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Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of the Ohio State University

By

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The Ohio State University

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possibly have survived the past four years. Special thanks go to Carol, a special friend, whose kindness, understanding and friendship in the past year have been largely responsible for the maintenance of the author's sanity.
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Studies in Physical Biochemistry: Professor K. C. Aune
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ABBREVIATIONS

chl  chlorophyll
DCIP  2,6-dichlorophenolindophenol
DCMU  3(4-dichlorophenyl)-1,1-dimethylurea
DPC  diphenylcarbazide
DPCN  diphenylcarbazone
EDTA  ethylenediamine tetraacetic acid
$\Delta G^\circ$  change in standard free energy
I  inhibitor concentration
$K_d$  dissociation constant for binding of ions
$K_I$  dissociation constant for inhibitor, I
LHPP  light-harvesting pigment protein
n  number of binding sites for ions (subscripts refer to different classes of binding sites)
PS I  Photosystem I
PS II  Photosystem II
$P_{700}$  Photosystem I phototrap
r  distance
s  sedimentation coefficient
SDS  sodium dodecylsulfate
SDBS  sodium dodecylbenzenesulfonate

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INTRODUCTION

The complex network of physical and chemical interactions which collectively we define as life constitutes a nonequilibrium, entropy-decreasing process. The maintenance of such a process requires an input of energy from an outside source to prevent the system from decaying in a spontaneous, entropy-increasing manner to an equilibrium state. The ultimate source of energy for sustaining life on this planet is solar radiation or light. Before light energy can be used for the maintenance of life, it must be converted into some biologically useful form of chemical energy. This is accomplished through the process of photosynthesis.

The process of photosynthesis is carried out by a variety of procaryotes and eucaryotes. Procaryotes having photosynthetic capabilities include blue-green algae, green sulfur bacteria (Chlorobacteriaceae), purple sulfur bacteria (Thiohodaceae), and purple nonsulfur bacteria (Athiohodaceae). Photosynthetic eucaryotes include euglenoids, diatoms, red, green and brown algae, in addition to higher plants.

Defined in the most general sense, photosynthesis is the biological conversion of light energy into chemical energy. The ultimate manifestation of the photosynthetic process is the light-
dependent assimilation of carbon dioxide into organic compounds. In green plants, the energy from light is used to produce carbohydrate from carbon dioxide and water. Oxygen is given off as a byproduct. The total process can be expressed as in Equation I.

\[
2 \text{H}_2\text{O} + \text{CO}_2 \xrightarrow{\text{light, cnl}} (\text{CH}_2\text{O}) + \text{H}_2\text{O} + \text{O}_2 \quad (I)
\]

For the synthesis of one hexose molecule, Equation I must be multiplied by six. Using standard free energies of formation \(\Delta G^0\) for the reaction as written is +144 kcal./mole. The energy required to run this reaction is provided by light.

In eucaryotes, the photosynthetic process is localized in specialized organelles known as chloroplasts. These organelles consist of an outer membrane and an inner membrane system. It is the inner membrane system with which the process of photosynthesis is associated. The inner membrane contains the pigment and enzyme systems responsible for collecting light and converting it into a useful form of chemical energy. The enzymes responsible for utilizing this chemical energy to carry out the fixation of carbon dioxide are located in the stromal space (the region between the inner membrane system and the outer membrane). A schematic representation of the chloroplast is shown in Figure 1.

The major pigments of the chloroplast inner membrane system are the chlorophylls. The most important of these is chlorophyll a (chl a). This pigment is involved in the collection of
Figure 1. Schematic representation of chloroplast.
Outer Membrane

Grana Stacks

Stroma Lamellae

Stroma
light and in the phototrails involved in the conversion of light into chemical energy. Chlorophyll b (chl b) and a variety of accessory pigments including carotenoids and phycobilins serve to broaden the spectral region over which light is collected and to protect essential components from photodegradation. The pigments involved in the collection of light are collectively termed light harvesting pigments of the light harvesting apparatus. Photons arriving in the light harvesting apparatus migrate through the apparatus until a phototrap is reached. At the phototrap, the energy of the photon may be trapped by the transfer of an electron from an excited state of the phototrap pigment to an acceptor molecule. The reduced acceptor and oxidized phototrap are then restored to their original state through electron transport reactions. However, not all the light collected by the light harvesting apparatus becomes trapped in this useful manner. Photons collected by the light harvesting apparatus may be lost before the photon reaches the phototrap through fluorescence or radiationless deexcitation (heat).

As mentioned above, the reaction at the phototrap involves the separation of oxidizing and reducing equivalents. The oxidizing equivalents generated in the photosynthetic process are of sufficiently positive oxidation-reduction potential to oxidize water to oxygen and the reducing equivalents sufficiently negative potential to reduce NADP⁺ to NADPH. Thus, the process of photosynthesis involves the light-dependent transfer of electrons from water to NADP⁺.
The most widely accepted view as to how electrons are transferred from water to NADP⁺ involves two light-dependent reactions. This view of photosynthetic electron transport has become known as the "Z" scheme. Although debate is still in progress regarding some regions of the scheme, a fairly current representation of the "Z" scheme is presented in Figure 2. Two photosystems are viewed as acting in series to move electrons from water to NADP⁺. Photosystem I generates a weak oxidant and a strong reductant capable of reducing NADP⁺ in a series of dark electron transfers. Photosystem II generates a weak reductant and a strong oxidant which is involved in oxygen evolution. The weak reductant formed by PS II brings about the reduction of the weak oxidant formed by PS I. This process involves an exergonic electron transport chain between the acceptor of the PS II phototrap and the donor to the PS I phototrap. ATP production is coupled to this portion of the electron transport chain. Electron transport may also take place in a cyclic manner around PS I, also resulting in photophosphorylation of ADP to ATP. Little is known concerning which components are involved in cyclic electron transport and cyclic photophosphorylation. The ATP and NADPH produced by the photosynthetic process associated with the chloroplast membrane can then be used in the enzymatic fixation of carbon dioxide into carbohydrate.

The above introduction is meant only to give the reader of this dissertation a background view of photosynthesis. The
Figure 2. "Z" Scheme of Electron Transport. Abbreviations are as follows. PS I, Photosystem I; PS II, Photosystem II; P$_{700}$, PS I phototrap; P$_{680}$, PS II phototrap; X, primary acceptor of PS I; Q, primary acceptor of PS II; Fd, ferredoxin; fp, ferredoxin:NADP$^+$ oxidoreductase; FRS, ferredoxin reducing substance; PC, plastocyanin; cyt f, cytochrome f; PQ, plastocyanin; cyt b$_{559}$, cytochrome b$_{559}$; cyt b$_6$, cytochrome b$_6$; E'$_o$, midpoint oxidation-reduction potential at pH 7.0.
concepts discussed above are covered in a number of textbooks (1-3), reviews (4-16), and books devoted to the subject of photosynthesis (17-37). For a review covering the historical development of these concepts, the reader is referred to a recent review by Myers (38).
LITERATURE REVIEW

In order for the "Z" scheme of photosynthetic electron transport to function at its maximum efficiency, an equal number of photons must be delivered to the phototrapps of PS I and PS II, i.e., both photosystems must receive equal excitation. If one photosystem is receiving and processing a larger number of photons than the other, the photosystem receiving the fewer photons will become rate-limiting and thus lower the efficiency for the overall process. Thus one would expect quantum yields for reactions involving both photosystems to be maximal in spectral regions where both photosystems absorb an equal number of photons and to drop significantly at wavelengths where only one photosystem absorbs. However, this is not found to be the case. Quantum yields do drop at far red wavelengths (> 670 nm) where only Photosystem I absorbs. (39) They are remarkably constant and maximal, however, throughout the remainder of the visible spectrum, including regions where only Photosystem II absorbs. (40, 41) This observation has led to the view that excitation energy (absorbed photons) may be partitioned between the two photosystems. It is this problem of excitation energy distribution and the control thereof with which this dissertation is concerned.
The distribution of excitation energy will be discussed later in this review. In order to follow much of the discussion regarding this topic, it is necessary to have some understanding of the structure of the chloroplast inner membrane system and the concept of the photosynthetic unit. Hence, these two topics will be dealt with before we move on to excitation energy distribution.

Chloroplast Membrane Structure

The structure of the chloroplast inner membrane system has been the subject of a number of recent reviews. (14, 16, 42-47) Hence, rather than exhaustively review this subject here, the discussion at this point is limited to matters which are specifically related to the subject matter of this dissertation. As will be discussed in a later section, the structure of the inner membrane and changes in this structure are intimately related to changes in the distribution of excitation energy between the two photosystems.

The inner membrane system can be divided into two regions. A portion of the membrane system consists of stacks of vesicular membranes known as thylakoids. These stacks are referred to as grana stacks or thylakoid stacks. The second portion of the membrane, the stroma lamellae, interconnects region of where grana stacking occurs. These two portions of the membrane system can be separated using the French press. (48) Stroma lamellae isolated in this manner possess only PS I activity while the grana stacks show the presence of both PS I and PS II activities. (48-50) It has been postulated that the detergent, digitonin, may also
fractionate the membrane in this manner (48), although more recent evidence indicates that this type of fractionation also removes PS I activities from isolated grana. (49, 51)

The extent of grana stacking has been shown to be strongly dependent on the ionic environment of the grana stacks. Izawa and Good (52, 53) and Anderson and Vernon (54) have shown that chloroplast grana become unstacked in Tricine and that the addition of divalent cation to the medium brought about a restacking of the unstacked grana. Gross and Prasher (55) have recently shown that, in the case of spinach chloroplasts, monovalent cations also serve to unstack stacked grana before the divalent cation induced restacking could be observed. More will be said about these changes in the review section concerning their association with changes in the distribution of excitation energy between the two photosystems.

The localization of the two photosystems within the chloroplast membrane is a topic which has received much attention. This topic has been recently reviewed by Trebst. (16) The evidence available indicates that the acceptor sides of PS I and PS II are located toward the outside of the membrane while the donor side of PS I is on the inside of the membrane. Evidence for the localization of the donor side of PS II on the inside and outside of the membrane is about equal.
Arntzen has recently shown that the primary photochemistry of PS II is inhibited by iodination with lactoperoxidase indicating that the PS II phototrap may be exposed to the outside of the membrane. (56)

Freeze-fracture electron microscopy has shown two sizes of particles distributed within the chloroplast membrane. (57-59) Small (110 Å) particles are localized in the outer half of the membrane and are suggested to be markers for PS I. Larger particles (175 Å) are located in the inner half of the membrane and may serve as markers for PS II. The exact nature of these particles and the identity of the components which make up the particles is unknown.

Divalent cation may also bring about changes in the internal structure of the chloroplast membrane. In two cases, it has been observed that divalent cations bring about a rearrangement of the 175 Å particles. The 175 Å particles may be related to grana stacking since they do not appear until the onset of grana stacking in greening systems (62) and appear to be rearranged in certain mutants which do not form grana stacks. (58, 61) Gross and Packer (63) and Gross and Prasher (55) have also observed a divalent cation induced shrinkage of the membrane in a direction perpendicular to the plane of the membrane.

Divalent cations have been found to affect the fluorescence from various fluorescent probes used to study membrane structure. (64, 65) Changes in absorption flattening monitoring structural changes in the membrane are also affected by divalent cations. (66-69) In several such studies the data obtained can be explained
by either the divalent cation induced stacking or a divalent cation induced alteration of the internal structure of the inner membrane system. These two alternatives may not be mutually exclusive. A divalent cation induced change in the structure of the membrane may be required in order for the macroscopic change in stacking to occur.

The Photosynthetic Unit

An understanding of the control of excitation energy distribution between the two photosystems (and within a single photosystem) is intimately related to the concept of the photosynthetic unit. For our purposes, the photosynthetic unit can be defined as containing a reaction center or phototrap and the light-harvesting apparatus which collects photons for that reaction center. Three basic concepts exist as to the nature of the photosynthetic unit. (70, 71)

1. The Separate Package Unit (70-73): This model suggests that each reaction center has associated with it a light harvesting apparatus serving only that reaction center. No interaction (other than at the electron transport level) is allowed between either like or unlike photosynthetic units in the separate package model. Thus, all photons absorbed in a given unit must be used for photochemistry at the reaction center or dissipated as heat or fluorescence within that unit. This model predicts a linear dependence of quantum yields for photochemistry or other
dissipative mechanisms on the number of active or open reaction centers.

2. The Matrix Unit (71, 74): In this model, the light-harvesting apparatus is not strictly associated with any given reaction center. The photosynthetic unit is viewed as a matrix of light-harvesting pigments with reactions located at various points within the matrix. In such a model, the term, photosynthetic unit, takes on a statistical meaning rather than a morphological meaning. Excitation energy absorbed anywhere in the matrix may serve any reaction within the matrix, although reaction centers closer to the site of light absorption may be favored. Mathematical treatments of the matrix model predict a non-linear dependence of quantum yields on the fraction of open reaction centers.

3. Connected and Intermediate Size Units: The above two models represent two opposite and limiting viewpoints as to how the photosynthetic unit may be constructed. Intermediate viewpoints are also available and may represent a situation which is closer to reality.

a. Connected Units (70, 71, 75-78): In this model, the photosynthetic units are viewed morphologically as separate package units which are arranged in a more compact manner. As a result of this more closely packed arrangement, excitation energy absorbed in one unit may be transferred to a neighboring unit,
provided the distance between the two units is less than the critical
distance for transfer of excitation energy between chlorophyll
molecules. This critical distance is defined as the separation
between two pigment molecules where the rate of transfer between
the two molecules is equal to the rate of the other deexcitation
processes. This distance is on the order of 60-70 Å. (70, 79, 81)
This transfer of excitation energy between two (or more) units
causes the photosynthetic unit to behave in manner expected for a
matrix-type unit while maintaining its morphological identity as a
separate package-type unit.

b. Intermediate Size Units (70): This model is similar to
the connected unit model except that it allows a limited contact
between units. Such units are viewed as having two to four reaction
centers and their associated light-harvesting apparatus arranged in
a "mini-matrix".

The evidence as to the in vivo nature of the photosynthetic
unit is contradictory at best. For both PS I and PS II, evidence is
available for almost any of the above models one might choose.
(76, 77, 82-88) One explanation which seems attractive to the
author is that the exact nature of the photosynthetic unit may not be
constant but may vary with varying conditions. There may in fact
be a morphological entity which corresponds to the separate package
photosynthetic unit. From the above discussion it can be seen that
interaction of various strengths between such units could produce
any of the other models. The strength of these interactions would increase as one moved from the separate package concept to the matrix concept. The strength and nature of the interactions between units may vary considerably with varying conditions, accounting for the different conclusions reached by different workers.

Evidence supporting such a variable interaction between photosynthetic units is beginning to appear. In the case of PS II, interactions between units have been suggested based on fluorescence induction curves and flash yields for oxygen evolution. (89, 90) It has also been shown that divalent cations are required for these interactions. (91, 92) In particular, fluorescence induction curves change from exponential to sigmoidal upon addition of Mg$^{++}$. (91) Furthermore, the dependence of the relative flash yield for oxygen evolution on the fraction of open phototrails changes from linear to non-linear when Mg$^{++}$ is added. (92) These findings would suggest that in the absence of divalent cations the photosynthetic units behave as separate package units and that the addition of divalent cations brings about an interaction between these units in such a manner as to cause them to no longer act as separate package units. Similar changes have been observed during the greening of dark grown Chlorella mutant indicating that PS II units represent separate package type units early in the greening process and that an interaction between units appears during
greening. The appearance of this interaction between PS II units is related to the appearance of chlorophyll b in the membrane. (93)

**Excitation Energy Distribution**

The observations discussed previously have led to the idea that excitation energy may be partitioned in some manner between the two photosystems. As early as 1963, Myers suggested that excess excitation energy from PS II may spill over into PS I. (94, 95) By excess excitation energy, it is meant that the intensity of light preferentially utilized by PS II is high enough to effectively maintain the PS II traps in a closed state at all times. Under such conditions, PS I would be rate-limiting. The proposed spillover mechanism would serve to at least partially relieve this rate-limitation.

Results obtained by Homann (96) and Murata (97-98) concerning the effects of divalent cations on chlorophyll a fluorescence support the spillover hypothesis. At room temperature, the chlorophyll a fluorescence is primarily from PS II. (99, 100) It was observed that if divalent cations were added to chloroplasts following the addition of DCMU (a treatment that closes the PS II traps by inhibiting electron transport on the reducing side of PS II), the intensity of fluorescence was found to increase. These results were interpreted as indicating that a part of the excitation energy from PS II may spillover into PS I and that the spillover mechanism is inhibited by divalent cations. Divalent cations were also found
to affect the relative quantum yields for DCIP and NADP\(^+\) reduction
in a manner suggesting that divalent cations inhibited the spillover
process. (97) Results from low temperature\((77^\circ K)\) fluorescence
studies, where fluorescence peaks from both PS II and PS I can be
observed (99, 100), also support the spillover hypothesis and the
proposed effect of divalent cations on spillover. (97, 101)
Support for this type of divalent cation control of excitation energy
distribution has also come from Mohanty et al. (102) and Briantais
et al. (91).

The effects of divalent cations on enhancement have led
Sun and Sauer (103) to a slightly different view concerning
spillover. They suggest that divalent cations serve to promote
spillover from PS I to PS II rather than inhibit spillover from PS II
to PS I. Similar observations have been made by Sinclair. (104)
In the case of Euglena chloroplasts, Jennings and Forti (105, 106)
have observed spillover in both directions.

An alternative suggestion has been made that the role of
divalent cations is to control the fraction of excitation energy being
delivered to a particular photosystem from a light-harvesting
apparatus which serves both photosystems. This viewpoint has
been supported by Ben-Hayyim and Avron (107) and Marsho and
Kok (92) and is related to the State I-State II light conversion of
Bonaventura and Myers (108) discussed below.
Bonaventura and Myers (108) have observed changes in fluorescence and PS II photochemistry in response to changes in intensity and wavelength of light. Light preferentially exciting PS II brought about a condition they termed State II. A change to light preferentially exciting PS I brought about a conversion to a different state, State I. Their interpretation was that a change in the nature of the exciting light could trigger a change in the partitioning of excitation energy in the light-harvesting apparatus between the two photosystems. An alternative explanation has been offered suggesting that the observations are also consistent with a variation of the equilibrium constant between the two photosystems. (109) The State I-State II conversion suggested by Bonaventura and Myers (108) has recently been related to the divalent cation effects discussed above. (110) It has been suggested that State I corresponds to the situation in the presence of divalent cations and State II to the situation in the absence of divalent cations.

Other workers have suggested that divalent cations may decrease the rate constant for radiationless deexcitation in PS II (111, 112) or increase the efficiency of the PS II phototrap. (113) It has also been suggested that the light-induced changes in fluorescence may be related to extrusion of divalent cations from the membrane. (114) Studies involving fluorescence induction indicate that both the light-harvesting apparatus and the PS II phototraps may be affected by divalent cations. (115, 116)
Mathematical and computer simulations developed by Malkin (117), Seely (118, 119), and Kitijima and Butler (120, 122) may be helpful in elucidating the nature of changes in excitation energy distribution. While each of these simulations has certain attractive features, the general applicability of any one of them is necessarily limited by certain assumptions made in each case.

Divalent cation-induced structural changes have been shown to accompany the changes in fluorescence and relative quantum yields indicating that these structural changes might be responsible for the changes in excitation energy distribution. (123, 124) Such divalent cation induced structural changes have been observed by numerous authors. (52, 53, 63, 125, 126) Jennings and Forti (127) have shown that these structural changes involve protein since a brief treatment with trypsin destroyed the divalent cation-induced fluorescence changes. The fluorescence changes were also destroyed by glutaraldehyde fixation. Washing the chloroplast membranes with EDTA did not affect these changes indicating that coupling factor was not involved in the structural changes. Other authors have found that EDTA washes do suppress the divalent cation induced fluorescence changes. (102) In the same paper, Jennings and Forti questioned the correlation between the fluorescence changes and the structural changes due to an apparent difference in the concentration of divalent cation required to produce the two effects. Vander Meulan and
Govindjee (128) have also questioned this correlation on kinetic grounds. These differences in interpretation may be due to the multiple levels of divalent cation-induced structural changes of the chloroplast membrane discussed in an earlier section. At this time, a case can be made for either the divalent cation induced stacking of unstacked membranes or the divalent cation-induced alteration of the membrane internal structure being responsible for the changes in excitation energy distribution. The two levels of structural changes may be related in that the changes in the internal membrane structure may be necessary in order for the more macroscopic changes in stacking to occur.

In this laboratory, it has been shown that the divalent cation-induced changes in fluorescence and membrane structure could not be observed unless low concentrations (less than 10 mM) of monovalent cations or a zwitterionic buffer were present. (101, 129) Monovalent cations were found to decrease the fluorescence yield and brought about changes in the 77° K emission spectrum indicating that monovalent cations seemed to act in a manner opposite that of divalent cations. The addition of divalent cations after this monovalent cation-induced decrease produced increases if the fluorescence yield indicating that spillover from PS II to PS I was inhibited. The interpretation was made that, under the low salt conditions in which chloroplasts for these experiments were prepared, spillover from PS II to PS I
was already inhibited. It was suggested that monovalent cations served to promote the spillover process after which it could be inhibited by divalent cations. This laboratory has also shown that, under the initial low salt conditions, the chloroplast membranes showed grana stacking. Concentrations of monovalent cations sufficient to promote spillover from PS II to PS I resulted in unstacking of the grana. The addition of divalent cations caused a restacking of the membranes into grana stacks. It was suggested that the unstacked state corresponded to one in which spillover from PS II to PS I was promoted and the stacked state to one in which such spillover was inhibited. (129) While the work in this laboratory has been done with chloroplasts from spinach, Vander Meulan and Govindjee (128) have observed that, using chloroplasts from peas or oats, the prior addition of monovalent cations is not necessary to observe the divalent cation effects. However, the isolation of chloroplasts from peas in a low salt medium such as that used in this laboratory produces chloroplasts in which the grana are initially unstacked. (130)

The divalent cation-induced changes in fluorescence and membrane structure have been correlated with the binding of divalent cations to the chloroplast membrane. (131) Two types of binding sites for divalent cations have been found by this laboratory on the chloroplast membrane. (131, 132) Site I ($K_d = 8 \mu M$, $n = 0.65 \mu$moles Ca$^{++}$ bound/mg chlorophyll) has
been shown to be responsible for the reversal of uncoupling of photophosphorylation by quaternary ammonium salts. (132) Site II \( (K_d = 51 \mu M, n = 0.5 \) moles Ca\(^{++}\) bound/mg chlorophyll\) has been shown to be involved in the divalent cation regulation of excitation energy distribution. (131)

Recently, Prochaska and Gross (133) have shown that the binding of divalent cations to the chloroplast membrane and the associated structural and fluorescence changes can be inhibited by treating the membranes with a water-soluble carbodiimide and a nucleophile, glycine ethyl ester. This has been taken to indicate that carboxyl groups of proteins are responsible for divalent cation binding to the chloroplast membrane. Prochaska and Gross (134) have since shown that the highest specific activity for divalent cation binding is located in the PS II fraction when chloroplasts are fractionated with Triton X-100. Studies are currently underway to determine which protein components become labeled with glycine ethyl ester in the presence of carbodiimide. It is hoped that this information will allow determination of which protein components are involved in the binding of divalent cations to the chloroplast membrane.

From the above discussion, it is abundantly clear that our knowledge concerning the control of excitation energy distribution is still in its infancy. It seems generally that divalent cations and/or light quality are controlling factors and that structural
changes are in some way responsible for changes in excitation energy distribution. Many of the conceptual differences as to how excitation energy is distributed between the two photosystems may result from our lack of knowledge concerning the exact nature of the photosynthetic unit. (See the previous section.) The separate package type unit clearly cannot exist under all conditions since under certain conditions there is a transfer of excitation energy between photosynthetic units of the same and different photosystems. This does not say that there are no conditions where the separate package concept is valid. The variable interaction between photosynthetic units suggested in the previous section may be intimately related to the changes which have been attributed to variations in excitation energy distribution. It is rather interesting that divalent cations seem to be involved in changes in interactions between both like and unlike units. The majority of evidence supporting changes in excitation energy distribution can be interpreted in varying manners according to any of the previously discussed models for the photosynthetic unit. The point to be made here (and in the previous section) is that it may be self-limiting to freeze our interpretations to fit any single model of the photosynthetic unit. The better determination of the nature of the photosynthetic unit and the further study of the possible interactions between units will undoubtedly aid our understanding of how excitation energy is distributed between the two photosystems.
Conversely, a fuller elucidation of the excitation energy distribution problem may lead to the ultimate understanding of interactions between photosynthetic units.

**Pigment-Protein Complexes and Subchloroplast Particles**

We shall now turn our attention to certain submembrane proteins and particles with which this dissertation is involved. These are 1) the light harvesting pigment (chl a-b) protein, (LHPP), 2) the P700 chlorophyll a protein, and 3) a PS II subchloroplast particle prepared using Triton X-100, (TSF II).

**The Light-Harvesting Pigment Protein**

Using the anionic detergent, sodium dodecylsulfate (SDS), sodium dodecylbenzene sulfonate (SDBS), it is possible to solubilize the chloroplast membrane. (135, 139) When an SDS extract of the chloroplast membrane is subjected to electrophoresis, three chlorophyll containing bands are separated. (136-139) The fastest moving of these bands represents free pigment which has been released during the solubilization process. This pigment represents about 35% of the total chlorophyll and is probably present in the form of a pigment-detergent micelle. (139) The slowest moving band contains predominately chlorophyll a as the pigment and is thought to be characteristic of PS I. (136, 137, 138) More will be said about this band in the next subsection. The middle band contains both chlorophyll a and chlorophyll b in equimolar amounts. (136-137) Originally thought to be characteristic of
PS II, this band is now recognized to be a part of the light-harvesting apparatus and has been shown not to be involved in PS II photochemistry. (140) The pigment-protein complex constituting this band is now called the light-harvesting pigment (chl a-b) protein. For convenience, we will abbreviate this as LHPP.

The original characterization of the light-harvesting pigment protein was done by Thornber and co-workers. (137, 138) They determined that this pigment-protein complex constituted about 50% of the membrane protein and contained equimolar amounts of chlorophyll a and chlorophyll b. (137-138) They also reported an amino acid composition and estimated a molecular weight of 22,000. (138) The value of the molecular weight has since been revised upward to a value of 32,000. (139, 140) Others than chlorophylls, carotenoids were found to be associated with the protein. (138) These carotenoids were predominately xanthophylls. The pigment-protein complex was observed to have a sedimentation coefficient of 2-3 S in a detergent containing medium. (138)

The association of chlorophyll b with this pigment-protein complex led Thornber and co-workers to suggest that this protein came from PS II. (137, 138) The presence of mutants which lack this protein but still carry out PS II reactions have brought about a reconsideration of the role of this protein. In a study involving a barley mutant which lacked chlorophyll b, the protein moiety and all other components normally associated with the protein were
found to be lacking. (140) Since this mutant could still carry out efficient PS II photochemistry, it was suggested that this pigment-protein complex was associated with the light-harvesting apparatus rather than the PS II phototrap. It has also been suggested that the light-harvesting pigment protein may contain all of the chlorophyll b present in the membrane. (140, 141) Evidence supporting this has been obtained by Genge et al. (142) in studies of mutants lacking chlorophyll b and plants grown under conditions where chlorophyll b synthesis is suppressed. It has also been shown that this protein is coded for by a nuclear gene. (143)

If lipids (including pigments) are removed from the membrane prior to solubilization with SDS, a much more complex pattern is observed showing at least 17 bands when stained for protein. (144-152) In such gels, at least three prominent bands fall into the 20-30,000 molecular weight range. (Accurate determinations of molecular weights for membrane proteins may not be obtained by SDS; polyacrylamide gel electrophoresis and such determinations should be viewed with some skepticism.) Two of these three bands have recently been shown to be related to the light-harvesting pigment protein discussed above. (153) This is of special interest in that it has been shown that mutants lacking these two bands do not form grana stacks. (147-149) These bands have thus been termed "stacking peptides" (147-149) The barley mutant lacking the light-harvesting pigment protein has also been shown to form fewer grana stacks than normal. (154)
stacking is intimately related to changes in the distribution of excitation energy between the two photosystems as suggested by Gross and Prasher (129), it is not unlikely that a protein involved in grana stacking may also be involved in the regulation of excitation energy distribution. Evidence that this is the case will be presented in this dissertation.

