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A PHOTOSYNTHETIC STUDY OF OLIVE NECROTIC 8147  
MUTANT AND NORMAL MAIZE (ZEA MAYS L.).

University of Illinois at Urbana-Champaign,  
Ph.D., 1972  
Biology

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A PHOTOSYNTHETIC STUDY OF OLIVE NECROTIC 8147 MUTANT  
AND NORMAL MAIZE (ZEA MAYS L.)

BY

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THESIS

Submitted in partial fulfillment of the requirements  
for the degree of Doctor of Philosophy in Biology  
in the Graduate College of the  
University of Illinois at Urbana-Champaign, 1972

Urbana, Illinois

UNIVERSITY OF ILLINOIS AT URBANA-CHAMPAIGN

THE GRADUATE COLLEGE

May, 1972

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY  
SUPERVISION BY MAARIB DARWISH LUTFI BAKRI  
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BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR  
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Lastly, I want to thank my family for their patience, encouragement and help during my graduate career.

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## CHAPTER I

### INTRODUCTION

Present day models for photosynthesis incorporate two hypotheses. The first is that of the "Photosynthetic Unit" proposed on the basis of the experiments of Emerson and Arnold [1] on the amount of  $O_2$  evolved (or  $CO_2$  fixed) by Chlorella in response to brief saturating flashes of light. Results of these experiments led to the current definition of Photosynthetic Unit (PSU) as a set of several hundred chlorophyll molecules that cooperate in harvesting light quanta and delivering this energy to a photochemical reaction center. The second hypothesis is Van Niel's concept of the primary photochemical act in photosynthesis which states that the trapped light energy provides a separation of reducing (H) and oxidizing entities (OH); the first supplies the reducing power for  $CO_2$  fixation, while the second is disposed of either through conversion into  $O_2$  (in higher plants and algae) or through oxidation of a substrate (in bacteria) [2].

#### 1. Photosynthetic Unit

The classical experiments of Emerson and Arnold (1) established that a set of 2400 chlorophyll molecules cooperate in evolving one molecule of oxygen; this corresponds to 300 chlorophyll (Chl) molecules per electron transferred as there are 8 primary events ( $2400/8 = 300$ ). Another estimate of the size of the unit is 200 Chl molecules per electron, since the chloroplasts contain one molecule of Cytochrome (Cyt) f [3], one of P700 [4], and one of plastocyanin (PC) [5] per 400 Chl molecules. However, smaller units have been identified in Chl deficient mutants of tobacco [6,7], pea [8], Lespedeza [9] and some algae including Chlorella [10,11]. Part of this

research will be devoted to the study of a mutant of Zea mays which seems to have smaller photosynthetic units compared to normal maize. This type of mutant in Zea mays has not been described earlier.

Chl molecules are divided between two functional units assigned to each photosynthetic system and known as PS I and PS II, where the Roman numeral denotes the type of reaction they perform. Each PSU consists of a light energy collector (or antenna), to which the majority of the pigments belong (bulk) and a light energy converter (trap) which transforms the electronic excitation into chemical energy. Since each PSU contains accessory pigments in addition to Chl a, the total pigment complements are called pigment system I and pigment system II. The bulk pigments consist of Chl a and Chl b (higher plants and chlorophytes), Chl c (bacillariophytes and phaeophytes), phycobilins (cyanophytes and rhodophytes) and carotenoids, i.e., xanthophylls and carotenes, (all plants). These pigments enrich the plants with wider absorption bands, and therefore, with a more efficient photon-harvesting apparatus.

Both pigment systems I and II [12] contain accessory pigments as well as Chl a, but they differ in the relative abundance of these pigments in them (Figure 1). Pigment system I contains a larger proportion of the long wavelength forms of Chl a (Chl a 680, Chl a 685-705, the numbers referring to their red absorption maxima), a smaller proportion of the short wavelength form of Chl a (Chl a 670) and of Chl b (or phycobilins, etc.), most of the carotenes, and all of the reaction center Chl a molecules having one of its absorption bands at 700 nm, (P700, ref. 13). This system does not have a fluorescence transient and is weakly fluorescent at room temperature [14,15]. The pigment system II [12] contains a larger

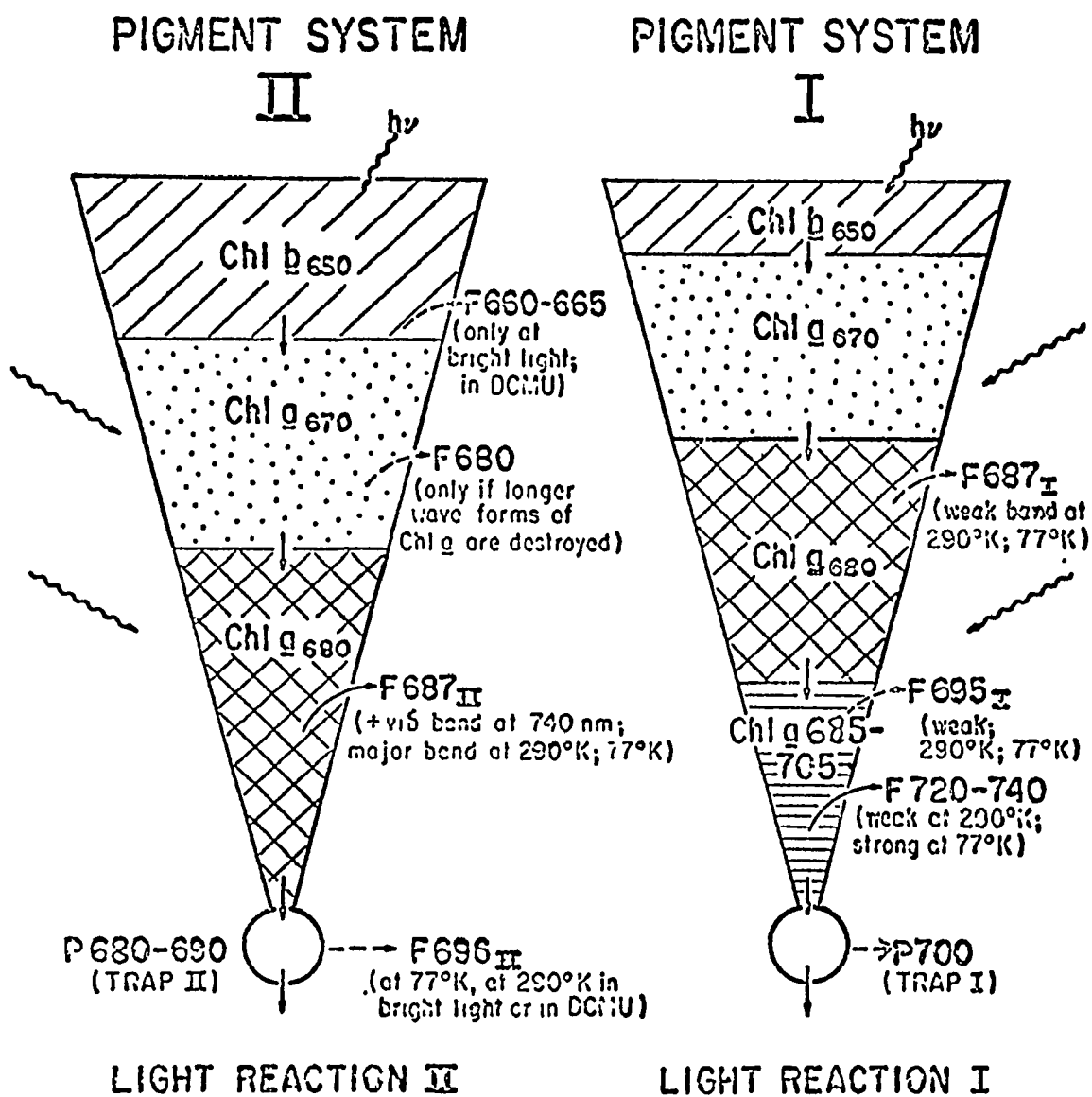


Figure 1. Diagrammatic distribution of pigments between PS I and PS II. (From Govindjee (1971) presented in the Particle Workshop in Göttingen, West Germany.)

proportion of Chl a 670, of Chl b (or phycobilins), most of the xanthophylls, a smaller proportion of the long wavelength forms of Chl a, and all of the reaction center Chl a molecules P680-690 (discovered by Doring et al. [16]). This system, relative to system I, is strongly fluorescent and shows the fluorescence transient [14,17]. The main fluorescence peak of plants at 685-687nm originates in Chl a 680 of this system [18]. Most of the data on the time course of Chl fluorescence yield reflect the changes in the redox level of system II.

The localization and the arrangement of the two pigment systems in vivo is still an unsettled question. However, there is evidence that they exist in separate "packages" that are opposed to each other within a "unit" membrane with system I being on the outer side and system II on the inner side [19,20].

As to the arrangement of the photosynthetic units which belong to the pigment system II on these membranes, two models have been proposed [21]: 1) the "lake" model in which reaction centers are embedded in a lake of pigments and hence interunit energy transfer is facilitated when some of the traps are non-operative, and 2) the "isolated puddles" model in which each unit has its own reaction center and no energy transfer between the units occurs. Evidence for the interunit energy transfer in system II was obtained by Joliot and Joliot [22]. For photosystem I there is evidence for the lake model as was shown in the red algae Cryptopleura violacea [23], and for the isolated puddle model in isolated spinach chloroplasts [24].

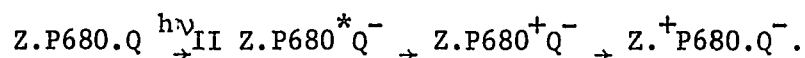
For the intersystem energy transfer, two hypotheses have been suggested: 1) the "spill over" hypothesis (terminology of Myers [25]), in which the excitation energy absorbed but not utilized by pigment system II

is transferred to system I; 2) the "separate package" hypothesis in which the two photosystems are physically separated and energy transfer from PS II to PS I is absent. Support for the separate package hypothesis has come from work of Joliot and Joliot [24]. Contrary to this, Murata [26], Malkin [27], and Ben-Hayyim and Avron [28], have presented evidence in support of the excitation energy spill over. In recent years, a new picture has begun to emerge from studies in various laboratories. These studies indicate that the degree of "spill over" of quanta depends on the conformational status of the membranes that bear the two pigment systems. Duysens [29]; (for a physical picture, see [30]) proposed a model in which he suggested that spill over of energy from PS II to PS I is facilitated when the two pigment systems are close to each other and hence the fluorescence yield of PS II is quenched, while the reverse would occur in the case where the two pigment systems are separated. Regulation of these conformational changes has been shown to be mediated by mono and divalent cations [26].

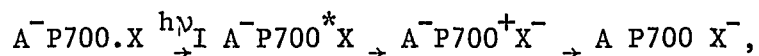
## 2. Two Light Reactions in Photosynthesis

It is generally accepted now that photosynthesis in green plants and algae requires the cooperation of two light reactions, sensitized by two pigment systems. The earliest experimental observations supporting this picture were: the "Red Drop," discovered by Emerson and Lewis [31], which refers to the decline in efficiency of photosynthesis at long wavelengths (>685 nm) where the long wavelength form of Chl a is the only absorbing pigment; and the enhancing effect of shorter wavelength on far red illumination (Emerson Enhancement Effect) [32].

A model which involves the cooperative interaction of two light reactions acting in series was first presented by Hill and Bendal [33]. Schematic presentation of a modified model is shown in Figure 2. A series model postulates that the first light reaction, arbitrarily called photosystem II, forms a strong oxidant ( $Z^{4+}$ ), which is formed only after four primary reactions and then it reacts with water to evolve  $O_2$  and a weak reductant ( $Q^-$ ) is postulated to be formed as follows:



A second reaction, arbitrarily called photosystem I, forms a weak oxidant (A) and a strong reductant ( $X^-$ ):



where A is a pool of intermediates which include plastoquinone, cytochromes and plastocyanin, and X [34] is a low potential energy acceptor. Then A picks up an electron from  $Q^-$ , and  $X^-$  reduces  $CO_2$  into carbohydrates via a series of steps.

The primary electron acceptor of PS II has been recently suggested to be a component distinguishable from cytochromes, whose photoreduction is observed as a decrease in absorbance with a maximum at 550 nm [35]. The Photoreduction of this component labelled as C550 proceeds effectively only in short-wavelength system II light, is insensitive to low temperature, does not require plastocyanin, and is resistant to inhibition by DCMU. Erixon and Butler [36] have equated this absorbance change with Q because of its similar oxidation-reduction potential.

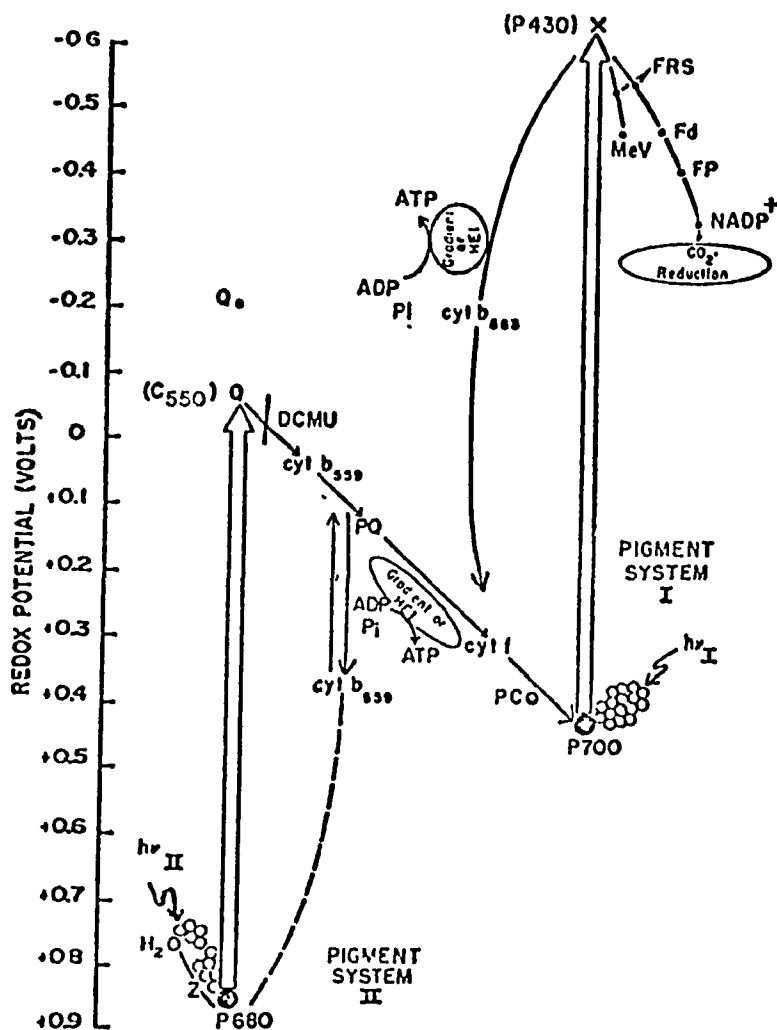


Figure 2. Two light reactions in photosynthesis. Z, primary electron donor of light reaction II; P680, energy trap of pigment system II; Q, primary electron acceptor of system II--probably equivalent to C550; PQ, plastoquinone; PC, plastocyanin; P700, energy trap of pigment system I; X, primary electron acceptor of system I--equivalent to P430?; Fd, Ferredoxin; Fp, flavoprotein,  $\text{NADP}^+$ , Nicotinamide adenine dinucleotide phosphate; FRS, Ferredoxin reducing substance; ATP, adenosine triphosphate; HEI, high energy intermediate, MeV, methyl viologen.



The identity of the primary electron acceptor of PS I (X) has been studied for many years, yet no definitive description of its nature has been reached. Yocum and San Pietro [37] have isolated from spinach a factor termed ferredoxin reducing substance (FRS), that was required for photo-reduction of  $\text{NADP}^+$ , ferredoxin, and methylviologen by chloroplast fragments. Photoreduced FRS could serve as a reductant for the dark reduction of  $\text{NADP}^+$  or ferredoxin. This suggested that FRS could be the primary electron acceptor of PS I. Regitz et al. [38] reported a similar substance. However, Hiyama and Ke [39,40] have recently suggested that the primary electron acceptor of PS I is a component designated as "P430." From studies on PS I particles from spinach and blue green algae, and utilizing the technique of flash kinetic spectrophotometry, they were able to demonstrate a broad absorption band around 430 nm, which was kinetically different from P700. P430 has been suggested to be the primary electron acceptor of PS I for the following reasons: 1) it is bleached as fast as P700; 2) its recovery in the dark is accelerated by ferredoxin and by various artificial electron acceptors with redox potentials as low as -521 mV; 3) the recovery kinetics are identical to those of a concomitant reduction of several of the artificial electron acceptors used; 4) the back flow of electrons is a direct reduction of the photooxidized P700 by the P430 formed; and 5) the quantum yield and effective wavelengths for P430 formation are identical with those for P700 photooxidation.

The two photosystems are connected by a chain of electron carriers, through which reduced Q and oxidized P700 interact. Plastoquinone (PQ) Cyt b, Cyt f, plastocyanin (PC) and P700 have been implicated as components of this chain. Controversy exists as to whether Cyt f or PC interacts

directly with P700. Support for direct interaction of PC with P700 comes from experiments with plastocyanin deficient Chlamydomonas reinhardi mutants which were unable to photooxidize Cyt f [41] and also from the observation by Hind [42] that PC stimulated the rate of Cyt f photooxidation in Triton-treated chloroplasts. Contrary to this, the work of Fork [43] with fraction 2 (grana stack) particles prepared by the French press shows that it is unlikely for PC to function between Cyt f and P700. He found that although these particles contained no PC [44] they showed the rapid Cyt f oxidation, and the addition of PC had no effect on this absorbance change.

The electron flow from Q (0.02 V) to P700 (+0.4 V) is coupled with ATP formation. This has been denoted as non-cyclic photophosphorylation (PP) to distinguish it from a second type, the cyclic PP. This latter type involves the photochemical activity of only system I through which electrons move in a closed circuit without net oxidation or reduction. The cyclic PP is evidenced only when exogenous redox couples are added to the system. The occurrence of cyclic photophosphorylation in vivo is thought to supply the balance of ATP required for CO<sub>2</sub> fixation. The site of ATP formation according to the "cross over" approach of Chance and Williams [45], has been shown to precede cytochrome f in the chain [46]. Recently Cramer et al. [47], using the same approach, have shown that a phosphorylation site between plastoquinone (PQ) and Cyt f must exist. Others, however, have suggested two sites in the chain in subchloroplast particles [48].

Two forms of Cyt b559, which can be interconverted, have been postulated to exist in chloroplasts [47]. These are: 1) a high potential Cyt b559 (hydroquinone reducible) which is predominant in coupled chloroplasts,

and 2) a low potential Cyt b559 (ascorbate reducible, but not hydroquinone reducible) which exists in uncoupled chloroplasts. The Cyt b559 which is oxidized by PS I has been shown to be a low potential Cyt b559. The authors suggest that the rate of non-cyclic electron transport may be partly regulated by the distribution between the low and the high potential states of Cyt b559.

In spite of the accumulated evidence in support of the Z scheme (more appropriately called the N-scheme) (Figure 2), Kanff and Arnon [49] have questioned its validity on the basis of results obtained in their laboratory (detailed discussion of this model will be presented in Chapter V). Knaff and Arnon have suggested the presence of three light reactions and two pigment systems (Figure 3A). According to them, there are two light reactions of PS II (non-cyclic PP) and one of PS I (cyclic PP). Unlike the two traditional light reactions, these two photosystems are presumed to be working independently in parallel rather than in series. The two system II light reactions are called 2a and 2b and operate in series in a manner similar to the Z scheme. The 2b reaction is responsible for H<sub>2</sub>O oxidation, whereas 2a is responsible for Cyt b559 oxidation and reduction of NADP<sup>+</sup>.

Another concept of three light reactions in photosynthesis has arisen (Sane et al. [50]) recently from results of studies with chloroplast particles obtained by mechanical methods, sonication [51] and French press [52] followed by differential centrifugation. These studies and others (see [53]) presented good support for the occurrence of two PS I reactions and one PS II reaction in chloroplasts. Whether the two PS I reactions qualitatively differ from each other is still not known. The PS I reaction located in the stroma lamellae is physically separated from PS II and may

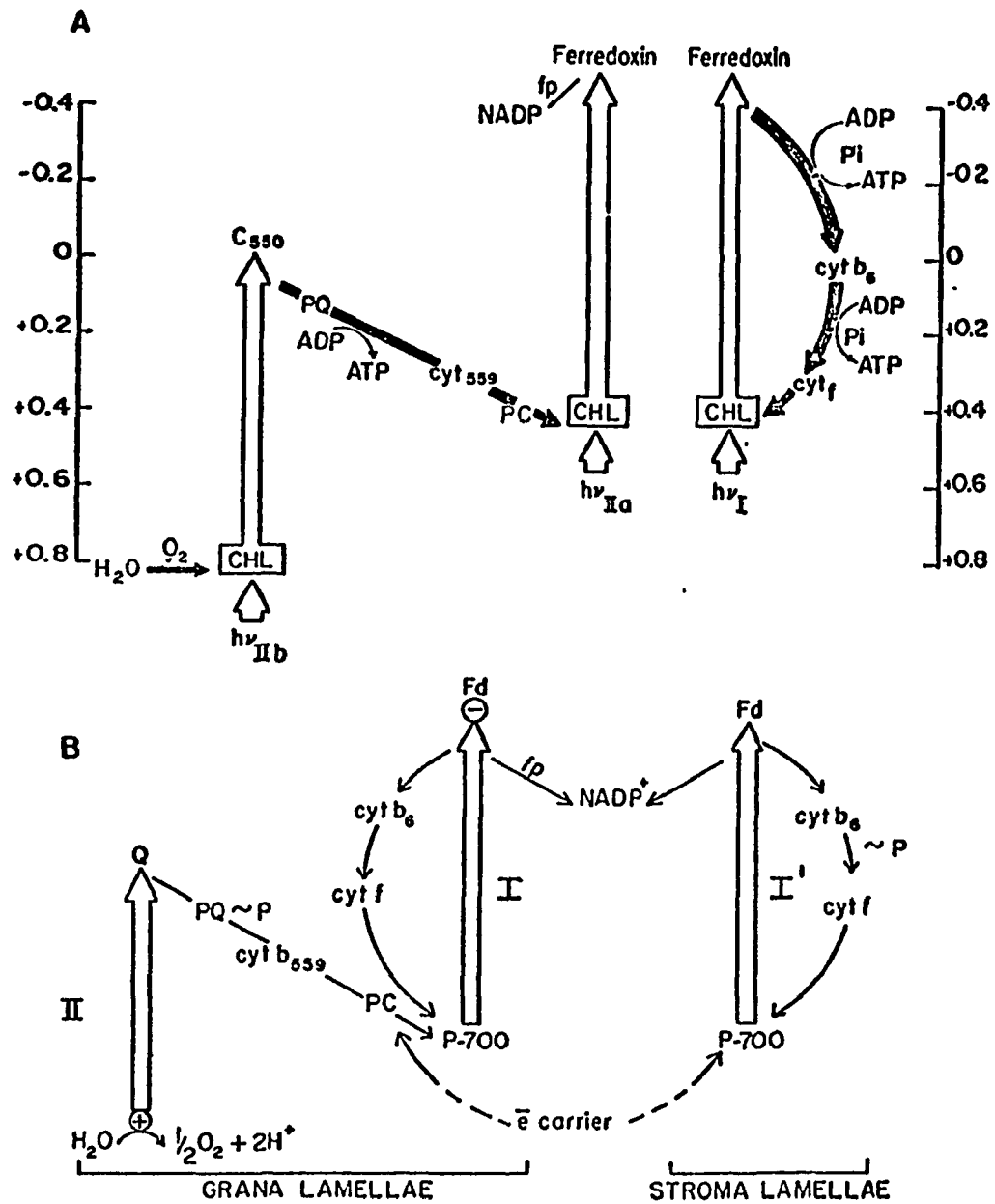


Figure 3. Electron transport in higher plant chloroplasts. A, according to Knaff and Arnon [49]; B, according to Sane and Park [50].

be primarily involved in cyclic PP. The PS I in the grana may be largely involved in non-cyclic electron transport to  $\text{NADP}^+$ .

### 3. Carbon Dioxide Fixation in $C_4$ Plants

Since maize and maize mutants have been used in this study, it is pertinent to discuss briefly the pathway of  $\text{CO}_2$  fixation in  $C_4$  species which possess the  $C_4$ -dicarboxylic pathway of photosynthetic  $\text{CO}_2$  fixation (see [54]). This alternate process of  $\text{CO}_2$  fixation, also referred to as the  $\beta$ -carboxylation pathway [55], is present in a variety of tropical grasses (e.g., maize, sugar cane, sorghum) as well as in species from several dicot families and is correlated with several anatomical and biochemical characteristics. The unique feature of photosynthetic  $\text{CO}_2$  fixation in  $C_4$  species is that it occurs by two linked, but spatially separated cycles, one involving  $C_4$  acids in the mesophyll plastids and the other the Calvin cycle in bundle sheath chloroplasts. The mechanism linking the two cycles has not been definitely established in all  $C_4$  species, but it is presumed to involve the movement and subsequent enzymatic decarboxylation of  $C_4$  acids in the bundle sheath chloroplasts, followed by re-fixation by Ribulose diphosphate (RuDP) carboxylase [55]. Plate 1 [56] diagrammatically represents the compartmentation of carbon metabolizing reactions in  $C_4$  photosynthesis between the mesophyll and bundle sheath chloroplasts of the malate-former [57] Zea mays. Assessment of the requirements for ATP and NADPH in this cycle is possible if certain assumptions are made. If RuDP is the acceptor for the C-4 Carboxyl of dicarboxylic acids, then 5 ATP and 2 NADPH would be required for the incorporation of each  $\text{CO}_2$  into carbohydrate. However, if a diose or diose phosphate were the acceptor, the

Plate 1. The compartmentation of carbon metabolizing reactions in  $C_4$ -photosynthesis between the mesophyll (Ms) and bundle sheath (Bs) chloroplasts of the 'malate-former' Zea mays L. Solid lines represent enzymatic reactions; dashed lined indicate movement of photosynthetic intermediates. Courtesy of R. Chollet.

