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Title: INVESTIGATION OF THE OXIDIZING SIDE OF PHOTOSYSTEM II

THROUGH MUTANT ANALYSIS

Abstract approved: Redacted for privacy
                    (Norman I. Bishop)

The approach of mutant analysis has been taken in an effort to add
to the current sparse knowledge concerning the biochemical composition
of the water-splitting apparatus of photosynthetic organisms. Several
low°flourescent mutants of the green alga Scenedesmus obliquus D_3 with
altered oxygen-evolving capacities are characterized. The mutants LF-1,
LF-3 and LF-5 are shown to be unable to use water as an electron source
in photoreductions. Whole cell and cell free photoreactions using al-
ternate electron donors proceed at rates comparable to those of the
wild-type strain. These data, plus the ability of a photosystem II
electron donor to restore the variable component of the flourescence
yield to whole cells of the mutants, indicate that the functional al-
teration of the photosynthetic apparatus is restricted to the oxidizing
side of photosystem II. The thylakoids of these mutants are lacking
about 60 percent of the manganese normally bound to the membranes of
the wild-type strain. Analysis of the cytochrome content of thylakoids
of LF-1 reveals a loss of the high potential form of cytochrome b-559

but not of total b-type cytochromes or cytochrome f.

The mutant LF-2 is shown to be temperature dependent in the expression of its mutant phenotype. When grown at $20^{\circ}C$, LF-2 strongly resembles the wild-type organism. However, when grown at $34^{\circ}C$, it shows a severe loss of photosynthetic activity. The functional alteration in the photosynthetic apparatus of LF-2 ($34^{\circ}C$) is again demonstrated to be restricted to the oxidizing side of photosystem II. LF-2 shows a progressive loss of thylakoid-bound manganese and of the high potential form of cytochrome b-559 as the growth temperature is raised. Photosynthetic capacity of cells of LF-2 ($34^{\circ}C$) can be restored by incubation for a few minutes in 1 mM Mn^{+2}. It is concluded that LF-2 is altered in its ability either to take up or incorporate manganese ion into the thylakoids and that the photosynthetic apparatus itself has not been affected. Restoration of photosynthetic activity in LF-2 ($34^{\circ}C$) also results in a reappearance of the high potential form of cytochrome b-559, and indicates that the loss of this cytochrome in LF-1 would be a consequence of its manganese deficiency.

Analysis of the polypeptide composition of the thylakoid membranes of the wild-type and the mutants LF-1, LF-3, and LF-5 by lithium dodecyl-sulfate polyacrylamide gel electrophoresis reveals a shift in mobility of one major protein band from an apparent molecular weight of 34 kilodaltons in the wild-type to 36 kilodaltons in the mutants. The polypeptide patterns of the mutant LF-2 are identical to those of the wild-type. It is hypothesized that the 36 kilodalton polypeptide represents a non-processed precursor of the physiologically active 34 kilodalton polypeptide of the wild-type.

The data suggest that the 34 kilodalton polypeptide is required for the activity of the oxidizing side of photosystem II and for the binding of the manganese involved in water photolysis.

Investigation of the Oxidizing Side of
Photosystem II Through Mutant Analysis

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LIST OF ABBREVIATIONS

A	Absorbance
Asc	Ascorbate
ATP	Adenosine triphosphate
b-559	Cytochrome b-559
b-563	Cytochrome b-563
chl	Chlorophyll
cm	10^{-2} meters
Cyto f	Cytochrome f
DCMU	3-(3,4-dichlorophenyl)-1,1 dimethyl urea
DPC	Diphenyl carbazide
DPIP	2,6-dichlorophenol-indophenol
E_m	Midpoint oxidation-reduction potentials
EDTA	Ethylenediaminetetraacetic acid
Fd	Ferredoxin
Fe·S	A non-heme iron-sulfur center
HQ	Hydroquinone
HP	High potential
kd	10^3 daltons
keV	10^3 electron volts
LDS	Lithium dodecyl-sulfate
LP	Low potential
M	Molar
ma	10^{-3} amperes
MV	Methyl viologen
N	Normal

NADP	Nicotinamide adenine dinocleotide phosphate
nm	10^{-9} meters
PAGE	Polyacrylamide gel electrophoresis
PC	Plastocyanin
PCV	Packed cell volume
PQ	Plasto quinone
PSI	Photosystem I
PSII	Photosystem II
Q	The primary electron acceptor of Photosystem II
R	The secondary electron acceptor of Photosystem II
SDS	Sodium dodecyl-sulfate
STK	Sucrose-tricine-KCl
TKE	Tricine-KCl-EDTA
Tris	Tris (hydroxymethyl) aminomethane
V/V	Volume/volume
W/V	Weight/volume
X	The primary electron acceptor of Photosystem I
X-430	The secondary electron acceptor of Photosystem I

INVESTIGATION OF THE OXIDIZING SIDE OF
PHOTOSYSTEM II THROUGH MUTANT ANALYSIS

I. INTRODUCTION

Photosynthesis can properly be viewed as the conversion of the
electromagnetic energy of light into the chemical energy used in biologi-
cal reactions. In green plants, eukaryotic algae and the cyanobacteria,
a more specific description would be that light is used to drive the
transfer of electrons against a thermodynamically unfavorable energy
gradient from water to nicotinamide adenine dinucleotide phosphate
(NADP); additional energy conservation occurs along the way through
formation of adenosine triphosphate (ATP) from adenosine diphosphate
(ADP) and inorganic phosphate. Molecular oxygen is released as a by-
product of the process.

Although some forms of bacteria also convert light energy into
chemical energy, they possess a type of photosynthetic system distinct
from that of the aforementioned organisms in that they lack the ability
to use water as a reductant. As a consequence, they do not evolve oxy-
gen and require a major source of an alternate electron donor.

The assimilatory power generated by the 'light reactions' can be
utilized in many of the energy requiring cell processes. A major use is
that of reduction of CO_2 to carbohydrate, but nitrate or sulfate re-
duction or nitrogen fixation as well as other reactions can consume
large amounts of this energy. These so called 'dark reactions' of
photosynthesis can occur independently of the light of substrate
quantities of reductant and ATP are provided. Additionally, they occur
in the stroma of the chloroplast, or water matrix of the cyanobacterial
cell, whereas the electron transport activities associated with the

energy trapping system are restricted to the photosynthetic membranes.

This thesis is an attempt to add to the present knowledge concerning the biochemical makeup and nature of the water-splitting apparatus of oxygen evolving photosynthetic organisms. The 'dark reactions' and bacterial photosynthetic systems will not be discussed except where specific examples are required for clarity.

The Z Scheme--General Aspects

Most of the models currently presented to represent the components and energetics of the photosynthetic electron transport pathway in oxygen evolving organisms use the basic Z scheme of Duysens et. al. (1961) superimposed on an oxidation-reduction potential energy scale. This scheme indicates that there are two distinct photochemical reaction centers connected in series through an electron transport chain and with an electron donor system on the high potential side and an electron acceptor system on the low potential side. Alternative, or cyclic, pathways around the reaction centers are also included. Figure 1 is an attempt to incorporate current knowledge into the Z scheme format. It is based on the work of countless early investigators (see recent reviews by: Bishop, 1971a; Govindjee, 1975; Trebst and Avron, 1977) and also recent work by Butler (1978) and Malkin and Posner (1978).

While the scheme is presented in a two-dimensional, energetic format, it should be recalled that the system exists in a dynamic, three-dimensional biological membrane system probably possessing qualities described by Singer (1974). His fluid mosaic model for the membrane implies a somewhat free lateral mobility for protein and protein aggregates but a more restricted transmembrane mobility, providing a rationale

for the observed asymmetry of the photosynthetic membranes.

Most of the studies of photosynthesis have been done using higher plants, and most of these with a few representative species such as spinach, maize, peas and lettuce. It is assumed, and experimental data seem to verify, that there is a considerable homology of mechanism among the higher plants and between higher plants and the green algae. The relationship to the cyanobacteria is of course more distant and greater differences would be expected. In the description of the components of the photosynthetic chain it is assumed that the general features apply equally to the green algae and to higher plants except where specific differences are mentioned.

The pathway for electrons in photosynthesis as shown in Figure 1 can be separated into two distinct sections, designated photosystem I (PSI) and photosystem II (PSII). That this is not an arbritrary distinction has been demonstrated by the physical isolation of PSI and PSII enriched preparations, originally by Boardman and Anderson (1964) and subsequently by many others.

Also, there are many examples of the independent operation of the photosystems in vivo. For example, the heterocysts of nitrogen-fixing cyanobacteria appear to posses only PSI activity, allowing for generation of reducing power and ATP without production of oxygen which would poison the nitrogenase enzyme (Douze et. al., 1972). Also, some members of the green algae, such as Scenedesmus, after a period of anaerobic adaptation, can reduce CO_2 using hydrogenase gas as an electron source in a strictly PSI dependent reaction (Bishop, 1967).

The essential features of the scheme can be summarized as follows: Light is absorbed by an array of chloroplyll molecules associated with
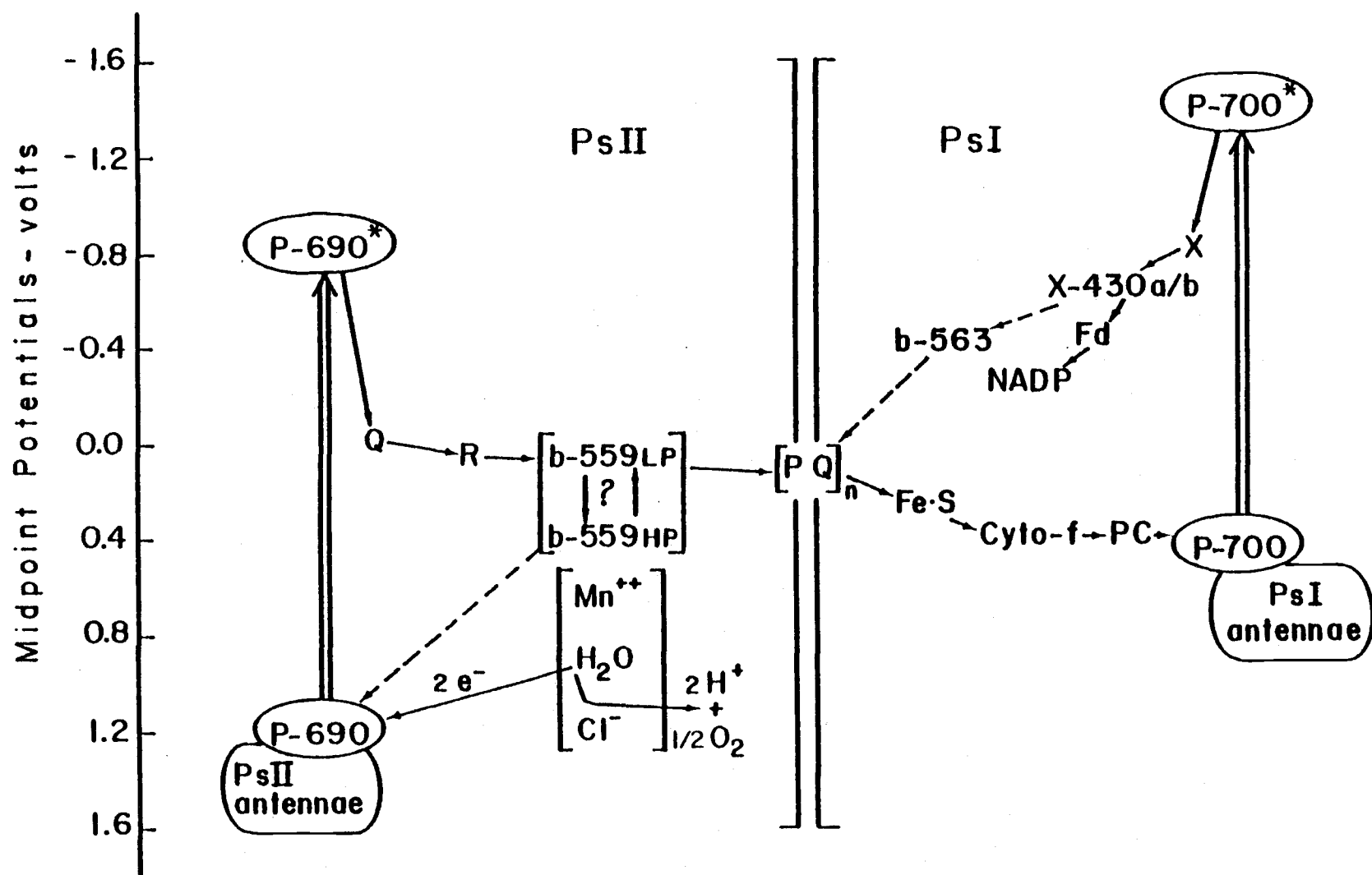
Figure 1.   The Z Scheme for photosynthetic electron transport.

PSI (PSI antennae chlorophylls) and is transferred to the reaction center resulting in a charge separation which produces a strong reductant capable of reducing NADP, and a weak oxidant which is reduced by electrons traversing down (in an energetic sense), a chain of redox-couples. The source for these electrons is the reaction center of PSII, which has used light absorbed by its antennae chlorophylls to initiate a charge separation analogous to that of PSI. The photoact of PSII results in the production of a weak reductant and a strong oxidant. The oxidant removes electrons sequentially from water resulting in the release of oxygen. Since the reactions which result in oxygen evolution are oxidations, initiated by the photoact of PSII, this portion of the chain is referred to as the oxidizing side of PSII.

Not shown in Figure 1 is the major light harvesting pigment-protein complex which absorbs most of the light energy used to drive photosynthesis. This complex plays no direct role in the photochemical reactions but transfers its absorbed energy to the antennae systems of the reaction centers (Thornber, 1975).

There are at least two sites along the electron transport chain at which there is a vectorial release of protons to the inside of the thylakoid membrane (Gould and Izawa, 1973). The first (site I) is between the photosystems and probably involves a proton shuttle mechanism mediated by plastoquinone. The second (site II) is now accepted to be at the water oxidizing site, where four protons are released as four electrons are removed from two water molecules, resulting in the release of an oxygen molecule.

This implies that there should be two protons released to the inner thylakoid space per electron traveling from water to the terminal PSI

electron acceptor. Proton to electron ratios of greater than two have been reported (Fowler and Kok, 1976), and recently Velthuys (1978), proposed that a third site for proton translocation exists. Based on the correlation of proton uptake with an indicator of electron flow between the photosystems (i.e., an absorbance change at 515 nm), he suggested the site was located on the reducing side of PSII near site I.

According to the chemiosmotic theory of Mitchell (1966), it is the proton gradient across the membrane which is utilized to provide the energy required for ATP formation. It should be noted that the term site refers only to the location of proton release and does not imply that ATP formation occurs at that location or that there is necessarily a strict stoichiometry between protons released and number of ATP molecules formed. It is now recognized that ATP formation occurs at a protein complex, termed the coupling factor and recognition site (CF_I and CF_0), which is not directly involved in electron transport.

Two nonlinear, or cyclic pathways have been included in the electron transport scheme. Cyclic electron flow, driven by the PSI photochemical reaction center, is thought to represent a means by which the cell can generate ATP independently of reductant production, as the electrons pass repeatedly through the proton translocating site I. The various energy and reductant demands of the cell could be satisfied by regulation of the non-cyclic and cyclic flow of electrons. Evidence for the in vivo occurrence of this process is provided by the light induced increase in ATP levels, in leaf discs and in algae, which is insensitive to inhibitors of non-cyclic electron transport (Forti and Parisi, 1963; Bedell and Govindjee, 1973). There is also an abundance of spectroscopic evidence with both broken (see Trebst, 1977 for review) and

intact chloroplasts (e.g., Slovacek et. al., 1979), indicating that a cyclic pathway driven by PSI and independent of PSII does exist. Cyclic flow around the PSII reaction center has also been demonstrated, with cytochrome b-559 as the carrier, both with isolated chloroplasts (see Cramer and Whitmarsh, 1977) and recently _in_ _vivo_, under conditions of restricted electron flow to PSI (Mende, 1980).

It should be noted that the Z scheme as presented in Figure 1 is intended to be used as an operational model only. While there is considerable experimental support for this scheme, others have been proposed. Knaff and Arnon (1969a) suggested that cyclic photophosphorylation occured at a reaction center complex (PSI) which was separate from the two reaction centers involved in linear electron flow (PSIIb and PSIIa). Huzisige and Yamamoto (1972) also propose that there are three reaction centers, but indicate that there are two PSII reaction centers which can donate electrons to PSI. Experimental support for these alternative schemes is not abundant at the present time and therefore, the data presented in this thesis will be discussed in terms of the Z scheme, but with the proviso that alternative interpretations may be possible.

Photosystem I

PSI and components associated with PSI function are diagrammed on the right-hand side of Figure 1. The PSI reaction center contains a special chlorophyll molecule, designated P-700, which is the site of the light induced charge separation (Kok, 1976). Recent work by Mullet et. al. (1980), indicates that approximately 110 chlorophyll a molecules may serve as light harvesting antennae specifically for this PSI reaction

center, with 40 serving as 'core' antennae and the others physically
more peripheral. The primary electron acceptor of P-700, whose chemical
identity is still not certain is indicated by an X. In bacterial sys-
tems the initial acceptor has been found to be a bacteriopheophytin
molecule (Parsons and Cogdell, 1975) and the possibility of an analogous
situation, i.e., a special chlorophyll molecule associated with P-700,
has not been excluded for the PSI reaction center. The next electron
acceptor is an iron-sulfur center or possibly two distinct centers,
designated P-430, A and B (due to an absorption change at 430 nm ob-
served upon their reduction). This chain of acceptors serves to pro-
duce a rapid and substantial charge separation across the membrane
(see Malkin and Bearden, 1978).

Electrons from P-430 can reduce ferredoxin, a low molecular weight,
water-soluble protein, first isolated from plant material by Davenport
et. al. (1952), which can serve as an electron donor in a number of
biological reactions. The two reactions of interest here are NADP
reduction, which is catalyzed by the flavoprotein Ferredoxin-NADP oxido-
reductase, and reduction of cytochrome b-563 to initiate cyclic elec-
tron flow around PSI.

The primary donor to P-700 is the copper protein plastocyanin
(Katoh et. al., 1961). Cytochrome f, a c-type cytochrome, is generally
believed to operate in a linear pathway between PSII and PSI and to
reduce plastocyanin rather than P-700 itself (see Cramer and Whitmarsh,
1977 for a review). There is conflicting evidence, however, and other
roles and locations for cytochrome f have been proposed (see Witt, 1979).
Some algae, in addition to the membrane bound cytochrome f, also
possess a soluble c-type cytochrome (C-553) which can replace

plastocyanin in its function under certain circumstances, most notably during growth in copper deficient media (Wood, 1978).

Malkin and Aparico (1975) recently found that photosynthetic membranes contain a high potential iron-sulfur center which probably functions in the electron transport chain between plastoquinone and cytochrome f. It is termed the Rieske center by analogy to a similar center of the mitochondrial chain (see Malkin and Bearden, 1978, for a discussion of its properties).

Plastoquinone was first demonstrated to be an essential component of the electron transport pathway by Bishop (1959), who used purified plastoquinone A to reconstitute Hill reaction activity to lypholized, petroleum ether extracted chloroplasts. In higher plants there are approximately ten plastoquinone molecules per PSII or PSI reaction center while in the eukaryotic algae the ratio can be as high as 100. Experiments using low concentrations of the PSII inhibitor 3-(3,4-dichlorophenyl)-1,1 dimethyl urea (DCMU) indicated that electrons from a single PSII complex can be donated to several different PSI complexes, and that the transfer is mediated through the plastoquinone pool (Siggel et. al., 1972). Electron transport through plastoquinone is thought to result in a translocation of protons from the stroma to the inner thylakoid space and it is included as part of the endogenous cyclic pathway which results in photophosphorylation.

The chloroplast membrane contains several lipophilic quinones other than plastoquinone. Several of these have been suggested to play a role in electron transport, however, at the present time all have been eliminated from direct participation with the possible exception of Vitamin E (α-tocopherol). Sicher (1977) investigated a mutant of <u>Scenedesmus</u>

which could not synthesize Vitamin E (Bishop and Wong, 1974), and con-
cluded that Vitamin E was not required for electron transport or phos-
phorylation in the alga. In contrast, Barr and Crane (1977), using
lipophilic, stable free radicles to remove specifically Vitamin E from
spinach chloroplasts, proposed that this quinone was required for
electron transport on the reducing side of PSII. More work is required
to resolve these discrepancies and therefore Vitamin E has not been
included in Figure 1.

Photosystem II--Reaction Center and Reducing Side

There is general agreement that PSII contains a single reaction
center operating in a manner analogous to that of PSI, although there
is currently less information available for this system. Döring et. al.
(1967), were the first to attribute a flash induced absorption change
at 690 nm to the bleaching of the PSII reaction center chlorophyll,
hence, the name P-690 (also called P-680). Presumably, P-690 derives
its special characteristics from association with a protein component
of the membrane and also posseses an array of antennae chlorophyll
molecules arranged in a manner similar to that described for P-700.
Recent improvements in gel electrophoresis techniques have allowed for
the isolation of pigment-protein complexes which may represent the
reaction center and the antennae of PSII (Delepelaire and Chua, 1978).

An analysis of the fluorescence yield changes in Chlorella cells
allowed Kautsky et. al. (1960), to deduce that there were two photo-
systems involved in the photosynthetic electron transport system and
that the level of fluorescence was influenced by the redox state of a
substance between the systems. Duysens et. al. (1961) extended this
work and introduced the term Q for this substance which would quench

fluorescence when in the oxidized state (i.e., when it was able to accept electrons from PSII). Today, Q refers specifically to the primary electron acceptor of PSII. The chemical identity of Q remains controversial, but there is considerable evidence to indicate that it may be a plastoquinone molecule operating as a one-electron carrier in a special environment. This hypothesis is based on the presence of an absorption change peaking at 320 nm (the compound being termed X-320), whose kinetics correlate with those of P-690 (Stiehl and Witt, 1968), and which corresponds to the absorption spectrum of the semi-quinone anion of plastoquinone (Van Gorkum, 1974). There are reports of primary acceptors other than X-320 (e.g., Eckert, et. al, 1979), but their roles in PSII are uncertain.

β-carotene is closely associated with the reaction centers of both PSII and PSI. Its presence has been associated with a light-induced absorption change at 550 nm (C-550) which was thought to be due to the reduction of the primary acceptor of PSII. However, Malkin and Knaff (1973) demonstrated PSII activity in its absence and C-550 is now regarded to be an indirect effect of the charge separation across the membrane generated by the PSII photoact, i.e., a band-shift phenomenon. It is likely that β-carotene serves a photoprotective role and may also be required for maximum efficiency in the trapping of excitation energy at the reaction center (Searle and Wessels, 1978).