The **P700-Chlorophyll a Protein**

The slow-moving chlorophyll containing band on SDS gels has been suggested to arise from PS I. (136-139) This pigment protein complex has also been characterized by Thornber and coworkers. (137, 138) Original isolations of this pigment-protein complex from higher plants using SDS resulted in a complex which did not show photochemical activity. (138) Using a blue-green alga as the starting material, it was possible to obtain the protein in a photochemically active form which showed P700 (PS I phototrap) activity. (155-157) Recently, a protein complex showing P700 activity has been isolated from higher plants using Triton X-100. (158) This complex also contains cytochromes b6 and f in addition to P700. Other than the presence of photochemical activity and the presence of the cytochromes, this protein is analogous to that previously isolated using SDS.

This protein contained predominately chlorophyll a and constituted about 25% of the membrane protein. (137) The amino acid composition of this protein has also been reported. (138)
The molecular weight of this protein has been estimated at 100,000-160,000 at various times and has been suggested to be composed of subunits of 35,000 molecular weight. (138-139) Using the SDS preparation, a sedimentation coefficient of 9 S was determined. (138) The Triton preparation resulted in a larger complex having a sedimentation coefficient of 13 S. (158) The addition of SDS to the Triton preparation resulted in a loss of photochemical activity and shifted the red band of chlorophyll a to shorter wavelengths. (158)

A variety of other pigment-protein complexes have been studied by a number of authors. (159-167) We have chosen to work with the Thornber preparations for a number of reasons. First, the isolation procedure allows one to obtain a reasonable amount of material to work with in a rather short time. Second, these preparations are better characterized than some of the other preparations. Third, the presence of these two pigment-proteins can be quickly and accurately determined using SDS: polyacrylamide gel electrophoresis. At least one of the preparations (the light-harvesting pigment protein) has been shown to be related to the ability of the chloroplast membrane to form grana stacks. Other reasons for our working with these proteins will be developed in the section dealing with results.

Triton PS II Particles

Subchloroplast particles characteristic of PS II have been prepared by a variety of methods, including solubilization with
Triton X-100 (168-174), or digitonin (51, 57, 159, 167, 175-180) and French press treatment (48, 49, 181, 182) and sonication. (50, 63, 69, 181, 182) The preparation we have worked with is the Triton preparation of Vernon and coworkers. (168-174) This type of preparation is hereafter referred to as TSF II, a nomenclature suggested by Vernon et al. (170)

The TSF II particle shows a chlorophyll a/chlorophyll b ratio of 2.0. (170) This particle is photochemically active in that it can carry out the light-dependent reduction of DCIP using DPC as an electron donor. (183) This reaction is characteristic of PS II and can be observed in chloroplasts in which electron donation from water to PS II has been blocked. (184-186) Cytochrome b559 has been found to be associated with particles prepared in this manner. (169, 173)

The TSF II particle contains the PS II phototrap and a portion of the light-harvesting apparatus. (171) A smaller particle can be prepared from the TSF II particle by passage through a Bio-Glass column. (171) This smaller particle is called TSF IIa since the only chlorophyll it contains is chlorophyll a. (171, 173, 175) The TSF IIa particle has many of the properties which one would expect from a PS II phototrap. (171, 173, 187) The chlorophyll b present in the TSF II preparation is suggested to be associated with the light-harvesting apparatus rather than the phototrap. (171) SDS gel electrophoresis of the TSF II particle shows an enrichment of peptides in the 20,000-30,000 molecular weight range. (150, 188)
These proteins have been previously related to the light-harvesting pigment protein. (See the previous section.) The TSF-II particle has also been shown to be enriched in the ability to bind divalent cations. (188) The presence of salts has been shown to be required for the preparation of PS II particles by either detergent methods (189) or by sonication (50) The requirement for salts in order to isolate subchloroplast particles may be due to an ion-induced alteration in the structural arrangement of the chloroplast membrane.

Summary

In this literature review, an attempt has been made to relate a number of areas in which photosynthetic research is rapidly progressing. None of the areas reviewed can be by any means considered completely solved. As pointed out in the section dealing with excitation energy distribution, progress in any one of these areas may lead to rapid progress in any one of the other areas. From this review of the literature, the following statements can be made.

1) There is a large amount of evidence that excitation energy can be partitioned in some manner between the two photosystems.

2) Factors such as light and the ionic composition of the medium serve to regulate the distribution of excitation energy between the two photosystems.

3) The changes in excitation energy distribution are accompanied by a change in the structure of the chloroplast
membrane. These structural changes may involve changes in grana stacking and/or changes in the internal structure of the chloroplast membrane.

4) Changes in interactions between photosynthetic units have been suggested. In some cases, these variable interactions have been related to the presence or absence of divalent cations in the medium. The extent to which such interactions between photosynthetic units is related to changes in excitation energy distribution remains to be clarified.

5) If grana stacking is intimately related to changes in excitation energy distribution, then components involved in the stacking process may also be involved in the regulation of excitation energy distribution. One such component is the light-harvesting pigment protein which has been related to a protein fraction required for grana stacking.

6) Considerable study is needed concerning the possibility and nature of interactions between components related to PS I, PS II, or the light-harvesting apparatus. These studies need to involve both the interactions between the various components which occur in the membrane and the interactions occurring between the individual components in isolated form. Studies involving isolated components may help to elucidate not only the interactions between various components but also the constraints placed upon the interactions by the localisation of the components in a membrane system.
Statement of Problem

In light of the previous literature review, we have studied the light-harvesting pigment protein hoping to elucidate its possible involvement in the control of excitation energy distribution. We have asked the following questions.

1) What evidence can be obtained indicating that the light-harvesting pigment protein is involved in the regulation of excitation energy distribution?

2) Does the light-harvesting pigment protein bind divalent cations with binding parameters similar to those found on the chloroplast membrane?

3) Does the binding of divalent cations to the light-harvesting pigment protein bring about any interaction between protein molecules?

4) Does the protein interact with itself in the absence of divalent cations?

5) What effect do the interactions mentioned in 4) and 5) have on the absorption and fluorescence properties of the chlorophylls associated with the light-harvesting pigment protein?

6) Can interactions between the light-harvesting pigment protein and either PS I or PS II be detected using components isolated from the chloroplast membrane?

7) How might such interactions be involved in the regulation of excitation energy distribution or interactions between
photosynthetic units in the intact chloroplast membrane system?

While this dissertation does not provide definitive answers to all of the above questions (work on questions 6 and 7 is only in the preliminary stages), it does provide a starting point for future studies of interactions which may be involved in the regulation of excitation energy distribution. Furthermore, studies of this type may give information concerning the interactions between various components which give rise to a stable and functional membrane. Although studies of this nature may be filled with pitfalls due to differences in the environment between a given component in its isolated form and its native form in an intact membrane system, they may yield valuable information concerning limitations and constraints placed upon these components by placing them in a membrane system.
EXPERIMENTAL METHODS

Preparation of Washed Chloroplast Lamellae

Unless otherwise stated, washed chloroplast lamellae were prepared according to the method of Gross (190). Fresh spinach was used as the source except in the case of the developmental studies done in collaboration with Dr. C. J. Arntzen's laboratory. For those experiments, pea seedlings were used.

The preparation of washed chloroplast lamellae consisted of grinding the leaves (washed in deionized water and devined) in a Waring blender in 50 mM Tris-Cl, pH 7.6-350 mM sucrose for about 20 seconds. The resulting brei was filtered through four layers of cheesecloth and centrifuged at 3000 x g for 10 minutes. The green pellet was then resuspended in 100 mM sucrose and recentrifuged at 12,000 x g for 15 minutes. The pelleted material was then resuspended in 100 mM sucrose and filtered through two layers of Kimwipes to remove any large particles. The resulting preparation was diluted with 100 mM sucrose to a final concentration near 1 mg chlorophyll/ml.

Preparation of SDS Extract of Washed Chloroplast Lamellae

Washed chloroplast lamellae prepared according to the method of Gross (190) were centrifuged at 17,300 x g for 15
minutes. The pelleted chloroplasts were resuspended in 50 mM Tris-Cl, pH 8.2 containing 1.0% SDS. The volume for resuspension was equal to the volume of the supernatant from which the chloroplasts were pelleted. The solution was stirred for 30-40 minutes at room temperature and then centrifuged at 17,300 x g for 15 minutes to remove any unsolubilized material. The pelleted material, consisting mostly of starch, was discarded. The SDS extract (supernatant) was used as the starting material for the preparation of the light-harvesting pigment protein (LHPP).

Preparation of LHPP

The light-harvesting pigment protein (LHPP) was prepared by a variation of the method of Kung and Thornber. (139) Washed chloroplast lamellae prepared according to the method of Gross (190) were used to prepare an SDS extract as described above.

Two 3 ml aliquots of the SDS extract were added to two centrifuge tubes containing 1 gram hydroxylapatite (Bio-gel HTP) and 22 ml 10 mM P_i, pH 7.0. After stirring briefly to form a slurry, the tubes were centrifuged for 5 minutes at 3000 x g. The supernatant was poured off and 25 ml of 10 mM P_i, pH 7.0 added to each tube and the contents stirred to reform the slurry. Centrifugation was then repeated as described for the original slurry. This washing procedure was repeated five times with 10 mM P_i, pH 7.0 and two times with 0.1 M P_i, pH 7.0. The 10 mM P_i washes remove unabsorbed and loosely absorbed pigment and protein and the major portion of the SDS.
The slurry was then washed with 0.3 M Pi, pH 7.0 until the green material eluted in this buffer was no longer in the supernatants. The green supernatants from this series of washes were pooled and labeled HA-I. The slurry was then washed with 0.4 M Pi, pH 7.0 containing 1.0 mM MgCl₂ and 0.05% SDS. The green supernatants from this series of washes were pooled and labeled HA-II.

Saturated ammonium sulfate was added to the HA-II to a final concentration of 35% and the solution centrifuged at 31,000 x g for 25 minutes. The pelleted green material was then resuspended in 50 mM Tris-Cl, pH 8.2 and dialyzed extensively against the same buffer.

After dialysis, the solution was centrifuged at 17,300 x g for 15 minutes to remove any material which precipitated during the dialysis. The LHPP was then stored at 4°C in the dark. Prior to use, the LHPP was dialyzed against 10 mM Tris-Cl, pH 8.2.

This preparation is different than that of Kung and Thornber (139) in two respects. First, we have substituted a hydroxylapatite slurry for their hydroxylapatite column. Second, we have omitted the 1.0 mM MgCl₂ from the buffer which they used to resuspend the LHPP precipitated by the ammonium sulfate addition. These two alterations have shortened the time required to isolate this protein and have enabled us to study the effects of divalent cations on the protein.

**Ca^{++} Binding to LHPP**

The Ca^{++} binding properties of the LHPP were studied by
equilibrium dialysis. One ml samples containing 1.0-7.0 ug chlorophyll/ml in various experiments were placed in dialysis bags and equilibrated for 18 hours against 20 ml of solutions containing 5.0 mM Tris-Cl, pH 8.2 and 2-250 uM CaCl₂. A constant amount, between 0.050 and 0.075 ucuries in various experiments, of \(^{45}\text{Ca}^{++}\) was placed in each tube and cold CaCl₂ added to obtain the desired Ca\(^{++}\) concentration in each tube. Specific activities, cpm/umole Ca\(^{++}\), were calculated for each tube prior to the beginning of the dialysis. After equilibration the contents of the dialysis bags and 1 ml samples of the dialysis medium from each tube were plated on planchets and counted using a Nuclear-Chicago Gas Flow Planchet Counter. The difference between the number of counts inside and outside the dialysis bag was taken as a measure of the amount of Ca\(^{++}\) bound by the LHPP. The free Ca\(^{++}\) concentration was determined from the number of counts/minute in the dialysis medium at the end of the equilibration.

Data were treated assuming identical and independent binding sites. The equation derived using these assumptions is given in Equation II.

\[
(\text{smoles Ca}^{++} \text{ bd/mg chl})^{-1} = \frac{nK_d^{-1} (\text{Ca}^{++}) \text{ free}}{1 + K_d^{-1} (\text{Ca}^{++}) \text{ free}}
\]

(II)

The derivation of this equation can be found in a number of sources. (191-193) We have plotted the data we have obtained in double reciprocal form according to Equation III.
In this equation, \( n \) is the number of binding sites and \( K_d \) is the dissociation constant for binding to these sites. Plotting \( \frac{1}{\text{pmoles Ca}^{++} \text{ bound/mg chlorophyll}} \) against \( \frac{1}{\text{Ca}^{++} \text{free}} \), one can determine \( n \) and \( K_d \) from the intersections with the ordinate abcissa, respectively.

Inhibition constants for the inhibition of \( \text{Ca}^{++} \) binding by Mg and Na were determined as above except that a constant concentration of \( \text{MgCl}_2 \) (5 or 20 uM) or \( \text{NaCl} \) (1 or 2 mM) was added to each tube in addition to the \( \text{CaCl}_2 \). Inhibition constants for competitive and noncompetitive inhibition of \( \text{Ca}^{++} \) binding were determined from Equations IV and V, respectively.

\[
\left( \frac{1}{\text{pmoles Ca}^{++} \text{ bound/mg chlorophyll}} \right) = \frac{1}{n} + \frac{K_d}{n} \left( 1 + \frac{I}{K_I} \right) \left( \frac{1}{\text{Ca}^{++} \text{free}} \right) \quad (\text{IV})
\]

\[
\left( \frac{1}{\text{pmoles Ca}^{++} \text{ bound/mg chlorophyll}} \right) = \frac{1}{n} \left( 1 + \frac{I}{K_I} \right) + \frac{K_d}{n} \left( 1 + \frac{I}{K_I} \right) \left( \frac{1}{\text{Ca}^{++} \text{free}} \right) \quad (\text{V})
\]

In these equations, \( I \) is the concentration of the inhibitor and \( K_I \) is the dissociation constant for the inhibitor.

The data for the Dixon plot were obtained by determining the amount of \( \text{Ca}^{++} \) bound/mg chlorophyll as a function of \( \text{MgCl}_2 \) at two different concentrations of \( \text{CaCl}_2 \). Data were fitted according to Equation VI.
\[
\frac{1}{(\text{umoles Ca}^{++} \text{ bd/mg chl})} = \frac{1}{n} + \frac{K_d}{n} \left(\text{Ca}^{++}\right)_{\text{free}} + \frac{K_d}{n K_I} \quad \text{(VI)}
\]

\(K_I\) values were determined from plots of \(1/\text{umoles Ca}^{++} \text{ bound/mg chlorophyll}\) against \((\text{Mg}^{++})\).

**SDS Content of LHPP**

The SDS content of LHPP was determined by adding \(^{35}\text{S}-\text{SDS}\) at places in the isolation procedure where SDS was required. Specific activities in the ranges 0.02-0.04 and 0.22-0.25 ucuries/umole SDS were used. Details are presented in the results section.

**Analytical Ultracentrifugation Studies Involving LHPP**

The concentrations of LHPP with which we were dealing were not sufficient to produce Schlieren patterns in the ultracentrifuge. We have been able to carry out sedimentation velocity experiments by taking advantage of the fact that LHPP is green due to the presence of chlorophyll. By setting the analyzer angle for the optical system of the ultracentrifuge to 90°, we have been able to follow the sedimentation of LHPP by following the movement of pigment away from the meniscus as the centrifugation proceeds. Thus, we have effectively been able to use absorption optics to follow the sedimentation of LHPP by recording a moving boundary on glass photographic plates (2" x 10" Kodak metallographic plates). At the beginning of the run, the area above the meniscus is more darkly exposed than the area representing the solution.
since a portion of the light passing through the solution is absorbed by the chlorophylls on the LHPP. As the run proceeds, and the LHPP sediments, the boundary of the more darkly exposed region moves into the solution. This is due to the removal of pigmented material away from the meniscus as the LHPP sediments. By measuring the rate of movement of this boundary with time, we have been able to determine sedimentation coefficients for the LHPP under various conditions.

All runs were made in a Beckman Model E analytical ultracentrifuge using a 30 mm single sector cell. Rotor speed was 48,000 rpm for all runs. Photographs were taken on Kodak metallographic plates at 8 minute intervals. Data was plotted according to Equation VII. (194, 195)

\[
\ln \frac{r}{r_0} = \frac{2}{3} S (t - t_0)
\]

(VII)

In this equation, \( r \) is the distance of the boundary from the center of rotation at time, \( t \). Likewise, \( r_0 \) is the distance of the boundary from the center of rotation at some reference time, \( t_0 \). The reference time, \( t_0 \), was uniformly taken as the time at which the first photograph was taken. The angular velocity is represented by \( w \) and is defined as \( \frac{2 \pi (\text{rpm})}{60} \) radians/second. Under the conditions of our runs, (rpm = 48,000), \( w = 5026.5 \) radians/second and \( w^2 = 2.527 \times 10^7 \) radians²/second². The sedimentation coefficient, \( S \), is in units of seconds⁻¹. The sedimentation
coefficients have been expressed in terms of Svedberg units where
$$1 \text{S} = 1 \times 10^{-13} \text{ seconds}^{-1}.$$  

Data was plotted according to the above equation. Plots of \( \ln \left( \frac{r}{r_0} \right) \) against \((t-t_0)\) were found to be linear (see the results section) and the sedimentation coefficient determined from the slope of these plots. LHPP concentrations from 1.0 to 8.0 \(\mu g\) chlorophyll/mg were used in these studies. All runs were made in 10 mM Tris-Cl, pH 8.2. In studies where ions were added, the additions were made just prior to placing the solution in the ultracentrifugation cell. Identical results were obtained, however, if the LHPP solution was dialyzed overnight against a buffered solution containing the desired concentration of the desired ion.

**Absorption Spectrum of LHPP as a Function of LHPP Concentration**

Visible absorption spectra were recorded for concentrations of LHPP ranging from 1.0 to 7.0 \(\mu g\) chlorophyll/ml. The absorbance at 436, 464, 652, and 668 nm was plotted as a function of LHPP concentration. The linear portion of this curve observed at low LHPP concentrations was extrapolated to higher concentrations to calculate an "ideal" absorbance at any LHPP concentration. The difference between the observed absorbance and the calculated "ideal" absorbance was taken as the deviation from ideality. We have expressed this deviation from ideality as a percentage of the ideal absorbance according to Equation VIII.
\[
\text{Deviation from ideality (\%)} = \frac{A_{\text{obs}} - A_{\text{calc}}}{A_{\text{calc}}} \times 100 \quad (VIII)
\]

All spectra were recorded using an Aminco-Chance spectrophotometer in the split beam spectral mode of operation. Spectra were commonly run on the 1.0 absorbance scale. The spectra for samples having low concentrations of LHPP were also run on more expanded scales to verify the linearity observed at low LHPP concentrations.

**Fluorescence Emission and Excitation Spectra**

The chlorophyll fluorescence emission spectrum of LHPP was determined using an Amonco-Bowman spectrofluorimeter. The excitation wave length was set at 435-440 nm to irradiate chlorophyll a or 464-470 nm to irradiate chlorophyll b. The emission spectrum in each case was determined by either scanning from 600 to 720 nm or making readings of the fluorescence level at 5 nm intervals between 600 and 720 nm.

Excitation spectra for chlorophyll fluorescence from LHPP were determined in a similar manner. The emission wave length was set at 650, 660, or 670 nm and the excitation wave length varied from 400 to 550 nm.

Emission spectra obtained using concentrations of LHPP ranging from 1.0 to 7.0 ug chlorophyll/ml were used to study the effect of LHPP concentration on the chlorophyll fluorescence properties of LHPP. Excitation spectra were determined for
emission at 675 and 650 nm at each concentration. The emission at 675 nm was predominately from chlorophyll a while that at 650 nm showed a considerable contribution from chlorophyll b. The ratio of the ability of chlorophyll b to excite fluorescence at either 650 or 675 nm to the ability of chlorophyll a to excite fluorescence at either emission wave length was expressed as $F_{465} \rightarrow 650/F_{435} \rightarrow 650$ and $F_{465} \rightarrow 675/F_{435} \rightarrow 675$. These ratios were plotted as a function of LHPP concentration. The readings used in calculating these ratios were taken directly from the excitation spectra. All studies were done in 10 mM Tris-Cl, pH 8.2. The effects of ions on the fluorescence properties of LHPP was studied in the same manner with ions being added just prior to the fluorescence measurements. The effects of ions on both the emission and excitation spectra were studied. Emission spectra were most often used since one could make simultaneous measurements of the abilities of chlorophyll a and chlorophyll b to excite fluorescence at a given emission wave length. Concentration curves for the divalent cation effects on fluorescence from LHPP were also determined from excitation spectra.

**Material for Developmental Studies**

Pea seedlings for the developmental studies were grown in the laboratory of Dr. C. J. Arntzen of the University of Illinois. Seedlings were grown in the dark for one week and then transferred to an intermittent light system which alternated 2 minutes of light
with two hours of darkness for a period of two days. If ungreened plants were desired, the leaves of the seedlings were used at this time to make chloroplasts. If greening was desired, the seedlings were placed in continuous white light for the desired time, up to 48 hours, immediately upon removal from the intermittent light system.

Measurements of divalent cation effects on chlorophyll a fluorescence at room temperature and liquid nitrogen temperature as a function of greening time for plants grown in the intermittent light system were done by Mr. Paul Armond of Dr. Arntzen's laboratory at the University of Illinois. Mr. Armond also took and analyzed the electron micrographs used to follow the development of grana stacking as a function of greening time for plants grown in the intermittent light system.

**Development of Pigment-Protein Complexes During Greening of Pea Seedlings Grown in the Intermittent Light System**

Chloroplasts from plants grown in the intermittent light system and then greened for various lengths of time were prepared according to the method of Gross. (190) SDS extracts were prepared from these chloroplasts as described for the preparation of SDS extracts from which the LHPP was isolated. Samples of the SDS extracts from various greening times were subjected to SDS; polyacrylamide gel electrophoresis on gels consisting of 7.5% acrylamide-0.375% N,N'-methylenebisacrylamide. The buffer consisted of 50 mM Tris-Cl, pH 8.2-0.1% SDS. Electrophoresis
was carried out at 1.5 milliamps/tube (constant current) for two hours.

The polyacrylamide gels were prepared by mixing 9 ml of a solution of 20% acrylamide containing 1% N,N'-methylenebisacrylamide with 3 ml of water and 12 ml of 100 mM Tris-Cl, pH 8.2-0.2% SDS. While stirring, 0.05 ml of TEMED, tetramethylethylenediamine, was added. Finally, 1.0 ml of 5 mg/ml ammonium persulfate was added to start the polymerization. Aliquots of 1.9 ml of the gelling solution were rapidly pipetted into 12 capped gel electrophoresis tubes and 0.01-0.02 ml of water layered on top to assure the formation of a flat gel surface. One hour was allowed for polymerization. Gels were equilibrated electrophoretically at 150 volts for 15 minutes prior to electrophoresis of the samples.

After electrophoresis, the gels were scanned at 670 nm using a Gilford spectrophotometer equipped for gel scanning. The gel scans were traced on tracing paper and the relative area under each pigmented band determined gravimetrically using a Mettler H10 balance. This was done by cutting out the area underneath the gel scan and weighing it. The individual peaks were then cut out and weighed. The results were expressed as the percentage of the weight of the individual peak of the total weight of the area under the gel scan. This method gives us an estimate of the percentage of chlorophyll a associated with each of the three pigmented bands.
Ca++ Binding to Chloroplast Membranes Isolated During the Greening of Pea Seedlings Grown in the Intermittent Light System

Ca++ binding curves for chloroplasts from pea seedlings grown in the intermittent light system and then greened for 48 hours or left ungreened were done according to the method of Gross and Hess. (131) Chloroplasts were incubated with 30 ml of a solution containing 100 mM sucrose and enough Tris base to titrate to pH 8.0 plus 5-500 uM CaCl₂. A constant amount of 45Ca++, about 0.3 curies, was added to each tube and specific activities determined for each individual tube prior to the addition of chloroplasts. The chloroplasts and the bound Ca++ were removed from suspension by centrifugation, 12,000 x g, 15 minutes. The pelleted material was resuspended in a minimal amount of water (about 1 ml) and plated on planchets prior to counting the radioactivity using a Nuclear-Chicago Gas Flow Planchet Counter. Using the specific activity determined for each tube, the number of umoles Ca++ bound/mg chlorophyll was determined. Samples of the supernatants were also counted to determine the free Ca++ concentration. Data was plotted in double reciprocal form as described previously for the binding of Ca++ to the LHPP. Complete binding curves of this type were done for chloroplasts grown in the intermittent light system and greened for either 0 or 48 hours.

The variations in Ca++ binding to chloroplast membranes as a function of greening time for plants grown in the intermittent light system was followed by comparing the amount of Ca++
bound in a reaction mixture containing 1 mM CaCl$_2$ to the amount bound in a reaction mixture containing 20 uM CaCl$_2$. The 20 uM reaction mixture should saturate Site I while the 1 mM CaCl$_2$ reaction mixture should saturate both Site I and Site II. By setting up a ratio of the amount bound in the 1 mM reaction mixture to the amount bound in the 20 uM reaction mixture, we obtain an estimate of the quantity ($n_I + n_{II}$)/$n_I$. The amount of Ca$^{++}$ bound in each reaction mixture was determined by the method discussed above for the construction of complete binding curves.

**Preparation of Photosystem II Particles (TSF-II)**

Photosystem II particles (TSF-II) were prepared according to the method of Vernon et al. (169) Chloroplasts prepared according to the method of Gross (190) served as the starting material. These chloroplasts were incubated in a medium of 0.5 M sucrose, 0.1 M NaCl, 30 mM Tris-Cl, pH 7.6 containing Triton X-100 sufficient to obtain a Triton/chlorophyll ratio of 40. After an incubation of one hour, the solution was centrifuged 15 minutes at 10,000 x g to remove any unsolubilized material. Any pelleted material was discarded. The supernatants were then centrifuged for one hour at 144,000 x g in a Beckman L2-65B preparative ultracentrifuge at a temperature of 4° C. The pelleted material from this centrifugation (TSF-II) was resuspended in 50 mM Tris-Cl, pH 8.2 containing 0.5 M sucrose. The TSF-II prepared in this manner could be stored for at least two weeks in the dark at 4° C with no loss of activity.
Assay of TSF-II Particles

TSF-II particles were assayed using a modification of the assay developed by Vernon et al. (183) involving the photoreduction of DCIP by PS II using diphenylcarbazide (DPC) as an electron donor. The assay medium consisted of 0.5 mM DPC, 50 uM DCIP, 0.5 M sucrose, and 1 mM Tris-Cl, pH 8.2. The photoreduction of DCIP was followed using an Aminco-Chance spectrophotometer in the split beam, kinetic mode of operation. Absorbance changes due to DCIP reduction were followed at 600 nm and a millimolar extinction coefficient of 20 was used to determine the amount of DCIP reduced. Illumination was provided from a projector supplying excitation light at right angles to the measuring beam of the spectrophotometer. The blank was made up exactly as was the sample except that it was not illuminated. For most experiments, excitation was provided through a broad band red cut off filter. The intensity of illumination provided in this manner was $7.2 \times 10^4$ ergs/cm$^2$/second. In some experiments, Baird Atomic interference filters were used in place of the broad band filter. Interference filters having maximum transmissions at 650, 680, and 710 nm were used. The intensities obtained with these filters and the half band widths for the interference filters are given below.
Regardless of which filter was used to screen the excitation, a broad band blue filter was placed between the sample chamber and the photomultiplier to protect the photomultiplier from the excitation light. The rate of DCIP reduction was measured from the absorption change at 600 nm which occurred when the excitation light was turned on. These changes were followed kinetically and were linear throughout the first minute of illumination. The extent of the change after one minute of illumination was used to calculate the rates of DCIP reduction. No back reaction was observed when the illumination was terminated. TSF-II particles were added in the dark just prior to the assay. The chlorophyll concentration was between 9 and 15 ug chlorophyll/ml for all assays. The effects of ions and DCMU on the rate of DCIP photoreduction by TSF-II particles were studied in the same manner. Ions, in the form of chloride salts, or DCMU were added to the individual assay tubes from stock solutions to the concentrations desired.