- 1- Phosphopyruvate carboxylase
- 2-  $NADP^+$ - malate dehydrogenase
- 3- Asp: $\alpha$ -KG aminotransferase
- 4- "Malic" enzyme
- 5- Ribulose-1,5-diphosphate carboxylase
- 6- Sequence of phosphoglycerate kinase and  $NADP^+$ -glyceraldehyde phosphate dehydrogenase
- 7- Triosephosphate isomerase
- 8- Pyruvate, Pi dikinase



requirement for ATP would be reduced to 4 or 3 depending upon the source of these compounds [54].

#### 4. Chlorophyll a Fluorescence in the Study of Photosynthesis

Fluorescence provides a non-destructive tool to monitor the transformation of the pigments in photosynthesis (for further discussion see [12] and [30]). In spite of the low yield of Chl a fluorescence in living cells (3%) and the complexity due to the overlapping of the absorption and fluorescence spectra of the various pigments, a systematic analysis of the intensity and spectrum of fluorescence has yielded important information concerning the composition of the two pigment systems and the primary mechanism of photosynthesis. The fluorescence spectrum of Chl a and the action spectrum of its excitation are indicative of the properties of the two pigment systems.

A light quantum, absorbed by the pigment molecule in the "bulk" of one of the two pigment systems, faces three alternatives. Within the lifetime of its excited state it may be lost by "internal conversion" into thermal energy, it may be re-emitted as fluorescence, or it may be transferred to other pigment molecules. The energy quanta that reach a trap get converted into chemical energy by an "uphill" electron transfer. The efficiency of quantum conversion in the traps is close but not quite 100% when the electron transport chain operates at its highest rate (see theoretical discussion in [58] and [59]).

The quanta re-emitted as fluorescence are presumed to originate mainly in the bulk of the photosynthetic unit. However, when the trap is less efficient in converting the trapped energy into chemical energy, most--but



not all--unused quanta have a good chance to return to the "bulk" and be emitted there; some may originate in the trap II under that situation. At very low temperatures, the trap has a greater chance of fluorescence because of the lower probability of "back transfer" into the bulk.

### 5. Fluorescence at Low Temperatures

Measurements of fluorescence at low temperatures have several advantages: 1) sharpening of the bands is observed and hence, overlapping of the fluorescence and absorption bands is reduced; and 2) the quantum efficiency of fluorescence is considerably increased at low temperatures because both photochemical and internal conversion deactivations are slowed down.

The emission spectrum of chloroplasts at room temperature has a peak around 685 nm with a broad shoulder at 720-740 nm. However, upon cooling to 77°K, this emission spectrum becomes a three-banded structure with maxima at 685 (F685), 695 (F695) and 735 (F735) (see [12]). The longwave F735 band is usually broader and more intense than the other two bands, probably consisting of more than one component. The F735 band has been assumed to originate mainly from pigment system I and F685 mainly from pigment system II [60,61,62]. The F695 band has been suggested to originate from the trap of system II as it appears when the traps are closed [61,63]. Recently, Mohanty et al. [64] have re-investigated the emission characteristics both at room and 77°K of dilute suspension (2-5% absorption) of a purified system I Chl-a-protein complex obtained from Phormidium luridum and of purified PS II particles. The F695 band was observed in the emission spectra of both system I complex and PS II at 77°K and at room temperature.

At 77°K the Chl a fluorescence at 685 nm was nearly as intense as that at 720 nm in dilute samples of System I complex. The authors attribute the distorted shape of emission spectra of published PS I particles [65] to the reabsorption of 685 nm fluorescence in relatively thick suspensions. Thus, it was suggested that all the three bands at 77°K originate in both pigment systems I and II, but greater portion of F735 originates in the first and greater portion of F685 and F695 is from the second.

## 6. Fluorescence Transients

The Chl a fluorescence yield in vivo undergoes characteristic but reproducible changes with time (transient changes) during which the intensity [66] and the spectral composition [67,68] of the fluorescence are altered. When dark-adapted photosynthetic organisms are subjected to continuous intense illumination, the fluorescence yield rises instantaneously to an initial level (O) from where it proceeds biphasically to a maximum (P), thereafter declining at a slower rate to the semi-stationary level (S). The notation OPS for the fluorescence transient was first used by Lavorel [69]. In algae, the biphasic rise includes an intermediate maximum (I), and a dip (D) (Munday and Govindjee [70]), and there are further changes--a rise to a maxima (M) and a decline to a terminal steady state (T) (Papageorgiou and Govindjee [71]). Higher plant chloroplasts, in the absence of added oxidants, show the biphasic rise (OIDP, often referred to as  $F_0$  to  $F_\infty$  rise) but no clear dip (D) is observed [72]. Many authors refer to the first point as  $F_i$  (i for initial) as they are not sure if they are measuring true "0". The decay of P to S is extremely slow in such chloroplasts. However, in the presence of oxidant [73] or cofactors [74,75] of the cyclic electron transport, Chl a fluorescence yield in chloroplasts decays to a low level.

The intensity of fluorescence at the level 0 is referred to as "constant" and the difference in the fluorescence yield between P and 0, as "variable" fluorescence. It is generally assumed that it is only the variable fluorescence that reflects changes in photochemistry. The yield of variable fluorescence increases with light intensity saturating at high intensity, but the yield of constant fluorescence is independent of intensity. The nature of fluorescence at 0 is not yet clear. Its yield is assumed to remain constant when photochemistry changes. It is assumed to originate from the bulk chlorophylls of system I and II before the energy is trapped at the reaction centers. For a detailed review of the fluorescence transient, see [30].

#### 7. Chloroplast Structure and Photosynthesis from Studies with Mutants

Structure-function correlations are generally difficult to make. Attempts to correlate lamellar structure to function have evolved from studies in mutants of higher plants and algae. From such studies, two possible kinds of structure-function relationship could be cited: 1) correlation between the presence of grana (partition regions) in chloroplasts and good PS II activity, and 2) correlation between structural changes of chloroplasts and changes in the light harvesting capacity of the plant. The first correlation has mainly evolved from structural and functional studies on a mutant strain of tobacco (NC-95 var) [76]. Chloroplasts from the yellow sections of the leaves of this mutant exhibit no stacked lamellae and showed essentially no PS II activity as measured by the Hill reaction with ferricyanide, but they carried out high rates of Phenazinemetosulfate (PMS) cyclic PP. In other mutants of higher plants whose structure and functions have been studied, such as barley [77],

peas [8], and soybeans [78], such a clear cut correlation between good PS II activity and the presence of grana could not be established. However, Woo et al. [79] showed that the granal bundle sheath chloroplasts of the  $C_4$  plant sorghum had no Hill reaction as measured by  $NADP^+$  reduction, but they carried out  $NADP^+$  reduction from ascorbate. Though data from higher plants fit this general picture of correlation between the existence of partitions and the abundance of PS II, there are discrepancies in the algae. A pigment deficient mutant strain of C. reinhard, ac-31 has normal PS II activity and normal photosynthesis, although no stacking of lamellae is observed in its plastids [80].

As for the second type of structure-function relationship, examples are the Chl b-less mutants of barley [77], certain mutant strains of tobacco [81], and the Chl-deficient mutants of peas [8]. As compared to normal plants, photosynthesis in these mutants saturates at higher intensities, and their chloroplasts show limited stacking of lamellae.

#### 8. Objectives of the Study

This research project started as an attempt to use necrotic higher plant mutants to study the nature and properties of the unknown intermediates Q, Z, and X (Figure 2) in the electron transport chain of photosynthesis. The mutants were supplied by the Plant Genetics Laboratory of the University of Illinois. Initially, these mutants were assumed to be necrotic because their photosynthesis was impaired due to lack of some electron carrier intermediates. It was further assumed that addition of subfractions of PS I or PS II, isolated from normal chloroplasts, to chloroplasts of the mutant, would restore their photosynthetic activity. This

was to be followed by a detailed study of the properties of fractions, which would be equivalent to the unknown intermediates Z, Q or X as the case may be.

The results of the screening--that took nine months of my study--of six necrotic mutants of Z. mays (nec. 8376, Sienna 80813, nec. 6697, Olive nec. 80810, Olive nec. 8147, and yellow green EPB) showed that none were impaired in photosynthesis. On the contrary, one of these mutants (ON8147) showed at saturating light intensity higher photosynthetic rates on chlorophyll basis than wild plants. As a result, I decided to investigate this mutant in detail to discover the cause of its high photosynthetic activity, and abandoned the original project.

In the first part of this investigation I studied the pigment contents, the spectral characteristics, and the photochemical activities of chloroplasts of the maize mutant (ON8147) and the wild plant. Attempts to correlate the functional and structural characteristics of this mutant were made to test the hypothesis that PS I is associated with unstacking of lamellae in chloroplasts (see [53]). A summary of part of this research was presented earlier [82]. Since the mesophyll chloroplasts of the mutant were enriched in PS I relative to PS II, as evidenced from fluorescence measurements at 77<sup>o</sup>K, the fluorescence transient and Chl a/b ratio etc., and because bundle sheath chloroplasts of C<sub>4</sub> plants were found to lack PS II [79], I investigated the pigment composition, the spectral characteristics including absorption and fluorescence, and partial photosynthetic reactions of both types of chloroplasts from the normal maize, in order to further understand the photosynthetic process in the mutant. My work on mesophyll and bundle sheath chloroplasts was complete [84] before the work of other investigators

[84-86] became available to me. In this thesis, I will point out where my data show differences in results from the previously published data. I will also present unpublished information (e.g. quenching of fluorescence of atebtrin, chlorophyll a fluorescence emission and excitation spectra at 298°K, the excitation spectra of thick samples at 77°K, the time course of Chl a fluorescence, the degree of polarization of fluorescence, and calculation of the efficiency of energy transfer from carotenoids and Chl b to Chl a) in bundle sheath and mesophyll chloroplasts.

My results of the study of the mutant ON8147 showed that the high photosynthetic saturation rates per chlorophyll in this mutant compared to normal maize is due to smaller photosynthetic units. Also it appears that the efficiency of system I and II reactions, even on protein basis, are larger in the mutant than in normal maize. Thus the mutant is more efficient in more than one way. When structure and function are correlated in ON8147 the result gives further support for the idea that PS I is associated with stroma lamellae of chloroplasts.

Results of a study of bundle sheath and mesophyll chloroplasts showed that Bs chloroplasts are about 40% deficient in PS II compared to Ms chloroplasts contrary to earlier [79] reported results of presence of traces of PS II activity in these chloroplasts of maize. Furthermore, my results show that the electron transport chain from H<sub>2</sub>O to NADP<sup>+</sup> in Bs chloroplasts is intact contrary to the findings of Bishop et al. [87]. Results of absorption spectra at 298°K and of Chl a fluorescence excitation and emission spectra at 298°K and 77°K showed that Bs chloroplasts are enriched in longwave forms of Chl a relative to Chl b. The degree of polarization of fluorescence indicated that Bs chloroplasts contain relatively

higher amounts of oriented longwavelength forms of Chl a than in Ms chloroplasts. In both types of chloroplasts, the efficiency of energy transfer from Chl b and carotenoids to Chl a are calculated to be 100 and 50% respectively. Furthermore there is no significant difference in the efficiency of energy transfer between the two types of chloroplasts. Results of quenching of fluorescence of atebtrin in Bs and Ms chloroplasts indicated that fluorescence quenching of atebtrin could not be attributed completely to the proton gradient as suggested by Schuldiner and Avron [88]. Instead it reflects some energy state that leads to ATP production.

A separate part of this dissertation was an attempt to investigate the three light-reaction scheme of photosynthesis presented by Knaff and Arnon [49] challenging the conventional Z-scheme. Tris treated sonicated chloroplasts---as used by the earlier workers---were isolated and their absorption, fluorescence excitation and emission spectra at 77<sup>0</sup>K were measured. This was done to test whether sonication causes preferential loss of PS I, as was shown in Chlorella [89], leading to an action spectrum of Cyt b559 photooxidation matching that of PS II, and thus to Knaff and Arnon's scheme. Also in this part of the dissertation, attempts have been made to test the hypothesis presented by Govindjee et al. [90] for the photoreduction of NADP<sup>+</sup> by PS II with the supply of energy, in the form of HEI or ATP, either from PS I or respiration depending on the physiological state of the cell.

## CHAPTER II

## MATERIALS, METHODS AND TECHNIQUES

1. Plant Material

The mutant of maize (Olive necrotic 8147)--provided by the Plant Genetics Laboratory, University of Illinois--was obtained as a spontaneous mutation. It is a nuclear recessive mutation located on chromosome 1. The homozygous mutant segregates in a Mendelian ratio (Dr. Lambert, personal communication). The plants were grown in a controlled temperature green house in a photoperiod (16 hours light, 8 hours darkness) and a temperature of 21° C. (day) and 16° C. (night). Normal maize plants (single cross hybrid GSC 50) were grown under similar conditions. The mutant is pale green in color and necrotic symptoms appeared in 10-15 days depending on the growth season. Experiments were made with leaves of 10-day old plants.

2. Preparation of Mutant and Normal Maize Chloroplasts

In the comparative study of the mutant and the normal maize, no attempts were made to separate the bundle sheath (Bs) and mesophyll (Ms) chloroplasts. However, the method employed for chloroplast extraction yielded only mesophyll chloroplasts. Microscopic examination of the chloroplast preparation confirmed that our comparison was limited to mesophyll chloroplasts. For the preparation of chloroplasts from plants, only healthy portions of the leaves were used. Eight gm fresh weight of the mutant and 5 gm of normal plants were separately ground in a chilled mortar with 25 ml of 0.02M (Tris(Hydroxymethyl) Aminomethane - HCl) buffer (pH 7.8), containing 0.4M sorbitol [91], 0.01M NaCl and 6 mg/ml Carbowax 4000 [92]. The brei was filtered through eight layers of cheesecloth, centrifuged at



200xg for 1 minute to remove cell debris, and centrifuged again at 1000xg for 8 minutes to pellet the chloroplasts. The chloroplast fraction was washed once in the homogenizing mixture and finally the chloroplasts were suspended in various buffers depending on the type of assay performed. For measuring 2,6- dichlorophenol indophenol (DCPIP) reduction and determination of the concentration of P700 and cytochrome f, chloroplasts were suspended in 1.5 ml of 0.01M HEPES buffer [93] pH 6.7 and 0.001M  $MgCl_2$ , while for measurements of rates of methyl viologen, they were suspended in 0.02M TES buffer pH 7.8 containing 0.16M sucrose and 0.01M NaCl [94]. For fluorescence measurements, chloroplasts were suspended in 0.05M phosphate buffer (pH 7.2). Both types of chloroplasts (ON8147 and normal) were isolated simultaneously, and assays were run alternately.

### 3. Separation of Mesophyll and Bundle Sheath Chloroplasts

For obtaining bundle sheath and mesophyll chloroplasts, seedlings of Zea mays (single crosshybrid GSC 50) were grown in a greenhouse, as described earlier, for 4-5 weeks. Bundle sheath chloroplasts were separated according to a modified method of Woo et al. [79]. The isolation medium contained 20 mM tris-HCl buffer, pH 7.8, 0.33M sorbitol, 1 mM  $MgCl_2$ , and 6 mg Carbowax 4000 per ml of reaction mixture. The use of 0.5% bovine serum albumin, 2% polyclar AT and mercaptoethanol in our homogenizing mixture did not increase the chloroplast activity and hence, these chemicals were omitted in the experiments reported here. To isolate mesophyll chloroplasts, leaves were homogenized in a Servall Ominimixer for 15 seconds at 70% of the line voltage, followed by a pause for 30 seconds, then blended for 5 seconds at 100% of the line voltage. After filtration through a double layer of Miracloth

(obtained from Calbiochem Co.), the debris, which contained bundle sheath strands and mesophyll cells, was further homogenized for 4 minutes at 100% of the line voltage (30 second pauses introduced after every 30 seconds of homogenization, needed to obtain active chloroplast preparation). The slurry was then filtered through one layer of miracloth and the debris was washed several times with the homogenizing buffer to remove any mesophyll cell contaminants. The remaining steps of separation were similar to that of Woo et al. [79]. This method of preparation yields fragments of bundle sheath and mesophyll chloroplasts. Examination of our preparations with iodine staining under the light microscope indicated less than 2% contamination of bundle sheath chloroplasts with mesophyll chloroplasts and vice versa.

#### 4. Isolation of Chloroplasts and Preparation of Photosystem II Particles

Spinach was used as a source of chloroplasts to prepare photosystem II particles. Spinach leaves (250gm) were homogenized in a chilled, one gallon stainless steel commercial Waring blender in 0.02M tris-HCl buffer pH 7.2 containing 0.4M sucrose, 0.01M NaCl and 0.001M MgCl<sub>2</sub>. Chloroplasts were washed twice, and they were then suspended in 0.02M tris-HCl buffer pH 7.2 to obtain chloroplast fragments.

System II particles were prepared according to the method of Huzisige et al. [95]. However, for these experiments, the 1.2 GT fraction (following their terminology) was used; the final step of purification of PS II particles by centrifugation on sucrose gradient was skipped. The ratio of Chl a to Chl b in this fraction varied between 2.0 and 2.3. The fact that we might have some PS I contamination in this preparation does not affect

our results as a 15 minute sonication treatment is involved in this method of preparation of PS II particles, and from earlier work [96] it has been shown that plastocyanin is removed from chloroplasts during sonication. This implies that system I particles, if present in this preparation, are not connected to PS II and hence, any reduction of  $\text{NADP}^+$  from water through system I cannot take place without re-addition of plastocyanin. My result on the photoreduction of  $\text{NADP}^+$  from  $\text{H}_2\text{O}$  in 1.2 GT (PS II) fraction without addition of PC confirms this (see Table 10, Chapter V).

#### 5. Preparation of Tris-Treated-Sonicated Chloroplasts ( $\text{T}_{20}\text{S}_2$ )

Chloroplasts were prepared from spinach according to Whatley and Arnon [97] followed by tris treatment according to a modified method of Yamashita and Butler [98] which was used by Knaff and Arnon [99] to study photooxidation of Cyt b559. Tris treatment was followed by sonication in a Branson Sonifier model W185D for either 1, 2 or 5 minutes. Throughout this study, chloroplasts treated this way will be referred to as  $\text{T}_{20}\text{S}_2$  where T stands for the time of incubation of the chloroplasts in 0.8M tris buffer pH 8.0 and S refers to the time of sonication in minutes.

#### 6. Preparation of Photosynthetic Pyridine Nucleotide Reductase

For measurement of  $\text{NADP}^+$  photoreduction in isolated chloroplasts, crude Photosynthetic Pyridine Nucleotide Reductase (PPNR) enzyme preparation was used. This enzyme was prepared according to method A of San Pietro and Lang [100]; purification of the enzyme was carried out through the protamine sulfate precipitation step. Determination of the amount of protein in the enzyme was made according to the method of Warburg and Christian [101].

## 7. Absorption Measurements

Absorption measurements were made with Bauch and Lomb (Spectronic 505) recording spectrophotometer equipped with an integrating sphere. The optical cell had a path length of 1 cm. The absorbance values for the path length (d) of the excitation light in the sample were obtained by multiplying the absorbance value at 1cm path length by d. These absorbance values were converted to percent absorption values for calculations of the yields. For measurements of difference absorption spectra between two chloroplast suspensions, the optical density of the two samples were matched at one wavelength. The samples were placed inside the integrating sphere, one in the reference position and the second in the measuring position. A spectrum of the difference was recorded between 750-400 nm. A base line for this region was obtained by placing buffer in both the reference and the measuring cuvettes.

## 8. Pigment Analysis

Total chlorophyll and the individual concentrations of Chl a and b were determined in 80% acetone using the equations of MacKinney [102] and the method of Arnon [103]. For the determination of pigments in whole leaves, pre-weighed leaf material was thoroughly ground in aqueous 80% (v/v) acetone using a Ten Broeck homogenizer. The homogenate was centrifuged for five minutes at 2000 x g in a Servall centrifuge (type SS-1). The residue obtained after centrifugation was twice washed with 80% acetone, the washings combined with the original supernatant, and the solution taken to a known volume with 80% acetone. Absorbances of the acetone extracts were measured at 460, 645, and 663 nm in a Bauch and Lomb recording

light or dark with the help of cathetometers. Pre and post-illumination gas exchange was measured and an average of the two readings was taken as a measure of dark  $O_2$  uptake. Photosynthesis was calculated as the difference between the average of the last five readings taken in 8-minute periods of light and dark. Measurements were made at  $20^{\circ}C$  under white saturating light ( $25 \times 10^5$  ergs/cm<sup>2</sup>.sec.).

#### 10. Measurements of Partial Electron Transport Reactions in Isolated Chloroplasts

Photosystem II activity was followed spectrophotometrically using water as reductant and DCPIP as oxidant.\* Samples were excited for 30 seconds with saturating white light provided by 1000 W projection lamp. Heat from the lamp was absorbed by a 5-inch thick water bath. Absorbance at 603 nm was recorded before and after illumination of the sample and the difference between these absorbance values is taken as a measure of DCPIP photoreduction by chloroplasts. The reference cuvette in dark, contained 0.05 M phosphate buffer pH 7.0, 0.01N NaCl and chloroplasts while DCPIP was added in addition to these in the sample cuvette. The molar extinction coefficient ( $20.6 \times 10^3$ ) for DCPIP was used to calculate DCPIP reduction rates at pH 7.0 [109]. For measurement of rates of DCPIP reduction at various light intensities, calibrated metal screens of various mesh sizes were placed before the exciting light.

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\*The fast (55 msec) kinetics of DCPIP reduction shows a contribution of both PS I and PS II to the reduction of this dye; however, the slow (3 sec) kinetics represent mainly the reduction by PS II [108].

Photosystem I and both PS I and PS II activities were followed amperometrically using DCPIP<sub>2</sub> and water as donors respectively and methyl viologen as acceptor. When water was used as a donor, 2% ethanol and excess of catalase were added to the reaction mixture. This treatment destroys H<sub>2</sub>O<sub>2</sub>, but does not allow O<sub>2</sub> evolution from H<sub>2</sub>O<sub>2</sub>. For the determination of oxygen uptake by a reduced low-potential dye, methyl viologen, reduced by reactions of photosynthesis, a Clark type concentration electrode was used [110]. The temperature was 20°C. The cells were illuminated from both sides with two 300-W incandescent lamps; the intensity of this illumination was high enough to saturate photosynthesis  $4 \times 10^5$  ergs/cm<sup>2</sup>.sec. Light energy measurements were made with a Yellow Spring radiometer (model 63). For measurement of the light intensity curve of this reaction, light intensity was lowered by using the same screens described for DCPIP reduction light curve.

#### 11. Measurement of Nicotinamide Adenine Dinucleotide Phosphate Photoreduction

NADP<sup>+</sup> photoreduction was followed by measuring absorbance change at 340 nm, using a Cary 14 spectrophotometer fitted with a side illumination attachment. In this "Cross-beam" method, the actinic beam was obtained from a 1000 Watt projection lamp filtered through a water bath to remove the heat, and an interference filter with a peak at 655 nm and 37 nm half band width. The energy incident on the sample was  $4.8 \times 10^4$  ergs/cm<sup>2</sup>.sec. The photomultiplier was shielded from the actinic beam by two Corning (C.S. 7-60) filters. The reference cuvette in dark contained chloroplasts (100µgm chlorophyll) suspended in 3 ml of 0.05M phosphate buffer pH 7.1. In addition to the buffer, the reaction cuvette contained 2 µmoles NADP<sup>+</sup>

and 6 mg protein in 3 ml reaction mixture. Since the enzyme was not added to the reference cuvette, the difference in scattering between the measuring and the reference cuvettes had to be compensated by lowering the intensity of the reference beam with a 64% transmittance Balzer's neutral density filter. Measurement of absorbance change at 340 nm was recorded while the sample was illuminated. Both initial and steady state rates were recorded; however, all the results which are reported here represent steady state rates. For calculation of rates of  $\text{NADP}^+$  reduction in  $\mu\text{moles/mg chl/hr}$ , a molar extinction coefficient of  $6.22 \times 10^3$  [111] was used.

#### 12. Measurements of Cytochromes and P700

Light induced absorbance changes were measured by a split-beam differential spectrophotometer which is built and designed by Prof. Sybesma and it is described by Sybesma and Fowler [112]. The measuring beam had a half band width (1/2 B.W.) of 6.6 nm in all experiments unless mentioned otherwise. The intensities of the incident light on the sample were measured with a photocell placed directly in the sample position. The photocell had been previously calibrated by an Eppley thermopile.

