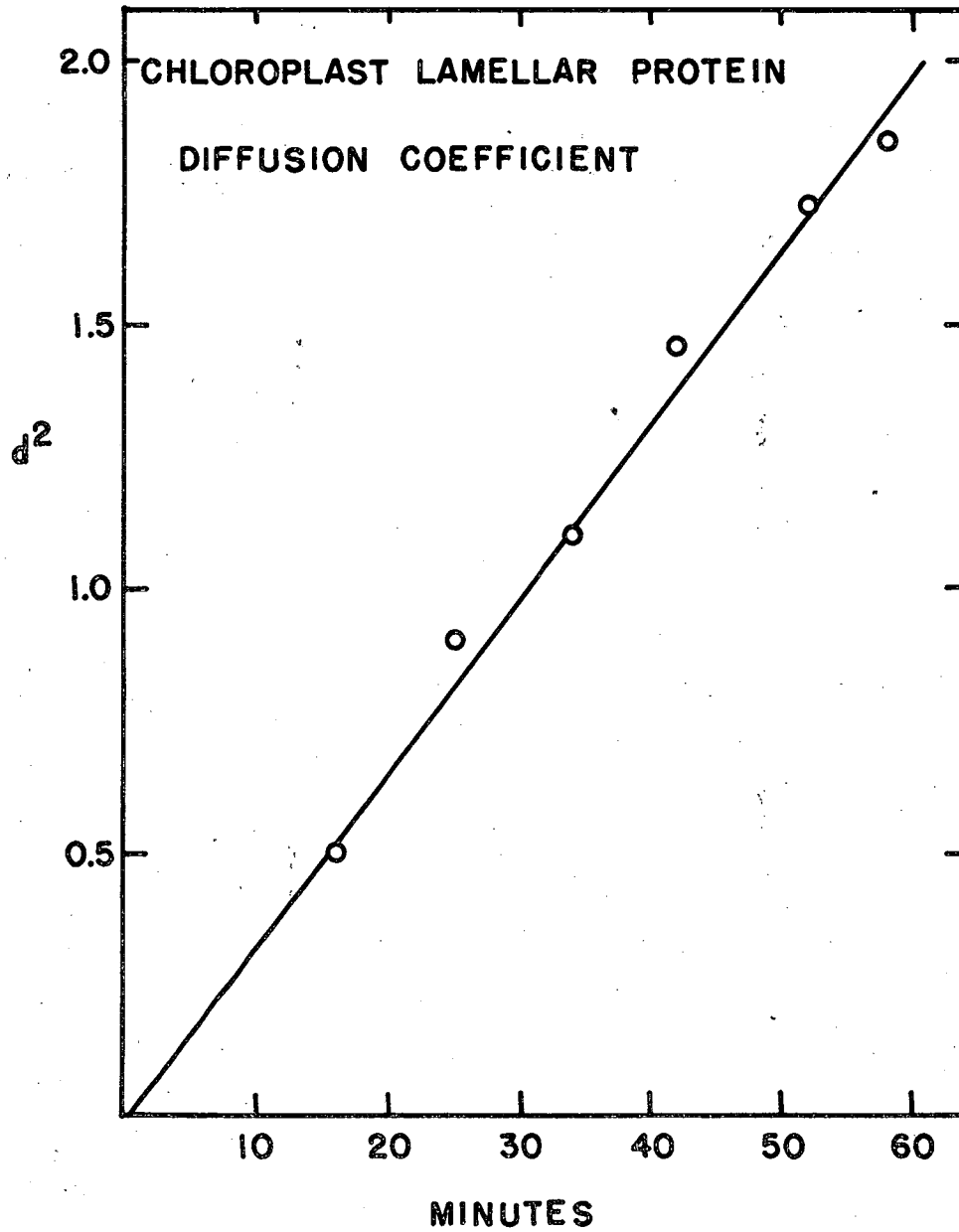
Fig. 23. Spreading of a synthetic boundary of the chloroplast
lamellar protein fraction at low speed in the analytical
ultracentrifuge. Conditions as described in the text and
Fig. 21.

no large aggregates formed and sedimented from the synthetic boundary.

5. Summary of data on the protein fraction

Treatment of purified chloroplast lamellae with dodecylsulfate and butanol effectively strips the protein free of lipid. The lipid-free protein fraction was studied by spectrophotometry and analytical ultracentrifugation.

The protein fraction was found to consist of a large quantity of heme-free protein and the two photosynthetic cytochromes f and b . The absorption maxima of the α and β bands of the ferrocytochromes do not appear to be modified by low concentrations of detergent.

In the analytical ultracentrifuge, the protein fraction behaved as a single component exhibiting an association-dissociation equilibrium. The mean sedimentation coefficient at infinite dilution was found to be 2.3 S and the diffusion coefficient 9.1×10^{-7} cm sec . Hence, the average molecular weight of the proteins is about 22,000 g-mole^{-1}. This value can only be considered approximate, owing to the hazardous extrapolation of the sedimentation velocity data and that a partial specific volume of the proteins is assumed. Assuming the proteins to be spherical, the radius of the proteins is about 20 Å. It is suggested that these comprise a fundamental repeating structural unit in the lamellae.

Discussion

From the measured physical constants of the chloroplast lamellar protein fraction it is possible to compute the average molecular weight. Insertion of the sedimentation coefficient, $s = 2.3 \times 10^{-13}$ sec, diffusion coefficient, $D = 9.1 \times 10^{-7}$ cm^2 sec^{-1}, and $\bar{v} = 0.722$ (assumed)

into the Svedberg equation, $M = \dfrac{S}{D} \cdot \dfrac{RT}{1-\bar{v}p}$, gives the molecular

weight $\simeq$ 22,000 g mole^{-1}.

The behaviour of the protein fraction in the analytical ultra-
centrifuge is typical of a system exhibiting an association-dissociation
equilibrium and the tendency for aggregation is very high. This is
expected for a protein fraction derived from a cell membrane, where
structural integrity is mandatory for biochemical activity. The
insolubility of the protein fraction at physiological pH and the
process of solubilization by detergent leads one to conclude that
the protein species are not covalently bound. The proteins are hydro-
phobic in nature and the membrane structure is possibly maintained
through the repulsion of water rather than by other attractive forces
or covalent bonds.

The properties and molecular dimensions of this chloroplast
lamellar protein fraction bear a striking similarity to the structual
protein of mitochondria described by Criddle et al.(1962). However,
they found that the sedimentation coefficient of the structural protein
is not dependent upon concentration, in contrast to the result found
in this study. A possible explanation of this discrepancy is that the
two studies were conducted in different concentrations of detergent.
Criddle, et al. used 0.2% Na=dodecyl sulfate, whereas the sedimentation
velocity determinations described in this study were conducted in
0.002% Na-dodecylsulfate. It is likely that the higher concentration
of detergent used by Criddle et al sufficiently eliminated interaction
of the protein, even at high protein concentrations. Low detergent
concentrations were used in this study in order to avoid possible
ambiguities arising from operating above critical micellar concentrations.

From the sedimentation velocity studies it can be concluded that all the molecular species in the lamellar protein fraction have a similar sedimentation coefficient, as only one boundary was detected. Assuming that other hydrodynamic parameters are also similar, then the proteins are of approximately the same molecular weight, i.e., about 22,000.

If the molecular weight of a quantasome is two million and is composed of 50% protein, then there are about 40 of the lamellar proteins per quantasome, two of them being the two photosynthetic cytochromes f and b_6. The radius of a sphere of molecular dimensions similar to those of the lamellar protein fraction is about 20 Å. Hence, the total volume of protein in a quantasome is approximately 1.34×10^6 Å^3. The quantasome volume is in the order of 2.4×10^6 Å^3.

There have been other reports of preparations of proteins from the lamellae of chloroplasts. Criddle and Park (1964) prepared a protein from an acetone powder of lamellae by solubilization with large quantities of cholate and deoxycholate followed by salt fractionation. The protein is heme-free, the sedimentation coefficient is 2.2 S and the molecular weight 23,000 in good agreement with the results reported here. The binding properties of the protein were studied and it was found that the protein forms complexes with ATP, phospholipid, chlorophyll and myoglobin. By analogy to the protein isolated from mitochondria (Criddle et al, 1962) it was termed "structural protein".

Weber (1963) solubilized chloroplast lamellae in formic acid and extracted the lipid with ether. After fractionation, he isolated a protein with a sedimentation coefficient of 6 S. A similar protein was isolated from lamellae by Thornber, et al. (1964) by treatment with 70% acetic acid followed by dialysis against 3% acetic acid.

The soluble fraction after dialysis contained 20% lipid and was shown to have a sedimentation coefficient of 6 S and to consist of six components on electrophoresis on polyacrylamide gel. Neither relative concentrations of the six components nor detailed behavior of the protein in the ultracentrifuge were given.

Pertinent information concerning lamellar sub-structure comes from observations on the effect of detergents on the chloroplasts and the formation of pigment-protein complexes. Treatment of chloroplast lamellae with digitonin or bile salts (2 to 3%) leads to the formation of a particle with a sedimentation coefficient of 13.8 Svedgergs and a molecular weight of about 265,000 (Smith and Pickles, 1941). Itoh, et al. (1964) showed that treatment of grana with 3×10^{-3} M dodecylbenzene sulfonate formed a pigment-protein with a sedimentation coefficient of 2.9 S, and higher concentrations led to the formation of 1.2 S component. This effect is very similar to that induced by dodecyl sulfate where a 2.5 S component is formed with 0.25% detergent, and higher concentrations yield a 1.69 S component (Smith and Pickles, 1941). Furthermore, in both cases the pigment remains bound to the protein in the presence of these anionic detergents, whereas bile salts and digitonin extract the pigments (Smith, 1941).

Thus, it appears from this study of the properties of the protein fraction of chloroplast lamellae, other reports in the literature and the effect of detergents on lamellae, that the principal protein in the membrane in the chloroplast is in the order of 22,000 molecular weight and 40 Å in diameter. That this is a fundamental repeating sub-unit is also suggested by the work of Menke (1963). He studied the low angle X-ray scattering of chloroplast lamellae

and found a 36 Å to 40 Å periodicity in the plane of the lamellae.

It also appears that various complexes of the lamellar protein can be isolated. Unfortunately, there are no analytical data on the 13.8 S complexes formed by treatment of the lamellae with digitonin or bile salts, but they represent one-fourth of the quantasome protein. Park and Biggins (1964) showed electron micrographs of shadowed lamellar sonicates which clearly indicated that the quantasome could comprise of four sub-units. It is possible that these may represent specific complexes of the cytochromes and the, as yet unknown, enzymic apparatus responsible for oxygen evolution.

I. C. General comments on the structure of chloroplast lamellae

Investigations by a large number of workers during the last
decade have demonstrated the importance of membranes in energy trans-
foramtions within the living cell. One notable feature of cell
membranes that has emerged from these investigations is the inextri-
cable association between membrane structure and enzymic function to
the extent that the loss of one invariably results in the loss of the
other. This is true of mitochondrial cristae and chloroplast lamellae.
Both are structural mosaics of protein and lipid and their electron
transfer mechanisms are facilitated by the participation of several
unique electron carriers operating in a sequential fashion. It is
presumed that the geometric relationships of these intermediates are
critical in the mechanism and are determined by the structure of the
membrane itself.

Chloroplast lamellae and mitochondrial cristae are very similar
in composition and function. Both contain cytochromes, structural
protein, quinones, non-heme iron, copper and phospholipid (Green, 1961;
Park and Pon, 1962). Both have electron transport steps coupled to
the transport of electrons between oxygen and NAD or NADP. However,
mitochondrial cristae catalyze a series of exergonic reactions whereas
chloroplast lamellae catalyze the reverse process by means of energy
derived from the quantum conversion process.

The apparatus responsible for the absorption of quanta, their
migration to a reaction center and conversion to chemical potential,
is a further complication of chloroplast lamellar structure. Apart
from the intimate geometric configuration demanded by the electron

transport system in the photosynthetic unit, one important requisite
possibly facilitated by structure is the spatial separation of
oxidized and reduced sites in the primary quantum conversion act (Calvin,
1959). Such a spatial separation would make immediate back reactions
unlikely.

Goëdheer (1955) and Sauer and Calvin (1962) showed that the
majority of the chlorophyll in chloroplast lamellae is unoriented, but
Calvin (1959) has suggested that the porphyrins could be tilted on
three axes by analogy to the crystal structure of nickel phthalocya-
nine. Such an organization would account for the low dichroism
observed by Goëdheer. Sauer and Calvin (1962), in a more detailed
study of the electric dichroism and electric birefringence of quanta-
somes, demonstrated a dichroism anomaly at 695mu. They interpreted
the dichroism as resulting from a small fraction of chlorophyll a
which is oriented at a particular site in the quantasome. They
suggested the possibility of its equivalence with the long wave frac-
ion (5%) of chlorophyll a absorbing in the same spectral region (Brown
and French, 1959). Sauer and Calvin proposed a mechanism whereby such
an oriented fraction of chlorophyll a participates in the collection
and trapping of quanta absorbed by pigments in the photosynthetic unit
and conversion to chemical potential. Absorbed quanta migrate by
resonance transfer to the quantum conversion site, the quantatrope,
which contains the oriented chlorophyll, an oriented cytochrome and
an acceptor molecule. The excited state transfers an electron to
the acceptor molecule and the hole which remains on the chlorophyll
then migrates through a conduction band of the oriented pigments until
it reaches a molecule adjacent to the oriented cytochrome. Here the
cytochrome transfers an electron to the chlorophyll and is converted

to its oxidized form. They support their model by pointing out that such events could occur at very low tempertures (-150°C) since the migration of chemical species is not involved and the reversal of the process is rendered improbable by the spatial separation of the acceptor molecule and the cytochrome. After such a charge separation, subsequent electron transfer reactions are chemical and have a temperature coefficient. As yet there is no evidence for the existence of an oriented cytochrome or acceptor molecule. However, it does appear possible that P_{700} (Kok and Hoch, 1961) is the oriented chlorophyll a adjacent to the cytochrome, and cytochrome f oxidation in leaves has now been demonstrated at very low temperatures (Chance and Bonner, 1963).

The quantasome, as described by Park (1962), is a fundamental morphological repeating unit of chloroplast lamellae. Evidence that it may also be a unit of photosynthetic function as originally suggested by Park (1962), has been recently reviewed by Park and Biggins (1964). The possibility appears attractive on the basis of the quantasome size and the concentration of photosynthetic pigments and electron transport intermediates. For instance, the quantasome of molecular weight, 2×10^6 g-mole^{-1}, contains 230 chlorophylls, one P_{700} and one cytochrome f. These are the concentrations of constituents in the photosynthetic unit determined by kinetic experiments (Kok and Businger, 1956), oxidation-reduction titration (Kok and Hoch, 1961) and difference spectrophotometry (see I, B.2). That each quantasome is a photosynthetic unit can only be unequivocally settled by the isolation of a monodisperse preparation of quantasomes which is active in quantum conversion and electron transport and is physiologically further indivisible. As yet, aggregates of up to eight quantasomes

have been shown to be active in quantum conversion. Consideration of the size distribution as outlined in I. A. 4 and activity of the lamellar fragments (see II. C for results and discussion), suggests the possibility that the smallest fragments of lamellae consisting of two or three quantasome are active, unless the large aggregates in the preparations display an unusually high specific activity.

From the investigations of Park and Pon (1961) and this study (see also Part II and Sauer and Biggins, 1964) on the photochemical activity of quantasome aggregates, it appears that the critical structural requirement necessary in photosynthetic electron transport processes is the geometric arrangement of the constituents within the photosynthetic unit rather than the distribution of photosynthetic units in chloroplast lamellae. Thus the localization of components within the quantasome is important in our understanding of the photosynthetic mechanism.

From an investigation of lipid-extracted chloroplast lamellae by electron microscopy, Park and Pon (1963) showed that there is a considerable amount of residual structure suggesting that the protein exists in macro-units embedded in a lipid matrix. As the unextracted lamellae appear fairly smooth by comparison (except in regions of membrane breakage) this would indicate that a considerable portion of lamellar lipid is located on the outer surface of the membrane. Recent investigations (Park, 1964) show that the pigment complement is also associated with this lipid, as a similar residual structure is also apparent after complete extraction of the lamellar pigments. That the pigments are completely extracted was shown by spectrophotometry of electron microscope grids prior to observation.