Studies of Chlorophyll a Fluorescence from TSF-II Particles

The effects of ions and DCMU on the steady state level of chlorophyll a fluorescence from TSF-II particles were studied using an Aminco-Bowman spectrofluorimeter. Excitation was
provided at either 435 nm (to irradiate chlorophyll a) or at 470 nm (to irradiate chlorophyll b). Chlorophyll a fluorescence was measured at 680 nm. Fluorescence was recorded as a function of time using a Sargent-Welch recorder. All studies were done in 0.5 M sucrose, 1 mM Tris-Cl, pH 8.2. These conditions were chosen to be as close to the conditions used in the studies of the effects of ions and DCMU on the DPC-dependent reduction of DCIP by TSF-II particles discussed previously. TSF-II was added in the dark just prior to the experiments to a concentration of 3-6 μg chlorophyll/ml. After a steady state level of fluorescence was reached, ions or DCMU were added from stock solutions to the concentrations desired using Oxford samplers. Experiments were done in which ions or DCMU were added before and after the addition of DPC to close the PS II phototrails from the oxidizing side of the PS II phototrap.

Preparation of the P₇₀₀-Chlorophyll a Protein

The P₇₀₀-chlorophyll a protein was isolated according to the method of Shiozawa et al. (158) from spinach leaves. This is the only time when chloroplasts were not prepared according to the method of Gross (190). We have used the procedure of Shiozawa et al. (158) in its entirety, including the preparation of chloroplasts. Spinach leaves were washed and packed into a Waring blender and homogenized for 20-25 seconds in 200 ml of 0.5 M sucrose, 0.1 M NaCl, 20 mM sodium ascorbate, 50 mM Tris-Cl,
pH 8.2. The homogenate was filtered through four layers of cheesecloth and then centrifuged for 10 minutes at 3000 x g. The pelleted material was resuspended in 0.1 M NaCl, 1 mM EDTA, 20 mM sodium ascorbate, 50 mM Tris-Cl, pH 8.2. The resulting suspension was centrifuged at 12,000 x g for 15 minutes. The chloroplast pellet was resuspended in a solution of 20 mM sodium 50 mM Tris-Cl, pH 7.4. At this point the total chlorophyll concentration was determined. For each 25 mg of chlorophyll two ml of Triton X-100 was added and the solution stirred for 15 minutes at 4°C. The suspension was then centrifuged at 12,000 x g for 10 minutes and the supernatant applied to a hydroxylapatite column which had been previously equilibrated with 10 mM Pi, pH 7.0. The column was washed with 10 mM Pi, pH 7.0 until the eluate was colorless. A volume of 1% Triton X-100, 20 mM sodium ascorbate, 50 mM Tris-Cl, pH 7.4 equal to one and one half times the volume of the loaded material was then passed through the column, followed by another wash with 10 mM Pi, pH 7.0 until the eluate was colorless. The column was finally washed with 0.2 M Pi, pH 7.0 and the green band representing the P700-chlorophyll a protein collected. The collected material was dialyzed against 50 mM Tris-Cl, pH 8.2 (three liters, one liter/change).

Assay of P700-Chlorophyll a Protein

The P700-chlorophyll a protein was assayed for electron transport activity using the disproportionation of diphenylcarbazone
(DPCN). This assay has been shown by Shneyour and Avron (196) to be characteristic of a PS I reaction. We have used this assay because it can be carried out in a low salt medium which facilitates the study of ions on the system. Soluble factors (plastocyanin, ferredoxin, etc.) and reductants do not have to be added to the system in order for the assay to work. Our assay medium consisted of 2 mM Tris-Cl, pH 8.2 containing 1 mM DPCN. The disproportionation of DPCN was followed at 485 nm assuming a millimolar extinction coefficient of 2.7. An Aminco-Chance spectrophotometer in the split beam kinetic mode of operation was used for all measurements. Illumination was provided at right angles to the measuring beam of the spectrophotometer. The blank was made up exactly as was the sample except that it was not illuminated. Illumination was provided through either a broad red cut off filter or an interference filter having a maximum transmission at 650, 680, or 710 nm. The intensities obtained using these filters were presented in the section dealing with the DCIP reduction assay for TSF-II particles. A broad band blue filter was kept in front of the photomultiplier to protect it from the excitation light. The rate of DPCN disproportionation was followed from the absorption change at 485 nm which occurred when the excitation light was turned on. These changes were followed kinetically and were linear throughout the first minute of illumination. The extent of the absorbance change at 485 nm
after one minute of illumination was used to calculate the rate of DPCN disproportionation. The $P_{700}$-chlorophyll $a$ protein was added in the dark just prior to the assay of an individual tube. The concentration of chlorophyll was 8-10 ug chlorophyll/ml. The effects of ions of DPCN disproportionation by the TSF-II chlorophyll $a$ protein were also studied in this manner. Ions, in the form of chloride salts, were added to the individual assay tubes to the concentrations desired.

**Effects of Trypsin Treatment on Divalent Cation Induced Fluorescence Changes in Chloroplasts**

The effects of trypsin on the divalent cation induced fluorescence changes in chloroplasts were also studied using an Aminco-Bowman spectrophotometer. Chloroplasts for these studies were prepared according to the method of Gross. (190) The assay medium consisted of 100 mM sucrose, 10 uM DCMU plus 0.4 mM Tris base (enough to titrate to pH 8.0). Chloroplasts were added to a concentration of 6.7 ug chlorophyll/ml. As a control for these experiments, the extent of both the monovalent cation induced decreases in chlorophyll $a$ fluorescence and the divalent cation induced reversal of these decreases were measured. This was done by adding 5 mM NaCl after allowing the fluorescence to reach a steady state level. After the new steady state level was attained, 3.3 mM CaCl$_2$ (or MgCl$_2$) was added to reverse the monovalent cation induced fluorescence decreases. The effects of
trypsin on the monovalent and divalent cation induced fluorescence was studied by adding $2.5 \times 10^{-5}$ mg of trypsin to the chloroplasts in the assay medium prior to the addition of either NaCl or CaCl$_2$. The incubation time before the addition of salts was varied. Experiments were also done in which the trypsin was added after the addition of NaCl but before the addition of CaCl$_2$. In this case, the grana would be unstacked while in the previous case, grana would be stacked. (129)

The effects of trypsin on the structural changes accompanying the fluorescence was studied by following changes in turbidity or 180° light scattering by monitoring absorbance changes at 540 nm. This was done using an Aminco-Chance spectrophotometer in the split beam, kinetic mode of operation. The assay medium, chlorophyll concentration, and additions of trypsin and ions were the same as described for the fluorescence studies.

The effects of the trypsin treatment on the pigment protein complexes was studied by the technique described for following the development of the pigment protein complexes during the greening of pea seedlings grown under intermittent light conditions. Chloroplasts treated with trypsin were solubilized in SDS and subjected to SDS:polyacrylamide gel electrophoresis. The gels were scanned at 680 nm using a Gilford spectrophotometer equipped for gel scanning. The relative area under each of the pigmented bands was determined gravimetrically. Trypsin concentrations used in these studies were $1.0 \times 10^{-5}$ mg/ml and $2.0 \times 10^{-5}$ mg/ml.
The results obtained with trypsin treated chloroplasts were compared with results obtained with untreated chloroplasts. A comparison was also made of the effects of trypsin on the pigment protein complexes when the chloroplasts were in the stacked and unstacked state. This was done by adding the trypsin before or after unstacking by NaCl and then solubilizing in SDS.

**Difference Spectra for Extraction of Chlorophyll from LHPP by 80% Acetone**

The difference spectrum for the extraction of chlorophyll from LHPP was recorded using the Aminco-Chance spectrophotometer in the split beam spectral mode of operation. The sample cuvette contained 1 ml of LHPP (10 µg chlorophyll/ml) plus 4 ml acetone. The reference cuvette contained 1 ml of LHPP plus 4 ml of water. The baseline was determined by placing 1 ml of LHPP and 4 ml of water in both the sample and reference cuvettes. The difference spectra for the extraction of chlorophyll from the LHPP were determined for LHPP in 10 mM Tris-Cl, pH 8.2, 10 mM borate buffer, pH 8.2 and 10 mM borate buffer, pH 10.2. The concentration dependence for the acetone extraction was determined by running difference spectra as described above for the same concentration of LHPP with different acetone concentrations.

**Chlorophyll Determinations**

The chlorophyll content of the various types of materials used in this dissertation was determined by the method of
For samples containing 0.5-1.5 ug chlorophyll/ml, 0.05 ml of the sample was added to 5.0 ml of 80% acetone and centrifuged in a desk top centrifuge to remove precipitated protein. Absorbances were determined at 645 nm and 663 nm against an 80% acetone blank. The chlorophyll a and chlorophyll b content was determined from Equation IX and Equation X.

\[
\text{chlorophyll a (mg/ml)} = 1.27 (A_{663}) - 0.269 (A_{645}) \quad \text{(IX)}
\]

\[
\text{chlorophyll b (mg/ml)} = 2.29 (A_{645}) - 0.468 (A_{663}) \quad \text{(X)}
\]

For more dilute samples, 1 ml of the sample was mixed with 4 ml of acetone to achieve the 80% acetone concentration. The cofactors in Equations IX and X were adjusted accordingly.

**Protein Determinations**

Protein was determined according to a modification of the Lowry protein determination suited for insoluble proteins. (198) Chlorophyll was extracted and protein precipitated with 80% acetone. The protein pellet was resuspended and solubilized in 1 ml of 1 M NaOH. From this point the standard Lowry method was followed with the exception that no more NaOH was added. Standard curves were constructed using bovine serum albumin as a standard. While this method was found to give reproducible results with chloroplast membranes, difficulties were encountered in its application to the LHPP. This was most likely due to the low concentrations of LHPP with which we are dealing.
RESULTS AND DISCUSSION

Reasons for Studying the Light-Harvesting Pigment Protein

We chose to study the possible involvement of the light-harvesting pigment protein with regard to ion-induced changes in excitation energy distribution for a number of reasons. First, the LHPP constitutes a major portion of the chloroplast membrane protein, representing about 50% of the total lamellar protein. Due to the large number of divalent cation binding sites on the chloroplast membrane (131), a protein present in this large an amount must be considered a likely candidate for divalent cation binding sites. Second, this protein is thought to be associated with the light-harvesting apparatus (140) where control of excitation energy distribution could be most easily manipulated. A model proposed by Seely (118, 119) predicts that a change in orientation of as few as six chlorophylls in a light-harvesting apparatus of 344 chlorophylls could result in a large change in the distribution of excitation energy between the two photosystems. Third, the LHPP is the smallest unit thus far isolated from photosynthetic membranes of higher plants which still has pigment attached. The LHPP has a molecular weight of 33,000 and contains one molecule each of tightly bound chlorophyll a and chlorophyll b. (137-141)
The interactions of the chlorophyll molecules with the protein moiety and the interactions between pigment protein complexes can thus be studied using the LHPP. It is not hard to imagine that such interactions could be involved in changes in excitation energy distribution, since the efficiency of such energy transfer depends on the distance and orientation between pigment molecules. (80, 81)

The interactions of LHPP in the presence and absence of ions discussed in this dissertation could easily be responsible for changes in distance and/or orientation between pigment molecules. Recent evidence has indicated that under certain growth conditions and in a variety of mutants where chlorophyll b is absent, the entire protein moiety and all of its constituents are also missing. (142, 147-149, 153) Fourth, Prochaska and Gross (134) have shown that the highest specific activity for Ca$^{2+}$ binding is present in the TSF-II (PS II) particle when chloroplast membranes are fractionated using Triton X-100. This particle is enriched in LHPP. (188) Fifth, SDS polyacrylamide gel electrophoresis of chloroplast membranes treated with a carbodiimide plus a nucleophile, glycine ethyl ester, and then solubilized in SDS show the incorporation of the major portion of the glycine ethyl ester into the LHPP. (134) The treatment of chloroplast membranes with the carbodiimide and nucleophile has previously been shown by Prochaska and Gross (133) to inhibit Ca$^{2+}$ binding to the chloroplast membrane and the divalent cation induced structural and fluorescence changes. Finally, the
LHPP has recently been shown to be equivalent to a protein fraction which appears to be related to the ability of the chloroplast membrane to form grana stacks. (147-149, 153) If the changes in excitation energy distribution are related to changes in grana stacking as has been suggested by Gross and Prasher (129), the implication that LHPP is involved in grana stacking may also imply its role in the regulation of excitation energy distribution.

**LHPP Isolation**

We have isolated the light-harvesting pigment protein (LHPP) in a manner similar to that of Kung and Thornber. (139) The alterations we have made on their procedure consist of substituting a hydroxylapatite slurry for their hydroxylapatite column and omitting MgCl\(_2\) from the buffer they used to resuspend the pigmented material precipitated in the ammonium sulfate precipitation. Details of the isolation procedure are described in the section on experimental procedures.

We originally attempted to isolate the LHPP without these modifications and encountered two problems. First, using the column instead of the slurry, we obtained smaller amounts of material than we had hoped to obtain. This problem could be partially solved by overloading the column. The second problem encountered was that the LHPP obtained using the column was often of variable quality. In some cases, it appeared as if most of the chlorophyll a had been converted to pheophytin (had lost its Mg\(^{++}\)). This type of change is characterized by the shift of the
Soret band of chlorophyll a from 436-440 nm to the 410-420 nm region of the visible spectrum. Both of these problems could be overcome to a large extent by substituting a slurry for the hydroxylapatite column. Using such a slurry, we are able to both load more material and elute the LHPP from the slurry in a shorter time. Although we still occasionally obtain preparations which show signs of pheophytin, the occurrence of this problem is less frequent than that encountered using the column method. Preparations showing signs of pheophytin are routinely discarded. We are not sure as to why the slurry works better than the column, but we feel that part of the pheophytin problem may be related to the length of time the LHPP is on the hydroxylapatite.

Although the slurry method does allow us to obtain a larger amount of LHPP in less time, this method is not without problems. The fraction from the 0.3 M P$_i$, pH 7.0 washes of the hydroxylapatite slurry (HA-I) should correspond to the PS I chlorophyll a protein complex. This does not appear to be the case, however. This fraction consistently has a chlorophyll a/chlorophyll b ratio of between 1.5 and 2.5, indicating a considerable contamination with LHPP. This may occur due to the fact that we overload the hydroxylapatite slurry with the SDS extract. Excess LHPP which cannot be bound at the normal sites on the slurry may thus bind loosely to the sites where the chlorophyll a protein would be bound. This observation may be related to the findings of
Bernardi et al. (199) concerning the presence of two classes of binding sites for proteins on hydroxylapatite. They have found that basic proteins can be eluted from hydroxylapatite by NaCl or KCl as well as by phosphate buffers. Neutral or acidic proteins can often be eluted by salts of divalent cations, however. They suggest that the two hydroxylapatite binding sites involve the interaction of basic residues with phosphate groups on the hydroxylapatite and the interaction of acidic residues with the calcium ion in the hydroxylapatite. In this light, it is interesting to note that a divalent cation is required to elute LHPP from the hydroxylapatite slurry (except for the small portion which comes off in the HA-I washes). The divalent cation is not sufficient, however, to elute the protein. The other components, 0.4 M P_i, pH 7.0 and 0.05% SDS of the elution buffer are also required.

Due to the contamination by LHPP, we have generally discarded the HA-I washes. There is a possibility though that the contamination of this fraction with LHPP may indicate some real interaction between the LHPP and the chlorophyll a protein. This type of interaction and evidence for it will be discussed in the section regarding the P_700 chlorophyll a protein.

We have also tried a number of other possible ways to isolate LHPP. These include sequential solubilization with low concentrations of SDS, passage of the SDS extract through a Sephadex G-150 column, and extraction of LHPP from SDS.
polyacrylamide gels. Only the later method gave material showing the characteristics of LHPP. The amount of LHPP which could be prepared in this manner was not worth using for the preparation of the pigment-protein complex. Preparative gel electrophoresis might provide more material but if this technique is attempted in the future SDS content should be determined again under the conditions at which the electrophoresis is performed.

Properties of LHPP

Due to the fact that LHPP is an integral membrane protein and is rich in hydrophobic residues, the LHPP has a low solubility in aqueous solution. Most LHPP preparations we have made have a concentration of 7-10 μg chlorophyll/ml. Occasionally, a preparation is found to have a concentration as high as 20 μg chlorophyll/ml but such preparations are the exception rather than the rule. Such preparations show no other differences from the preparations having lower concentrations. Attempts to concentrate the LHPP using an Amicon Ultrafiltration Cell resulted in precipitation of the LHPP unless SDS was added.

The possibility was considered that the omission of MgCl$_2$ from the final buffer might affect the solubility of the LHPP. This is apparently not the case. After resuspending the pigmental material from the ammonium sulfate precipitation in 50 mM Tris-Cl, pH 8.2, we have split the preparation and dialyzed against the same buffer containing 0, 0.1, and 1 mM MgCl$_2$. In each case, it was
observed that a similar amount of precipitate was present at the end of the dialysis. The chlorophyll concentration and the chlorophyll a/chlorophyll b ratio was the same for all three dialysates at the end of the dialysis indicating that the absence of MgCl₂ does not affect the solubility of LHPP. The material which precipitates during dialysis has the same chlorophyll a/chlorophyll b ratio and protein/chlorophyll ratio as the LHPP indicating that the precipitate may consist of LHPP which has dropped out of solution.

The chlorophyll a/chlorophyll b ratio for LHPP is found to be 1.0 ± 0.2 which agrees with earlier reports by Thornber and coworkers. (137-141) The visible spectrum of LHPP is shown in Figure 3. Spectral peaks from chlorophyll a are found in the 435-440 nm and 668-671 nm regions of the spectrum. Chlorophyll b peaks are found in the 465-470 nm and 650-654 nm regions of the spectrum.

We have encountered problems in determining accurate protein/chlorophyll ratios for the LHPP. Based on a molecular weight of 33,000 and two moles of chlorophyll per mole of protein, the protein/chlorophyll ratio (w/w) can be calculated to be 18.3. Thornber et al. (138) have obtained similar numbers using protein nitrogen determinations for protein determinations. We have attempted to use a modification of the Lowry protein determination suited for insoluble proteins. (198) This method is described in the experimental section. The best values we have been able to
obtain (using BSA as a standard) for protein/chlorophyll ratios have been between 13 and 15. Often ratios less than 10 and as low as 3 have been obtained. The problems associated with this method may include interference by lipids, solubility of a portion of the protein in the 80% acetone used to remove the chlorophyll, and the fact that the concentration we are working with is low and accurate readings are difficult to obtain. The best values of the protein/chlorophyll ratio have been obtained from preparations which have been concentrated in the presence of 0.1% SDS.

**Ca^{++} Binding to LHPP**

The initial question we wished to ask concerning LHPP was whether this protein could bind divalent cations with a similar affinity to that observed for binding sites on the chloroplast membrane. To answer this question, we have studied Ca^{++} binding to LHPP by the technique of equilibrium dialysis. The procedure used is described in detail in the experimental section.

A time course for the establishment of equilibrium is shown in Figure 4. In the first two hours of dialysis, there is a rapid uptake of Ca^{++} into the dialysis bag, possibly due to charge imbalance across the dialysis membrane. Following this initial uptake, the amount of Ca^{++} inside the dialysis bag decreases to an equilibrium value after about 10 hours. This equilibrium level is stable for up to 24 hours after the start of the dialysis. Low concentrations of buffer do not appear to affect the equilibrium level.
Figure 3. Visible Absorption Spectrum of LHPP. The absorption spectrum of LHPP was recorded using an Amico-Chance spectrophotometer in the split-beam spectral mode of operation. The LHPP concentration was 7.9 μg chlorophyll/ml.
The same amount of Ca\(^{++}\) was bound in 1, 5, and 10 mM Tris-Cl, pH 8.2. Ca\(^{++}\) binding to LHPP did appear to be totally inhibited however in 50 mM Tris-Cl, pH 8.2.

Data from Ca\(^{++}\) binding studies covering the concentration range of 2-250 µM CaCl\(_2\) was routinely plotted in double reciprocal form according to Equation XI.

\[
\frac{1}{\text{µmoles Ca}^{++}\text{bd/mg chl}} = \frac{K_d}{n} + \frac{1}{n} \frac{1}{(\text{Ca}^{++}\text{free})}
\]

(XI)

In this equation, \(n\) is the number of binding sites and \(K_d\) is the dissociation constant for binding to the sites. Values of \(n\) and \(K_d\) can be determined from the intersections of the line drawn through the data points with the ordinate and abcissa respectively. The intercept with the ordinate is \(1/n\) and the intercept with the abcissa is \(-1/K_d\) when \(1/(\text{µmoles Ca}^{++}\text{bd/mg chl})\) is plotted against the reciprocal of the free Ca\(^{++}\) concentration.

Figure 5 shows such a double reciprocal plot covering the concentration range 2-250 µM CaCl\(_2\). Two classes of binding sites are apparent, as indicated by the two straight line segments in Figure 5. We have labeled these Site I and Site II with Site I being the site with the higher affinity (or lower dissociation constant) for the binding of divalent cations. The labeling of these sites should not be confused with Sites I and II on the chloroplast membrane (131, 132) although Site II on the LHPP may be related to Site II on the membrane. The binding parameters for the two classes of divalent cation binding sites on the LHPP are presented in Table 1.
Figure 4. Time Course for the Establishment of Equilibrium During Dialysis of LHPP Against Ca\(^{++}\). LHPP, \(\mu g\) chl/ml, was dialyzed against 200 \(\mu M\) CaCl\(_2\). Ca\(^{++}\) binding was determined as described in the experimental methods section after dialyzing for various lengths of time.
Ca$^{++}$ Binding to LHPP

($\mu$ moles Ca$^{++}$ bd/mg chl)
Figure 5. Double Reciprocal Plot of Ca\textsuperscript{++} Binding to LHPP.

Ca\textsuperscript{++} binding curves were determined and plotted in double reciprocal form as described in the experimental methods section. LHPP concentration was \( \mu \text{g} \) chlorophyll/ml. All binding experiments were done in 5 mM Tris-Cl, pH 8.2.
Ca$^{++}$ BINDING TO LHPP
($\mu$ moles Ca$^{++}$/bd/mg chl)$^{-1}$
Table 1. Ca\textsuperscript{2+} binding parameters for LHPP.

<table>
<thead>
<tr>
<th></th>
<th>Site I</th>
<th>Site II</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_d$ (uM)</td>
<td>$2.5 \pm 0.6$</td>
<td>$32 \pm 9.4$</td>
</tr>
<tr>
<td>$n$ (umoles Ca bd/mg chl)</td>
<td>$1.5-4.0$</td>
<td>$9.5 \pm 1.5$</td>
</tr>
<tr>
<td>$K_f$, Mg</td>
<td>$5.1 \mu$M (c)</td>
<td>$31\mu$M (c)</td>
</tr>
<tr>
<td>$K_f$, Na</td>
<td>$1.0$ mM (c)</td>
<td>$1.4$ mM (n)</td>
</tr>
</tbody>
</table>

$Ca^{2+}$ binding to LHPP was determined as described in the experimental procedures section. Values for $K_d$, $n$, and $K_f$'s were determined from double reciprocal plots. The values for the dissociation constants are those obtained by extrapolation to zero protein concentration. The letters in parentheses refer to competitive (c) and noncompetitive (n) inhibition of $Ca^{2+}$ binding. The $K_f$'s are the average of the values obtained with three independent preparations of LHPP.
Site II has a dissociation constant which is similar to that observed for Site II on the chloroplast membrane. Site II on the chloroplast membrane has been shown to be responsible for the regulation of excitation energy distribution. It is this class of binding sites in which we are especially interested which appears to be related to divalent cation effects on the LHPP to be discussed later. We have been unable to correlate any divalent cation effect on LHPP with Site I.

Figures 6 and 7 indicate that Ca\(^{++}\) binding to both Site I and Site II is inhibited in a competitive manner by MgCl\(_2\). The same figures also show that although Ca\(^{++}\) binding to Site I is inhibited in a competitive manner by NaCl, Ca\(^{++}\) binding to Site II appears to be inhibited in a noncompetitive manner. Inhibition constants for competitive and noncompetitive inhibition of Ca\(^{++}\) binding to LHPP have been determined from equations presented in the Experimental Methods section. The inhibition constants, K\(_I\), for Mg\(^{++}\) and Na\(^+\) are shown in Table 1 along with the Ca\(^{++}\) binding parameters for the two classes of divalent cation binding sites on LHPP. In the case of Site II, we have verified the competitive nature of inhibition of Ca\(^{++}\) binding by Mg\(^{++}\) using a Dixon plot as shown in Figure 8. Values of 1\(\mu\)moles Ca\(^{++}\) bd/mg chl) are plotted against Mg\(^{++}\) concentration at two concentrations of Ca\(^{++}\). The fact that the two lines intersect above the abcissa is indicative of competitive inhibition. From this type of plot the K\(_I\) for Mg\(^{++}\) was determined
Figure 6. Mg$$^{++}$$ and Na$$^+$$ Inhibition of Ca$$^{++}$$ Binding to Site I on LHPP. Ca$$^{++}$$ binding curves were determined as described in the experimental methods section except that 1 mM NaCl on 5 M MgCl$_2$ was present in addition to CaCl$_2$. LHPP concentration was 1.5 µg chlorophyll/ml. All inhibition studies were done on 5 mM Tris-Cl, pH 8.2. Inhibition constants, K$_I$'s, for Na and Mg were determined using Equation IV presented in the experimental methods section.
INHIBITION OF Ca** BINDING TO LHPP SITE I
(μ moles Ca** bd/mg chl)^1

\[ \frac{1}{[Ca^{++}]} (mM^{-1}) \]

- 0.1
- 0.3
- 0.5
- 0.7
- 0.8

\[ [Ca^{++}] = 1.5M + 0.4 \]

- No Addition
- 5μM MgCl2
- Initial No Cl
Figure 7. Mg$^{++}$ and Na$^{+}$ Inhibition of Ca$^{++}$ Binding to Site II on LHPP. Inhibition studies were done as described in Figure 6 except that NaCl (5mM) or MgCl$_2$ (20 μM) were present in addition to CaCl$_2$. LHPP concentration was 4.8 μg chlorophyll/ml. Values of $K_1$ were determined from Equation IV or V.
INHIBITION OF Ca** BINDING TO LHPP SITE II
(µmoles Ca** bd/mg chl)-1
Figure 8. Dixon Plot of Mg$^{++}$ Inhibition of Ca$^{++}$ Binding to Site II on LHPP. Ca$^{++}$ binding was determined as described in the experimental methods section. Ca$^{++}$ concentrations were 10 and 100 µM and the Mg$^{++}$ concentration varied between 0 and 75 µM. LHPP concentration was 4.8 µg chlorophyll/ml. Data were plotted according to Equation VI presented in the experimental methods section.
to be 31 uM. This value agrees well with the value in Table I which was determined using double reciprocal plots.

SDS Content of LHPP

One matter which concerned us was the possibility that the observed Ca\(^{++}\) binding to LHPP might be due to the presence of bound anionic detergent, SDS, used in the isolation of this protein. SDS is introduced at two points in the isolation of LHPP. The first is in the preparation of the SDS extract in 50 mM Tris-Cl, pH 8.2, 1% SDS. The second is in the final buffer for washing the hydroxylapatite slurry (0.4 M P\(_i\), pH 7.0, 1 mM MgCl\(_2\), 0.05% SDS). By adding \(^{35}\)S-SDS (specific activity = 0.02-0.02 \(\mu\)curies/\(\mu\)mole SDS) in each of these places in independent experiments and recognizing that the maximal amount of bound detergent would be the sum of the amount of SDS bound in each experiment, we have determined that less than 1 \(\mu\)mole of SDS is bound per mg chlorophyll. Although low number of counts/min was low (40-60 cpm over background), we were able to obtain rough estimates that the SDS content was in the range of 0.3-0.8 \(\mu\)moles SDS/mg chlorophyll. For these determinations all of the number of counts per minute were at least twice background and all data were corrected for the background level.

Due to the low specific activity used in these experiments and the low number of counts obtained, we felt that bound SDS could not be responsible for the observed Ca\(^{++}\) binding properties
of Site II on the LHPP although residual SDS could not be unambiguously excluded as a source of Site I binding.