Based on analysis of fluorescence increases induced by dithionite or DCMU in chloroplasts with inactivated water-splitting systems and provided with alternate electron donors, Velthuys and Amesz (1974) proposed that there is a secondary acceptor (R) operating between X-320 and the large plastoquinone pool which links the photosystems. They

observed a period of two in the fluorescence pattern and suggested that two negative charges must accumulate at R, prior to its reacting with the plastoquinone pool. The identity of R is unknown at this time, but it is possibly another special plastoquinone molecule. Barr and Crane (1977) suggest it may be Vitamin E. R donates either to plastoquinone or possibly to cytochrome b-559.

It must be noted that definition of the electron transport system of PSII has relied heavily upon kinetic observations, on fluorescence and on electron paramagnetic resonance (EPR) analyses and it is, therefore, difficult to present a concise picture of this mechanism. Direct biochemical studies on the electron transport components of this system have been difficult because of the general lability of PSII. For this reason, the proposed functions for the PSII components remains somewhat arbitrary.

Photosystem II--The Oxidizing Side

Water Oxidation

The equation for whole plant photosynthesis as originally defined by de Saussure in the early 19th century is:

$$H_2O + CO_2 \longrightarrow CH_2O + \frac{1}{2}O_2.$$
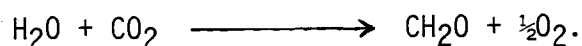
The equation does not indicate the origin of the free oxygen product and for many years it was assumed that the source was carbon dioxide. However, the experiment in 1940 by Hill and Scaresbrick demonstrating oxygen evolution by isolated, broken chloroplasts using 'artificial' electron acceptors indicated that carbon dioxide was not involved. Ruben et. al. (1941), using ^{18}O labeled water attempted to obtain direct evidence that the molecular oxygen originated from water. However, their results were ambiguous due to the high rate of exchange reactions

between water and bicarbonate (Brown and Frenkel, 1953; and Metzner, 1975). Their experiments were repeated by Holt and French (1948) using 'cleaner' chloroplast preparations, and one of the central dogmas of photosynthesis mechanistics was directly substantiated, i.e., water is the source of photosynthetic oxygen.

Since diatomic oxygen originates from water photolysis, a minimum of two water molecules must be involved per oxygen molecule released:

$$2H_2O \longrightarrow 4\ e^- + 4H^+ + O_2.$$
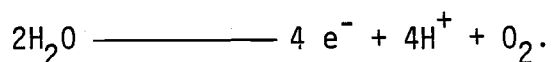
Since each photoact transfers a single electron at a time through a reaction center, a minimum of four quanta (i.e., four photoacts) are required per oxygen released (and eight are required for electrons traversing both photosystems).

In 1955, Allen and Franck noticed that with anerobically adapted algae, the oxygen yield produced by flashes of light (0.5 ms/flash) was greater on the second than the first flash. This work was extended by Joliot and Joliot (1968), who used aerobic, dark adapted algae and a sensitive oxygen probe to study the effect of brief saturating flashes of light on oxygen evolution. They found that little or no oxygen was released on the first two flashes, the greatest amount on the third and an intermediate amount on the fourth. With subsequent flashes, this pattern was repeated although the amplitudes dampened out to a steady state rate of oxygen production after many flashes. Kok et. al. (1970) proposed a model for water oxidation which incorporated these observations. Their scheme indicates that the oxygen evolving system can exist in five states (S_0, S_1, S_2, S_3 and S_4), termed the S-states, which differ in the amount of positive charge stored in the total system. To explain the observed periodicity in the flash-yield pattern, they predicted that four sequential photoacts result in four electrons being

removed from the system. Oxygen is released from the center only after the fourth positive charge of the set has reached the center. This also results in the $S_4 \longrightarrow S_0$ transition and the center is ready to begin the water oxidation process again.

To explain why the maximum yield occurs on the third flash for dark adapted algae, they hypothesized that only states S_0 and S_1 are stable in the dark, while states S_2 and S_3 are unstable and decay to S_1. Therefore, most of the centers are in the S_1 condition when the first flash is received. Dampening of the oscillation in O_2 yield is thought to occur through the operation of two processes, double hits, and, misses. Double hits result from the actinic flash being of longer duration than the photoact, allowing some reaction centers to undergo two transitions during one flash. The misses are probably due either to a failure of some reaction centers to undergo a charge separation, or to a back reaction occurring prior to stabilization of the charge separation.

Renger (1977 and 1978) proposed a detailed hypothetical model for the molecular mechanism of this water oxidation process which incorporates the kinetic data and also provides a rationale for involvement of cytochrome b-559 (see later sections). Confirmation of its validity awaits further investigation.

Manganese

Pirson (1937) found that manganese deficiency in Chlorella results in a decrease in photosynthetic efficiency which can be easily and rapidly reversed by simple addition of manganese salts to the suspending medium. This easy reversibility makes manganese deficiency unique among the essential trace elements. Kessler (1959) determined

that manganese was not required for photoreduction of CO_2 by hydrogen in adapted algae (an exclusive PSI type reaction) and predicted that it was required only for the oxygen-evolving reactions. Subsequent investigations have verified his interpretations and, in light of the current concepts, have placed its site of function on the oxidizing side of PSII (see Cheniae and Martin, 1970; and Cheniae, 1970). It is estimated that there are five to eight manganese atoms per PSII reaction center in Scenedesmus membranes, and five to six per reaction center in spinach (Cheniae and Martin, 1971a). It has been shown that oxygen-evolving capacity is abolished when approximately two-thirds of the membrane bound manganese is removed by means of Tris buffer or hydroxylamine extraction (Cheniae and Martin, 1970). The remaining one-third is more tightly bound to the thylakoids than is the larger pool. The function of this tightly bound manganese is not certain. Its presence seems to be required for oxidation of artificial electron donors to PSII, although this may be an indirect effect, as the structure of the thylakoids is disrupted by such extreme manganese deficiencies. Some evidence exists that manganese is tightly bound to polypeptides associated with light-harvesting pigment-protein complex (Lagoutte and Duranton, 1975; Foyer and Hall, 1979), but the relationship of this to the manganese required for PSII activity is still uncertain.

Most workers have assumed that the larger pool is involved directly with water oxidation, either by complexing of intermediates or via charge accumulation through oxidation of the manganese ion itself. The fact that a variety of oxidation states are known for manganese, some of which possess highly positive oxidation-reduction potentials in both free and bound states, certainly makes it an attractive candidate for participation in these reactions.

Hydrated Mn^{++} in free solution exhibits a distinct six-line elec-
tron paramagnetic spectrum. Chelation of any sort, such as with EDTA or
binding to protein, results in the disappearance of this spectrum.
Studies using this phenomenon have verified that inhibition of oxygen
evolution by Tris treatment is accompanied by the release of two-thirds
of the membrane manganese and indicate that it is released to the inner
thylakoid space--demonstrating that it is bound to the same side of the
membrane as the water oxidation site (Lozier et. al., 1971; and Blanken-
ship and Sauer, 1974).

Cheniae and Martin (1969 and 1971b) studied the reactivation pro-
cess in manganese deficient cells of Anacystis nidulans. They concluded:
(1) that uptake of manganese ion by the cells can occur in the dark, but
is greatly stimulated by light in a process assumed to be linked to
photophosphorylation; (2) binding of the manganese ion to the active
site in the water-splitting apparatus can occur in the dark ; and (3) a
PSII driven, multiquantum photoactivation process is required to form
centers capable of water photolysis. Homan (1967) had also noted the
stimulatory effect of dim light on the manganese reactivation process.

With the exception of Tris inhibited isolated chloroplasts, reacti-
vation has been reported only for intact algal cells. Significantly,
Yamashita and Tomita (1974) observed that Tris-washed chloroplasts
(from spinach) can be reactivated in the dark without the addition of
exogenous manganese. However, more extensively depleted chloroplasts
(i.e., with the manganese removed from the inner thylakoid space) re-
quired both light and externally supplied manganese for reactivation.
These observations lead Blankenship et. al. (1975), to suggest that in
higher plants, as well as for the algae, the incorporation of manganese

is a two-step process; a light driven transport into the thylakoid and a dark binding to the site responsible for activity.

Chloride Ion

The original observation of Warburg and Lüttgens (1944) that the Hill reaction of isolated chloroplasts required the presence of Cl^- has been confirmed by many workers. Recently, Kelley and Izawa (1978) concluded that the Cl^- requiring step is specifically associated with the oxidizing size of PSII, and further that it probably acts as a cofactor in the hypothesized Mn-containing water-splitting enzyme. In contrast to the specificity for manganese shown by this system, other anions can replace Cl^-, although with less efficiency. Kelley and Izawa (1978) listed the following anion series for stimulation of Hill reaction activity in broken, EDTA washed chloroplast particles: $Cl^- > Br^- > NO_3^- \geq I^- \geq HCOO^- > HCO_3^- > F^-$ (no effect).

Most of the studies on the Cl^- effect have been done _in vitro_ and it has recently been suggested that the _in vivo_ requirement is still uncertain (Terry, 1977). However, the results of Terry (1977) have been criticized (Kelley and Izawa, 1978) and at the present time Cl^- retains its status as one of the two (along with manganese) identified factors required for the functioning of the oxidizing side of PSII.

Plastoquinone

Plastoquinone is known to be an electron carrier operating on the reducing side of PSII (Bishop, 1959) but a possible involvement in the oxidizing side has also been proposed (Okayama, 1974; and Sadewasser and Dilley, 1978). The extraction of at least 75 percent of the total plastoquinone pool results in an inhibition of electron transport on the

oxidizing side of PSII as well as between the photosystems. Restoration of activity can be obtained by reactivation with plastoquinone A or C, or Vitamin E in addition to β-carotene. The conclusion was drawn that plastoquinone _in vivo_ acts as a structural component necessary for the full activity of the oxygen-evolving site and that it is not directly involved in electron transport on this side of PSII.

Copper

Plastocyanin is the only known component of the photosynthetic membrane which requires copper for its activity; however, it accounts for only one-half of the copper associated with the membrane. Renger et. al. (1967) suggested that copper which was accessible to the complexing agent salicylaldoxine, was required for PSII activity. Barr and Crane (1976) extended his work and concluded that a copper-protein is active on the oxidizing side of PSII. It has also been reported that Cu^{++} (as well as Mn^{++}) was effective in restoring Hill reaction activity to sodium cyanide inhibited chloroplasts (Takahashi and Asada, 1976). A scheme for the oxidizing side of PSII which includes a copper-protein was proposed by Holdsworth and Arshad (1977), based on their isolation from a diatom of a large Cu-Mn-pigment-protein complex which demonstrated PSII activity. In spite of these efforts, copper is not at the present time included as a required component of PSII by most workers.

Inhibitors

There are many treatments, both physical and chemical, which selectively inhibit the oxidizing side of PSII (see Cheniae, 1970; Izawa, 1977; and Trebst, 1974 for reviews). Many of the treatments, such as mild heating, washing with 0.8 M Tris or hydroxylamine or chaotropic

agents, result in the loss of the loosely-bound manganese of PSII.

Tris treated chloroplasts can be reactivated by washing the membranes with ascorbate and reduced indophenol dyes (Yamashita et. al., 1971). Additional Mn^{++} is not required since the ion remains trapped in the inner thylakoid space for several hours and is available for reincorporation (Cheniae and Martin, 1978).

Hydroxylamine is an effective inhibitor at low concentrations, and when used in the dark it specifically removes the manganese associated with water-oxidation. Hydroxylamine treated algae can be reactivated but attempts to reactivate isolated membranes have been unsuccessful (Cheniae and Martin, 1972).

Chaotropic agents (such as quanidine, thiocyanate and urea) apparently interupt the hydrogen bounding structure of water, probably resulting in disorganization of the hydrophobic region of the membrane involved in water photolysis (Lozier et. al., 1971).

Cytochrome b-559

Photosynthetic membranes contain a b-type cytochrome whose alpha absorption band, of the reduced state, absorbs light maximally at 559 nm (i.e., cytochrome b-559). There are between two and three cytochrome b-559 proteins per PSII reaction center. The assignment of a physiological role for this cytochrome has been a difficult problem and is without resolution to date (see Cramer and Whitmarsh, 1977 for review). Most of the cytochrome b-559 shows a close physical association with PSII and at liquid nitrogen temperatures it can donate electrons to P-690 (Knaff and Avron, 1969b). At various times it has been proposed that this cytochrome may act as: (1) a component in the electron transport chain between the photosystems; (2) as a mediator of cyclic

electron flow around PSII; and (3) as an electron acceptor in water oxidation (and of course, electron donor for P-690[+]). Arguments against these suggested roles stem primarily from the slow kinetics of oxidation and/or reduction of b-559 seen spectroscopically under physiological conditions. An additional factor which complicates their analyses is the presence of more than one oxidation-reduction potential form. Isolated, intact chloroplasts, which are able to fix CO_2, possess both low potential (LP) at + 70 mV, and high potential (HP) at + 370 mV, forms of b-559 in nearly equal proportions (Heber et. al., 1976). Wada and Avron (1971) observed that inhibition of the water-splitting apparatus results in the appearance of a form of b-559 with an intermediate redox potential. Horton and Croze (1977) extended this work and found that removal of the loosely bound manganese associated with water photolysis resulted in the lowering of the redox potential of the HP form to approximately 240 mV. This conversion to the intermediate potential paralleled the loss of manganese and the inhibition of oxygen evolution. The presence of positive ionic environments (such as one influenced by Mn^{++}) has been suggested as a factor which could explain the unusually high potential of this b-type cytochrome (Cramer and Horton, 1975).

Disruption of the membranes to the extent that PSII cannot oxidize artificial donors results in the conversion of all of the b-559 to a low potential form, and it is likely that the cytochrome is able to assume a range of potentials between the HP and LP forms rather than being restricted to one intermediate state (Horton and Croze, 1977).

Reversible conversion of the redox states of cytochrome b-559 has been demonstrated by Okayama and Butler (1972). Extraction of chloroplasts with heptane resulted in loss of oxygen evolution and of b-559

(HP). Readdition of plastoquinone and β-carotene caused partial restoration of oxygen evolution and of the HP form of b-559.

Although it is clear that the presence of the high potential form of b-559 is an excellent indicator of PSII activity with high quantum efficiency, none of the hypotheses for its suggested mode of action in PSII has overwhelming experimental support. As an alternative to the earlier suggestions, Butler (1978) has proposed a model for the role of b-559 in which it functions on both sides of PSII, acting as an electron carrier between the photosystems and serving to stabilize one or more of the water-splitting steps via proton binding. However, his proposal awaits experimental verification, and the position of this cytochrome shown in Figure 1 is therefore speculative.

The Search for Proteins Directly Involved in Water Oxidation

It is assumed that there are specific proteins or protein complexes which contain the catalytic, active site where water is oxidized and molecular oxygen is released. Early workers suggested known enzymes which possessed related activities such as catalase, hydrogenase, cytochrome f, peroxidase or superoxide dismutase as candidates. However, these suggestions have not been substantiated (see Brown and Frenkle, 1953). Current information suggests that at least part of the water-splitting apparatus will bind manganese and that it will be oriented towards the inner surface of the thylakoid. A major problem in the attempt to identify components involved in water oxidation is the extremely fragile nature of the process. Procedures used to isolate portions of the electron transport chain have generally resulted in irreversible loss of water-splitting activity and also the release of manganese

bound to the active site. An exception to this rule is the recent report of the isolation of a system II particle with oxygen evolving activity from a cyanobacterium (Stewart and Bendall, 1979). Hopefully, the polypeptide composition of this preparation will soon be determined and published.

It is, however, a possibility that the proteins required for water photolysis will remain associated with PSII particles, although in an inactive form. Several reports of the polypeptide composition of isolated PSII particles have been published (e.g., Kuwabara and Murata, 1979; Newman and Sherman, 1978; and Novak-Hofer and Siegenthaler, 1977). While none of these reports have suggested the association of individual polypeptides with the oxidizing side of PSII, they do provide a reference source when considering specific proteins for such a role.

There have been a few reports of proteinaceous factors which were stated, or implied, to be involved either directly or indirectly in water photolysis. Black (1969) reported restoration of oxygen-evolving activity in spinach chloroplasts, which had been inactivated by freezing and thawing, by addition of a concentrated protein factor released from the membrane during the treatment. No further reports have emerged from his study. Huzisige et. al. (1968) reported the isolation of a protein released by membranes during long-term Tris treatment (12 hours), which could enhance Hill reaction activity in inhibited, isolated chloroplasts. They named this protein the oxygen-evolution factor, however, its direct role in this reaction has not been demonstrated.

A large pigment protein complex was isolated from a diatom by Holdsworth and Arshad (1977) which possessed PSII activity with an artificial electron donor. The complex contained about 40 polypeptide

subunits, all of a molecular weight of near 25,000, and since it contained manganese (as well as copper), they suggested that it contained the apparatus required for water photolysis. However, the lack of oxygen-evolving ability and the similarity in molecular weight of the subunits to those of the light-harvesting pigment-protein complexes which also bind manganese (Foyer and Hall, 1979) suggests that their conclusion may be premature.

Immunological studies by Schmid et. al. (1976) indicate that a protein fraction with molecular weights near 11,000, is involved on the oxidizing side of PSII. Since the proteins are accessible to antibodies on the outer surface of the membrane, they suggest that the inhibitory effect on PSII may be indirect.

The strongest evidence for the direct involvement of a protein in the water-oxidation process was presented by Spector and Winget (1980). They isolated a 65,000 dalton, manganese containing protein from spinach chloroplasts that restored oxygen-evolving capacity when added to preformed liposomes containing thylakoids depleted of this protein. This protein was inactivated by Tris treatment, while a similar treatment of the membranes prior to readdition of the protein had no effect. This is a very recent development and it utilized a rather specialized technique for the diagnostic assay (liposome production). Verification of its results could prove it to be a significant advance in photosynthesis research.

However, at the present time, no proteinaceous component of photosynthetic membranes has been identified which is generally acknowledged to participate in a direct manner in the water-splitting reactions. It is also not known if there is a series of carriers in a water to reaction chain, or if there is direct donation to P 690^+ by the water-

splitting enzyme.

In this thesis, evidence obtained through analyses of select mutants of the green alga <u>Scenedesmus</u> <u>obliquus</u> will be presented which indicates that a membrane polypeptide, with a molecular weight of 34,000 daltons, is involved with the oxidizing side of PSII.

II. MATERIALS AND METHODS

Mutant Isolation and Identification

The photosynthetic mutants used in this study were induced by x-ray irradiation of cells of Scenedesmus obliquus strain D according to the techniques described by Bishop (1971b). An initial selection and isolation of a group of mutants possibly blocked on the oxidizing side of PSII was performed by Dr. Bishop using the general procedures outlined in Bishop et. al. (1977b) for the low flourescent PSII type mutants. Further screening resulted in the decision to use the mutants designated LF-1, LF-2, LF-3 and LF-5 for further analysis of their PSII oxidizing side characteristics.

In the final period of this study two additional mutants were selected which lacked chlorophyll b; one was obtained from the normal wild type strain (named KØ-9) and the second from LF-1 (designated LF-1(a/b-)).

Algal Culture

Scenedesmus obliquus strain D_3 (termed the wild type or WT) and the mutant strains derived from it were cultured heterotrophically on nitrate medium (Kessler, Arthur and Brugger, 1957) supplemented with 0.5 percent glucose and 0.25 percent yeast extract. The cells were maintained in the dark on 250 ml of media in 500ml screw-cap Erlenmeyer flasks while agitated on a rotary shaker (New Brunswick Scientific Gyratory Shaker Model G-25) at 28°C. For growth at 33°C cells were usually cultured in 100 ml of media in bubble tubes which were submersed in a temperature-regulated water bath. Air was introduced into the tubes from the bottom, and the cultures were kept in the dark. Cells grown at 20°C were either maintained in bubble tubes or in culture bottles in a small rotary incubator (New Brunswick

Model G-24), placed in a walk-in cold room.

Two-day old (28^{0}C) cultures were routinely used in all experiments. These cultures were near the end of the logarithmic growth phase and possessed maximal photosynthetic activity (Berzborn and Bishop, 1973). For cultures grown at 20^{0}C or 33^{0}C, the cells were harvested at an equivalent stage of growth (seven to ten μl PCV/ml of medium).

Packed cell volume (PCV) of the algal cultures was determined by centrifuging an aliquot of the sample in a cytocrit centrifuge tube (SGA Scientific Inc.) using a Sorvall table top centrifuge (Model GLC-1) for five minutes at 300 xg.

Growth medium with greater than normal concentrations of Mn^{++} was prepared by addition of $MnCl_2$ from sterilized 100x stock solutions to autoclaved, room temperature standard medium. Growth medium containing radioactive manganese-54 at a high specific activity was prepared by excluding manganese from the trace metal additions to the normal medium and adding enough ^{54}Mn (as $MnCl_2$-carrier free from New England Nuclear) to yield a 0.08 μCi/ml solution. Cells grown in complete medium were used as an inoculum source.

Chloroplyll Determinations

Whole cells of _Scenedesmus_ were extracted repeatedly with boiling methanol until all of the pigments had been removed. The cellular debris was removed by centrifugation (five minutes at 300 xg).

For the determination of the chlorophyll concentration in homogenized chloroplast suspensions, samples were prepared by dilution of small aliquots in methanol followed by centrifugation to remove the precipitate. Optical densities of the samples at specific wavelengths were

determined with Zeiss PMQ 2 spectrophotometer. The equations developed
by Holden (1965) were used to convert the optical densities into chloro-
phyll concentrations (mg/l):

Chlorophyll (a + b) = $25.5 A_{650} + 4.0 A_{665}$

Chlorophyll a   = $16.5 A_{665} - 8.3 A_{650}$

Chlorophyll b   = $33.8 A_{650} - 12.5 A_{665}$

These equations are based on the extinction coefficients of chlorophylls
a and b in methanol as determined by MacKinney (1941). For conversion
to molar values, an average molecular weight for chlorophyll (a + b)
of 906 was used.

When dilute chloroplast suspensions were to be assayed, the method
of Arnon (1949), using 80 percent acetone, was employed since the
aqueous portion of the sample was of necessity appreciable.