When measurements were made in the blue and the green regions of the spectrum the photomultiplier (Amperex 56 AVP with a S11 photocathode) was protected by using two Corning filters C.S. 4-96 and C.S. 4-94. For measurements of cyt f, the actinic beam was passed through an interference filter with peak transmission at 696 nm (1/2 B.W., 6.5 nm) providing an incident intensity of  $52 \times 10^4 \text{ ergs/cm}^2 \cdot \text{sec}$  on the sample. Light minus-dark difference spectra of chloroplasts suspensions were measured between 400-450 nm. These spectra were characteristic of that produced upon

excitation of an f-type cytochrome with a large negative solet band at 422 nm and a positive band near 405 nm. Hence the light induced absorbance changes at 422 nm were used to calculate the amount of cyt f in the samples. For measurement of cyt  $b_3$  in Ms and Bs<sup>-</sup> chloroplasts, light induced absorbance changes at 559 nm were determined. Samples were excited with light passed through interference filters with peak transmissions at 655 nm (1/2 B.W., 37 nm) and 710 nm (1/2 B.W., 10.5 nm). The intensities of the incident beams were  $8 \times 10^4$  ergs/cm<sup>2</sup>.sec and  $0.15 \times 10^4$  ergs/cm<sup>2</sup>.sec respectively.

For measurements in the near infra red region of the spectrum a S1 photomultiplier (Amperex 56 CVP) was used. The P700 was measured by following light-induced absorbance changes at 703 nm. The measuring beam, obtained from a tungsten light source, was passed through a Corning filter C.S. 2-62 before entering the measuring monochromator set at 703 nm. The measuring slits had a half-band width of 16.5 nm. A narrow band (1/2 B.W., 12.5 nm) interference filter with a peak transmission at 703 nm was placed before the photomultiplier to allow measurements of absorbance changes of P700 only, and not those due to the fluorescence of Chl a at 685 nm. Samples were excited with interference filters with peak transmissions at 480 nm (1/2 B.W., 11 nm) and 436 nm (1/2 B.W., 8 nm). The intensities of the incident beams on the sample were  $2.0 \times 10^4$  ergs/cm<sup>2</sup>.sec and  $1.5 \times 10^4$  ergs/cm<sup>2</sup>.sec respectively.

All samples used for measurements of P700 and cytochromes had 0.6 absorbance units at 678 nm.

### 13. Fluorescence Measurements

The emission and the excitation spectra of Chl fluorescence were measured with a spectrofluorometer which was designed and built by Govindjee



and Spencer. A detailed description of it is reported elsewhere [113]. For measurements of excitation spectra of fluorescence at 298<sup>o</sup>K, the exciting slit was set at 1.5 mm (1/2 B.W., 4.95 nm) and the measuring slit was set at 3 mm (1/2 B.W., 9.9 nm). A Corning filter (C.S. 7-69) was placed before the analyzing monochromator for measurements of excitation spectra of fluorescence at 740 nm and longer wave lengths at both 298<sup>o</sup>K and 77<sup>o</sup>K. At 77<sup>o</sup>K, the measuring slit was set at 2 mm (1/2 B.W., 6.6 nm) and the exciting slit was 1 mm (1/2 B.W., 3.3 nm). Excitation spectra were corrected for the variations in the incident quanta at different wavelengths.

For measurements of emission spectra, the exciting slit was set at 2 mm (1/2 B.W., 6.6 nm) and the measuring slit was 1 mm (1/2 B.W., 3.3 nm) unless mentioned otherwise. The emission spectra were corrected for the spectral sensitivity of the photomultiplier (EMI 9558B) and the transmission efficiency of the analyzing monochromator. The samples which were used for measurements at 298<sup>o</sup>K and 77<sup>o</sup>K were adjusted to have absorbance of 0.45 and 0.01 respectively at 678 nm in 1 cm pathlength. Dewar vessel which contained a sample of 3 ml had a pathlength of 0.374 cm at room temperature. At 77<sup>o</sup>K, it was difficult to estimate the pathlength. For details of measurements at low temperature, see Cho [114].

For measuring variable fluorescence, samples were excited with a high-intensity  $2.0 \times 10^5$  ergs/cm<sup>2</sup>.sec blue-green light; the exciting band-- provided by a combination of two Corning filters (C.S. 3-75 and C.S. 4-96) placed in front of a tungsten lamp--had a maximum at 500 nm and a half maximum bandwidth of 120 nm [115]. Fluorescence was observed at 685 nm (half band width, 6.6 nm). A Corning C.S. 2-58 filter, placed at the entrance slit of the analyzing monochromator, eliminated stray exciting light.

The degree of polarization of fluorescence of mesophyll and bundle sheath chloroplasts was measured as described by Mar [116].

#### 14. Measurement of Light-Induced Quenching of Atebrin Fluorescence in Isolated Chloroplasts

Atebrin has a broad emission band with a maximum around 505 nm. To measure the fluorescence of atebrin at 505 nm, samples containing 6 nmoles of atebrin solution were excited with a weak 420 nm monochromatic beam (1/2 B.W., 13.2 nm) (the current set at 17 amps), filtered through a Corning filter (C.S. 5-60). The analyzing monochromator was set at 505 nm and its slits were set at 2 mm (1/2 B.W., 6.6 nm). A Corning filter (C.S. 4-72) was placed before the analyzing monochromator to prevent the stray exciting light. The actinic beam was filtered through a Corning filter (C.S. 2-62).

The method used in measuring the quenching of fluorescence of atebrin in Chloroplasts by light was as follows: the level of atebrin fluorescence in the presence of chloroplasts before shining the actinic beam was recorded and taken as the initial level of fluorescence of atebrin. (The initial level of atebrin without chloroplast suspension was higher than in its presence because of the filtering effect of light by chlorophyll in the suspension.) The actinic light was turned on and the kinetics of the quenching of fluorescence were recorded. The difference between the levels of fluorescence before and after shining the actinic beam on the sample, divided by the level of fluorescence in dark, was taken as the degree of quenching of fluorescence of atebrin induced by light absorbed in the chloroplasts.

## CHAPTER III

SPECTRAL CHARACTERISTICS AND PHOTOCHEMICAL ACTIVITIES OF THE  
MUTANT OLIVE NECROTIC 8147 AND NORMAL MAIZE1. Introduction

Several chlorophyll deficient mutants of higher plants and algae have been described [117]. The majority of these mutants are lethal or their photosynthetic rates are lower than that of the wild type. However, several viable mutants of this type have been described [9,78,118]. These mutants share in common certain structural and functional characteristics. The leaves of these plants contain reduced amount of total chlorophyll and carotenoids compared to normal green plants. However, the deficiency in carotenoids is relatively less than that in chlorophyll and thus yielding relative enrichment in carotenoids per unit chlorophyll [9,78,81]. Also, the deficiency of chlorophyll a is often less than that of chlorophyll b, resulting in higher chlorophyll a/b ratios compared to normal green plants [8,76,78,119].

In these mutant plants, photosynthesis saturates at the same [119-121] or higher [8,9,122] light intensities than in the wild type. At light saturation the rates of CO<sub>2</sub> fixation or O<sub>2</sub> evolution are 2-10 times those of the controls per unit chlorophyll and at least equal to the wild type rates on a leaf area or weight basis. The high photosynthetic efficiency at saturating light intensities in these mutants has been attributed to factors, such as a smaller size of the photosynthetic unit [7,8,9,123], a larger pool size and faster turnover of plastoquinone [120], or an increased activity of RUDP carboxylase [124]. Compared to the wild type, the chloroplasts of these mutants exhibit fewer lamellae and grana per chloroplast,

a reduced number of lamellae per grana, and a significant increase in single, unstacked lamellae [8,9,77,78,81].

The mutant ON8147 of Zea mays which is used for this study is similar in many respects to these chlorophyll-deficient viable mutants, however the former mutant is lethal. The cause of lethality does not seem to be related to inhibition of photosynthetic ability, but no definite conclusion could be made here because no attempts have been made to study the nature of lethality. Certain predictions could be made in this respect and could be tested in experiments. This mutant may be similar to the lethal chloroplast mutants of barley [125] which were shown to be deficient in certain amino acids which are required for the synthesis of chlorophyll. If this is the case, then growth of these mutants in a medium supplied with these amino acids should alleviate the cause of necrosis. This is suggested because the amount of total chlorophyll decreases drastically before senescence, suggesting probable exhaustion of reserves which are required for chlorophyll synthesis in the seeds.

## 2. Results and Discussion

### a. Pigment Analysis of Leaves

Pigment concentrations, expressed on fresh weight basis, in leaves of normal and mutant plants are shown in Table 1. The amount of total Chl is one-third that in normal leaves. The ratio of Chl a/b, in acetone extracts of leaves, ranged from 10-12 in the mutant to 3.4-4.0 in normal leaves. The ratio of Chl a/b in the extract of normal maize leaves is slightly higher than that in spinach (a C<sub>3</sub> plant). This is attributed to the presence of both bundle sheath and mesophyll cells in the homogenate of maize (a C<sub>4</sub> plant)

Table 1

Pigment Content and Rate of Photosynthesis in Leaves of ON8147 and Normal Maize

Leaf Material	<u>mg Total Chlorophylls</u> g Leaf Fresh Wt.	<u>mg Total Carotenoids*</u> g Fresh Wt.	Chl a/b	<u>Total Carotenoids</u> <u>Total Chlorophylls</u>	Rate of Photosynthesis $\mu\text{mole O}_2$ evolved/mg Chl/hr
Normal maize	1.85	0.27	3.5±0.5	0.15	100
ON8147	0.62	0.18	11.4±1.4	0.29	540

\*Mainly an estimate of  $\beta$ -carotene and lutein.

leaves used here. Chloroplasts of bundle sheath cells have higher Chl a/b ratio (see Table 5 in Chapter IV) than mesophyll chloroplasts, thus resulting in higher ratio of Chl a/b in maize leaves. The amount of total carotenoids is 1.5 times more in the normal than in mutant plant. However, the ratio of carotenoids to total chlorophyll is about 2 times more in mutant than in normal plants because of a greater deficiency in the chlorophylls.

b. In Vivo Absorption Spectra and Protein Content of Chloroplast Suspensions

In Figure 4 are presented the absorption spectra--"normalized" at 710 nm--of suspensions of chloroplasts isolated from normal and mutant maize leaves. These spectra are consistent with the results obtained from acetone extraction in leaves. At 678 nm, Chl a is the main absorbing pigment while at 650 nm absorption by Chl b is dominant. The ratio of optical densities (absorbance) at 678 nm and 650 nm is 2.3 in normal chloroplasts and 3.1 in the mutant. This results in a higher Chl a/b ratio in the mutant. The two spectra differ in the position of the absorption maxima in the blue region; there is a 9 nm red shift of maxima of this band in the mutant compared to normal chloroplasts. This shift could partly be due to a larger proportion of (or different) carotenoids to Chl a in the mutant. In the normal, the absorption maxima are at 436 nm and 470 nm while they are at 445 nm and about 480 nm in the mutant.

The dashed curve represents the difference absorption spectrum of both samples which were adjusted to have equal absorbance between 710-715 nm. This curve shows clearly that in the mutant there is less Chl b and Chl a 673 relative to the long wave form of Chl a. In the difference spectrum, there are more carotenoids in the mutant (represented by bands with peaks

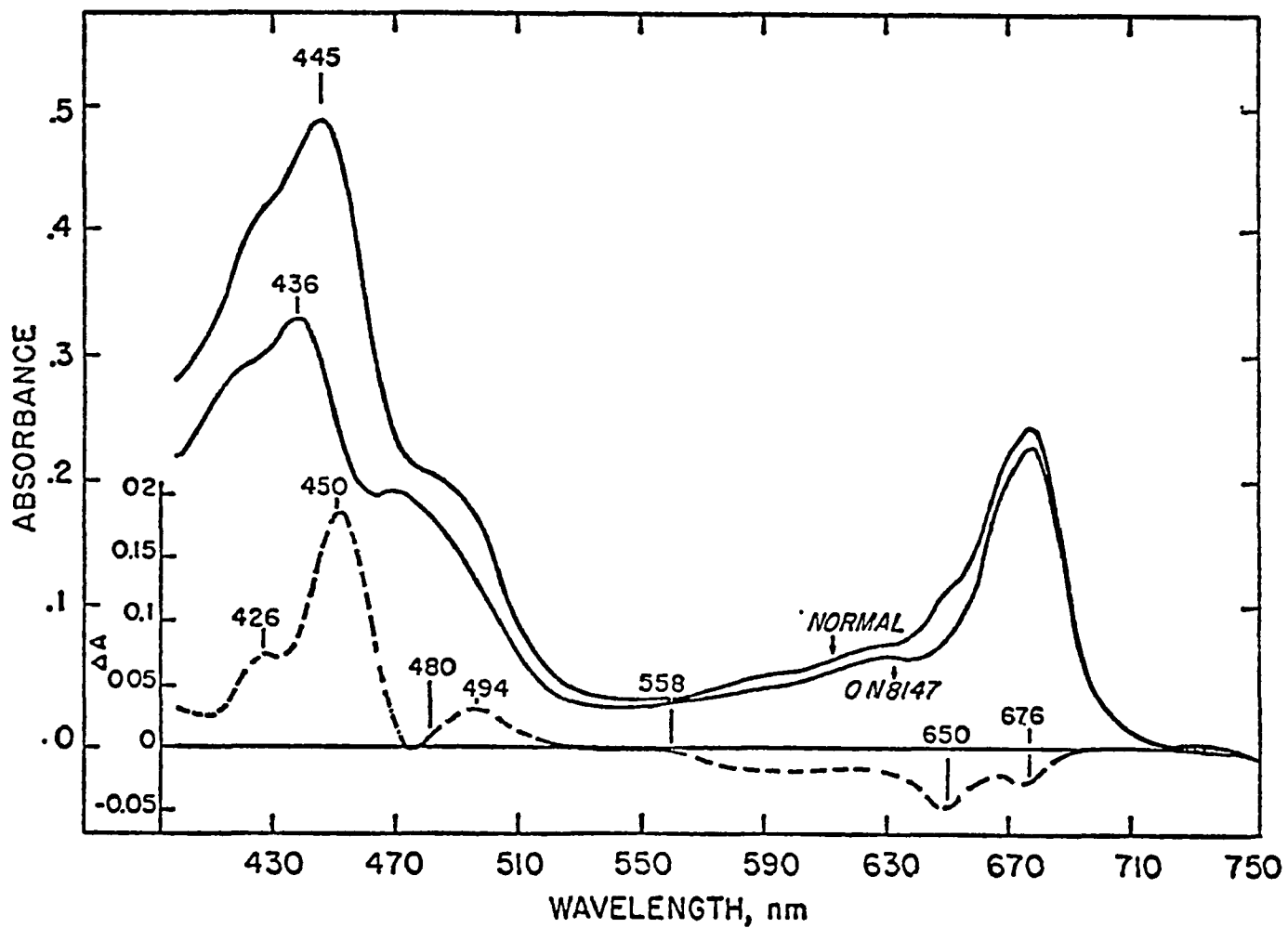


Figure 4. Room temperature absorption spectra of chloroplast suspensions. Dashed curve depicts the difference absorption spectrum (mutant-normal).

at 426, 450 and 494 nm) relative to Chl a and Chl b. The dip at 475 nm is due to lower Chl b/Chl a ratio in the mutant.

Protein content of chloroplast fragments isolated from ON8147 and normal maize was determined. The fragments were washed twice to free them of soluble proteins of the stroma. In normal chloroplasts 22mg protein/mg Chl was measured compared to 57mg protein/mg Chl in ON8147. The ratio of the latter value to the former is about 2.6.

In summary, on fresh weight basis the mutant ON8147 has three times less total chlorophylls, one and a half times less carotenoids but twice the amount of carotenoids per total Chl, two and a half times higher protein per Chl, and about three times higher Chl a/b ratio compared to normal maize. Furthermore, the ratio of Chl a 673 to the long wave form of Chl a is less in the mutant than in normal maize chloroplasts. Although both Chl a 673 and Chl b are present in PS I and PS II, they are in excess in PS II (see Figure 1). Because of the lower content of Chl a 673 and Chl b relative to other forms of Chl a and the high Chl a/b ratio in ON8147, it is suggested that pigments of PS II relative to pigments of PS I are less in the mutant. Fluorescence yield at 298°K of chloroplasts of ON8147 and normal maize (presented later, p. 56) further support this conclusion.

### c. Photosynthesis of Leaf Discs

Rates of net oxygen evolution with white saturating light (intensity,  $25 \times 10^5$  ergs/cm<sup>2</sup>.sec) in leaf discs of mutant and normal maize have been determined manometrically. Photosynthesis is light saturated at  $25 \times 10^5$  ergs/cm<sup>2</sup>.sec in mutant and normal leaf discs because similar rates of oxygen evolution were obtained as light intensity was lowered to 11% of the full intensity. The rates of photosynthesis in  $\mu$ moles O<sub>2</sub> evolved/mg Chl/hr



were 540 for the mutant and 100 for normal maize (Table 1). These values are averages of two experiments. These rates are equivalent to 4.5 and 9.5  $\mu\text{moles O}_2/\text{mg protein/hr}$  in normal and mutant leaf discs respectively (Table 2). This suggests that in the mutant the observed high saturation rates are due to the presence of about twice as many (or twice as efficient) dark limiting enzymes as in normal plants. Hence at saturating light intensity limitation on the dark enzymatic reactions of photosynthesis is reduced in the mutant, resulting in its higher rates compared to normal plants. A typical saturation rate, in continuous light, of 180 or 120  $\text{O}_2$  evolved/hr/Chl was calculated by Rabinowitch [126], on the basis of data of Emerson and Arnold [1] on rates of photosynthesis in a flashing light experiment. These theoretical calculations of saturation rates of photosynthesis match the result of Willstatter and Stoll [127] who found that healthy plants in the presence of abundant supply of  $\text{CO}_2$  and light can produce 1  $\text{O}_2$  molecule every 20-30 seconds per chlorophyll molecule. Similar saturation rates of photosynthesis in continuous light (150  $\text{O}_2$  evolved/Chl/hr) were calculated by Kok and Cheniae [108]. This was based on the finding that the rate limiting dark enzymes have a turnover time of about 10 msec ( $K = 100 \text{ sec}^{-1}$ ), and the concentration (C) of the dark enzymes is 1/2500 chlorophyll molecules according to a photosynthetic unit of 2500 molecules of chlorophyll for one  $\text{O}_2$  evolved [128]. Hence the maximum photosynthetic rate in continuous light is equivalent to  $K.C$  which is equivalent to 144  $\text{O}_2$  evolved/hr/Chl.

Thus to account for a saturation rate of 540  $\mu\text{moles O}_2$  evolved/mg Chl/hr in ON8147, one has to postulate either a high concentration of C i.e., a smaller PSU) or a shorter turnover time. Our data do not allow us to make the distinction. In order to see whether high saturation rates of

Table 2

Saturation Rates per mg Total Lamellar Protein for PS II, PS I and Photosynthesis  
in Normal and Olive Necrotic Maize

Material Used for Measurement	PS II $\mu\text{moles DCPIP reduced}$ $\text{mg}^{-1} \text{protein hr}^{-1}$	PS I $\mu \text{ eq } \text{O}_2 \text{ consumed}$ $\text{mg}^{-1} \text{protein hr}^{-1}$	Photosynthesis $\mu\text{moles } \text{O}_2 \text{ evolved}$ $\text{mg}^{-1} \text{protein hr}^{-1}$
Normal maize chloroplasts	7.0	3.6	-
ON8147 chloroplasts	7.2	8.0	-
Normal maize leaf discs	-	-	4.5
ON8147 leaf discs	-	-	9.5

photosynthesis are accompanied by parallel increases in saturation rates of partial photosynthetic reactions (e.g., PS I and PS II), it was necessary to determine activities of PS I and PS II and measure their light dependence curve in mutant and normal maize chloroplasts.

#### d. Photochemical Activities of Isolated Chloroplasts

1) Photosystem II: dichlorophenol indophenol reduction.--Light curves of the Hill reaction in isolated chloroplasts of normal and mutant maize are presented in Figure 5. In normal chloroplasts, the rate of reduction of DCPIP saturated around  $2 \times 10^5$  ergs/cm<sup>2</sup>.sec of white light. In contrast, in mutant chloroplasts the rate saturated above  $6 \times 10^5$  ergs/cm<sup>2</sup>.sec of white light. At  $30 \times 10^5$  ergs/cm<sup>2</sup>.sec of white light, the mutant chloroplasts reduced DCPIP at the rate of 412  $\mu$ moles/mg Chl/hr compared with 186  $\mu$ moles/mg Chl/hr for normal chloroplasts. The ratio of the rate of DCPIP reduced per mg Chl per hour of mutant and normal chloroplasts is 2.2 at saturating intensity. However, this ratio decreases to one at  $1 \times 10^5$  ergs/cm<sup>2</sup>.sec. At intensities below  $1 \times 10^5$  ergs/cm<sup>2</sup>.sec, the quantum efficiency of the Hill reaction in the mutant chloroplasts should be lower than for normal chloroplasts because of the presence of less chlorophyll per photosynthetic unit (i.e., the probability of absorption within one unit is lower) in the mutant than in normal maize. In the mutant there is about two and a half-fold increase in pigments of PS II relative to PS I (as it is evident from results of acetone extraction of chlorophylls, and in vivo absorption spectra of chloroplasts). If saturation rates of PS II activities of mutant and normal maize were expressed on the basis of chlorophyll in PS II, they would be 1442 and 372  $\mu$ moles/ChII/hr respectively. The

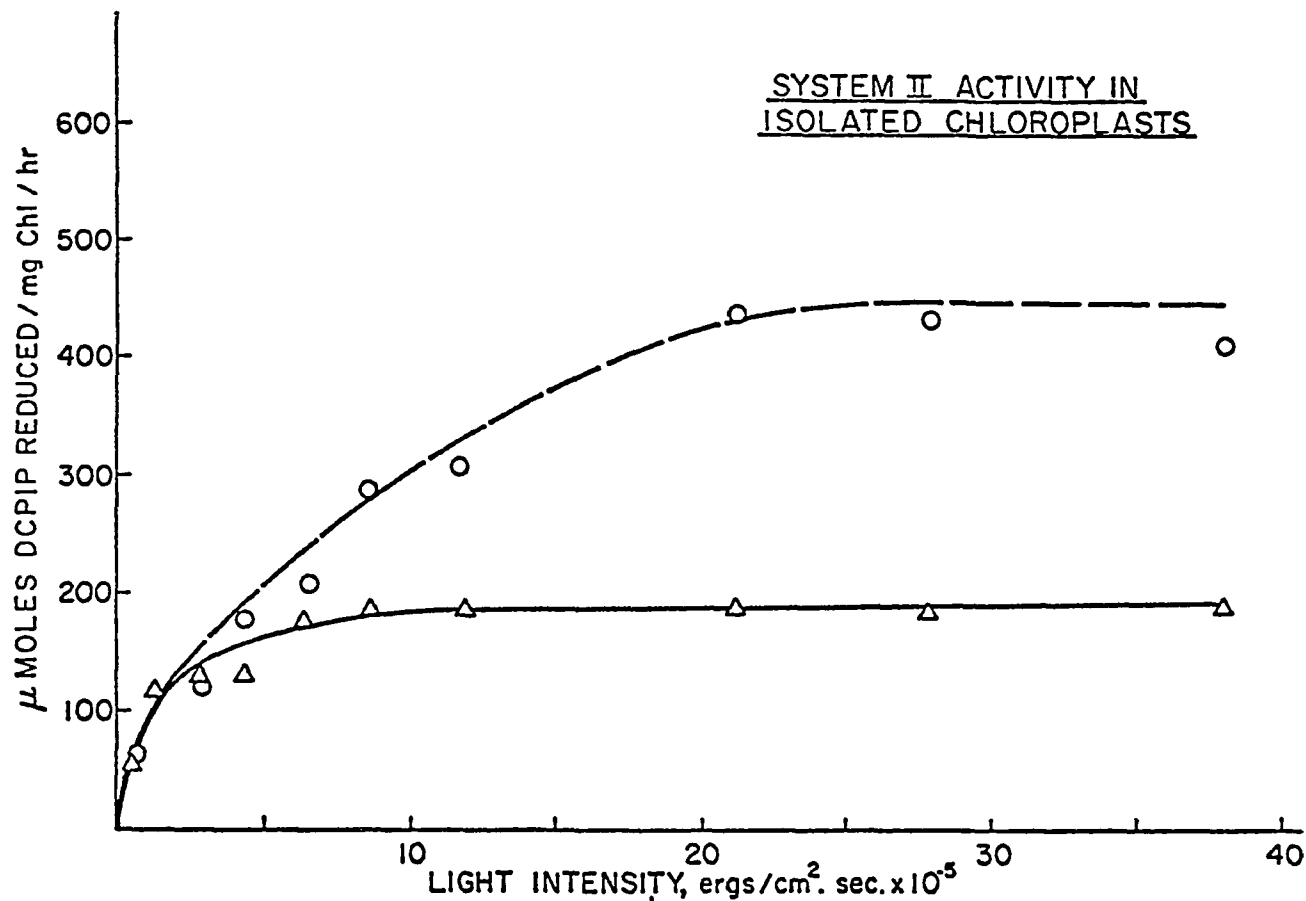


Figure 5. Light intensity curves for the reduction of DCPIP by isolated chloroplasts of mutant --o-- and normal --Δ-- plants. The reaction mixture contained (in 3 ml) chloroplasts containing 10 μg chlorophyll and (in μmoles): phosphate buffer (pH 7.2), 50; NaCl, 10; MgCl<sub>2</sub>, 0.003; DCPIP, 0.048. The reaction rates were measured by the decrease in absorbance at 603 nm after illumination for 30 sec.