To show unambiguously that neither Site I or Site II Ca\(^{++}\) binding is due to bound SDS, we have repeated these experiments using a ten-fold higher specific activity (0.22-0.25 \(\mu\)curies/umole SDS). This has given us a high enough counting rate (400-500 cpm above background) to determine more accurately the SDS content of LHPP. With specific activities in this range, we have found the SDS content of LHPP to be 0.2-0.3 \(\mu\)moles SDS/mg chlorophyll. This indicates that, with the lower specific activity, we may have been slightly overestimating rather than underestimating the SDS content due to the error inherent in using a lower number of counts. The SDS content determined using the higher specific activity is clearly less than the number of divalent cation binding sites for either Site I or Site II on LHPP. This shows that the binding of divalent cations to LHPP is not due to the presence of the anionic detergent, SDS, used in the isolation of this protein.

**Dependence of Site I on LHPP Concentration**

In our initial studies on Ca\(^{++}\) binding to LHPP, we occasionally performed a binding experiment which showed the presence of only one class of binding site. Such an experiment is shown in Figure 9. The binding parameters for this site were equivalent to those obtained for Site II indicating that Site I was not present. A re-evaluation of the existing data showed that in binding experiments were less than 2.5 \(\mu\)g chlorophyll/ml were used both classes of
Figure 9. Double Reciprocal Plot of Ca\(^{++}\) Binding to LHPP. Ca\(^{++}\) binding was determined as described in Figure 5 except that the LHPP concentration was 2.6 \(\mu g\) chlorophyll/ml.
Ca^{++} BINDING TO LHPP
\( (\mu \text{moles Ca}^{++} \text{ bd/mg chl}^{-1}) \)
binding sites were seen. However, at concentrations greater than 2.5 μg chlorophyll/ml, only Site II was present. We thus felt that a study of Ca++ binding as a function of LHPP concentration might be informative.

Figure 10 shows the results of such a study. The dissociation constants for both Site I and Site II have been plotted as a function of the LHPP concentration (expressed in terms of chlorophyll) at which the dissociation constants were determined. The dissociation constant for Site II appears to be independent of LHPP concentration in the range shown in this figure. A later figure (Figure 16) shows that the dissociation constant is the same at concentrations as high as 7.0 μg chlorophyll/ml. Site I, however, shows a striking dependence on LHPP concentration. At low LHPP concentrations, the dissociation constant for Ca++ binding to Site I can be extrapolated to 2.5 μM Ca++. The dissociation constant for Site I increases as the LHPP concentration is increased, being about equal to the dissociation constant for Site II at 3.0 μg chlorophyll/ml. Above this concentration, Site I could not be observed. No change in the number of binding sites was noted for Site II in this concentration range. See however Figure 16. The number of binding sites for Site I was also constant at all concentrations where this site could be observed. At the present time we can assign no function to Site I.
Figure 10. Dependence of $K_d$'s for $Ca^{++}$ Binding on LHPP Concentration. Values of $K_d$ for Site I and Site II were determined from a series of $Ca^{++}$ binding curves similar to those shown in Figures 5 and 9. LHPP concentrations from 0.5 to 3.5 $\mu$g chlorophyll/ml were used in these studies.
Dependence of $K_d$ for Ca$^{2+}$ Binding on LHPP Concentration

$K_d$ (µM) vs. [chl] (µg/ml) for Site I and Site II.
Ultracentrifugation Studies of LHPP: Evidence for Cation Independent Association

Such a dependence of the dissociation constant for Ca\(^{++}\) binding to Site I on LHPP concentration is most reasonably explained by an association of the protein which alters the environment and thus the affinity for divalent cations of the residues responsible for Site I binding. Such behavior has been observed and quantitated in the case of hemerytherin where the affinity for thiocyanate was found to be dependent on protein concentration (200). To see if an associative phenomenon could be responsible for the changes in the Site I dissociation constant, we have resorted to sedimentation velocity experiments in the analytical ultracentrifuge.

The major problem we encountered with this type of experiment was that the low concentrations of LHPP with which we wished to work were not sufficient to produce Schlieren peaks. We have been able to avoid this problem by taking advantage of the fact that the LHPP contains chlorophyll and is thus green. Measuring the movement of the pigment away from the meniscus as described in the experimental methods section, we have determined sedimentation coefficients for various concentrations of LHPP. Plots for data treated in this manner are linear as shown in Figure 11. Sedimentation coefficients were determined from the slopes of such plots.
Figure 11. Sedimentation Velocity Runs with Two Concentrations of LHPP. Sedimentation velocity runs were performed as described in the experimental methods section. Sedimentation coefficients were determined from the slopes of plots of $\ln (L/r_0)$ vs. $(r/r_0)$ such as those shown in this figure. LHPP concentrations are 3.2 (■) and 6.4 (●) ug chlorophyll/ml. All ultracentrifugation studies were done in 10 mM Tris-Cl, pH 8.2.
To see if association of the LHPP could be responsible for the observed changes in the dissociation constant for Ca\(^{++}\) binding to Site I on this protein, we determined sedimentation coefficients for LHPP at several protein concentrations. The results are shown in Figure 12. As usual, we have expressed LHPP concentration in terms of chlorophyll concentration. Extrapolated to zero protein, the sedimentation coefficient is 2.7 S. This agrees with the value reported by Thornber et al. (138) in the presence of SDS and most likely represents the sedimentation coefficient of the monomer. In the absence of any protein-protein interaction, one would expect that a plot of sedimentation coefficient against protein concentration should be linear with a negative slope. (201) This is due to the fact that the frictional coefficient increases with protein concentration resulting in a decrease in the sedimentation coefficient. Figure 12 shows that this type of behavior is not observed with LHPP. Instead of decreasing with increasing protein concentration, the sedimentation coefficient increases with increasing LHPP concentration. Such behavior is indicative of a reversibly associating system. (201) In the case of LHPP, the sedimentation coefficient increases with increasing protein concentration, finally plateauing at concentrations above 6.0 µg chlorophyll/ml at a value of 6.2-6.3 S. We believe that such an association of the LHPP could be responsible for the observed effect of LHPP concentration on the dissociation constant for Site I
Figure 12. Dependence of Sedimentation Coefficient on LHPP Concentration. Sedimentation coefficients were determined at a number of LHPP concentrations between 1 and 7 μg chlorophyll/ml from plots such as those in Figure 11. Ultra-centrifugation studies were done as described in the section on experimental methods.
Dependence of $S$ on LHPP Concentration

$S$ vs. $[\text{chl}]$ ($\mu g/\text{ml}$)
Ca\(^{+ +}\) binding. The shape of the curve in Figure 12 suggests that the association may proceed in two stages: one stage being represented by the slow, nearly linear rise occurring at concentrations less than 5 \(\mu g\) chlorophyll/\(ml\) and the other by the apparent transition between 5 and 6 \(\mu g\) chlorophyll/\(ml\). Evidence to be presented later supports the idea that there are two stages on LHPP association.

The associative phenomenon shown in Figure 12 takes place in the absence of added cations. We shall refer to this type of association as cation independent association of LHPP to distinguish it from a cation dependent association to be discussed next. The cation independent association is separated into two stages as suggested above. Stage I refers to the cation independent association occurring at concentrations less than 5 \(\mu g\) chlorophyll/\(ml\) which appears to be related to the observed changes in the dissociation constant for Site I Ca\(^{+ +}\) binding. Stage II is characterized by the transition occurring between 5 and 6 \(\mu g\) chlorophyll/\(ml\) in Figure 12. More will be said about the two stages of cation independent association of LHPP in later sections dealing with the cation dependent association of LHPP, the spectral properties of LHPP, and the fluorescence properties of LHPP.

**Effects of Ions on Sedimentation Coefficient for LHPP: Evidence for Cation Dependent Association of LHPP**

Having observed that an association of LHPP occurred in the absence of ions, we wished to ask the following questions. Do
cations also produce an association of the LHPP? If a cation
dependent association of LHPP is possible, is it affected by the
cation independent association of the LHPP? Can such a cation
dependent association be related to the divalent cation binding by
LHPP?

To see if the presence of ions brought about an association
of the LHPP, we determined the sedimentation coefficient in the
presence and absence of various ions. These initial studies were
done at LHPP concentrations sufficient to place us on the plateau
region of Figure 12. Thus, the sedimentation coefficient is no
longer changing with protein concentration and interference due to
the cation independent association can be negated. A second
advantage of conducting the initial studies in this region is that the
concentration on the plateau region is more than twice the highest
concentration at which Site I can be observed. It is thus unlikely
that any divalent cation effect observed in this concentration range
could be attributable to Site I binding. By working at high con-
centrations we were thus able to study the effects of Site II
binding without interference from Site I. Site II is the binding site
in which we are particularly interested since its dissociation
constant (32 μM) is near that reported for the binding site on the
chloroplast membrane responsible for divalent cation induced
alterations in excitation energy distribution. (131)
Table 2 shows the effects of a number of ions on the sedimentation coefficient of LHPP in the concentration range discussed above. It is evident from this table that divalent cations increase the sedimentation coefficient dramatically whereas monovalent cations have no effect. Divalent cations raised the sedimentation coefficient from 6.2 to 9.6-9.8 S when added alone. When added in the presence of monovalent cations, however, divalent cations increased the sedimentation coefficient to 12.3-12.6 S. There was no specificity for divalent cation as Ca$^{++}$, Mg$^{++}$, and Mn$^{++}$ all produced similar changes. Neither Na$^{+}$ or K$^{+}$ produced any change in the absence of divalent cations. These results are indicative of a divalent cation induced association of the LHPP. The data presented in Table 3 indicate that the divalent cation induced association is reversible in the presence or absence of monovalent cations. This is shown by the observation that upon dialysis to remove the divalent cation the sedimentation coefficient returns to the value observed in the absence of divalent cations.

To see if the trends shown in the plateau region extend throughout the entire concentration range we have studied, we have determined the sedimentation coefficient in the presence and absence of ions at several LHPP concentrations. The results of such a study are shown in Figure 13. The curve obtained in the absence of added ions is the same as that in Figure 12 and will not be discussed again here. In the presence of 0.5 mM Ca$^{++}$, the
Table 2. Effects of ions on the sedimentation coefficient of LHPP.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Sedimentation Coefficient (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 10 mM Tris-Cl, pH 8.2</td>
<td>6.2</td>
</tr>
<tr>
<td>2. Same as 1. + 0.5 mM MgCl₂</td>
<td>9.8</td>
</tr>
<tr>
<td>3. Same as 1. + 0.5 mM CaCl₂</td>
<td>9.8</td>
</tr>
<tr>
<td>4. Same as 1. + 0.5 mM MnCl₂</td>
<td>9.6</td>
</tr>
<tr>
<td>5. Same as 1. + 5.0 mM NaCl</td>
<td>6.3</td>
</tr>
<tr>
<td>6. Same as 1. + 5.0 mM KCl</td>
<td>6.4</td>
</tr>
<tr>
<td>7. Same as 1. + 5.0 mM NaCl + 0.5 mM CaCl₂</td>
<td>12.3</td>
</tr>
</tbody>
</table>

Sedimentation coefficients were determined as described in the experimental methods section. Ions were added to LHPP in 10 mM Tris-Cl, pH 8.2 just prior to the centrifuge run. The LHPP concentration was 6.4 µg chlorophyll/ml.
Table 3. Reversibility of divalent cation induced changes in sedimentation coefficient for LHPP.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Sedimentation Coefficient (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 10 mM Tris-Cl, pH 8.2</td>
<td>6.2</td>
</tr>
<tr>
<td>2. Same as 1. + 0.5 mM CaCl₂</td>
<td>9.6</td>
</tr>
<tr>
<td>3. Same as 2. except dialyzed vs.</td>
<td></td>
</tr>
<tr>
<td>10 mM Tris-Cl, pH 8.2</td>
<td>6.6</td>
</tr>
<tr>
<td>4. 10 mM Tris-Cl, pH 8.2 +</td>
<td></td>
</tr>
<tr>
<td>5.0 mM NaCl</td>
<td>6.3</td>
</tr>
<tr>
<td>5. Same as 4. + 0.5 mM CaCl₂</td>
<td>12.6</td>
</tr>
<tr>
<td>6. Same as 5. except dialyzed vs.</td>
<td></td>
</tr>
<tr>
<td>10 mM Tris-Cl, pH 8.2 + 5.0</td>
<td></td>
</tr>
<tr>
<td>mM NaCl</td>
<td>6.4</td>
</tr>
</tbody>
</table>

Sedimentation coefficients were determined as described in the section on experimental methods. Ions were added prior to the centrifuge run. Following the centrifuge run, a like sample containing the same concentration of ions was dialyzed as indicated overnight and a centrifuge run was then made using the dialyzed sample.
Figure 13. Ion Induced Changes in the Sedimentation Coefficient for LHPP as a Function of LHPP Concentration.

Sedimentation coefficients were determined as described in the experimental methods section at various concentrations of LHPP in the presence and absence of salts as indicated.
sedimentation coefficient is much greater than in the absence of ions, indicative of a divalent cation dependent association of the protein. The shape of the curve in the presence of Ca\(^{2+}\) is approximately a mirror image of the curve obtained in the absence of added ions. The transition between 4 and 6 \(\mu\)g chlorophyll/ml still occurs but the transition is in the opposite direction. This indicates that the second stage of cation independent association of LHPP decreases the extent of the divalent cation dependent association of the protein.

The data presented in Figure 13 also show that if Ca\(^{2+}\) is added in the presence of a monovalent cation (5 mM Na\(^{+}\)), a different behavior is noted. The sedimentation coefficient is nearly constant and maximal over the entire concentration range studied. The addition of NaCl alone produced no change in the sedimentation coefficient observed on either side of the transition between 4 and 6 \(\mu\)g chlorophyll/ml in the absence of divalent cations. It thus seems unlikely that monovalent cations reverse the cation independent association of the LHPP.

Two possible explanations can be advanced for the observation that there is a greater divalent cation dependent association in the presence of monovalent cations than in their absence at high concentrations of LHPP. The divalent cation binding site responsible for the divalent cation dependent association of LHPP
may consist of a combination of specific and nonspecific binding sites with similar binding parameters. Binding to the specific sites might result in association while binding to nonspecific sites would be nonproductive. In such a system, monovalent cations might serve as regulators, altering the distribution of specific and nonspecific sites. Alternatively, a number of divalent cation binding sites might be buried due to the cation independent association of the LHPP. Monovalent cations may make these buried sites available by either loosening the association responsible for burying the sites or by causing a conformational change exposing the buried sites. In a later section, it is shown that a number of divalent cation binding sites do become unaccessible due to the second stage of cation independent association of the protein. The observation that monovalent cations do not bring about a decrease in the sedimentation coefficient, however, argues against a monovalent cation reversal of the cation independent association. We are thus in the rather unenviable position of suggesting without proof that monovalent cations may produce a conformational change in the LHPP which results in the exposure of buried binding sites. The exposure of these sites would then be responsible for the increase in the divalent cation induced association of LHPP observed in the presence of monovalent cations at high LHPP concentrations. An ionic strength effect cannot be ruled out.
Ca\(^{++}\) Concentration Dependence for Ca\(^{++}\) Induced LHPP

To show that the divalent cation dependent association of LHPP is related to binding to Site II, we have done Ca\(^{++}\) concentration curves for the Ca\(^{++}\) induced increases in the sedimentation coefficient of LHPP. These curves are shown in Figures 14 and 15. Figure 14 shows the Ca\(^{++}\) concentration curve obtained at a LHPP concentration on the plateau region of Figure 13. The half maximal effect was at 45 \(\mu\)M CaCl\(_2\). We feel that this value provides a reasonable correlation with the dissociation constant for Ca\(^{++}\) binding to Site II on the LHPP (32 \(\mu\)M). The difference between the two numbers can be attributed to the fact that in plotting the Ca\(^{++}\) binding data, the free Ca\(^{++}\) concentration was used, whereas the total Ca\(^{++}\) concentration was used in plotting curves for the Ca\(^{++}\) induced changes in sedimentation coefficient.

To see if the concentration where the half-maximal effect was noted was affected by the second stage of cation independent association, we have repeated this experiment at a LHPP concentration lower than that where the transition is observed in Figures 12 and 13. This experiment is shown in Figure 15. The half-maximal effect is still found near 45 \(\mu\)M CaCl\(_2\), indicating that the dissociation constant for Site II is unaffected by the second stage of cation independent association of LHPP.
Figure 14. Concentration Dependence for Ca\(^{++}\) Induced Increase in the Sedimentation Coefficient of LHPP---High LHPP Concentration. Sedimentation coefficients were determined for LHPP (6.4 \(\mu\)g chlorophyll/ml) as described in the experimental methods section. Ions were added to the concentration desired just prior to the ultracentrifugation run.
\[ [\text{ch}l] = 6.4 \mu g \text{ chl/ml} \]
Figure 15. Concentration Dependence for Ca$^{++}$ Induced Increases in the Sedimentation Coefficient of LHPP—Low LHPP Concentration. Conditions were identical to those in Figure 14 except that a LHPP concentration of 3.2 $\mu$g chlorophyll/ml was used.
[Ca^{++}] (\mu M) vs. [chl] 3.2 \mu g chl/ml

- 0 NaCl
- 5mM NaCl
Figures 14 and 15 also show the Ca\(^{++}\) concentration dependence for the Ca\(^{++}\) induced changes in the sedimentation coefficient in the presence of NaCl. The major difference between the two curves is due to the fact that they were done on opposite sides of the transition in Figure 13. This difference is attributable to the second stage of cation independent association of LHPP. At either LHPP concentration, however, the half-maximal effect is found at 70-75 μM CaCl\(_2\). It is difficult to rationalize this change in the half-maximal concentration with the noncompetitive nature of Na\(^{+}\) inhibition observed for Site II. (See Table 1 and Figures 6 and 7.) If the inhibition of Ca\(^{++}\) binding to Site II is strictly non-competitive, one would expect a change in the extent of association but no change in the concentration where the half-maximal effect is observed. We feel that the noncompetitive inhibition of Ca\(^{++}\) binding to Site II by Na\(^{+}\) may not be real. The concentration curves presented in Figures 14 and 15 are suggestive of competitive rather than noncompetitive inhibition. If the inhibition of Ca\(^{++}\) binding to Site II on LHPP is competitive rather than noncompetitive, an estimate of the K\(_I\) for Na\(^{+}\) inhibition can be made from the concentrations required to produce the half-maximal effect in the presence and absence of Na\(^{+}\). Such an estimate gives a competitive K\(_I\) for Na\(^{+}\) to be 6.7 mM. This value is presented only as an alternative to the noncompetitive, lower value obtained from the Ca\(^{++}\) binding studies. It is possible that both are real and
represent separate effects. On the other hand, if one looks at the lines drawn for the Na\(^+\) inhibition of Ca\(^{++}\) binding to Sites I and II in Figures 6 and 7, one notes that they share a nearly common intercept with the abcissa. Thus, it is also possible that the non-competitive nature of the Na\(^+\) inhibition of Site II may be due in part to interference from the competitive inhibition of Site I.

**Ca\(^{++}\)** Binding to LHPP at Higher LHPP Concentrations

To determine if the second stage of cation independent association of LHPP affects the Ca\(^{++}\) binding properties as suggested in the previous section, we have done Ca\(^{++}\) binding curves on both sides of the transition in Figure 13. These curves are presented in double reciprocal form in Figure 16. The common intersection of the two lines with the abcissa indicates that the dissociation constant, \(K_d = 32 \mu\text{M}\), is not affected by the second stage of cation independent association of the LHPP. This agrees with the above observation that the second stage of cation independent association does not affect the concentration dependence of the divalent cation induced increases in the sedimentation coefficient of LHPP.

The different intercepts of the two lines with the ordinate in Figure 16 shows that although the dissociation constant is unaffected, the number of Site II Ca\(^{++}\) binding sites is decreased by the second stage of cation independent association. At a concentration of 4.3 \(\mu\text{g}\) chlorophyll/ml, the number of Site II Ca\(^{++}\)
Figure 16. \(\text{Ca}^{++}\) Binding to LHPP at Higher LHPP Concentrations. \(\text{Ca}^{++}\) binding was determined as described in the experimental methods section and Figures 5 and 9. Higher concentrations of LHPP (4.3 and 7.0 \(\mu\)g chlorophyll/ml) were used in this study.
binding sites is 10 μmoles Ca\(^{++}\) bound/mg chlorophyll. (Identical curves are obtained at concentrations between 2.6 and 4.3 μg chlorophyll/ml. Below 2.6 μg chlorophyll/ml, two classes of binding sites are seen as discussed previously.) At a concentration of 7.0 μg chlorophyll/ml the number of Site II Ca\(^{++}\) binding sites is decreased to 5.7 μmoles Ca\(^{++}\) bound/mg chlorophyll. It thus appears that the second stage of cation independent association conceals about 43% of the total number of Ca\(^{++}\) binding sites in Site II on the LHPP. This is in agreement with the ultracentrifugation studies which showed that the second stage of cation independent association decreased the extent of the divalent cation induced association of the LHPP.

**Effects of Cation Independent Association on Absorption Spectrum of LHPP**

From the data discussed in the previous sections concerning the ultracentrifugation studies and Ca\(^{++}\) binding to LHPP, we are able to suggest that there are two stages of cation independent association of LHPP. The first stage brought about the observed change in the dissociation constant for Ca\(^{++}\) binding to Site I and the second stage decreased the number of binding sites in Site II. It was thus of interest to see if these two stages of cation independent association affected the environment of the chlorophylls on the LHPP.

To determine if this was the case, we have run absorption spectra of several concentrations of LHPP. These spectra are
shown in Figure 17. This figure shows that there is no shift in the absorption peaks for either chlorophyll a or chlorophyll b in the concentration range where the two stages of cation independent association occur. The association of the LHPP in the absence of cations thus does not involve the formation of chlorophyll dimers or oligomers of the type studied by Katz's laboratory. (202, 203)

Such chlorophyll:water:chlorophyll oligomers show a shift of the red absorption maximum to wave lengths greater than 730 nm. The \((\text{chlorophyll:chlorophyll})_n\) oligomer (204) is also not involved since the formation of this chlorophyll species would result in a shift of the red absorption maximum to 680 nm or above. Since we see no bandshifts in the absorption spectrum characteristic of chlorophyll-chlorophyll interaction in the presence or absence of water, we conclude that the formation of chlorophyll oligomers is not involved in the association of LHPP.

A plot of absorbance against LHPP concentration shows a positive deviation from Beer's Law in the absorption bands from both chlorophyll a and chlorophyll b. This deviation is shown in Figure 18 for the absorption peaks due chlorophyll a. If one extends the line through the points obtained at low LHPP concentrations to higher concentrations, the points at higher LHPP concentrations lie above the line. We have considered this line to represent the ideal (or calculated) absorbance at a given concentration and have taken the difference between this
Figure 17. Absorption Spectra of LHPP at Various Concentrations. Absorption spectra were recorded for LHPP concentrations ranging from 1.0 to 7.0 μg chlorophyll/ml using an Amico-Chance spectrophotometer in the split beam spectral mode of operation.
Figure 18. Beer's Law Plots for Chlorophyll a Peaks on LHPP Absorption Spectrum. Plots of A vs. LHPP concentration were constructed from spectra such as those shown in Figure 17. The "ideal" absorbance at any LHPP concentration was determined as described in the text.
0.6
0.5
0.4
0.3
0.2
0.1

Absorbance

[Chl] (µg/ml)

436nm
436nm (Ideal)

668nm
668nm (Ideal)
calculated value and the observed value as the deviation from ideality. We have expressed this deviation from ideality as a percentage of the calculated value according to Equation XII.

\[
\text{Deviation from ideality (\%)} = \frac{A_{\text{obs}} - A_{\text{calc}}}{A_{\text{calc}}} \times 100 \quad (\text{XII})
\]

Figure 19 shows a plot of the deviation from ideality for both chlorophyll a and chlorophyll b as a function of LHPP concentration. The peaks at 464 and 652 nm are due to chlorophyll b and those at 436 and 668 nm due to chlorophyll a. From this figure, it appears that chlorophyll b shows a deviation from ideality in the two concentration ranges where the two stages of cation independent association of the LHPP take place. Chlorophyll a, on the other hand, shows a deviation from ideality corresponding only to the second stage of cation independent association of the LHPP. These results thus suggest that the first stage of cation independent association of the LHPP affects only the environment of chlorophyll b whereas the second stage affects the environment of both chlorophyll a and chlorophyll b. It is not possible at this time to state the nature of the change in the environment of the chlorophylls due to the association of the LHPP since so little is known about the nature of the environment of the chlorophylls and the nature of their binding to the protein. We can only speculate that the changes in the environment of the chlorophylls may be due to either changes in the exposure of the chlorophylls to the solvent or the introduction
Figure 19. Deviation from Ideality in Beer's Law Plots as a Function of LHPP Concentration. The deviation from ideality was determined according to Equation XII as described in the text. The deviation for ideality was expressed as a percentage of the "ideal" absorbance.
of additional amino acid residues into the environment of the chlorophylls as the LHPP associates.

**Model for Cation Independent and Divalent Cation Induced Association of LHPP**

Based on the evidence discussed above, we are able to suggest a model for the association of LHPP observed in the presence and absence of ions. The cation independent association appears to take place in two stages. The evidence for these two stages is summarized in Table 4.

We shall discuss the model for the cation independent association first. While we have not determined molecular weights, the sedimentation coefficients obtained at a function of LHPP concentration in the absence of divalent cations (see Figure 12) are consistent with a monomer $\rightarrow$ dimer $\rightarrow$ tetramer conversion when compared with the sedimentation coefficients for a number of proteins listed in the CRC Handbook of Biochemistry and Molecular Biology. (205) We have used this monomer $\rightarrow$ dimer $\rightarrow$ tetramer conversion as the basis of our proposed model of the cation independent association of LHPP.

The model we suggest to account for the observations in Table 4 is shown in Figure 20. In this model we suggest that the LHPP molecule is divided into two portions; one representing a highly hydrophobic region of the protein (shaded) and the other portion being more hydrophilic (unshaded). The division between
Table 4. Evidence for the two stages of cation independent association of LHPP.

<table>
<thead>
<tr>
<th>Stage I</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Concentration range; 1-4 ug chlorophyll/ml</td>
<td></td>
</tr>
<tr>
<td>2. Increase in $K_d$ for divalent cation binding to Site I</td>
<td></td>
</tr>
<tr>
<td>3. Non-negative slope of plot of sedimentation coefficient against LHPP concentration</td>
<td></td>
</tr>
<tr>
<td>4. Change in environment of chlorophyll b</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stage II</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Concentration range; 4-6 ug chlorophyll/ml</td>
<td></td>
</tr>
<tr>
<td>2. Decrease in the number of Site II divalent cation binding sites</td>
<td></td>
</tr>
<tr>
<td>3. Transition in plot of sedimentation coefficient against LHPP concentration</td>
<td></td>
</tr>
<tr>
<td>4. Difference in the extent of the divalent cation induced increase in sedimentation coefficient on opposite sides of the above mentioned transition</td>
<td></td>
</tr>
<tr>
<td>5. Changes in the environment of both chlorophyll a and chlorophyll b</td>
<td></td>
</tr>
</tbody>
</table>
Figure 20. Model for Cation Independent Association of LHPP. Details of the model are discussed in the text; a and b refer to chlorophyll a and chlorophyll b respectively. The shaded areas correspond to hydrophobic regions of the LHPP.
these two portions of the molecule is probably not as clear cut as indicated in this figure. The hydrophilic and hydrophobic regions are probably intermixed rather than constituting two distinct halves of the molecule. The divalent cation binding sites are most likely associated with the hydrophilic regions of the LHPP. We suggest that the two stages of cation independent association involve interactions between the hydrophobic portions of the LHPP. This would allow a greater percentage of the surface to be covered with hydrophilic residues in the associated form of the protein. Such a situation would be energetically favorable and could provide the driving force for the cation independent association of LHPP. The first stage of cation independent association would involve the hydrophobic region in the neighborhood of chlorophyll b and would not significantly affect the number of Site II divalent cation binding sites. The second stage of cation independent association involves the hydrophobic regions in the vicinity of both chlorophyll a and chlorophyll b and results in a substantial decrease in the number of divalent cation binding sites in Site II. This decrease in the number of binding sites may be due to either the burial of sites in regions inaccessible to ions or the introduction of residues from a neighboring unit into the environment of the residues responsible for divalent cation binding thus altering the dissociation constant for some of the sites.
The divalent cation induced association of the LHPP can also be treated in terms of this model. This is shown in Figure 21. The sedimentation coefficients observed in the presence of Ca\(^{++}\) at LHPP concentrations less than 4 μg chlorophyll/ml are consistent with an interaction between 3-4 of the tetramer units described above. At LHPP concentrations greater than 6 μg chlorophyll/mL, the sedimentation coefficients observed in the presence of Ca\(^{++}\) are consistent with an association between 2-3 of the tetramer units. We suggest that the monomer and dimer forms interact due to Ca\(^{++}\) binding (possibly due to the bridging of carboxyl groups on adjacent units). Such an interaction involving the hydrophilic portion of the molecule would result in a greater exposure of the hydrophobic portions of the molecule to the solvent. To alleviate this problem, hydrophobic interactions would bring about an additional association of the protein similar to the cation independent association already discussed. The tetramer is capable of the divalent cation induced association to a lesser extent due to the previous observation that a substantial percentage of the Site II divalent cation binding sites become unavailable as a result of the second stage of cation independent association of the LHPP. As suggested previously, monovalent cations might cause some type of conformational change or alteration in the interactions between units which would expose these unavailable sites thus allowing the divalent cation induced association of the LHPP to proceed to its fullest extent.
Figure 21. Model for Divalent Cation Induced Association of LHPP. Details of the model are given in the text and in Figure 20.
While the model presented here is grossly oversimplified and highly speculative, it does serve to explain the data we have obtained. Further work is needed to establish the exact nature of the protein-protein interactions involved in the association of LHPP in the presence and absence of ions and the nature of the changes in the environment of the chlorophylls resulting from these interactions.