Amperometric Measurement of Hydrogen and Oxygen

Oxygen and hydrogen concentrations were followed in a 1.4 ml dual
electrode cuvette similar to that described by Jones and Bishop (1976).
The instrument incorporates the circuitry for the hydrogen electrode
described by Wang et. al. (1971). The cuvette was maintained at $28^{o}C$
and the instrument's responses to O_2 and H_2 were calibrated with water
in equilibrium with air or ten percent hydrogen using standard values
for H_2 and O_2 solubility in water. Illumination was provided by a
quartz-iodide source with an intensity of 49 mW/cm^2 after passing
through 2.5 cm of two percent (w/v) $CuSO_4$.

For photosynthesis and respiration measurements, cells were sus-
pended in 0.05 m $KH_2-K_2HPO_4$ buffer (pH 6.5) at five μl PCV/ml and rates

of O_2 uptake and production measured for an average of three dark-light cycles.

In experiments where metal ions were to be added to cell suspensions prior to analysis, cells were collected from the growth medium, washed and suspended in five mM PIPES buffer (pH 6.8). Aliquots were placed in 25 ml Erlenmyer flasks and incubated, with shaking in the water bath of a Gilson respirometer exposed to room light. The appropriate metal ions were added from concentrated stock solutions and the cells incubated for various times before analysis.

For photohydrogen production, cells (200 μl PCV) were suspended in 20 ml of 0.05 m KH_2-K_2HPO_4 buffer (pH 6.5) in a 50 ml Erlenmyer flask sealed with a serum stopper. The flasks were flushed with argon or helium for five minutes (using syringe needles) and then incubated for a minimum of four hours in the dark with shaking in the water bath of a Gilson respirometer at 28^OC. Samples were removed with a syringe and injected into the cuvette which had been pre-flushed with the inert gas.

When additions, such as DCMU or PSII electron donors, were to be made, 200 μl (PCV) of the algae were incubated in 15 ml buffer. Then 1.5 ml of the cell suspension was injected into a second sealed flask which contained 0.5 ml of the desired solution and an inert gas atmosphere. This flask was thoroughly shaken and 1.5 ml of the suspension was transferred to the cuvette as before.

Photoreduction

Photoreduction was performed at 28^OC using a Gilson differential respirometer (Bishop, 1972). Cells (100 μl PCV) were collected by centrifugation, washed and resuspended in 2.7 ml of 0.05 m KH_2-K_2HPO_4

buffer (pH 6.5); 0.2 ml of 6×10^{-5} M DCMU or 0.2 ml of H_2O was placed in the sidearm's well. The reaction vessels were flushed with a gas mixture of 96 percent H_2-4 percent CO_2, sealed and the cells incubated for a minimum of four hours in the dark. This procedure allowed for activation of their hydrogenase enzyme. The H_2O or DCMU was then added from the sidearm and the samples were illuminated from below at a light intensity of 9 mW/cm^2. Rates were converted to μmoles CO_2 consumed per hour per milligram of chlorophyll.

Chloroplast Isolation and Absorption Spectra

Chloroplast fragments were prepared from the Scenedesmus strains according to the method of Berzborn and Bishop (1973) with the following modifications. The glass beads used were 1.5 mm in diameter rather than 0.35 mm. The filtrate collected after the breakage of the cells with the glass beads was centrifuged at 6,000 x g for five minutes. The pellet, consisting of unbroken cells, starch grains and other cell debris was discarded and the green supernatant recentrifuged at 54,000 x g for 30 minutes. For chloroplast reactions and for cytochrome analysis the resulting membrane pellet was suspended using a glass homogenizer, and diluted to the appropriate chlorophyll concentration in a solution consisting of 20 mM Tricine-KOH pH 7.5; 30 mM KCl; 0.4 M sucrose (STK solution).

Absorption spectra of the isolated chloroplasts of the WT and mutant strains were determined in an Aminco DW-2 spectrophotometer operating in the split-beam mode. The chloroplasts were diluted to 20 μgm chl/ml in the STK solution with the reference cuvette containing only STK.

Chloroplast Purification

Chloroplast fragments were prepared as for use in partial reactions (see chloroplast isolations); however, the membrane pellet was homogenized in TKE solution (20 mM Tricine/KOH pH 7.5; 10 mM KCl; 0.05 mM EDTA) which also contained sucrose (0.4M).

This was layered onto a step gradient of sucrose solutions (all in the TKE solution), consisting of five ml of 1.5 M, 3 ml of 1.2 M and 3 ml of 1.0 M sucrose. This was then overlayered with the 15 ml of TKE solution and centrifuged at 55,000 x g_{max} for 30 minutes in a SW-27 rotor at 4°C.

After centrifugation the tube contained a turbid region at the top (probably lipoprotein) followed by a clear region, then a very pale green layer (original sample location), the dark green chloroplast region (in the 1.0 - 1.2 M sucrose zone), the clear or pale green 1.5 M sucrose layer and a small pellet of white and some green material. The upper layers were removed by gentle suction and the dense green membrane containing layer collected by use of a syringe. This fraction was then diluted with TKE solution (about 25 ml) and the membranes pelleted by centrifugation at 54,000 x g for 30 minutes. The supernatant was discarded and the membranes used either immediately or stored frozen in the centrifuge tube for no longer than one week prior to use.

Chloroplast Reactions

Chloroplasts were isolated as indicated and all reactions were performed in the standard STK solution. The methodology of Harvey (1974) was used for the water to methyl viologen assay. The reaction mixture contained 0.1 mM methyl viologen, 0.3 mM sodium azide and the chloroplasts at 45 ug/ml of chlorophyll. This reaction was originally

described by Kok et. al. (1965), and has been shown to require both photosystems.

Photoreduction of the oxidized form of the dye, 2,6-dichlorophenol-indophenol (DPIP), was also used as an assay of electron transport capability (Holt et. al., 1951), using both water and artificial electron donor systems. Chloroplasts were diluted to 5 μgm/ml (chlorophyll), DPIP added to make a 10 μM solution and the sample divided between two cuvettes which were placed in an Aminco DW-2 spectrophotometer operating in the split-beam mode. Illumination of the sample cuvette was provided by a high pressure Xenon lamp focused on the top of the cuvette, the reference cuvette being shielded from the light. The actinic light was passed through a Corning #2403 cut-off filter and had an intensity at the sample of 30 mW/cm^2. The photomultiplier tube was protected from the actinic light by a Schott 601 interference filter. Dye reduction was followed at 601 nm and a value of 21×10^{-3}/μmole-cm was used as the extinction coefficient for DPIP (Armstrong; 1964).

DPIP reduction was also used to assay electron donation to PSII as a diphenylcarbazide (DPC), (Vernon and Shaw). DPC was added as a concentrated (50 mM) ethanolic solution to the previous reaction mixture, yielding a solution that contained 1 mM DPC and 1.6 percent ethanol. Control chloroplasts were prepared by treating wild type chloroplasts to inhibit specifically their water splitting capacity. Both gentle heating (Katoh and San Pietro, 1967), and washing with Tris (Yamashita and Butler, 1968a) were employed. Heat treating involved placement of dilute chloroplast suspension in a water bath (T = 52°C) for 10 minutes; the procedure caused approximately 98 percent inhibition of the H_2O-DCIP reaction. The Tris inhibition was accomplished by homogenization of the

isolated chloroplast membranes in ice cold 0.8 M Tris, pH 8.0 followed by incubation in the dark for 20 minutes. The membranes were then collected by centrifugation (50,000 x g for 30 minutes) and resuspended in STK solution. Photosystem I activity of isolated chloroplast membranes was assayed using ascorbate-DPIP as the electron donor and methyl viologen as the acceptor in chloroplasts with PSII inhibited by DCMU. The reaction mixture contained: 50 μM DPIP, 0.1 mM methyl viologen, 5 mM sodium ascorbate, 0.3 mM sodium azide, 7.5 μm DCMU and chloroplasts at 25 μg/ml of chlorophyll. The reaction was followed as light-dependent oxygen uptake.

Fluorescence Measurements

Fluorescence measurements were made with the instrumentation and methods developed by Senger and Bishop (1972). A projector lamp directed through a Bausch and Lomb high-intensity monochromator set at 436 nm provided the actinic light source with an intensity at the sample cuvette of 0.1 mW/cm^2. Variable yield fluorescence was measured by noting the effects of 650 nm and 712 nm wavelengths of light, each with an incident intensity of 0.02 mW/cm^2, on the fluorescence yield induced by the 436 nm light. The cells were used at a final concentration of 1 μl/PCV/nl in 0.05 M KH_2-K_2HPO_4 buffer (pH 6.5) or in non-supplemented growth medium. When indicated, DCMU was added to a final concentration of 5 x 10^{-6} M.

When the PSII specific electron donor system of ascorbate reduced hydroquinone was used, the final concentrations were 3 mM sodium ascorbate and 2 mM hydroquinone.

Cytochrome Analysis

Concentrations of the various cytochromes of chloroplast prepa-
rations were estimated by analysis of chemically-induced difference
spectra measured in an Aminco DW-2 spectrophotometer. The procedure,
equations and molar extinction coefficients were taken from Henningsen
and Boardman (1973) and from Bendall et. al. (1971). The use of the
ferrocyanide-ferricyanide difference spectra, which indicates the
presence of the highest potential form of cytochrome b-559, was adopted
from the work of Horton and Croze (1977). The chloroplast suspensions
were usually diluted to 90 µg/ml of chlorophyll. The final concen-
trations of potassium ferricyanide, potassium ferrocyanide, hydroquinone
and sodium ascorbate were 3.2 mM (0.1 ml of 0.1 M stock solutions added
to 3.0 ml of sample one to three minutes prior to recording the spectra).
Sodium dithionite was added as crystals at least ten minutes prior to
determination of the spectra.

Cytochrome f and cytochrome b-559 (HP) were determined from the
readings at 554 nm and 559 nm in a hydroquinone reduced minus ferri-
cyanide oxidized difference spectrum, using the following equations:

$$\text{cyt f (µM)} = 53.1 A_{554} - 24.3 A_{559}$$

$$\text{cyt b-559 (HP)(µM)} = 58.4 A_{559} - 18.3 A_{554}$$

These equations assume molar extinction coefficients of 2.2×10^4 for
cytochrome f at 554 nm (Forti et. al., 1965) and 2.0×10^4 for cytochrome
b-559 at 559 nm (Boardman and Anderson, 1967). Total b-type cytochrome
was determined from the reading at 561 nm in a dithonite reduced minus
ferricyanide oxidized difference spectrum using a molar extinction
coefficient of 2.0×10^4. Cytochrome b-563 was estimated from the
reading at 563 nm in dithonite reduced minus ascorbate oxidized difference

spectra using 2.0×10^4 as the molar extinction coefficient. The values for total b-559 were obtained by subtraction of b-563 from those for the total b-type cytochromes.

Manganese Analysis by Neutron Activation

Thylakoids were prepared as described in the earlier section on chloroplast isolation with the following modifications: The membrane pellets were homogenized in 30 ml of TKE solution and repelleted by a second high-speed centrifugation, all solutions were prepared with deionized double glass distilled water, and all glassware was washed with 3 N HCl and rinsed with deionized water. The final washed membrane pellets were homogenized in deionized water, samples removed for chlorophyll determinations and aliquots dispensed in 1.5 ml polyethylene vials (1 ml/vial). The samples were heated to dryness in an oven and the vials sealed by heating with a quartz rod which had been held in the flame of a Bunsen burner. The sample vials were then placed in 7.5 ml polyethylene vials (two per vial) which were also heat-sealed. Standards were prepared in a similar fashion using aqueous $MnCl_2$ freshly diluted from a concentrated stock solution.

The samples and standards were then placed in the rotating rack of the TRIGA nuclear reactor at the Radiation Center on campus. They were exposed to a flux of thermal neutrons ($\sim 10^{12}$ neutrons/cm^2-sec) for one or two hours (reactor operating at 1 megawatt of power).

The resulting nuclear reaction of interest is ^{55}Mn (n,γ) ^{56}Mn. The manganese-56 isotope is unstable with a half-life of 2.6 hours. Its decay to stable ^{56}Fe is accompanied by the emission of a gamma ray with an energy of 846 keV and an intensity of 100 percent. A lithium drifted-

germanium semi-conductor detector, held at liquid nitrogen temperature, in conjunction with a 1,000 channel pulse height analyzer was used to determine the area under the 846 keV photopeak, which was well-isolated from other photopeaks. Comparison of the peak areas to a standard curve, which was linear over the range of concentrations present in the samples, allowed for calculation of the manganese concentration in the samples.

Manganese and Copper Analysis by Atomic Absorption

The method suggested in procedure E of the Analytical Method Committee's Report (1967) was used as a guide for the digestion of organic matter prior to analysis by atomic absorption (see also Down and Gorsuch, 1967). Membranes were prepared as described in the previous neutron activation section except that the final washed pellet was homogenized in TKE solution. A volume of the suspension containing 2.5 mg of chlorophyll was pipetted into a test tube and made to five ml with TKE. The sample volume was then reduced to approximately 2 ml by mild heating. Concentrated H_2SO_4 (2.0 ml) was added and the samples were placed in a sand bath at about 150°C for 20 to 30 minutes which produced a black, boiling solution. Samples were removed individually and 50 percent H_2O_2 (Fisher Scientific Co.) was added one drop at a time to the hot solution. The samples were gently stirred on a vortex mixer, and care was taken not to add the peroxide too rapidly. When the solution was almost clear, 50 percent H_2O_2 was added to make a total of 3.5 ml of this reagent added per sample. The samples were returned to the sand bath for 20 minutes. After cooling overnight, the samples were gravity filtered through acid washed Whatman #42 filter paper, using five ml of one percent HCl for a rinse solution. After adjusting the volume to

ten ml with deionized water the colorless samples were ready for analysis.

Standards were prepared by dilution of acidic solutions (one percent V/V HCl) of manganese prepared from Mn powder (Matheson, Coleman and Bell, Co.) or of copper prepared from pure copper wire.

The instrument used was the aa/ae Spectrophotometer by Instrumentations Laboratories, Inc. Double distilled, millipored water was used as the zero, and 1.0 ppm Mn or Cu standards as a reference during the analysis. An average of three readings were taken for each sample and standard. The instrument was adjusted to measure absorbance at 279.6 nm for manganese and 324.6 nm for copper.

Polyacrylamide Gel Electrophoresis

General Methods

The polypeptide composition and chlorophyll-protein complexes of purified thylakoid membranes were examined by polyacrylamide gel electrophoresis (PAGE) in the presence of either sodium dodecyl-sulfate (SDS) or lithium dodecyl-sulfate (LDS).

Both methods are based on the discontinuous SDS gel system described by Laemmli (1970) which consists of an Ornstein (1964) and Davis (1964) stacking system with SDS added. The electrophoresis was carried out using the verticle dual slab gel apparatus manufactured by Bio-Rad Laboratories and based on the design of Studier (1973). Model 220 (10 cm long x 14.0 cm wide) and Model 221 (28.0 cm long x 14.0 cm wide) were used and the gel thickness was kept at a uniform 1.5 mm in all cases. The stacking gels were 1.0 cm long and had an acrylamide concentration of 4.5 percent. The running gels were either of a uniform ten percent acrylamide

concentration or consisted of a linear acrylamide gradient of 7.5 percent to 15 percent accompanied by 5-17.5 percent sucrose gradient in the gel (Chua and Bennoun, 1975). Electrophoresis was carried out in the dark without pre-electrophoresis, under regulated temperature conditions. Current was provided by a Buchler power supply and maintained at 17.5 ma/slab.

Following electrophoresis, the gel slabs were photographed to record the occurrence and position of the chlorophyll-protein complexes and then placed in a staining solution of 0.2 percent Coomassie brilliant blue (R-250) seven percent acetic acid and 25 percent methanol. Gels were usually stained overnight and then destained by successive washes in a solution of seven percent acetic acid and 50 percent methanol. The stained gels were photographed and either stored at 4^OC in plastic bags or dried onto Whatmann 3 mm filter paper using a gel slab drier (Hoefer Scientific Instruments).

Sodium Dodecyl-sulfate Polyacrylamide Gel Electrophoresis

For sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) the sample solubilization method of Chua and Bennoun (1975) was used. The membrane pellet was homogenized in a solubilization solution (50 mM Na_2CO_3, 50 mM dithiothreitol, two percent SDS and sucrose , 12 percent w/v) at room temperature, the chlorophyll concentration determined and the sample diluted with additional solubilization solution to attain 1 mg chl/ml. This yields a SDS/chl ratio of 20:1. After a minimum of one-half hour at room temperature, the samples were loaded on the gel (usually 15 μl into a 10 mm x 1.mm slot). The core of the apparatus was maintained at 12^OC by circulating water from a constant temperature water bath.

Lithium Dodecyl-sulfate Polyacrylamide
Gel Electrophoresis

LDS-PAGE was carried out using the method of Delepelaire and Chua (1979) except that a 4.5 percent stacking gel was used and that the electrophoresis was carried out at a constant current of 17.5 mA/slab. Solubilization of the purified thylakoids was also as described by Delepelaire and Chua (1979). The temperature of the cell was maintained at 4°C during the electrophoresis by placing the apparatus in a walk-in cold room and use of a circulating water bath for the core of the cell.

Molecular Weight Estimation

The molecular weights of the polypeptides separated by SDS or LDS-PAGE were estimated by comparison of their migration distances to those of protein standards of known molecular weights (Weber and Osborn, 1969; Neville, 1971). The protein standards (from Sigma Chemical Co.) and their molecular weights were: Bovine serum albumin (66,000), oval-bumin (45,000), DNase I (31,000), trypsinogen (24,000), β-lactoglobulin (18,400) and lysozyme (14,300). These were dissolved in the same solubilization solution as used for the membrane preparations, checked for purity by electrophoresis, and a standard mix prepared. The standards and the membrane samples were electrophoresed on the same slab gel (using a uniform ten percent acrylamide separating gel), which was stained and dried on to filter paper. A curve was prepared by plotting migration distance (from the start of the separating gel to the protein band) versus the logarithm of the molecular weight of the standards. The molecular weights of membrane proteins separated on the same gel were estimated directly from their migration distances.

Lithium Dodecyl-sulfate Polyacrylamide Gel Electrophoresis of ^{54}Mn Labeled Membranes

Purified chloroplast membranes were prepared by the standard method from cells grown in media containing ^{54}Mn (see algal culture section). The membranes were solubilized and electrophoresed as described for LDS-PAGE. The gel was then cut into strips, each containing the separated proteins of one of the samples. Each strip was then cut into pieces (0.5 cm long) and these were placed in 13 x 100 mm polyethylene tubes. The tubes were capped and placed in the rack of a Packard Model 5230 Auto-gamma Scintillation Spectrometer and their activity determined. The authenticity of the ^{54}Mn was verified by comparison of the energy spectrum to published spectra. Counting efficiency of the instrument for ^{54}Mn was estimated to be near 45 percent.

Re-electrophoresis of Protein-Gel Slices

The procedure outlined by Cleveland et. al. (1977) was used as a guide when proteins or chlorophyll-protein bands separated by electrophoresis were to be re-electrophoresed into another gel. After the initial separation using the LDS-PAGE technique, the bands of interest were either cut out of the gel immediately, if the bands were pigmented, or after being stained, if they were not pigmented. A one-half hour staining period, followed by a brief (five-minute) destaining period was sufficient to locate the protein bands. The band was cut from the gel, placed in a solution of 50 mM Na_2CO_3, 50 mM dithiothreitol, 12 percent sucrose and 0.1 percent LDS--and immediately frozen for later use. For re-electrophoresis a second gel was prepared, ten µl of

solubilization solution (with two percent LDS) layered into each slot, the gel piece (freshly thawed) cut to size and carefully pushed to the bottom of the slot and another 10 μl of solubilization solution layered over the gel pieces. Electrophoresis was then performed as before. To heat treat the samples prior to re-electrophoresis, the gel pieces were placed in small test tubes containing the 0.1 percent LDS solution and heated in a water bath at the temperature and for the times indicated in the figures.

Isolation of a 33 kd Spinach Thylakoid Protein and Initial Application of the Technique to Scenedesmus

A protein with an apparent molecular weight of 33 kd was isolated from spinach thylakoids according to the methods of Kuwabara and Murata (1979), with the omission of the final isoelectric focusing step. In a preliminary experiment, the feasibility of using their technique to isolate a polypeptide from Scenedesmus was tested by applying their PSII particle preparation procedure to isolated membranes of the mutant LF-1(a/b-). The membrane pellets of the mutant were suspended in 0.05 M NaCl, 0.3 M sucrose, 0.05 M sodium phosphate buffer pH 6.8 and Triton X-100 was added from a 20 percent V/V stock solution to yield a Triton/chl ratio of 50. The sample was stirred on ice in the dark for 30 minutes then centrifuged for ten minutes at 6,000 x g. The supernatant was subjected to a second spin at 75,000 x g for 30 minutes. The pale green pellet was homogenized in 0.01 M Tricine/NaOH buffer (pH 7.6) and recentrifuged (75,000 x g for 30 minutes). The pellet from this spin was equivalent to the PSII particle preparation and was analyzed by LDS-PAGE.

RESULTS AND DISCUSSION

Selection of the Mutants

Yamashita and Butler (1968a) observed that inhibition of the oxi-
dizing side of PSII results in a decrease in the fluorescence yield in
isolated spinach chloroplasts. This is in sharp contrast to the large
increase in the fluorescence yield which results from interruption of
electron flow between the photosystems. Epel and Levine (1971) demon-
strated that this phenomenon could be exploited as a selection technique
and isolated several, low fluorescent mutants of Chlamydomonas which
were altered specifically in their water-splitting ability. Dr. Bishop,
in the initial selection of a group of mutants of Scenedesmus from
which the strains used in this study were chosen, used the following
criteria: the mutants must (1) exhibit normal growth on enriched medium
either in light or dark; (2) be incapable of photoautotrophic growth;
(3) demonstrate photoreduction when grown heterotrophically; and (4)
possess a low fluorescence yield. These criteria exclude mutants
which are (1) blocked in later reactions of the Calvin-Benson cycle;
(2) have an induced light-sensitivity of the photosynthetic apparatus;
(3) possess a deficiency or modification of photophosphorylation
capacity; and (4) are auxotrophic strains (e.g., arginine requiring).

Further screening resulted in the decision to use the mutants LF-1
and LF-2 for detailed analysis. Later, LF-3 and LF-5 were included
since these independently isolated mutants possessed a phenotype essenti-
ally identical to that of LF-1 and served to reinforce a conclusion
as to the nature of the genetic alteration in LF-1. LF-2 was one of a
group of mutants which were essentially similar to LF-1 but were capable
of producing compensation point levels of photosynthetic oxygen when

cultured at 28°C. When it was discovered that many of these mutants were temperature sensitive, LF-2 was selected for further study because it exhibited the simplest responses to this variable.