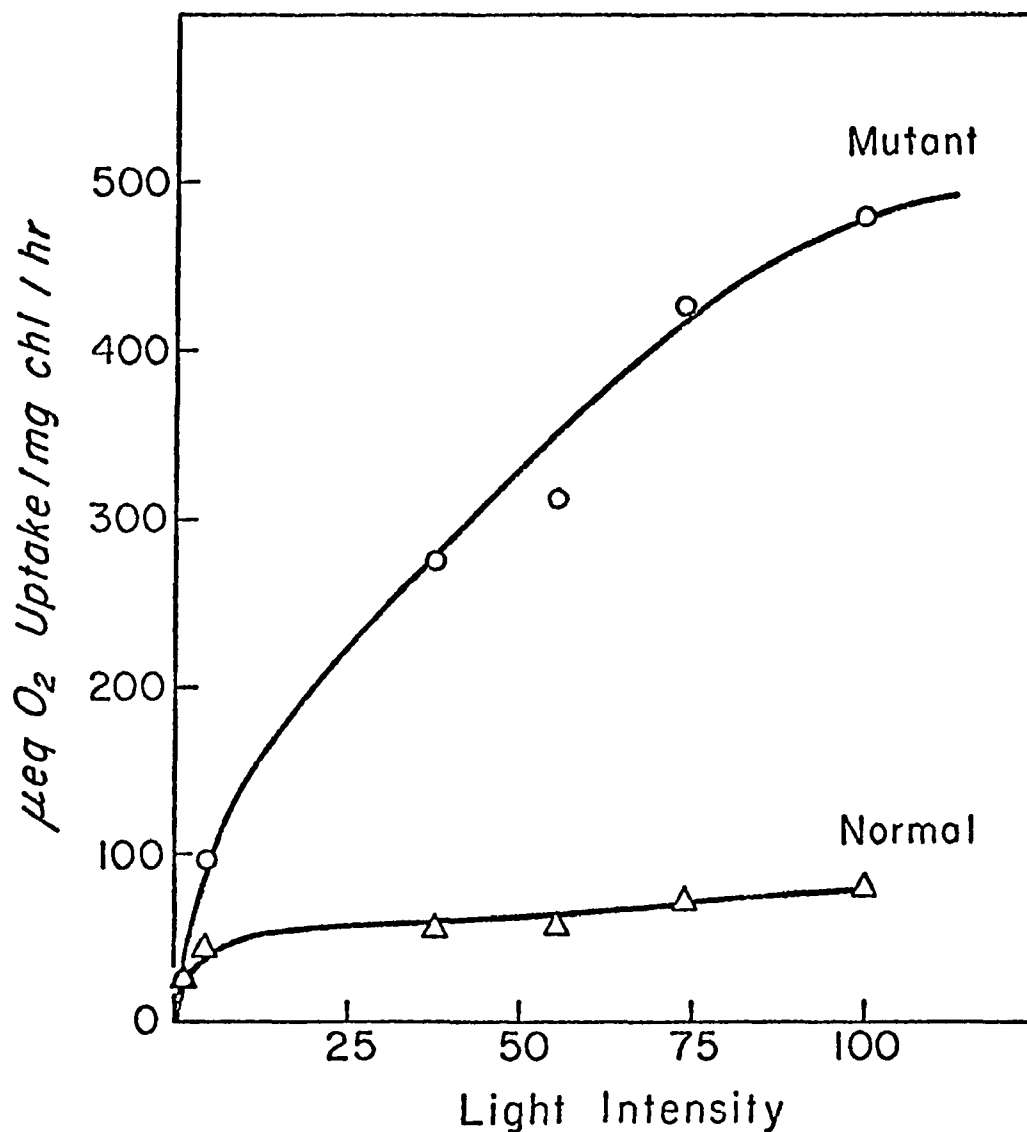


Figure 6. Light intensity curves for the reduction of methyl viologen by isolated chloroplasts. The reaction mixture contained (in 4 ml) chloroplasts containing 40  $\mu\text{gm}$  chlorophyll, and (in mM): TES buffer (pH 7.3), 50; Sucrose, 0.2; Methyl viologen, 0.01; DCPIP, 0.1; Ascorbate, 3; DCMU, 0.01. The reaction rates were determined by measuring  $\text{O}_2$  uptake by reduced methyl viologen. The intensity at 100% corresponds to  $4 \times 10^5$   $\text{ergs/cm}^2\text{.sec}$ .

values of these ratios disappears if rates of PS I and PS II activities are expressed per chlorophyll in each system. In the mutant, there is about two and a half-fold more pigments in PS I relative to PS II (as discussed in Sections 1 and 2). The saturation rates of PS I per chlorophyll of PS I are 680  $\mu\text{eq}$  of  $\text{O}_2$  consumed/hr, and 160  $\mu\text{eq}$  consumed/hr for ON8147 and normal maize respectively. The ratio of these rates is about four. A similar value was obtained for the ratios of rates of PS II, expressed on the basis of chlorophyll in PS II, of mutant and normal chloroplasts.

The saturation rates of PS I per mg total protein (Table 2) are 8.0  $\mu\text{eq}/\text{mg}$  protein/hr and about 3.6  $\mu\text{eq}/\text{mg}$  protein/hr for mutant and normal chloroplasts respectively. If we make the same assumption, as we made earlier that the distribution of proteins between the two systems follows that of chlorophyll, then the system I activity per mg protein is about 1.5 times higher in the mutant than in the normal.

To summarize: the high photosynthetic capacity of leaf discs (at saturating light intensities, per mg Chl as well as per mg protein) of the mutant compared to normal is also reflected in the photochemical activity of isolated chloroplasts. In isolated chloroplasts of the mutant, PS II shows 2.2 fold higher rates per total Chl while PS I shows about six-fold higher rates per total Chl than in normal maize. When rates of PS I and PS II were expressed per Chl in each system four-fold higher light saturation rates were obtained for both PS I and PS II of mutant compared to PS I and PS II of normal chloroplasts. However, the ratio of the rates of mutant and normal maize decreases from 2.2 for PS II and 6.0 for PS I at  $6 \times 10^5$   $\text{ergs}/\text{cm}^2 \cdot \text{sec}$  to a ratio of 1 in both cases at  $1 \times 10^5$   $\text{ergs}/\text{cm}^2 \cdot \text{sec}$ .

suggesting that at intensities below  $10^5$  ergs/cm<sup>2</sup>.sec, a lower quantum efficiency in the mutant may be observed. Light saturation rates of PS II per mg total protein are similar in the mutant and normal chloroplasts, while rates of PS I by isolated chloroplasts on a protein or Chl basis and photosynthesis in leaf discs are about two-fold higher in the mutant compared to normal maize. Assuming the same distribution of protein between the two pigment systems as that for chlorophylls, both the PS I and the PS II, activities per mg protein in each system are higher in the mutant.

#### e. Estimation of the Size of Photosynthetic Unit

It was suggested earlier that in order to account for high photosynthetic saturation rates per mg Chl in the mutant, a smaller PSU, or faster turnover rate of dark enzymes (or both) compared to normal maize had to be postulated. To estimate the size of the PSU in mutant and normal chloroplasts, the concentrations of P700/Chl and Cyt<sub>f</sub>/Chl were measured. The definition of PSU in this study is not the classical one [1]. It is defined as the minimum number of Chl molecules required for the transport of an electron from H<sub>2</sub>O to NADP<sup>+</sup>. The ratios of Chl/P700 and Chl/Cyt f in normal and mutant chloroplasts are presented in Table 3. In normal chloroplasts, the Chl/P700 is 490 and this agrees with a reported value in spinach chloroplasts [4]. However, in the mutant the Chl/P700 is 250. Thus, PSU I in the mutant is about half as small as in normal chloroplasts calculated on the basis of P700. If, however, the size of PSU I is expressed on the basis of Chl a of system I only, we estimate it to be 250 for normal and 160 for the mutant. The Chl/Cyt f content in normal chloroplasts is 450, similar to a reported value in spinach chloroplasts [129], and 113 in mutant chloroplasts. Here, again, the estimated size per

Table 3

Number of Chlorophyll Molecules per P700 and Cyt f in  
Chloroplasts of ON8147 and Normal Maize

Chloroplasts	Chl/P700	Chl/Cyt f
Normal	490	450
ON8147	250	113

chlorophylls of system I is 225 for normal and 75 for mutant. The ratio of Chl/Cyt f in normal to that in mutant chloroplasts is about three to four. In chloroplasts, there is one molecule of P700 [4], and one of Cyt f [3] per 400 chlorophyll (total) molecules. Since in the mutant the size of PSU, measured on the basis of Cyt f, is smaller than that on P700 basis, there must be either a larger pool of Cyt f, or the rate of turnover of Cyt f (reoxidation by PSI) is faster than in normal maize. Thus, a higher light induced change of Cyt f relative to P700, per Chl, would be observed. No distinction could be made between the two cases, at this stage, since the fast kinetics of reduction by PS II and oxidation by PS I of Cyt f were not measured.

In conclusion, the high photosynthetic saturation rates, per Chl, of ON8147 compared to normal maize is probably due to smaller photosynthetic units. Also, it appears that the efficiency of system I and II reactions, even on protein basis, are larger. Thus, the mutant is more efficient in more than one way.

In order to give a physical picture for the PSU in ON8147 and normal maize, it is important to keep in mind the unequal distribution of



chlorophyll molecules between the two pigment systems of the mutant. On the basis of differences in relative fluorescence yield of Chl of mutant and normal chloroplasts, and from fluorescence emission data, it is suggested that PS I has two and a half-fold more chlorophyll relative to PS II. Hence the chlorophyll content of PS II should be about two-fifth that of PS I. The high photosynthetic saturation rates are due to smaller and more efficient PSU in the mutant than in normal chloroplasts. Furthermore, the PSU of PSII in the mutant is smaller than that of PS I. Hence, in the mutant the saturation rate of oxygen evolution in photosynthesis and of PS I in chloroplasts will be limited by the dark limiting enzymes of PS I. If the PSU I of mutant was as small as the PSU II, the saturation rates of overall photosynthesis would even be higher.

#### f. Time Course of Chlorophyll Fluorescence

The time course of Chl fluorescence yield of normal and mutant chloroplasts obtained by illumination with  $2 \times 10^5$  ergs/cm<sup>2</sup>.sec of blue light are presented in Figure 7. The two curves are normalized at the initial level of fluorescence  $F_0$ . The curve for normal chloroplasts shows the typical characteristics of the fluorescence induction [130]: an initial fast rise to a value  $F_0$ , and a slower biphasic rise to a final level  $F_\infty$ . In the mutant one observes a slower fluorescence rise to a very low  $F_\infty$  level than in normal chloroplasts. However, both curves reach their  $F_\infty$  levels at 5 seconds. The percent variable to total fluorescence

$(\frac{F_\infty - F_0}{F_\infty})$  is 63 in normal chloroplasts compared to a value of 31 in

mutant chloroplasts. The relative quantum yield of variable to constant

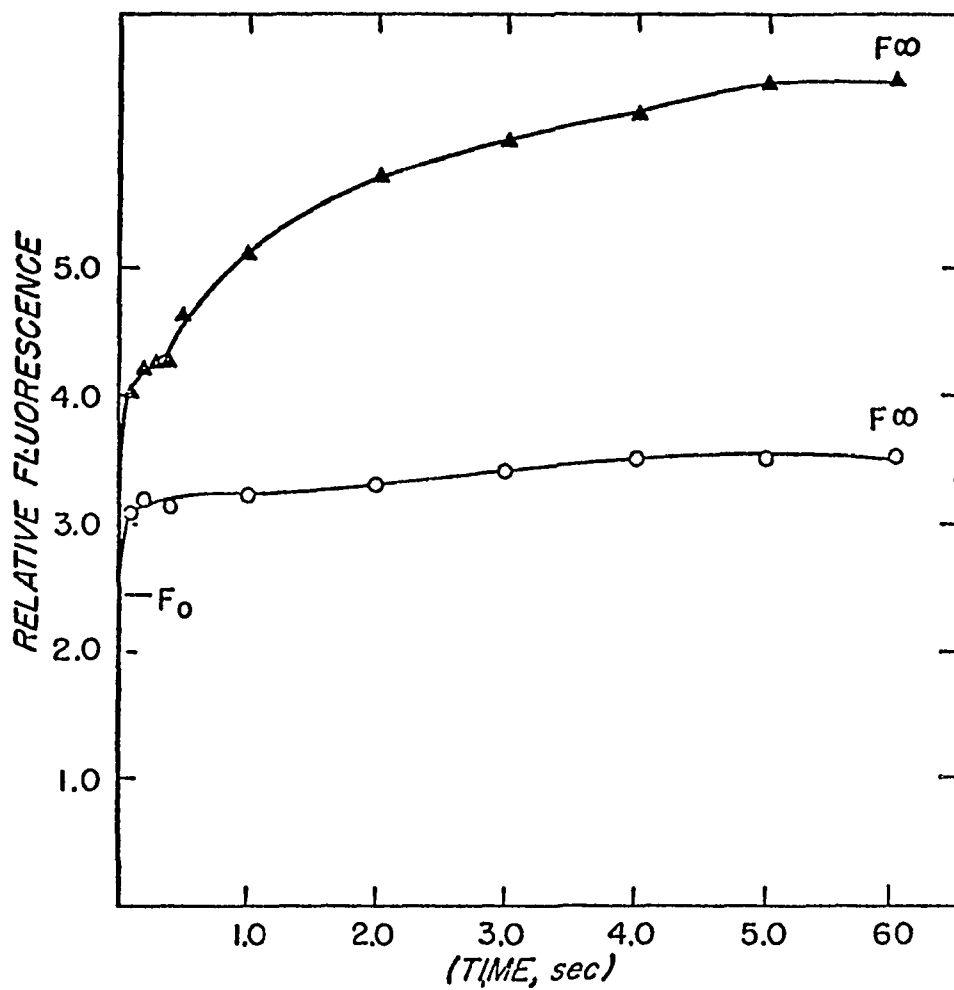


Figure 7. Time course of fluorescence of chloroplasts from ON8147 -- o -- and normal --  $\Delta$  -- plants. Chloroplasts are suspended in 0.02 M tris-HCl (pH 7.20; 0.4 M sorbitol and 10 mM NaCl to give a chlorophyll concentration of 1.5  $\mu$ M. Incident light,  $\lambda_{\max} = 480$  nm,  $2 \times 10^5$  ergs/cm<sup>2</sup>.sec.

fluorescence  $\left(\frac{F_{\infty} - F_0}{F_0}\right)$  is 1.69 in normal chloroplasts and 0.46 in the mutant.

This shows clearly that variable fluorescence in the mutant is one-third that in normal chloroplasts. The ratio  $(F_{\infty}/F_0)$  is 2.7 in normal compared to 1.45 in mutant chloroplasts.

The rise in Chl fluorescence yield of isolated chloroplasts on illumination has been attributed to the photoreduction of a primary electron acceptor Q by system II [131]. Reduced Q is reoxidized in darkness, or by far red light absorbed predominantly by pigment system I. A decreased variable fluorescence in mutant chloroplasts could be attributed to either one of four alternatives: 1- lower quantum efficiency of PS II induced electron transport in chloroplasts of the mutant. Assuming Q acts as a quencher of Chl a fluorescence, the yield will remain low. 2- If one assumes that in the mutant photosystem I absorbs more quanta from the incident light than pigment system II. Such an unequal distribution of quanta could occur if there were more chlorophyll molecules associated with PS I, than with system II, as has been suggested for the Chl b-less mutant of barley [132]. Indeed, mutant has a lower ratio of Chl b/Chla than the normal. 3- If the concentration of Q in system II of the mutant is less than that in normal chloroplasts, then a lower variable to constant fluorescence is expected in the mutant compared to normal chloroplasts. 4- The presence of a larger pool of (Q + A) could cause slow fluorescence rise, but the  $F_{\infty}$  level in such a case should be the same as in normal chloroplasts.

The first possibility is ruled out because of the earlier results which showed that chloroplasts of the mutant possessed, at least, two times higher DCPIP photoreduction activity than normal chloroplasts at saturating light intensities. The second alternative is consistent with results of

absorption spectra (Figure 4), fluorescence spectra at room temperature and at 77°K of the chloroplasts, which suggest the presence of more pigments of PS I relative to pigments of PS II in the mutant. In measuring the time course of Chl fluorescence, samples were excited with broad band blue-green light which excited pigments of both PS II and PS I (to a lesser extent). Hence, if less pigments are present in PS II relative to PS I, then the rate of QH reoxidation by PS I would be faster than its formation. Thus keeping a considerable part of the primary pool of oxidants (Q) in its oxidized state. Further support for fast reoxidation of QH by pool A in the mutant comes from the slower rise of the  $F_0$  to I level in the mutant than in normal chloroplasts; assuming that in the  $F_0 \rightarrow I$  phase, interaction between A pool and Q is operating (as was shown by Munday and Govindjee [70] for Chlorell).

The third possibility is ruled out because in such situations the photochemical activity of PS II, even at saturating light intensities, would be reduced compared to normal case. This is not observed in the mutant. As for the fourth possibility, although a slow fluorescence rise of  $F_0 \rightarrow I$  phase is observed in the mutant, the  $F_\infty$  level never reached that of normal chloroplasts. Hence the presence of a larger pool of (Q + A) could not by itself account for the low variable fluorescence of the mutant. (The decline from  $F_\infty$  to steady state level in Figure 8 has a different reason, see below.)

In Figure 8 is presented the slow fluorescence induction curves in normal and mutant chloroplasts. In both cases, the fluorescence intensity rises to  $F_0$  in 12 seconds; thereafter, a slow decline in fluorescence intensity is observed in normal chloroplasts. Finally the steady state

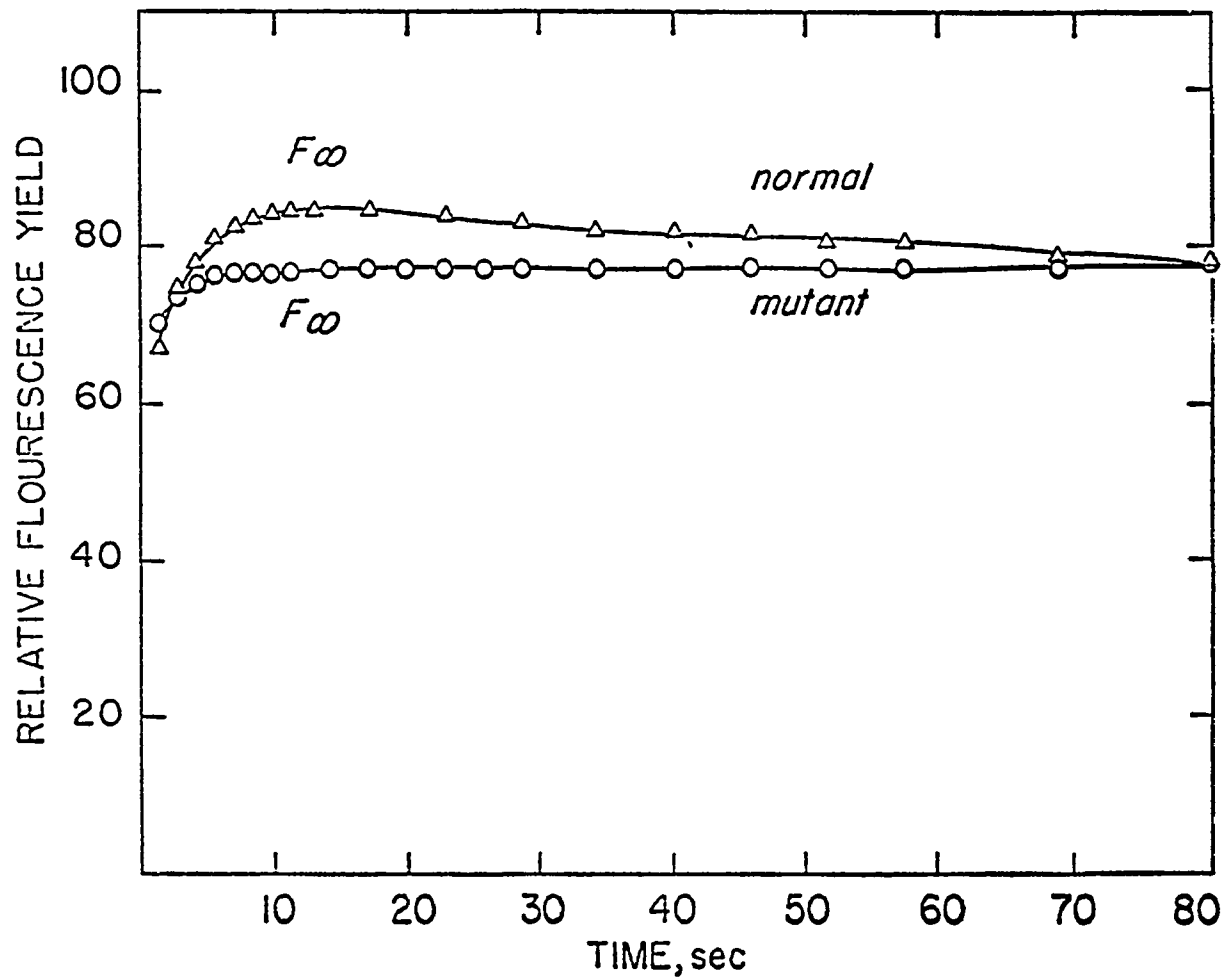


Figure 8. Time course of slow fluorescence changes in isolated chloroplast fragments of normal --  $\Delta$  -- and mutant -- o -- plants. Conditions as in Figure 7.

level approaches that of the mutant in 1 minute. In chloroplasts, this decline in fluorescence intensity at later times has been attributed by Malkin and Kok [133] to slow reoxidation of QH by oxygen through Mehler [134] type reactions. The decline of fluorescence intensity at  $F_{\infty}$  is appreciable in normal compared to mutant chloroplasts (see Figure 8). The absence of decline in fluorescence intensity at  $F_{\infty}$  in the mutant could be explained if one assumes that the reoxidation of QH by far red light is faster than that in normal chloroplasts, so that a great portion of the Q pool in the steady state stays in the oxidized form.

#### g. Relative Chlorophyll Fluorescence Yields of Chloroplasts

The relative Chl fluorescence yields at 685 and 710 nm were determined in the chloroplasts of mutant and normal maize. Samples were excited either with a monochromatic beam at 430 nm (1/2 B.W. 4.95 nm) or with a broad blue band with a peak maximum at 480 nm (1/2 B.W. 120 nm). When samples were excited with 430 nm light, where Chl a absorbs more than Chl b, the relative fluorescence yield at 685 nm was 57 in normal chloroplasts and 37 in the mutant (see Table 4). The ratio of the fluorescence yields of normal to mutant chloroplasts was 1.54. However, when relative fluorescence yields were measured at 710 nm, a similar value of 10 was obtained in both mutant and normal chloroplasts; thus the ratio of their yields was one. The ratio of the relative fluorescence yields at 685 nm and 710 nm is 3.7 in mutant chloroplasts compared to 5.7 in normal. Similar results were obtained when samples were excited mainly by PS II light.

In summary, these results show: 1- The ratio of the relative fluorescence yields at 685 nm to 710 nm is lower in the mutant than in normal chloroplasts. 2- In the mutant, there are more pigments of PS I relative

Table 4  
Relative Fluorescence Yield of Chl at Room Temperature

Source of Chloroplasts	Exciting wavelength, nm	Relative fluorescence yield at 685 nm	Relative fluorescence yield at 710 nm	F685/F710
Normal	430	57	10	5.7
ON8147	430	37	10	3.7
Normal	480	36	5	7.2
ON8147	480	23	5	4.6

to pigments of PS II than in normal chloroplasts. Thus, these results agree with the interpretation given earlier for lower variable fluorescence in the mutant than in normal chloroplasts.

#### h. Emission Spectra of Chlorophyll a

Emission spectra at 77°K (Figure 9) were measured to confirm the conclusions reached earlier on the preponderance of pigment system I relative to pigment system II in the mutant. The ratio of fluorescence at 735 nm to 685 nm was 1.5 in normal chloroplasts and 3.2 in the mutant. The ratio in normal chloroplasts approached that which was reported for very thin samples (about 5% absorption) in spinach chloroplasts at 77°K [61].

The F735 band has been assumed to originate mainly from pigment system I, and F685 and F693 mainly from pigment system II. Cho and Govindjee [62] working with intact algal cells and Mohanty et al. [64] with separated pigment complexes and particles have shown that all the three fluorescence bands at 77°K originate in both pigment systems. However, a greater proportion of F735 originates in PS I, while the main portion of F685 and F696 originated in PS II. Therefore, the differences in the ratios of F735 to F685 in normal and mutant chloroplasts indicate that there are more pigments of PSI relative to pigments of PS II in the mutant than in normal maize. The emission band at 695 nm is not very clear since narrower slits should have been used to see it. However, one can observe in these spectra an assymetry of the 685 nm band which is due to presence of 695 band. Also, the half-band width of this band is too broad for it to be from F685 only.