**Fluorescence Properties of LHPP**

We have also examined the chlorophyll fluorescence properties of the LHPP. The emission maximum is found between 670 and 675 nm when excited with light absorbed by chlorophyll a (435-440 nm) or light absorbed by chlorophyll b (464-470 nm).

An emission spectrum for chlorophyll fluorescence from LHPP is shown in Figure 22. The most surprising feature of this figure is the shoulder occurring on the low wavelength side of the emission band. The fact that this shoulder is more prominent when the fluorescence is excited by chlorophyll b leads us to believe that this shoulder may be due to fluorescence from chlorophyll b. This idea is supported by the excitation spectra for fluorescence at various wavelengths within the emission band shown in Figure 23. At 670 nm, the fluorescence is excited to a greater extent by chlorophyll a than by chlorophyll b. At 660 nm the fluorescence is excited to a nearly equal extent by either chlorophyll a or chlorophyll b. However, at 650 nm, the fluorescence excited by
Figure 22. Emission Spectra for Chlorophyll Fluorescence from LHPP. Emission spectra were determined using an Aminco-Bowman spectrofluorimeter. Excitation was provided at either 438 nm (to irradiate chlorophyll a) or 464 nm (to irradiate chlorophyll b).
Figure 23. Excitation Spectra for Chlorophyll Fluorescence from LHPP. Excitation spectra were determined using an Aminco-Bowman spectrofluorimeter for the fluorescence measured at 670, 660, and 650 nm.
chlorophyll a has decreased below the level of fluorescence excited by chlorophyll b. These results are consistent with a portion of the fluorescence from LHPP arising from chlorophyll b.

The observation that the chlorophyll b of the LHPP is slightly fluorescent is interesting since chlorophyll b is non-fluorescent in vivo. (206, 207) This is due to the fact that in vivo chlorophyll b transfers its excitation energy to chlorophyll a with 100% efficiency. Chlorophyll b fluorescence has been observed in green algae under high light conditions in the presence of DCMU, conditions which should effectively close the PS II phototrails. (208) The observation of chlorophyll b fluorescence from the LHPP would indicate that the efficiency of excitation energy transfer from chlorophyll b to chlorophyll a in this pigment-protein complex is less than 100%. This is most likely due to either the distance between or orientation of the chlorophylls being such that excitation energy transfer between the chlorophyll molecules does not take place with 100% efficiency. The observation that the transfer of excitation energy from chlorophyll b to chlorophyll a is less than 100% efficient does not necessarily mean that the structure of the LHPP as isolated is greatly different than that in vivo. It is possible that the chlorophyll b of the LHPP transfers its excitation energy with high efficiency in vivo to chlorophyll a other than the chlorophyll a on the LHPP.
Chlorophyll Fluorescence as a Function of LHPP Concentration

The unusual chlorophyll fluorescence properties of LHPP led us to investigate these properties as a function of LHPP concentration. This was done to see if these properties are affected by the two stages of cation independent association of the LHPP. To do this, we have looked at the relative abilities of chlorophyll a and chlorophyll b to excite the observed chlorophyll fluorescence at 650 and 675 nm. As discussed in the previous section, the fluorescence at 675 nm is predominately from chlorophyll a while that at 650 nm has a substantial contribution from chlorophyll b. The results of such a study are shown in Figure 24.

For the 675 nm emission, the ratio, \(F_{465} \rightarrow 675/F_{435} \rightarrow 675\), is constant up to LHPP concentrations of 4 µg chlorophyll/ml. Above this concentration, this ratio increases, leveling off at concentrations greater than 6 µg chlorophyll/ml. This transition between 4 and 6 µg chlorophyll/ml is in the same concentration range as that observed for the second stage of cation independent association of the LHPP. The observed increase in \(F_{465} \rightarrow 675/F_{435} \rightarrow 675\) indicates that there is an apparent increase in the ability of chlorophyll b to excite chlorophyll a fluorescence as a result of the second stage of cation independent association of the LHPP. This would mean that the second stage of cation independent association of the LHPP may bring chlorophyll a and chlorophyll b molecules from neighboring units together in such a manner that
Figure 24. Chlorophyll Fluorescence from LHPP as a Function of LHPP Concentration. The ratios, $F_{465} \rightarrow 675/F_{435} \rightarrow 675$ and $F_{465} \rightarrow 650/F_{435} \rightarrow 650$, were determined from excitation spectra for fluorescence at 675 or 650 nm at various LHPP concentrations.
the distance and orientation of the chlorophylls is more conducive to excitation energy transfer from chlorophyll b to chlorophyll a. Alternatively, it is possible that the observed increase in this ratio may be due to an increase in the contribution of chlorophyll b fluorescence to the emission at 675 nm.

For the emission at 650 nm where there is a greater contribution from chlorophyll b, the ratio, $F_{465} \rightarrow 650/F_{435} \rightarrow 650$, increases in an apparently biphasic manner with LHPP concentration, corresponding to the two stages of cation independent association of the LHPP. This is consistent with the conclusion reached from the studies of the absorption spectrum of LHPP as a function of concentration which indicated that both stages of cation independent association affected the environment of chlorophyll b.

The effects of LHPP concentration on the ratios, $F_{465} \rightarrow 675/F_{435} \rightarrow 675$ and $F_{465} \rightarrow 650/F_{435} \rightarrow 650$, can be explained in terms of the model presented in Figure 20 for the cation independent association of LHPP. In the monomer unit, excitation energy from chlorophyll b may be transferred to chlorophyll a or dissipated as heat or fluorescence within a LHPP unit. As the LHPP associates, the transfer of excitation energy to chlorophylls on neighboring units becomes possible. Since both stages of cation independent association are suggested to involve the chlorophyll b regions of the LHPP unit, it is possible that the
association of the LHPP may enable excitation energy from chlorophyll b in one unit to be more readily transferred to a chlorophyll b in a neighboring unit, thus increasing the probability of chlorophyll b fluorescence. A simpler explanation would state only that the chlorophyll b is made more fluorescent due to the changes in environment resulting from the association of the protein. Similar arguments can be invoked to account for the correlation of the LHPP concentration dependence of the $F_{465} \rightarrow 675/F_{435} \rightarrow 675$ ratio with the second stage of cation independent association of the LHPP.

**Effects of Cations on Chlorophyll Fluorescence from LHPP**

We were also interested in studying the effects of ions on chlorophyll fluorescence from LHPP to see if the divalent cation induced association of the protein produced any changes in the emission characteristics of fluorescence from LHPP. The effects of divalent cations on the emission spectrum of LHPP (excited at 435 nm) is shown in Figure 25. Both Mg$^{++}$ and Ca$^{++}$ decreased the observed fluorescence. Ca$^{++}$, however, produced a significantly larger decrease than did Mg$^{++}$.

The effects of Mg$^{++}$ on chlorophyll fluorescence from LHPP in the presence and absence of Na$^+$ are shown in Table 5 for two concentrations of LHPP. The two concentrations used should correspond to the monomer and tetramer forms in the previously discussed model of cation independent association of
Figure 25. Effects of Cations on Emission Spectrum for Chlorophyll Fluorescence from LHPP. Emission spectra were recorded using an Aminco-Chance spectrofluorimeter. Excitation was provided at 438 nm. The LHPP concentration was 4.25 µg chlorophyll. Ions were added as indicated just prior to the fluorescence measurements.
Fluorescence Intensity (Relative Units)

438 nm excitation

---

No Salt

0.5 mM MgCl₂

0.5 mM CoCl₂

X (nm)

Fluorescence Intensity (Relative Units)
Table 5. Effects of ions on chlorophyll fluorescence from LHPP at two LHPP concentrations.

A. LHPP = 1.7 ug chlorophyll/ml

<table>
<thead>
<tr>
<th>Conditions</th>
<th>$F_{465} \rightarrow 675/F_{435} \rightarrow 675$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 10 mM Tris-Cl, pH 8.2</td>
<td>0.803</td>
</tr>
<tr>
<td>2. Same as 1. + 1 mM MgCl$_2$</td>
<td>0.909</td>
</tr>
<tr>
<td>3. Same as 1. + 10 mM NaCl</td>
<td>0.799</td>
</tr>
<tr>
<td>4. Same as 1. + 1 mM MgCl$_2$ + 10 mM NaCl</td>
<td>0.891</td>
</tr>
</tbody>
</table>

B. LHPP = 6.4 ug chlorophyll/ml

<table>
<thead>
<tr>
<th>Conditions</th>
<th>$F_{465} \rightarrow 675/F_{435} \rightarrow 675$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 10 mM Tris-Cl, pH 8.2</td>
<td>0.864</td>
</tr>
<tr>
<td>2. Same as 1. + 1 mM MgCl$_2$</td>
<td>1.026</td>
</tr>
<tr>
<td>3. Same as 1. + 10 mM NaCl</td>
<td>0.864</td>
</tr>
<tr>
<td>4. Same as 1. + 1 mM MgCl$_2$ + 10 mM NaCl</td>
<td>1.000</td>
</tr>
</tbody>
</table>

The ratio of $F_{465} \rightarrow 675/F_{435} \rightarrow 675$ was determined from excitation spectra for fluorescence at 675 nm in the presence and absence of ions as indicated. All excitation spectra were determined using an Aminco-Bowman spectrofluorimeter. Ions were
added to LHPP just prior to the fluorescence measurements. All such studies were done in 10 mM Tris-Cl, pH 8.2. Similar results were obtained with two independent preparations of TSF-II.
association of LHPP. At both concentrations, 1 mM MgCl₂ increased the ratio, \( F_{465} \rightarrow 675/F_{435} \rightarrow 675 \), to a similar extent in the presence and absence of 10 mM NaCl. NaCl (10 mM) alone had no effect on the ratio, \( F_{465} \rightarrow 675F/435 \rightarrow 675 \), at either LHPP concentration. An increase in the ratio, \( F_{465} \rightarrow 675/F_{435} \rightarrow 675 \), may have one of two interpretations. It might indicate an increase in the transfer of excitation energy from chlorophyll b to chlorophyll a. Alternatively, it may indicate a decrease in the ability of chlorophyll a to excite chlorophyll a fluorescence due to an increase in radiationless de-excitation.

It is this later explanation which best fits the data discussed below concerning the effects of ions on the excitation spectrum for chlorophyll a fluorescence.

The excitation spectra for chlorophyll a fluorescence at 675 nm from LHPP at three concentrations of Mg ++ are shown in Figure 26. The peak at 435 nm is from absorption by chlorophyll a and the peak at 465 nm due to absorption by chlorophyll b. The largest effect is clearly on the height of the peak at 435 nm. This peak is decreased by the addition of divalent cations to the medium. The 465 nm peak in the excitation spectrum is hardly affected by the same concentrations of divalent cations. This would indicate that the transfer of excitation energy from chlorophyll b to chlorophyll a is not affected by divalent cations. The implication can also be made that the transition giving rise to
Figure 26. Effects of MgCl$_2$ on Excitation Spectrum for Chlorophyll Fluorescence from LHPP. Excitation spectra for fluorescence measured at 675 nm were recorded using an Aminco-Bowman spectrofluorimeter. LHPP concentration was 6.3 $\mu$g chlorophyll/ml. MgCl$_2$ was added to the concentration indicated just prior to the fluorescence measurements.
Fluorescence at 675 nm (Relative Units)

No Salt

0.4 mM MgCl₂

1 mM MgCl₂
the chlorophyll a fluorescence (from the first excited state of chlorophyll a to the ground state) is unaffected. If this were not the case, one would expect a decrease in chlorophyll a fluorescence when either chlorophyll a or chlorophyll b was excited. The decrease in the 435 nm peak in the excitation spectrum is not due to a decrease in absorption by chlorophyll a. Divalent cations produce no changes in the absorption spectrum of the LHPP.

In Figure 27, we have summarized these results in terms of a model based on the energy levels of the chlorophyll a and chlorophyll b of the LHPP. Since divalent cations produce no changes in the absorption spectrum of LHPP, the parameters, $a_1$ and $b_1$, must be unaffected by divalent cations. For reasons described in the preceding paragraph, we also believe that all of the parameters for chlorophyll b and the parameters for transfer of excitation energy from chlorophyll b to chlorophyll a are also unaffected by divalent cations. These results would tend to indicate that the decreases observed when divalent cations are added to the medium involve the second excited state of chlorophyll a. The divalent cation induced decreases in chlorophyll a fluorescence would then most likely be indicative of an increase in the rate of radiationless de-excitation, process $a_2$. The possibility that the decreases in chlorophyll a fluorescence may be due to the formation of "quenching centers", $k_q$, cannot be ruled out at this time. Similar divalent cation induced decreases
Figure 27. Model to Explain Fluorescence Changes

Observed with LHPP. Details are described in the text.
in chlorophyll a fluorescence have been noted in a PS II particle prepared using the detergent Triton X-100 (TSF-II particles) which are enriched in LHPP. These results are presented in the section of this dissertation covering the effects of ions on TSF-II particles. It has been suggested that such decreases in fluorescence as have been observed in the LHPP and the TSF-II particle may be due to a quenching of fluorescence by molecular oxygen. This possibility is also discussed in the section on the effects of ions on TSF-II.

The suggestion we have made here that the second excited state of chlorophyll a is involved in the divalent cation induced decreases in fluorescence could be tested by using wave lengths between 650 and 670 nm to excite the fluorescence. Such excitation would excite only the first excited state of chlorophyll a and chlorophyll b. If only the second excited state of chlorophyll a is responsible for the divalent cation induced decreases in chlorophyll a fluorescence, one would expect divalent cations to have no effect on fluorescence excited by wave lengths in this range. We have not attempted such experiments because to do so would require alterations in our present spectrofluorimeter.

To see if the divalent cation induced decreases in chlorophyll fluorescence were related to the divalent cation induced association of the LHPP, we have done concentration curves for the divalent cation induced fluorescence changes. The
first of these concentration curves is shown in Figure 28. The curves in this figure were done at a LHPP concentration of 6.4 µg chlorophyll/ml. From these curves, it is apparent that at this concentration, the fluorescence at 675 nm is decreased by divalent cations to a greater extent when chlorophyll a is excited than when chlorophyll b is excited. The same is true for the fluorescence at 650 nm. From these curves, it appears that the concentration where half-maximal decreases in chlorophyll fluorescence are found is on the order of 150-250 µM MgCl₂. This is significantly different from either the dissociation constant observed for divalent cation binding to the LHPP and the half-maximal concentration for the divalent cation induced increases in the sedimentation coefficient of LHPP. It is thus unlikely that the divalent cation induced decreases in chlorophyll fluorescence observed with LHPP are related to the divalent cation induced association of the LHPP. At concentrations less than 100 µM MgCl₂ there appears to be a slight increase in chlorophyll fluorescence which takes place. It is possible that this slight increase is related to the divalent cation induced association of the LHPP. Due to the small nature of these changes and the scatter inherent in using such small changes, we have been unable to determine an accurate estimate of the concentration where this increase is half-maximal.
Figure 28. Concentration Curves for Divalent Cation Induced Changes in Chlorophyll Fluorescence from LHPP—High LHPP Concentration. The fluorescence at 675 and 650 nm was determined with excitation at either 435 or 465 nm using an Aminco-Bowman spectrofluorimeter. MgCl₂ was added to the concentrations indicated just prior to the fluorescence measurements. The LHPP concentration was 6.3 μg chlorophyll/ml.
The above concentration curves were done at a concentration high enough that we were considering the tetramer state in the model suggested earlier. It was of interest to us to see if similar results were obtained at lower concentrations. Figure 29 shows a similar set of experiments done at a concentration of 1.8 μg chlorophyll/ml. There are two rather striking differences between the curves shown in this figure and those shown in Figure 28. First, for the fluorescence at either 675 or 650 nm, divalent cations cause a decrease in the level of fluorescence excited with light absorbed by both chlorophyll a and chlorophyll b. The effect is greater, however, when chlorophyll a is excited directly. Second, the magnitude of the divalent cation induced decreases in chlorophyll fluorescence is greater in this figure than in Figure 28. The slight increases noted at concentrations less than 100 μM MgCl₂ in the previous figure are also missing although this may be due to the scatter in the data at low concentrations of divalent cation.

The differences between Figure 28 and Figure 29 are most likely due to the cation independent association of the LHPP discussed earlier. At low concentrations such as those used in Figure 29, the monomer form would predominate according to the model presented earlier. In this form, it appears that divalent cations produce larger decreases in chlorophyll fluorescence excited by either chlorophyll a or chlorophyll b. At higher
Figure 29. Concentration Curves for Divalent Cation Induced Changes in Chlorophyll Fluorescence from LHPP--Low LHPP Concentration. MgCl$_2$ concentration curves were determined as in Figure 28 except that the LHPP concentration was 1.8 µg chlorophyll/ml.
Fluorescence (Relative Units)
concentrations, however, decreases of this nature are produced only when the fluorescence is excited by chlorophyll a. Even then, the decreases are smaller than those observed at lower LHPP concentrations. We feel that these results indicate that divalent cations may affect both chlorophyll a and chlorophyll b in similar manners, possibly involving the second excited state. As the LHPP associates, chlorophyll b must become largely inaccessible to divalent cations of the medium since divalent cation induced decreases in chlorophyll fluorescence are not found when chlorophyll b is excited at high LHPP concentrations. Chlorophyll a must also become partially concealed since the magnitude of the chlorophyll a fluorescence changes decreases with increasing LHPP concentration. This is consistent with the model presented in Figure 20. Thus, although the divalent cation induced changes in the chlorophyll fluorescence properties of the LHPP do not appear to be directly related to the divalent cation induced association of the LHPP, they are consistent with the model presented to account for the cation independent association of the LHPP.

Summary of LHPP Data

The LHPP has been shown to have two classes of binding sites for divalent cations. Site I binds Ca\(^{++}\) with a dissociation constant of 2.5 \(\mu\)M. Site II binds Ca\(^{++}\) with a dissociation constant of 32 \(\mu\)M. The dissociation constant for Site I and the
number of binding sites for Site II vary with LHPP concentration in a manner indicative of two stages of cation independent association of the LHPP. Analytical ultracentrifugation studies also indicate two stages of cation independent association of the LHPP as do studies of the absorption and fluorescence spectra of LHPP as a function of LHPP concentration.

Divalent cations also produce a divalent cation specific association of the protein. The extent of the divalent cation induced association varies in a manner consistent with the proposed cation independent association of the LHPP. The concentration dependence for the divalent cation induced association of the LHPP indicates that the binding of divalent cations to Site II is responsible for the divalent cation induced association.

Divalent cations also bring about decreases in chlorophyll fluorescence from LHPP. The concentration dependence for this phenomenon, however, is much different from the concentration dependence observed for the divalent cation induced association of the LHPP and the dissociation constant for Site II divalent cation binding. The divalent cation induced decreases in chlorophyll fluorescence thus do not appear to be a direct result of the divalent cation induced association of the LHPP. It has been suggested that such decreases may indicate that divalent cations bring about an increase in radiationless de-excitation or create "quenching centers" in the involving the LHPP. Although the
divalent cation induced decreases in chlorophyll fluorescence do not appear to be directly related to the divalent cation induced association of the LHPP, the observed changes are consistent with the proposed model for the cation independent association of LHPP in which the chlorophyll molecules has less exposure to solvent in the associated form of the protein.

The studies here represent the first observations of protein-protein interactions involving the LHPP. We have shown that LHPP interacts with itself in both the presence and absence of divalent cations. A model has been presented to account for both the cation independent and divalent cation specific association of the LHPP.

At this point, one might question whether or not the protein-protein interactions involving the LHPP observed in solution are related to the function of this protein in the chloroplast membrane. The LHPP would clearly have more constraints placed upon it in the membrane than in solution. We do find it interesting that the LHPP binds divalent cations to Site II with an affinity similar to that of the binding site on the chloroplast membrane which regulates excitation energy distribution. Furthermore, the divalent cation binding to the LHPP is not a static binding process, but rather brings about a divalent cation induced association of the protein. We feel that such interactions (although possibly on a more limited scale due to constraints placed on the LHPP by the
membrane) could be responsible for the divalent cation control of excitation energy distribution since the transfer of excitation energy is dependent upon both the distance between and orientation of pigment molecules. (80, 81) These parameters would undoubtedly be affected by the protein-protein interactions we have described for the LHPP. Such changes may be reflected in the alterations in the absorption and fluorescence spectra of LHPP which were correlated with the two stages of cation independent association of the protein. Such effects may also be present due to the divalent cation induced association of the LHPP but be obscured by the apparently unrelated divalent cation induced decreases in chlorophyll fluorescence.

Although our studies on LHPP have provided considerable information concerning protein-protein interactions involving the LHPP, they have not shown that LHPP is directly related to the cation control of excitation energy distribution as observed in the chloroplast membrane. We have been able to show that this protein is involved in the control of excitation energy distribution through a collaboration with the laboratory of Dr. C. J. Arntzen of the University of Illinois. These experiments are described in the next section.

Developmental Studies

The best evidence we have been able to obtain concerning the involvement of the light harvesting pigment protein in
excitation energy distribution comes from a collaborative study done with the laboratory of Dr. C. J. Arntzen of the University of Illinois. This study involved the development of the light-harvesting pigment protein and excitation energy distribution during the greening of pea seedlings grown in intermittent light.

Work in Dr. Arntzen's laboratory had shown that if etiolated pea seedlings were grown in an intermittent light system, (12 cycles of 2 min light followed by 2 hr darkness), the plants developed chloroplasts which showed the presence of PS I, PS II, and proton pump activities. This was taken to indicate that the phototrap of the two photosystems, the electron transport components, and the phosphorylating machinery were present in plants grown in this manner. Such plants did not, however, show grana stacking or the divalent cation-induced changes in chlorophyll a fluorescence indicative of changes in excitation energy distribution. Light saturation curves indicated that the chloroplasts from such plants also showed smaller photosynthetic unit sizes than normal. SDS: polyacrylamide gel electrophoresis showed that chloroplasts from plants grown in this manner were deficient in the light-harvesting pigment protein. If plants grown in the intermittent light system were greened in continuous light for 24-48 hr, the chloroplasts formed grana stacks, developed a larger photosynthetic unit size, showed the ability to produce the divalent cation-induced fluorescence changes, and showed the presence of the light-harvesting pigment protein.
The above observations led us to believe that it might be profitable to look at the time course of appearance for the light-harvesting pigment protein and the divalent cation-induced fluorescence changes during the greening of pea seedlings grown in the intermittent light system. This author went to Dr. Arntzen's laboratory and such a study was carried out by Mr. Paul Armond, a graduate student of Dr. Arntzen, and myself. Figures 30-32 represent work performed by Paul Armond and are presented here with the permission of Mr. Armond and Dr. Arntzen.

Figure 30 shows the time course of appearance of the divalent cation-induced changes in chlorophyll a fluorescence as studied at room temperature during the greening of pea seedlings grown in the intermittent light system. The curve consists of two phases. There is a large increase in the extent of the Mg$^{++}$-induced fluorescence increase which occurs in the first 8 hr. Following this initial phase is a slower phase in which the Mg$^{++}$-induced change increases at a slower rate.

To show that the distribution of excitation energy between the two photosystems is affected, fluorescence studies were also done at 77° K. At such low temperatures, fluorescence at 687 nm is largely from PS II and fluorescence at 735 nm from PS I. Figure 31a shows the ratio of fluorescence at 735 nm to that at 687 nm in the presence and absence of Mg$^{++}$ as a function of
Figure 30. Time Course for Appearance of Divalent Cation Induced Fluorescence Changes During the Greening of Pea Seedlings Grown in Intermittent Light--Room Temperature Studies. Experiment performed by and figure courtesy of Mr. Paul Armond and Dr. C. J. Arntzen of the University of Illinois. See the text for details.
Figure 31. Time Course for Appearance of Divalent Cation Induced Fluorescence Changes During the Greening of Pea Seedlings Grown in Intermittent Light--Liquid N₂ Temperature Study. Experiment performed by and figure courtesy of Mr. Paul Armond and Dr. C. J. Arntzen of the University of Illinois. See the text for details.
greening time in continuous light after removal from the intermittent light system. In the presence or absence of Mg\(^{++}\), this ratio was near zero for plants just removed from the intermittent light system indicating that in such plants divalent cations do not affect the distribution of excitation energy between the two photosystems. As the plants were allowed to green, this ratio was found to increase in both the presence and absence of Mg\(^{++}\). In the absence of Mg\(^{++}\), however, the extent of the increase of this ratio was considerably larger, indicating that in the absence of Mg\(^{++}\) the distribution of excitation energy is poised toward PS I while in the presence of Mg\(^{++}\) the poise is more toward PS II. The change in excitation energy distribution can be represented as the difference between the two curves in Figure 31a. This difference is plotted as a function of greening time in continuous light in Figure 31b. The difference in \(F_{735}/F_{687}\) in the presence and absence of Mg\(^{++}\) shows the same biphasic time course of appearance as that shown in Figure 30 for the development of the divalent cation-induced changes in fluorescence observed at room temperature.

The appearance of grana stacking was also followed as a function of greening time in continuous light after removal from the intermittent light system. Electron micrographs of leaf tissue greened for various lengths of time were analyzed according to the statistical method of Goodenough and Levine. (209) Figure
31a shows the probability of having a grana stack containing n membranes per stack. Figure 32b shows the average number of membranes per grana stack as a function of greening time. The appearance of grana stacks also shows a biphasic nature similar to that shown for the divalent cation induced changes in chlorophyll a fluorescence at room temperature and 77°K.

Our contribution to this collaboration consisted of determining the time course of appearance of the light-harvesting pigment protein and studying the divalent cation binding characteristics of the developing membrane system. These studies will now be described.

Time Course of Appearance of LHPP During the Greening of Pea Seedlings Grown in Intermittent Light

Previous workers have shown that SDS extracts of chloroplast membranes form three pigmented bands when subjected to SDS: polyacrylamide gel Electrophoresis. (See the literature review.) In order of their increasing distance from the origin of the gel, these bands represent a pigment protein complex characteristic of PS I, the light-harvesting pigment protein (previously thought to be associated with PS II), and a free pigment or pigment-detergent micelle band. As discussed above, the light-harvesting pigment protein had been shown to be absent in pea seedlings grown under intermittent light conditions but appeared if such plants were then greened in continuous light. It was thus of
Figure 32. Time Course for Appearance of Grana Stacking During the Greening of Pea Seedlings Grown in Intermittent Light. Experiment performed by and figure courtesy of Mr. Paul Armond and Dr. C. J. Arntzen of the University of Illinois. See the text for details. \( N \) is the number of grana stacks containing \( n \) thylakoids.
interest to see if the time course of appearance of LHPP paralleled the time course of for the divalent cation effects on fluorescence and the appearance of grana stacking. If this protein is related to these effects a similar time course of appearance should be noted. To answer this problem, SDS extracts were prepared from chloroplasts isolated from plants grown in the intermittent light system and then greened for varying lengths of time in continuous light. The SDS extracts were then subjected to SDS-polyacrylamide gel electrophoresis as described in the experimental section. Following electrophoresis, the gels were scanned at 670 nm using a Gilford spectrophotometer equipped for gel scanning. The gel scans were then traced and the various bands cut out and weighed on a Metler H-10 balance. The weight of each individual band was expressed as the percentage of the total weight of the area under the tracing. This allowed a quantitative determination of the relative percentage of the total chlorophyll present in each of the three pigmented bands.