Sastry and Kates (1964 a) provide indirect evidence supporting

the notion that a portion of the lamellar lipid is localized on the outer surface of the membranes. In an investigation of the nature of bean leaf lipids, they found that mono- and di-galactosyl lipids are absent in water homogenates and chloroplast preparations although they are present in high concentrations in whole leaves. They suggested and found (1964 b) that the leaves contain galactolipid-hydrolyzing enzymes which act as soon as the plant cells are broken. They showed that the galactolipases are localized in the cell sap and chloroplasts and suggested that the reported losses of lipids during chloroplast isolation (Mego and Jagendorf, 1961) may have been caused by these enzymes. These observations indicate that the chloroplast-lamellar galactolipids (Wintermans, 1962) are at locations on the membrane which are quite accessible to galactolipase hydrolysis. Therefore, for reasons of simplicity it is suggested that they are at, or very near to, the lamellar surface. If so, it is possible that they are oriented with their sugar residues to the outer, hydrophilic stroma.

From a consideration of these experimental observations we can tentatively reason that a considerable proportion of lipid including the photosynthetic pigments, is localized on the outer surface of the membrane.

Part II. Photochemistry of the lamellae

A. Historical survey of electron transport

The evolution of minute traces of oxygen by expressed juices of plant material was demonstrated in the late nineteenth century (Englemann, 1881; Ewart, 1896; Beijerinck, 1901, and Molisch, 1904). However, Hill (1939) first examined the process quantitatively and he found that freshly isolated chloroplasts would evolve oxygen for an extended period of time when illuminated in the presence of an electron acceptor such as ferric ions. The reaction was subsequently called the "Hill reaction", and a variety of other artificial oxidants were found to be effective such as p-benzoquinone, ferricyanide and indophenol dyes. Finally, a physiological oxidant, NADP, was found to be effective but only when an appropriate enzyme, photosynthetic pyridine nucleotide reductase (San Pietro and Lang, 1958) or the chloroplast stroma (Arnon, et al., 1957) was added back to the photosynthetic membrane.

Arnon, et al. (1957, 1959) demonstrated the coupling of phosphorylation to the reduction of a Hill oxidant such as ferricyanide and to NADP in what they termed non-cyclic photophosphorylation. In addition, they also demonstrated the occurrence of another type of photophosphorylation which proceeds in the absence of any gas exchange, but only in the presence of an added cofactor such as PMS or vitamin K, i.e., cyclic photophosphorylation. It thus became apparent that chloroplast lamellae are able to reduce NADP, form ATP, and evolve oxygen in what is now generally referred to as photosynthetic electron transport. In photosynthetic electron transport, electrons from water are raised to a sufficiently negative redox potential ($E_o^1 = -0.37$ volts) to reduce NADP. This endergonic process is driven by light absorbed by the pigment system of a photosynthetic unit.

The two cytochromes F and b_6 are found exclusively in photosynthetic tissues (Hill, 1954; Hill and Bonner, 1961). The possible participation of these components in photosynthetic electron transport, by analogy with respiratory electron transport, led Hill and Bendall (1960a) to propose a working hypothesis for photosynthetic electron transport driven by at least two separate light reactions. They suggested that the two light reactions probably act in series and are separated by an exergonic, dark reaction that could possibly include a phosphorylation step. A variety of experimental devices provides evidence in support of their model, from a study of quantum yields and action spectra in vivo (Emerson and Lewis, 1943; Emerson, et al., 1957) action spectra in vitro (Govindjee, et al., 1963) differential absorption spectroscopy (Duysens, et al., 1961) and biochemical investigations with isolated chloroplasts (Vernon and Zaugg, 1960; Losada, et al., 1961).

Emerson and Lewis (1943) studied the quantum yield of photosynthesis as a function of the wavelength of actinic illumination and found that there was a pronounced decrease at wavelengths longer than 670 mμ but still within the absorption bands of the photosynthetic pigments (first Emerson effect, or "red drop"). Subsequent investigations (Emerson, et al., 1957) showed that the low quantum yields in the long wavelength region could be considerably enhanced by the addition of supplementary light of shorter wavelength (second Emerson effect, or "enhancement"). These results indicated the participation of more than one pigment system in the light reactions of photosynthesis.

Duysens, et al. (1961) demonstrated the reversible photo-oxidation of a c-type cytochrome in Porphyridium cruentum by differential absorption spectroscopy. The action spectrum for the oxidation showed two

maxima—one at 680 mµ (System I) and one at 560 mµ (System II). Since
the kinetics of the oxidation in monochromatic beams of 560 mµ and 680 mµ
light were found to be markedly different, the action spectrum indicated
and was interpreted as the result of the interaction of at least two
pigment systems. The interaction of the two systems was investigated
by an experiment in which two actinic beams of different wavelengths
were used. Light at 680 mµ was found to oxidize the cytochrome, whereas
additional 560 mµ illumination reduced the cytochrome. They also found
that inhibitors of O_2 evolution inhibited the cytochrome reduction,
indicating that System II is associated with the photooxidation of
water.

Vernon and Zaugg (1960) showed that chloroplasts, whose ability to
photooxidize water is impaired by ageing, or inhibited by the addition
of DCMU, are able to photoreduce NADP if supplied with ascorbate and a
catalytic amount of DPIP. Jagendorf and Margulies (1960) showed that
PMS could also couple ascorbate to the photoreduction of NADP in a DCMU-
inhibited system. Losada, et al. (1961) showed that during the course
of NADP reduction by DCMU-poisoned chloroplasts in the presence of
DPIP/ascorbate, photophosphorylation also proceeded with an overall
stoichiometry similar to that of noncyclic photophosphorylation. They
also showed that the addition of catalytic amounts of DPIP to a system
reducing ferricyanide in the Hill reaction resulted in no photophos-
phorylation—only the photooxidation of water. Losada, et al. inter-
preted their findings in terms of the Hill and Bendall model and pro-
posed that the indophenol dye, in the oxidized and reduced form, reacts
with an intermediate in the electron transport chain. If the photo-
oxidation of water is inhibited by DCMU, then reduced DPIP acts as

electron donor for System I, the reduction of NADP. If the DPIP is maintained in the oxidized form, by the addition of substrate amounts of ferricyanide, only System II, O_2 evolution, is operative and the electron transport is truncated prior to the phosphorylation step (i.e., DPIP is electron acceptor). That the two light reactions had been biochemically separated was supported by subsequent work of the same type (Arnon, et al., 1961), where monochromatic illumination was used to activate the reactions. System II, the photooxidation of water, was shown to proceed most efficiently at 644 mμ, whereas System I, the photoreduction of NADP and coupled phosphorylation. occurred with maximum efficiency at about 670 mμ, indicating that the two systems are sensitized by different reaction centers.

The second Emerson effect (enhancement) was shown to occur for NADP reduction by isolated chloroplasts (Govindjee, et al. 1962, 1963), with water as electron donor, implying the participation of more than one photo-event in overall photosynthetic electron transport.

The model for the two light reactions in higher plant photosynthetic electron transport also encompasses bacterial electron transport. Frenkel (1954) showed that bacterial chromatophores can produce ATP when illuminated, and the type of photophosphorylation is similar to the higher plant cyclic photophosphorylation. It was later shown (Nozaki, et al., 1961) that bacterial chromatophores are also capable of non-cyclic photophosphorylation with NAD as electron acceptor if a hydrogen donor such as succinate is added. The current unifying concept therefore is that the bacterial electron transport is analogous to the higher plant photosystem I with coupled phosphorylation. The photooxidation of water, the higher plant photosystem II, is absent

in bacteria, and the addition of an external electron hydrogen donor
other than water is necessary for non-cyclic transtort. These con-
cepts can be summarized by means of the following diagram:

NADP   NAD

cyclic   cyclic
   processes   processes

        H   H

photo-   donors   donors
system
  I

 phosphorylation   phosphorylation

      photo-
      system
        II

$H_2O \longrightarrow O_2$

higher plants   photosynthetic bacteria
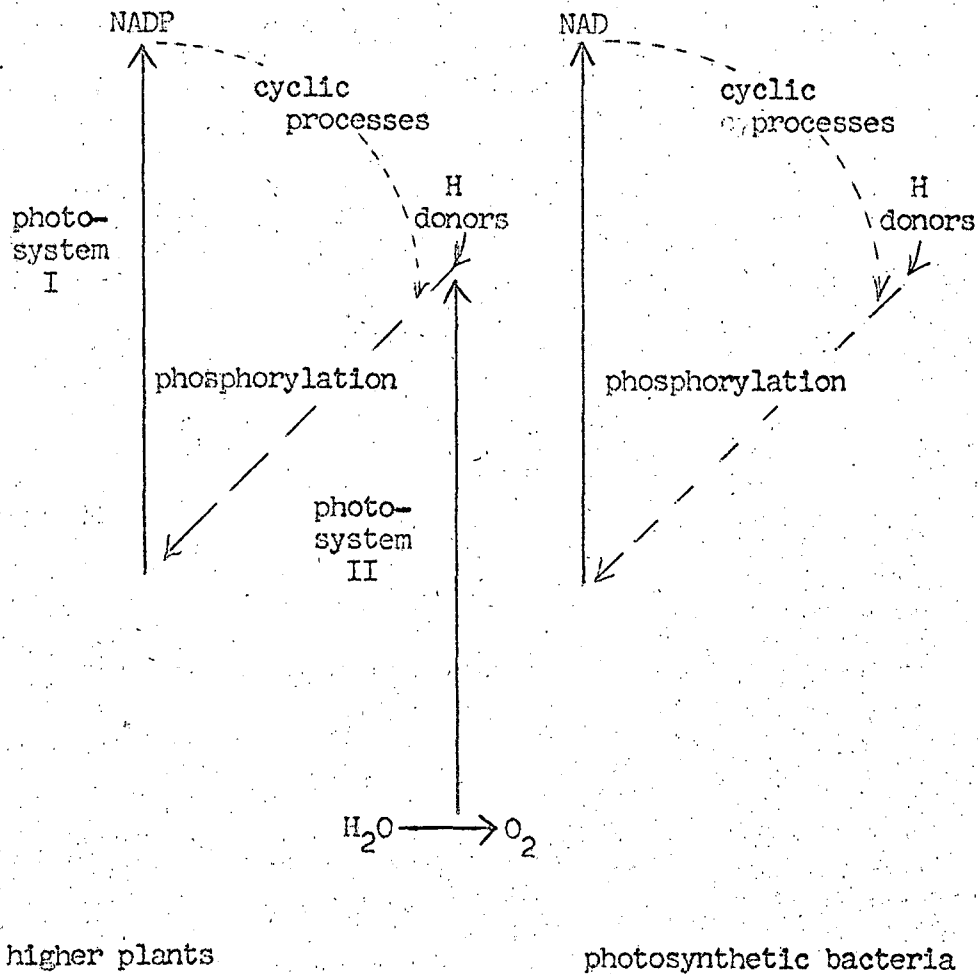
The exact nature of the pigments and reaction centers involved
in the primary photochemical events in Systems I and II is still un-
known. Kok found a pigment with absorption maxima at 700 mμ and
430 mμ which reversibly beaches with illumination. This pigment,
termed P_{700}, behaves in the dark as a one-electron transferring agent

with an $E_o^{\frac{1}{2}}$ of +0.43 volts, the absorbing form being the reduced molecule (Kok, 1961). The oxidation of P_{700} is caused by far red illumination (System I) and short wavelengths cause the reduction which is inhibited by DCMU. In an investigation of the kinetics of the oxidation of P_{700} and cytochrome f induced by rapid flashes, Witt, et al. (1961b) found that the P_{700} oxidation occurred more rapidly than the oxidation of the cytochrome. This is consistent with the possibility that the cytochrome reduces P_{700} as suggested by Kok. It is appealing to think that P_{700} and cytochrome f are intimately arranged with the oriented fraction of chlorophyll a which absorb in the long red region (Sauer and Calvin, 1962; Brown and French, 1959; Butler, 1961) and serve as an energy trap for photosystem I as discussed in Part I.

As yet, there is no evidence concerning the nature of the active center of photosystem II, although action spectra of photosynthesis in certain algae and chloroplasts tend to indicate the participation of chlorophyll b or other accessory pigments.

Part II of this work is a study of NADP and ferricyanide reduction by isolated chloroplasts and lamellar fragments. We have attempted to characterize pigment Systems I and II more definitely by measuring the wavelength dependence and quantum requirements of the individual light reactions in chloroplast electron transport. In particular, we have i) compared the photoreduction of NADP with water as electron donor in a DCMU-poisoned system, i.e., a comparison of Systems I and II operating together and System I only; and ii) compared the Hill reaction with ferricyanide as terminal electron acceptor with that when a catalytic quantity of indophenol dye is added. The ability of quantasome aggregates and various membrane fractions to reduce NADP is also described.

B. Experimental

Preparation of chloroplasts and lamellar fragments

Chloroplasts were prepared from Spinacia oleracea L. by minor modifications of procedures according to Hoch and Martin (1963) or according to Arnon, et al. (1956). Lamellar fragments were prepared as described in Part I.

Preparation of enzymes

Photosynthetic pyridine nucleotide reductase was prepared from Spinacia oleracea L. according to the procedure of San Pietro and Lang (1958) through the 75% acetone precipitation step. The protein was redissolved, centrifuged and dialyzed against 5×10^{-3} $\underline{M}$ tris-chloride pH 8 overnight. The protein was further purified through the first DEAE-cellulose column treatment of Hill and Bendall's method (1960b). This partially purified preparation contained chloroplast ferredoxin (Tagawa and Arnon, 1962) and ferredoxin-NADP reductase (Shin, et al., 1963), as shown by the following assays.

Enzyme assays

The preparation of chloroplast ferredoxin and ferredoxin-NADP reductase was not further purified. It was assayed in the photochemical reduction of NADP by isolated chloroplasts. The reaction mixture, 4 ml, contained the following in μmoles/ml: tris-Cl, pH 8.0, 50; NADP, 0.5; ADP, 1.0; $MgCl_2$, 7.5; chlorophyll, $OD^{1\ cm}_{678}$ = 0.25; enzyme, variable.

The reactions were carried out aerobically in stoppered vessels in an Aminco Warburg Apparatus (Amer. Instr. Co., Maryland). The illumination was provided by eight photoflood lamps mounted underneath the tank and the intensity was adjustable by means of a Variac. The voltage used was 85 volts, giving a light intensity of 2000 foot candles measured

at the point of illumination of the sample. Reactions were carried out for 10 min at 15°C. The samples were then withdrawn and ultra-centrifuged at 40,000 rpm (Spinco Model L ultracentrifuge #40 rotor) to remove the chloroplast membranes. The clarified supernatants were then assayed spectrophotometrically for $NADPH_2$ against a similarly treated control with no enzyme.

Fig. 24 shows the enzyme activity of such a preparation.

The preparation was also assayed for $NADPH_2$-diaphorase activity by a procedure according to Shin, et al. (1963). The reaction mixture (2.5 ml) contained the following in μmoles/ml: tris-Cl pH 9.0, 50; $NADPH_2$, 0.1; trichlorophenol indophenol, 0.12; and enzyme, variable. The reaction was carried out in 10 x 3 mm cuvettes and was started by the addition of 10 μl $NADPH_2$. The absorbance decrease at 620 mμ was monitored spectrophotometrically after addition of the enzyme. A Cary Model 14R automatic recording spectrophotometer was used for this purpose, and the initial rates were taken as the reaction velocities.

Fig. 25 shows the $NADPH_2$-diaphorase activity of the preparation which is taken as an indication of the presence of $NADPH_2$-ferredoxin reductase.

This enzyme preparation was used in saturating concentrations in the quantum yield determinations for NADP reduction by isolated chloro-plasts.