In addition, two mutants designated KØ-9 and LF-1 (a/b-) were selected to aid in one portion of this study. These mutants were chosen for their inability to synthesize measurable amounts of chlorophyll b, KØ-9 being derived directly from the WT, while LF-1 (a/b-) was derived from LF-1.

Location of the Block in Electron Transport in LF-1, LF-3 and LF-5

Whole cells of the mutants LF-1, LF-3 and LF-5 show only a trace of photosynthetic oxygen evolution as measured with the oxygen electrode system (Table 1). Actually this is more correctly described as a transitory decrease in respiratory oxygen uptake upon illumination and probably does not represent water photolysis. The ability of the mutants to carry on rates of photoreduction comparable to the WT strain demonstrates that PSI and the CO_2 fixation machinery in these mutants is unimpaired. The time course of photoreduction for WT and LF-1 cells with and without the addition of DCMU is shown in Figure 2. The absence of DCMU has no effect on photoreduction rate in the mutants since no oxygen is produced, whereas with the WT strain its presence is required to suppress the production of oxygen which inactivates this system. The photoreductive characteristics of LF-3 and LF-5 were strictly comparable to those of LF-1.

The source of electrons for photohydrogen production has been demonstrated to be water (Bishop et. al., 1977a). The anaerobically adapted cells of the WT strain exhibit a low level of dark H_2 production and an

Table 1. Whole Cell Reactions of the Wild Type <u>Scenedesmus</u> and the
Mutants LF-1, LF-2, LF-3, and LF-5 Grown at 28°C.

	WT	LF-1	LF-2	LF-3	LF-5
Photosynthesis[a]	210	trace	60	trace	trace
Respiration[b]	58	52	50	49	47
Photoreduction[c]	51	49	52	48	48

The rates are given as:

[a] Total µmoles of O_2 evolved/mg chl-hr (i.e., net O_2 production
in the light plus a correction for dark respiratory uptake).

[b] µmoles O_2 uptake/mg chl-hr in the dark.

[c] Steady state total µmoles CO_2 uptake/mg chl-hr in sample
treated with DCMU.
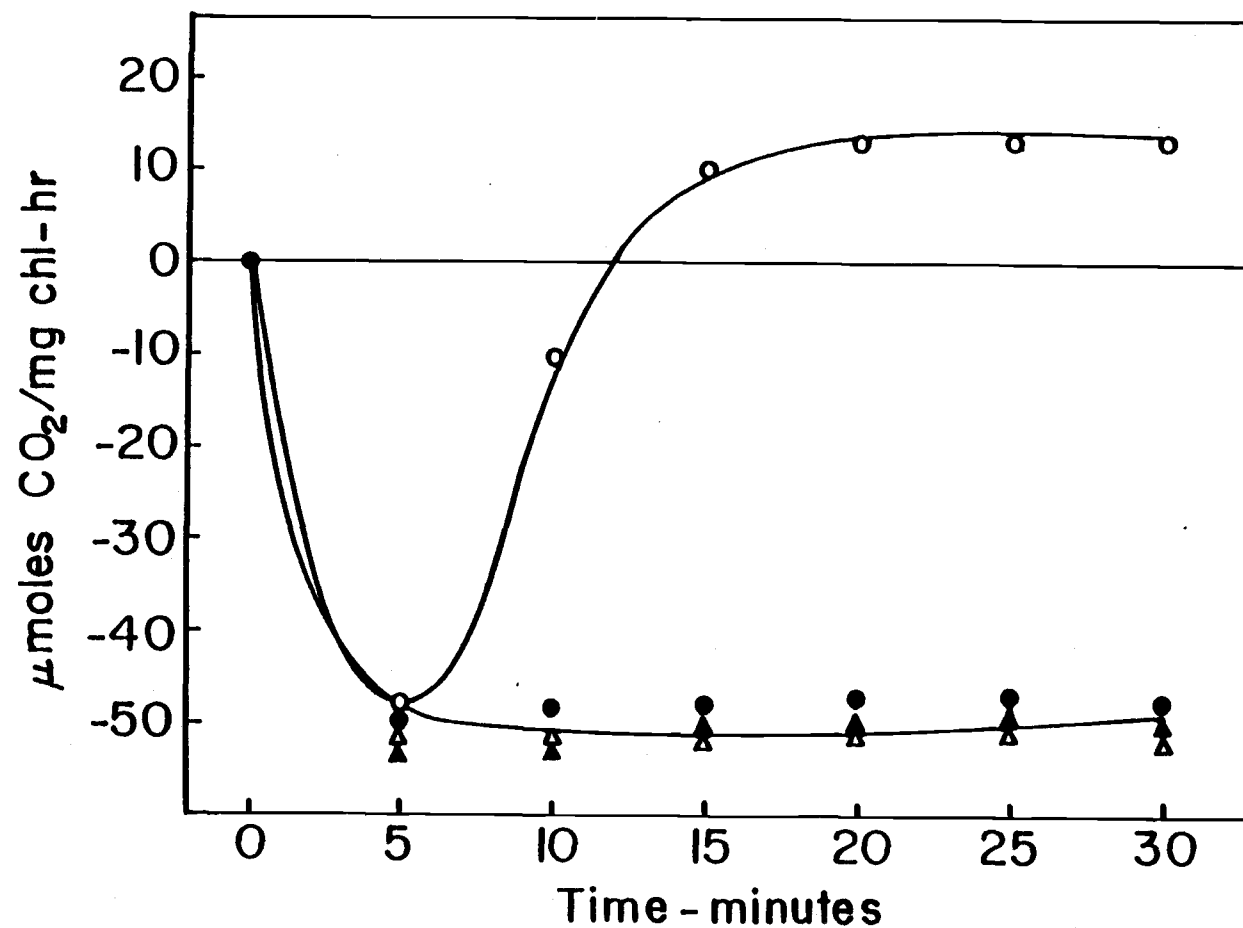
Figure 2.   The time course of photoreduction for whole cells of the wild-type <u>Scenedesmus</u> with DCMU (●――●) and without DCMU (o――o), and for the mutant LF-1 with DCMU (▲――▲) and without DCMU (△――△).

initial rapid rate of H_2 evolution upon illumination (Figure 3a). This net production is soon replaced by a net decrease in the H_2 due to the action of an uptake system and also due to inactivation of the hydrogenase by photosynthetic oxygen. As expected, adapted cells of LF-1 produced only minute amounts of H_2 when illminated (Figure 3b). It had been demonstrated by Yamashita and Butler (1968a) that hydroquinone when reduced by ascorbate (HQ/Asc) acts as a PSII specific electron donor for isolated chloroplasts. Addition of HQ/Asc to whole cells of LF-1 caused a partial restoration of the photohydrogen production with features similar to those observed in the WT (Figure 3c).

The partial reactions of isolated chloroplasts of the WT and LF-1 (Table 2) provide further evidence that the only alteration in electron transport system of this mutant is on the oxidizing side of PSII. Reactions requiring water as the electron source ($H_2O \longrightarrow$ DPIP and $H_2O \longrightarrow$ Mv) are blocked in LF-1. The minor amounts of water photolysis suggested by whole cell analysis is not observed with the isolated chloroplasts. The PSI reaction (DPIP/Asc - MV) indicates that this system in LF-1 is unaffected by the mutation as was already noted for the whole cell photoreduction. When the oxidizing side of PSII of the WT chloroplasts is inhibited by Tris treatment or by mild heating, the PSII reaction center oxidizes DPC and uses the electrons to reduce DPIP, although less efficiently than the intact membranes using water as an electron source (Vernon and Shaw, 1969). The rates observed for the DPC to DPIP reaction in LF-1 chloroplasts is equivalent to that of the inhibited WT membranes.

Growth of LF-1, LF-3 and LF-5 at $20^{\circ}C$ had no effect on their photosynthetic capacities (Table 3). Additionally, growth of LF-1 in medium

Table 2. Partial Reactions of Isolated Chloroplast Particles of the Wild Type Scenedesmus and Mutants LF-1 and LF-2.

	$H_2O \longrightarrow Mv^*$	$H_2O \longrightarrow DPIP^{**}$	$DPC \longrightarrow DPIP^{**}$	$DPIP/Asc \longrightarrow Mv^*$
WT(18°)	57	63	ND	247
WT(28°)	59	63	21	330
WT(34°)	65	72	ND	231
LF-1(28°)	0	0	26	350
LF-2(18°)	46	52	ND	191
LF-2(28°)	18	23	ND	217
LF-2(34°)	9	11.5	33	198

The rates are given as:

* μmoles of O_2 uptake/mg chl-hr

** μmoles of DPIP reduced/mg chl-hr.

ND = not determined. See Materials and Methods for conditions used for each experiment.
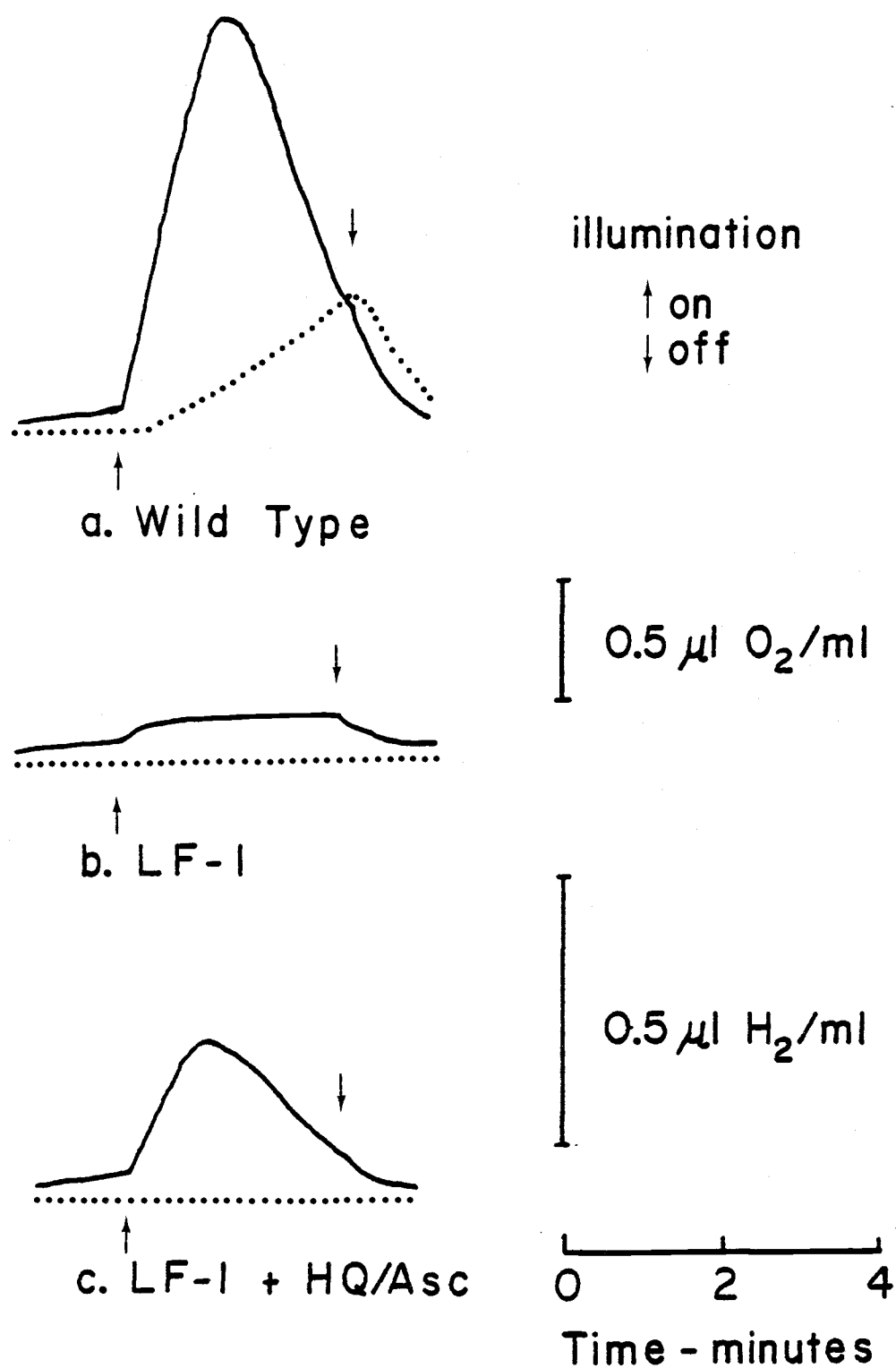
Figure 3. Photoproduction of hydrogen (——) and oxygen (.....) by
anaerobically adapted cells of: (a) the wild-type _Scenedesmus_;
(b) the mutant LF-1; and (c) LF-1 plus hydroquinone and ascor-
bate.

supplemented with up to 10 mM Mn^{++} did not restore oxygen-evolving capacity.

Temperature Dependent Nature of the Phenotype of LF-2, and the Localization of the Block in Electron Transport

Whole cells of LF-2, when grown at $28^{o}C$ on standard medium, generally produce photosynthetic oxygen at a rate nearly equal to respiratory oxygen uptake (i.e., near the compensation point). However, a significant variation in this rate from experiment to experiment led to the discovery that the expression of the mutant phenotype in LF-2 is temperature dependent. The data in Table 3 indicate that when cells are grown at $20^{o}C$ they produce oxygen photosynthetically at nearly WT levels; growth at $34^{o}C$ reduces oxygen production to levels well below the compensation point. The rate of oxygen production in LF-2 ($34^{o}C$) cells is also quite variable although consistently low (see the next section on reconstitution of LF-2). Continued growth at $34^{o}C$ caused alteration of chloroplast pigments in both LF-2 and the WT; hence all cultures for $34^{o}C$ growth used cells maintained at $28^{o}C$ for inoculum and were harvested after two days of growth.

Simple incubation (for two hours in buffer) of cells of LF-2 ($20^{o}C$) at $34^{o}C$, or of LF-2 ($34^{o}C$) at $20^{o}C$ did not reverse the photosynthetic characteristics acquired during the prior growth periods (Table 4). Later findings that the LF-2 ($34^{o}C$) membranes are manganese deficient indicate that the lack of restoration of activity after the transfer of LF-2 ($34^{o}C$) cells to $20^{o}C$ could be due to continued deficiency of this metal. However, the retention of high activity of LF-2 cells grown at $20^{o}C$ when transferred to $34^{o}C$ indicates that the photosynthetic membranes of LF-2 once formed are not affected by the higher temperature.

Table 3.   Whole Cell Reactions of the Wild Type <u>Scenedesmus</u>
           LF-1 and LF-2 Grown at 20°C or 34°C.

	20°C			34°C	
	WT	LF-1**	LF-2	WT	LF-2
Photosynthesis*	200	trace	160	190	39
Respiration*	45	40	50	49	54

* Photosynthesis given as μmoles O_2 produced/mg chl-hr. Respiration
given as μmoles O_2 uptake mg/chl-hr (see Table 1 for an explanation).

** Rates observed for LF-3 and LF-5 were equivalent to those of LF-1.

Table 4. Photosynthesis* of Whole Cells of WT and LF-2 Grown at Either 20°C or 34°C and Then Assayed at Both Temperatures.

	Incubation and Assay Temperature	
	20°C	34°C
WT (20°C)	175	185
LF-2 (20°C)	140	150
WT (34°C)	180	165
LF-2 (34°C)	30	28

NOTE: Cells were washed and suspended in phosphate buffer (0.05 M pH = 6.5) and incubated in the dark for two hours at the indicated temperatures prior to analysis).

* Photosynthesis measured as μmoles O_2 evolved/mg chl-hr (see Table 1 for explanation). The electrode was recalibrated prior to analysis at the different temperatures.

Efforts to localize the site of the block in the electron transport system of LF-2 (34°C) utilized the same procedures as described for LF-1 in the preceding section. High rates of photoreduction indicated an intact PSI and CO_2 fixation system. Partial reactions of membranes isolated from cells grown at various temperatures (Table 2) indicate that as the temperature is raised those reactions requiring water as the electron donor are inhibited while PSI is unaffected. Addition of DPC to LF-2 (34°C) membranes results in an enhancement of DPIP photoreduction that is equivalent to the rate observed in Tris inhibited WT membranes. This observation indicates the PSII reaction center remains functional during growth at the restrictive temperature.

Reactivation of Photosynthetic Oxygen Production in LF-2 (34°C)

It has been demonstrated that the uptake of manganese by algal cells is an active process that can be inhibited by uncouplers of phosphorylation (Pirson, 1937; Homan, 1967). Also, this process can occur in the dark, but is greatly stimulated by illumination. DCMU, at a concentration sufficient to completely inhibit the Hill reaction, results in only 50 percent inhibition of Mn^{++} uptake. The uptake process is totally separable from the photoactivation of the oxygen-evolving system (Cheniae and Martin, 1969). The possibility that the phenotype exhibited by LF-2 (34°C) is due to an impairment of manganese uptake rather than an alteration of the photosynthetic apparatus itself was therefore investigated. An initial experiment indicated that addition of excess Mn^{++} to the standard growth medium could reverse the effect of high temperature on LF-2. The optimum concentration of Mn^{++} was near one mM. The presence of five mM Mn^{++} in the medium proved to be toxic for the WT

strain (based on a chlorotic appearance of the culture), but was tolerated by LF-2 (34°C) although there was a slight color change noted in the culture. It was then found that cells of LF-2 (34°C) which had been grown on standard medium and which retained only 20 percent (approximately) of the wild type photosynthetic activity, could be rapidly reactivated by addition of Mn^{++} just prior to analysis. Again the optimum concentration was found to be near one mM Mn^{++}; over 80 percent of the WT rate of oxygen production could be regained within a few minutes following manganese addition (Figure 4). Addition of high concentrations of Mn^{++} to the phosphate buffer normally used in whole cell analysis resulted in the formation of a precipitate, therefore, PIPES buffer at pH 6.8 was used for all reactivation experiments (Good et. al., 1966). The conditions resulting in reactivation were qualitatively the same as those described for reactivation of manganese deficient alga (Homan, 1967; and Cheniae and Martin, 1967). Addition of the manganese to cells exposed to dim light (room light) and further incubation in the dim light resutls in rapid reactivation. Addition of one mM Mn^{++} to cells in the dark, followed by illumination with the intense light of the oxygen electrode system was not effective in sustained restoration of oxygen production. The inhibition of the photoactivation process by intense light has been noted previously by Cheniae and Martin (1969).

The concentration of Mn^{++} required for optimum restoration of activity in LF-2 (34°C) is approximately 1,000 times greater than that reported for the reactivation of manganese deficient Scenedesmus (Homan, 1967). Also, the Mn^{++} was equally effective when added as the chloride or sulfate salts, indicating that the restoration is not due to an effect of the chloride ion.
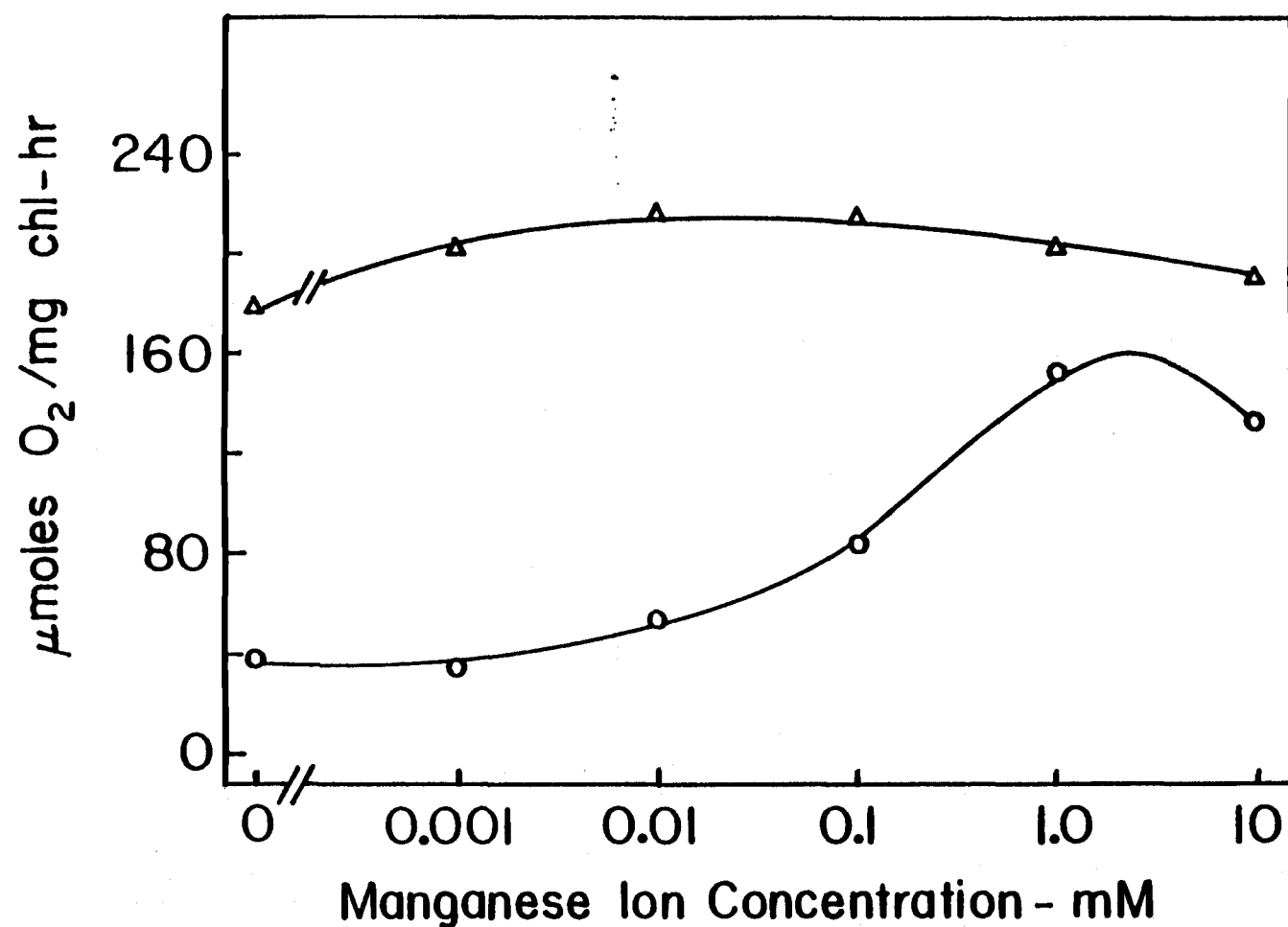
Figure 4.   Effect of manganese ion (as $MnCl_2$) on whole cell photosynthetic oxygen production of the wild-type Scenedesmus (△——△) and the mutant LF-2 (34°C) (o---o).   Cells were suspended in 5 mM PIPES buffer (pH 6.8) containing the $MnCl_2$ and incubated in dim light for several minutes prior to analysis.