The results of this study are shown in Figure 33. The most striking feature of this figure is that the appearance of the LHPP parallels the appearance of the divalent cation effects on fluorescence and the appearance of grana stacking. The percentage of the total chlorophyll in the light harvesting pigment protein position on the gels increases sharply in the first eight hours of greenning. This initial increase is followed by a slower increase
Figure 33. Time Course for the Appearance of Pigment-Protein Complexes During the Greening of Pea Seedlings Grown in Intermittent Light. The relative percentage in each of three pigmented bands obtained upon SDS: polyacrylamide gel electrophoresis was determined as described in the experimental methods section. SDS extracts were prepared from chloroplasts isolated from pea seedlings grown in the intermittent light system and then greened for the stated times. Symbols are as follows: PS I chlorophyll a protein (.), LHPP (x), Free Pigment (o).
which continues up to 48 hours of greening. The PS I pigment protein complex does not show this biphasic response during greening. The percentage of chlorophyll in the PS I band does however appear to increase slightly during the greening process. This would imply that the PS I complex may also be synthesized during the greening period. If this were not the case, one might expect the percentage of chlorophyll present in the PS I band to decrease as the other pigment protein is synthesized due to a dilution of the PS I band chlorophyll with respect to the total chlorophyll. Such a dilution may be reflected in the free pigment band which shows a significant decrease in the percentage of total pigment as the plant greens. (It is as yet unclear whether this band represents true free pigment or pigment which may be loosely associated with various proteins and is dissociable in SDS.) Alternatively, the possibility must be considered that the decrease in free pigment may be due to the incorporation of free pigment into the LHPP band. Against this, it can be argued that the time course for the decrease in the percentage of chlorophyll in the free pigment band does not exactly correspond to that for the appearance of the LHPP band. The chlorophyll a/chlorophyll b ratio for plants grown in the intermittent light system indicates that little, if any, chlorophyll b is present. Other authors (140, 142) have shown that when the LHPP protein is absent, all of its constituent pigments are also absent. Thus, the incorporation of free pigment into the LHPP seems unlikely.
Divalent Cation Binding Characteristics of the Developing Membrane System

It has been previously shown in this laboratory that two classes of binding sites for divalent cations are present on the chloroplast membrane. (131, 132) Site I \( (K_d = 8 \mu M) \) has been shown to be responsible for the reversal of uncoupling of photophosphorylation by quaternary ammonium salts. Site II \( (K_d = 50 \mu M) \) has been shown to be responsible for divalent cation control of excitation energy distribution and the structural changes associated with this control process. It was thus of interest to determine the divalent cation binding characteristics of chloroplast membranes from plants grown in the intermittent light system and then greened in continuous light for varying lengths of time.

Complete binding curves (in double reciprocal form) are shown in Figures 34 and 35 for pea seedlings grown in the intermittent light system and for plants grown in the same manner but greened for 48 hours in continuous light. The binding parameters determined from a number of such binding curves are given in Table 6. The data presented in this table indicate that, although there is no significant change in the dissociation constant for either site, there is an increase in the ratio, \( n_{II}/n_{I} \) as a result of the greening process. Thus, during the greening of plants grown in the intermittent light system, there appears to be a preferential synthesis of Site II compared to Site I.
Figure 34. Ca$^{++}$ to Chloroplasts from Pea Seedlings Grown in Intermittent Light. Pea seedlings were grown in the intermittent light system of Dr. C. J. Arntzen, University of Illinois. Chloroplasts were prepared according to the method of Gross. (190) Ca$^{++}$ binding to the chloroplast membranes was done according to the method of Gross and Hess (131) as described in the experimental methods section.
Ca$^{++}$ Binding to Chloroplasts From Pea Seedlings – 0 hr. Greening

$(\mu$ moles Ca$^{++}$/bd/mg chl$^\text{-1}$ vs. $[\text{Ca}^{++}]^{-1}$ (mM)$^{-1}$)
Figure 35. Ca\(^{++}\) Binding to Chloroplasts from Pea Seedlings Grown in Intermittent Light and then Greened for 48 Hours. Experiment was performed as in Figure 34 except that the pea seedlings used were greened in continuous white light for 48 hours after removal from the intermittent light system.
Ca++ Binding to Chloroplasts From Pea Seedlings - 48 hr. Greening

(μmoles Ca++ bd/mg chl)^{-1}

[Ca++]^{-1} (mM)^{-1}

0 0.4 0.8 1.2 1.6

-100 -150 -100 -50 0 50 100 150 200 250
Table 6. Comparison of Ca$^{++}$ binding parameters for chloroplasts from pea seedlings grown in an intermittent light system with those grown in the same manner and then greened for 48 hours.

<table>
<thead>
<tr>
<th>Growth Conditions</th>
<th>Intermittent Light</th>
<th>Intermittent Light plus 48 hours greening</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_d, Site I</td>
<td>9.7 μM (5.7-15)</td>
<td>4.6 μM (4.0-5.0)</td>
</tr>
<tr>
<td>K_d, Site II</td>
<td>50 μM (44-56)</td>
<td>47 μM (44-50)</td>
</tr>
<tr>
<td>n_II/n_I</td>
<td>0.73 (0.60-0.83)</td>
<td>1.17 (1.08-1.34)</td>
</tr>
</tbody>
</table>

Etiolated pea seedlings were grown in an intermittent light system (2 min light followed by 2 hours dark, 12 cycles). Chloroplasts were prepared according to the method of Gross (190) from plants grown in the intermittent light system and plants grown in the same manner but greened for 48 hours in continuous white light. Ca$^{++}$ binding was measured using $^{45}$Ca$^{++}$ as described in the experimental section. Values of K_d and n_II/n_I presented represent an average of four independent determinations. The values in parentheses give the range of values obtained in each case.
The variation of Site II Ca\(^{++}\) binding as a function of greening time was studied by comparing the amount of Ca\(^{++}\) bound in a reaction mixture containing 1 mM CaCl\(_2\) to the amount bound in a reaction mixture containing 20 μM CaCl\(_2\). The 20 μM reaction mixture nearly saturates Site I while the 1 mM reaction mixture should saturate both Site I and Site II. A ratio of Ca\(^{++}\) bound in the 1 mM reaction mixture to that bound in the 20 μM reaction mixture should thus give a measure of \((n_I + n_{II})/n_T\). The results of this study are shown in Figure 36. In this figure, the difference between the ratio obtained with plants greened for 48 hours and that obtained for ungreened plants is set at 100%. As can be seen, about one fourth of the change occurs on a time scale similar to that observed for the appearance of LHPP, the appearance of the divalent cation effects on fluorescence, and the formation of grana stacks. This change in the first few hours of greening is followed by a slower change extending throughout the remainder of the greening period. This would suggest that in the first few hours of greening, there is a preferential synthesis of Site II relative to Site I. The similarity between Figure 36 and Figures 30-34 indicate that a portion of the Site II binding sites which develop during the greening of plants grown in the intermittent light system are related to the divalent cation control of excitation energy distribution, the ability of the membranes to form grana stacks, and the presence of the light-harvesting pigment protein.
Figure 36. Time Course for Changes in Ca++ Binding During the Greening of Pea Seedlings Grown in Intermittent Light.

Ca++ binding was determined as described in the experimental section using reaction mixtures containing 20 μM and 1 mM CaCl₂. Chloroplasts were prepared from pea seedlings grown in the intermittent light system and then greened for various lengths of time.
Change in \( \mu \text{moles Ca}^{++} \text{ bd/mg chl in 1mM CaCl}_2 \) vs. Greening time (hr)
It was somewhat surprising to find that any Site II binding sites were present in chloroplasts grown in the intermittent light system since such plants did not show the divalent cation-induced changes in fluorescence and chloroplast structure. We feel that this indicates that the Site II observed on fully developed chloroplast membranes may consist of two subclasses of similar sites. One subclass (about 2/3 of the total number of sites) is present in plants grown in the intermittent light system and may not be related to the divalent cation control of excitation energy distribution. The second subclass appears when plants grown in the intermittent light system are subjected to further greening in continuous light. This subclass (about 1/3 of the total number of sites) appears to be intimately related to divalent cation control of excitation energy distribution.

Summary of Developmental Studies

The appearance of divalent cation control of excitation energy distribution and the appearance of grana stacking during the greening of pea seedlings grown in an intermittent light system has been shown to coincide with the appearance of the light-harvesting pigment protein. The Ca\(^{++}\) binding capacity of Site II on the chloroplast membrane also increases in a manner parallel to the appearance of divalent cation control of excitation energy distribution.
Each of the above phenomena shows a biphasic time course of appearance—a rapid accumulation in the first few hours, followed by a slower phase lasting several hours. This would suggest that in the first few hours of continuous light, the photosynthetic units synthesized during the intermittent light are being completed. This would include a filling-out of the light-harvesting apparatus and the development of excitation energy distribution control. After this phase (and possibly throughout this phase) new photosynthetic units are being synthesized in their entirety, thus accounting for the apparent biphasic characteristics observed. The fact that grana stacking appears and divalent cation binding increases in a similar biphasic manner strengthens the idea that large structural changes of the chloroplast membrane and divalent cation binding to the chloroplast membrane are related to the divalent cation control of excitation energy distribution.

This study shows that divalent cation control of excitation energy distribution and grana stacking are closely related to the presence of the light-harvesting pigment protein (LHPP) in the membrane. This represents the first time that a pigment-protein has been suggested in the control of excitation energy distribution. The correlation between the appearance of grana stacking and the light-harvesting pigment protein was not unexpected since this protein has been previously related to a protein fraction which is absent in various mutants which do not form grana stacks. (147-
149) This study strengthens the view that this protein fraction is intimately involved in the stacking process by showing that the protein and grana stacking appear simultaneously in a greening system.

The above studies indicate that during the greening of pea seedlings grown in an intermittent light system, a specific protein fraction, the light-harvesting pigment protein, is incorporated into the chloroplast membrane. The incorporation of this protein into the membrane brings about an increase in the relative number of Site II divalent cation binding sites, responsible for divalent cation control of excitation energy distribution, and facilitating the formation of grana stacks.

Studies on PS II Particles Prepared Using Triton X-100

We have also studied the effects of ions on a PS II particle prepared using the detergent Triton X-100. This particle was isolated as described by Vernon et al. (168-173) Following Vernon's nomenclature, we shall refer to this particle as TSF-II. (170)

We have studied this particle in particular for a number of reasons. First, the particle can be easily assayed for PS II activity by following the photoreduction of DCIP using DPC as an electron donor. (183) This reaction has been shown to be characteristic of PS II in subchloroplast particles and in chloroplasts treated in such a manner as to disrupt the flow of electrons from
water to the PS II phototrap. (183) Second, the TSF-II particle is enriched in chlorophyll b and the major protein components are those in the 20,000-30,000 molecular weight range which have been related to the light-harvesting pigment protein (LHPP). (188, 142) Thus, LHPP appears to be associated with the PS II phototrap. Third, it is in this particle that Prochaska and Gross have found the highest activity for Ca\(^{++}\) binding to subchloroplast particles prepared with Triton X-100. (134) They have also observed cation-induced changes on chlorophyll a fluorescence in both this particle and the PS I particle prepared using Triton X-100. (134) Fourth, ions have been shown to affect the DPC-dependent photo-reduction of DCIP by the TSF-II particle. This effect of ions has been suggested to be due to an ionic strength effect. (183) This particle thus appeared to be a good subject to determine if the effects of cations on LHPP (discussed earlier) might affect the transfer of excitation energy from the light-harvesting apparatus to the PS II phototrap.

**Preliminary Studies**

The assay we have used to measure PS II activity in TSF-II particles is essentially the same as that of Vernon and Shaw. (183) We have however changed the buffer system from 30 mM \(P_i\), pH 6.7 to 1.0 or 10 mM Tris-Cl, pH 8.2. In addition to buffer all assay mixtures contained 0.5 M sucrose, 0.5 mM DPC and 50 uM DCIP. Ions were added as indicated.
Using the isolation procedure of Vernon et al. (169) we have obtained TSF-II particles which show PS II activity in the DPC-dependent photoreduction of DCIP. Chlorophyll a/chlorophyll b ratios have been found to be in the range of 1.4 to 2.0. However, preparations having an a/b ratio of less than 1.8 showed little or no activity. Such preparations were routinely discarded. Preparations with a/b ratios between 1.8 and 2.0 showed activities from 10 to 20 μmoles DCIP reduced/mg chlorophyll/hour. Other than the loss of activity in preparations having a/b ratios less than 1.8, there was no correlation between the activity of the preparation and the a/b ratio.

The DPC concentration dependence for the photoreduction of DCIP by TSF-II particles is shown in Figure 37. DPC concentrations above 0.2 mM appear to be saturating. Lineweaver-Burk plots, such as the one shown in Figure 39, give a $K_m$ for DPC of 35-45 μM.

The PS II inhibitor, DCMU, is generally believed to inhibit photosynthetic electron transport on the reducing side of PS II. (210-212) Some authors have obtained evidence however that DCMU may inhibit on the oxidizing side of PS II or at the PS II phototrap itself. (213-215) The similarity between the structures of DPC and DCMU also brings about the possibility that DCMU may inhibit DCIP photoreduction by competing for the site of electron donation by DPC. It was thus of some interest to look briefly at the DCMU inhibition of DCIP photoreduction by TSF-II particles.
Figure 37. DPC Concentration Dependence for DPC Dependent Photoreduction of DCIP by TSF-II. The assay medium consisted of 1 mM Tris-Cl, pH 8.2, 0.5 M sucrose, 50 µM DCIP, plus DPC to the concentrations indicated. TSF-II was added to a concentration of 16 µg chlorophyll/ml and DCIP reduction determined as described in the experimental methods section. The same experiment was repeated in the presence of 100 µM DCMU.
$v \ (\mu \text{ moles DCIP Reduced/mg chl/hr})$
Figure 37 also shows that DCMU inhibits the photoreduction of DCIP by TSF-II particles at all DPC concentrations. A concentration curve for the inhibition of DCIP photoreduction by DCMU is shown in Figure 38. The concentration of DCMU required for half-maximal inhibition is clearly less than 10 uM. An inhibition study shown in Figure 39 shows that at a concentration of 5 uM inhibition by DCMU is noncompetitive with respect to DPC. From this figure, a $K_i$ for DCMU is estimated to be 3.6 uM. The noncompetitive nature of the inhibition with respect to DPC indicates that DCMU does not compete with DPC for the binding site where DPC donates electrons to PS II. Additional data concerning the effects of DCMU of TSF-II will be discussed in the section dealing with chlorophyll a fluorescence from TSF-II particles.

Effects of Ions on DPC-Dependent Photoreduction of DCIP by TSF-II

Since one of our major interests has been the study of divalent cation effects on the light-harvesting pigment protein, we began our studies of ion effects on TSF-II by looking for divalent cation effects. The data presented in Table 7 show that 1 mM MgCl$_2$ inhibits DCIP reduction by TSF-II particles. Because we had altered the buffer used by Vernon and Shaw (183), we felt it wise to see if this divalent cation effect could be observed under conditions similar to those used by Vernon and Shaw. As Table 7 indicates, the buffer had no effect on the extent of inhibition by
Figure 38. Concentration Curve for DCMU Inhibition of DPC Dependent Photoreduction of DCIP by TSF-II. The assay medium contained 1 mM Tris-Cl, pH 8.2, 0.5 M sucrose, 0.5 mM DPC, and 50 μM DCIP. DCMU was added to the desired concentration. TSF-II was added to a concentration of 16 μg chlorophyll/ml. The reduction of DCIP was followed as described in the experimental methods section.
\( v \) (\( \mu \) moles DCIP Reduced/mg chl/hr)
Figure 39. Lineweaver-Burk Plot of Inhibition of DPC Dependent DCIP Reduction by DCMU. DCIP reduction was determined as a function of DPC concentration in the presence and absence of 5 μM DCMU. The assay medium was as described in Figure 37. Data were plotted in double reciprocal form; 1/V vs. 1/(DPC).
Table 7. Effect of Mg$^{++}$ on DCIP photoreduction by TSF-II particles in different buffer systems.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Relative Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 30 mM P$_4$, pH 7.0</td>
<td>100</td>
</tr>
<tr>
<td>Same + 1 mM MgCl$_2$</td>
<td>68</td>
</tr>
<tr>
<td>2. 10 mM Tris-Cl, pH 7.0</td>
<td>100</td>
</tr>
<tr>
<td>Same + 1 mM MgCl$_2$</td>
<td>72</td>
</tr>
<tr>
<td>3. 10 mM Tris-Cl, pH 8.2</td>
<td>100</td>
</tr>
<tr>
<td>Same + 1 mM MgCl$_2$</td>
<td>67</td>
</tr>
<tr>
<td>4. 1 mM Tris-Cl, pH 8.2</td>
<td>100</td>
</tr>
<tr>
<td>Same + 1 mM MgCl$_2$</td>
<td>69</td>
</tr>
</tbody>
</table>

In addition to buffer, all samples contained 0.5 M sucrose, 0.5 mM DPC, and 50 uM DCIP. TSF-II concentration was 15 ug chlorophyll/ml. Illumination was provided at right angles to the measuring beam in the sample cuvette through a broad band red cut off filter. The intensity of illumination was 7.2 x 10$^4$ erg/cm$^2$/second. The reference cuvette was not illuminated. Under each set of conditions the rate obtained in the absence of MgCl$_2$ was taken as 100%. The rates obtained in the presence of MgCl$_2$ were expressed as a percentage of the rate obtained in the absence of MgCl$_2$. 
The extent of the divalent cation inhibition was the same in both 30 mM $P_4$, pH 7.0 and 10 mM Tris-Cl, pH 7.0. In 10 mM Tris-Cl, the same extent of inhibition was observed at pH 7.0 and pH 8.2. Finally, the same extent of inhibition was observed with 1 and 10 mM Tris-Cl, pH 8.2. Unless otherwise indicated, all subsequent studies were buffered with 1 mM Tris-Cl, pH 8.2.

It was also of interest to see if other ions could produce effects similar to those observed with MgCl$_2$. The inhibition of DCIP reduction by various ions is shown in Table 8. MgCl$_2$ appears to be slightly more effective than CaCl$_2$ in the inhibition of DCIP reduction by TSF-II particles. MnCl$_2$ produced the largest inhibition of the divalent cations tested. In the case of MnCl$_2$, however, there was an appreciable back reaction when the light was turned off. Such a back reaction was not observed with any of the other ions tested. Two possible explanations can be offered for the inhibition observed with MnCl$_2$. First, Mn$^{++}$ may be oxidized on the oxidizing side of PS II. The Mn$^{+++}$ formed may then be reduced to Mn$^{++}$ on the reducing side of PS II competing with DCIP for reducing equivalents. Second, the reduced DCIP may reduce the Mn$^{+++}$ thus giving low readings for the amount of DCIP reduced. From our data, it is not possible to decide between these two alternatives.
Table 8. Effects of various ions on DCIP reduction by TSF-II particles.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Relative Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No salt additions</td>
<td>100</td>
</tr>
<tr>
<td>1 mM MgCl₂</td>
<td>54</td>
</tr>
<tr>
<td>1 mM CaCl₂</td>
<td>66</td>
</tr>
<tr>
<td>1 mM MnCl₂</td>
<td>25</td>
</tr>
<tr>
<td>100 mM NaCl</td>
<td>54</td>
</tr>
<tr>
<td>100 mM KCl</td>
<td>68</td>
</tr>
</tbody>
</table>

All assay mixtures contained 1 mM Tris-Cl, pH 8.2, 0.5 M sucrose, 0.5 mM DPC, and 50 uM DCIP. Illumination was provided as described in Table 7. Rates in the presence of ions were expressed as a percentage of the rate obtained in the absence of ions.
Table 8 also shows that monovalent cations inhibit the DPC-dependent photoreduction of DCIP by TSF-II particles. Much higher concentrations are required to achieve the same extent of inhibition. NaCl appears to be slightly more effective than KCl.

Concentration Dependence for MgCl\textsubscript{2} Inhibition of DCIP Photoreduction by TSF-II Particles

The DPC-dependent photoreduction of DCIP was studied as a function of MgCl\textsubscript{2} concentration. MgCl\textsubscript{2} was found to inhibit DCIP reduction in two concentration ranges, indicating the possibility of two separate effects. The first stage of the MgCl\textsubscript{2} inhibition occurs at concentration considerably less than 1 mM MgCl\textsubscript{2}. A concentration curve for this stage of divalent cation inhibition of DCIP reduction is shown in Figure 40. Half-maximal inhibition occurs between 70 and 90 uM MgCl\textsubscript{2} in various preparations of TSF-II. The second stage of divalent cation inhibition of DCIP photoreduction occurs at concentrations greater than 1 mM MgCl\textsubscript{2}. A concentration curve for this second stage of divalent cation inhibition is shown in Figure 41. Half-maximal inhibition is obtained at a concentration of about 5 mM MgCl\textsubscript{2} after correction is made for the inhibition occurring below 1 mM MgCl\textsubscript{2}. This second stage of inhibition will shortly be shown to be an ionic strength effect.
Figure 40. Concentration Curve for Divalent Cation Specific Inhibition of DCIP Reduction by TSF-II Particles. The assay medium consisted of 1 mM Tris-Cl, pH 8.2, 0.5 M sucrose, 0.5 mM DPC and 50 μM DCIP. MgCl₂ was added to the desired concentration. TSF-II was added to a concentration of 16 μg chlorophyll/ml. DCIP reduction was followed as described in the experimental methods section.
Figure 41. Concentration Curve for Second Stage of Divalent Cation Inhibition of DCIP Reduction by TSF-II Particles. Conditions were as described in Figure 40 except higher concentrations of MgCl₂ were added.
$v$ (µ moles DCIP Reduced/mg chl/hr)
Concentration Dependence for NaCl Inhibition of DCIP Photoreduction by TSF-II Particles

A concentration curve for the NaCl inhibition of the DPC-dependent photoreduction of DCIP by TSF-II particles in shown in Figure 42. Half-maximal inhibition is obtained at a concentration of 10 mM NaCl. In some preparations, there is a slight stimulation of the DCIP reduction rate in this concentration range. (See Figure 44 for an example of this kind of behavior.) This stimulation does not appear in all preparations and is often small in preparations where it is observed. We have not tried to obtain this NaCl stimulation reproducibly and have only noted that it is present in some preparations and not in others. The elucidation of this NaCl stimulation remains a subject for future study. The NaCl inhibition of DCIP photoreduction does however appear reproducibly in all preparations. This NaCl effect is apparently related to the second stage of divalent cation inhibition discussed in the previous section. As well be shown in the following section, it also appears to be an ionic strength effect.

Effect of Ionic Strength of DCIP Photoreduction by TSF-II Particles

The similarity between the NaCl inhibition of DCIP reduction and the second stage of MgCl₂ inhibition led us to believe that these two effects might be related. Figure 43 shows a plot of the relative rate of DCIP reduction as a function of ionic strength. Ionic strength was varied with either NaCl or MgCl₂.
Figure 42. Concentration Curve for Monovalent Cation Inhibition of DCIP Reduction by TSF-II Particles. Conditions were as described in Figure 40 except that NaCl was added instead of MgCl₂.
$v (\mu\text{moles DCIP Reduced/mg chl/hr})$
Figure 43. DCIP Photoreduction by TSF-II Particles as a Function of Ionic Strength. Data such as that presented in Figures 40-42 is plotted as a function of ionic strength.
DCIP Reduction (% Control Rate)

Ionic Strength (mM)

NaCl

MgCl₂

0 20 40 60 80 100 120 140 160

0 20 40 60 80 100 120 140 160
as indicated. Relative rates were determined by dividing the rate obtained in the presence of added ions by the rate obtained in the absence of added ions. This ratio was multiplied by 100 and expressed as a percentage. As can be seen in Figure 43 there is a similar dependence between either the NaCl inhibition of the second stage of MgCl₂ inhibition and the ionic strength of the assay medium. This is probably equivalent to ionic strength effects previously observed by Vernon and Shaw. (183) If one subtracts the first stage of divalent cation inhibition of DCIP reduction (that occurring at concentrations less than 1 mM MgCl₂) from the NaCl curve, the two curves are nearly identical. This strengthens the supposition that the second stage of divalent cation inhibition and the monovalent cation inhibition represent an ionic strength effect.

Figure 44 shows a curve similar to that shown in Figure 43. There are some important differences however. First, the ionic strength has been varied by adding either NaCl or MgCl₂ after adding enough MgCl₂ to saturate the lower concentration divalent cation effect. The points obtained by varying the ionic strength in these two ways fall on the same line. This provides further evidence that these two effects are the same and represent an ionic strength effect. Second, the preparation on which this series of experiments was done showed one of the best examples of the NaCl stimulation obtained at low NaCl concentrations.
Figure 44. DCIP Photoreduction by TSF-II Particles as a Function of Ionic Strength. Data such as those presented in Figures 40-42 are plotted as a function of ionic strength. Symbols are as follows: (•) NaCl only, (○) MgCl₂ only, (□) NaCl in the presence of 1 mM MgCl₂.
This stimulation causes an apparent shift of the NaCl inhibition of DCIP reduction to slightly higher concentrations. (Compare this figure with Figure 43.) Third, even though the NaCl stimulation of DCIP reduction is evident in this preparation, the stimulation is not observed when NaCl is used to vary the ionic strength in the presence of 1 mM MgCl₂. This may indicate that the NaCl stimulation of DCIP reduction may be inhibited or prevented by the presence of divalent cations. The reason why we always observe the divalent cation effect while only sometimes observe the NaCl stimulation is unclear.

Since the second stage of divalent cation inhibition and the monovalent cation inhibition of DCIP reduction appear to represent an ionic strength effect, we have applied Debye-Huckel limiting law to the data to gain further knowledge concerning the nature of the interaction affected by ionic strength. The Debye-Huckel limiting law is expressed in Equation XIII.

\[ \log k = \log k_0 + 1.018 \frac{Z_a Z_b}{1} I^{1/2} \]  

(XIII)

In this equation, \( k \) is the rate constant at a given ionic strength and \( k_0 \), the rate constant in some reference solution of low ionic strength. \( I \) is the ionic strength of the medium and \( Z_a \) and \( Z_b \) are the charges on the species involved in the interaction affected by ionic strength. Rearranging this equation such that all rate constants are on the left hand side, one obtains Equation XIV.

\[ \log \frac{k}{k_0} = 1.018 \frac{Z_a Z_b}{1} I^{1/2} \]  

(XIV)
This is the equation we have used to plot the data presented in Figures 45 and 46. Because the rates we obtain when the light is turned on are linear with time, we can equate $v$ and $v_0$ with $k$ and $k_0$, respectively. ($v = kt$, $v_0 = k_0 t$, $v/v_0 = k/k_0$). All rates were measured one minute after the start of illumination. A linear rate was observed throughout the illumination period. Thus what we have plotted in Figures 45 and 46 is $\log v/v_0$ against $t^{1/2}$. Figure 45 shows such a plot for the case where the ionic strength was varied by the addition of MgCl$_2$. The reference rate, $v_0$, was the rate obtained using the usual assay medium plus 1 mM MgCl$_2$. This enables us to observe only the ionic strength effect since the specific divalent cation effect is saturated well below 1 mM MgCl$_2$. The slope of this plot (0.917) allows us to calculate the product, $Z_a Z_b$, to be -0.9. This is taken to indicate that the charged species affected by ionic strength are univalent and oppositely charged. It appears that the inhibition of DCIP reduction by TSF-II particles by increasing ionic strength may involve the disruption of an ion pair consisting of two univalent, oppositely charged groups. Figure 46 shows a similar plot obtained by varying the ionic strength with NaCl instead of MgCl$_2$. For this plot the reference rate, $v_0$, was the rate obtained with the usual assay medium and no added ions. The data are more scattered in this plot than in the one constructed from the MgCl$_2$ data. This may be the result of the second NaCl effect (the NaCl
Figure 45. \( \ln \left( \frac{v}{v_0} \right) \) vs (Ionic Strength)\(^{1/2} \) for MgCl\(_2\) Data. This plot was constructed from data such as those presented in Figure 43, the inhibition of DCIP reduction by MgCl\(_2\). Reference conditions were the normal assay medium containing 1 mM MgCl\(_2\) to saturate the divalent cation specific inhibition of DCIP reduction.
MgCl₂ Data

\[ \log \frac{k}{k_0} \]

\[ \sqrt{I.S.} \]
Figure 46. $\ln \left( \frac{v}{v_0} \right) \text{ vs } (\text{Ionic Strength})^{1/2}$ for NaCl Data. This plot was constructed from data such as those presented in Figure 43 for the inhibition of DCIP reduction by NaCl.
stimulation discussed previously). A small amount of this second effect in the same concentration range could be expected to result in a scattering of the data. From the slope of this plot (0.82), the product, $Z_aZ_b$, was calculated to be 0.8. This is in reasonable agreement with the value obtained using the MgCl$_2$ data considering the scatter present in the NaCl data due to possible interference from a second NaCl effect.