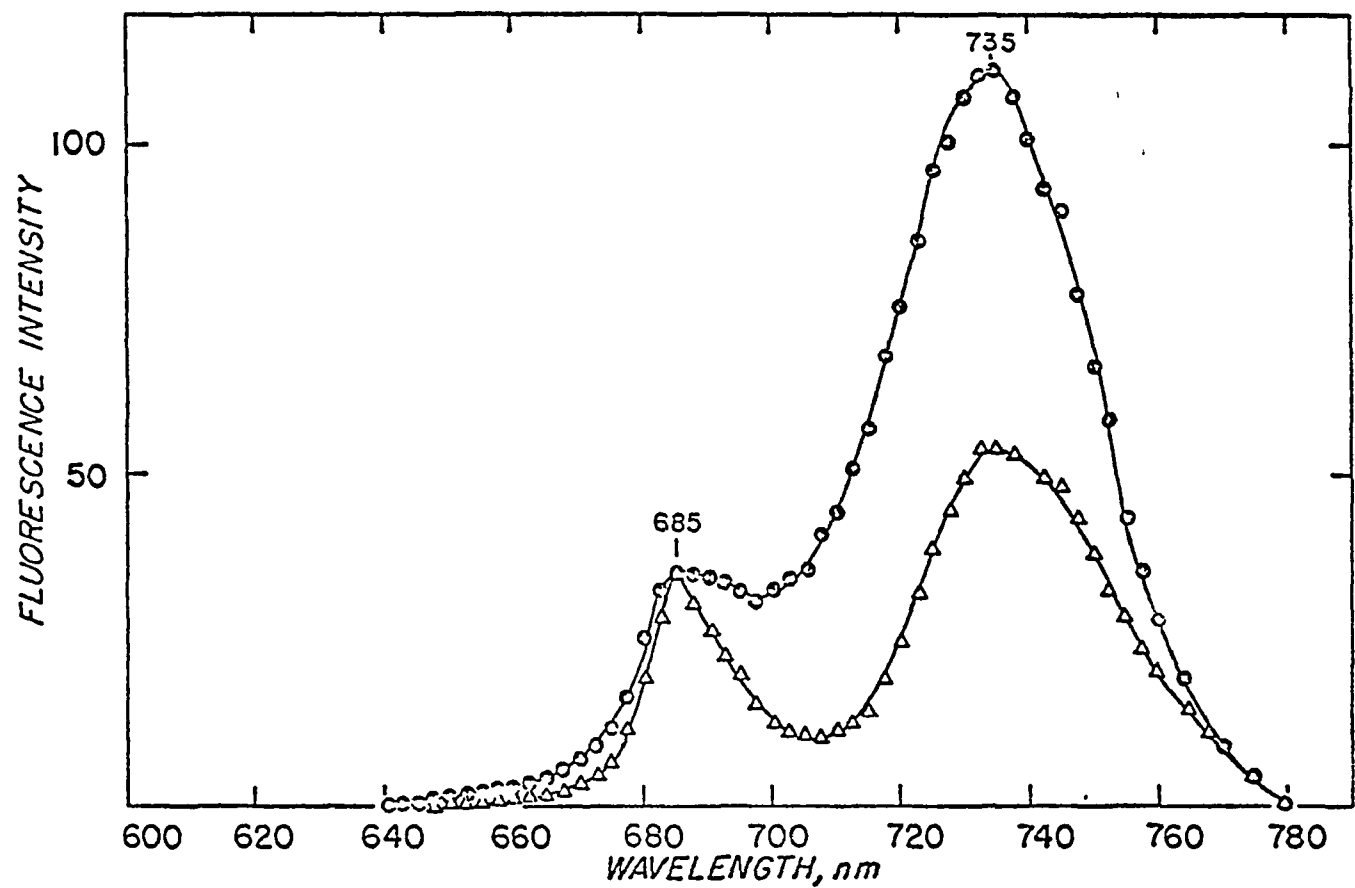


Figure 9. Fluorescence emission spectra at 77°K of chloroplasts from ON8147 -- o -- and normal -- Δ -- plants. Excitation wavelength, 440 nm; light intensity,  $6 \times 10^3$  ergs/cm<sup>2</sup>.sec; 0.01 absorbance cm<sup>-1</sup> at 678 nm.

### i. Fine Structure of Plastids

For the purpose of comparison of the fine structure of plastids in ON8147 mutant with that of normal plastids in maize, electron micrographs of normal mesophyll and bundle sheath plastids in the primary leaves are presented\* [56]. Mesophyll chloroplasts (Plate 2) possess an extensive grana network system, ribosomes, DNA-like fibrils, osmiophilic granules, starch grains, and a well-developed peripheral reticulum [135,136]. The bundle sheath plastids (Plate 3) are similar except that they contain an extensive system of single, parallel lamellae with limited areas of stacking into small grana and frequently possess numerous starch grains.

The fine structure of plastids in the ON8147 mutant has been studied by Professor Paolillo (unpublished). Electron micrographs of mesophyll and bundle sheath plastids of primary leaves of ON8147 were made available to me for use in this study by Professor Paolillo. This was sought in order to correlate the structural and the photosynthetic activity of the ON8147 mutant which has been used in this study. Interpretation of the fine structure of the micorgraphs, kindly made by Professor Paolillo (personal communication), follows.

According to Paolillo, the phenotype of plastids in the ON8147 mutant approaches normal, immature maize plastids in its most normal extreme, while it is abnormal in its most affected extreme.

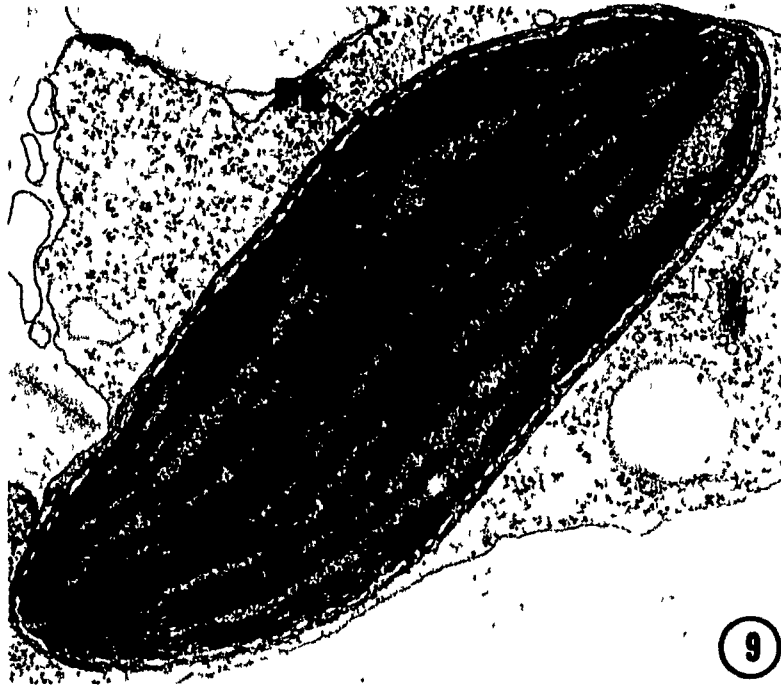
The two extreme cases of the structure, the most advanced and the least advanced of mesophyll plastids in the ON8147 mutant are presented

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\*with the permission of the author.

Plate 2. Mesophyll chloroplasts of normal maize (courtesy of R. Chollet).

Plate 3. Portion of a bundle sheath cell of normal maize (courtesy of R. Chollet.)



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(Plate 4 and Plate 5). Bundle sheath plastids in ON8147 are not presented here since they approach the structure of normal bundle sheath plastids.

The mesophyll plastids of the primary leaves of ON8147 in the most normal extreme (Plate 4) has widely spaced grana stacks of 2 - 6  $\pm$  compartments. The grana are loosely connected by "frets" and the compartments of a granum are interconnected at the margins.

In the more abnormal plastids, the small lamellar profiles are numerous. Grana are more widely spaced and diverse in size and shape. In the profile, one observes regions where the lamellae seem to "focus" or come together. In the extreme cases, there are few grana of any significance (Plate 4).

Other peculiarities in plastids of ON8147 have been observed by Paolillo; these are: grana of normal size are spaced far apart and are joined by numerous parallel frets. Accumulation of tubular or spherical vesicles (which are not prolamellar bodies) are also observed.

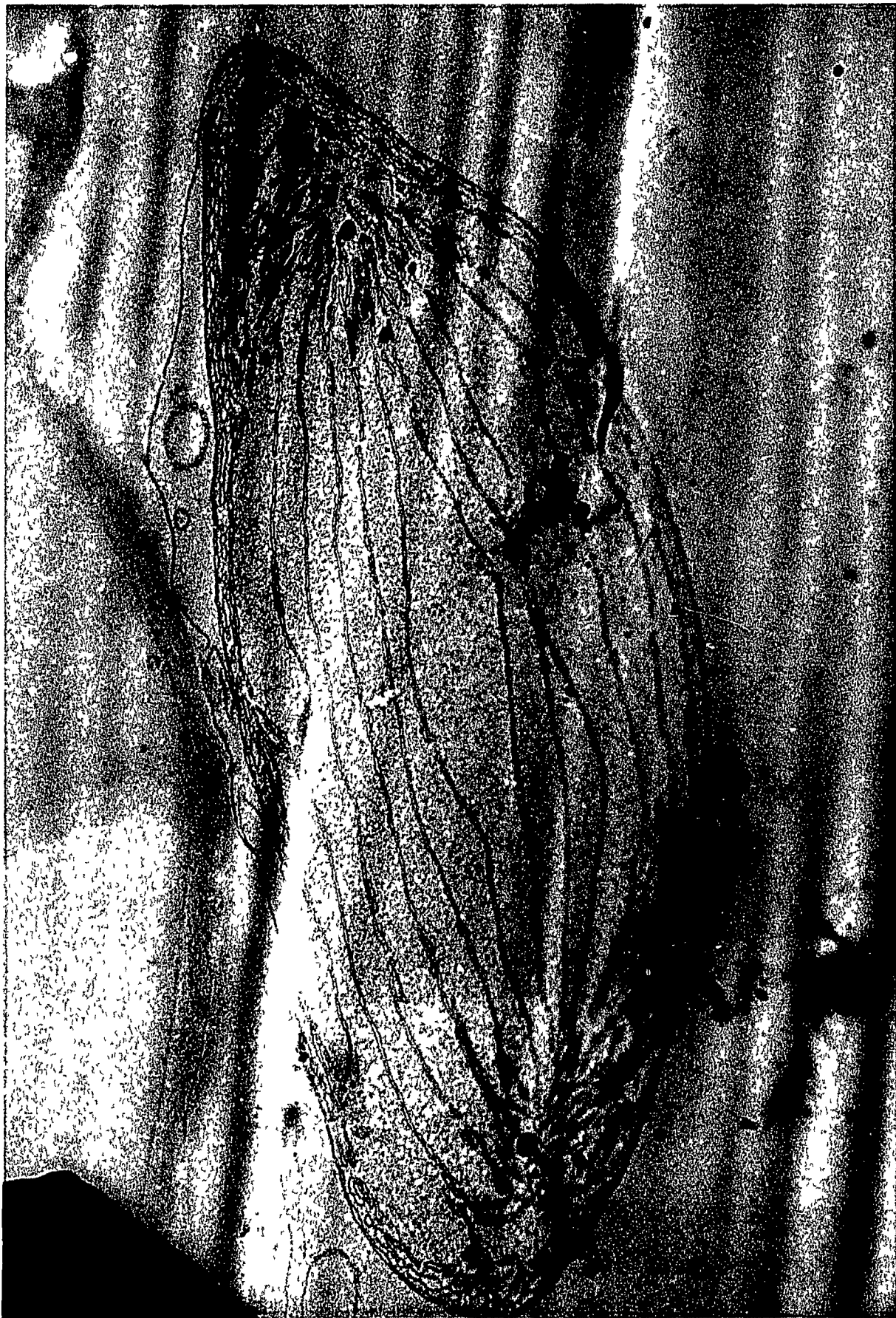
The fine structure of the mesophyll plastids of ON8146 seems to be similar to that of Chl b-less mutant of barley [77], Su/su mutant of tobacco [81], Chl-deficient mutant of pea [8] and the heterozygous light green (LG mutant of soybean [78]). Compared to the wild types, the chloroplasts of these mutants, in general, exhibit a reduced number of lamellae per grana, and a significant increase in single, unstacked lamellae. Associated with such structural changes these mutants show high Chl a/Chl b ratios than wild types. However, only in the ON8147 mutant of maize and barley mutant there have been altered proportions of the two photosystems; increase in pigments of PS I relative to pigments of PS II, in comparison to the wild type.

Plate 4. The most advanced type of mesophyll chloroplasts of mutant ON8147 of maize (courtesy of Prof. D. J. Paolillo, unpublished data).



Plate 5. The least advanced type of mesophyll chloroplasts of ON8147  
(courtesy of Prof. D. J. Paolillo, unpublished data.)





Recently, several lines of evidence have given support to the idea of PS I being associated with stroma lamellae of chloroplasts (see review [53]). In ON8147, the high Chl a/b ratio, the lower fluorescence yield at 685 nm relative to 710 nm, the low level of variable fluorescence, and the high F735/F685 at 77<sup>0</sup>K, compared to normal chloroplasts are associated with a structure of plastid with a greater proportion of stroma to grana lamellae.

The mesophyll chloroplasts of ON8147 share in common all these characteristics with bundle sheath chloroplasts of normal maize which are enriched in PS I relative to PS II (results presented in Chapter IV). However, they differ in that bundle sheath chloroplasts of Zea mays show 60% (or more) decreased activity of PS II relative to that in mesophyll chloroplasts. On the other hand, the mutant ON8147 shows at least equal rates of PS II induced electron transport at  $1 \times 10^5$  ergs/cm<sup>2</sup>.sec as normal chloroplasts.

In conclusion, when structure and function are correlated in the mutant ON8147, the result gives further support for the idea that PS I is associated with stroma lamellae of chloroplasts.

### 3. Summary and Conclusions

Leaves of ON8147 mutant of maize contain 30% of total chlorophylls and 70% of total carotenoids of normal plants. However, because of a greater deficiency in the chlorophylls, there is a two-fold increase in carotenoids per unit chlorophyll in the mutant compared to normal maize plant. The high Chl a/b ratio, the low ratio of Chl a673 to longwave form of Chl a, the lower fluorescence yield at 685 nm relative to 710 nm, the

low level of variable fluorescence, and the high F735/F685 at 77°K, compared to normal chloroplasts suggest the presence of more pigments of PS I relative to pigments of PS II in ON8147.

The high photosynthetic capacity of leaf discs (at saturating light intensities, per mg Chl as well as per mg protein) of the mutant compared to normal is also reflected in the photochemical activity of isolated chloroplasts. In isolated chloroplasts of the mutant, PS II shows 2.2 fold higher rates per total Chl while PS I shows about six-fold higher rates per total Chl than in normal maize. When rates of PS I and PS II were expressed per Chl in each system four-fold higher saturation rates were obtained for both PS I and PS II of mutant compared to PS I and PS II of normal chloroplasts. However the ratio of the rates of mutant and normal maize decreases from 2.2 for PS II and 6.0 for PS I at  $6 \times 10^5$  ergs/cm<sup>2</sup>.sec to a ratio of 1 in both cases at  $1 \times 10^5$  ergs/cm<sup>2</sup>.sec suggesting that at intensities below  $10^5$  ergs/cm<sup>2</sup>.sec, a lower quantum efficiency in the mutant may be observed. Light saturation rates of PS II per mg total protein are similar in the mutant and normal chloroplasts, while rates of PS I and photosynthesis in leaf discs are about two-fold higher in the mutant compared to normal maize. Assuming the same distribution of protein between the two pigment systems as that for chlorophylls, both the PS I and the PS II, activities per mg protein in each system are higher in the mutant. The high photosynthetic saturation rates, per Chl, of ON8147 compared to normal maize is probably due to smaller photosynthetic units. PSU I in the mutant is about half as small as in normal chloroplasts calculated on the basis of P700. Also, it appears that the efficiency of system I and II reactions, even on protein basis,

are larger. Thus, the mutant is more efficient in more than one way. Due to the presence of two and a half-fold higher pigments in PS I relative to PS II, the PSU of PS II in the mutant is smaller than that of PS I. Hence, in the mutant the saturation rate of oxygen evolution of photosynthesis and of PS I in chloroplasts will be limited by the dark limiting enzymes of PS I. If the PSU I of mutant was as small as the PSU II, the saturation rates of overall photosynthesis would even be higher.

Recently, several lines of evidence have given support to the idea of PS I being associated with stroma lamellae of chloroplasts. In the mutant, the high Chl a/b ratio, the lower fluorescence yield at 685 nm relative to 710 nm, the low level of variable fluorescence, and the high F735/F685 at 77°K, compared to normal chloroplasts are associated with a structure of plastid with a great proportion of stroma to grana lamellae. In conclusion, when structure and function are correlated in the mutant ON8147, the result gives further support for the idea that PS I is associated with stroma lamellae of chloroplasts.

## CHAPTER IV

SPECTRAL CHARACTERISTICS AND PHOTOCHEMICAL ACTIVITIES  
OF MESOPHYLL AND BUNDLE SHEATH CHLOROPLASTS1. Introduction

Plants which fix  $\text{CO}_2$  via the  $\text{C}_4$  dicarboxylic acid pathway have been shown to contain two distinct types of chloroplasts contained in separate cells: bundle sheath and mesophyll [137;cf 138]. Chloroplasts of the mesophyll cells contain grana, but those of the bundle sheath cells show various degrees of granal development, depending on the species and growth conditions. Methods of separating the two types of chloroplasts by sucrose gradient centrifugation [139], by non-aqueous density fractionation [140], and by a method based on the differential resistance of the bundle sheath and mesophyll cells to breakage [79,141] have been reported. Woo et al. [79] have shown that the agranal bundle sheath chloroplasts are deficient in photosystem II (PS II); they lack cytochrome b559 and the fluorescence bands associated with PS II while granal mesophyll and bundle sheath chloroplasts exhibit normal PS II activity. Bishop et al. [78] have confirmed the results of Woo et al. in which they observed the inability of bundle sheath chloroplasts of Zea mays to photoreduce  $\text{NADP}^+$  (with  $\text{H}_2\text{O}$  as reductant) but they found that these chloroplasts were able to obtain photoreduction of DCPIP, ferricyanide and cytochrome c with  $\text{H}_2\text{O}$  as reductant. They, however, concluded that the bundle sheath chloroplasts have active PS I and PS II but lacked a soluble electron carrier connecting the two systems. In a preliminary note [83] on photochemical activity and spectral characteristics of bundle sheath and mesophyll chloroplasts of Zea mays, we reported the presence of an intact electron transport chain in bundle sheath chloroplasts with PS II activity of 20-50% that of mesophyll

chloroplasts. In a subsequent paper, Bishop et al. [142] reported that their inability to obtain  $\text{NADP}^+$  photoreduction in agranal chloroplasts of Zea mays and Sorghum bicolor was due to the loss of plastocyanin during preparation of chloroplasts. In their reconstituted system they obtained rates of  $\text{NADP}^+$  photoreduction comparable to those obtained from grana-containing mesophyll chloroplasts; thus, the presence of fully active PS II in the agranal chloroplasts of these species is implied!

Mayne et al. [84] reported results on isolated bundle sheath and mesophyll cells of Digitaria sanguinalis which agree with our independent results on bundle sheath and mesophyll chloroplasts of Zea mays in that there is a complete electron transport from water to  $\text{NADP}^+$  in both chloroplasts with quantitative difference in the distribution of PS I and PS II components: three-fold higher PS I/PS II ratio in the bundle sheath cells than in the mesophyll cells. During the preparation of this manuscript, Anderson et al. [86] reported uncoupled rates of ferricyanide reduction in bundle sheath chloroplasts equivalent to 17% of the rates of mesophyll chloroplasts. This agrees with our earlier note [83] but is contrary to their own earlier report [79] where they obtained traces of PS II activity in bundle sheath chloroplasts as measured by the amount of  $\text{O}_2$  evolved when DCIP is photoreduced. They concluded that bundle sheath chloroplasts in maize contain more PS II than those in sorghum, but they are deficient in PS II when compared with mesophyll chloroplasts.

In this chapter our independent results on the chloroplasts, from both mesophyll and bundle sheath cells of maize are presented. We present data on the pigment composition, the time course, the emission and the action spectra of Chl fluorescence, at 298°K and 77°K. Action spectra of the relative quantum yields of Chl fluorescence at 298°K are also measured and

the relative efficiency of energy transfer from the accessory pigments to Chl a has been calculated. Data on the degree of polarization of Chl fluorescence and on delayed light emission are presented. In addition, results are presented for the photochemical activities of PS II (DCPIP reduction), PS I (methyl viologen reduction in the presence of DCMU and DCPIHP<sub>2</sub>), PS I and II (methyl viologen reduction using water as a donor). Furthermore, light induced absorbance change at 559 nm (Cyt b<sub>3</sub>) and the antagonistic effects of PS I and PS II lights on this change, and the light-induced quenching of atebirin fluorescence are also presented.

## 2. Results and Discussion

### a. Pigment Composition and In Vivo Absorption Spectra

Table 5 presents data on pigment contents of mesophyll and bundle sheath chloroplasts of Zea mays. In bundle sheath chloroplasts, the ratio of Chl a/Chl b is 5.0 compared to 3.0 for mesophyll chloroplasts. This agrees with ratios published for other C<sub>4</sub> plants [84,86]. The total carotenoid(s) content in bundle sheath chloroplasts is 15% less than that of mesophyll chloroplasts. The ratio of total Chl/Carotenoids (mg/mg) in bundle sheath chloroplasts is 7.0 compared to 6.0 for mesophyll chloroplasts. In S. bicolor (C<sub>4</sub> plant), Anderson et al. [86] have reported similar ratios in both mesophyll and bundle sheath chloroplasts but the latter were enriched in  $\beta$ -carotene and contained less xanthophylls compared to the former; this is to be expected as system I is known to be enriched in carotenes and not xanthophylls [143].

The absorption spectrum at room temperature of bundle sheath chloroplasts (Figure 10) shows a shift of 1 nm towards longer wavelengths in the red band compared to mesophyll chloroplasts; it is at 679 nm in bundle

Table 5  
The Contents of Pigments in Mesophyll  
and Bundle Sheath Chloroplasts

Type of Chloroplasts	Chl a/b *	Total Carotenoids ** mg/mg Chl	Chl/Carotenoids mg/mg
Mesophyll	3.0	0.166	6.0
Bundle Sheath	5.0	0.155	7.0

\* Average of 8 experiments; \*\* average of 2 experiments



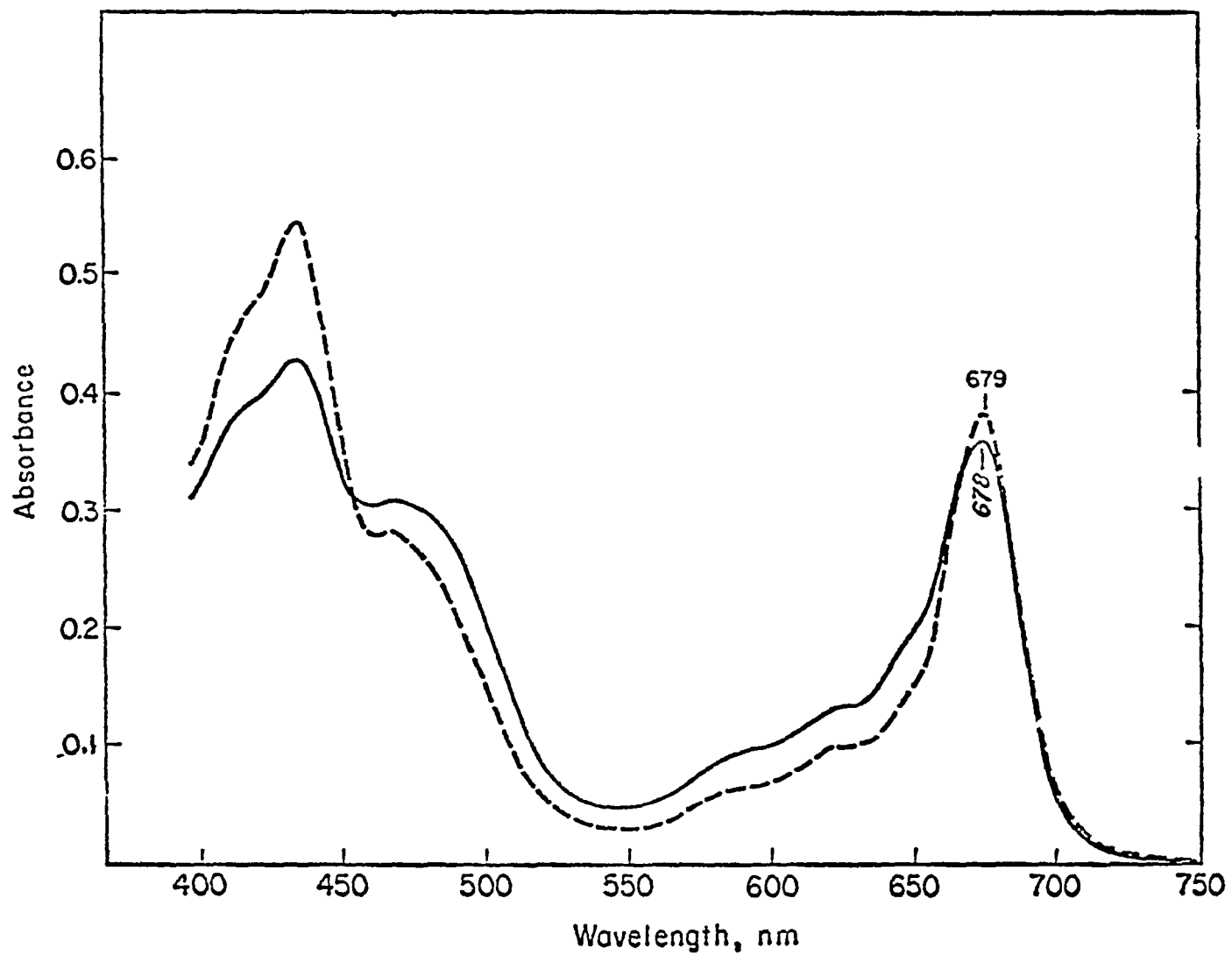


Figure 10. Room temperature absorption spectra of Ms (—) and Bs (----) chloroplast suspensions.