Organization of Chlorophyll in LF-1 and LF-2

Although it was once assumed that the pigments of thylakoids were simply dissolved in the lipid bilayer, the current view is that most, if not all of these hydrophobic molecules are associated in a very ordered manner with protein components of the membrane. This is based on low temperature absorption and fluorescence spectra, which indicate the presence of several distinct micro-environments for the pigments and the separation of pigment-protein complexes by detergent polyacrylamide gel electrophoresis (see Thornber et. al., 1979 for a review). Several lines of evidence indicate that the organization of chlorophylls and other pigments in LF-1 and LF-2 is not different from that of the WT strain.

The amount of chlorophyll per ml of packed cell volume, and the chlorophyll a to b ratio of LF-1 and LF-2 ($28^{o}C$) are within the same range as is seen in the WT (Table 5). Also, the room temperature absorption spectra of isolated membranes and from the WT and mutants are virtually identical. Additionally, the spectra of membranes from WT and LF-2 grown at $20^{o}C$ and at $34^{o}C$ as described in the materials and methods section are also identical.

Previous work by Bishop and Oquist (1980b) demonstrated that the low temperature fluorescence spectrum of LF-1 retained all of the emission bands (at 686, 696 and 716 nm) which are characteristic of the WT strain. Additionally, during the many electrophoretic separations of the membranes (see the section on gel electrophoresis), no difference in the pigment-protein complexes between the mutants and the WT were ever observed when the samples were electrophoresed under identical conditions.

Table 5. Chlorophyll Content and Chlorophyll a to Chlorophyll b
Ratios of Whole Cells of Wild Type Scenedesmus and Mutants
LF-1 and LF-2 from 28°C Cultures.

| | Chlorophyll | |
	(a + b) mg/ml PCV	a/b
WT	4.2 ± 0.8	4.7 ± 0.9
LF-1	5.1 ± 0.9	4.3 ± 1.3
LF-2	4.8 ± 0.7	4.0 ± 0.7

Each value is the average of five measurements, with the standard
deviation indicated after each number.

Variable Yield Fluorescence

Analyses of the fluorescence characteristics of illuminated algae and isolated chloroplasts have been instrumental in forming the current concepts of the photosynthetic process. The room temperature emission spectrum of thylakoids is essentially that of the first electronic excited state of chlorophyll a. This indicates a one-way transfer of energy from other pigments to this molecule and that the higher excited states decay rapidly, by non-radiative means to the lowest energy excited state. Fluorescence is only one of several de-excitation pathways available to the excited chlorophyll a molecule. Under optimal conditions, as much as 85 percent of this energy can be used to drive photosynthetic electron transport, with the high energy electron leaving the chlorophyll molecule altogether and the original ground state being restored by donation of an electron from some other molecule.

Other de-excitation pathways include collisional processes in which the energy is eventually converted to heat and conversion to the excited triplet state, which can decay by radiationless means or by emission of light (phosphorescence). Since these are competitive pathways, the percentage of molecules which are de-excited by a certain process will be influenced by the activity of the other processes. Kautsky et. al. (1960) initially postulated that the fluorescence yield (i.e., quanta emitted/quanta absorbed) of thylakoids could be related to photosynthetic electron transport. They suggested that under conditions of free electron flow the fluorescence yield would be low, whereas when this pathway was blocked the fluorescence yield level would rise. Duysens and Sweers (1963) extended their work and proposed a slightly different model. They suggested that the electrons from water

traversed two photosystems, linked in series, on their way to NADP.
The level of fluorescence emanating from the thylakoid was controlled
by a hypothetical substance, the so-called Q, which could quench fluor-
escence yield when in the oxidized state (i.e., when the photochemical
pathway for chlorophyll de-excitation was available) but not when in the
reduced state (i.e., when it could not accept electrons from the
reaction center). Therefore, blockage of the electron flow between the
photosystems by addition of inhibitors such as DCMU, or by mutation
results in a large and invariant increase in the fluorescence yield.

In Figures 5 and 6 are recorded the variable yield fluorescence
patterns of the WT and the mutants under various conditions. The
samples are dilute suspensions of whole cells and are analyzed in an
apparatus which selectively amplifies only the fluorescence signal orig-
inating from a modulated 436 nm exciting light. The variation from the
base level fluorescence are induced by illumination of the sample with
continuous 650 nm or 712 nm light, the fluorescence induced by these
wavelengths of light is not detected by the instrument but rather only
their effect upon the photosynthetic apparatus causing changes in the
redox levels of Q or on the variable yield component of the fluorescence.
The energy of the 650 nm light is directed primarily towards PSII with
some going to PSI, while the 712 nm light exclusively drives PSI. In the
WT cells the addition of 650 nm light results in a large increase in the
fluorescence yield above the base level as the activity of PSII in-
creases and the intermediates between the photosystems become reduced.
When the 650 nm light is turned off, the fluorescence drops rapidly to
the base level. When the 650 nm light is on and PSI light (712 nm) is
added the fluorescence level drops as PSI oxidizes the intermediates.
The addition of 712 nm light alone also results in a drop in the

Figure 5. Comparison of the influence of PSII (650nm) and PSI (712nm) wavelengths of light on the variable yield fluorescence of whole cells of the wild-type and mutant LF-1 with and without the addition of hydroquinone/ascorbate.
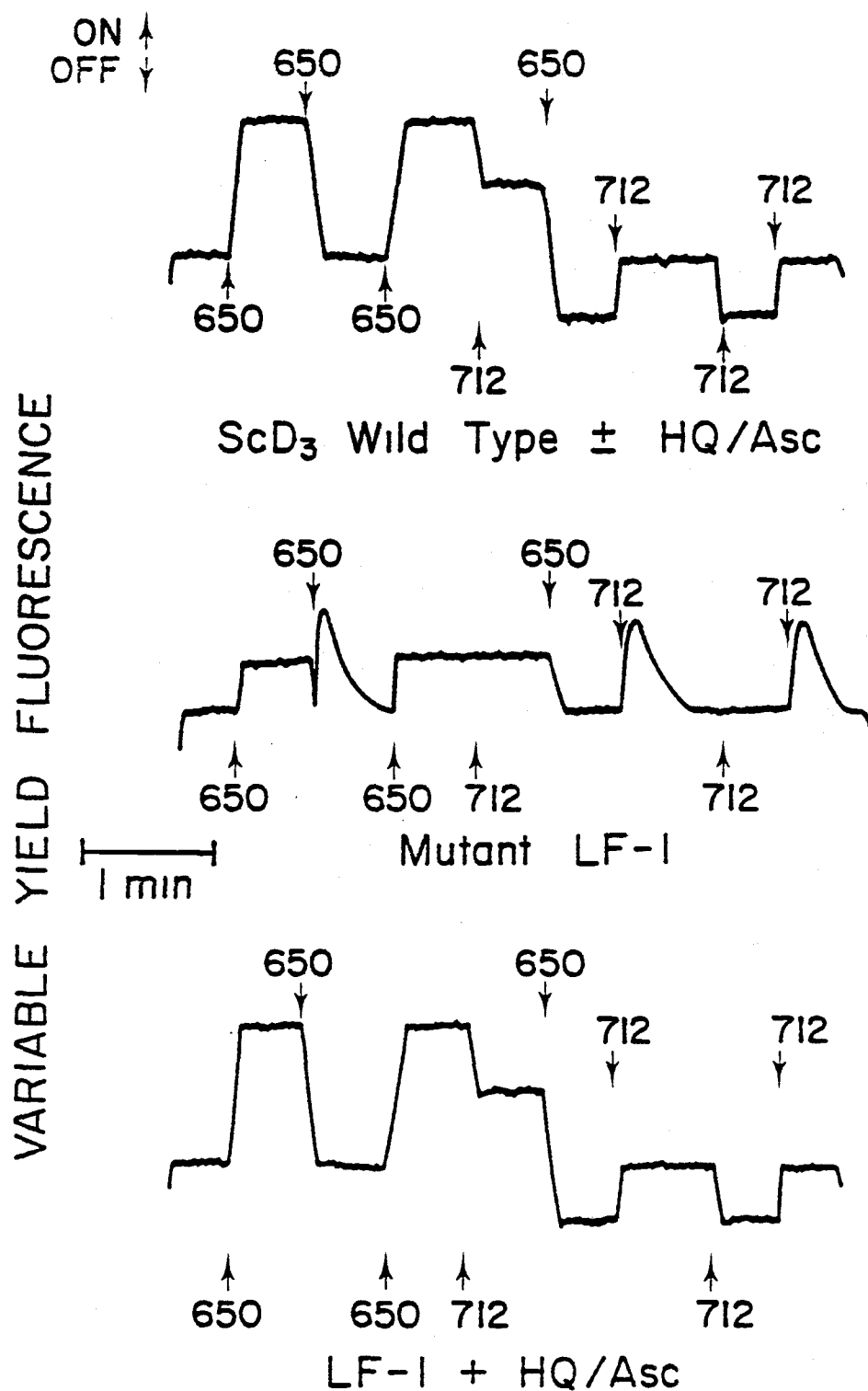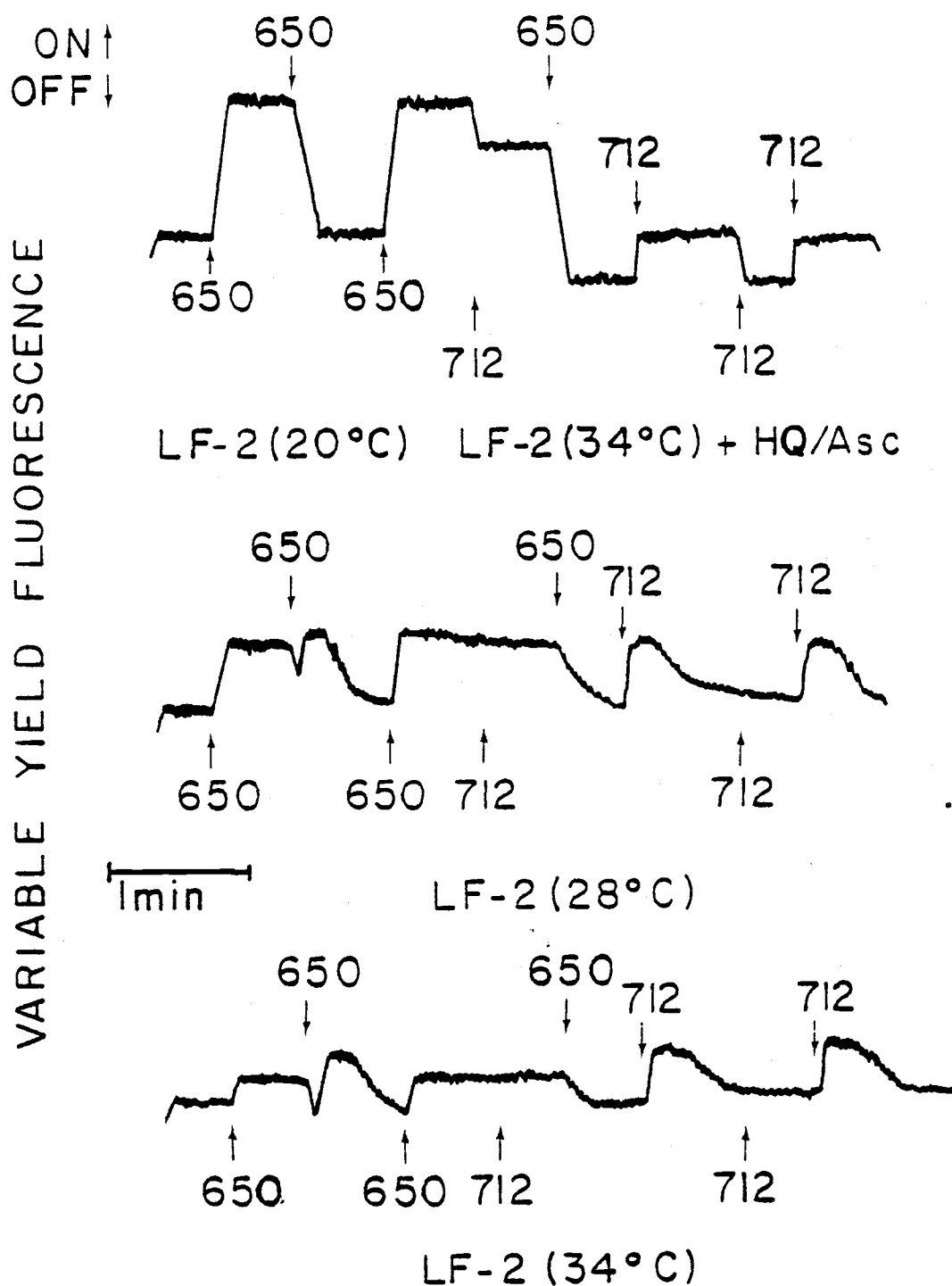
Figure 6. Comparison of the influence of PSII (650nm) and PSI (712nm) wavelengths of light on the variable yield fluorescence of whole cells of LF-2 (grown at 20°C, 28°C or 34°C), and LF-2 (34°C) to which hydroquinone/ascorbate had been added.

flourescence, in this case to below the base level. When the mutant
LF-1 is subjected to the same routine, several variations from the
WT pattern are observed. First, the rise in the flourescence yield
upon addition of 650 nm light is only about one-quarter of the increase
seen in the WT. This phenomenon was initially reported by Yamashita
and Butler (1968 a and b; and 1969) for isolated chloroplasts which had
been inhibited on the oxidizing side of PSII by treatment with Tris,
UV or mild heating. A rationale for this response was provided later
when it was discovered that the oxidized reaction center chlorophyll
(P-690$^+$) acts as a quencher of flourescence possibly by P-690$^+$ still
absorbing excitation energy which could facilitate a back reaction with
Q$^-$ (Butler et. al., 1973). When electron flow from water is interrupted,
the reaction centers cannot be reduced and P-690$^+$ accumulates, quenching
flourescence. The lack of electron flow between the photosystems in
LF-1 is indicated by the total absence of an effect of addition of 712
nm light on the flourescence level. The origin of the strong light off
transient observed in LF-1 is unknown but may be related to conformational
changes of the chloroplast. The data indicate that it is associated
solely with PSI, since removal of the 650 nm light while illuminating
with 712 nm did not elicit the response; while the 650 nm or the 712 nm
lights alone did.

Perhaps the strongest evidence showing that electron flow in
LF-1 is blocked only on the oxidizing side of PSII, while the rest of
the photosynthetic apparatus is identical to the WT strain, is that
addition of the PSII specific electron donor system hydroquinone-ascorbate
(see Methods section) restores a WT variable yield flourescence
(Figure 5, bottom). Yamashita and Butler (1968a) introduced this PSII

donor and demonstrated that its addition to isolated chloroplasts in-
hibited by Tris could partially restore the variable-yield fluorescence.
A higher concentration of HQ/Asc (ten times the amount used by Yamashita
and Butler) was used in this experiment since whole cells were employed
instead of isolated membranes. The responses of the mutants LF-3 and
LF-5 in these experiments are essentially identical to those of LF-1,
both without and with the addition of HQ/Asc. The fluorescence pattern
of WT cells was not altered when HQ/Asc was added.

The data in Figure 6, showing the variable yield fluorescence pat-
tern of LF-2, can be interpreted in much the same manner as for LF-1.
When grown at $20^{o}C$ the cells of LF-2 exhibit fluorescence responses
similar to the WT. Growth at $34^{o}C$ on standard media results in cells
whose fluorescence pattern strongly resembles that of LF-1, including
the light off transient. The WT pattern in LF-2 ($34^{o}C$) can be restored
in this case, either by addition of HQ/Asc or by reactivation of the
water-splitting apparatus through addition of one mM Mn^{++} to the cells
prior to analysis. The pattern from cells grown at $28^{o}C$ represents a
fluorescence response intermediate to those of the $20^{o}C$ and $34^{o}C$ cells.
Again, this localizes the specific site of the block in LF-2 ($34^{o}C$) as
being on the oxidizing side of PSII, and indicates that the balance
of the photosynthetic system is intact.

Manganese and Copper Content of Isolated Membranes

In Table 6 are listed the results of the manganese analyses for
isolated membranes of the WT and mutants. Two methods were used in the
analyses (neutron activation and atomic absorption) and they yielded
comparable results. Total data was combined to obtain the values

Table 6. The Influence of Growth Temperature on Manganese and Copper
Content of Isolated Membranes of the Wild Type <u>Scenedesmus</u>
and the Mutant Strains.

	chl/Mn			Mn/400 chl	%WT	chl/Cu		
	X*	S	n			X	S	n
WT 18°C	128	± 8	3	3.1	100	275 ±	44	2
28°C	106	± 22	8	3.8	100	214 ±	13	3
34°C	143	± 60	2	2.8	100	115		1
LF-1 28°C	255	± 25	4	1.6	42	212 ±	13	2
LF-2 18°C	209	± 37	2	1.9	61	168 ±	9	2
28°C	464	± 204	5	0.9	45	212 ±	13	2
34°C	905	(8664)	1	0.4	16	127		1
20°C - 34°C**	704		1	0.6	21		ND	
34°C - 20°C**	222		1	1.8	58		ND	
LF-3 28°C	278 ±	3	2	1.4	37		ND	
LF-5 28°C	270		1	1.5	39		ND	

* X = mean; S = standard deviation; n = number of independent samples.

** Cells from cultures grown at the first temperature listed were used to
inoculate fresh medium placed at the second temperature, grown to late
log phase and harvested for analysis.

listed. In both cases, the concentration of manganese was determined
by comparison to a standard curve which was linear over the region of
interest (0 to 2 ppm). A plot of manganese content versus various
quantities of WT membranes (using chlorophyll concentration as an indi-
cator) was also linear.

The values for WT ($28^{o}C$) varied from 3.1 to 5.3 manganese atoms
per 400 chlorophyll molecules with a mean value of 3.8 for eight samples.
Cheniae and Martin (1971a), also working with Scenedesmus obtained
values that were somewhat higher (five to eight Mn/400 chlorophylls) but
still in general agreement with the values obtained for this work.

Cheniae and Martin (1970) developed the concept that this membrane
bound manganese exists in two distinct pools differing in functional and
physical properties. Two-thirds of the manganese is loosely bound to
the membrane and is thought to be directly involved in the water-splitting
reactions. The other third is more tightly bound and its function is
less clear, though it may also be associated with PSII activity. Of
particular interest for this study is that the membranes of LF-1, LF-3
and LF-5 all show a decrease in manganese content relative to the WT
and that this represents a loss of about 60 percent of the normal mang-
anese level. The possibility that these mutants are unable to bind
manganese specifically involved in water photolysis is certainly sug-
gested by the coincidence of the percentages, and by the total and
specific inhibition of the water-splitting reactions in these mutants.

The decrease in manganese in the membranes of these mutants is
probably not due to an impairment of the uptake of the ion from the media.
Growth in excess manganese did not restore their photosynthetic activity
and also the level of manganese present in the mutant membranes was
fairly constant at 38 to 44 percent of WT levels.

Analysis of membranes from LF-2 grown at $28^{O}C$ also indicated that a reduction in the manganese levels occurs in this mutant. When it was discovered that LF-2 was temperature dependent with respect to its phenotype, samples were prepared from cells grown at the lower ($18^{O}C$) and higher ($34^{O}C$) temperatures, along with WT controls. It is evident from the data of Table 6 that the loss of photosynthetic activity observed in LF-2 as a function of increasing growth temperature is accompanied by a large reduction in the manganese bound to its thylakoids. The chl/Mn values for LF-2 range from 209 for cells grown at $18^{O}C$ which possess nearly 80 percent of the WT photosynthetic rates, to 905 from cells grown at $34^{O}C$ which have only ten to 20 percent of the WT activity. As was noted for the photosynthetic activities, at the higher temperatures the values for LF-2 showed much greater variation than the WT or LF-2 ($18^{O}C$) data. A second analysis of the membranes from LF-2 ($34^{O}C$) indicates a very low Mn content--in parentheses in the table, a chl/Mn ratio of 8664--and probably represents a combination of variability in the manganese content in these membranes plus the inaccuracy of the analytical method at extremely low manganese levels.

The reversible nature of the manganese deficiency of LF-2 is indicated by the loss of manganese when cells from $20^{O}C$ cultures are grown for two days at $34^{O}C$ (chl/Mn = 704) and the recovery of the element when $34^{O}C$ cells are used to inoculate media, and cultured at $20^{O}C$ (chl/Mn = 222).

The WT controls grown at $18^{O}C$ and $34^{O}C$ indicated that there may be some decrease in the manganese content of the membranes. However, this decrease (or variability) is very small when compared to the large alterations observed in the mutants.

Since it has been suggested that copper may play an essential role on the oxidizing side of PSII (see Introduction), its content in the thylakoids of the strains used in this study were also analyzed (Table 6). The chl/Cu ratios for membranes from 28°C cultures of WT, LF-1 and LF-2 were remarkably similar (214, 212, and 212, respectively). The samples from cells grown at 18°C and 34°C showed greater variation, but again, no correlation with the photosynthetic activity could be found (e.g., both WT and LF-2 showed increases in copper content at 34°C). It is known that plastocyanin contains much of the membrane copper, and that the amount of plastocyanin bound to the membranes can be affected by growth conditions and membrane isolation methods (see Introduction). Therefore, part of the variability of copper seen in these analyses may be related to plastocyanin levels. However, the similarity of the values for the WT, LF-1 and LF-2 (28°C) samples, and the WT and LF-2 (34°C) samples certainly suggests that the copper content of the membranes cannot be related to the phenotypes of the mutants.

Cytochrome Analyses

The concentrations of the cytochromes present in photosynthetic membranes can be estimated, in situ, by analysis of chemically induced difference spectra. (Table 7 contains the chlorophyll to cytochrome molar ratios obtained from such analyses of isolated membranes of WT and the mutants LF-1 and LF-2.) The problems associated with the determination of cytochrome levels in photosynthetic membranes are well known (see Bendall et. al., 1971; and Cramer and Whitmarsh, 1977 for discussions). The problems arise from lack of knowledge of accurate alpha band extinction coefficients for the b-type cytochromes, from overlapping

(or mixed) spectra, the existence of several redox potential forms of b-559, and from variations in accessibility of the membrane bound cytochromes to the chemicals used in the assays. Given these limitations, the analyses still provide the best means of estimating cytochrome concentrations and are especially useful in revealing differences between samples which can be subjected to nearly identical assay conditions.