Light intensity measurements

Incident light intensity measurements were made at the point of sample illumination by means of a silicon solar cell (Hoffman, type 120CG). The wavelength dependence of the silicon solar cell was deter-mined by use of a Bausch and Lomb 500 mm grating monochromator with a
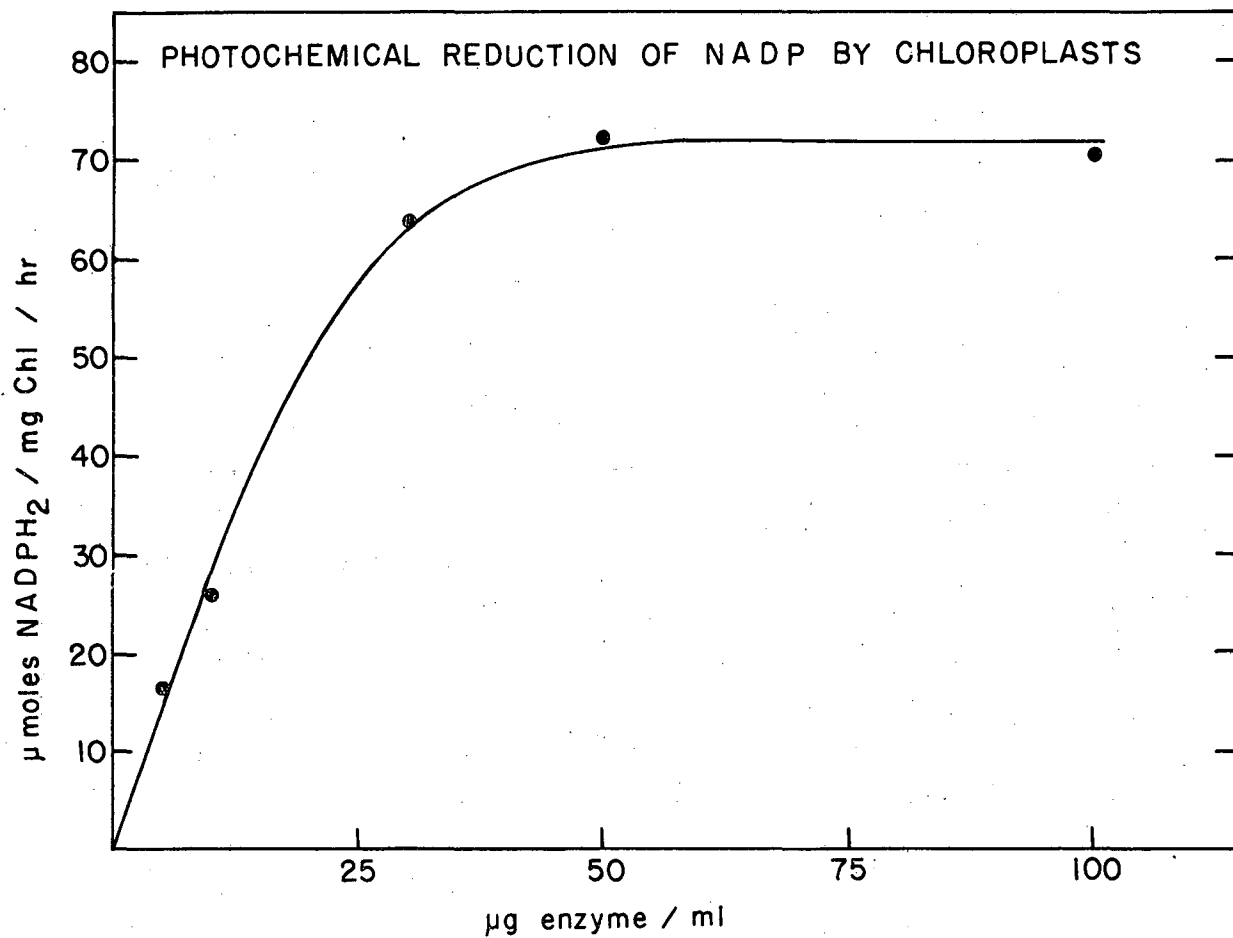
Fig. 24. Enzyme activity of chloroplast ferredoxin and ferredoxin-
   NADP reductase in the photochemical reduction of NADP by
   spinach chloroplasts.
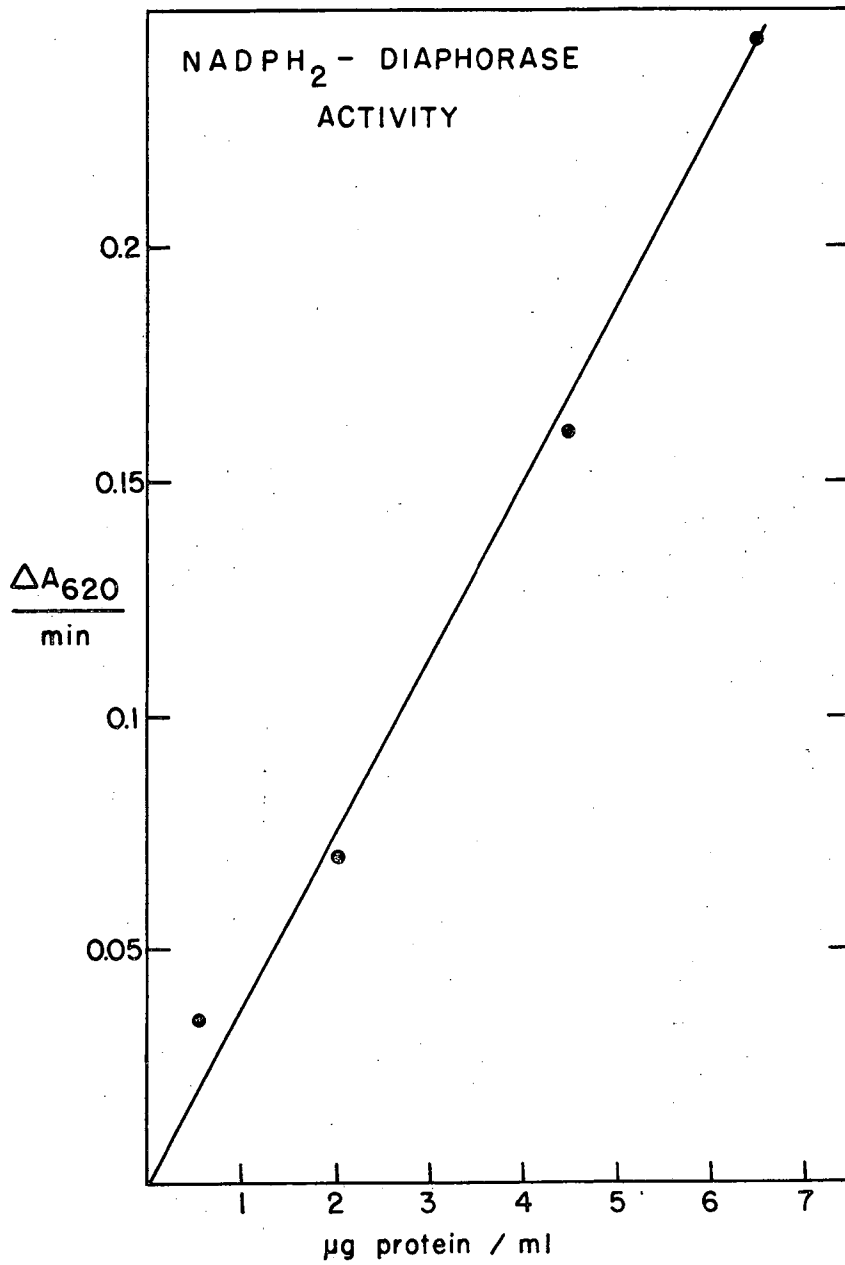
Fig. 25. Diaphorase activity of ferredoxin-NADP reductase using
2, 3, 6-trichlorophenolindophenol as electron acceptor.

tungsten source and a Reeder thermopile (C. M. Reeder Co., Detroit)
which had been calibrated using a standard lamp (National Bureau of
Standards, Washington, D. C.). Corrections were applied to the results
for the 5% measured reflection loss on the cuvette sides.

Absorbed intensity calculation

For the accurate determination of the quantum yield of a photo-
chemical reaction the intensity of radiation absorbed must be known.
This is especially difficult in the case of chloroplasts at wavelengths
longer than 690 mμ, owing to the relatively large contribution of light
scattering. The use of the measured transmission of the sample may
lead to large errors. The technique for the measurement of absorbed
light intensities in this study is based upon a report by Sauer and
Park (1964) on the spectral characteristics of chloroplast and quanta-
some suspensions.

Fig. 26 shows the absorption spectrum of a single chloroplast sus-
pension measured using three techniques: direct absorption using a
Cary Model 14R recording spectrophotometer, the opal glass technique
(Shibata, et al., 1954), and the scattered transmission method with a
red-sensitive photomultiplier. The result using the opal glass technique
is identical to that using the scattered-transmission method. However,
the opal glass technique is unreliable at wavelengths longer than 700 mμ
(Sauer and Park, 1964).

Fig. 27 shows long wavelength absorption spectra of concentrated
chloroplasts and quantasome aggregates measured by the scattered-
transmission method. The absorption maximum is 678 mμ. It is apparent
from these data that measurements of chloroplasts in the long wave-
length "tail" of the chlorophyll a absorption band are unreliable, even
when the scattered-transmission method is used. However, the quantasome
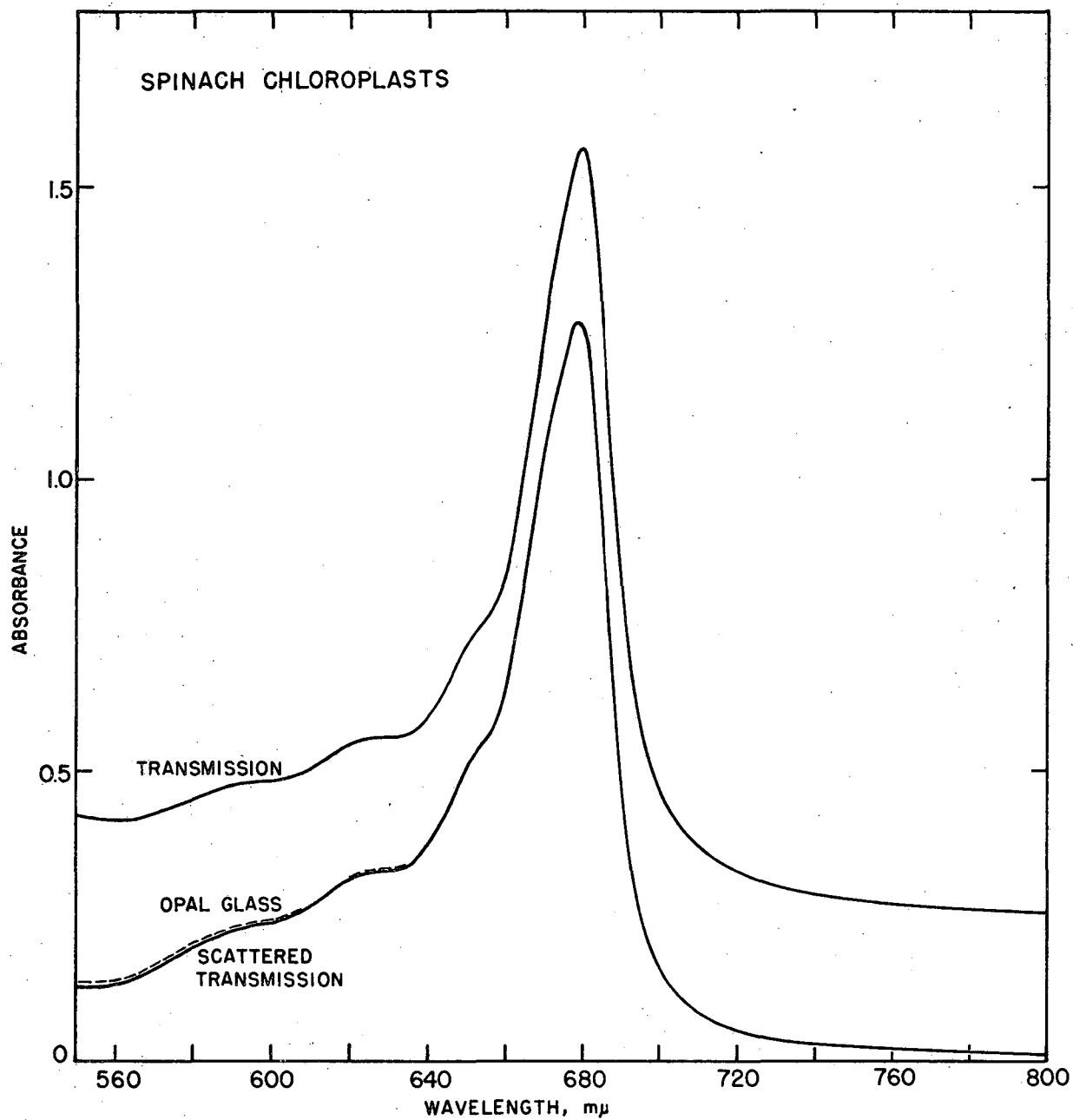
Fig. 26. Absorption spectra of spinach chloroplasts from 550 mμ
to 800 mμ. A comparison of three measuring techniques.
All measurements were made on the same chloroplast sus-
pension.
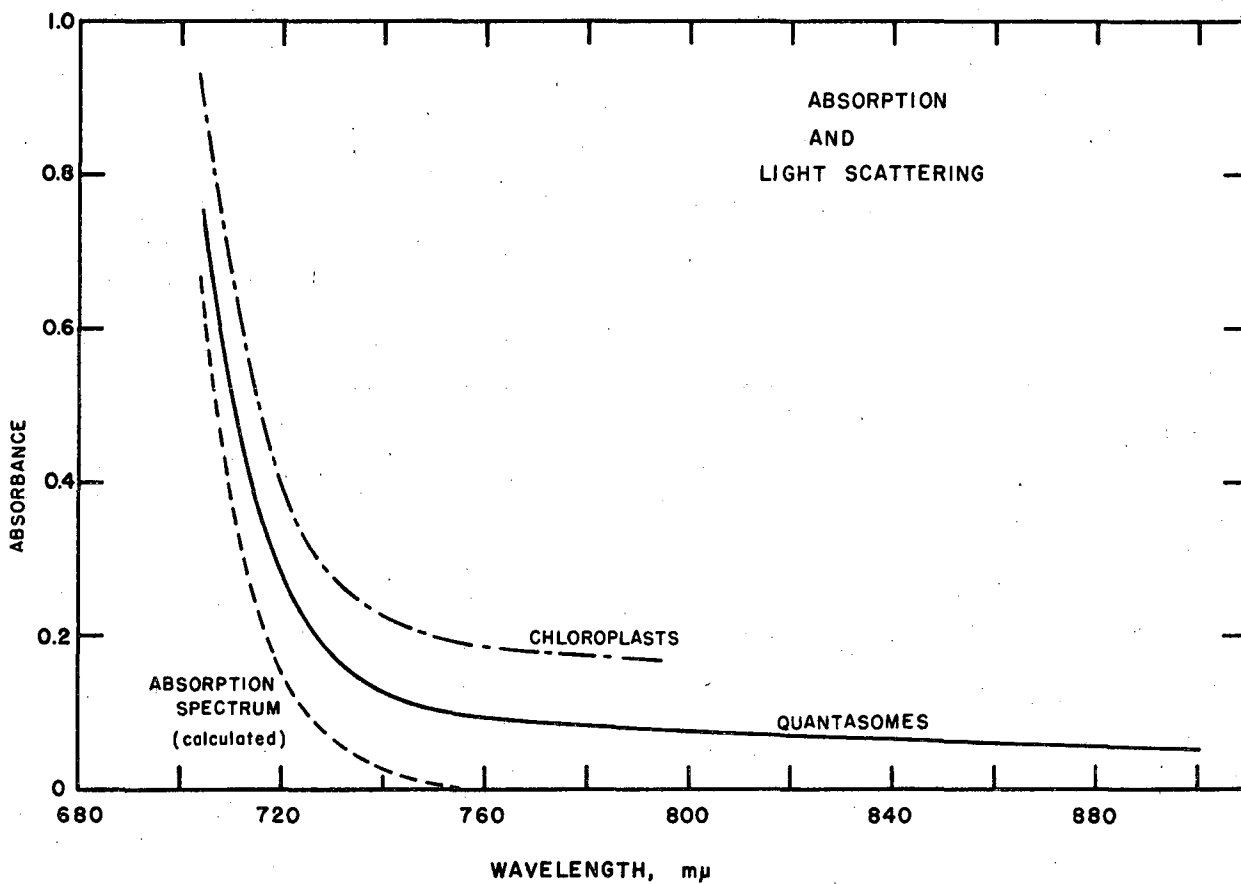
Fig. 27. Absorption spectra of spinach chloroplasts and
quantasomes at long wavelengths using Cary 14 Spectro-
photometer with Model 1462 Scattered-Transmission
Accessory and Dumont 6911 photomultiplier. Curves are
normalized to an absorbance of 10.0 at 678 mμ. Lower
curve (dashed): computed quantasome absorption corrected
for light scattering.

aggregates scatter considerably less and a correction, based on turbidity measurements from 760 mμ to 900 mμ, can be applied.

Fig. 28 shows the measured optical density of a quantasome preparation from 735 mμ to 900 mμ plotted versus $1/\lambda^4$. The relationship is linear in the region 775 mμ to 900 mμ and passes through the origin as is expected for light scattering by small particles. This line was extrapolated to shorter wavelengths and the contribution due to this Rayleigh scattering, which is quite small, was subtracted out of the measured scattered-transmission spectrum to give a reliable true absorption spectrum as shown in Fig. 27 (lower curve).

If one assumes that the true absorption spectrum of chloroplasts is identical to that of quantasome aggregates, then it is possible to compute the true absorption of a chloroplast suspension from the corrected quantasome absorption spectrum. The validity of the assumption that the spectral characteristics of quantasome and chloroplasts are similar is discussed by Sauer and Park (1954).