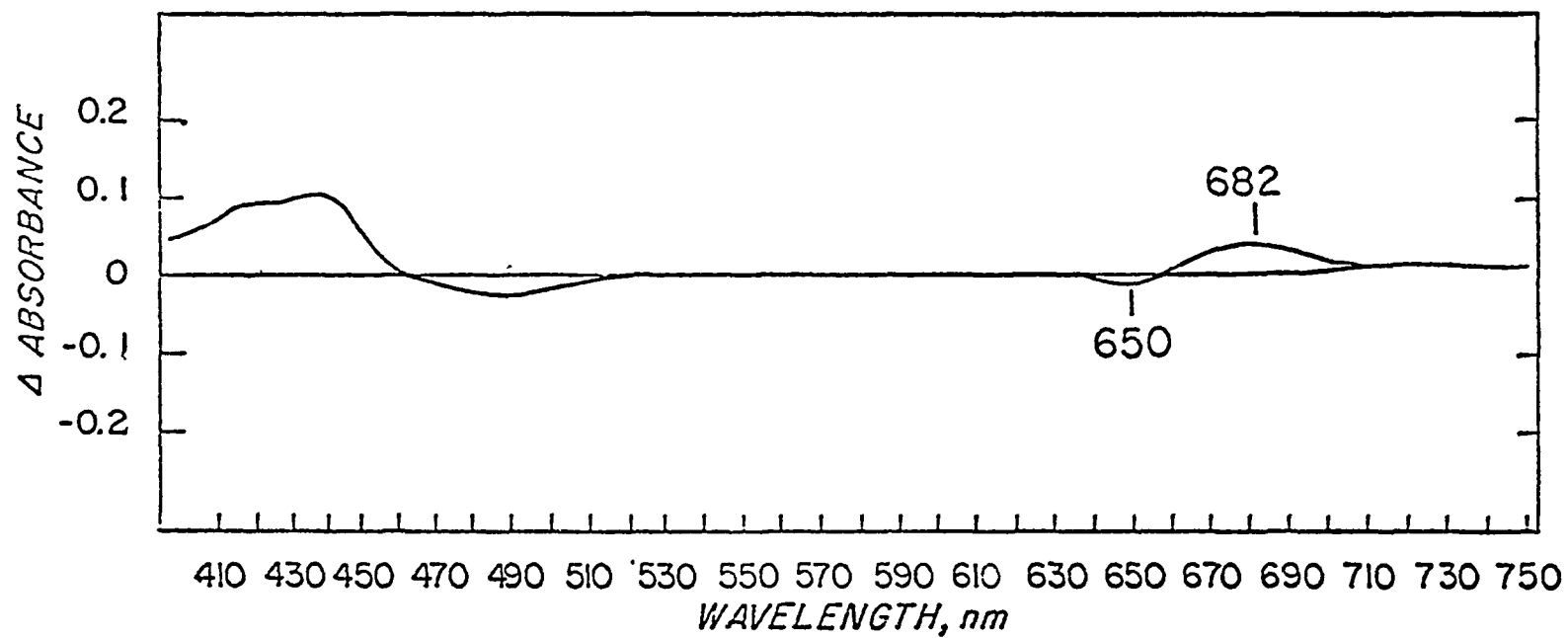


Figure 11. Difference Absorption spectrum of (Bs minus Ms) chloroplast fragments.

### b. Photochemical Activity

In Table 6 are presented results of Hill reactions with DCPIP and methyl viologen as oxidants in isolated chloroplasts of mesophyll and bundle sheath cells of Zea mays. The rates reported are averages of five experiments. The ratios of the rates of bundle sheath chloroplasts to those of mesophyll chloroplasts are 0.40 for DCPIP reduction and 0.39 for methyl viologen reduction. These ratios are higher than those reported by Woo et al. [79] who suggested the presence of traces of PS II activity in bundle sheath chloroplasts of S. bicolor and Zea mays compared to normal chloroplasts. Bishop et al. [87] measured ferricyanide, DCPIP, and cytochrome c reduction in bundle sheath and mesophyll chloroplasts of maize and they obtained ratios of 0.4, 0.58, and 0.66 respectively. Recently Anderson et al. [86] measured uncoupled rates of ferricyanide reduction in bundle sheath and mesophyll chloroplasts of maize and obtained a ratio of 0.17. Mayne et al. [84] measured rates of benzoquinone and methyl viologen reduction in isolated chloroplasts of mesophyll and bundle sheath cells of D. sanguinalis (C<sub>4</sub> plant). The ratio of the rates of benzoquinone reduced in bundle sheath cells to that in mesophyll cells was 0.51 and for methyl viologen reduction, this ratio was 0.53. In a very recent report (which came to my attention during the preparation of this manuscript) Anderson et al. [86] reported higher uncoupled rates than those reported previously [85] for both DCPIP reduction and ferricyanide reduction in bundle sheath fragments of Zea mays. Their earlier reported ratio for rates of ferricyanide reduced by bundle sheath to that by mesophyll chloroplasts was obtained from rates measured at a light intensity of  $40 \times 10^3$  lux. In their recent paper [86] they report that the rates of Hill reactions in bundle sheath chloroplasts saturate at a higher light intensity than those

Table 6

Photochemical Activities of PS I, PS II and  
PS I + PS II of Mesophyll and Bundle Sheath Chloroplasts

Type of Chloroplasts	PS II $\mu\text{moles DCPIP red.}$ $\text{mg}^{-1} \text{Chl hr}^{-1}$	Ratio BS/MS	$\mu\text{moles O}_2$ consumed $\text{mg}^{-1} \text{Chl hr}^{-1}$			
			PS I + PS II $\text{H}_2\text{O} \rightarrow \text{MV}$	Ratio BS/MS	PS I $\text{DCPIP} \rightarrow \text{MV}$	Ratio BS/MS
Mesophyll (MS)	150		141		241	
		0.4		0.39		3.2
Bundle Sheath (BS)	60		56		778	

For measurement of DCPIP reduction (PS II activity), 3 ml reaction mixture containing chloroplasts (10-15  $\mu\text{gm}$  chlorophyll), and (in mM): phosphate buffer (pH 6.8), 50; NaCl, 0.01; and DCPIP, 0.045. For measurement of electron transport activity from  $\text{H}_2\text{O}$  to MV (PS I + PS II activity), 4 ml reaction mixture containing chloroplasts (50  $\mu\text{gm}$  chlorophyll), and (in mM): TES buffer (pH 7.3), 50; sucrose, 0.2; MV, 0.01; 2% ethanol and excess of catalase. For measurement of electron transport activity from  $\text{DCPIP} \rightarrow \text{MV}$  (PS I activity), 4 ml reaction mixture containing the same as for measurement of PS I + PS II plus the following (in mM): DCPIP, 0.1; Ascorbate, 3.; DCMU, 0.01.

in mesophyll chloroplasts. When true saturation rates of DCPIP reduction in bundle sheath chloroplasts (*i.e.*, rates measured at  $118 \times 10^3$  lux), a ratio of 0.34 is obtained for bundle sheath fragments compared to mesophyll chloroplasts. These ratios are even higher when the chloroplasts are coupled; they are 0.34 for rates of DCPIP photoreduction measured at  $40 \times 10^3$  lux and 0.58 for rates measured at  $118 \times 10^3$  lux.

In this study, the method employed for separation of bundle sheath and mesophyll chloroplasts produces mainly fragments of the chloroplasts. The rates of DCPIP reduction (Table 6) are obtained without the addition of an uncoupler and are measured at  $30 \times 10^6$  ergs/cm<sup>2</sup>sec for both types of chloroplasts. In comparing the results reported here with those of Anderson *et al.*, the above mentioned should be considered. Under such conditions, the ratio of the rate of DCPIP reduced by bundle sheath to that of mesophyll chloroplasts is 0.4, compared to a ratio of 0.58 reported by Anderson *et al.* We consider this difference not to be very significant.

I also measured PS I activity in isolated chloroplasts of both types of cells in maize (Table 6). When DCMU and reduced DCPIP were added to the chloroplasts, I obtained rates of methyl viologen reduction three times higher in bundle sheath than in mesophyll chloroplasts. Woo *et al.* [79] have shown rates of PS I activity in bundle sheath chloroplasts comparable to that in mesophyll chloroplasts. In  $\mu$ moles  $\text{NADP}^+$  reduced per mg Chl per hour, they reported 41 for bundle sheath and 43 for mesophyll chloroplasts. Bishop *et al.* [87] also measured PS I activity, with  $\text{DCPIP}_2$  as the electron donor, in both chloroplasts of maize; they obtained rates of  $\text{NADP}^+$  reduction in bundle sheath to be 35% of that of mesophyll chloroplasts. These results disagree with ours on PS I activity in bundle sheath chloroplasts, which show enrichment of these chloroplasts in PS I compared to

mesophyll chloroplasts as should be expected from other data. Our results are consistent with the amount of P700/Chl reported for bundle sheath chloroplasts of  $C_4$  plants [146,85].

The ratio of the rate of methyl viologen reduction by water in bundle sheath to that of mesophyll chloroplasts agree quite well with those for DCPIP reduction (Table 6). This shows that the electron transport from  $H_2O$  to methyl viologen is intact in bundle sheath chloroplasts, contrary to an earlier report by Bishop *et al.* [87].

Figure 12 shows the antagonistic effect of PS II and PS I lights on absorbance changes at 559 nm in bundle sheath and mesophyll chloroplasts (electron transfer from diphenyl carbazide (DPC)  $\rightarrow$  methyl viologen (MeV)). The data presented here are not quantitative, since they are not corrected for percent absorption by the sample and light intensities of the exciting beams. In both cases when the samples are excited by 655 nm ( $8 \times 10^4$  ergs/cm<sup>2</sup>.sec) light, an increase in absorbance at 559 nm is observed indicating reduction of Cyt b559. However, when 710 nm ( $0.15 \times 10^4$  erg/cm<sup>2</sup>.sec) light is imposed on top of 655 nm light, a decrease in absorbance indicating oxidation of Cyt b559 is obtained. When 655 nm light is turned off, only oxidation of Cyt b by PS I light occurs and hence a further decrease in absorbance is obtained. In bundle sheath chloroplasts when 655 nm light was turned off the slope of the decrease was larger than in the case of mesophyll chloroplasts. Although these samples had similar chlorophyll concentration, the percent absorbance at 710 nm in bundle sheath chloroplasts is higher than in mesophyll chloroplasts due to the presence, in the former, of high ratio of longwave form of Chl a to Chl b.

### c. Fluorescence Quenching of Atebrin In Chloroplasts

Recently Kraayenhof [147,148] reported a quenching of the fluorescence of the uncoupler atebrin in isolated chloroplasts. This quenching was induced by the operation of the electron transport, ATP hydrolysis or a pH gradient. It was suggested that the extent of quenching may be used to measure the "energy state" of the chloroplast. However, Schuldiner and Avron [88] have shown that, upon illumination, atebrin was distributed between the inside of the chloroplast and the solution according to the ratio of proton concentration. They suggested that the mechanism of uncoupling of atebrin, which has two basic amine groups, is similar to that of ammonium chloride uncoupling in chloroplasts. This implies that upon illumination of chloroplasts, atebrin is distributed between the inside of the chloroplast and the solution according to the ratio of proton concentration. Thus they suggested that the fluorescence quenching of atebrin may serve as a tool for following the proton gradient across the chloroplasts and not as a means to measure the energy state  $X^VI$ . However, they suggest that the quenching of atebrin fluorescence as it moves into chloroplasts is partially due to the "inner filter" effect by chlorophyll molecules on lamellae.

We used the quenching of atebrin fluorescence as a tool to measure the "energy state" or the proton gradient (?) induced upon illumination of both types of chloroplasts in Zea mays. The  $H^+$  gradient has been suggested (see review, 149) to be the driving force for ATP formation because of the similarity between the light stage of  $X_E$  and the proton gradient in terms of kinetics of formation and decay, pH optimum, and the effect of electron flow carriers.

Several other lines of evidence suggest that the energy coupling mechanism in chloroplasts is strongly dependent on the formation of a

light-induced proton gradient (for a recent review see ref. 150). However, with subchloroplast particles, the situation seems to be different, and massive proton movement is not correlated with ATP formation. McCarty [151] has shown that ATP formation in sonically prepared subchloroplast particles was active after proton uptake was largely inhibited by  $\text{NH}_4\text{Cl}$  or nigericin and  $\text{K}^+$ . Further support for this came from the work of Nelson et al. [152] with digitonin-devoid subchloroplast particles, and recently from the work of Arntzen et al. [153] with stroma lamellae, obtained by the French pressure treatment [52] and subsequent differential centrifugation, and with various chloroplasts having different amounts of stromal and granal membranes.

In Table 7 is presented the quenching of atebtrin fluorescence upon illumination with red light in mesophyll and bundle sheath chloroplasts in the presence of the cyclic electron flow carrier (PMS), and the non-cyclic electron carrier (diquat). When diquat is added to the chloroplasts, the proton gradient across the membrane is assumed to be due to non-cyclic electron transport. When DCMU, ascorbate and PMS are added to the chloroplasts, the proton gradient is due to cyclic electron transport. Bundle sheath chloroplasts show 8-14% quenching of atebtrin fluorescence compared to 85% in mesophyll chloroplasts when diquat is added. If this quenching is associated with the non-cyclic ATP formation, then this result shows again the presence of less PS II activity in bundle sheath chloroplasts than in mesophyll chloroplasts in Zea mays. This quenching is sensitive to DCMU as both types of chloroplasts showed no quenching upon its addition. When non-cyclic electron flow from PS II was stopped by DCMU and ascorbate was added as the electron donor to PS I with PMS as a "cyclic" electron carrier, both chloroplasts showed similar percentage of quenching.



measurements of both Anderson *et al.* [85] and Arntzen *et al.* [153]. This indicates that fluorescence quenching of atebirin could not be attributed completely to the proton gradient as suggested by Schuldiner and Avron [88]. Instead, it reflects some "energy state" that leads to ATP production; it could reflect that membrane potential ( $\Delta\Psi$ ) across the membranes. I plan to make several measurements (pH, atebirin fluorescence, and ATP) on the same samples to check this conclusion.

#### d. Fluorescence Transients

The time course of Chl fluorescence at 685 nm was measured in suspensions of Bs and Ms chloroplasts of *Zea mays* (Figure 13). The two curves are normalized at "0" (or  $F_0$ , the initial level of fluorescence which reflects the amount of fluorescence emitted by the bulk pigment of PS I and PS II). The yield of total fluorescence ( $F_\infty/F_0$ ) in Ms chloroplasts is 3.35 compared to 2.0 in Bs chloroplasts. The relative fluorescence yield of variable with respect to constant fluorescence [ $(F_\infty - F_0)/F_0$ ] for Ms is 2.35 and 1.0 for Bs chloroplasts. However, the quantum yield of the constant fluorescence in Ms chloroplasts of maize is 1.3 times that of Bs chloroplasts.

The rise in Chl fluorescence intensity of isolated chloroplasts on illumination has been attributed to the photoreduction of a primary electron acceptor Q in photosynthesis by PS II [131]. Reduced Q is reoxidized in darkness, or by far red light absorbed predominantly by pigment system I. The relative decrease in the yield of variable fluorescence in Bs compared to Ms chloroplasts could be due to either one of the following several alternatives (for discussion of different possibilities, see p. 52, Chapter 3).

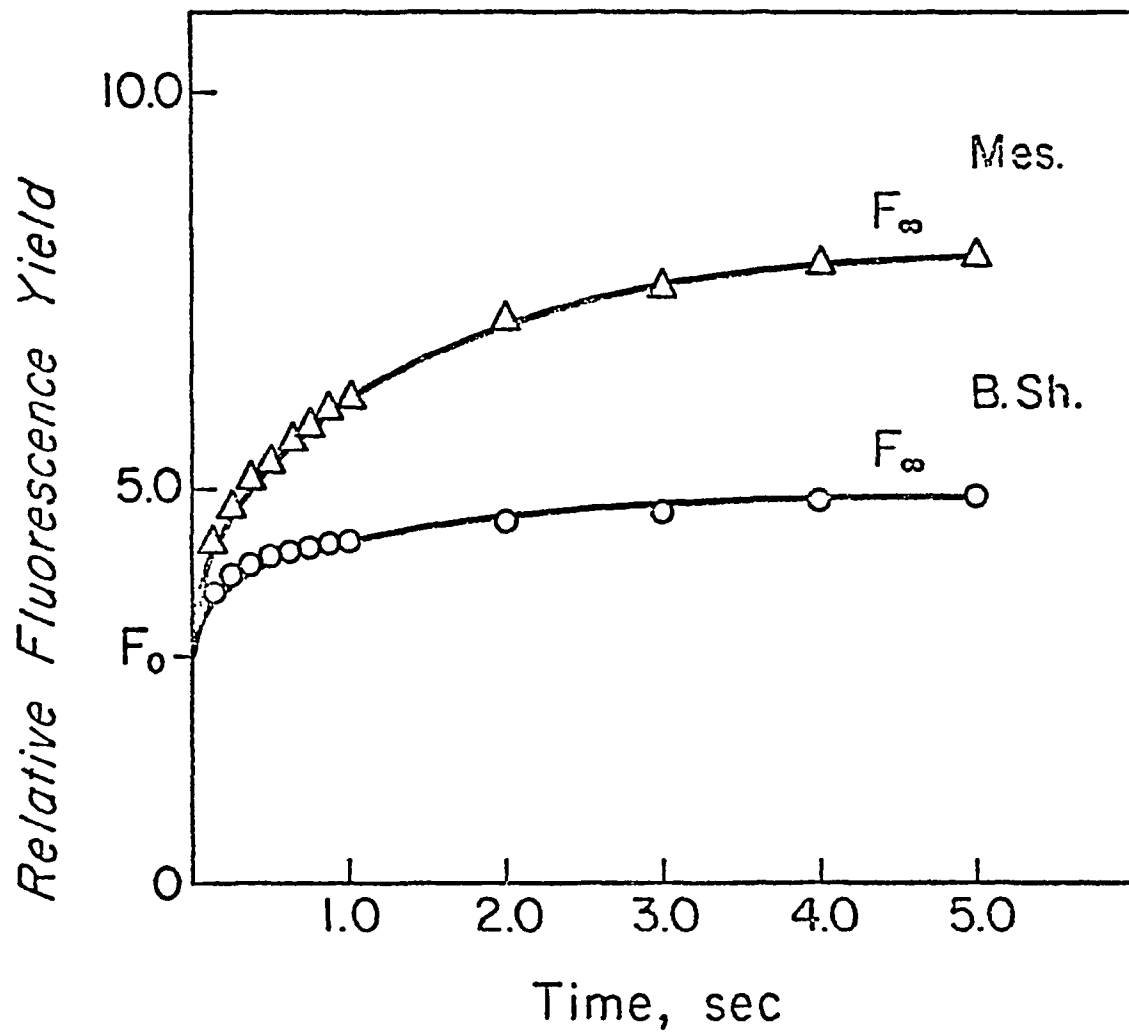


Figure 13. Time course of Chl fluorescence of mesophyll (- $\Delta$ -) and bundle sheath (-o-) chloroplast fragments normalized at  $F_0$ . Conditions as in Figure 7.

The possibility of the presence of inactive PS II in *Bs* is unlikely to explain the decrease in the relative yield of variable with respect to constant fluorescence in *Bs* chloroplasts in spite of a parallel decrease observed in PS II activity of these chloroplasts. The activity of PS II in *Bs* is 40% of that of PS II in *Ms* chloroplasts (see Table 6). This decrease in activity, most likely, is not due to inactivity of PS II in *Bs*, but rather to deficiency in PS II pigments and their associated electron transport carriers. However, unless a quantitative estimation of concentration of these intermediates to determine the actual decrease in their concentration relative to *Ms* chloroplasts is made, the above mentioned possibility could not be ruled out completely.

The presence of more pigments in PS I relative to PS II could explain the relative decrease of variable with respect to constant fluorescence in *Bs* compared to *Ms* chloroplasts. From the ratio of Chl a/b, the difference absorption spectrum, and the emission spectra at 77°K and 298°K, it is evident that there are more pigments of PS I relative to PS II in *Bs* chloroplasts.

The possibility of larger (Q + A) pool could be ruled out because of the absence of slow fluorescence intensity rise to  $F_{\infty}$  in *Bs* chloroplasts; an expected result if larger pool of (Q + A) was present in these chloroplasts. The time required to reach  $F_{\infty}$  is the same in *Ms* and *Bs* chloroplasts.

The possibility of decrease in concentration of Q in *Bs* compared to *Ms* could also explain the decrease in the relative yield of variable fluorescence in *Bs* chloroplasts. If PS II activity is decreased due to deficiency in PS II components, then one would expect the concentration of Q to be reduced in these chloroplasts. Also because of presence of more pigments in PS I than in PS II, even the small concentration of QH formed

would be reoxidized quickly upon illumination of chloroplasts. Hence a combination of these two factors: less content of Q and higher PSI relative to PS II, would produce low variable fluorescence in Bs., compared to Ms chloroplasts.

#### e. Excitation (or Action) Spectra of Chl<sub>a</sub> Fluorescence

I have measured the fluorescence excitation spectra of both types of chloroplasts in Zea mays at room temperature (RT) and at 77°K for the following reasons: (1) the difference absorption spectrum of both types of chloroplasts (Figure 11) showed that the bundle sheath chloroplasts in comparison to mesophyll chloroplasts were enriched in Chl a 678, Chl a 680 and, perhaps, Chl a 670 relative to Chl b. Fluorescence excitation spectra at 77°K should resolve these differences clearly. (2) To study and compare the efficiency of energy transfer from Chl b to Chl a and from carotenoids to Chl a in the two types of chloroplasts; for this, the relative quantum yields of fluorescence of Chl a excited by Chl b, Chl a or carotenoids in both types of chloroplasts need to be calculated from the fluorescence excitation spectra.

Figure 14 shows RT excitation spectra of Chl fluorescence at 740 nm ( $F_{740}$ ) for mesophyll and bundle sheath chloroplast fragments normalized at 675 nm for Chl a. The ratio of fluorescence excited by 675 nm light ( $F_{675}^{740}$ ) to that excited by 650 nm light ( $F_{650}^{740}$ ) is 1.7 in mesophyll compared to 2.4 for bundle sheath chloroplasts. At 675 nm, Chl a is the main pigment contributing to  $F_{740}$  while at 650 nm Chl b contribution is dominant. The higher ratio of 2.4 bundle sheath compared to the lower ratio of 1.7 in mesophyll chloroplast agrees with the higher ratio of Chl a/b obtained from acetone extraction, and also from the in vivo measurement of the absorption spectrum of these chloroplasts.

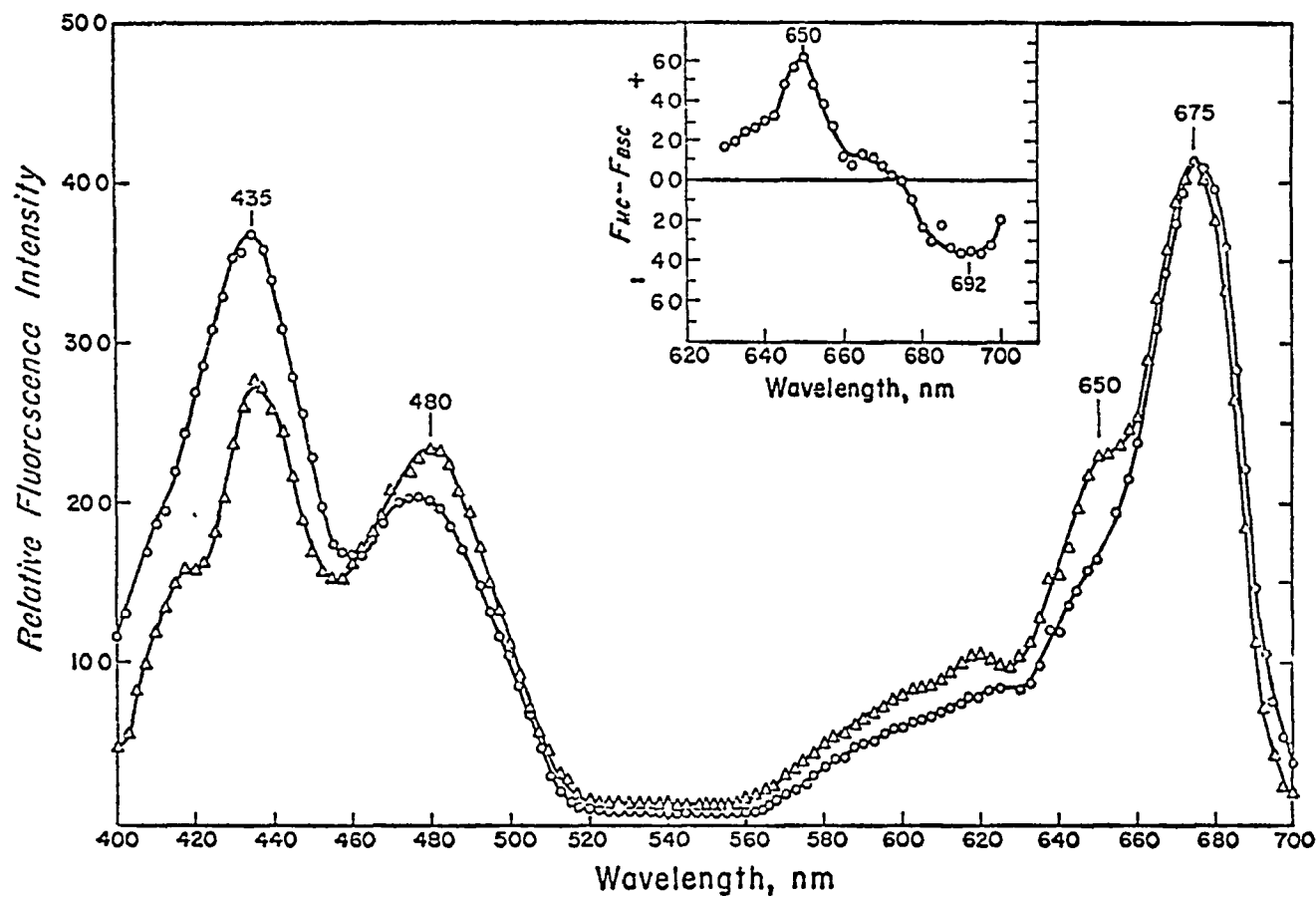


Figure 14. Fluorescence excitation spectra of F740 of mesophyll (MS) ( $-\Delta-$ ) and bundle sheath (BS) ( $-o-$ ) chloroplast fragments, normalized at 675 nm. Insert, the difference excitation spectrum. Chloroplasts (absorbance at 678 nm, 0.17) were suspended in 0.02 M tris-HCl buffer pH 7.8, 0.4 M sorbitol and 0.01 M NaCl.