While we have used the Debye-Huckel ionic strength theory to treat data, this theory may not be truly applicable to our situation and hence the results and interpretations presented here must be questionable at best. Objections to the application of Debye-Huckel theory rest on two grounds. First, Debye-Huckel theory is usually considered applicable at ionic strengths less than 0.01 M. The concentrations of salts we have used give higher ionic strengths than 0.01 M. Second, the application of this formulation to reactions involving large particles is in itself questionable. The Debye-Huckel theory rests on a Poisson distribution of ions around a central ion. Such a situation may not be attainable in a large particle such as the one with which we are dealing. In spite of these problems we have obtained an amazingly good fit (at least in the case of the MgCl$_2$ data) to Equation XIV which follows from the Debye-Huckel theory. The results and interpretations presented should be viewed with a certain amount of caution.
Light Intensity Curves for DCIP Photoreduction by TSF-II in the Presence and Absence of Ions

Previous workers have shown that there is a hyperbolic relationship between the rate of a photosynthetic Hill reaction and the intensity of the illumination. \(216-218\) This relationship is expressed in Equation XV.

\[
\frac{1}{v} = \frac{1}{K_d} + \frac{1}{k_l} \frac{1}{I} \quad (XV)
\]

In this equation, \(v\) is the reaction rate, \(K_d\) is the reaction rate at infinite light intensity, \(k_l\) is the rate constant for the rate-limiting light dependent step, and \(I\) the light intensity. It was suggested that factors affecting the light dependent steps would result in a change in \(k_l\) while factors affecting steps other than those depending directly on light (dark electron transport reactions) would affect \(K_d\). More recently, it has been shown that \(k_l\) may also be affected by factors affecting electron transport on the side of the phototrap which is not rate limiting. \(218\)

We have applied this type of analysis to data concerning the effects of ions on the DPC-dependent photoreduction of DCIP by TSF-II particles. Light intensity curves for this reaction were done in the presence and absence of ions. Light intensity was varied by changing the voltage to the lamp providing the illumination. Illumination was provided through a broad band red cut off filter. Plots of \(1/v\) against \(1/I\) were constructed in the presence and absence of added ions.
Figure 47 shows such a plot of $1/v$ against $1/I$ in the presence and absence of 1 mM MgCl$_2$. Figure 48 shows a similar plot in the presence and absence of 100 mM NaCl. In both of these plots the lines obtained in the presence and absence of added salt intersect on the ordinate and not on the abcissa. This indicates that although $K_d$ is apparently unaffected by the addition of salts, $k_I$ is affected by the addition of ions to the assay mixture. The concentrations used for these studies would indicate that both the specific divalent cation effect (the inhibition of DCIP reduction observed at concentrations less than 1 mM MgCl$_2$) and the ionic strength effect bring about a change in the light dependent steps but not in the dark electron transport reactions. These data suggest that the inhibition of DCIP photoreduction by either the specific divalent cation effect or the ionic strength could be overcome by a higher intensity of illumination. Unfortunately, we are not equipped to obtain intensities sufficient to saturate with respect to light intensity in either the presence or absence of added ions.

These results would indicate that the effects of ions on DCIP reduction by TSF-II particles may involve either the reaction center of PS II or the transfer of excitation energy from the light-harvesting apparatus to the reaction center.
Figure 47. Double Reciprocal Plot of DCIP Reduction as a Function of Light Intensity in the Presence and Absence of MgCl₂. DCIP reduction was measured as a function of light intensity in the presence and absence of 1 mM MgCl₂. The conditions of the assay were as described in Figure 40. Light intensity was varied by altering the voltage to the light source using a Variac regulator. The intensity of illumination was measured using a Model 65 Radiometer (Yellow Springs Instruments). Illumination was through a broad band red cut off filter as described in the experimental methods section.
Figure 48. Double Reciprocal Plots of DCIP Reduction as a Function of Light Intensity in the Presence and Absence of 100 mM NaCl. DCIP reduction was measured as a function of light intensity in the presence and absence of 100 mM NaCl. Conditions were as described in Figure 47.
The graph shows the relationship between the reaction rate and the inverse of the light intensity. The x-axis represents \((I \times 10^{-4})^{-1} \text{ (erg/cm}^2\text{/sec})^{-1}\) and the y-axis represents \(v' (=\mu \text{ moles DCIP Reduced/mg chl/hr})^{-1}\). Two lines are plotted: one for 100mM NaCl and another for No Salt. The data points for No Salt are represented by circles, and for 100mM NaCl, they are represented by squares.
Comparison of Ion Effects on DCIP Photoreduction by TSF-II Particles with Different Wave Lengths of Illumination

It was also of interest to see if the wave length of illumination affected the nature of the effects of ions on DCIP reduction by TSF-II particles. This was done by comparing the extent of inhibition obtained when ions were added to the assay medium and illumination provided through a broad band red cut off filter to the extent of inhibition obtained in the presence of ions when illumination was provided through an interference filter having a transmission maximum of 650, 680, or 710 nm. The results of such a study are presented in Table 9.

The data presented in this table seem to indicate that the wave length of illumination has little effect on the extent of inhibition of DCIP reduction by either the specific divalent cation effect or the ionic strength effect. In the case of the specific divalent cation effect (1 mM MgCl₂), there is almost no difference between the values obtained with any of the interference filters. The small changes observed in the case of the ionic strength effect (10 mM MgCl₂ or 100 mM NaCl) are not considered significant. These results indicate that the points of inhibition by divalent cation and ionic strength effects are such that the reaction is inhibited to the same extent regardless of the wave length of illumination driving the reaction. This indicates that the inhibition of DCIP reduction by divalent cations and ionic
Table 9. Comparison of different illumination on DCIP reduction by TSF--II particles.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>RCO</th>
<th>650 nm</th>
<th>680 nm</th>
<th>710 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>No salt additions</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1 mM MgCl₂</td>
<td>77</td>
<td>69</td>
<td>71</td>
<td>71</td>
</tr>
<tr>
<td>10 mM MgCl₂</td>
<td>34</td>
<td>37</td>
<td>43</td>
<td>43</td>
</tr>
<tr>
<td>100 mM NaCl</td>
<td>80</td>
<td>79</td>
<td>86</td>
<td>71</td>
</tr>
</tbody>
</table>

Conditions were as described for Table 8. Illumination was provided through a broad band red cut off filter (RCO) or an interference filter having the desired wave length. Intensities of illumination were as described in the experimental methods section. Using each filter, rates in the presence of ions were expressed as a percentage of the rate obtained in the absence of ions.
strength effects may be due to an inhibition or inactivation of the PS II phototrap or reaction center. If transfer from the light-harvesting apparatus to the reaction center were being preferentially inhibited, one would expect a greater inhibition when illumination was provided through the 650 nm interference filter than when illumination was provided through the 680 nm interference filter. This is not observed to be the case with either the specific divalent cation effect or the ionic strength effect. Effects on the light-harvesting apparatus cannot be totally excluded however. The absorption due to the PS II reaction center is much smaller at 680 nm than the absorption due to the light-harvesting apparatus. The larger absorption by the light-harvesting apparatus might cause an effect on the light-harvesting apparatus to predominate at this wavelength even though the absorption maximum for the PS II phototrap is located at 680 nm.

**Effects of DCMU on Chlorophyll a Fluorescence from TSF-II Particles**

We began our studies on chlorophyll a fluorescence from TSF-II particles by looking at the effect of the PS II inhibitor, DCMU. In whole chloroplasts, this inhibitor acts on the reducing side of PS II and raises chlorophyll a fluorescence to its maximal level. This happens because a block on the reducing side of PS II effectively closes the reaction centers and excitation energy which might otherwise drive a photochemical
reaction must be dissipated as fluorescence or radiationless de-excitation. This produces an increase in chlorophyll a fluorescence when DCMU is added to chloroplasts. The effects of DCMU on the DPC-dependent photoreduction of DCUP were described in an earlier section.

Two interesting observations can be made concerning the effect of DCMU on chlorophyll a fluorescence from TSF-II particles. First, in these particles, the addition of DCMU produces a decrease rather than an increase in chlorophyll a fluorescence. Second, the nature of this decrease is dependent upon whether or not DPC is present in the medium. These two observations are shown in Figure 49. The top half of this figure shows that DPC causes chlorophyll a fluorescence to rise slightly and reach a steady state level. If DCMU is added after this steady state level in the presence of DPC has been achieved, the chlorophyll a fluorescence drops rapidly (in less than 3 seconds) to a new steady state level. The bottom half of this figure shows the case where the additions are reversed. If DCMU is added first, there is only a slight decrease in chlorophyll a fluorescence. This decrease is much slower than the decrease observed if DCMU is added in the presence of DPC. If DPC is added following DCMU, there appears to be another slight decrease in chlorophyll a fluorescence. In all of these experiments, the excitation wavelength was 435 nm. The same results were observed when 470
Figure 49. Effect of DCMU on Chlorophyll a Fluorescence from TSF-II Particles. Steady state chlorophyll a fluorescence levels were recorded using an Aminco-Bowman spectrofluorimeter. Excitation was provided at 435 nm and fluorescence measured at 680 nm. DCMU was added to a concentration of 33 μM before or after the addition of 333 μM DPC. The assay medium consisted of 1 mM Tris-Cl, pH 8.2, 0.5 M sucrose. TSF-II concentration was 6.7 μg chlorophyll/ml.
Fluorescence at 680nm

33 μM DCMU

33 μM DPC

33 μM DCMU

33 μM DPC

30 sec
nm was used as the excitation wave length.

The TSF-II particle can be considered "open" at both ends in the absence of added electron donors and acceptors. It is likely that the rise to a steady state level observed upon the addition of DPC is due to a closing of the phototaps from the oxidizing side. The observation that the change in chlorophyll a fluorescence is not observed when DPC is added following DCMU indicates that DCMU interferes in some manner with either the phototrap itself or the ability to close the phototrap from the oxidizing side. It was earlier shown that DCMU is not a competitive inhibitor of DPC. (See Figure 39.) The observation that similar results were obtained when chlorophyll a (435 nm) or chlorophyll b (470 nm) was excited indicates that transfer of excitation energy from chlorophyll b to chlorophyll a is not affected. It appears that in order for the large, rapid decrease in chlorophyll a fluorescence to be observed, the phototaps must be closed with DPC. All of these results indicate that the point of inhibition by DCMU in TSF-II particles involves an inhibition of the ability to maintain the phototrap in the closed state. While the data suggest the phototrap as the site of DCMU inhibition in the TSF-II particles, the above observations do not constitute a solid argument that other factors (transfer from the light-harvesting apparatus to the phototrap, possible sites of interaction between the site of DPC and the phototrap, etc.) may not also be involved. The site of inhibition
does not appear to be on the reducing side of the phototrap however. The exact nature of the inhibition by DCMU provides an interesting topic for future study.

**Effects of Ions on Chlorophyll a Fluorescence from TSF-II Particles in the Presence of DPC**

We have also studied the effects of ions on chlorophyll a fluorescence from TSF-II particles. Figure 50 shows that low concentrations of both mono- and divalent cations produce decreases in chlorophyll a fluorescence when the phototraps are closed with DPC. The same type of effect is observed when chlorophyll a is excited at 435 nm or chlorophyll b is excited at 470 nm. These changes appear to be similar in nature to those observed when DCMU is added to DPC and may indicate that the fluorescence changes caused by DCMU and low concentrations of ions are related. Jennings and Forti have shown that DCMU is apparently able to interact with the site for the Mg$^{++}$-induced fluorescence changes observed on the chloroplast membrane. (106) We may be observing a related phenomenon here. The concentration of MgCl$_2$ used in these studies is sufficient to saturate the divalent cation specific inhibition of DCIP reduction discussed earlier but not high enough to be in the range where inhibition due to the ionic strength effect is observed. The observation that low concentrations of NaCl produce similar fluorescence changes to those observed with divalent cations but do not produce an
Figure 50. Effects of Ions on Chlorophyll a Fluorescence from TSF-II Particles in the Presence of DPC. MgCl₂ or NaCl were added to concentrations of 0.33 mM and 6.7 mM, respectively, following the addition of 0.33 mM DPC. Fluorescence measurements were done as described in Figure 49. All measurements were made in 1 mM Tris-Cl, pH 8.2. 0.5 M sucrose.
Excitation $\lambda$: 435 nm
Emission $\lambda$: 680 nm

0.33 mM DPC
0.33 mM MgCl$_2$

0.33 mM DPC
6.7 mM NaCl

30 sec
inhibition of DCIP reduction (other than the inhibition associated with the ionic strength effect) may indicate that the rapid decrease in chlorophyll a fluorescence observed when ions are added following DPC is not related to the divalent cation specific inhibition of DCIP reduction by TSF-II particles. Concentration curves for the ion induced changes in chlorophyll a fluorescence in the presence of DPC may help to clarify this situation.

The addition of ions at concentrations sufficient to observe the ionic strength effect following the addition of DPC shows a slightly different change in chlorophyll a fluorescence. This is shown in Figure 51. The rapid (less than 3 seconds) decrease observed with lower concentrations on ions and with DCMU is still present. However, the fluorescence does not level off immediately when ions are added at concentrations sufficient to observe the ionic strength effects as it did in the other cases. In this figure, a much slower decrease follows the initial rapid decrease. This second decrease takes 4-5 minutes to reach completion and decreases the chlorophyll a fluorescence to approximately one half its initial level. The half-time for this slow decrease appears to be on the order of 1 minute. Similar results are obtained with both MgCl$_2$ and NaCl and with both 435 and 470 nm excitation. This second, slow decrease in chlorophyll a fluorescence may be related to the previously discussed ionic strength effect.
Figure 51. Effects of Ions on Chlorophyll a Fluorescence from TSF-II Particles in the Presence of DPC. MgCl₂ or NaCl was added to the concentrations of 33.3 mM following the addition of 0.33 mM DPC. Fluorescence measurements were made as described in Figure 49.
Excitation λ: 435 nm
Emission λ: 680 nm

0.33 mM DPC
33.3 mM MgCl₂

0.33 mM DPC
33.3 mM NaCl
Effects of Ions on Chlorophyll a Fluorescence from TSF-II Particles when Added Prior to DPC

When ions are added prior to the addition of DPC, a different pattern of fluorescence changes is observed. Figure 52 shows the case where low concentrations of ions are added prior to DPC and excitation is provided at 435 nm. The addition of either mono- or divalent cations caused a slow decrease in chlorophyll a fluorescence. This decrease appears much different than that observed when the same concentrations of ions were added following DPC. Compare this figure with Figure 50. The addition of DPC following the addition of ions resulted in an increase similar to that observed if DPC were added alone. This would indicate that the addition of ions to the medium does not interfere with the ability of DPC to close the phototrails from the oxidizing side. This is different than the behavior observed with DCMU where DPC added after DCMU could not close the phototrails from the oxidizing side. Thus although the sites of interaction of ions and DCMU with the TSF-II particle may be related or on close proximity, they do not appear to be identical.

If similar experiments are carried out using 470 nm excitation (exciting chlorophyll b instead of chlorophyll a), another difference is noted. Figure 53 shows such an experiment. Low concentrations of both mono- and divalent cations still produce the slow decrease in chlorophyll a fluorescence.
Figure 52. Effects of Ions on Chlorophyll a Fluorescence from TSF-II Excited at 435 nm when Added Prior to DPC. 
MgCl₂ or NaCl were added to concentrations of 0.33 mM and 6.7 mM, respectively, prior to the addition of DPC to a concentration of 0.33 mM. Steady state fluorescence levels excited at 435 nm and measured at 650 nm were recorded using an Aminco-Bowman spectrofluorimeter. The TSF-II concentration was 6.7 μg chlorophyll/ml. The assay medium consisted of 1 mM Tris-Cl, pH 8.2, 0.5 M sucrose.
Excitation $\lambda$: 435 nm
Emission $\lambda$: 680 nm

0.33 mM MgCl$_2$
0.33 mM DPC

6.7 mM NaCl
0.33 mM DPC

30 sec
Figure 53. Effects of Ions on Chlorophyll a Fluorescence from TSF-II Excited at 470 nm when Added Prior to DPC. Conditions were as described in Figure 52 except that the excitation wave length was 470 nm instead of 435 nm.
Excitation λ: 470 nm
Emission λ: 680 nm

0.33 mM MgCl₂

0.33 mM DPC

6.7 mM NaCl

0.33 mM DPC

30 sec
However, it appears that there is a slight increase in chlorophyll a fluorescence prior to the slower decrease. A comparison of this figure with Figure 52 shows that this transitory increase is not present when chlorophyll a is excited directly at 435 nm. This may indicate that divalent cations serve to quench chlorophyll a fluorescence preferentially and also promote the transfer of excitation energy from chlorophyll b to chlorophyll a. A similar preferential quenching of chlorophyll a fluorescence was observed for the light-harvesting pigment protein. The data concerning the effects of ions on the fluorescence properties of the light-harvesting pigment protein were discussed earlier.

The case where higher concentrations of ions (in the range where the ionic strength effect is observed) are added prior to DPC is shown in Figures 54 and 55. With excitation of 435 nm, it appears that both mono- and divalent cations produce a biphasic decrease in chlorophyll a fluorescence. With excitation at 470 nm, however, there appears to be only a monophasic decrease in chlorophyll a fluorescence when either mono- or divalent cations are added. In either case, the ability of DPC to close the phototrap from the oxidizing side appears to be unimpaired.

The effects of ions on chlorophyll a fluorescence from TSF-II in the absence of added electron donors and acceptors has been studied in more detail by L. Prochaska in our laboratory. There are differences between the results the two of us have
Figure 54. Effects of Ions on Chlorophyll a Fluorescence from TSF-II Excited at 435 nm when Added Prior to DPC. Conditions were as described in Figure 52 except that MgCl$_2$ and NaCl were added to a concentration of 33.3 mM prior to the addition of DPC.
Excitation $\lambda$: 435nm
Emission $\lambda$: 680nm

33.3 mM MgCl$_2$

0.33 mM DPC

33.3 mM NaCl

0.33 mM DPC

30 sec
Figure 55. Effects of Ions on Chlorophyll a Fluorescence from TSF-II Excited at 470 nm when Added Prior to DPC. Conditions were as described in Figure 54 except that the excitation wavelength was 470 nm instead of 435 nm.
Excitation λ: 470 nm
Emission λ: 680 nm

33.3 mM MgCl₂
0.33 mM DPC

33.3 mM NaCl
0.33 mM DPC

30 sec
obtained. First, the fluorescence changes studied by Prochaska are larger than those which I have observed. Second, he has detected no differences in the nature of the fluorescence changes which appear to be related to the wave length at which chlorophyll a fluorescence was excited. Third, he has not been able to detect the transient increase in chlorophyll a fluorescence when low concentrations of ions are added and chlorophyll b is excited at 470 nm. The differences between our two sets of observations concerning the effects of ions on chlorophyll a fluorescence may be attributable to a number of factors. There is a difference in the conditions under which the two of us have looked for ion effects on chlorophyll a fluorescence. The conditions for the studies done by this author are identical to the conditions used for the studies concerning the effects of ions on DCIP reduction by TSF-II particles (1 mM Tris-Cl, pH 8.2, 0.5 M sucrose). Prochaska's observations were made under conditions similar to those used in divalent cation binding studies (0.2 mM Tris, pH 8.0, 100 mM sucrose). There was also a difference in the photochemical activity of the preparations used by the two of us. The preparations used by this author had a higher activity in the DPC-dependent reduction of DCIP than those used by Prochaska. In some cases his preparations showed the decreases in chlorophyll a fluorescence even though they showed no photochemical activity. This may mean that the changes observed by Prochaska are not dependent
on PS II photochemistry and represent changes in the nature or organization of the light-harvesting apparatus. A closer study of the effects of ions on chlorophyll a fluorescence from TSF-II particles as a function of buffer and sucrose concentrations and photochemical activity may be required to resolve the different observations from these two studies.

Summary of Data on TSF-II Particles

Our studies of the effects of ions on the DPC-dependent photoreduction of DCIP by TSF-II particles has shown the presence of at least two types of ionic effects. At low concentrations, there is a divalent cation specific inhibition of DCIP reduction. Half-maximal occurred at concentrations between 70 and 90 uM MgCl₂. No corresponding effect was noted for monovalent cations. At higher concentrations, salts of both mono- and divalent cations inhibit DCIP reduction due to an ionic strength effect. These two types of effects appear to be independent since the ionic strength effect can be observed even after the divalent cation specific effect has been saturated. Light intensity curves indicate that light requiring steps, rather than dark electron transport reactions, are the sites of inhibition by both the divalent cation specific and ionic strength effects. The inhibition of DCIP reduction by the ionic strength effect appears to involve the disruption of a charge pair consisting of two univalent, oppositely charged species.
The PS II inhibitor, DCMU, also inhibits the DPC-dependent photoreduction of DCIP. This inhibitor does not compete with DPC for the site of electron donation to PS II even though the structures of DCMU and DPC are similar. The effects of DCMU on chlorophyll a fluorescence tend to indicate that in this particle DCMU may inhibit at some point other than the reducing side of PS II. The similarity between the fluorescence changes induced by DCMU and those induced by low concentrations of divalent cations are suggestive of a possible relationship between the sites of interaction of DCMU and divalent cations. However, the difference in the ability of DPC to produce fluorescence changes which may be indicative of closing the PS II phototrap in the presence of DCMU and divalent cations indicates that although the sites of interaction for DCMU and divalent cations may be related or in close proximity, they are not identical.

The effects of ions on chlorophyll a fluorescence from TSF-II particles appears to be complex and to depend upon a number of factors including the presence of an electron donor, the state of the PS II phototrap, the wave length of excitation, and possibly the medium in which the effects are observed. It is possible that ions may affect chlorophyll a fluorescence at the level of both the PS II phototrap and the light-harvesting apparatus. Further work is needed to elucidate the exact nature of these fluorescence changes, the relationship between the ion-induced
decreases in chlorophyll a fluorescence and the observed inhibition of DCIP reduction by the divalent cation specific and ionic strength effects, and the relationship of the ion-induced fluorescence changes to possible structural changes which may be involved in the regulation of PS II activity or the distribution of excitation energy between the two photosystems in the intact membrane.

One attractive explanation for the observation that low concentrations of divalent cations decrease both the rate of DCIP reduction and chlorophyll a fluorescence by TSF-II particles is that divalent cations may create quenching centers in the light-harvesting apparatus. The location of such quenching centers in the light-harvesting apparatus would enable them to compete effectively with PS II photochemistry and the dissipation of excitation energy by fluorescence or radiationless de-excitation. It has been suggested to us by Dr. S. Lien of Dr. A. San Pietro's laboratory at Indiana University that molecular oxygen may be involved in such a quenching process. Excitation energy might be quenched at such centers by reducing oxygen to the level of peroxide or superoxide. We have not looked for oxygen uptake by TSF-II particles in the presence and absence of ions which might be indicative of such a quenching phenomenon. It is hoped that such a study can be made in the near future to see if such a phenomenon is related to the observed inhibition of DCIP reduction and the decreases on chlorophyll a fluorescence brought about by ions.
Interactions between pigment proteins such as those described earlier for the light-harvesting pigment protein could be responsible for the creation of such quenching centers. In this light, it is interesting to note that the ion-induced fluorescence changes observed using the TSF-II particle are similar to those observed with the light-harvesting pigment protein. Thus, any study of oxygen uptake as a function of ions should be extended to include the light-harvesting pigment protein as well as the TSF-II particle.

Another means of localizing the ion effects in the light-harvesting apparatus would involve the preparation of a PS II reaction center which lacks the light-harvesting apparatus. The TSF-IIa particle which can be prepared from the TSF-II particle used in these studies would be an interesting subject for such studies. The TSF-IIa particle contains only chlorophyll a and is able to carry out the DPC-dependent photoreduction of DCIP. (173) The light-harvesting pigment protein is not present in the TSF-IIa particle. Thus, any effects observed using TSF-II particles which involve interactions of ions with the light-harvesting apparatus should be absent in the TSF-IIa particle. The use of plants grown under conditions where the light-harvesting apparatus is deficient (such as the intermittent light system used for growing peas used in the developmental studies done with Dr. C. J. Arntzen's laboratory) could help to elucidate whether any effects
observed in the TSF-IIa particles which might be indicative of effects on the reaction center or dark electron transport reactions are observed in vivo in the absence of the light-harvesting apparatus. There is clearly enough work remaining concerning the effects of ions on PS II particles to keep a number of investigators busy for some time. It is hoped that the studies described here may provide a useful starting point for the more extensive studies necessary to resolve this problem.

Studies Involving the P700-Chlorophyll a Protein

We have also begun studies on the P700-chlorophyll a protein. This pigment protein complex has been isolated and characterized from a variety of plants by Shiozawa et al. (158) We have used spinach as our starting material. Spinach was not among the plants used by Shiozawa et al. (158)

Using the isolation procedure of Shiozawa et al. (158), we have obtained a protein fraction similar to their P700-chlorophyll a protein from spinach. Our preparations from spinach, however, do not appear to be as clean as those previously reported. (We have not tried to repeat the isolation of the P700-chlorophyll a protein from the same plant material used by Shiozawa et al. Thus, we cannot at this time state whether or not the differences observed by us is due to a real difference in the starting material or due to some unnoticed variation in our preparative method.) The nature of the differences between our preparations allows us to make
some interesting observations which might have otherwise have
gone undetected. These observations are discussed below.

The major difference observed in our preparation is the
presence of a small amount of the light-harvesting pigment protein
(LHPP). In our preparations, we have obtained chlorophyll a/
chlorophyll b ratios ranging from 4.4 to 7.0. Shiozawa et al.
(158) report that their preparations contain no chlorophyll b. We
have arbitrarily divided our preparations into two classes: those
having an a/b ratio of less than 5.0 and those with a ratio of
greater than 5.0. We have designated these classes as follows.

Preparation A  (chl) = 0.524 mg/ml  a/b = 5.93
Preparation B  (chl) = 0.516 mg/ml  a/b = 4.50

The visible spectrum of a typical preparation is shown in
Figure 56. The presence of chlorophyll b (or LHPP) is indicated
by the absorbance at 465 nm and 650 nm. The spectrum presented
by Shiozawa et al. (158) shows a lower absorbance at these two
wave lengths than does the spectrum presented in Figure 56.

The P_{700}-chlorophyll a protein which we have isolated
from spinach shows photochemical activity as evidence by its
ability to carry out the disproportionation of DPCN. This reaction
is characteristic of PS I. (196) Activities obtained with prepara-
tion A were in the range of 115-140 umoles DPCN dispropor-
tionated/mg chlorophyll/hr. Preparation B showed activities
between 65 and 100 umoles DPCN disproportionated/mg
Figure 56. Visible Absorption Spectrum of $P_{700}$-Chlorophyll a Protein. The visible absorption spectrum of the $P_{700}$-chlorophyll a protein was recorded using an Aminco-Chance spectrophotometer in the split-beam spectral mode of operation. The chlorophyll concentration was 0.501 mg chlorophyll/ml and the chlorophyll a/chlorophyll b ratio was 5.96. The buffer was 50 mM Tris-Cl, pH 8.2.
chlorophyll/hr. These activities were determined using a broad band red cut off filter. The intensity of illumination for such determinations was $7.2 \times 10^4$ ergs/cm²/second.

The addition of ions to the assay medium (2 mM Tris-Cl, pH 8.2, 1 mM DPCN) stimulated the rate of DPCN disproportionation with both Preparation A and Preparation B. This is shown in Table 10. The interesting feature of this effect is that it is greatest in the preparation which shows the lower a/b ratio or the greatest LHPP content. This would tend to imply that the presence of ions may increase the transfer of excitation energy from the LHPP to the P700 PS I phototrap.