Actinic illumination

i) Light from a tungsten projection lamp (G. E. DFK, 1000 W) powered with a Variac was collimated by means of a spherical mirror and passed through 4 cm of water, a Corning 1-69 infrared absorbing glass, one or more cut-off filters and an interference filter. Table 7 shows the various filter combinations used to isolate these monochromatic beams (approx. 10 mμ band widths).

ii) Alternatively, a Bausch and Lomb 500 mm grating monochromator was used instead of the arrangement of filters. However, only low light intensities were then possible.
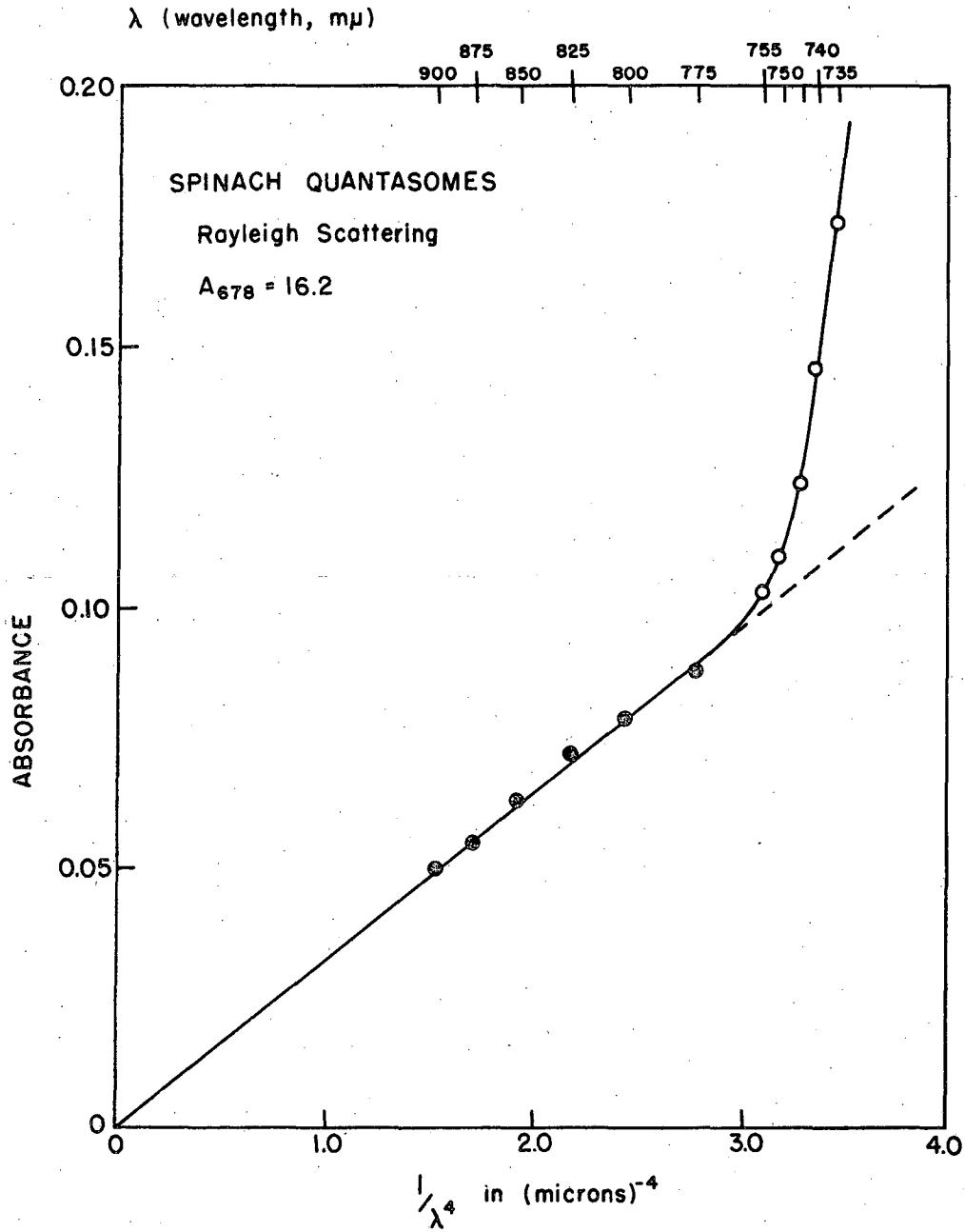
Fig. 28. Rayleigh scattering of spinach quantasomes from 760 mμ to 900 mμ. Turbidity of quantasomes versus inverse fourth power of wavelength.

Table 7

band isolated by interference filter[a] mμ	supplementary cut-off filter[b]
550	3-68
600	3-68
649	2-58
660	2-58
679	2-58
688	2-64
703	2-64 + 4-77
708	2-64 + 4-77
720	2-64 + 4-77
730	2-64 + 7-59

a. Baird Associates Type B-1.
b. Corning.

iii) For measurement of the action spectrum of ferricyanide reduction (II.E.) an interference wedge filter (Veril-200, Schott and Gen., Mainz, Germany) was used instead of the single interference filters and cut-off filters, to simultaneously provide excitation wavelengths from 592 mμ to 716 mμ. This was used in conjunction with a specially designed multi-compartment reaction vessel, to be described later.

C. NADP reduction by preparations of chloroplast lamellae

Introduction

Prior to the studies on the quantum requirement and wavelength dependence for NADP reduction by chloroplast preparation about to be described, it was necessary to select the conditions most favorable for the preparation of lamellae. It was also of interest to investigate the effect of various treatments on the membrane enzymology such as sonication and extensive aqueous washings. Park & Pon (1961) showed that lamellar fragments, consisting of up to 8 quantasomes as seen in the electron microscope, are active in quantum conversion and support the fixation of carbon dioxide. This shows that the actual integrity of the grana areas or stroma lamella as such is not essential and implies that the major structural requirement necessary is the intimate arrangement of the constituents within the photosynthetic unit.

Antimycin A is an effective inhibitor in respiratory electron transport and prevents the oxidation of cytochrome b by cytochrome c in mitochondria (Chance & Williams, 1956). Cyclic photophosphorylation in chloroplasts is insensitive to Antimycin A when vitamin K is cofactor (Arnon, et al., 1956), but when ferredoxin is cofactor, the reaction is severely impaired. This implies that cytochrome b_6 may be an intermediate in electron transfer during ferredoxin-catalyzed cyclic photophosphorylation and may be by-passed when vitamin K is cofactor. Bacterial non-cyclic photosynthetic electron transport is also sensitive to Antimycin A when succinate is electron donor, but not when ascorbate/DPIP is donor (Nozaki, et al., 1961). The effect of Antimycin A on NADP reduction by chloroplast lamellae was studied when water and reduced indophenol are electron donors.

In this study whole chloroplasts were isolated and were then washed free of the soluble stroma. The membranes were then sonicated and the sonicate clarified by differential centrifugation. The various fractions were assayed in the photochemical reduction of NADP in the presence of added chloroplast ferredoxin and ferredoxin-NADP reductase.

Procedure

Chloroplast lamellar fractions were prepared as outlined in Fig. 4 (Part I). The fractions assayed were P_1, whole chloroplasts washed with dilute buffer to remove the soluble stroma fraction; P_1-sonicated, to investigate the effect of sonication; S_2, the supernatant after the removal of large lamellae from the sonicate; and P_2, a pellet of very small fragments obtained by ultracentrifugation of S_2. The reason for sedimenting the small fragments to form the pellet P_2 was to free the lamellar fragments of any components detached from the lamellae by sonication.

The reaction mixture was 4.15×10^{-4} $\underline{M}$ - NADP, 0.04 $\underline{M}$ tris-Cl pH 8.0, 100 μg enzyme/ml and lamellae adjusted to give an absorbance of 2 at 678 mμ (1 cm path). The final volume was 5 ml. The reactions were carried out in 30-ml flat-bottomed glass vessels illuminated from below with saturating white light in an Aminco Warburg Apparatus as described in Section II.B. The NADPH$_2$ formed after 10 min was measured spectrophotometrically after removal of the lamellae by ultracentrifugation (10 min, 105,000 g, Spinco Model L ultracentrifuge). In the case of the sonicated preparations, where the particles were exceedingly small, their sedimentation was greatly facilitated by the addition of a small volume of concentrated NaCl (5 $\underline{M}$) to bring the solution ionic strength to at least 0.1 prior to ultracentrifugation. Using this technique, fraction P_2 was sedimented at 105,000 g after 10 min. (Spinco

Model L, #40 rotor).

Results

Table 8 shows the capacity of various lamellar fractions to photoreduce NADP in saturating white light. The data show that sonication of the lamellae results in about a 30% loss in photochemical activity. Removal of the large membranes and isolation of the small particles results in a preparation, P_2, which is just as active as the sonicate, indicating that large lamellar fragments are unnecessary for NADP reduction. Only the addition of ferredoxin and ferredoxin–NADP reductase is necessary.

Table 8

Photochemical activity of various chloroplast lamellar fractions

Preparation of lamellae	NADPH$_2$ formed umoles/mg Chl./hr		% activity
	plus enzyme	minus enzyme	
control P_1	65.7	3.1	100
sonicate	44.2	–	67
S_2	53.1	–	80
P_2	43.0	–	67

Table 9 shows data demonstrating the insensitivity of the NADP-reducing systems to Antimycin A.

Table 9

Effect of Antimycin A on chloroplast NADP-reducing systems

Electron donor	umoles NADPH$_2$/mg Chl./hr.	
	+ Antimycin A	–
water	62	60
ascorbate/DPIP/DCMU	59	56

Chl. A_{678} = 0.21 (fraction P_1), enzyme 100 μg/ml, NADP
4.15 x 10^{-4} $\underline{M}$, 0.05 $\underline{M}$ tris pH 8, and, when added, ascor-
bate 20 μM, DPIP 0.2 μM, DCMU, Antimycin A, 10 γ per ml.

Discussion

These results show that under conditions of white light saturation
small lamellar fragments consisting of up to 8 quantasomes are capable
of photoreducing NADP in the presence of added chloroplast ferredoxin
and ferredoxin-NADP reductase. The photoreduction of NADP is insensi-
tive to inhibition by Antimycin A in the normal system when water is
electron donor, and in the DCMU-poisoned system when ascorbate/DPIP is
electron donor.

Antimycin A is a powerful inhibitor of respiratory electron trans-
port and prevents the oxidation of cytochrome b by cytochrome c (Chance
and Williams, 1956). Bacterial photophosphorylation and higher plant
ferredoxin-catalyzed cyclic photophosphorylation (Tagawa, et al., 1963)
are both Antimycin A sensitive, but the cyclic photophosphorylations
catalyzed by FMN, PMS or vitamin K are insensitive. This suggests the
participation of a b-type cytochrome in ferredoxin-catalyzed cyclic
photophosphorylation but not in the non-cyclic transfer of electrons
from water to ferredoxin with NADP as terminal acceptor (see Section
II.F. for full discussion).

The results obtained for the photochemical activity of the small
lamellar fragments confirm and extend the previous data of Park and
Pon on carbon dioxide assimilation (1961) and Hill reaction activity
(1963) by similar preparations. In the experiments described here in
this study, the lamellae were washed free of soluble stroma protein

quite rigorously prior to sonication. Park and Pon (1961) sonicated whole chloroplasts and then isolated a high 'g' fraction of lamellar fragments that would fix carbon dioxide only in the presence of the final supernatant which contained the stroma proteins and other co-factors. They also found (1963) that the Hill reaction with ferricyanide as oxidant was unimpaired after washing lamellae with hypotonic buffer. However, the capacity for photophosphorylation was destroyed by washing and they suggested that a coupling factor was removed during such treatment.

The results reported here in this section show that under conditions of white light saturation the photoreduction of NADP proceeds only in the presence of added ferredoxin and a flavo-enzyme, ferredoxin-NADP reductase. Ferredoxin is normally removed from the lamellae by washing with hypotonic buffer (Tagawa and Arnon, 1962), whereas the flavo-enzyme is bound more strongly (Avron and Jagendorf, 1956) and a prolonged incubation at room temperature is necessary to remove it completely. The effect of sonication in this connection is unknown, but it is possible that 9 Kc sonic rupture could remove the enzyme from the surface of the lamellae.

The results on the quantum efficiency of NADP reduction conducted under conditions of limiting light intensity, described in the next section, show that the small lamellar fragments are, at best, half as efficient as the intact membranes.

D. Action spectra and quantum yields for NADP reduction by chloroplasts and quantasomes[*]

Introduction

If the working hypothesis for photosynthetic electron transport in chloroplasts envisaged by Hill and Bendall (1960a) is correct, then the photoreduction of NADP, using water as reductant, should proceed with a minimum quantum requirement of two per electron throughout the spectral regions where photosystem II is not limiting. However, if electrons generated by system II are replaced by the ascorbate-indophenol couple (Vernon and Zaugg, 1960) and the reaction is therefore driven by photosystem I only, then the reduction of NADP should proceed with a minimum quantum requirement of one per electron transferred. Furthermore, if photosystem I is truly sensitized by chlorophyll a only, then this maximum quantum yield should be obtained where the primary light absorber in the photosynthetic unit is only chlorophyll a, i.e., at wavelengths longer than 710 mμ, where absorption by chlorophyll b is approaching zero.

For the normal reduction of NADP by chloroplasts with water as electron donor, several reports have recently appeared (Black, et al., 1963; Hoch and Martin, 1963, and Govindjee, et al., 1963) and for NADP reduction with ascorbate-indophenol as electron donor, one report (Hoch and Martin, 1963). For the normal system with water as donor, there is considerable disagreement among the results. For instance, at 700 mμ the reported quantum requirements in einsteins/equivalent NADP reduced are 14 (Black, et al., 1963), 2.9 (Hoch and Martin, 1963), and 6.3 (Govindjee, et al., 1963).

This study is a comparison of the photochemical properties of NADP

[*] Sauer and Biggins, 1964.

reduction by isolated chloroplasts when water and reduced indophenol are electron donors. It was undertaken in the hope that measurements of the quantum efficiency and wavelength dependence of the two reactions would bear pertinent information concerning the two photosystems in photosynthetic electron transport in higher plants.

Procedure

Our initial analytical method for studying the photoreduction of NADP by chloroplast preparations was similar to that of Black, et al. (1963) and Hoch and Martin (1963). Samples were illuminated for a fixed period of time, the chloroplasts removed by ultracentrifugation and the supernatant analyzed spectrophotometrically for $NADPH_2$. In our hands we found this technique very unreliable owing to the very active first-order reoxidation of $NADPH_2$. We subsequently showed that this activity was associated with our enzyme preparation and the chloroplast stroma fraction, and we could not remove it by short-term ultracentrifugation.