The insert in Figure 14 shows the difference excitation spectrum of F740 of the two curves normalized at 675 nm. The negative band with a peak maximum at 692 nm has a 1/2 band width of 22 nm. According to the reported [145] half bandwidths of Chl a forms in vivo, one can suggest that another form of Chl a besides Chl a 692 has more contribution to F740 in bundle sheath chloroplasts than in mesophyll chloroplasts. This is further suggested from the slight shoulder at 683 nm. (These bands could have been resolved if narrower exciting slits were used.) These results again show clearly that in comparison to mesophyll chloroplasts, bundle sheath chloroplasts are enriched in Chl a forms absorbing at long wavelengths relative to Chl b. The shoulder at 665 nm suggests that besides Chl b, bundle sheath chloroplasts also contain different amount of Chl a form absorbing at 660 nm. This form of Chl a has been suggested by Brody [154] to be the short-wave band of a longwave length aggregate form of Chl a present in pigment system I.

In the blue region, the peaks are at 435 nm and 480 nm in both chloroplasts. However, the ratio of  $F_{435}^{760}$  to  $F_{480}^{760}$  is different in the two cases; it is 1.2 in mesophyll compared to 1.6 in bundle sheath chloroplasts. Qualitatively these ratios are consistent with those obtained in the red portion of the fluorescence spectrum for Chl a/b, but the numerical value of the ratios differ because of the carotenoids contribution in this portion of the spectrum. It is interesting to compare the ratio of the intensity of fluorescence at 740 nm excited by 678 and 435 nm ( $F_{435}^{740}/F_{678}^{740}$ ) in bundle sheath and in mesophyll chloroplasts. It is 0.9 in the first and 0.67 in the second. The difference in these values between the two types of chloroplasts could be attributed to either one of the following reasons: 1) if the bundle sheath chloroplasts contained more carotenoids

than mesophyll chloroplasts then this is expected since spectra of Figure 14 are not quantum yield spectra. 2) If the amounts of carotenoids are the same in both chloroplasts, then this difference could be due to an increase in the efficiency of energy transfer from carotenoids to Chl a in bundle sheath chloroplasts compared to mesophyll chloroplasts.

The amount of total carotenoids in both types of chloroplasts was determined (Table 5); 13-15% less total carotenoids were found in bundle sheath compared to mesophyll chloroplasts. In spite of this finding which would probably rule out the second interpretation, a check for possible differences in efficiency of energy transfer from carotenoids to Chl a or from Chl b to Chl a in the two types of chloroplasts was considered an important problem to investigate. Results of such experiments will be presented later in this chapter.

No significant differences in efficiency of energy transfer from carotenoids to Chl a was observed between the two types of chloroplasts. Because carotenoids are 15% less in Bs than in Ms chloroplasts, the higher ratio of  $F_{435}^{740}/F_{678}^{740}$  in the former could be attributed to the blue band of the longwave form of Chl a which is present in higher concentration relative to other forms of Chl a in Bs chloroplasts compared to mesophyll chloroplasts.

#### f. Fluorescence (or Emission) Spectra at 77°K and 298°K

I measured the fluorescence spectra of thin suspensions (absorbance at 678 nm, 0.004 for measurement at 77°K and 0.15 for room temperature measurement) of chloroplast fragments of mesophyll and bundle sheath cells of Zea mays excited by 440 nm at 77°K (Figure 15) and 298°K (Figure 16). At 77°K, in both chloroplasts, we obtain three banded spectra, characteristics of fluorescence spectra of species with grana chloroplasts like spinach (see [12]), with maxima at 685 nm, 696 nm (not clearly resolved,

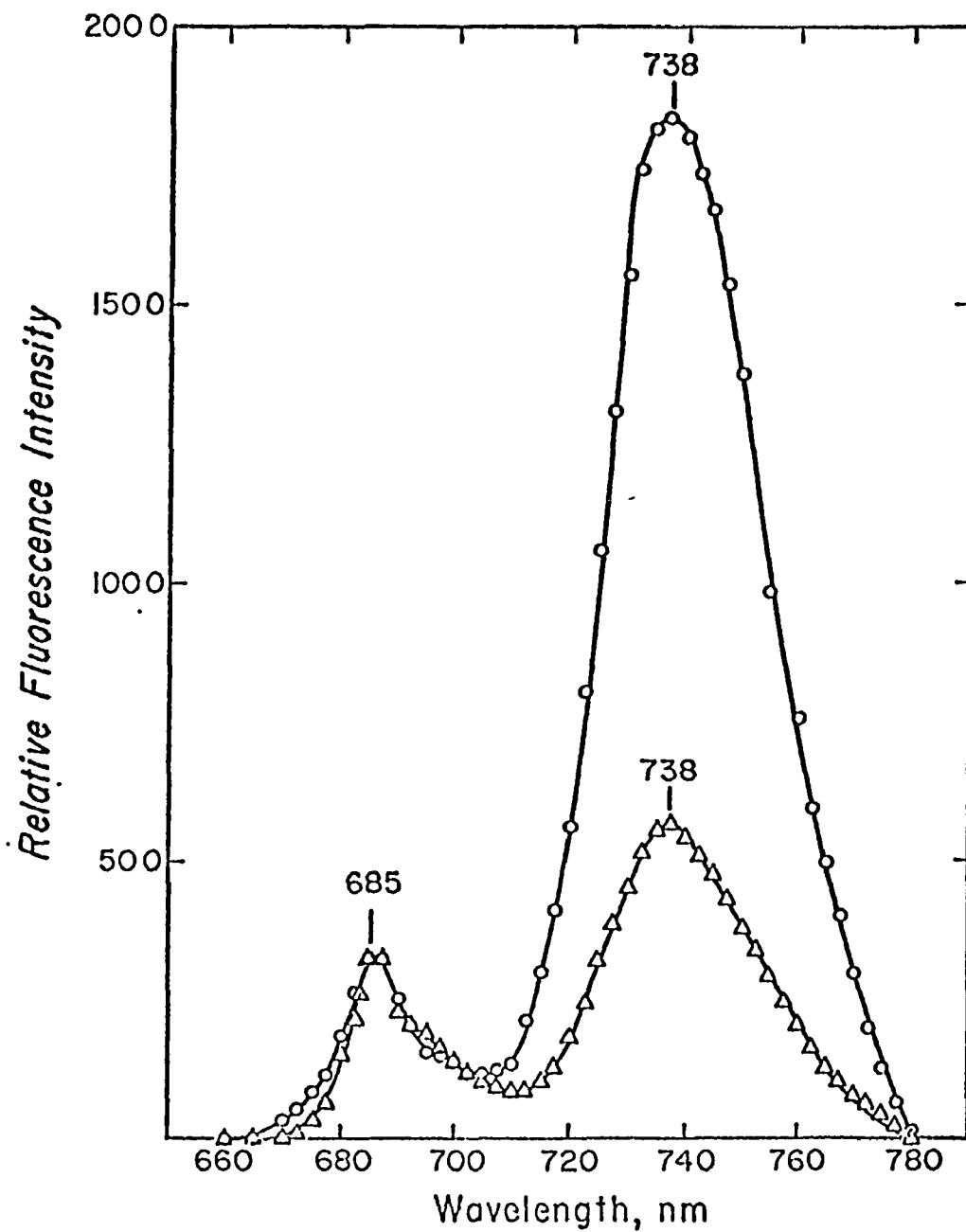


Figure 15. Fluorescence emission spectra at 77°K of mesophyll (-Δ-Δ-) and bundle sheath (-o-o-) chloroplast fragments, normalized at 685 nm. Details as in the legend of Figure 14. Exciting wavelength, 440 nm.



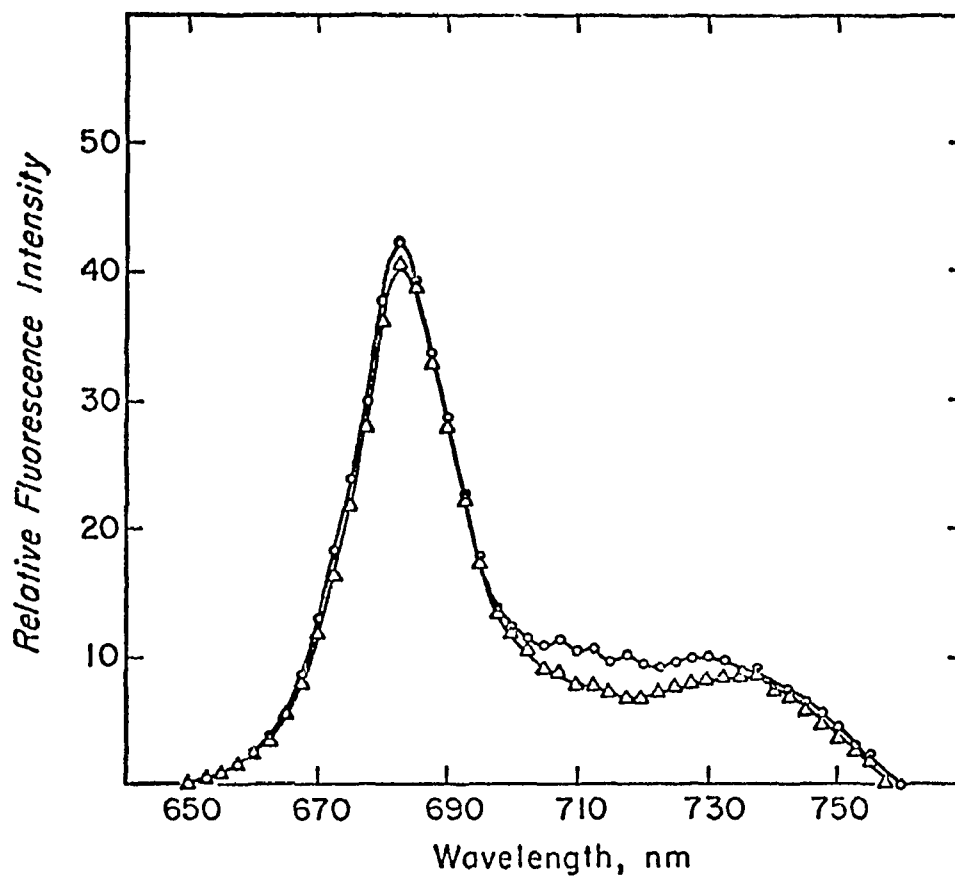


Figure 16. Fluorescence emission spectra at 298°K of mesophyll (-Δ-) and bundle sheath (-o-) chloroplast fragments. Conditions as in Figure 13. Exciting wavelength, 440 nm.

subchloroplast particles, have shown that F715-730 is mainly excited by PS I while F740-760 is excited by both PS I and PS II. From the above ratios, it is clear that bundle sheath chloroplasts are enriched in long wavelength forms of Chl a with respect to Chl a 678. The slightly higher ratio of F715/F683 to F740/F683 in bundle sheath chloroplasts indicate that there is more PS I with respect to PS II in these chloroplasts. These ratios, however, do not differ significantly in the case of mesophyll chloroplasts.

From fluorescence spectra at 298°K, I calculated the fluorescence yield to be about 40% less in bundle sheath compared to mesophyll chloroplasts. Most of the fluorescence at room temperature is emitted by pigments of PS II. Since bundle sheath chloroplasts contain about 40% the photochemical activity of PS II in mesophyll chloroplasts, then a 40% decrease in fluorescence yield at room temperature is expected in bundle sheath chloroplasts. This result is confirmed by Elkin and Park (personal communication to Govindjee) who measured the fluorescence of intact chloroplasts in situ of C<sub>4</sub> grass leaf cross sections, by using infrared color fluorescence photography. They observed that the total bundle sheath fluorescence intensity is approximately half that of the mesophyll; and the red fluorescence peak is between 1/8-1/4 the intensity of the mesophyll peak.

#### g. Excitation Spectra of Chl a Fluorescence in Thick Samples

Excitation spectra of fluorescence at 760 nm of thick samples (100% absorption at 678 nm) of mesophyll and bundle sheath chloroplasts at 77°K were measured in order to detect the differences in the amount of Chl a 705 between the two types of chloroplasts in Zea mays. This band can not be observed in thin suspensions because of its low concentration. Indication

of such differences comes from fluorescence emission spectra at 77°K of both chloroplasts.

From study of fluorescence excitation spectra and absorption spectra of leaves at 77°K, Butler [159] showed the presence of a 705 nm absorbing component of Chl a which fluoresces at 720 nm. The presence of this weakly fluorescent band at room temperature is inferred from the "red drop" of Chl a fluorescence [160,161]. This form of Chl a is present in low concentrations in green leaves and its fluorescence yield increases strongly at low temperatures. The enhancement of this band in very dense suspensions is probably due to the "detour" factor as suggested by Govindjee and Yang [61] who confirmed the existence of Chl a 705 band in isolated spinach chloroplasts. In spite of the use of thick samples, re-absorption of fluorescence when measuring at long wavelength is not a problem because there is no significant absorption at 760 nm. Chl a 705 has been variously identified with Chl a 700 [159,162], an oriented form of Chl a [163], or a Chl a dimer [164]. Various experimental results [17,165] indicate that the substance emitting at 730 nm is neither identical with P700 nor responsible for energy transfer from fluorescent Chl a to P700; it is a form of Chl a present in pigment system I (see [12] and [89]).

Figure 17 shows fluorescence excitation spectra at 760 nm of mesophyll and bundle sheath chloroplasts at 77°K. Since both samples have 100% absorption at 678 nm, equal absorption in both samples is assumed and hence, the amount of fluorescence at 760 nm excited by 680 nm and shorter wavelengths is the same. However, one can observe the difference in the two samples at wavelengths longer than 690 nm; the  $F_{760}^{705}$  in bundle sheath chloroplasts is 60 compared to a value of 32 for mesophyll chloroplasts. The differences between the assumed curves for Chl a 678 and the experimental curves are

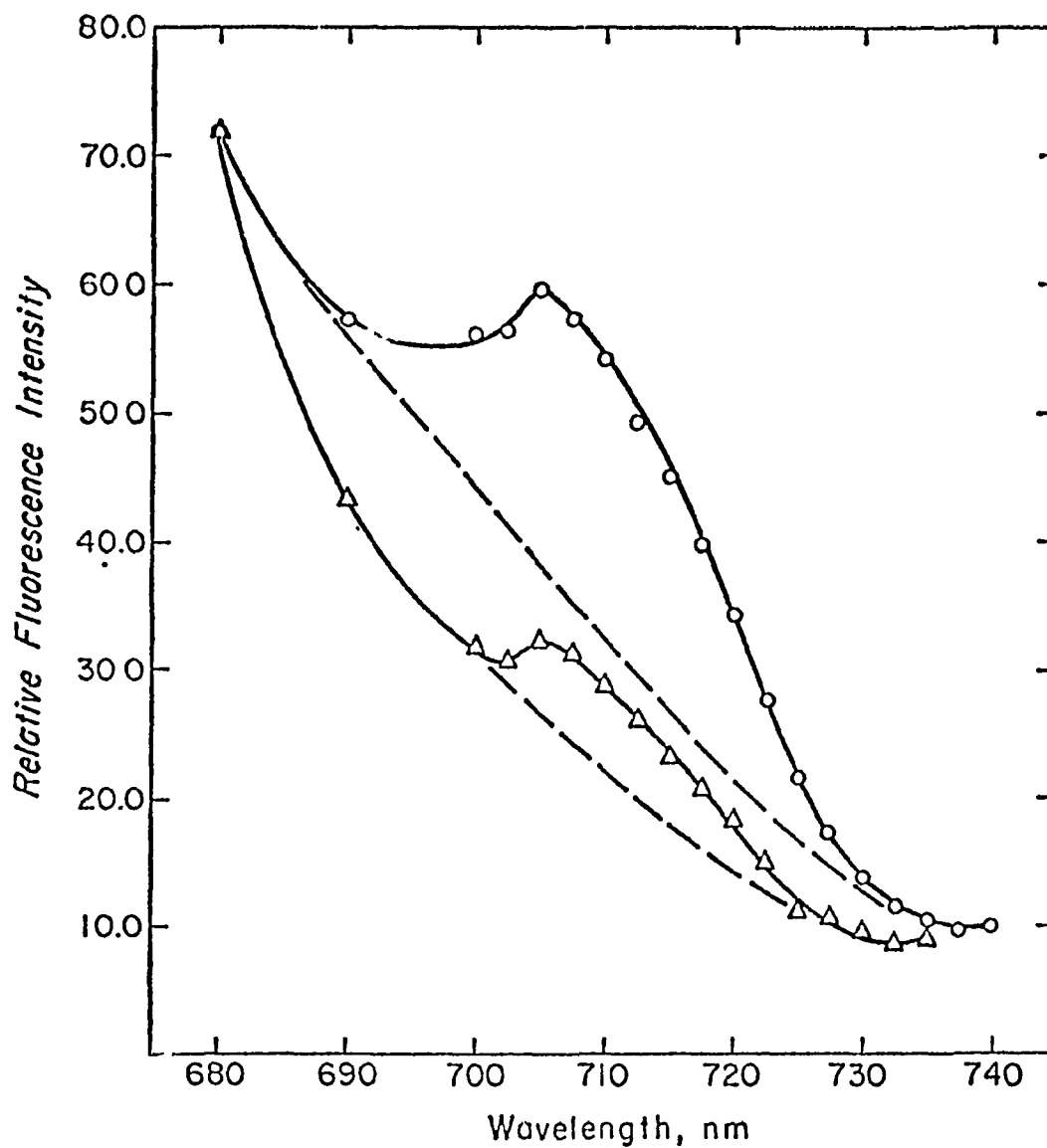


Figure 17. Excitation spectra of F760 at 77°K of thick samples (100% absorption at 680 nm), of mesophyll (-Δ-) and bundle sheath (-o-) chloroplast fragments. Conditions as in Figure 15.

20 and 5 for Bs<sub>1</sub> and Ms chloroplasts respectively. This shows clearly that the bundle sheath chloroplasts have more than twice Chl a 705 than the mesophyll chloroplasts. This result is consistent with the higher fluorescence emission at 735 nm relative to 685 nm at 77°K in bundle sheath compared to mesophyll chloroplasts.

Further support for higher content of Chl a 705 relative to Chl a 678 in bundle sheath than the mesophyll chloroplasts comes from the measurement of the polarization of fluorescence in the two types of chloroplasts. The degree of polarization of Chl fluorescence is 6% in bundle sheath chloroplasts and 4% in mesophyll chloroplasts. This implies that there is higher ratio of oriented to unoriented chlorophylls in bundle sheath chloroplasts. Chl a 705 has been shown to be highly oriented [163].

#### h. The Relative Quantum Yield of Fluorescence (F740) of Chl a Excited by Chl b and Carotenoids

The relative quantum yields of Chl a fluorescence measured at 740 nm, and excited by Chl b and carotenoids for mesophyll and bundle sheath chloroplasts were calculated from the fluorescence excitation spectra of Chl a (F740) of these chloroplasts. The fluorescence intensities of Chl a at 740 nm excited by Chl b and carotenoids were divided by percent absorption of the samples at the wavelength of excitation. Table 8 presents the relative yields of Chl a fluorescence at 740 nm excited by 435 nm (Chl a), 480 (Chl b), and 500 nm--90% absorption by carotenoids [165]--, in the blue region and at 650 nm and 675 nm in the red region.

From these values in Table 8, the efficiency of energy transfer from carotenoids to Chl a was calculated in both bundle and mesophyll chloroplasts. The ratio of the relative fluorescence yield at 740 nm which is excited by 500 nm to that excited by 675 nm, represents the efficiency of

Table 8

The Relative Quantum Yield of Fluorescence (F740) of Chl a  
Excited by Chl b and Carotenoids

Wavelength of Excitation (nm)	The Relative Quantum Yield of Fluorescence of Chl <u>a</u> (F740) in:	
	Mesophyll Chloroplasts	Bundle Sheath Chloroplasts
435	0.89	0.87
480	0.97	0.86
500	0.68	0.59
650	1.35	1.23
675	1.35	1.23

energy transfer from carotenoids to Chl a since at 500 nm 90% of incident quanta is absorbed by carotenoids, while at 675 nm absorption by Chl a is dominant [165]. In the second column of Table 9 is presented the results for both mesophyll and bundle sheath chloroplasts. The ratios obtained were 0.51 for the former and 0.48 for the latter. The ratio of these values is 1.06 which indicates that the efficiency of energy transfer from carotenoids to Chl a is almost the same in both types of chloroplasts.

The efficiency of energy transfer from Chl b to Chl a has also been compared in both types of chloroplasts. In the third column of Table 9 are presented the ratios of the fluorescence yield at 740 nm which is excited by 480 nm (Chl b) to that excited by 440 nm (Chl a) for both types of chloroplasts. These values are 0.99 for bundle sheath chloroplasts and 1.1 for mesophyll chloroplast; the ratio of these values is 1.11 which again indicates the similarity in the efficiency of energy transfer from Chl b to Chl a in both types of chloroplasts. From the last column, where the ratio  $F_{650}^{740}/F_{675}^{740}$  is presented, it is clear that the efficiency of energy transfer from Chl b to Chl a is 100% in maize chloroplasts. And, from column 2, it can be inferred that the efficiency of energy transfer from carotenoids (absorbing at 500 nm) to Chl a may be as high as 50% (it compares well with 50% in *Chlorella*).

### 3. Summary and Conclusions

In conclusion, my results indicate that in the bundle sheath chloroplasts of maize, there is an intact electron transport chain from water to  $\text{NADP}^+$ ; they are not devoid of PS II but contain about 40% the amount of PS II present in the mesophyll chloroplasts; and they contain about three times more PSI than in the mesophyll chloroplasts. Results on % quenching of fluorescence of atrazine, induced by cyclic and non-cyclic electron

Table 9

Efficiencies of Energy Transfer from Carotenoids and Chlorophyll b  
to Chlorophyll a in Mesophyll and Bundle Sheath Chloroplasts

Type of Chloroplasts	$\frac{F_{740}^*}{F_{500}}$ $\frac{F_{740}}{F_{435}}$	$\frac{F_{740}}{F_{500}}$ $\frac{F_{740}}{F_{675}}$	$\frac{F_{740}}{F_{480}}$ $\frac{F_{740}}{F_{435}}$	$\frac{F_{740}}{F_{650}}$ $\frac{F_{740}}{F_{675}}$
Bundle Sheath	0.72	0.48	0.99	1.0
Mesophyll	0.76	0.51	1.1	0.99

\* Obtained from action spectra of F740.



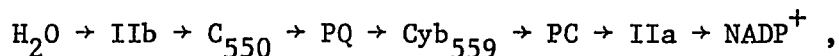
transport in bundle sheath and mesophyll chloroplasts, indicated that fluorescence quenching of atebirin could not be attributed completely to the proton gradient as suggested by Schuldiner and Avron [88]. Instead it reflects some energy state that leads to ATP production.

Measurements of the absorption spectra, the fluorescence excitation spectra at 298°K and 77°K and the fluorescence emission spectra, at 298°K and 77°K, show that the bundle sheath chloroplasts contain, relative to other chlorophylls, more Chl a (705, 693, 685, and probably Chl a 670, and less Chl b) than in mesophyll chloroplasts. From the measurements of relative quantum yield of Chl fluorescence, measured at 740 nm and excited by Chl b and carotenoids, of both types of chloroplasts, the efficiency of energy transfer from Chl b and carotenoids to Chl a are calculated to be 100 and 50% respectively. Furthermore, there is no significant difference in the efficiency of energy transfer between the two types of chloroplasts. The relative quantum yield of Chl a fluorescence at 298°K is lower by 40% in bundle sheath compared to that in mesophyll chloroplasts. The fluorescence spectra at 298°K as well as at 77°K show enrichment of the bundle sheath chloroplasts in F735 relative to F685 and F696. The degree of polarization of fluorescence is higher in bundle sheath (6%) than in mesophyll chloroplasts (4%). This result is consistent with the presence of relatively higher amounts of oriented longwavelength form of Chl a in bundle sheath compared to mesophyll chloroplasts.

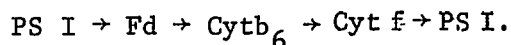
## CHAPTER V

PRELIMINARY STUDIES ON ALTERNATE PATHWAYS TO THE  
Z-SCHEME OF PHOTOSYNTHESIS1. The Concept of Three Light Reactions and Its Analysis

An alternate model to the Z-scheme has been proposed by Knaff and Arnon [49]. They proposed the existence of three light reactions (see Figure 3) in photosynthesis where the electron transport chain of system II is:



and the electron transport chain for system I is:



Evidence claiming support of this proposal [49,166] comes from the following observation by Arnon and his coworkers:

1) The absence of Emerson enhancement in  $\text{NADP}^+$  and ferricyanide reduction in isolated chloroplasts in contrast to the clear enhancement effect for  $\text{CO}_2$  assimilation by chloroplasts. These results disagree with earlier reports by R. Govindjee et al. [168], Govindjee and Bazzaz [169], Joliot et al. [170] and Avron and Ben-HayYim [28] who reported enhancement effects in both ferricyanide and  $\text{NADP}^+$  reduction (with the exception of [28] where enhancement in ferricyanide reduction was not found). Recently Sun and Saur [171] and Sinclair [172] confirmed the existence of enhancement of  $\text{NADP}^+$  reduction in chloroplasts. They clearly showed the

controlling role of  $Mg^{++}$  ions in the enhancement values of  $NADP^+$  reduction in chloroplasts. Sane and Park [173] found that the presence and absence of enhancement depends also on the kind of enzymes used in measuring  $NADP^+$  reduction. If crude preparation of PPNR is used, enhancement is observed, while no enhancement is observed when pure enzyme is used.