To verify that transfer from the LHPP to the phototrap was affected by the addition of either mono- or divalent cations to the medium, we have looked at the stimulation of DPCN disproportionation by ions at different wave lengths of illumination. These results are also shown in Table 10 for both Preparation A and Preparation B. We have compared the amount of stimulation when ions are added to the assay medium and illumination provided through either a 650, 680, or 710 nm interference filter. It is clear that for both preparations the greatest stimulation is obtained when illumination is provided through the 650 nm filter. Smaller effects are noted as one illuminates with light of longer wave lengths. This is consistent with the above suggestion that ions affect the transfer of excitation energy from the LHPP to the
Table 10. Comparison of different illuminations on DPCN disproportionation by the P700-chlorophyll a protein in the presence and absence of ions.

Preparation A: chlorophyll a/chlorophyll b = 5.93

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Relative Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RCO</td>
</tr>
<tr>
<td>No additions</td>
<td>100</td>
</tr>
<tr>
<td>1 mM MgCl₂</td>
<td>384</td>
</tr>
<tr>
<td>10 mM NaCl</td>
<td>252</td>
</tr>
</tbody>
</table>

Preparation B: chlorophyll a/chlorophyll b = 4.50

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Relative Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RCO</td>
</tr>
<tr>
<td>No additions</td>
<td>100</td>
</tr>
<tr>
<td>1 mM MgCl₂</td>
<td>765</td>
</tr>
<tr>
<td>10 nM NaCl</td>
<td>360</td>
</tr>
</tbody>
</table>

DPCN disproportionation rates were determined as described in the section on experimental methods. Illumination was provided through either a broad band red cut off filter (RCO) or an interference filter of the desired wave length. The light intensities obtained using these filters are also given in the experimental methods section. Using each filter, the rates observed in the
presence of ions were expressed as a percentage of the rates obtained in the absence of ions. The assay medium consisted of 2 mM Tris-Cl, pH 8.2, 1 mM DPCN. The \( P_{700} \)-chlorophyll a protein was added to a concentration of 8 ug chlorophyll/ml just prior to the assay.
to the PS I phototrap. Illumination at 650 nm should excite primarily chlorophyll b of the LHPP. The $P_{700}$-chlorophyll a protein spectrum presented by Shiozawa et al. (158) shows little absorbance at 650 nm compared to our preparations. The 680 nm illumination may excite the chlorophyll a of both the LHPP and the $P_{700}$-chlorophyll a protein. Illumination at 710 nm should excite primarily the phototrap of PS I but may also excite the accessory chlorophyll a associated with the phototrap complex. The fact that we still observe a considerable stimulation of DPCN disproportionation by ions when illumination is provided with 710 nm light may indicate that ions also affect either the phototrap itself or electron transport near the phototrap. It is not possible to determine which is the case until we are able to clean up our preparation to remove the contaminating LHPP.

We have constructed concentration curves for the stimulation of DPCN disproportionation by both mono- and divalent cations for Preparation A and Preparation B. The concentration curve for the stimulation of this reaction by $Mg^{2+}$ is shown in Figure 57. The half-maximal stimulation was noted at a concentration of 0.8 mM $MgCl_2$. Although this curve indicates only the data from Preparation A, the concentration dependence exhibited by Preparation B was identical. The concentration dependence for the stimulation of DPCN disproportionation by $Na^+$ using Preparation A is shown in Figure 58. The half-maximal
Figure 57. Mg\textsuperscript{2+} Stimulation of DPCN Disproportionation by the P\textsubscript{700}-Chlorophyll a Protein. DPCN disproportionation was determined as described in the experimental methods section. The assay medium consisted of 2 mM Tris-Cl, pH 8.2, 1 mM DPCN. The P\textsubscript{700}-chlorophyll a protein was added to a concentration of 8.0 µg chlorophyll/ml. MgCl\textsubscript{2} was added to the desired concentrations. Illumination was provided through a broad band red cut off filter as described in the experimental methods section.
Stimulation of DPCN Disproportionation by Mg^{++}
Figure 58. Na⁺ Stimulation of DPCN Disproportionation by the P₇₀₀-chlorophyll a Protein. Conditions were as described for Figure 57 except that NaCl, rather than MgCl₂ was added to the desired concentration.
Stimulation of DPCN Disproportionation by Na⁺
stimulation is found at a concentration of 15 mM NaCl. The same concentration dependence was found using Preparation B.

Although the concentration dependence is different (higher concentrations are required to observe the effect), the effects of mono- and divalent cations appear similar to results observed recently in our laboratory concerning the effects of ions on the relative quantum yield of DPCN disproportionation by chloroplasts in low salt medium. (219) The results obtained in both cases are indicative of an increase in the transfer of excitation energy from the LHPP to the PS I phototrap when ions are added to the medium.

We are currently trying to obtain the $P_{700}$-chlorophyll a protein in a purer state lacking the LHPP contamination. Our initial attempt to accomplish this involved recycling the $P_{700}$-chlorophyll a protein through a column of hydroxylapatite using the same washing procedure as used in the original isolation of the protein. This attempt proved unsuccessful. If we can obtain the $P_{700}$-chlorophyll a protein free of LHPP contamination, we can see just what the effects of ions on the PS I phototrap are and then add back the isolated form of LHPP to see if the effects observed can be reconstituted. Two other types of PS I preparations have appeared since we began these studies. (220, 221) These preparations might also be useful in determining the effects of ions on PS I.
Miscellaneous Observations

Other Observations on LHPP

While working with LHPP, we have made some observations which may relate to the state of the chlorophyll in this pigment-protein complex.

When stored at 4°C in the dark, the LHPP will "go bad" in 2-3 weeks. This is marked by a shift of the Soret band of chlorophyll a to lower wave lengths (410-420 nm) and a broadening of the red band of chlorophyll a. The absorption spectrum of such a bad preparation is shown in Figure 59. Such changes are indicative of conversion of the chlorophyll a to pheophytin. When this occurs, the LHPP was no longer used for the protein-protein interaction studies described in detail earlier. The position and intensity of the chlorophyll b bands do not appear to change during this period of time.

If an SDS extract from which the LHPP is prepared is stored at 4°C in the dark for one month and then subjected to the same treatment used to isolate the LHPP, a protein fraction is obtained which shows an absorption spectrum which is enriched in chlorophyll b. Such a spectrum is shown in Figure 60. While we have not characterized this fraction, it seems likely that it is LHPP which has lost a considerable portion of its chlorophyll a. Such a system might be worthwhile for studying the environment of chlorophyll b in the LHPP if the residual chlorophyll a can be
Figure 59. Visible Absorption Spectrum of an Aged LHPP Preparation Which Has Gone Bad. The visible absorption spectrum of an aged LHPP preparation was recorded using an Aminco-Chance spectrophotometer in the split-beam spectral mode of operation. The LHPP concentration was 50 μg chlorophyll/ml in 10 mM Tris-Cl, pH 8.2.
Figure 60. Visible Absorption Spectrum of LHPP Prepared from a One-Month Old SDS Extract. LHPP was isolated as described in the experimental methods section from an SDS extract which was aged for one month at 4°C in the dark. The visible absorption spectrum was recorded using an Aminco-Chance spectrophotometer in the split-beam spectral mode of operation.
removed. Reconstitution experiments involving the readdition of chlorophyll a to such a protein may also shed light on the environment of chlorophyll a and the nature of its binding to the LHPP.

The above considerations tend to indicate that either chlorophyll b is in a relatively more protected environment than chlorophyll a or that the binding of chlorophyll b to the protein is stronger in nature than the binding of chlorophyll a. Since the only difference between chlorophyll b and chlorophyll a is the location of a formyl group on chlorophyll b in the position where there is a methyl group on chlorophyll a, the increase in the affinity of LHPP for chlorophyll b relative to chlorophyll a could result from the differences in this portion of the molecule. The formyl group of chlorophyll b may also provide a point of recognition for the pigment by the protein.

One possibility which has seemed attractive to this author is that the formyl group of chlorophyll b may be recognized and bound to the protein via hydrogen bonding to an amino group on the protein. By raising the pH, one might expect to observe the formation of a Schiff's base type linkage between the chlorophyll b and the protein which could be trapped by reduction with sodium borohydride. Thus far attempts to trap such a linkage have proved unsuccessful.

The only evidence we have been able to obtain which might possibly implicate an amino group in the binding of chlorophyll b
to the LHPP comes from data on the extraction of the chlorophylls from the LHPP with 80% acetone. A difference spectrum (80% acetone-2 mM Tris-Cl, pH 8.2) for the extraction of the chlorophylls from the LHPP is shown in Figure 61. The changes on absorption at four wave lengths are plotted as a function of acetone concentration in Figure 62. All of these curves are biphasic in nature. The first phase may indicate the unfolding of the protein as the acetone concentration increases and the second stage the actual release of the chlorophylls into the solvent. Similar curves have been observed using chloroplasts. (222) Figure 63 shows difference spectra similar to that shown in Figure 61. These spectra were observed in 10 mM sodium borate buffer at two different pH values. At pH 8.2, the difference spectrum obtained in borate buffer is qualitatively similar to that observed in Tris-Cl buffer of the same pH. Thus, changing the buffer does not appear to affect the extraction of the pigments by 80% acetone. At pH 10.2, however, there is a significant difference from the spectra observed at pH 8.2 in either Tris-Cl or borate buffer. The spectral region characteristic of chlorophyll a is affected about equally in all cases. The chlorophyll b regions of the spectrum, however, appear to be less affected at pH 10.2 than at pH 8.2. This may indicate that the chlorophyll b is not being released into the medium. Two possible explanations can be put forth to explain such an observation. First, it is possible that a Schiff's base is
Figure 61. Difference Spectrum for Extraction of Chlorophylls from LHPP in Tris-Cl Buffer by 80% Acetone. One ml of LHPP (10 µg chlorophyll/ml in 10 mM Tris-Cl, pH 8.2) was mixed with either four ml of acetone or four ml of water. The difference spectrum was recorded using an Aminco-Chance spectrophotometer in the split-beam, spectral mode of operation. The base line was determined using two samples containing 1 ml of the LHPP and four ml of water.
80% Acetone-2 mM Tris Cl pH 8.2

\[ \Delta A \]

\[ \lambda (\text{nm}) \]

490 nm, 430 nm, 410 nm, 458 nm, 640 nm, 660 nm, 676 nm

400 500 600 700
Figure 62. Extraction of Chlorophylls from LHPP as a Function of Acetone Concentration. Difference spectra were recorded as described in Figure 61 except that different concentrations of acetone were used. From these difference spectra, the absorbance changes at 458, 490, 660, and 676 nm were determined. These values were plotted as a function of acetone concentration. The signs in parentheses, (+) and (-), refer to the direction of the change at the stated wave length.
Figure 63. Difference Spectra for Extraction of Chlorophylls from LHPP in Borate Buffer by 80% Acetone. Difference spectra were recorded as described in Figure 61. The only difference was that the LHPP was in either 100 mM sodium borate buffer, pH 8.2 or pH 10.2.
being formed at the higher pH even though attempts to trap this linkage have proved unsuccessful. Second, it may be more difficult to denature the protein and release the pigment at the higher pH. At this time we cannot determine the exact nature of these differences. In the viewpoint of this author, studies of this nature are worth continuing at some future date.

Effect of Trypsin on Excitation Energy Distribution

It has been reported by Jennings and Forti (127) that the fluorescence changes characteristic of changes in excitation energy distribution are not present in chloroplasts treated with trypsin or fixed with glutaraldehyde. This was taken as evidence that the structural changes responsible for producing the fluorescence changes are protein based.

We have further studied the effects of trypsin on the structural and fluorescence changes produced by ions in chloroplasts. Figure 64 shows that trypsin does decrease the ability of divalent cations to reverse the monovalent cation induced decrease in chlorophyll a fluorescence. The monovalent cation induced decrease in fluorescence is due to a bias of excitation energy transfer in favor of PS I and has been correlated with an unstacking of grana stacks. The bias in favor of PS I may involve a spillover of excess excitation energy from PS II to PS I. The divalent cation reversal of the monovalent cation induced decreases in fluorescence has been shown to be related to the
Figure 64. Effect of Trypsin on the Divalent Cation Induced Fluorescence Changes in Chloroplasts. Steady state fluorescence levels were determined using an Aminco-Chance spectrofluorimeter. The assay medium consisted of 0.4 mM Tris base, 100 mM sucrose, and 10 μM DCMU. Chloroplasts were added to a concentration of 6.7 pg chlorophyll/ml. The volume of the assay mixture was 3 ml. Trypsin (2.5 x 10^-5 mg) was added and the mixture allowed to incubate for 0 (●), 1 (▲), or 4 (□) minutes prior to the addition of NaCl.
Fluorescence at 680 nm (Relative Units)
re-stacking of unstacked thylakoids and may represent an inhibition of spillover from PS II to PS I. Figure 64 shows that a short incubation with trypsin is sufficient to destroy the ability of divalent cations to produce the divalent cation induced reversal of the monovalent cation induced decreases in fluorescence. A one minute incubation (prior to the addition of Na$^+$) was sufficient to destroy about one-third of the divalent cation effect. A four minute incubation destroyed nearly the entire divalent cation effect.

In the preceding experiment, trypsin was added prior to the addition of the monovalent cation. The chloroplast membranes were thus in the stacked state. (129) Quite different results were obtained if the grana were unstacked by adding the monovalent cation prior to the trypsin. This is shown in Figure 65. From this figure, it is apparent that in the case where trypsin is added after Na$^+$, the divalent cation reversal of the monovalent cation induced decreases in fluorescence is still present. This is somewhat surprising in that one might expect the sites of trypsin action resulting in the inhibition of the divalent cation response to be at least as exposed, and possibly more so, in the unstacked state as in the stacked state. This however does not appear to be the case. We believe that this observation is indicative of a structural change within the membrane which accompanies the unstacking process. This structural change appears to limit
Figure 65. Effect of Trypsin on Divalent Cation Induced Fluorescence Changes in Chloroplasts. Assay conditions were as described in Figure 64. The only alteration was that NaCl was added prior to trypsin. After an incubation on the presence of NaCl and trypsin as indicated, CaCl$_2$ was added. Trypsin incubations were 0 ( ), 1 (□), or 3 (○) minutes prior to the addition of CaCl$_2$. 
Fluorescence at 680nm (Relative Units)

5mM NaCl

3.3 mM CaCl₂

2.5 x 10⁻⁵ mg trypsin added here

Fluorescence at 680nm (Relative Units)

0 1 2 3 4 5 6 7 8 9

0 20 40 60 80 100

1 (min)
access to the sites of divalent cation interaction such that large molecules like trypsin cannot gain access to them.

We have also looked at the effect of trypsin on the structural changes accompanying the divalent cation induced fluorescence changes. We have done this by following changes in the absorbance at 540 nm, a measure of turbidity or 180° light scattering which is indicative of large structural changes. The results are shown in Figure 66. Even after trypsin treatment, there appears to be a considerable reversal of the monovalent cation induced decreases in turbidity. It is interesting, however, that this reversal is only about 75% of the reversal obtained in untreated chloroplasts. We cannot tell at this time if this reversal in the trypsin treated chloroplasts is indicative of the restacking of unstacked thylakoids as it is in untreated chloroplasts. Electron micrographs would be required to determine if this is the case. If the divalent cation induced structural changes observed in the trypsin treated chloroplasts which do not show the divalent cation induced fluorescence changes do represent a restacking of unstacked thylakoids, this system may be useful in determining whether it is the large structural changes associated with the stacking and unstacking of grana or the smaller changes within the membrane which are responsible for the observed alterations in excitation energy distribution.
Figure 66. Effect of Trypsin on the Divalent Cation Induced Structural Changes in Chloroplasts. Structural changes were monitored by following absorbance changes at 540 nm (indicative of changes in turbidity on 180° light-scattering) using an Aminco-Chance spectrophotometer in the split-beam kinetic mode of operation. Assay conditions were as described in Figure 64 for the fluorescence measurements.
[Diagram showing the effect of adding 3.3 mM CaCl₂ and 2.5 mg trypsin on the absorbance (ΔA₅₄₀) over time (t, min)].
Since our major interest throughout this dissertation has been pigment-protein complexes, it was of interest to see if the inhibition by trypsin of the divalent cation induced structural and fluorescence changes affected the pigment-protein complexes. To do this we have solubilized membranes which have been treated with trypsin and compared the percentages of pigment associated with each band when the SDS extract was subjected to gel electrophoresis with the values obtained using untreated membranes. The procedure used was the same as that described and used in the developmental studies: solubilize in SDS, run gels, scan at 680 nm, and determine gravimetrically the relative percentage of pigment in the PS I chlorophyll a protein, the LHPP, and the free pigment bands. The results of such a study is shown in Table 11. It appears that there are no significant changes in the percentages of pigment associated with either of the three bands due to the trypsin treatment. Similar results are obtained regardless of whether the trypsin was added before or after Na⁺. The position of the bands in the gels was also unchanged indicating that large portions of the pigment-protein complexes were not being removed by trypsin. This evidence does not indicate that the pigment-protein complexes are not involved in the control of excitation energy distribution. Other proteins which may be necessary to observe the manifestation of the divalent cation induced responses could be the site of trypsin action. Trypsin
Table 11. Effect of trypsin on pigment protein complexes.

<table>
<thead>
<tr>
<th>Relative Pigment (Chl a) Content (%)</th>
<th>PS I chl a Protein</th>
<th>LHPP</th>
<th>Free Pigment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23.0</td>
<td>27.8</td>
<td>50.4</td>
</tr>
<tr>
<td>Control + 1 x 10^-5 mg trypsin</td>
<td>20.6</td>
<td>28.7</td>
<td>47.6</td>
</tr>
<tr>
<td>Control + 2 x 10^-5 mg trypsin/ml</td>
<td>20.4</td>
<td>29.4</td>
<td>47.8</td>
</tr>
<tr>
<td>Control + 5 mM NaCl + 1 x 10^-5 mg trypsin/ml</td>
<td>22.2</td>
<td>28.7</td>
<td>49.3</td>
</tr>
<tr>
<td>Control + 5 mM NaCl + 2 x 10^-5 mg trypsin/ml</td>
<td>21.6</td>
<td>27.8</td>
<td>49.6</td>
</tr>
</tbody>
</table>

The relative percentage of pigment (chlorophyll a) was determined as described in the experimental methods section. The procedure was the same as that used to follow the development of the pigment protein complexes during the greening of pea seedlings grown in the intermittent light system. Chloroplasts were treated with trypsin as indicated in the presence or absence of NaCl prior to solubilization with SDS.
has been shown to have many effects on chloroplast membranes in addition to the one discussed here. These effects, especially the inhibition of PSII electron transport by trypsin, have been shown to require an unstacking of the grana stacks before the effect could be observed. (223) This is in contrast to the data presented here. It is possible that some of these other effects may be contributing to the excitation energy distribution problem in indirect ways such as electron transport inhibition and alterations in membrane integrity.
CONCLUSIONS

With regard to the light-harvesting pigment protein (LHPP), we have shown that this protein has two classes of binding sites for divalent cations. Site I has a dissociation constant of 2.5 uM (extrapolated to zero protein concentration) while Site II has a dissociation constant of 32 uM. The dissociation constant for divalent cation binding to Site I and the number of binding sites in Site II vary with protein concentration in a manner indicative of two stages of cation independent association of the LHPP. Supporting evidence for the two stages of cation independent association of LHPP have come from analytical ultracentrifugation and studies of the absorption and fluorescence spectra as a function of LHPP concentration. While we have not determined molecular weights, the observations are consistent with a monomer-dimer-tetramer conversion and a model based on this has been proposed.

Divalent cations also bring about an association of the LHPP. The extent of this association varies in a manner consistent with the two stages of cation independent association of the LHPP. The concentration dependence for the divalent cation induced association of the LHPP indicates that divalent cation binding to Site II is responsible for the divalent cation specific...
association of the protein. We have not been able to assign any function to Site I.

We have also found that divalent cations bring about decreases in chlorophyll fluorescence from LHPP. The concentration dependence for these divalent cation induced decreases in chlorophyll fluorescence is sufficiently different from that observed for the divalent cation induced association of the LHPP to indicate that the two are not directly related.

An appreciable amount of evidence has accumulated in recent years regarding the ability of divalent cations to control excitation energy distribution between the two photosystems. Divalent cations have also been implicated in the cooperation between PS II photosynthetic units. (For a full discussion of these phenomena, see the literature review section.) We feel that the protein-protein interactions described in this dissertation could be related to these phenomena. An association of the LHPP would undoubtedly affect both the distance between and orientation of pigment molecules, changes which could greatly alter excitation energy distribution.

The best evidence we have obtained implicating LHPP in the divalent cation regulation of excitation energy distribution has come from our collaborative studies with the laboratory of Dr. C. J. Arntzen of the University of Illinois. They had found that pea seedlings grown in an intermittent light system did not show the
divalent cation induced changes in fluorescence characteristic of alterations in excitation energy distribution. Furthermore, chloroplasts from such plants did not form grana stacks and lacked the LHPP. When plants grown in the intermittent light system were greened for 24-48 hours, the LHPP was present, grana stacks were present, and the divalent cation induced fluorescence changes were present. In our studies with the laboratory of Dr. Arntzen, we followed the time course of appearance of these parameters, as well as changes in the divalent cation binding parameters of the chloroplast membrane, as a function of greening time after removal from the intermittent light system. These studies indicated that during the greening of plants grown in the intermittent light system, the LHPP is synthesized and incorporated into the chloroplast membrane. The incorporation of this pigment-protein complex into the chloroplast membrane brings about an increase in the relative number of Site II divalent cation binding sites on the chloroplast membrane resulting in the ability of divalent cations to produce changes in excitation energy distribution and the ability of the membranes to form grana stacks.

We have also looked at the effects of ions on a sub-chloroplast particle characteristic of PS II (TSF-II) which is enriched in the LHPP. This particle is photochemically active as evidenced by its ability to carry out the DPC dependent photoreduction of DCIP, a PS II reaction. In studying the effects of ions on
this reaction, we have found two distinct types of effects. Divalent cations, at low concentrations, inhibit DCIP reduction by the TSF-II particles. This inhibition is half-maximal at concentrations of 70-90 uM MgCl₂. Light intensity curves indicate that light dependent steps, rather than dark electron transport reactions, are affected. High concentrations of monovalent or divalent cations also inhibit DCIP reduction by TSF-II particles, apparently due to an ionic strength effect. Light intensity curves also show that this ionic strength effect involves light, rather than dark, reactions. A Debye-Huckel analysis of the ionic strength data is consistent with the inhibition by the ionic strength effect being due to the disruption of a +1:-1 charge pair. The results observed for the inhibition of DCIP reduction by TSF-II particles by the divalent cation specific and ionic strength effects are consistent with either an inhibition of PS II photochemistry or the transfer of excitation energy from the light-harvesting apparatus to the PS II phototrap. It is possible that effects at both of these levels exist.

Ions were also found to produce decreases in chlorophyll a fluorescence from TSF-II particles. The divalent cation specific and ionic strength effects produce different types of fluorescence changes. The nature of the cation induced fluorescence decreases in TSF-II particles is complex and depends on a number of factors including the presence of an electron donor to PS II, the
state of the PS II phototrops, the wave length of excitation, and possibly the medium in which the system is studied. Under certain conditions, the divalent cation induced decreases in chlorophyll a fluorescence appear to be similar to those observed with the LHPP. The possibility that divalent cations may create "quenching centers" in the light-harvesting apparatus which are able to compete effectively with the PS II phototrops for excitation energy cannot be ruled out at this time. Such a phenomenon would be consistent with the observation that divalent cations cause both a decrease in the rate of DCIP photoreduction and a decrease in chlorophyll a fluorescence.

The effects of ions on DPCN disproportionation by the P700-chlorophyll a protein (characteristic of PS I) has also been looked into. Our preparation of this protein is contaminated with a small amount of LHPP. Thus far we have been unable to obtain a preparation of the P700-chlorophyll a protein from spinach which lacks this contamination. Both mono- and divalent cations stimulate the rate of DPCN disproportionation by our preparations of the P700-chlorophyll a protein. This stimulation appears to be due to an increase in the transfer of excitation energy from the LHPP to the PS I phototrap. The concentrations at which half-maximal stimulation is noted is 0.8 mM for divalent cations and 15 mM for monovalent cations.
The observations made here that divalent cations inhibit DCIP reduction by TSF-II particles and promote the transfer of excitation energy from the LHPP to the PS I phototrap are possibly related to the effects of ions on the relative quantum yields for PS I and PS II reactions recently observed by Gross (219) under low salt conditions in the absence of Tricine. In the presence of Tricine, divalent cations have been shown to decrease the relative quantum yield for PS I reactions while increasing the relative quantum yield for PS II reactions. (97, 219)

If Tricine is replaced with low concentrations (0.2-0.4 mM) of Tris, Gross (219) has observed much different effects. Under these conditions, divalent cations inhibit PS II reactions while stimulating PS I reactions. The dependence of the stimulation of PS I reactions on the wave length of illumination is consistent with an increase in the transfer of excitation energy from the light-harvesting apparatus to the PS I phototrap. The observations made in this dissertation that similar effects can be found in subchloroplast preparations containing only one of the two photosystems suggests that these effects may be characteristics of the individual photosystems. These results, along with those of Gross (219), may represent another level of divalent cation control of photosynthetic processes.

The data presented in this dissertation are consistent with the proposed role of divalent cations as regulators of excitation
energy distribution between the two photosystems. This control appears to be intimately related to the presence of LHPP in the membrane, the ability to form grana stacks and the binding of divalent cations to the chloroplast membrane. There also appears to be another level of regulatory effects of divalent cations recently observed by Gross. (219) These effects appear to take place at the level of the individual photosystems. Using sub-chloroplast preparations, where problems of the distribution of excitation energy between the two photosystems are not encountered since only one photosystem is present, we have found effects corresponding to the inhibition of PS II photochemistry and the promotion of excitation energy transfer from the light harvesting apparatus to the PS I phototrap observed by Gross. (219)

While we have answered many of the questions we set out to, we have created as many problems as we have solved. Further work is needed to determine the nature and cause of the divalent cation induced decreases in chlorophyll fluorescence observed with the LHPP. Experiments to settle this question are currently in the planning stages. These decreases do not appear to be directly related to the divalent cation induced association of the LHPP. The work we have begun with the TSF-II particle and the P700-chlorophyll a protein appears to be a very productive avenue for future study. The TSF-II particle shows a divalent cation specific and an ionic strength effect of the light dependent steps of
electron transport required for the reduction of DCIP. We have as yet not been able to establish whether these effects are at the level of the PS II phototrap or the light-harvesting apparatus. The possibility that divalent cations might create "quenching centers" in the light harvesting apparatus is now being considered. Experiments are in the planning stage involving oxygen electrode work, the ability of divalent cations to produce fluorescence changes under anaerobic conditions, and the preparation of a smaller PS II particle (TSF-IIa) which contains the PS II phototrap, but lacks a large portion of the light-harvesting apparatus including the LHPP. Chloroplasts from plants grown under conditions where the light-harvesting apparatus is deficient, such as the intermittent light system of Arntzen used in our developmental studies, may also prove useful in establishing whether these effects are at the level of the PS II phototamps or the light-harvesting apparatus. The preliminary studies on the P_{700}-chlorophyll a protein, slightly contaminated with LHPP, have shown that ions promote the transfer of excitation energy from the LHPP to the PS I phototrap. A cleaner preparation of the P_{700}-chlorophyll a protein lacking the LHPP contamination is clearly needed to establish if there are ion effects on the P_{700}-chlorophyll a protein itself. If such a clean preparation can be obtained, it should be possible to add back the LHPP in isolated form in an attempt to reconstitute the effects we have observed with our LHPP contaminated preparation of the P_{700}-chlorophyll a protein. Similar experiments should also be done regarding the interactions of LHPP with a TSF-IIa particle.
to see if the effects we have observed with the TSF-II particle can be reconstituted. Finally, an attempt could be made to reconstitute the $P_{700}$-chlorophyll a protein, the TSF-II a particle, and the LHPP into a system where changes in the distribution of excitation energy between the two photosystems might be observed. Such experiments will not be simple to do or interpret and a considerable investment of time may be required to fully reconstitute a working system. In the view of this author, such experiments are worth the effort. Such a line of study may not only provide information regarding the organization of the individual photosystems and the interactions within and between photosynthetic units, but may aid in our understanding of the structure and organization of photosynthetic membranes.
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