The data in Table 7 reveal that the isolated membranes of WT and LF-1 possess similar levels of total b-type (b-563 and total b-559) and cytochrome f. A major difference, however, exists in the measured amount of the highest potential form of cytochrome b-559 with the mutant containing much less than the WT. These data are in agreement with those of Epel et. al. (1972) who examined low flourescent mutants of Chlamydomonas which were also blocked on the oxidizing side of PSII. The question remains, however, as to whether the loss of the high potential form of 559 is due to an actual decrease in the amount of the cytochrome in the membrane or to an alteration of the membrane environment resulting in cytochrome conversion to a lower potential form. As was discussed in the introduction, such a conversion has been demonstrated to occur upon chemical inhibition of the water-splitting apparatus and can be correlated with the loss of manganese required for water oxidation (Horton and Croze, 1977). The hydroquinone-ferricyanide difference spectrum has classically been used to detect the high potential form of b-559; however, as Horton and Croze point out, hydroquinone may also be reducing some intermediate potential forms of b-559. They suggested that a better titrant would be ferrocyanide (E_{m7}ferri/ferro-cyaninde=+380 mV, E_{m7}hydroquinone/benzoquinone=+ 260 mV), which would be less likely to reduce any but the highest potential form of B-559 ($E_{m7.8}$=383 mV). Also, the

Table 7. The Influence of Growth Temperature on the Chlorophyll to
Cytochrome – mole ratios - of the Wild Type <u>Scenedesmus</u>
and Mutant Strains.

		Total b	b-563	Total b-559	b-559HP	Cyto-f
WT	20°C	185	347	400	613	656
	28°C	173	295	385	609	701
	34°C	165	323	333	650	692
LF-1	28°C	199	390	412	1855	878
LF-2	20°C	212	312	667	619	703
	28°C	208	401	435	774	774
	34°C	173	390	312	1141	874
34°C + Mn*		164	284	384	593	812

See Materials and Methods for the experimental detail. The values repre-
sent averages of two or three experiments.

* These membranes were from LF-2(34°C) cells which had been reactivated
by incubation with one mM MnCl$_2$ in 5 mM PIPES buffer, pH 6.8.

complication of cytochrome f (E_{m7} = + 365 mV), is not encountered with ferrocyanide as it is with hydroquinone. Figure 7 shows the ferro-ferricyanide difference spectra for WT and LF-1 membranes and reveals the large difference between the two. The membranes of LF-1 have been shown to be missing about two-thirds of the manganese normally present in WT membranes and it is a reasonable hypothesis that it is the lack of this manganese which has resulted in loss of the highest potential form of b-559 in this mutant. Supporting evidence for this hypothesis comes from the analysis of the cytochrome contents of the isolated membranes of LF-2. When grown at 20°C, LF-2 possesses high photo-synthetic ability and also has near WT levels of all measured cyto-chromes including b-559 HP (Table 7). As the growth temperature is raised to 28°C and then to 34°C, a large variation from the WT is seen only in the b-559 HP level. The difference spectra of membranes from LF-2 (34°C) cells grown under these conditions (see Materials and Methods) are very similar to those of LF-1. It is also noted that the loss of the high potential form of b-559 in LF-2 (28°C) and (34°C) is paralleled by a loss of membrane bound manganese (Table 6). However, LF-2 (34°C) can be rapidly reactivated by simple addition of manganese ions to the suspended cells and the reactivation is accompanied by a return of the b-559 HP to WT levels (Table 7). This, again, can be most clearly seen in the ferro-ferricyanide difference spectra (Figure 7), which shows the loss of the 559 nm peak in LF-2 (34°C) and its return upon reactivation with manganese. These data demonstrate that the altered cytochrome b-559 HP level observed in LF-1 could be due to a lack of manganese bound to the membrane.

WT

LF-1

LF-2 (34°C)

0.002A

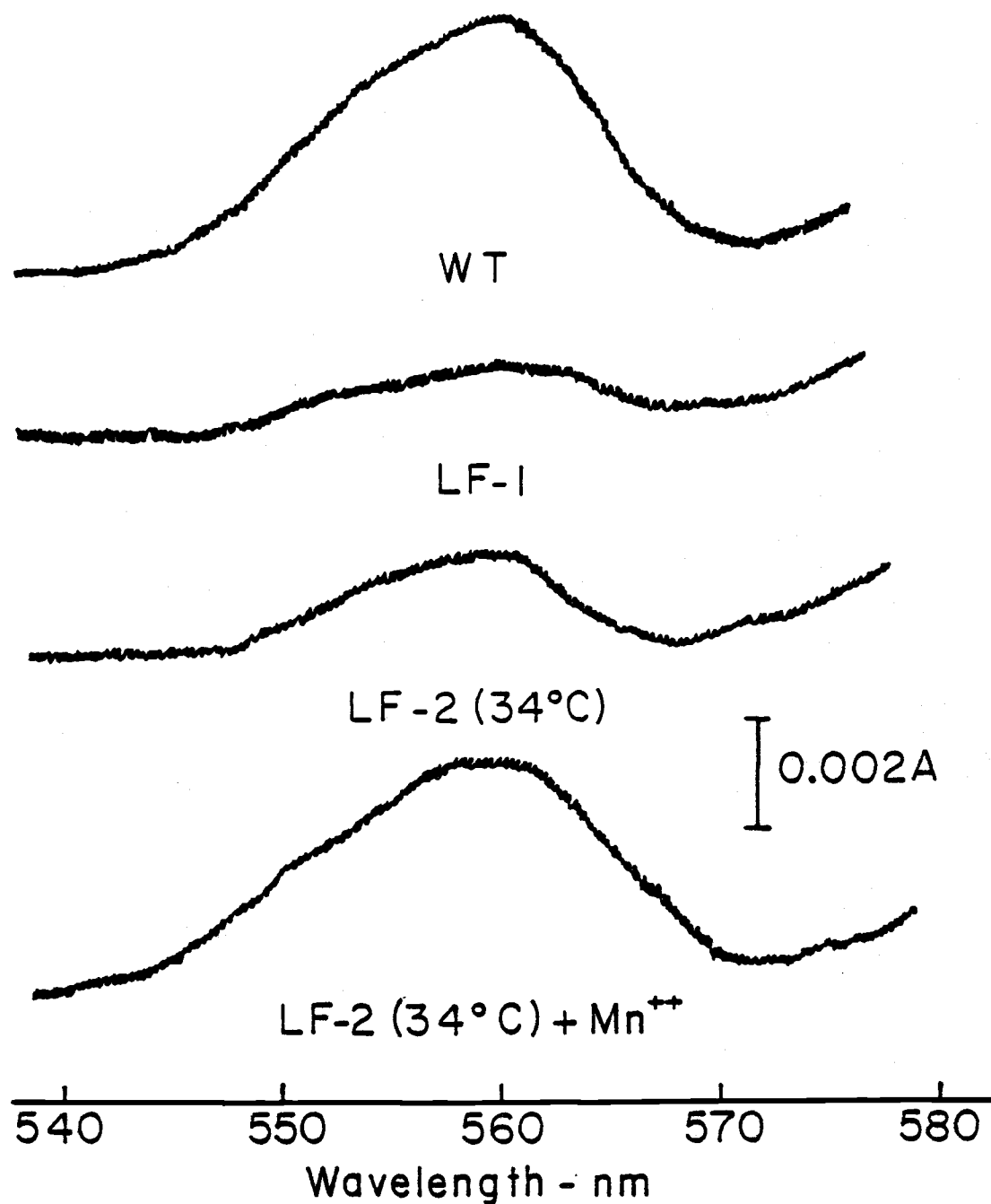LF-2 (34°C) + Mn^{++}

540 550 560 570 580

Wavelength - nm

Figure 7. Ferrocyanide minus ferricyanide induced absorbance difference spectra of membranes isolated from cells of the wild-type Scenedesmus and the mutants LF-1, LF-2 (34°C) and LF-2 (34°C) which had been reactivated by incubating in 1 mM MnCl$_2$.

Sodium Dodecyl-sulfate Polyacrylamide
Gel Electrophoresis

It has been a basic assumption in photosynthesis research that there is a protein or proteins which contain the 'active site' where the water-splitting reaction occurs. The complex and ordered kinetics, the requirements for chloride ion and for bound manganese ion, and the ability of various treatments to preferentially inhibit the process all indicate the involvement of an enzyme-type system distinct from the rest of the electron transport chain of PSII.

The primary reason for utilization of the mutant approach to this problem is that the possibility exists that the phenotype selected may be due to a specific alteration of a component of the membrane associated with the water-splitting reaction. The power of the method arises from the availability of the WT strain and numerous other phenotypes for direct comparison in all analyses.

The best technique available at the present time, for separation of water-insoluble membrane proteins is polyacrylamide gel electrophoresis of detergent solubilized samples. The technique is especially well suited for mutant analysis since the WT and mutant proteins can be separated under identical conditions and even minor alterations in the resulting protein banding patterns can be detected. Chua and Bennoun (1975) had demonstrated that isolated thylakoids of Chlamydomonas which had been purified on a sucrose step gradient, could be separated by SDS-PAGE to reveal at least 33 distinct polypeptide bands. A similar purification procedure for Scenedesmus membranes was developed to eliminate proteins from the sample which were not associated with the thylakoids. A critical feature of this purification procedure was that

thylakoids were found to retain between 80 and 90 percent of the photo-synthetic water-splitting activity (as H_2O-MV) of the membranes prior to this step. The membranes of the mutants demonstrated a banding pattern on the sucrose gradients which was identical to the WT membrane.

Figure 8 is an example of the polypeptide pattern observed when the WT and mutant (LF-1, LF-2, LF-3 and LF-5) membranes were solubilized using the method of Chua and Bennoun (1975) and electrophoretically separated in a slab gel apparatus using the gel and buffer system of Laemmli (1970). Close examination of this gel as well as many others using SDS as the detergent, revealed no difference in the polypeptide banding patterns between the WT and mutant strains. Variations were observed in the banding patterns from gel to gel, and when different concentrations of acrylamide were used; but the mutant and WT patterns were always identical when they were separated on the same gel.

The pigments associated with the thylakoids are not covalently attached to proteins and can be easily removed by extraction into or-ganic solvents. However, it was discovered that some pigment-protein complexes can survive detergent electrophoresis (Ogawa et. al., 1966; and Thornber et. al., 1966). The fragile nature of the association ensures that the number of complexes observed, and the amount of pig-ment retained will depend greatly on the specific conditions of the experiment. Many papers have appeared which describe methods to en-hance the appearance of more pigmented bands and reduce the percentage of the pigments which are stripped off of the proteins (see Thornber et. al., 1979 for a recent review). In all cases, heating the samples (at $100^{\circ}C$ for several minutes) results in total loss of pigment-protein association. Markwell et. al., 1979, recently developed an
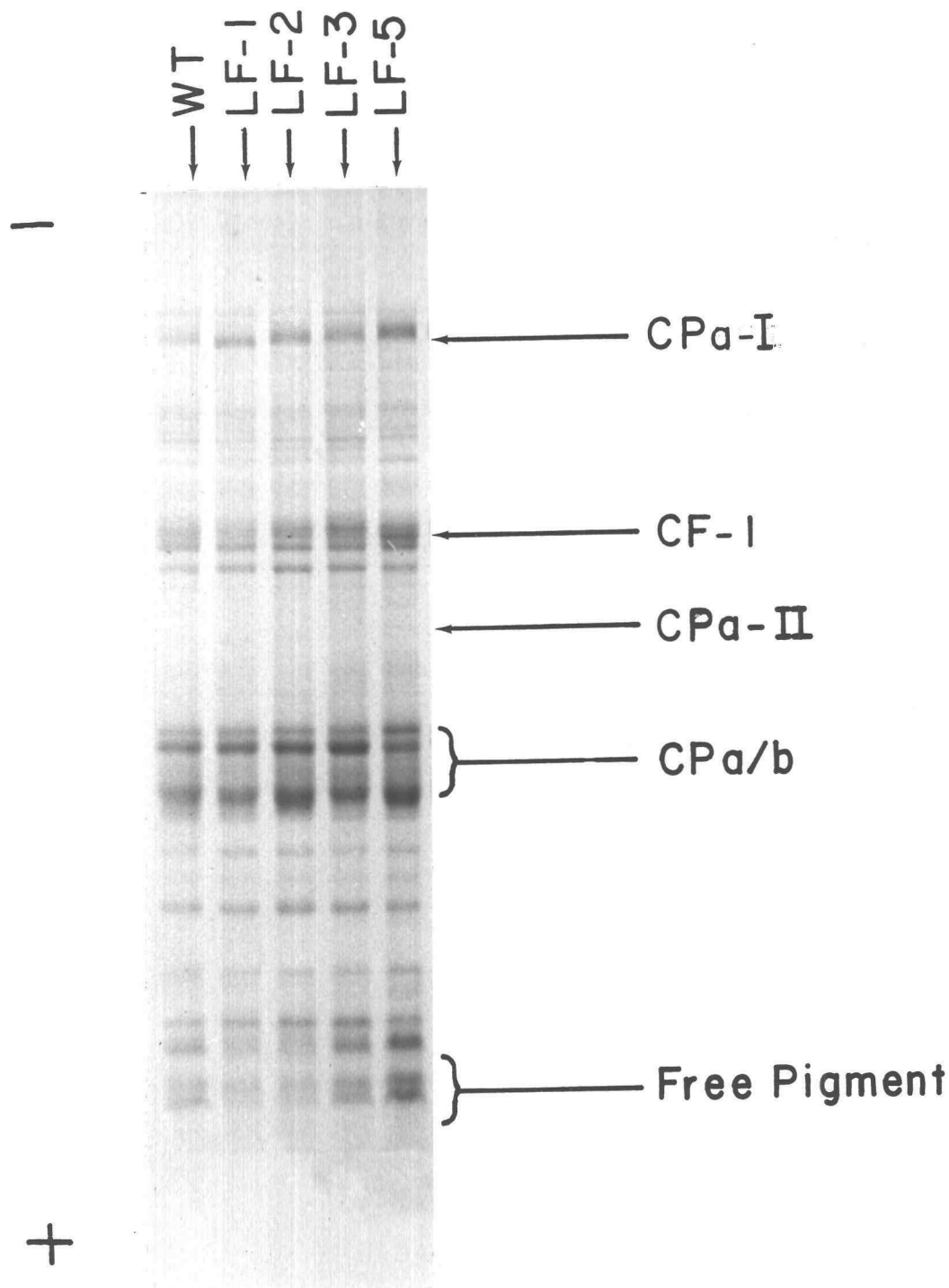
Figure 8.   Polypeptide profiles of thylakoid membranes of the wild-type
            Scenedesmus and the mutants LF-1, LF-2, LF-3 and LF-5.   The
            samples were isolated from cells grown at 28°C and analysis
            was by SDS-PAGE at 12°C using a 10% polyacrylamide concentra-
            tion in the separating gel.   The location of the chlorophyll
            protein complexes was noted and the gel stained with Coomassie
            brilliant blue R-250.

electrophoretic technique which demonstrated for the first time that all of the chlorophylls of the thylakoid are associated with protein, however, their system results in very diffuse protein banding with poor resolution of individual polypeptides.

The method used to produce the gel in Figure 8 results in the appearance of three pigment-protein bands, whose location on the gel prior to staining is indicated by the CP designations. CPa-I contains the PSI reaction center (P-700) and associated antennae chlorophylls as well as β-carotene while CPa/b is the light-harvesting pigment-protein complex and does not have a specific photochemical activity. CPa-II, a very faint band, was first described by Shutilova and Kutyurin (1976) and has been associated with the PSII reaction center. Additionally, there is a pigmented band not associated with protein but running near the electrophoretic front as micelles with the detergent (the free pigment zone). It must be noted that there is no standard nomenclature for these complexes and many systems are presently in use (Thornber et. al. 1979).

<div align="center">

Lithium Dodecyl-sulfate Polyacrylamide
Gel Electrophoresis

</div>

Delepelaire and Chua (1979) recently described a gel technique using lithium dodecyl-sulfate (LDS), which is more soluble than SDS at lower temperatures. This allows for the electrophoresis of whole membrane samples at $4^{o}C$ instead of $12^{o}C$ as in SDS-PAGE. Using membranes from Chlamydomonas, they found that two-pigment protein complexes appeared between CPI and CPa/b, and they presented evidence that both were related to PSII. This technique was used to analyze the membranes

isolated from the WT and LF-1. The pigmented bands described by Dele-
pelaire and Chua for Chlamydomonas were observed in these experiments
with Scenedesmus (Figure 9). The two PSII bands (Delepelaire and
Chua's CP-3 and CP-4 are labeled CPaII-1 and CPaII-2 in Figure 9),
were easily recognized. The CPa/b complex in our gels appeared to be
divided into two major regions with possibly several bands in each
region. CPa-I was always very clear and occasionally a band, probably
related to CPa-I appeared between CPa-I and the origin. As expected,
no difference between the WT and LF-1 was ever noted for these com-
plexes (LF-3 and LF-5 give identical results). However, when the gels
are stained to reveal the polypeptide pattern, a striking difference
between the WT and LF-1 is evident (Figure 10). A major polypeptide
band with an apparent molecular weight of 34 kilodaltons (kd) (see
section on molecular weight estimation) present with the WT is absent
in the LF-1 pattern, while a band of equal intensity but with an ap-
parent molecular weight of 36 kd is present in LF-1 but not the WT.
Careful examination of the gel reveals that this is the only detectable
alteration of any major or minor band.

An immediate hypothesis is that the genetic alteration in LF-1 has
resulted in the shift of the apparent molecular weight of a protein from
34 kd to 36 kd. This relationship between the 34 and 36 kd bands is
suggested by their proximity, their equal staining intensity and the
fact that this is the only alteration observed upon close examination
of the gels.

Delepelaire and Chua (1979) had reported that they occasionally
observed a green band in the region between CPaII-2 and the CPa/b
complex, however, in the gels which reveal the 34 and 36 kd bands,
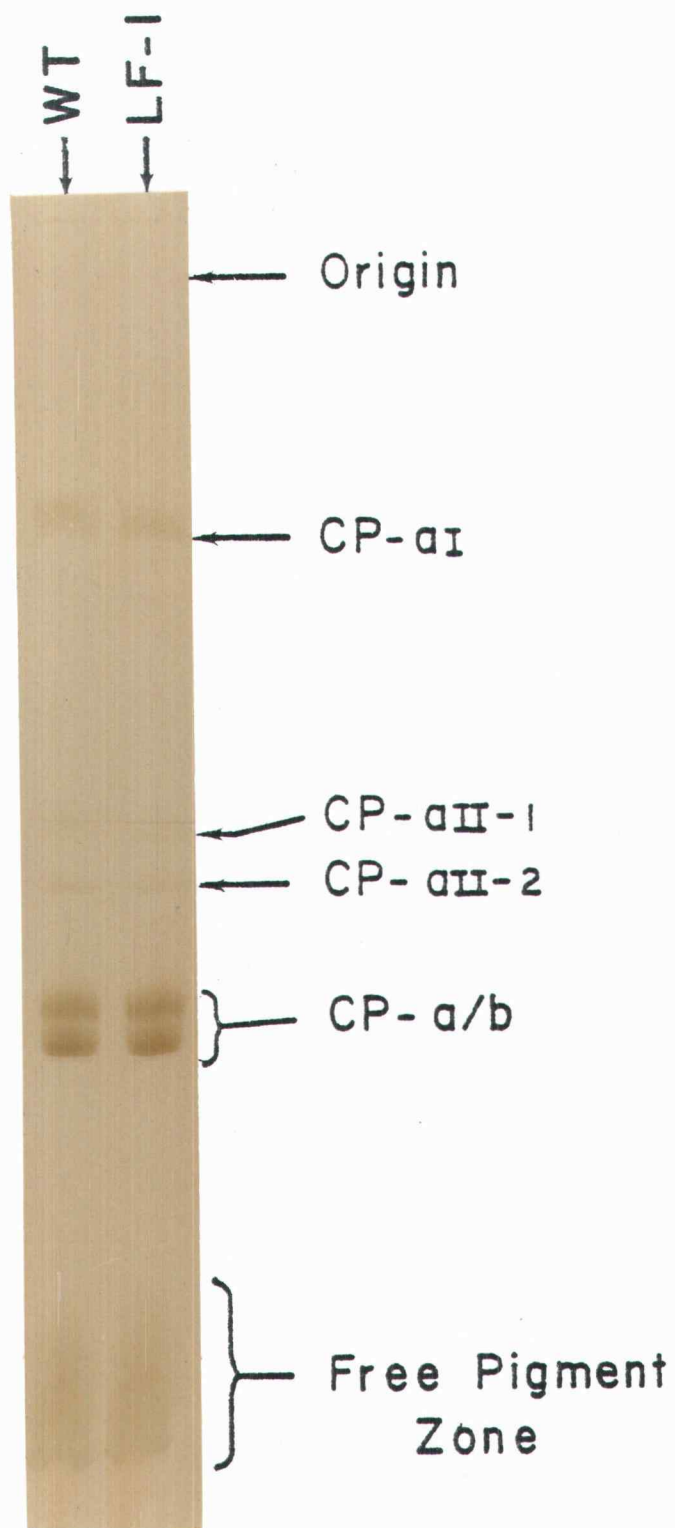
Figure 9.   Chlorophyll-protein complexes of the wild-type Scenedesmus and
            the mutant LF-1. The complexes were separated by LDS-PAGE at
            4°C on a 7.5% to 15% polyacrylamide gradient gel. The gel was
            photographed prior to staining.