2) Arnon and coworkers observed  $NADP^+$  reduction from  $H_2O$  in a chloroplast fraction which, they claimed, was devoid of P700 [174]. However, we note that they did not add plastocyanin, that is usually lost, when measuring P700.

3) At  $-189^\circ C$  there was more efficient photooxidation of Cytb559 with 664 nm (system I and II light) than with 715 nm (system I) light [166]. Photooxidation of Cytb559 was observed at room temperature but only with chloroplasts which lacked the ability to evolve  $O_2$  from  $H_2O$  (tris-treated 0.8M, pH 8.0 [175] or chloroplasts at alkaline pH [166]).

The failure to obtain photooxidation of Cytb559 in untreated chloroplasts, at room temperature, was explained as follows [166]: at physiological temperature, the photooxidation of this cytochrome in untreated chloroplasts is immediately balanced by its reduction by  $H_2O$  and the rapid sequence of photooxidation and reduction at room temperature escape detection with the recording technique used. In tris-treated chloroplasts the electron flow would already be impaired, at room temperature, by the tris treatment and hence, the photooxidation of Cytb559 would be measurable. This is supported by the fact that as soon as an electron donor to PS II, such as reduced P-phenylenediamine (PD) is added to the tris-treated chloroplasts, photooxidation of Cytb559 is sharply diminished. Further addition of Fd and  $NADP^+$  to the PD treatment restored the photooxidation.

prepared with the French press, showed absorbance changes ascribable to the oxidation of Cytb559. This oxidation was inhibited by DCMU and sensitized by PS II. However, this oxidation was too slow to account for the observed rates of Hill reaction. The author suggests that this Cytb559 does not function in the main pathway of electron transport.

4) Removal of plastocyanin from tris-treated chloroplasts by sonication impaired their capacity to photooxidize Cytb559 [179]. The addition of plastocyanin to the sonicated chloroplasts restored their capacity of photooxidation of Cytb559 with 664 nm (both system I and II) light.

Das and Govindjee [89] observed that at low pH (pH 4.0) aerobic sonication of Chlorella caused a preferential loss of a longwave form of chlorophyll which is weakly fluorescent at room temperature and highly fluorescent, with an emission maximum at 723 nm, at 77°K. Brody et al. [180] observed that the F735/F685 ratio, in particles obtained by sonication and differential centrifugation, decreases with the decrease of particle size. However, Boardman and Thorne [181] could not establish such a correlation; their spectra did not change upon decrease in size, confirming the earlier work of Butler and Baker [182] who observed that the shape of the emission spectrum does not change with the change in the size of the particles. (However, it is possible that the samples were too thick, and reabsorption of fluorescence was a problem in all their samples.) If sonication of chloroplasts causes preferential loss of longwavelength form of chlorophyll a, as was shown in Chlorella [89] and in particles obtained by sonication and differential centrifugation [180] in addition to causing loss of plastocyanin then it is possible to explain (partially) the results obtained by Arnon's group without need to propose two PS II light reactions.

In all experiments reported by Knaff and Arnon [166] on Cytb559 photooxidation by PS II, light of 664 nm wavelength is assumed to be exciting PS II only. This is incorrect as 664 nm excites both system I and system II almost equally. Action spectra of PS I and PS II, reported by several groups (see Govindjee [183]; Myers and Graham [184]) show that in green plants and green algae any exciting wavelength of light below 700 nm would not excite PS II alone. However, preferential excitation of PS I in green plants is possible by light of wavelengths above 700 nm. At 664 nm, PS II is only 12% more preferentially excited than PS I (calculated from action spectra of PS I and PS II reported by Joliot et al. [170]).

These above mentioned ideas considered together with the possible preferential destruction of the long wavelength form of Chl a during sonication could account for an action spectrum of photooxidation of Cytb559 which appears to match that of PS II.

Knaff and Arnon [179] present the following results on the role of plastocyanin (PC) in Cytb559 photooxidation to support their scheme. The removal of PC by sonication from tris-treated chloroplasts has little effect on the photooxidation of Cytf but seriously impairs the photooxidation of Cytb559 by 665 nm. However, no photooxidation of Cytb559 occurred in PS I (715 nm) light even when PC was added.

With this background in mind I checked the absorption and fluorescence characteristics of tris-treated, tris-treated and sonicated, and control chloroplasts to see whether these treatments actually caused any changes in the concentration and characteristics of the forms of the absorbing pigments.

Several preliminary experiments showed that no detectable changes in the absorption spectrum of the tris-treated chloroplasts were obtained, irrespective of the time of treatment. Hence in the results presented here, only data for tris-treated and sonicated particles are compared with normal chloroplasts. Although tris-treatment did not have an effect on absorption, sonicated chloroplasts could be used. However, I used tris-treated and sonicated particles in order to be consistent with the procedure of Knaff and Arnon [179].

## 2. Absorption Spectra of Tris-Treated and Sonicated Chloroplasts at 2980K

In Figure 18 are presented the absorption spectra between 610 - 750 nm of suspensions of untreated chloroplast fragments and tris-treated sonicated chloroplast fragments ( $T_{20}S_2$ ). In comparison with untreated fragments,  $T_{20}S_2$  fragments show a 2 nm shift of the red band towards shorter wavelengths. In aerobic sonicates of Chlorella, Das and Govindjee [89] observed a larger shift (5 nm) of the red band towards short wavelengths in comparison to non-sonicated Chlorella suspensions. In order to observe the differences in absorption of the two samples, the two curves were normalized at 670 nm and 715 nm relative to 670 nm is observed in  $T_{20}S_2$  chloroplasts compared to untreated chloroplasts. However, this is accompanied by an increase in absorption between 678 nm and 670 nm in  $T_{20}S_2$  compared to untreated chloroplasts. The cross-over of the two curves seems to be true because it was observed in several samples prepared on separate days.

The differences in absorption characteristics of  $T_{20}S_2$  and untreated chloroplasts are further confirmed by measuring their absorption difference spectrum as described in Chapter II (insert, top left of Figure 18). This

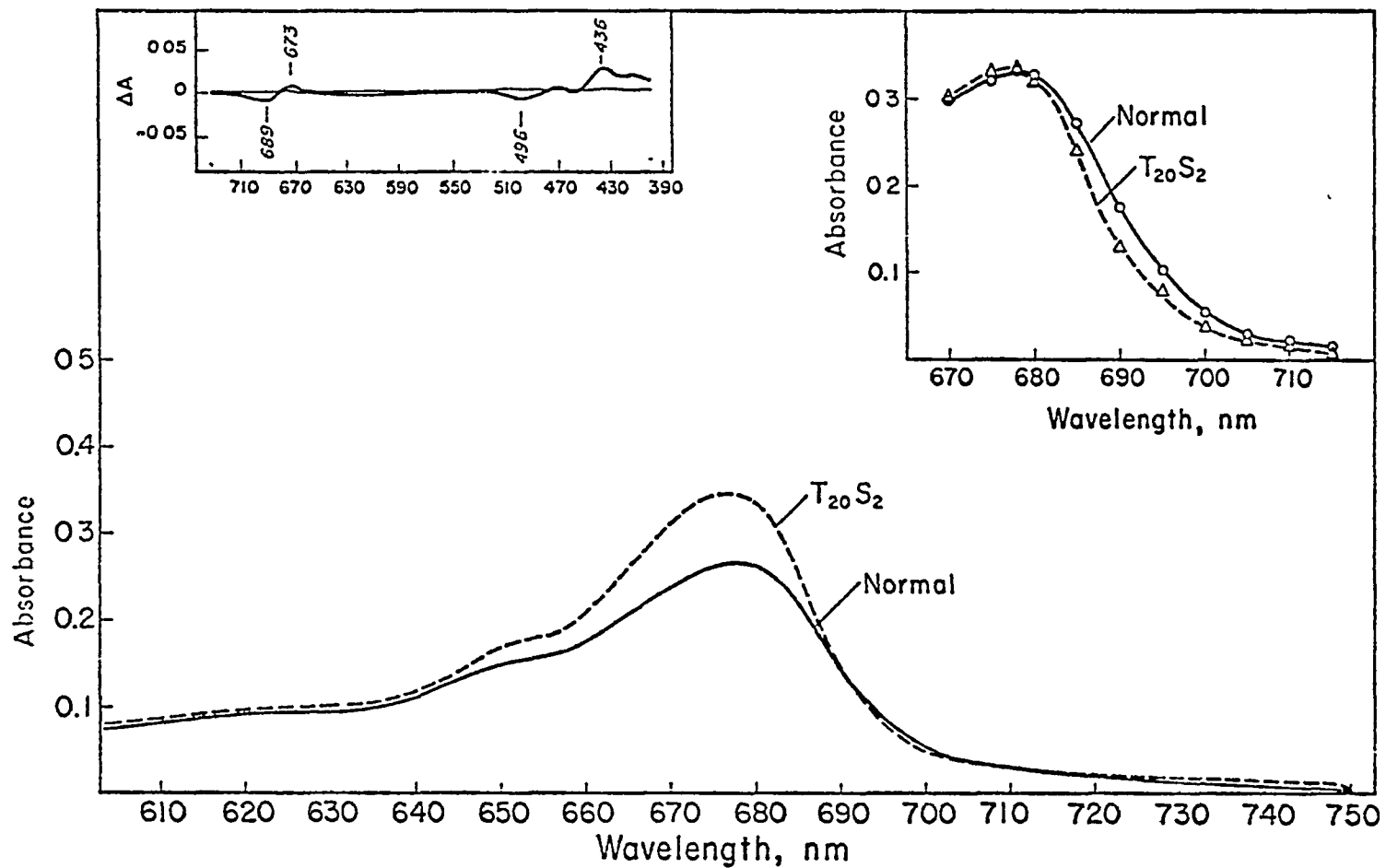


Figure 18. Room temperature absorption spectra of  $T_{20}S_2$  and untreated chloroplast suspensions. Top right insert, same spectra normalized at 678 nm. Top left insert, difference absorption spectra of (untreated-  $T_{20}S_2$  chloroplast fragments (note the direction of increasing wavelength is from right to left).

shows that, in comparison to untreated chloroplasts,  $T_{20}S_2$  have decreased amounts of a longwave form of Chl a with a peak maximum at 689 nm and increased amounts of Chl a 670 relative to other forms of chlorophyll. With this decrease in Chl a 690 relative to Chl a 670, there is a concomitant decrease in absorption at 496 nm relative to absorption at 530 nm. Since at 496 nm the main absorbing pigments are carotenoids therefore, sonication seems to lower the amounts of carotenoids relative to other pigments, and of Chl a 690 relative to Chl a 670. This result agrees with that of Das and Govindjee [89] obtained in aerobic sonicates of Chlorella.

### 3. Emission Spectra of Tris-Treated and Sonicated Chloroplasts at 77°K

From measurements of the difference absorption spectrum of both chloroplast samples (Figure 18, insert--top right), it is suggested that the amount of Chl a 690 decreases relative to short wavelength forms of Chl a in  $T_{20}S_2$ . Chl a 690 is known to be weakly fluorescent at room temperature but it fluoresces strongly at low temperatures with bands at 720 nm in Chlorella and at 735 nm in isolated chloroplasts. In all likelihood, there is a shift of this band to 700-705 nm at 77°K. To test that there was preferential loss of longwavelength form Chl a, the emission spectra of both samples were measured at 77°K. Both curves showed typical three-banded structure characteristics of emission spectra of chloroplasts at 77°K. However, the two curves differed in the ratios of the heights of the bands. In  $T_{20}S_2$  the ratio of  $\frac{F_{735}}{F_{685}} = 0.8$  compared to 1.9 in untreated chloroplasts, thus confirming the difference observed in the absorption spectra of  $T_{20}S_2$  and untreated chloroplasts. There is also slight decrease of  $F_{696}$  relative to  $F_{685}$  in  $T_{20}S_2$  compared to untreated chloroplasts.



#### 4. Excitation Spectra of Chlorophyll a Fluorescence at 760 nm of Thick Suspensions of Tris-Treated and Sonicated $T_{20}S_2$ Chloroplasts at 77°K

The change observed in the absorption spectrum of  $T_{20}S_2$  chloroplasts at long wavelengths is observed more clearly in the fluorescence excitation spectrum of Chl a measured at long wavelengths. Since absorption between 680 nm and 720 nm relative to absorption at 670 nm is lower in  $T_{20}S_2$  than in untreated chloroplasts, one would expect to observe a decrease in the excitation band of F760 excited by 705 nm in the former compared to the latter.

To observe the excitation band at 705 nm, thick samples were used (see Chapter IV, pp. 96 and 97). Results of fluorescence excitation measured at 760 nm at 77°K, are presented for both chloroplast samples in Figure 19. Because of the use of thick suspensions, the two samples showed equal amounts of Chl a fluorescence excited by any wavelength lower than 680 nm. However, the intensity of Chl a fluorescence excited at 705 nm differs greatly in both samples. The ratio of  $F_{760}^{705} / F_{760}^{680}$  in untreated chloroplasts is 1.17 compared to 0.89 in  $T_{20}S_2$  chloroplasts. This result is consistent with that of the absorption spectra at room temperature, and emission spectra at 77°K, in that preferential loss of Chl a 690-700 is observed in  $T_{20}S_2$ . Thus, it is likely that Arnon and coworker's system II a may simply be system I without the Chl a 670-700 that makes system I look like system II.

#### 5. Attempts to Study an Alternate Model of Photosynthesis

The series model (Figure 2) which is discussed in Chapter I, has influenced the thinking of most researchers in photosynthesis since all

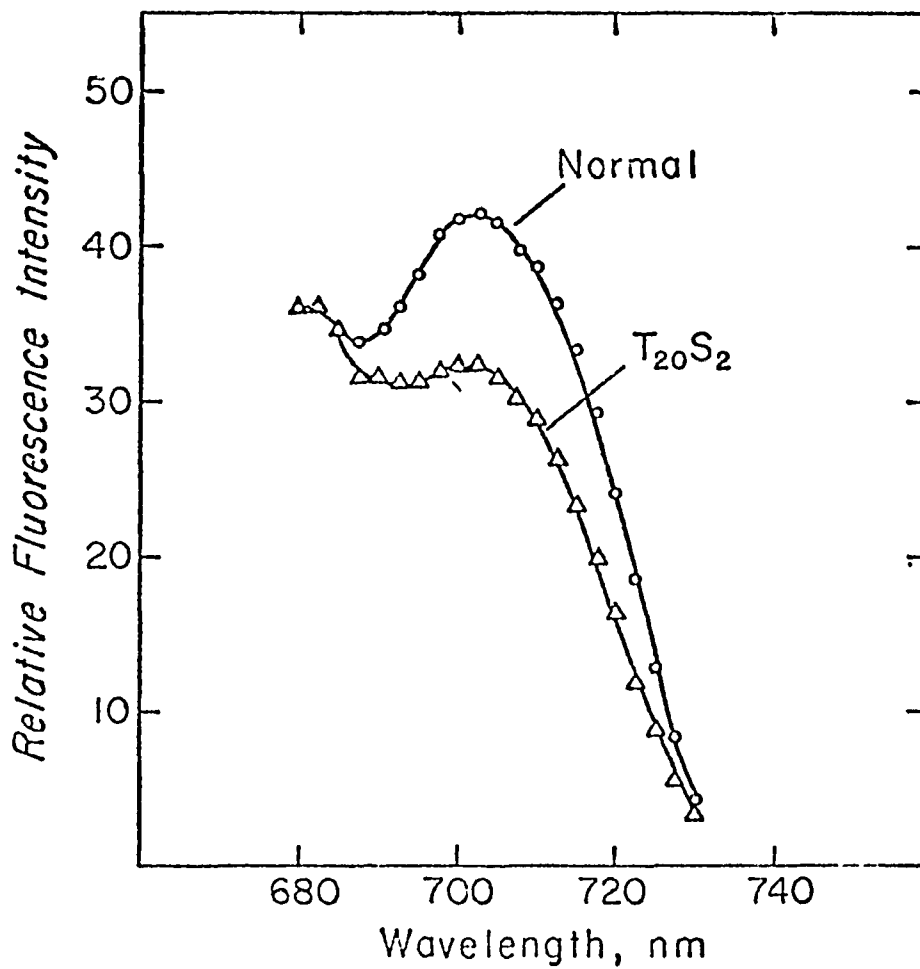


Figure 19. Excitation spectra of F760 at 77°K of thick samples (100% absorption at 680 nm), of normal and T<sub>20</sub>S<sub>2</sub>. Conditions as in Figure 15.

results obtained fit nicely into this scheme. Evidence for the mechanism of cooperation of the light reactions first came from spectrophotometric data presented on cytochrome *f* by Duysens et al. [185] and on P700 by Kok and Gott [186]. However, energy conserving processes have been found to be driven by light in the absence of oxygen evolution [187]. These processes have been shown to be sensitized mainly by far red light. It seems, therefore, that PS I has a role in energy conservation in addition to being part of the oxygen producing mechanism.

Several alternative models to the series scheme have been proposed [187-189]. (For a discussion of other models not cited here, see Clayton [190].) A model which does not postulate a direct interaction between the two photosystems, and which considers the supply of energy as the sole function of PS I, has been proposed by Hoch and Owens [187] and Govindjee et al. [188]. Such a model provides more direct association between photosynthesis and respiration than other schemes (see [191]. Govindjee et al. [188] presented their model to explain G. Bedell's (unpublished) experiments on the absence of enhancement in cultures of Chlorella vulgaris, which were grown under high light intensities and which showed high respiration rates. This model also explained the changes observed in the time course of fluorescence observed with far red preillumination.

Hoch and Owens [187] proposed their model on basis of experimental data obtained from studies on Anacystis nidulans. Their results showed that (1): system I light suppressed oxygen uptake while system II light increased oxygen uptake and (2) a combination of beams of PS I and PS II which increased oxygen production, decreased oxygen uptake. These results indicate that both enhancement effects and respiratory suppression are

## GOVINDJEE ET AL. (1966)

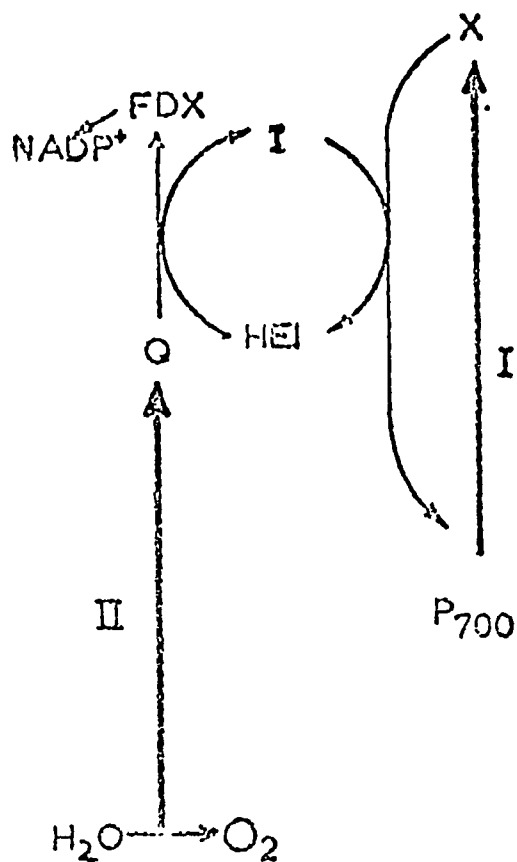


Figure 20. A model of photosynthesis proposed by Govindjee *et al.* [188]. Q<sup>-</sup>, primary reductant made in light reaction II; FDX, ferredoxin; X, primary acceptor of electron in photoact 1; HEI, high energy intermediate. The arrow from Q to FDX represents the reversed electron flow aided by HEI.

and Govindjee et al. [188]. These models could account for the presence and absence of enhancement of  $\text{NADP}^+$  reduction (Sun and Sauer [170] without need of proposing three light reactions as suggested by Knaff and Arnon [49]).

Rurainski et al. [194] utilized the technique of steady state relaxation spectrophotometry to measure the rates of turnover of P700 in isolated chloroplasts. The rates of  $\text{NADP}^+$  reduction was also measured in these samples. When isolated pea chloroplasts were suspended in 50mM tris buffer pH 7.8, the quantum yields of  $\text{NADP}^+$  reduction and of P700 turnover responded differently to increasing concentrations of  $\text{Mg}^{++}$ . When 50mM Tricine buffer (pH 8.0) was replaced by tris buffer, the lack of one-to-one correspondence between  $\text{NADP}^+$  reduction and P700 turnover was observed even in the absence of  $\text{Mg}^{++}$ .

In light of these results, I considered it important to test some of the results which are predicted if a scheme similar to the one proposed earlier [187,188] is operating in photosynthesis under certain physiological conditions of the plant. As a preliminary study to evaluate this model, I used an in vitro approach which involved a test for the ability of isolated system II particles to reduce  $\text{NADP}^+$  in the presence of a high energy compound, for example ATP. This is an obvious experiment if such a model operates in vitro. Difficulties of obtaining positive results in using this approach have been realized before experiments started. Nonetheless, these experiments were performed to check if the operation of such a model in vitro systems could be established.

In Table 10 is presented the ratios of Chl a/b, rates of DCPIP reduction and of  $\text{NADP}^+$  photoreduction in isolated chloroplasts, 1-2G

Table 10

Chl a/b Ratios and Photochemical Activities of Chloroplasts  
and Sub-Chloroplasts Fractions of Spinach

Fraction	Chl a/b	DCPIP reduced μmoles/mg Chl/hr		NADP <sup>+</sup> reduced μmoles/mg Chl/hr
		H <sub>2</sub> O donor	DPC donor	H <sub>2</sub> O → NADP <sup>+</sup>
G <sub>0</sub> (Chloroplasts)	2.8	312	-	75
1.2 G	3.1	-	-	-
1.2 GT	2.0-1.9	10	132	0

Reaction mixture for DCPIP reduction is as given in the legend of Figure 5. DPC, 1mM. For NADP photoreduction, the reaction mixture contained in 3 ml the following in μmoles: Tris-HCl (pH 7.2), 50; NADP, 5; PPNR, 1.2mg protein; 90μg Chlorophyll; DPC, 3.

(12,000 x g fraction from Digitonin treated chloroplasts) and 1-2GT (fraction 1-2G followed by TritonX-100 treatment) fractions. 1-2GT fraction has been used in these experiments as PS II particles. The photochemical activities of these particles are low compared to the chloroplast fractions. However, higher rates were obtained when DPC was used as an electron donor. No photoreduction of NADP<sup>+</sup> was observed in 1-2GT fraction. Isolated chloroplasts showed rates of NADP<sup>+</sup> photoreduction equivalent to 75 μmoles/mg Chl/hr. The main experiment which is involved in this study required a test for the ability of 1-2GT fraction to photo-reduce NADP<sup>+</sup> after addition of various concentrations of ATP.

Table 11  
Photoreduction of  $\text{NADP}^+$  in 1.2GT (PS II) Particles $\pm$  ATP

Addition	$\text{NADP}^+$ Photoreduction $\mu\text{moles/mg Chl/hr}$	
	$G_o$	1.2GT(PS II)
*Reaction Mix.	75	0
+0.5 mM ATP	71	0
+1.5 mM ATP	69	0

\*Reaction mixture is as in Table 10 for the photoreduction of  $\text{NADP}^+$  in presence of 1mM DPC.

In Table 11 is presented the results obtained from such an experiment. These particles (1-2GT) showed no ability to photoreduce  $\text{NADP}^+$  even in the presence of 0.5-1.5  $\mu\text{moles}$  of ATP. In my opinion, this negative result does not disprove the operation of such a model in vivo, but it probably rules out its function in vitro systems although there is no way to add HEI to the system. At the present, in spite of the inability to confirm the operation of such a model using PS II particles, one could envision the following to operate in whole Chloroplasts: that there is probably a control mechanism which switches PS I on and off depending on the redox state of Q (the primary electron acceptor of PS II). Cramer and Butler [195] have shown that Q has two redox states. Recently Joliot and Joliot [196] have obtained kinetic data which can be explained only if one assumes that two forms of Q exist in chloroplasts. Earlier, R. Govindjee et al. [197] interpreted their results on lyophylized maize chloroplasts in terms

that, indeed, a preferential loss of longwavelength forms of Chl a relative to other forms of Chl a in  $T_{20}S_2$  particles. Thus, it is likely that system IIa (Figure 3A) may simply be system I without the Chl a690-700 that makes system I look like system II.

In this chapter also an attempt was made to examine the hypothesis presented by Hoch and Owens [187] and Govindjee et al. [188] for the photo-reduction of  $NADP^+$  by PS II with exogenous supply of ATP. Particles of PS II were used to measure their ability to photoreduce  $NADP^+$  with addition of 1.5 and 0.5mM ATP as high energy intermediate. Although results of these tests were negative, the in vivo existence of a model of photosynthesis like the one proposed earlier [187,188] could not be ruled out. My results indicate that in vitro operation of such a model is hard to demonstrate as there is no way to add HEI to the system.



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