We overcame this difficulty by continuously assaying the photoproduction of $NADPH_2$ by spectrophotometry at 339 mμ. Using this technique, the initial velocities for the photoreduction were obtained from which quantum yields were calculated. A Cary Model 14R automatic recording spectrophotometer with a scattered transmission attachment was adapted for this purpose. Fig. 29 shows a diagram of the arrangement of the cuvettes and filters in the spectrophotometer. The beam of exciting light was directed through a hole in the side of the instrument. The interference and cut-off filters (IF_1 and CF_1) were attached to the spectrophotometer and the exciting beam was concentrated with a lens inside the compartment and directed on the side wall of the sample cuvette. An opaque barrier prevented the actinic beam from illuminating
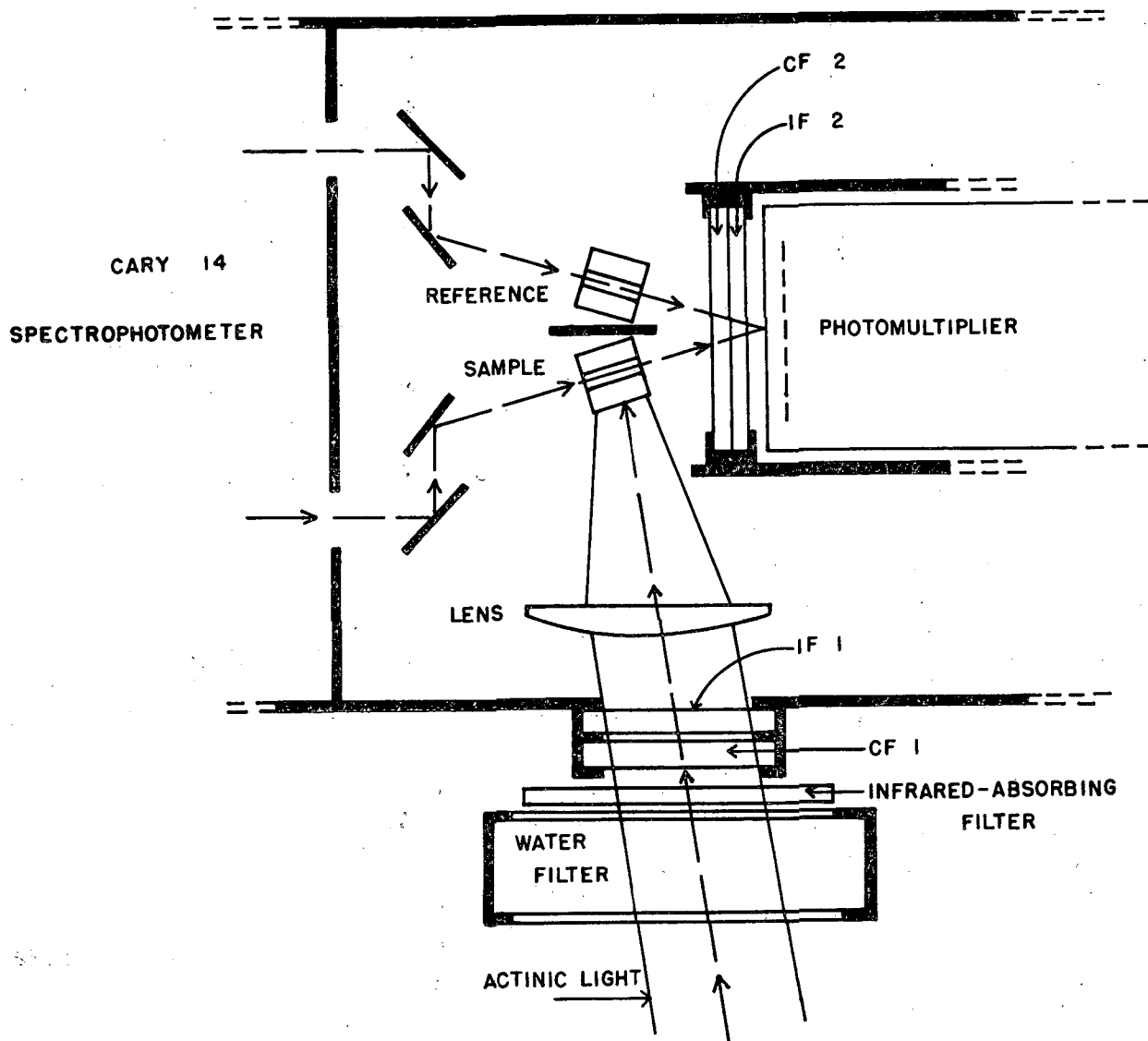
Fig. 29. Apparatus: sample compartment of the Cary Model 1462 Scattered-Transmission Accessory, showing the modifications for illumination of the sample cuvette. Filters IF 1 (or monochromator) and CF 1 select the actinic wavelengths from a tungsten lamp. Filters IF 2 and CF 2 prevent actinic light and chloroplast fluorescence from interfering with the photomultiplier.

the reference cuvette. An interference filter IF_2 (Baird Associates) and cut-off filter CF_2 (Corning, 7-60) were attached to the front of the photomultiplier (Dumont 7664) with masking tape to prevent the exciting light of longer wavelength and chlorophyll fluorescence interfering with the 339 mμ absorption measurement. The 0 to 0.2 optical density slide wire was used, and the chart speed was varied depending upon the reaction rate.

Reaction mixture

The reaction mixture contained the following in μmoles/ml: potassium phosphate pH 7.5, 50; NADP, 0.5; ADP, 1.0; $MgCl_2$, 7.5; enzyme, saturating quantities and chlorophyll, variable. In the DCMU-poisoned preparations, the above reaction mixture was used and, in addition, the following in μmoles/ml: DCMU, 0.01; Na-ascorbate, 5.0; and DPIP, 0.04. Solutions of these compounds were prepared immediately before use.

The reaction mixture (2 ml) was distributed equally between the reference and sample cuvettes. After the establishment of a stable base line in the dark, the sample cuvette was then illuminated with the actinic beam and the reaction allowed to proceed for about 8 min. Without modifying any of the instrument settings, the activity of the DCMU-poisoned preparation was studied immediately afterward (elapsed time ∿ 10 min). The activities of the two systems were studied in this way, as a function of incident intensity for wavelengths in the range 550 mμ to 730 mμ.

Course of the reaction

Fig. 30 shows an example of the time course of NADP reduction by illuminated chloroplasts, and the following features were invariably observed in these studies:
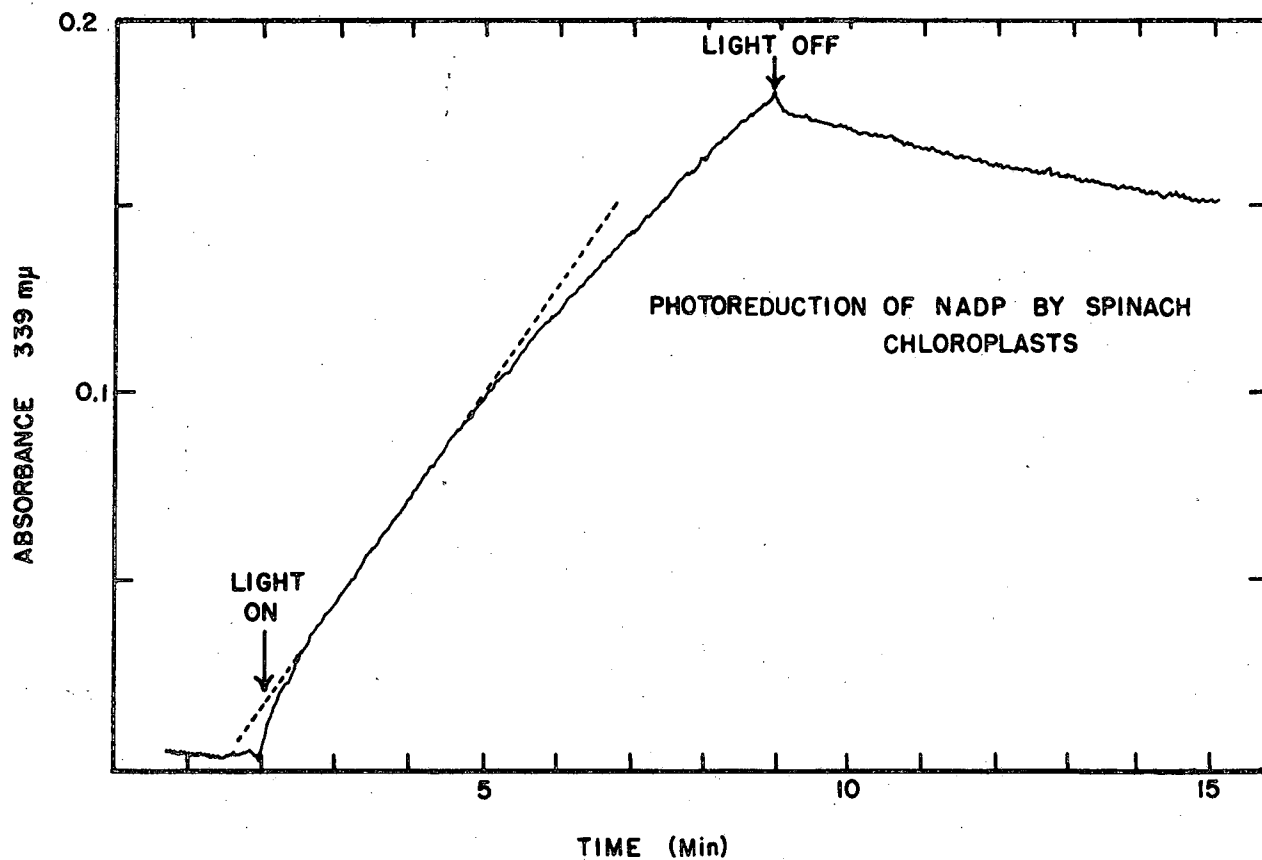
Fig. 30. A trace of the time course of the photoreduction of
NADP by isolated chloroplasts excited at 720 mµ.
(DCMU/DPIP/ascorbate system. A molar extinction co-
efficient of 6.2×10^3 1-mole^{-1}-cm^{-1} for NADPH$_2$ at 339 mµ
was used.

i) A stable level of absorbance at 339 mμ in the dark; ii) an initial rapid rise upon illumination during which about 0.2% of the NADP is reduced; iii) a period of slower but constant reduction of NADP; iv) a gradual decrease in reaction velocity after reduction of about 5% of the available NADP; v) a rapid decrease in absorbance upon cessation of illumination followed by, vi) a slower gradual decrease of absorbance. Stage ii) was not observed upon a second period of illumination even after a dark interval of several minutes. The steady initial velocity chosen for the reaction was the limiting slope of the nearly linear portion of stage iii), as shown in Fig. 30. In general, this could be determined unambiguously. After this stage, the reoxidation of the NADPH$_2$ became sufficiently great to cause a distinct decrease in the net rate of NADP reduction.

A number of samples were scanned from 300 mμ to 550 mμ before and after illumination in order to ascertain that other changes in absorbance in the system were not influencing the results. The spectra consisted of an absorption peak at 339 mμ and there was no evidence of any significant bleaching of the pigments or turbidity changes which would modify the base line.

Results

Quantum requirement and action spectrum

Quantum requirements for NADP reduction were calculated from the initial velocities and the calculated absorbed intensities. At each wavelength the incident intensity was varied over a tenfold range by adjusting the lamp voltage.

Fig. 31 and 32 show examples of the intensity dependence of the reaction. For all wavelengths studied and for both reactions, the re-
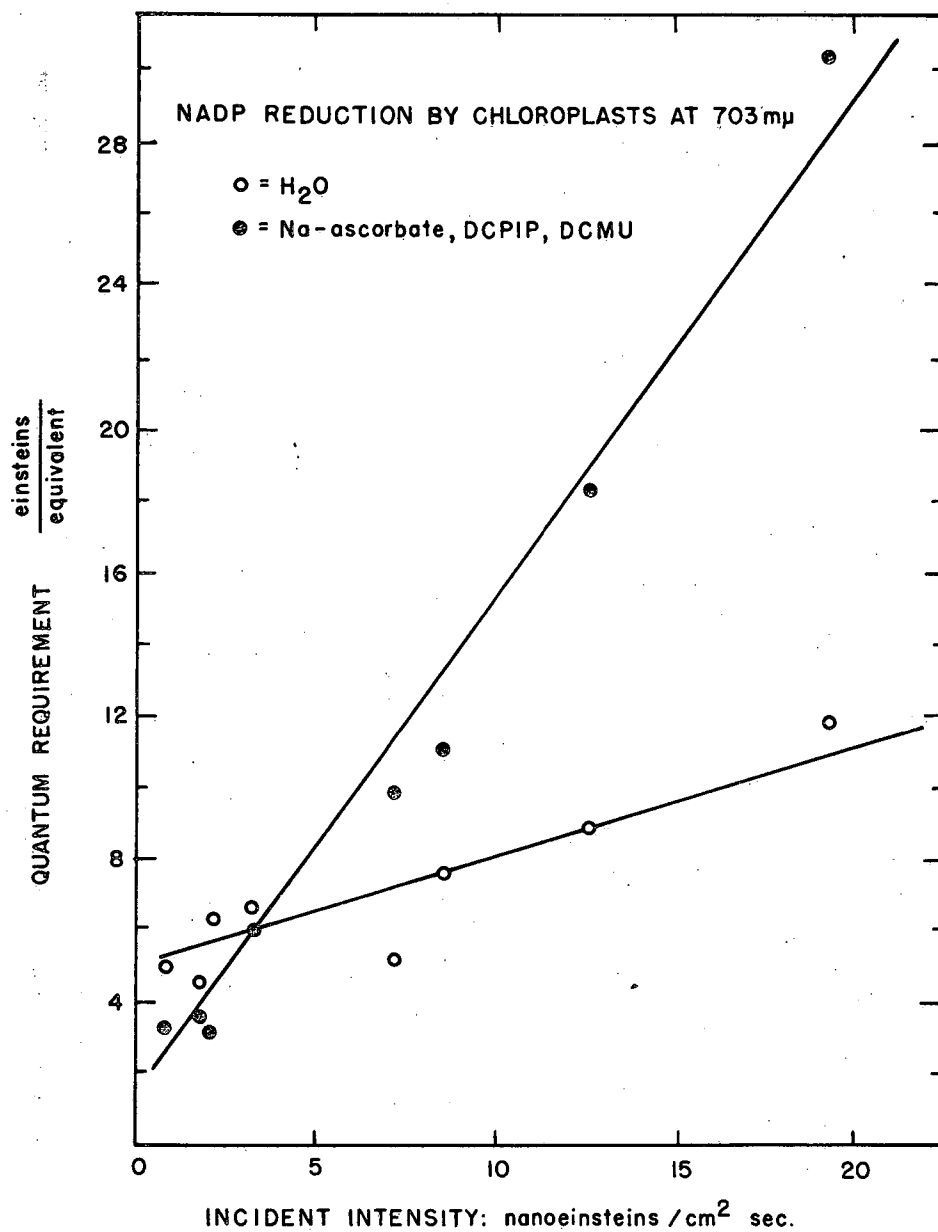
NADP REDUCTION BY CHLOROPLASTS AT 703 mμ

○ = H_2O

◉ = Na-ascorbate, DCPIP, DCMU

einsteins / equivalent

QUANTUM REQUIREMENT

INCIDENT INTENSITY: nanoeinsteins /cm^2 sec.

MUB-2805

Fig. 34. The relationship between the quantum requirement for
NADP reduction and incident intensity. ——○——, the
normal reaction with water as electron donor and ——◉——,
ascorbate/DPIP as electron donor in the DCMU-poisoned
system.

NADP REDUCTION BY CHLOROPLASTS AT 720 mµ

○ = H_2O

● = Na-ascorbate, DCPIP, DCMU

QUANTUM REQUIREMENT $\dfrac{\text{einsteins}}{\text{equivalent}}$

INCIDENT INTENSITY: nanoeinsteins / cm² sec.
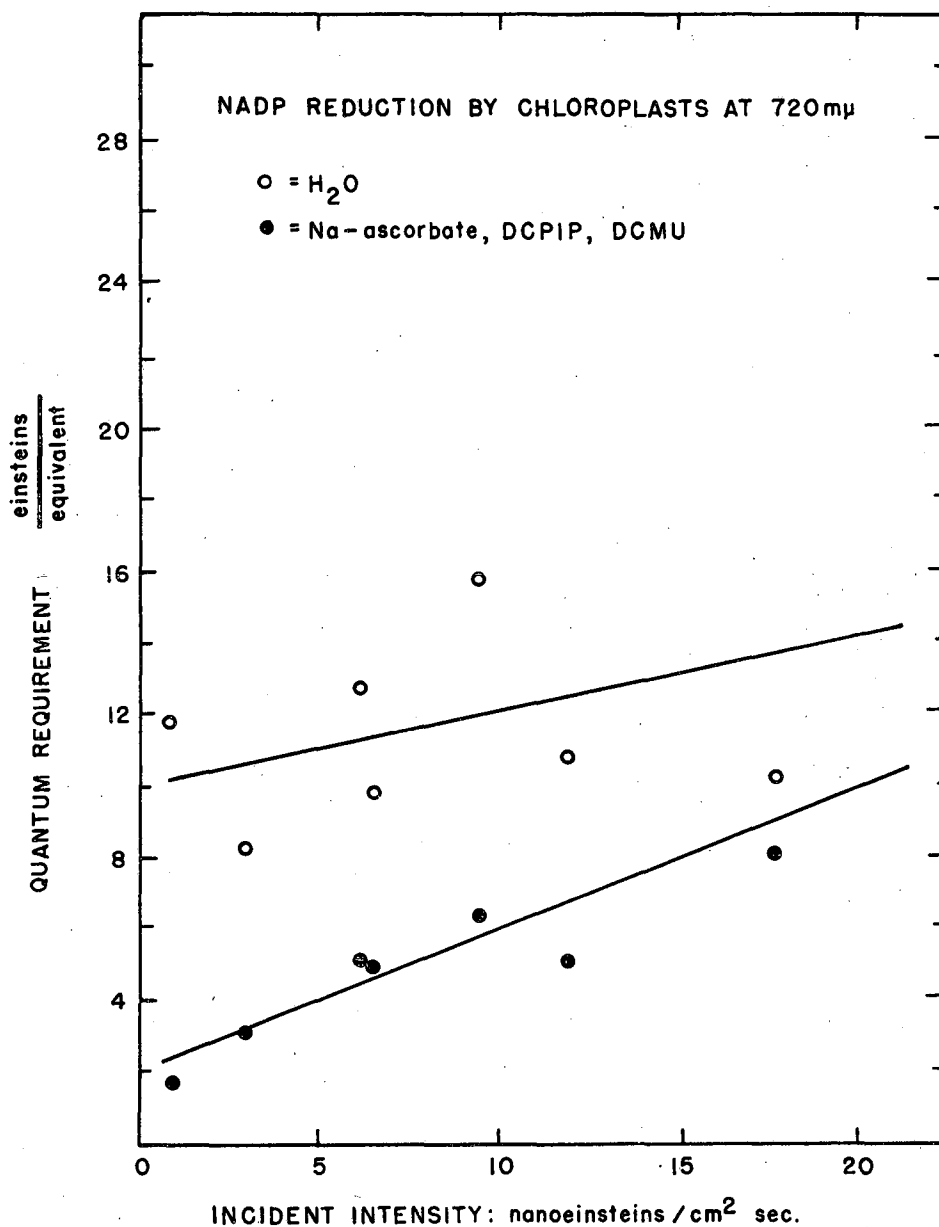
MUB-2806

Fig. 32. The relationship between the quantum requirement for
NADP reduction and incident intensity. ——○——, the nor-
mal reaction with water as electron donor and ——●——,
ascorbate/DPIP as electron donor in the DCMU-poisoned
reaction.

lationship is linear and the slope is positive. However, the relation-
ship between the dependencies of the two systems is irregular. The
zero intensity intercepts, obtained by extrapolation of the plots, are
shown as a function of exciting wavelength in the form of an action
spectrum in Fig. 33.