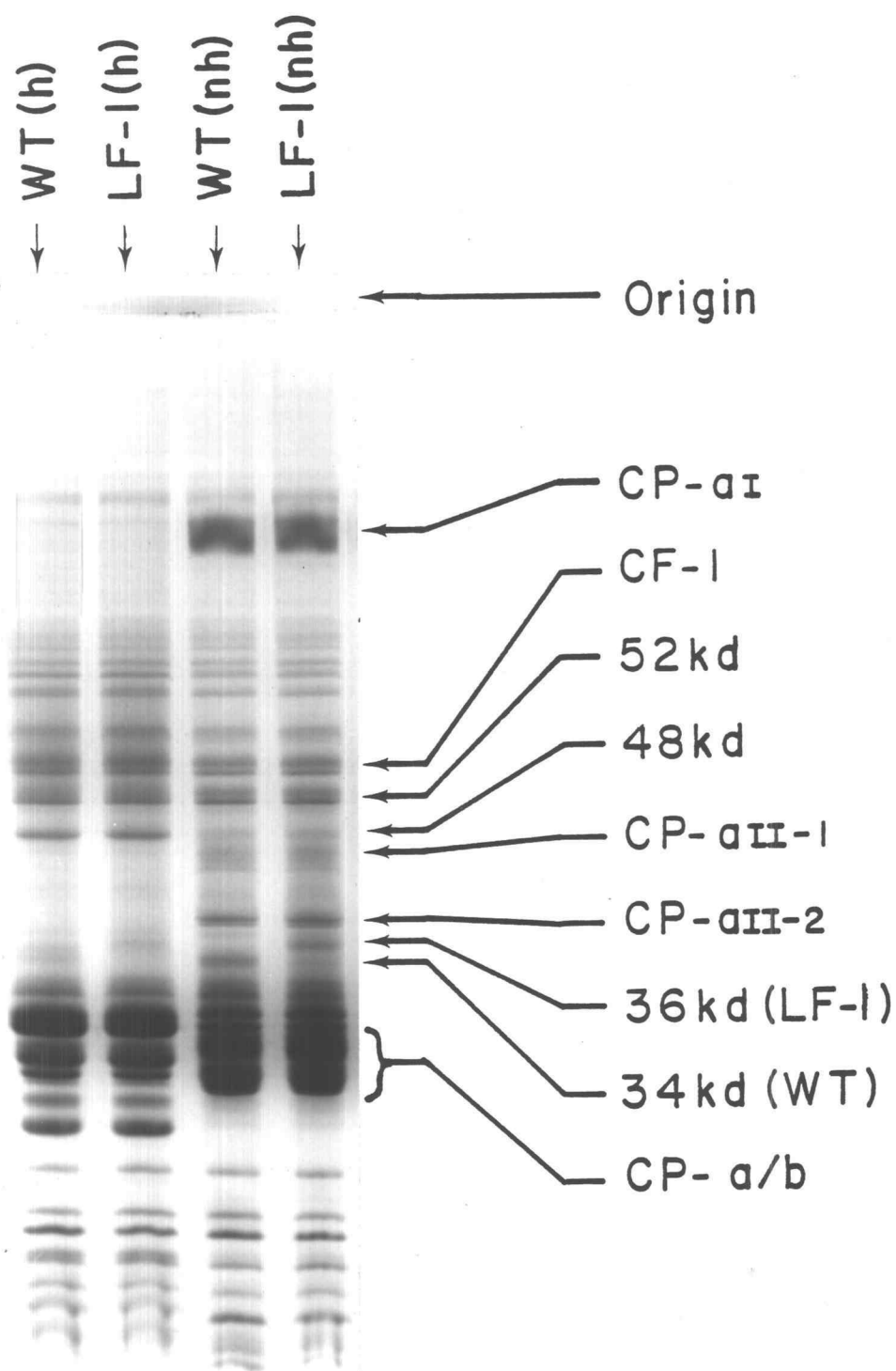Figure 10. Polypeptide profiles of thylakoid membranes from the wild-type
Scenedesmus and the mutant LF-1. Analysis was by LDS-PAGE at
$4^{o}C$ on a 7.5% to 15% polyacrylamide gradient gel. The solubil-
ized samples were either heated (h) for two minutes at $100^{o}C$ or
maintained at $4^{o}C$ (nh) prior to electrophoresis. The gel was
stained with Coomassie brilliant blue R-250.

there is no evidence of such a pigmented band. Heating of the samples

of WT and LF-1 (100°C for two minutes) prior to electrophoresis results

in the protein pattern observed in slots one and two of Figure 10.

The treatment leads to dissociation of the pigment-protein complexes

and the only colored area in the gel, prior to staining is the free

pigment zone. Of interest now, is that most of the protein associated

with the 34 and 36 kd bands is missing in these heated samples. How-

ever, it is significant that some staining is still evident and that the

difference between WT and LF-1 is retained. Close examination of the

gel does not reveal any other alterations in the protein patterns.

The reason for the disappearance of most of the protein of the 34 and

36 kd bands upon heating is at present unknown, however, the lack of

observable pigmentation in the regions in unheated samples and the

retention of traces of both bands in heat treated samples suggests

that it is not due to dissociation of pigment-protein complexes. Also,

other workers have noted that aggregation of polypeptides can occur

upon heating in the presence of SDS resulting in the loss of protein

bands (e.g., see Wessels and Borchert, 1978). That heating may induce

association with other membrane components is a distinct possibility,

but it must await further experimental evidence. Arguments against

the possibility that the 34 and 36 kd bands represent multimeric pro-

teins are found in later sections. The reasons why the difference

between the WT and LF-1, which is so obvious in LDS gels was never

observed after SDS-PAGE of the samples is also unknown, and requires

further investigation.

When membrane samples from LF-3 and LF-5 are subjected to LDS-

PAGE, along with LF-1, it is observed that the protein banding

patterns of all three mutants are identical (Figure 11). Although these mutants were independently isolated, it appears that the genetic alteration has resulted in an identical shift of the 34 kd protein to one of 36 kd.

Recently it has been found that some membrane proteins of mito-chondria and chloroplasts are synthesized and inserted into the mem-branes in a precursor form and subsequently processed by the removal of a small polypeptide chain to yield a physiologically active mem-brane component (e.g., Grebanier et. al., 1978 and Werner, 1974). That the 36 kd protein of LF-1, 3 and 5 represents a non-processed precursor form of the 34 kd protein of the WT is certainly suggested by the increase in the apparent molecular weight of the mutant poly-peptides and by the identical nature of the modification in several isolates of a generally similar phenotype.

Figure 12 shows the polypeptide pattern after LDS-PAGE of mem-branes of WT and LF-2 isolated from cells grown at 18° and 34^OC. The pigmented bands observed prior to staining were identical to those described for WT 28^OC samples with no difference between the WT and mutant samples. Close examination of the stained gel also revealed no alteration in the polypeptide patterns. The mutant possesses the 34 kd band of the WT and apparently in an equivalent amount. This is true for samples from cells grown at both the permissive and restrictive temperatures. The cells and membranes of LF-2 (34^OC) show a severe inhibition of photosynthetic electron transport which is localized on the oxidizing side of PSII (Table 2). However, this inhibition can be rapidly reversed by simple addition of manganese to a cell suspension (Figure 4). The lack of any observable difference between the WT and
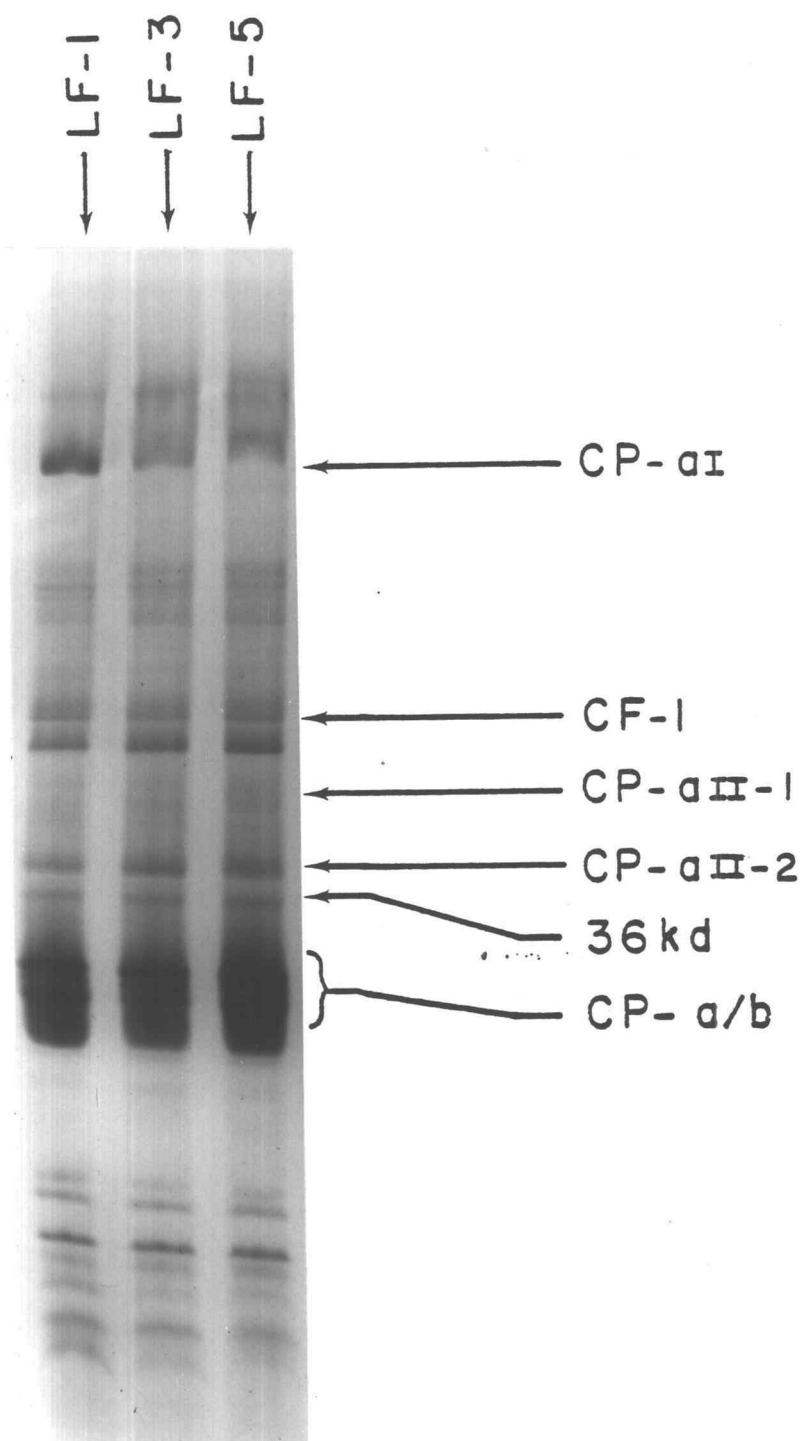
Figure 11. Polypeptide profiles of thylakoid membranes from the mutants LF-1, LF-3 and LF-5. Analysis was by LDS-PAGE at 4°C on a 7.5% to 15% polyacrylamide gradient gel. The samples were not heated prior to electrophoresis.
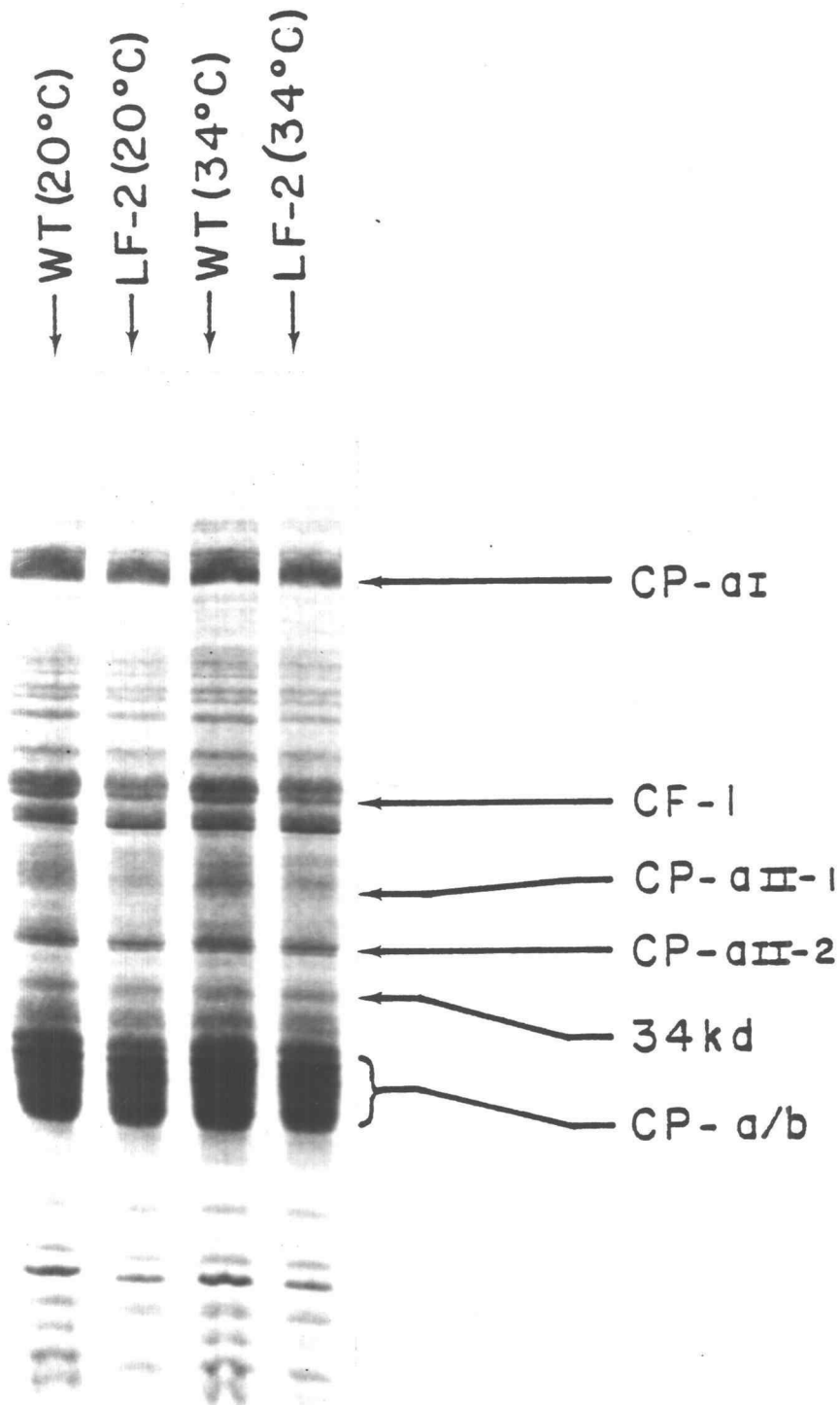
Figure 12. Polypeptide patterns of thylakoid membranes isolated from cells of the wild-type Scenedesmus and the mutant LF-2 grown at either 20°C or 34°C. The analysis was by LDS-PAGE of unheated samples on a 7.5% - 15% polyacrylamide gradient gel maintained at 4°C. The gel was stained with Coomassie brilliant blue R-250.

LF-2 in the SDS or LDS-PAGE polypeptide profiles provides further evidence that the components of the photosynthetic apparatus, and in particular, those components required for water photolysis, have not been altered in the mutant.

Molecular Weight Estimation

It has become a standard practice to estimate the molecular weights of polypeptide chains by comparison of their mobilities (i.e., migration distance x correction factors) during SDS-PAGE to the mobilities of proteins with known molecular weights (Weber and Osborn, 1969 and Neville, 1971). The molecular weights of the 34 kd and 36 kd bands of the WT and LF-1 were estimated by the procedure described in the Materials and Methods section and Figure 13 is the standard curve used for the determination. The actual figures obtained were 34.3 kd for the WT and 35.8 kd for the LF-1 proteins, but these were rounded off to the nearest whole kilodalton value. The assumption was made that LDS-PAGE was equivalent to SDS-PAGE for these determinations and this was verified by the nature of the standard curve. Also, direct migration distances, instead of mobilities were used since the samples and standards were electrophoresed on a single slab gel rather than on individual tube gels, hence, all correction factors were equivalent for all of the proteins. The gels have been run repeatedly and it has been noted that the 34 and 36 kd bands maintain the same relative relationship to the marker proteins. This is true on straight ten percent polyacrylamide gels as well as on the 7.5 percent to 15 percent polyacrylamide gradient gels.

A cautionary note must be added to these determinations, however,

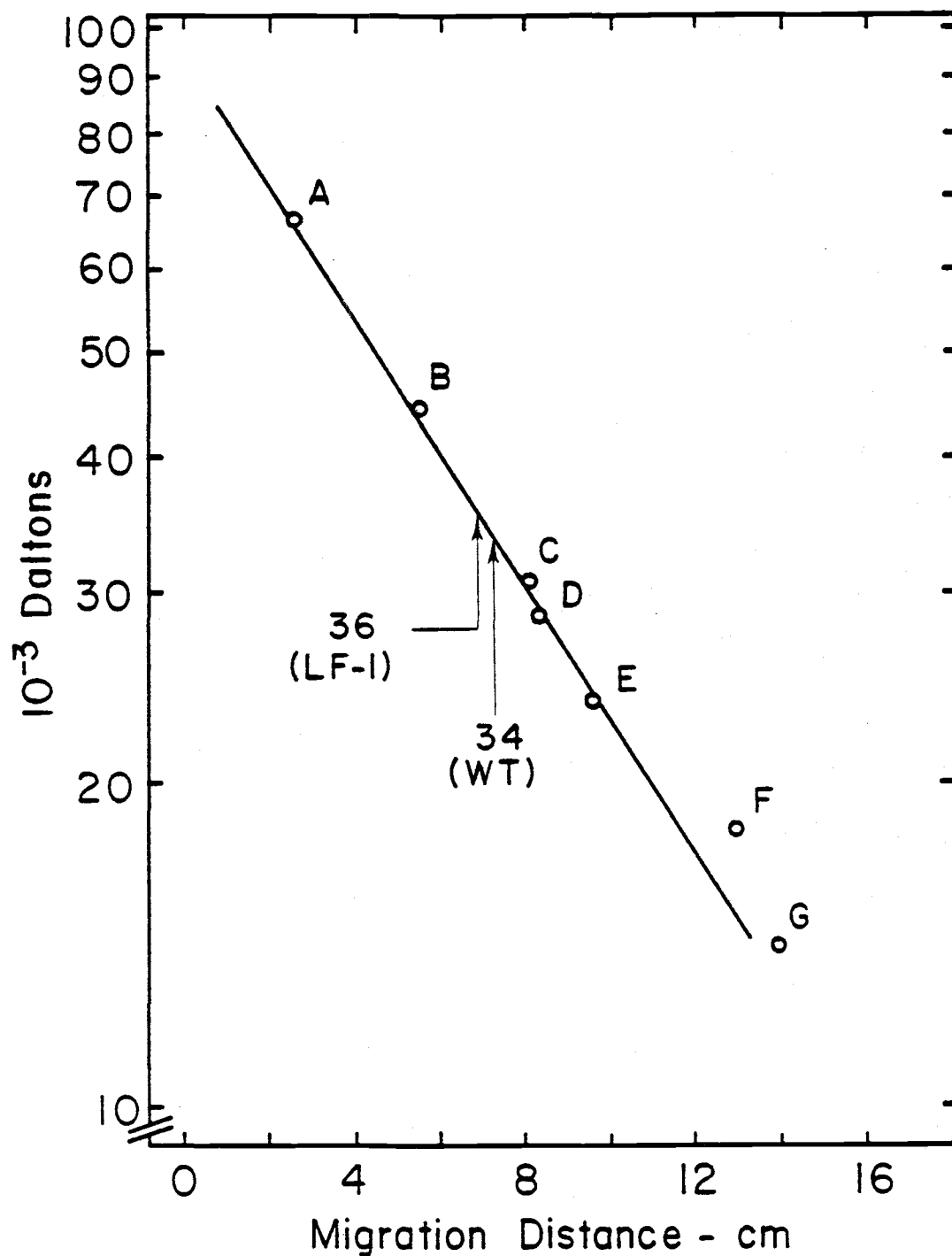Figure 13. The standard curve used to estimate the molecular weights of thylakoid polypeptides of the wild-type (34 kd) and LF-1 (36 kd). The protein markers were: A. bovine serum albumin (66,000); B. ovalbumin (45,000); C. DNaseI (31,000); D. carbonic anhydrase (29,000); E. trypsinogen (24,000); F. β-lacto-globulin (18,400) and G. lysozyme (14,300). See Materials and Methods for experimental details.

as there is evidence that factors other than molecular weight may affect migration rates in SDS-PAGE. Overall amino acid composition and sample treatment (Simon et. al., 1977) or even a single amino acid substitution in a protein (Noel et. al., 1979) can drastically alter the molecular weight estimated by this method. Therefore, it must be realized that the hypothesis that the proteins of WT and LF-1 differ by approximately 2,000 daltons in their molecular weights is based solely on a method that is usually reliable, but can be in error.

Analysis of ^{54}Mn Labeled Membranes

Since it has been reported that a 25 kd protein of Zea mays thylakoid retained significant amounts of manganese after SDS-PAGE, the possibility that a similar situation may exist for Scenedesmus was tested. Cells of WT and LF-1 were grown in medium which contained radioactive ^{54}Mn, and samples prepared for LDS-PAGE.

The samples were either not heated or heated (100° C for two minutes) and loaded onto a standard LDS-gel and electrophoresed. After the run, the presence of ^{54}Mn in sections of the gel was checked using a solid scintillation counter (see Methods section). Although the activities of the chloroplast membrane samples were high (32,000 cpm for WT and 3,200 cpm for LF-1), there was no indication of any manganese being retained by protein bands after electrophoresis. The activity of all the gel slices which contained protein was at background levels. The only manganese found in the gel migrated with the pigment-LDS micelles near the electrophoretic front (about eight percent of the total counts added to the gel). It had been hoped that a difference between WT and LF-1 could be detected; however, this was not the case under the conditions of the experiment.

Initial Attempts to Isolate the 34 kd and 36 kd
Polypeptides of the WT and LF-1 Strains

To more clearly define the relationship between the 34 kd protein of the WT and the 36 kd protein of the LF-1 strains, it is necessary to obtain them in a pure, isolated form. Once this had been accomplished, the precursor-product hypothesis could be tested using site specific proteases and SDS-PAGE techniques (Cleveland et. al., 1977). Furthermore, additional biochemical characterization of the proteins, such as amino acid composition and sequencing could be carried out.

However, the difference between WT and LF-1 was discovered when the deadline for this thesis project was not far away. Also a very unfortunate technical problem with a gel apparatus resulted in a frustrating halt to all progress for several months. Finally, however, this problem was solved and some preliminary steps were taken towards isolating these proteins.

Two general approaches were used: (1) re-electrophoresis of the proteins from gel slices; and (2) application of a method reported by Kuwabara and Murata (1979) for the isolation of a 33 kd protein of spinach.