The normal system for electron transport, with water as electron
donor, is qualitatively very similar to that obtained for complete
photosynthesis in higher plants and algae, in that there is an increasing
requirement for quanta at wavelengths longer than 680 mμ (i.e., first
Emerson effect, or "red drop"). From 550 mμ to 680 mμ the quantum re-
quirement increases slightly but is in the order of 2 to 3 einstein per
equivalent of NADP reduced. At longer wavelengths the quantum require-
ment reaches 14 einsteins/equivalent at 730 mμ. We consider it signifi-
cant that NADP reduction by isolated chloroplasts, using water as
electron donor, does occur at wavelengths longer 720 mμ and, particularly,
with requirements of only 14 einsteins/equivalent. We checked to ascer-
tain that the 339 mμ analyzing beam was ineffective in activating the
reaction at these long wavelengths by reducing the spectrophotometer
slit to zero for a period of time during several kinetic runs. Resumption
of the 339 mμ absorption measurement by reopening the slit showed that
the course of the reaction was uninterrupted. Also, by the introduction
of additional supplementary filters, we showed that no stray light of
shorter wavelength was sensitizing the reaction.

The DCMU-poisoned system, with added DPIP and ascorbate, behaves
very much like/normal system at wavelengths from 550 mμ to 680 mμ but
slightly more inefficiently. The quantum requirement is about 3 ein-
steins/equivalent NADP reduced. At wavelengths longer than 680 mμ,
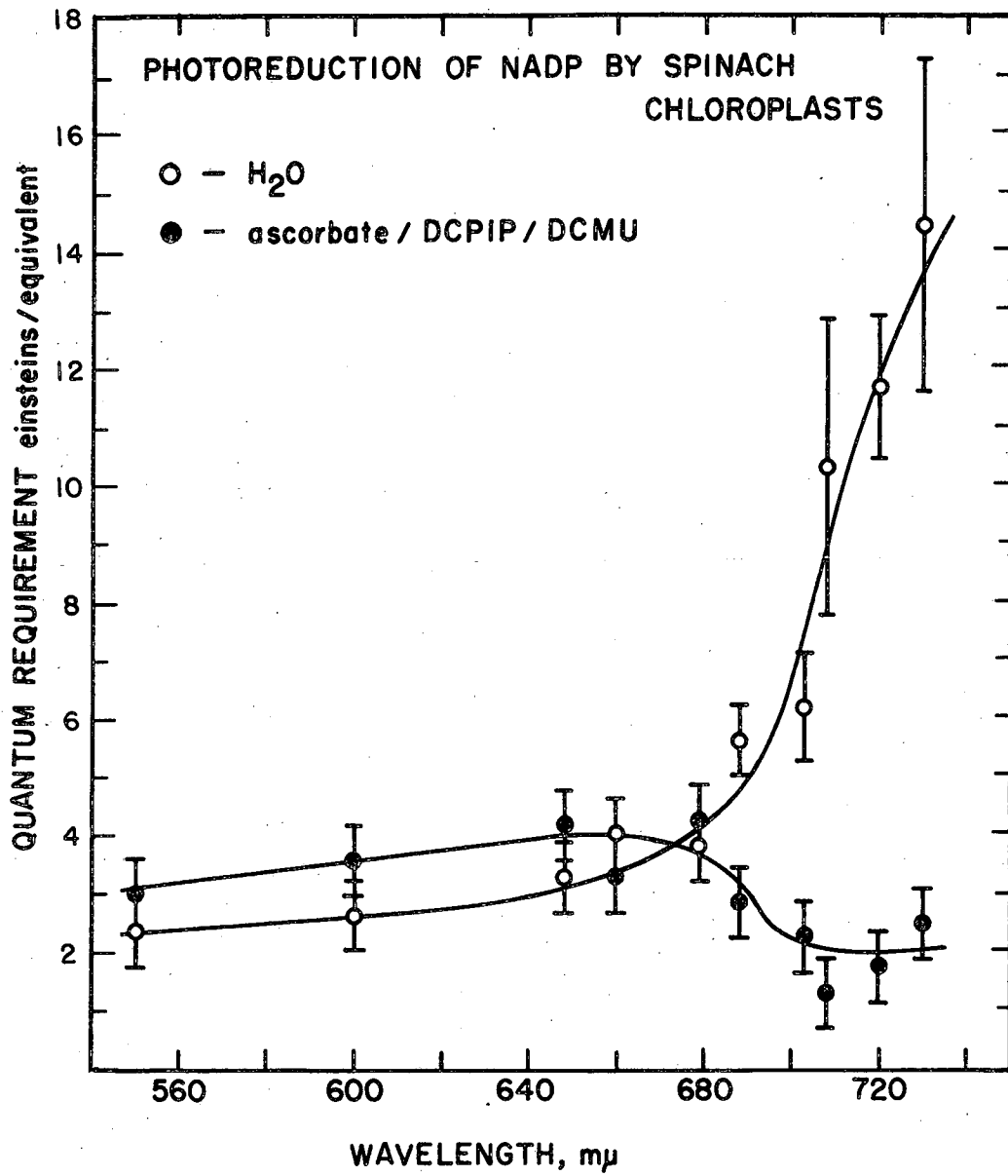
Fig. 33. · The action spectrum for NADP reduction by isolated
chloroplasts for the normal (———o———) and DCMU-
poisoned (———•———) systems. The quantum requirements
are values obtained from extrapolations to zero light intensity.

however, we found that the reaction proceeds very efficiently with quantum requirements of 1.5 einsteins/equivalent and, in general, the results were more reproducible than those for the normal system in this region of the spectrum.

Fig. 34 shows the dependence of NADP reduction on the concentration of DPIP in the reaction mixture for the DCMU-poisoned system at 680 mμ for the two incident intensities. The data show that the reaction saturates at DPIP concentrations of 0.04 μmoles/ml. This concentration was used in the reaction mixture and therefore the DPIP is not limiting in this reaction.

Discussion

Table 10 shows a comparison of the values obtained in this study for NADP reduction by isolated chloroplasts and quantasomes (Sauer and Biggins, 1964) in comparison with the literature values. For the normal system of NADP reduction with water as electron donor, our quantum requirements agree very well with those of Hoch and Martin (1963). However, our values are lower than those of Govindjee, et al. (1963) and Black, et al. (1963), particularly at 550 mμ and at wavelengths longer than 690 mμ. We attribute these discrepancies to differences in the measurement of absorbed intensities. Black, et al. (1963) used the opal glass technique for the measurement of chloroplast absorption. From the arguments outlined in Section II.B. concerning the errors introduced by inadequate corrections for scattered light, it would appear that they may have considerably overestimated the quantum requirements in regions where the relative contribution by scattering is large, i.e., at wavelengths longer than 700 mμ and the region of minimum absorption in the green. However, qualitatively the agreement is fairly good in that the
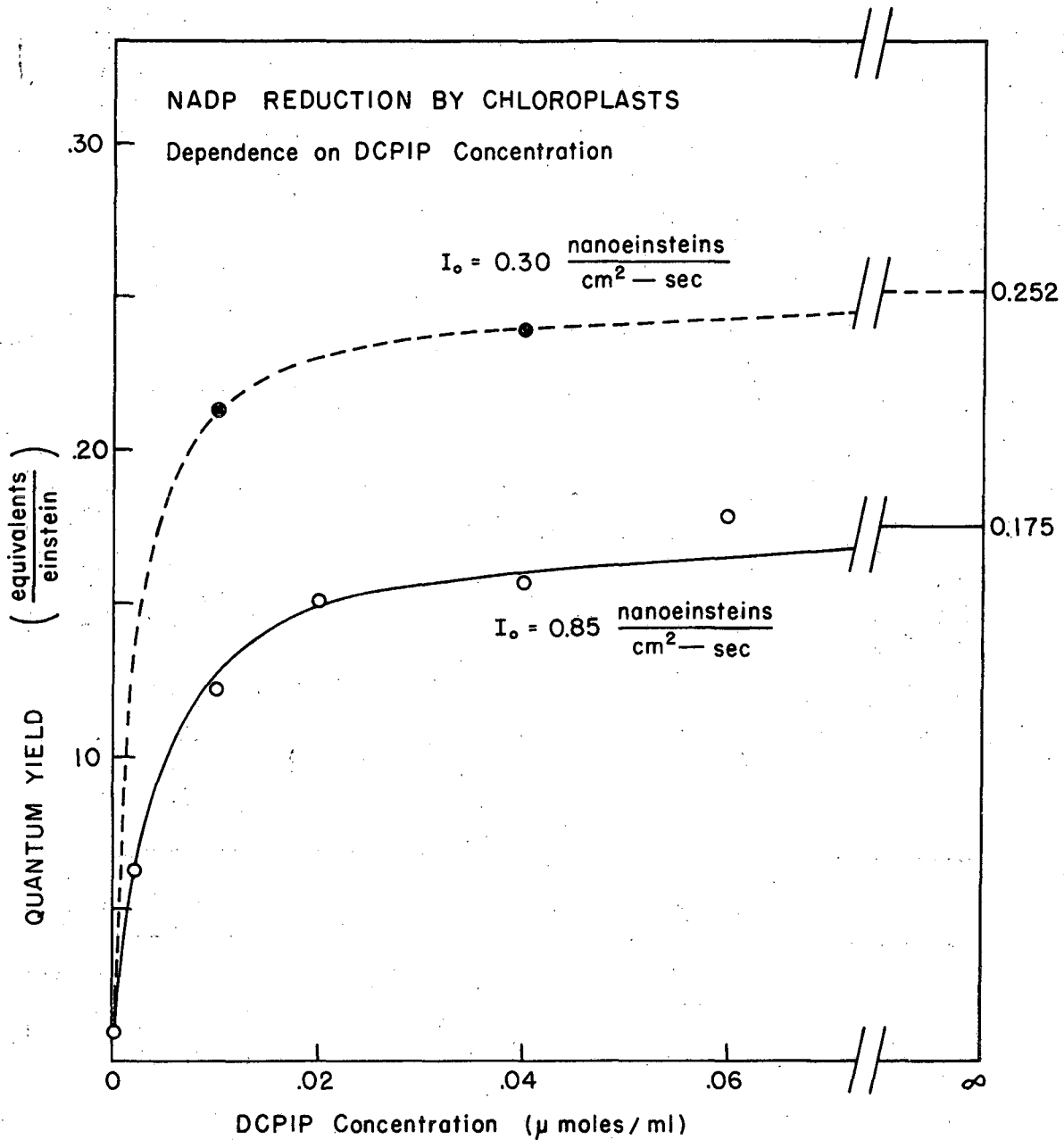
Fig. 34. The effect of the concentration of dichlorophenolindophenol on the quantum yield of NADP reduction by chloroplasts at 678 mμ. The standard reaction mixture was used with sufficient chloroplasts to give an absorbance of 0.8 at 678 mμ (1 cm path). Incident light intensities used were 0.30 (———●———) and 0.85 (———○———) nanoeinsteins/cm^2-sec.

Table 10

Quantum requirement for NADP reduction by spinach chloroplasts at wavelengths from 550 to 740 mμ—comparison with literature values. Q_o is the zero intensity quantum requirement in einsteins/equivalent at wavelength λ (mμ).

Normal System (H_2O Donor)

Black, Fewson, Gibbs & Gordon (1963)		Hoch & Martin (1963)			Govindjee, Govindjee & Hoch (1963)		This study (Sauer & Biggins) (1964)	
λ	Q_o	λ	Q_o		λ	Q_o	λ	Q_o
			Chlorophyll					
			10 μg/ml	50 μg/ml				
550	5.0						550	2.40
600	4.6						600	2.62
650	4.2	649	2.0				649	3.30
660	3.9	668	2.4				660	4.05
675	3.8	678	2.3		678	4.1	679	3.75
690	4.3	693	2.0	3.0	693	4.0	688	5.62
700	14.0	700	2.9	3.3	700	6.3	703	6.15
		710	6.2	5.5	714	10.0	708	4.60
		720		9.1	721	9.8	720	11.80
		730		8.7	730	12.5	730	14.40
		740		8.3	740	17.0		

DCMU-poisoned System (Ascorbate/DPIP Donor)

Hoch & Martin (1963)			This study (Sauer & Biggins) (1964)		
λ	Q_o		λ	Q_o	Q_o
	Chlorophyll			Chloroplasts	Quantasomes
	10 μg/ml	50 μg/ml			
			550	3.00	
			600	3.60	
649	3.7		649	4.20	9.3
668	4.3		660	3.30	
678	4.8		679	4.20	9.3
693	2.0	4.8	688	2.85	
700	1.8	2.7	703	2.25	6.0
710	1.6	2.1	708	1.35	
720		2.4	720	1.80	6.0
730		2.5	730	2.55	
740		3.1			

long wavelength decline in quantum efficiency appears to start at about 680 mμ.

For the DCMU-poisoned system, with ascorbate/DPIP as electron donor, our results agree with those of Hoch and Martin (1963). They used an integrating sphere spectrophotometer for the measurement of their chloroplast absorbances, which markedly reduces the effect of scattering at long wavelengths. However, although they were aware of the dark reoxidation of NADPH$_2$, it is not apparent from their report that a correction for it was applied. This factor could be expected to contribute to the discrepancies which remain between their data and ours for both systems studied. In particular, these differences appear to be greatest for the DCMU-poisoned system with added ascorbate/DPIP, where we find the reoxidation to be more pronounced.

The values for the normal system obtained by Govindjee, et al. (1963) are substantially greater throughout the long wavelength region. This discrepancy is puzzling since their measurements were carried out using the same techniques and reaction mixture as were those reported by Hoch and Martin (1963)!

At wavelengths shorter than 680 mμ our measurements for both the normal and DCMU-poisoned systems indicate a quantum requirement of about 3 einsteins/equivalent and not significantly wavelength dependent. Hoch, Martin and Black, et al. also report little change in quantum requirements from 648 mμ to 678 mμ. The relative quantum requirement obtained by Arnon, et al. (1961) for the DCMU-poisoned system with added ascorbate/DPIP shows a maximum at 661 mμ and lower values at longer and shorter wavelengths. This behavior is different from our study and the other previous studies. Arnon, et al. did not extrapolate their results to zero light intensity, and, in the absence of sufficient experimental

details in their report, the other possible reasons for the discrepancies cannot be discussed.

The quantum efficiencies obtained for NADP reduction by small aggregates of quantasomes (Fraction P_2, Fig. 4) are surprisingly high. In the DCMU-poisoned system with added ascorbate/DPIP, the quantum efficiencies are some 50% those of the unsonicated lamellae. However, under conditions of white light saturation (see Section II.C.) only 30% of the activity appears to be lost. This is possibly due merely to the conditions of light saturation and differences in experimental design.

The fact that the small fragments retain such a high activity, particularly after such drastic physical treatment, such as washing and sonication, reinforces our assumption that the fragments are physiologically representative of intact lamellae and, therefore, that their absorption spectrum is also representative of that of the true absorption spectrum.

The results obtained in this study for the reduction of NADP by chloroplasts with water or reduced indophenol as electron donors support, in part, the working hypothesis for photosynthetic electron transport formulated by Hill and Bendall (1960a) and adopted by Witt, et al. (1961a) Duysens, et al. (1961), and Kok and Hoch (1961). Our results can be accounted for in the framework of this two-pigment system mechanism by assuming that, at wavelengths longer than 700 mμ, pigment system I accounts for approximately 80% of the total absorption and pigment system II accounts for 20%. At wavelengths shorter than 680 mμ, each pigment system absorbs about 50% of the incident radiation. Assuming that electronic excitation energy cannot be transferred from one pigment to another, one can, therefore, conclude the following: For the normal

system with water as electron donor and driven by systems I and II, then at short wavelengths (i.e., <680 mμ) an <u>optimum</u> quantum require-ment of two einsteins/equivalent NADPH$_2$ would be obtained, since both systems are activated at equal rates; at longer wavelengths (i.e., >680 mμ) the quantum requirement will increase fourfold since the low absorbance of pigment system II at these long wavelengths limits the overall efficiency with which electrons can be obtained from water; for the DCMU-poisoned system, with added ascorbate/DPIP and sensitized by system I only, a quantum requirement of 2 would be obtained at short wavelengths since only half the absorbed quanta are trapped by system I; and at longer wavelengths, where system I becomes the major light ab-sorber, the quantum requirement should approach unity. These conclu-sions are very nearly verified by experiment in this study, and, if the hypotheses are correct, they lead to the observation that at very low light intensities the reactions observed here are operating at near optimum values.