Figure 14 shows the results of an experiment in which gel slices containing CPa-I, CPaII-1, CPaII-2, 34 kd and 36 kd bands were re-electrophoresed into a second LDS-gel along with the fresh membrane samples. The strains used for this experiment were the chlorophyll b-less mutants KØ-9 and LF-1(a/b-). These were used because neither retains chlorophyll in the CPa/b region of the gels. In WT and LF-1, the presence of chlorophyll in this area results in tailing of the proteins into the region which contains the 34 and 36 kd proteins.
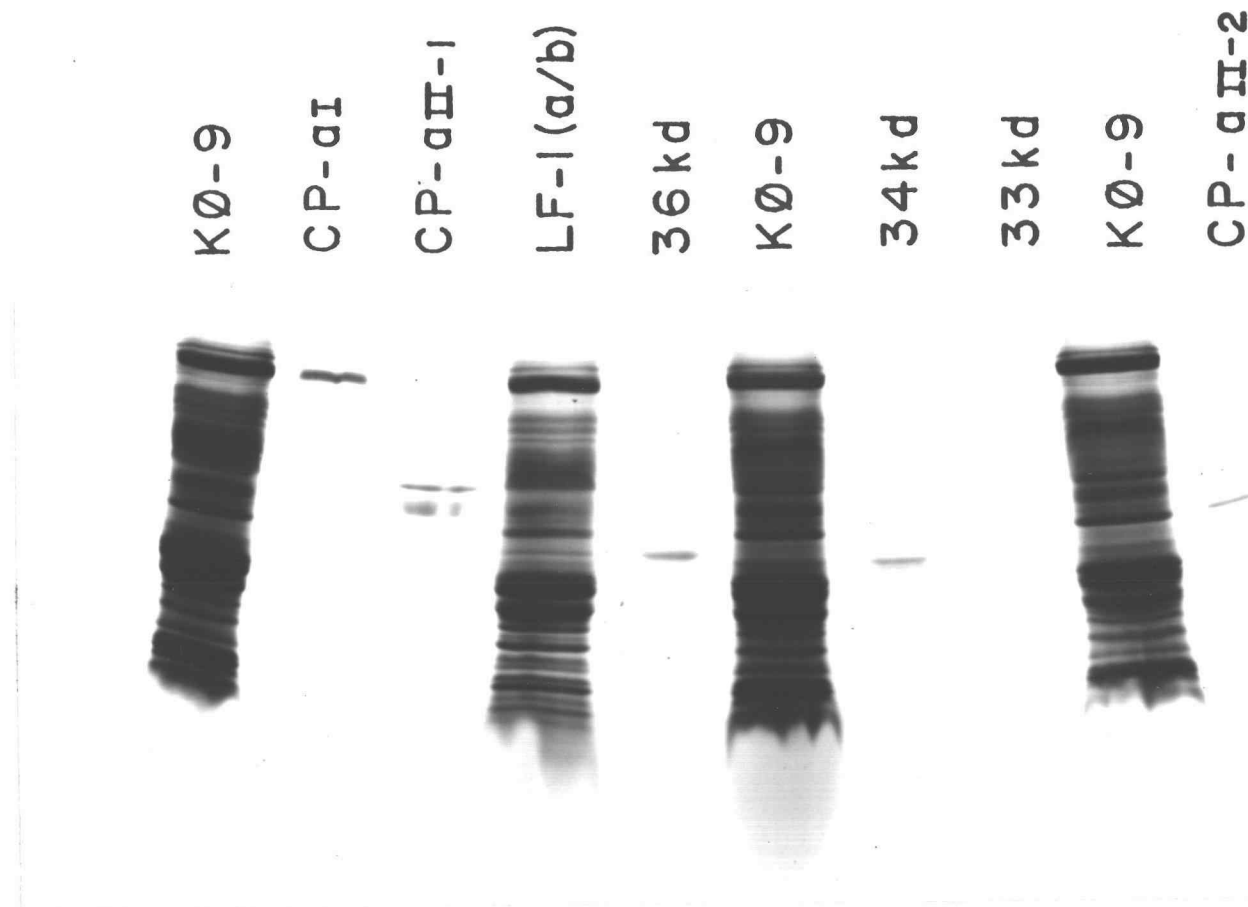
Figure 14. Re-electrophoresis of proteins and pigment-protein complexes of the mutants KØ-9 and LF-1 (a/b-). Gel slices containing the proteins of interest were cut from a LDS-polyacrylamide gel, subjected to a second electrophoretic separation along with whole membrane samples and the gel stained and photographed. The CP complexes and the 34 kd protein were taken from KØ-9 while the 36 kd protein was taken from LF-1 (a/b-). Additionally, a 33 kd protein isolated from spinach thylakoids was analyzed in this gel. The samples were not heated prior to electrophoresis. See Materials and Methods for experimental details.
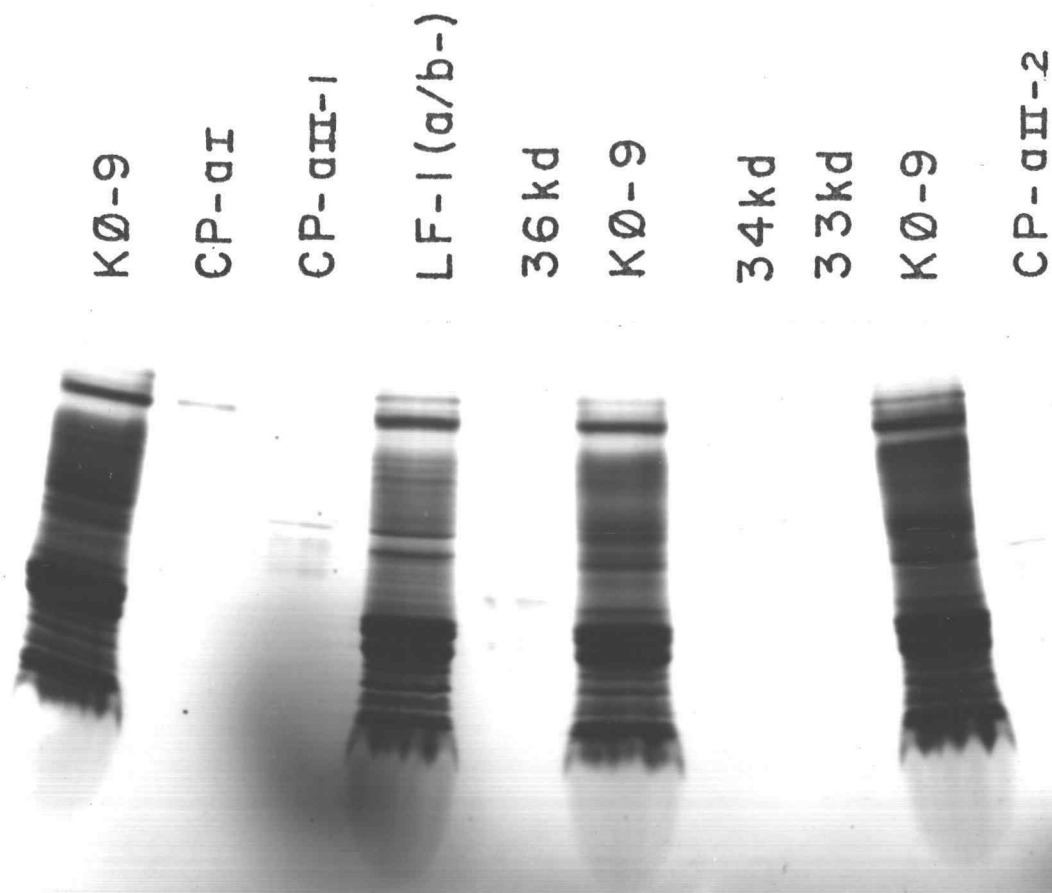
Figure 15. Re-electrophoresis of proteins and pigment-protein complexes of the mutants KØ-9 and LF-1 (a/b-). Conditions were as in Figure 14 except that all samples were heated (100°C for two minutes) prior to electrophoresis in the gel shown above. The CP complexes and 34 kd protein were taken from KØ-9 while the 36 kd protein was taken from LF-1 (a/b-). The 33 kd protein was isolated from spinach thylakoids. See Materials and Methods for experimental details.

The chlorophyll b-less mutants therefore allowed for less contamination from the CPa/b proteins in these experiments. The 34 kd protein of the WT is retained in KØ-9 and LF-1(a/b-) has the 36 kd protein of LF-1.

In Figure 14, it can be seen that the 34 and 36 kd proteins retain their original mobilities upon re-electrophoresis. They line up with the corresponding proteins from the whole membrane sample and the difference between them is retained. The 34 kd band shows some contamination from lower molecular weight proteins, which is expected since it runs so close to other proteins in the original gel. The gel slices and membrane samples in Figure 15 were heated prior to the second electrophoretic run. It can be seen that the mobility of the 34 and 36 kd proteins are unaffected by the heat treatment under these conditions.

It is also noted from the gel in Figure 14 that the CPaI, CPaII-1 and CPaII-2 complexes are more or less stable to re-electrophoresis with some conversion to the apoproteins seen in CPaII-2 and in CPaII-1. Heating the gel slices prior to re-electrophoresis results in total conversion to the apoproteins in CPaII-1 and CPaII-2 (see Delepelaire and Chua, 1979), while CPaI is still somewhat resistant, with no clear apoprotein band being identifiable.

Kuwabara and Murata (1979) recently described a technique for the purification of a 33 kd thylakoid protein from spinach chloroplasts or purified PSII complexes. Since the protein was associated with PSII particle preparations and had a molecular weight near to that of the 34 kd protein of the WT Scenedesmus membranes, it was believed that the two proteins could be homologous. The protein was isolated from spinach using their method with omission of the final isoelectric-

focusing step. The yield of the protein was very low; however, LDS-PAGE revealed that the protein was quite pure (i.e., one detectable band upon analysis by LDS-PAGE; Figures 14 and 15).

In a preliminary experiment, the procedure listed by Kuwabara and Murata for preparing PSII particles from spinach was applied to isolated membranes of LF-1(a/b-) Figure 16 shows a LDS-PAGE analysis of this preparation along with whole membrane samples. It is evident that a polypeptide is present in the PSII preparation which corresponds to the 36 kd polypeptide of the whole membrane sample of LF-1 (a/b-). Also, a sample of the PSII preparation was heated in the solubilization solution, a procedure that in whole membrane preparations of WT and LF-1 results in a drastic reduction in the amounts of the protein in the 34 and 36 kd bands. In this case, however, no loss of intensity of the 36 kd band is evident. This feature has also been noted by Dr. Bishop in his efforts to isolate the 34 kd polypeptide from the WT strain; i.e., once this polypeptide has been partially purified, heating does not affect its migration in LDS-PAGE. Based on these and other observations, it is believed that the 34 kd polypeptide of Scenedesmus is homologous to the 33 kd polypeptide isolated by Kuwabara and Murata from spinach. The indications are that the 34 kd and 36 kd bands represent single polypeptide chains and that with some modifications, the procedures used by Kuwabara and Murata may allow isolation of the algal proteins. However, this is speculation at this time, and will, of course, require experimental verification.
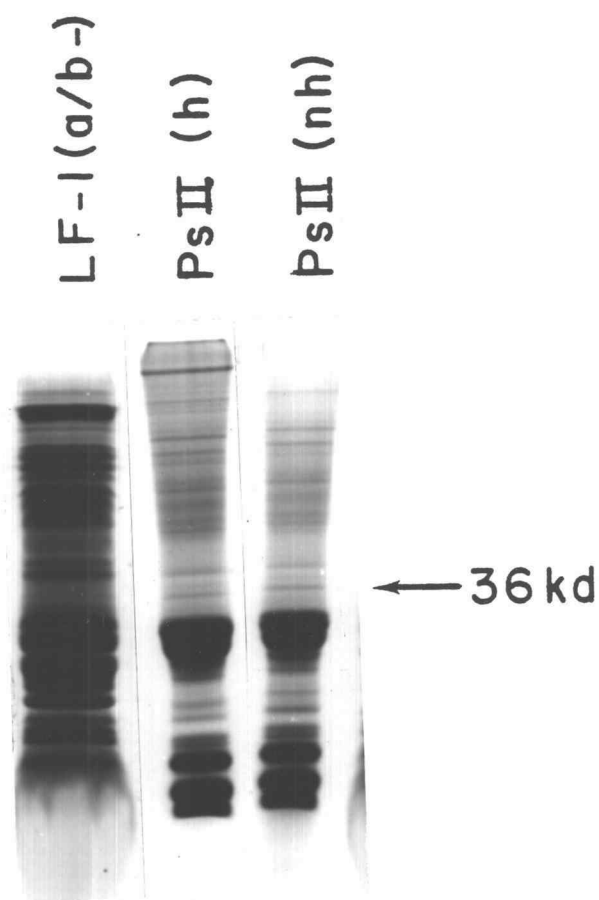
Figure 16.   LDS-PAGE of polypeptide patterns of whole membranes (non-
heated) and PSII particle preparations (nh = non-heated
and h = heated for two minutes at $100^\circ C$) from the mutant
LF-1 (a/b-).   See Materials and Methods for experimental
details.

IV. CONCLUSIONS

The Nature of the Genetic
Alteration in LF-2

The expression of the mutant phenotype in LF-2 has been shown to depend both upon the temperature at which it is grown and upon the concentration of manganese ion available to the cells. If cultured on standard media, the mutant exhibits high rates of photosynthetic activity when grown at $18^{o}C$, but severely restricted activity when grown at $34^{o}C$. The affected portion of the photosynthetic apparatus is initially restricted to a block in electron transport specifically on the oxidizing side of PSII. The location of the block, the decrease in the amount of manganese bound to the thylakoids as the growth temperature is raised, plus the ability of high concentrations of added manganese ion to restore photosynthetic activity to cells grown at $34^{o}C$, all indicate that it is a deficiency of this ion in the membranes which results in the loss of water-splitting capability.

The high rate of photosynthetic activity of either reconstituted cells, or those grown at the permissive temperature, plus the observation that simple transfer of cells from $18^{o}C$ to $34^{o}C$ does not lead to immediate inactivation suggest that the specific binding site for manganese ion on the oxidizing side of PSII has not been affected by the mutation. Additionally, no alteration of the polypeptide pattern of isolated thylakoids could be detected, although it is realized that the methods used may not be sensitive enough to reveal the minor changes in proteins which can result in a temperature-dependent phenotype. The absorption spectra of the thylakoids, the flourescence and

PAGE data all indicate that pigments of the membrane and the photosystems are organized within the chloroplast in a manner equivalent to that of the WT strain.

Furthermore, the data suggest that either the uptake of manganese ion or some step involved in its incorporation into the active site has been affected by the mutation. LF-2 should prove to be a useful tool for the further study of manganese uptake and incorporation into the photosynthetic membrane. The component affected by the genetic alteration in LF-2 is likely to be a protein present in minor amounts in either one of the membranes of the chloroplast or in the inner thylakoid space. This study would be greatly facilitated if techniques become available for the isolation of intact chloroplasts from Scenedesmus.

With respect to the investigation of the oxidizing side of PS II, the study of the characteristics of LF-2 has proven especially useful in providing a rationale for the behavior of the redox potential of cytochrome b-559 under conditions of induced manganese deficiency. Horton and Croze (1977) demonstrated that the conversion of the high potential (380 mV) form of this cytochrome to one with a lower potential (240 mV), could be correlated with the removal of manganese ion from the thylakoids and with inactivation of PSII following hydroxylamine treatment. The study of the mutant, LF-2, has verified their conclusion that it is specifically the loss of manganese which results in the lowering of the redox potential. Additionally, this study demonstrated for the first time that the high potential form can be completely restored when manganese is reincorporated into the membrane (Table 7). This is a crucial point, since the low fluorescent algal

mutants used in this study, as well as those described by Epel et. al.,
1972, show a significant loss of the b-559 high potential cytochrome.
The study of the characteristics of LF-2 suggests that the Chlamydomonas
mutants are most likely manganese deficient; if so, this would explain
the observed alterations of the redox state of cytochrome b-559.
Whether their mutants remain available for study is not known, however,
if they are, an analysis of their manganese content, and thylakoid poly-
peptide composition in light of the findings with the Scenedesmus
mutants would provide additional comparative data. Also, although not
extensively discussed in this thesis, several additional low fluoresc-
ent mutants of Scenedesmus with phenotypes comparable to LF-2, are
available and hopefully will be investigated at some future time.

The Nature of the Genetic Alteration in the Mutants LF-1, LF-3 and LF-5

The mutants, LF-1, LF-3 and LF-5, are drastically reduced in their
photosynthetic capacities. The site of the block in electron flow is
restricted to the oxidizing side of PSII with the balance of the photo-
synthetic apparatus intact and functional when provided with electron
donors other than water.

Analysis of the polypeptide composition of isolated thylakoids
of the WT and mutant strains by LDS-PAGE (Figures 10 and 11), revealed
that the mutants lack a 34 kd band which appears to have been replaced
by a 36 kd band. Based on the current evidence, it is hypothesized
that the 36 kd protein of the mutants represents a non-processed form
of the physiologically active 34 kd protein of the wild type. Many
examples of analogous precursor-product relationships have recently

been described for proteins which must either pass through or function in hydrophobic membranes (see Wickner, 1979 for a recent discussion of this phenomenon). Grebanier et. al. (1978) demonstrated that a 34.5 kd polypeptide, which is a major membrane associated, early product of protein synthesis in isolated maize chloroplasts is a precursor of a 32 kd protein present in chloroplasts of the intact cell. This example, as well as others (Werner, 1974 and Leto and Miles, 1980), has shown that precursor proteins can accumulate in the membranes prior to being processed. The genetic alteration which resulted in the phenotype exhibited by LF-1, LF-3 and LF-5 is hypothesized to have altered some portion of the mechanism involved in processing the 36 kd polypeptide to form the physiologically active 34 kd polypeptide observed in the WT.

These mutants also show a loss of the high potential form of cytochrome b-559 (380 mV). However, the total amount of b-559 does not appear to have been altered (Table 7). Also, a possible explanation for these data has been provided by the study of the mutant LF-2, which revealed that a similar cytochrome pattern results when there is a manganese deficiency in the thylakoids. The isolated membranes of LF-1, LF-3 and LF-5 contain only about one-third of the manganese normally associated with the WT membranes (Table 6). The constant nature of the deficiency plus the inability of high concentrations of this element to restore photosynthetic activity to the mutants indicates that this is not due to an impairment of manganese ion uptake. Rather, it is believed that the manganese ion is not being incorporated into the site on the oxidizing side of PSII at which it is specifically required for water photolysis. The evidence suggests that it

is the interaction between this manganese and cytochrome b-559 which allows for the appearance of the 380 mV redox form.

The question which has not been answered, is whether the b-559 HP form is itself required for the water-splitting reactions, or if it is just an indicator of the presence of manganese at the active site. It can only be concluded from this study, as it has been in many others where the experimental approaches have been less direct than those employed here, that the high potential state indicates the maximum potential of the chloroplast membranes for water photolysis.

Identity and Function of the 34 kd of the Wild Type Scenedesmus Strain

All of the data indicate that the 34 kd polypeptide of the wild type Scenedesmus thylakoids plays some essential role on the oxidizing side of PSII. Experiments with the mutants lacking this polypeptide have demonstrated that it is not required for the normal functioning of the rest of the electron transport system or for excitation transfer to the photochemical reaction centers. Kuwabara and Murata (1979) recently isolated a 33 kd protein from spinach, and preliminary experiments indicate that it may be homologous to the 34 kd protein in Scenedesmus. They suggested that the spinach protein functions in PSII either in electron transport or in excitation transfer, based solely on its specific association with PSII particles. Other workers have identified thylakoid proteins with molecular weights near 34 kd and associated with PSII particles in both higher plants (Anderson and Levine, 1974; Nolan and Parker, 1975; and Novak-Hofer and Siegenthaler, 1977) and in a cyanobacterium (Newman and Sherman, 1978), however, they did not propose any functional role for these proteins.

Cytochrome b-559 is known to be closely associated with PSII and it is present in PSII particle preparations. The mutant LF-1 is lacking the high potential form of this cytochrome, however, it retains WT levels of total b-559 (Table 7). The study of the cytochrome content of LF-2 ($34^{o}C$) before and after reactivation with manganese ion has provided a rationale for the loss of b-559 HP form in LF-1. Also, cytochrome b-559 has been isolated from spinach and found to be a lipoprotein (estimated molecular weight = 110,000), which contains only small polypeptide chains, all with a molecular weight of near 5,600 daltons (Garewal and Wasserman, 1974). It is therefore highly unlikely that the 34 kd polypeptide of Scenedesmus represents a portion of monomeric or oligomeric forms of this cytochrome. The other b-type cytochrome of the photosynthetic membranes (b-563) has also been isolated and partially characterized (Lach and Böger, 1977; and Stuart and Wasserman, 1975). It also contains only small polypeptide chains, with molecular weights estimated at 20,000 daltons or less. The bound form of cytochrome f (c-553), has a molecular weight of near 33 kd (Böhme et. al., 1980). However, it is thought to operate in the electron transport chain near PSI, and has not been associated with PSII particles. Additionally, cytochrome analysis indicates its presence in the LF- mutants.

Further evidence for the association of the 34 kd polypeptide with PSII comes from analyses of the composition of thylakoids of other mutants in this laboratory. Certain high-fluorescent mutant phenotypes apparently suffer a complete loss of the PSII apparatus. LDS-PAGE of their photsynthetic membrane reveals these mutants, in addition to missing the two PSII pigment-protein complexes (as well as the apo-

proteins) are also lacking the 34 kd protein (Bishop, Metz and Wong, 1980b).

There have been reports of chloroplast membrane proteins in higher plants which are synthesized as precursors which are processed by removal of a portion of the polypeptide chain (Grebanier et. al., 1978 and Weinbaum et. al., 1979). The molecular weights reported for these proteins are similar to the 36 and 34 kd proteins of LF-1 and WT Scenedesmus, however, since no evidence for physiological roles were included, establishment of a possible relationship among these proteins must await further and more detailed analysis.

At the present time, except for the requirements for manganese and chloride, knowledge concerning the biochemical composition of the oxidizing side of PSII is scanty. Spector and Winget (1980) recently presented evidence for the involvement of a 65 kd manganese-containing protein in the water-splitting reactions in spinach chloroplasts. However, the lack of a 65 kd protein in their original chloroplast preparations plus the specialized nature of their assay systems suggest that the report will require complete verification from other laboratories prior to general acceptance. Additionally, during the analyses in this laboratory of many mutants of Scenedesmus blocked on the oxidizing side of PSII, no alteration of polypeptides with molecular weights near 65 kd have been observed.

In this thesis, evidence acquired by mutant analysis was presented which indicates that a major membrane polypeptide of 34 kd is required for the activity of the oxidizing side of PSII. The alterations observed between the WT strain and the mutants LF-1, LF-3 and LF-5 can be explained if the 36 kd polypeptide in the mutants represents a

precursor to the physiologically active 34 kd polypeptide of the WT whose presence is required for water-splitting activity. Further, the loss of 60 percent of the membrane bound manganese in these mutants suggests that the 34 kd polypeptide of the WT Scenedesmus strain is involved in the binding of this ion at the site of water photolysis.

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