The quantum requirement for NADP reduction by chloroplasts with either water or reduced indophenol as electron donor increases with increasing light intensity. The relationship is linear and is observed at all wavelengths studied; however, the slopes of the plots of quantum requirement versus light intensity for both systems appear to be inde-pendent of wavelength.

This behavior has been reported previously by Hoch and Martin (1963) and similarly for the ferricyanide-medidated Hill reaction by Lumry, et al. (1957) and Rieske, et al. (1959). Lumry and Rieske (1959) pos-tulated several mechanisms for the interpretation of these results, and the following is one of the simpler ones also proposed by Kok and Busin-

ger (1956), which appears to be sufficient to explain our observations:

$$C \xrightarrow{\phi \ I} C^{*} \qquad\qquad 1)$$

$$C^{*} \xrightarrow{(1-p)} C \qquad\qquad 2)$$

$$C^{*} + T \xrightarrow{p} C + T^{*} \qquad\qquad 3)$$

$$T^{*} + \text{reactants (R)} \xrightarrow{k'_1} T + \text{products (P)} \qquad\qquad 4)$$
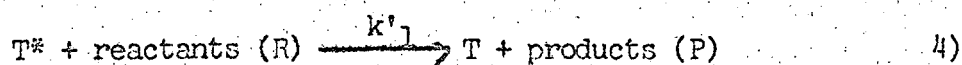
Here, light intensity I absorbed by pigment molecules C leads to excitation to reactive species C^{*} with a primary quantum efficiency ϕ (equation 1). At very low intensities, some fraction p of C^{*} transfers its excitation to a trapping site or reactive intermediate, T (equation 3). The remainder of C^{*} is deactivated according to equation 2, with an intrinsic probability $(1-p)$ and will not lead to the formation of products. The excited trapping sites, T^{*}, once formed, lead to the conversion of reactants, R (such as ferredoxin and water or reduced indophenol), to products, P (reduced ferredoxin and oxygen or oxidized indophenol), by a series of reactions summarized in equation 4. At high light intensities the steady-state concentration of the traps, T, will be reduced from the value T_o present in the dark or at very low (limiting light intensities, and the probability for the reaction described by equation 3 will decrease from p to $p(T/T_o)$.

Using the steady-state approximation for the concentration of the excited species C^{*} and T^{*}, one readily obtains for the reaction velocity the result

$$v = \frac{k_1 \ p \ \phi \ T_o \ I}{k_1 \ T_o + p \ \phi \ I} \qquad\qquad 5)$$

where $v = \dfrac{dP}{dt} = k'_1 \ RT^{*} \doteq k_1 \ T^{*}$. The pseudo first-order rate constant,

k_1, will be constant only during the early stages of the reaction before the concentration of reactants has decreased appreciably. The quantum requirement, Q, is then given by

$$Q = \frac{1}{V} = \frac{1}{p\phi} + \frac{I}{k_1 T_o} = Q_o + \frac{I}{k_D} \qquad \qquad 6)$$

Equation 6 predicts a linear relationship between the quantum requirement and the absorbed intensity with a positive slope. The intercept, Q_o, is the intrinsic quantum requirement for the overall reaction at zero intensity, and the slope $\frac{1}{k_D}$ is a measure of the rate parameter for reaction 4.

The experimental results of the dependence of the quantum requirement of NADP reduction on incident light intensity suggest that at high intensities an increasing fraction of the energy absorbed by the pigment system is not utilized in the quantum conversion process. The simplest explanation is that an intermediate or trap is present in low concentrations and the return of this trap to its unexcited state after activation is slow at high light intensities compared with the absorption of quanta. Under these conditions, the excess absorbed quanta are presumably dissipated and a high quantum requirement for the overall process is observed.

From a calculation of the pseudo zero-order rate constant, k_D, from all the results and assuming that there is only one trap per photosynthetic unit of about 160 chlorophyll $\underline{a}$ molecules, the decay time of the traps was estimated (Sauer and Biggins, 1964). The decay time for the trap in the DCMU-poisoned system is in the order of 150 msec, and for the trap in the normal system is about 50 msec. This latter value is in good agreement with the value 30 msec for the time between successive

effective quantum absorption acts determined in intermittent illumination studies by Emerson and Arnold (1932). The rate limiting step could be due to an intermediate endogenous to the photosynthetic unit bound in the membrane, or the enzyme system responsible for NADP reduction (ferredoxin and ferredoxin-NADP reductase).

An explanation for the decrease in efficiency of the small lamellar fragments in the photoreduction of NADP in the normal system, with water as electron donor, could be the loss of some factor during the isolation of the fragments. This factor, possibly a coupler of the two light reactions, could be a redox compound involved in the electron transfer process itself (plastocyanin) or some agent functioning in a structural capacity (a metal).

E. <u>Action spectrum of ferricyanide and ferricyanide/indophenol</u>

   <u>reduction</u>*

<u>Introduction</u>

In the current scheme for photosynthetic electron transport based on the participation of two distinct photoevents, the exact points of interaction of some of the Hill oxidants with the endogenous intermediates of the quantasome appear to be in doubt. Mayne and Brown (1963) report no enhancement (second Emerson effect) for ferricyanide reduction in isolated chloroplasts, implying that only one photosystem is involved. This is in contradiction to Bishop and Wittingham (1963), who suggest that both systems I and II are necessary.**

Losada, <u>et al.</u> (1961) believe that ferricyanide interacts at the same point as ferredoxin (i.e., two photosystems), and the inclusion of catalytic amounts of indophenol dye truncates the electron transfer chain between the phosphorylation step and system II. They furthermore believe that reduced indophenol acts as an electron donor at the same point.

Recent investigations by Gromet-Elhanan and Avron (1964), Keister (1963), and Shen, <u>et al.</u> (1963) show that oxidized indophenol, in concentrations greater than 10^{-4} $\underline{M}$, uncouple photophosphorylation. Shen, <u>et al.</u> suggest that catalytic amounts of indophenol do not modify the ferricyanide-Hill reaction as implied by Losada, <u>et al.</u> (1961), and that it merely acts as an inert uncoupler.

If the reduction of ferricyanide by isolated chloroplasts is a re-

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* Biggins and Sauer, 1964

** A similar contradiction applies for the quinone-mediated Hill

reaction.

sult of the operation of photosystems I and II, and indophenol truly truncates the electron transport chain to system II only, then the two reactions should exhibit quite different wavelength dependences. This report is a study of the wavelength dependence of the ferricyanide and ferricyanide/indophenol-Hill reaction to investigate the effect of the added indophenol.

Procedure

Chloroplasts were prepared by the procedure of Arnon, et al. (1956) and washed once with 0.035 $\underline{M}$ NaCl. The reaction mixture contained 0.525 µmole potassium ferricyanide/ml in 0.05 $\underline{M}$ tris-Cl buffer pH 8.0. In the ferricyanide/DPIP system, catalytic amounts of DPIP, i.e., 0.013 µmole/ml were included. Chloroplasts were added to give a final absorption at 678 mµ of 0.225.

Reactions at 12 wavelengths were run simultaneously in a multi-compartment vessel constructed of Epon (carbon-filled). Each compartment has a 3-cm light path and is 2 cc in volume. The compartments were made by milling the block of Epon. A glass microscope slide was attached to the lower surface, and the compartments were made leak-proof by the inclusion of a Parafilm gasket between the polished surface of the block and the glass slide.

The multi-compartment cell was placed directly over and in immediate contact with an Interference wedge filter (Veril-200, Schott and Gen., Mainz, Germany) such that each compartment received light of 10 mµ band width when illuminated from below. Illumination was provided by means of a tungsten lamp (General Electric DFK, 1000 W). The light was collimated, passed through 4 cm water and an infrared absorbing glass (Corning 1-69), and a short wavelength cut-off filter (Corning 3-72).

The spectral region studied was 592 mμ to 716 mμ, and incident light intensities were in the order of 10 nanoeinsteins/cm^2/sec.

The reactions were carried out at room temperature in the experimental arrangement described above. After illumination, which was usually 10 to 20 min, the reaction mixtures were rapidly withdrawn from the compartments and ultracentrifuged to remove the chloroplast lamellae. For this purpose, a Spinco Model L ultracentrifuge was used with a #40 rotor and 2-ml adapters (#303376), and ultracentrifugation conditions were 10 min at 105,000 g. The supernatants were analyzed spectrophotometrically at 420 mμ for residual potassium ferricyanide against dark controls using a Cary Model 14 spectrophotometer.

Incident light intensities were measured at the point of sample illumination and the absorbed light intensities were calculated by the procedure described in Section II.B. A correction for light absorption by oxidized DPIP was made by consideration of the inner filter effect, e.g., Kling, et al. (1963). From the corrected absorbed intensities and the amounts of ferricyanide reduced, quantum yields were calculated.

Results

Fig. 35 shows the wavelength dependence of the quantum yields for ferricyanide reduction and ferricyanide reduction in the presence of catalytic amounts of DPIP. Each reaction has a region of maximum efficiency at 650 mμ and a decline in quantum efficiency at wavelengths longer than 670 mμ. For identical experimental conditions, both the wavelength dependence and magnitude of quantum efficiency of the two systems is similar. These similarities imply that the ferricyanide is reduced by the same photoreaction in both systems and that the catalytic amount of DPIP does not significantly modify the wavelength dependence
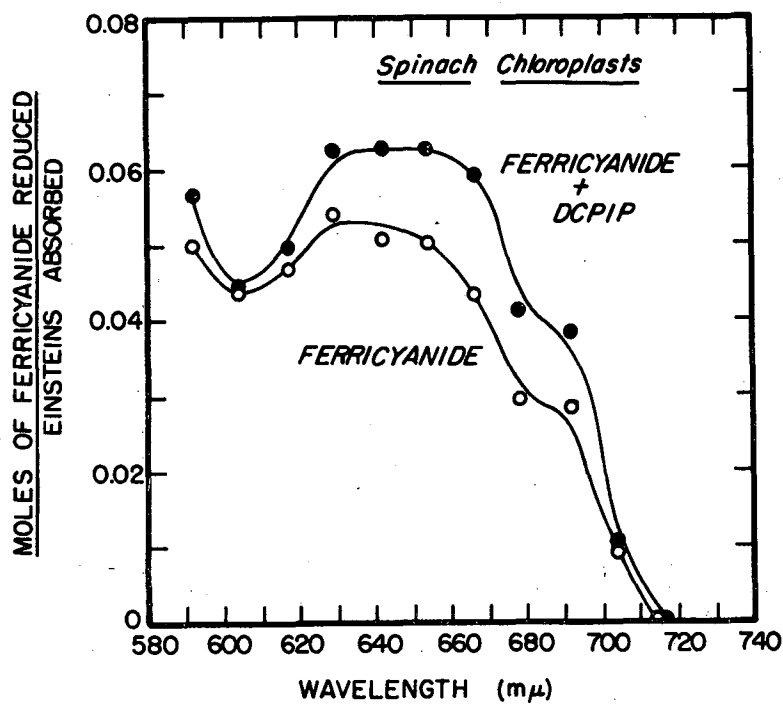
MU-33343

Fig. 35. Action spectra for the Hill reaction mediated by ferricyanide
and ferricyanide plus catalytic amounts of dichlorophenolindophenol.
Incident intensities were in the range 9-14 nanoeinsteins/cm^2/sec.
Illuminations were carried out at room temperature for 20 min.
Each reaction mixture contained sufficient washed chloroplasts to
give an absorbance of 0.225 at 678 mμ.

or photochemical efficiency of the ferricyanide reduction.

Discussion

These action spectra are characteristic of the short wavelength photosystem for photosynthesis (photosystem II). The data are qualitatively similar to data obtained by Lumry, et al. (1957), Arnon, et al. (1961), and Fork (1962) for oxygen evolution in the ferricyanide-mediated Hill reaction, and by Horio & San Pietro (1964) for ferricyanide reduction with coupled photophosphorylation. All investigators report a region of maximum efficiency at about 655 mμ.

The wavelength dependence and the magnitude of the quantum efficiencies of the ferricyanide and ferricyanide/DPIP systems are similar. These similarities imply that the two systems are sensitized by the same photoreaction and that the addition of catalytic amounts of indophenol to ferricyanide does not significantly modify the reduction of ferricyanide in the Hill reaction.

Shen, et al. (1963) showed that the ferricyanide and ferricyanide/DPIP systems have identical sensitivity to HOQNO, a powerful inhibitor of mitochondrial electron transfer, whereas the DPIP-Hill reaction is insensitive to similar concentrations of the inhibitor. Our findings in this study are consistent with their suggestion that the added indophenol is inert in the ferricyanide system. It appears, then, that the reduction of ferricyanide by isolated chloroplasts is sensitized by photosystem II in photosynthetic electron transport and includes a phosphorylation step.

Indophenol dye acts variously depending upon its concentration, oxidation state and condition of the chloroplasts. In concentrations

less than 10^{-4} $\underline{M}$, the oxidized dye acts as a terminal electron acceptor in the Hill reaction with coupled photophosphorylation. Such a reaction can be performed for an extended period of time if the very low concentration of indophenol is maintained in the oxidized condition by the inclusion of substrate amounts of an inert oxidant such as MnO_2 in the reaction mixture (Gromet-Elhanan & Avron, 1963). However, at concentrations higher than 10^{-4} $\underline{M}$, oxidized indophenol acts as an uncoupler of the phosphorylation it mediates! (Gromet-Elhanan and Avron, 1964; Keister, 1963; and Shen, et al., 1963).

Reduced indophenol is not an uncoupler of photophosphorylation and it can effectively act as an electron donor for photosystem I when NADP is the terminal electron acceptor (Vernon & Zaugg, 1960; Hoch & Martin, 1963, and this study). As the reduction of NADP with reduced indophenol as electron donor also results in a phosphorylation (Losada, et al., 1961), then the oxidized and reduced forms of indophenol must interact with different endogenous intermediates in the electron transport chain, assuming that the phosphorylation in question occurs at the same site. In addition, indophenol can act as a cofactor in a DCMU-insensitive cyclic photophosphorylation by chloroplasts (Trebst & Eck, 1961; Kok, et al., 1963).

F. Summary of Part II

Experiments concerning the photoreduction of NADP by preparations of chloroplast lamellae show that a 30% loss in activity occurs when the lamellae are sonicated. Lamellar fragments consisting of up to 8 quantasomes retain up to 50% the quantum efficiency of intact lamellae. The photoreduction of NADP was shown to be insensitive to Antimycin A.

A comparison of the absolute quantum requirement for NADP reduction by isolated chloroplasts as a function of wavelength was made between the normal system for NADP reduction with water as electron donor, and a DCMU-poisoned system with reduced indophenol as electron donor. Both reactions proceed with a quantum requirement of about three einsteins/ equivalent $NADPH_2$ from 550 mμ to 690 mμ. At longer wavelengths, the normal system is less efficient ("red drop") but the efficiency of the DCMU-poisoned system increases until the quantum requirement approaches one einstein/equivalent NADP reduced.

The quantum requirement for NADP reduction, with both water and reduced indophenol as electron donors, increases linearly with increasing light intensity. It is proposed that a photochemically generated inter- mediate, with an estimated lifetime of 50 msec in the normal system and 150 msec in the poisoned system, is responsible for the intensity dependence.

The action spectrum for the reduction of ferricyanide by lamellae in the Hill reaction suggests the participation of photosystem II only. The addition of catalytic amounts of indophenol does not significantly modify the photochemical efficiency or the wavelength dependence of the reaction, suggesting that the indophenol is inert and without effect.

G. General comments on photosynthetic electron transport

The generally accepted scheme for electron transport in higher plant photosynthesis at this time is summarized on an oxidation-reduction potential diagram in Fig. 36. The basic feature of this formulation is the participation of two separate light reactions connected in series such that the reductant formed by System II reacts through several intermediates with the oxidant formed by System I. However, it has been alternatively suggested that the two photosystems operate in parallel over the same range of potential and the products of the two reactions then dismute to form $NADPH_2$, ATP and oxygen. The results of French and Fork (1961) would tend to favor the parallel formulation. As yet, unequivocal evidence for either scheme or a possible intermediate situation has not been obtained.

Hoch (1964) doubts the series formulation from a consideration of quantum requirements. If the quantum requirement for CO_2 assimilation in vivo is assumed to be eight (see Kok, 1960 for review), then eight quanta result in the formation of $2NADPH_2$ and 2ATP, assuming the series formulation and the experimentally observed stoichiometries to be correct. The carbon reduction cycle, as is currently written (Bassham and Calvin, 1957), demands $2NADPH_2$ and 3ATP per carbon to reduce carbon dioxide to level of carbohydrate and regenerate the five carbon acceptor, ribulose 1,5-diphosphate. Hoch claims that although the additional ATP necessary may be obtained by cyclic photophosphorylation, in actual fact at least three more ATP's per carbon are necessary for overall growth of the organism and, therefore, the scheme as formulated requires revision. The validity of this particular argument can be questioned on several grounds. Assuming the energetic requirements of the carbon cycle to be true, then one hexose is generated at the expense of $12NADPH_2$ and 18ATP. However,
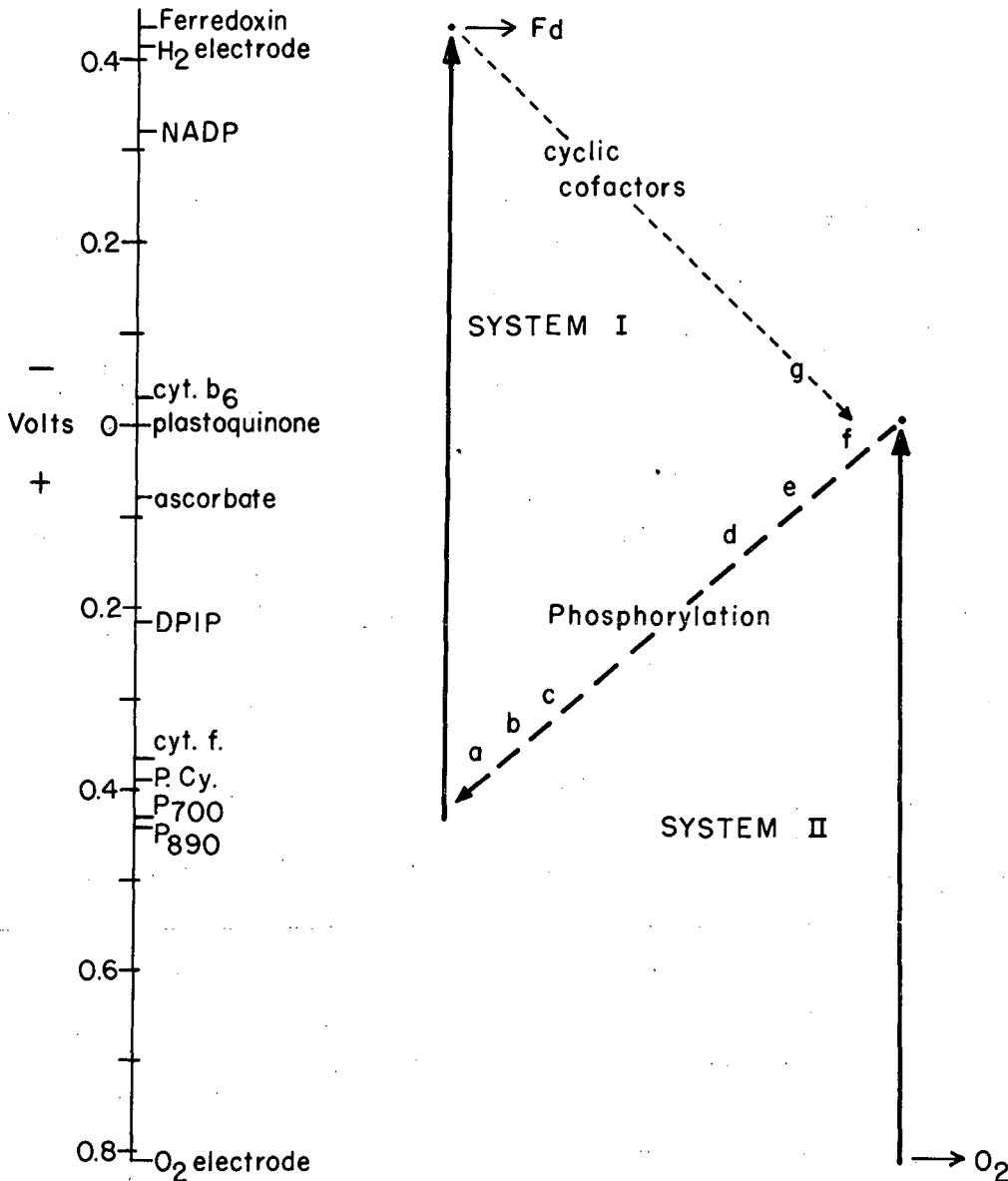
Fig. 36. Working scheme for photosynthetic electron transport in higher plants (after Hill and Bendall, 1960a). Letters a to g represent electron transfer intermediates in the photosynthetic unit. The known intermediates can be tentatively assigned as follows:

a, P_{700}; b, cytochrome f; e, plastoquinone; and g, cytochrome b_6. The points of interaction of the exogenous electron donors and Hill oxidants are as follows; d, reduced indophenol; f or e, PMS, K_3 & FMN; b or c, oxidized indophenol and ferricyanide. Antimycin A acts at g and HOQNO at b or c.

to polymerize the hexose into polysaccharide, only one ATP per hexose is required, i.e., 3-1/6 ATP per polysaccharide. The same is true for the synthesis of protein. One ATP is necessary for amino-acid activation for the formation of the s-RNA-amino-acid complex prior to incorporation into protein at the ribosome where about one GTP per peptide bond is required.

The supposition that carbon dioxide is always the ultimate oxidant during in vivo photosynthesis may also be misleading in these calculations. It is quite likely that during some of the time, the reduction of nitrate or sulfate occurs which only requires the participation of $NADPH_2$. During such circumstances any ATP formed could well be used for anabolic processes. The mid-day closure of stomata in some species of succulents, or during periods of water stress when the net uptake of carbon dioxide is thereby prevented, points to the possible variation and latitude in cellular metabolic processes during photochemical activities. Thus, there is a problem of cellular regulation involved. Furthermore, if necessary, "extra" ATP can readily be generated by high efficiency oxidative reactions, e.g., one triose when metabolized by the Krebs' cycle and oxidative electron transport in the mitochondrion, liberates in the order of 15 ATP!

Two major hypotheses concerning the interaction and regulation of the two photosystems have been proposed by Myers and Graham (1963) and are termed "spillover" and "separate package". The spillover hypothesis maintains that short wavelength quanta are absorbed by the pigments and energy transfer occurs within the photosynthetic unit. However, if the System II trap is excited, short wave quanta can migrate and excite Sys-

tem I since energy transfer can occur from short to long wavelengths. This mechanism would tend to maintain a fairly constant energy yield throughout the spectrum but a decrease occurs at long wavelengths owing to insufficient absorption by the accessory pigments. The separate package proposal is very similar except that the entire pigment compliment is separated equally into two groups. Energy transfer can occur within each of the two groups (Systems I and II) but the transfer of energy between them does not occur owing to a geometric or some other type of restriction. In this hypothesis, the overall yield is only maximal where the fractional absorption and yield for the two photosystems are equal. These two hypotheses represent limiting cases and it is possible that the overall process may depend upon some intermediate situation.

The data of Myers and Graham (1963) obtained for enhancement in Chlorella are consistent with the spillover hypothesis with the modification that the spillover mechanism itself operates with limited efficiency, thus resulting in yields intermediate between the predictions of the two limiting cases. Our own data in this study (Sauer and Biggins, 1964) favor the separate package hypothesis. In the quantum requirement determinations for NADP reduction in the DCMU-poisoned system with added ascorbate/DPIP (System I), we observed that the short wavelength requirement was double the requirement observed at long wavelengths (see Fig. 33). If energy transfer from System II to System I occurs as postulated in the spillover hypothesis, then one would predict much lower quantum requirements for System I in short wave light.

Bannister and Vrooman (1964) conducted a very thorough investigation of enhancement in vivo in Chlorella and concluded that their data could

be explained in terms of either hypothesis. Hence, as yet, there is no unequivocal evidence in support of either mechanism in the distribution of light quanta collected by the two component systems.

The relative positions of the known physiological electron transport components and points of interaction of some of the Hill reaction oxidants with the electron transfer chain can now be assigned with some degree of certainty. The primary oxidants of photosystems I and II are still unknown. However, chloroplast ferredoxin, a protein containing sulfur and non-heme iron, and related to a class of proteins found in hydrogenase-containing non-photosynthetic bacteria, is the first identifiable electron carrier to become reduced by photosystem I. Ferredoxin then acts as an electron donor in the reduction of NADP by ferredoxin-NADP reductase, a flavoenzyme recently crystallized by Shen, et al. (1963). The enzyme is specific for NADP. However, the enzyme also catalyzes the oxidation of $NADPH_2$, not only by the reverse process by ferredoxin, but also by a wide range of oxidants including NAD, FMN, FAD, indophenol dyes and menadione. Thus it has the properties of the FAD-containing $NADPH_2$-diaphorase of Avron and Jagendorf (1956) and the transhydrogenase of Keister, et al. (1960). Avron and Jagendorf found that the flavoenzyme is bound to the lamellae quite strongly and can only be removed by incubation of the lamellae at room temperature. The effect of sonication in this connection is unknown. Chloroplast ferredoxin (Tagawa and Arnon, 1962) is the same protein as photosynthetic pyridine nucleotide reductase (San Pietro & Lang, 1958) and the methemoglobin reducing factor (Davenport, et al., 1952). These reactions can be summarized as follows:

$$H_2 \xrightarrow{\text{Hydrogenase}} \underset{\underset{\text{System I}}{\uparrow}}{\overset{\overset{\text{methemo-}}{\underset{\text{globin, } O_2}{\uparrow}}}{\text{Ferredoxin}}} \underset{\text{reductase}}{\overset{\text{Fd-NADP}}{\underset{\longleftarrow}{\longrightarrow}}} \text{NADP} \xrightarrow[\text{reductase}]{\text{Fd-NADP}} \text{NAD, FMN, DPIP}$$

P_{700} appears to be the direct reductant of photosystem I (position a, Fig. 35) and, from the kinetic experiments of Witt, et al. (1961) and Chance and Bonner (1963), cytochrome f reduces P_{700} and therefore acts at position "b" in the scheme represented in Fig. 36. Evidence for the direct oxidant of photosystem II is still lacking, but many investigators have postulated plastoquinone as a likely candidate. Investigation of the reactions residual in chloroplasts after extraction of plastoquinone and restoration of activities after the addition of the pure compound back suggest that plastoquinone is unnecessary for NADP reduction when reduced indophenol is electron donor. However, it is mandatory in the ferricyanide-mediated Hill reaction with water as donor (Arnon and Horton, 1963) and for cyclic photophosphorylation catalyzed by PMS, vitamin K_3 and FMN (Whatley and Horton, 1963). These data suggest that plastoquinone is at position "e" and that electrons from reduced indophenol interact at "d", whereas those from PMS, vitamin K_3 and FMN interact at "f" or with the quinone directly.

From the accumulation of data on the photoreduction of ferricyanide and indophenol dyes, it can be concluded that in the Hill reaction they interact with the electron transfer chain subsequent to the phosphorylation step and are photoreduced at System II. Hence they probably interact with an endogenous intermediate at position "c" or with cytochrome f directly (position "b"). The ferricyanide and DPIP reductions are dissimilar in their sensitivity toward HOQNO, which suggests that

they either interact with different intermediates in the photosynthetic unit or their mode of binding is different.

Many redox compounds have been shown to be suitable as cofactors in the re-cycling of photosystem I, i.e., cyclic photophosphorylation. PMS, FMN, vitamin K_3 and DPIP are all effective and recently Tagawa, et al. (1963) showed ferredoxin to be a cofactor. A feature of the ferredoxin-catalyzed cyclic photophosphorylation not found in other cyclic photophosphorylations is its sensitivity to Antimycin A. This sensitivity is similar to the endogenous cyclic photophosphorylation observed in some bacteria and implies the participation of cytochrome b_6 in this mechanism. Evidently this site of Antimycin A inhibition is by-passed when PMS, etc. are used as cofactors. Furthermore, the results in the studies described here suggest that the site of Antimy- cin A inhibition is also by-passed in the overall transfer of electrons from water to NADP, suggesting that cytochrome b_6 is not an intermediate. Reference to the working scheme presented in Fig. 36 would suggest that "g" is the site of Antimycin A inhibition (or cytochrome b_6) and this is normally by-passed when cofactors such as PMS are included. Tagawa, et al. (1963) showed that the ferredoxin-catalyzed cyclic photophos- phorylation occurs readily in long wave light (708 mμ) and is inhibited when short wave light is supplied. However, short wave light is effective is System II is inhibited by the addition of DCMU. This indicates that excitation of System II reduces the normal acceptor for reduced ferredoxin in the cyclic process. Evidence for the par- ticipation of cyclic photophosphorylation in vivo has been provided by Forti & Parisi (1963).

As far as the mechanism for oxygen evolution is concerned, very little is known other than the necessity for manganese and chloride. The apparatus for oxygen evolution or some part of System II appears to be far more sensitive to denaturation than System I. For instance, System I resists heating at 55° C for 10 min, incubation at 25° C for 24 hours, 0.5% digitonin extraction, 60% acetone extraction, and partial lipase digestion, whereas System II is rendered completely ineffective by even less drastic treatment. This indicates a far more stringent structural requirement for System II.

<u>Concluding remarks concerning structure and function in photosynthesis</u>

The lamellae of chloroplasts are unique double unit membranes modified for quantum conversion in the photosynthetic process. From the amount of experimental evidence now accumulated, it appears possible that the quantasome, a repeating unit of lamellae, is a morphological expression of the physiological photosynthetic unit which contains 200 to 300 chlorophyll molecules. Unequivocal evidence concerning the validity of this notion would be the isolation of a photosynthetically active sub-unit of quantasome size which is further indivisible. With the development of large polymer beads such as polyacrylamide, for the exclusion chromatography of viruses and sub-cellular particles, the isolation of a homogeneous fraction of quantasome from a polydisperse preparation is now technically feasible. The development of more subtle methods of membrane fragmentation would be an additional benefit to this problem.

The lamellar protein fraction is still largely uncharacterized in terms of the exact nature of the constituent molecular species. From the studies reported here it seems that the species are of approximately the same molecular size, i.e., MW 22,000 and about 40 Å diameter. At the moment only three species have been identified; the two cytochromes and a large quantity of structural protein. It is possible that the mechanism for oxygen evolution requires the participation of one or more proteins with which manganese is presumably associated. A profitable experimental approach to the separation of the very insoluble protein fraction would be the use of cholate, deoxycholate and digitonin, as these detergents appear to denature less than dodecyl sulfate.

An end-terminal amino acid analysis of the protein fraction would be helpful in the estimation of the total number of polypeptides present.

The use of specific enzymes as an aid in the fragmentation of lamellae has not yet been fully exploited. Particularly useful enzymes would be those isolated from greening tissues where assembly of the photosynthetic apparatus takes place at a high rate. The use of these, e.g., chlorophyllase, specific proteases and lipases etc., would be of great value in the systematic degradation of intact lamellae particularly in conjunction with electron microscopy and photochemical activity measurements. The mechanism of oxygen evolution remains an unsolved problem in photosynthesis and the use of a protease for the mild degradation of lamellar protein fraction would be of considerable value in the investigation of the state of the manganese. If a manganese peptide could be isolated from such a proteolytic digest, the probability of identification and characterization of the manganese state would be considerably greater.

As far as future research in lamellar structure is concerned, it appears that owing to the high level of analytical sensitivity now reached in these problems, a far more stringent control of the physiological condition of the starting biological material is necessary. The minimum condition to be immediately strived for should be the level of physiological homogeneity of bacterial and algal populations now available.

Our knowledge of higher plant photosynthetic electron transport has increased tremendously owing to the biochemical advances which have permitted a study of the process extracellularly. In combination with investigations on intact organisms it seems clear now that the process is driven by two light acts sensitized by two pigment systems. However,

the relation between the two light acts remains unsolved. Although the "series" formulation appears to be well substantiated by a wide variety of experimental observations, the "parallel" formulation, or some intermediate situation, must still be considered.

The results obtained in this study confirm the quantum requirements obtained by the majority of workers in the field and also point to new parameters which require further investigation. These are the effect of light intensity and the distribution of absorbed quanta in the photosynthetic unit. These would most profitably be studied in connection with the problem of enhancement.

The reaction center for photosystem II, the role of accessary pigments, the primary oxidants and reductants for both light acts are unknown and knowledge concerning the nature of the exact coupling between electron transport and phosphorylation is still forthcoming.

It is anticipated that future work in photosynthesis will continue as independently defined problems of structure and function, but the findings of both approaches will, by the very nature of the process itself, tend to complement one another.

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