PHOTOPHOSPHORYLATION IN INTACT ALGAE

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THESIS

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It can be concluded on the basis of the data presented in this thesis that both the oligomycin treated (1.2×10^{-5} M) green alga Chlorella and the untreated blue-green algae Anacystis and Synechococcus show the presence of cyclic photophosphorylation under conditions in which the non-cyclic electron flow from photosystem II to photosystem I is blocked by the presence of 10^{-5} M 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). In Chlorella this was additionally supported by the results of similar experiments done in the absence of the terminal electron acceptor for the Calvin-Benson-Bassham cycle, carbon dioxide. Additionally, the light curve obtained in the presence of DCMU showed that in Chlorella, cyclic photophosphorylation saturates at about 1×10^{3} ergs cm^{-2} sec^{-1} and non-cyclic photophosphorylation (in the absence of DCMU) at about 1×10^{4} ergs cm^{-2} sec^{-1} red light (> 640 nm).

The level of cyclic photophosphorylation _in vivo_ in Chlorella seems to be primarily dependent on the previous growth conditions, varying from an average of less than 20 percent of the total photophosphorylation in regularly grown (moderate light) to 50 percent in cells exposed to very high white light intensities for the 24 hour period immediately preceeding the experiments. These high light exposed cells, also characteristically show a large dark respiration (oxygen uptake) level and an absence of the Emerson enhancement effect.

The addition of the photosystem I electron acceptor methylviologen (MV) to Chlorella cells treated only with oligomycin and to (untreated) Synechococcus cells indicates that non-cyclic photophosphorylation is the favored pathway in Chlorella, whereas cyclic photophosphorylation appears to be favored in the blue-green algae. In Chlorella this fact was further supported by the addition of the electron donors reduced dichlorophenol indophenol ($DCPIPH_2$) and reduced phenazine methosulfate ($PMSH_2$) in the presence of DCMU. In the presence or absence of MV the results showed that these two donors probably feed non-cyclically _in vivo_. Also in Chlorella, the addition of the cytoplasmic protein inhibitor cycloheximide in the presence of DCMU slightly stimulated the cyclic photophosphorylation level observed in the presence of DCMU alone. This would support the idea that photosynthetically produced ATP can be used for other than just carbon fixation reactions and that it can pass through the chloroplast membrane. That the control of the ATP levels in Chlorella cells is mediated primarily by the utilization processes rather than the limitation of the availability of ADP is supported by the fact that the control ΔATP (light minus dark ATP) level could be stimulated by more than 100 percent by the addition of MV. Completely untreated (no oligomycin or other poisons) Chlorella always showed negative ΔATP levels for up to 10 minutes illumination regardless of the suspension absorbance at 678 nm.

In the presence of 10^{-5} M DCMU, cyclic photophosphorylation in both Chlorella and Anacystis could be completely uncoupled by the addition of the powerful uncoupler S_{13} (5-Cl, 3-_t_-butyl, 2'-Cl, 4'-NO_2-salicylanilide, 10^{-6} M).

DEDICATION

This Thesis is Dedicated to the Memory

of

Therese Nicole Bedell

Born February 9, 1970
Died June 9, 1971

Her flashing brown eyes, dimpled smile and pats on my back as she hugged me and said, "Da, da," shall always be remembered and cherished.

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I. INTRODUCTION

A. General

Presently, most basic research on the photosynthetic processes is concerned with learning the mechanisms by which light energy is captured and transformed into the chemical energy sources, ATP (adenosine triphosphate) and $NADPH_2$ (nicotinamide adenine dinucleotide phosphate, reduced form), which are required for the photosynthetic CO_2 fixation reactions. The photosynthetic reactions must not only produce these chemicals, but they must be produced in sufficient quantity to fulfill the utilization requirements. The Calvin-Benson-Bassham cycle for the photosynthetic CO_2 fixation reactions occurring in most plants, requires a minimum of 3 ATP molecules and 2 $NADPH_2$ molecules to reduce one CO_2 molecule (1); the C_4 plants which use the Hatch-Slack-Johnson CO_2 fixation pathway require an additional ATP molecule (2). Furthermore, it has been shown that a minimum of 3 additional ATP molecules are required to incorporate already fixed carbon into cell material (3, 4). If to the preceding information we add the fact that many investigators find that only one ATP molecule is formed during the reduction of one $NADP^+$ molecule by non-cyclic photosynthetic electron transport (the P/e_2 ratio being one) (5, 6, 7), then an understanding of the origin of the ATP that is utilized *in vivo* in the light by photosynthetic CO_2 fixation and other ATP utilizing reactions becomes extremely important. A large amount of ATP is required by plant cells.

Although the presence of ATP was first observed in photosynthetic organisms in extracts from Chlorella cell suspensions in the early 1950's

(8, 9), there are very few publications on the direct measurement of ATP from whole plant cells (10, 11, 12, 13, 14, 15).

Most measurements of photophosphorylation were made on chloroplasts and bacterial chromatophores (16, 17, see review in 5). The results of these measurements have permitted photophosphorylation to be distinguished into three basic types: cyclic, pseudo-cyclic and non-cyclic.

The evidence for two light reactions in green plant photosynthesis has been thoroughly reviewed and elegantly discussed in terms of the Hill and Bendall scheme for photosynthesis (18, 19). The modified Hill and Bendall scheme (Figure 1) has been developed into the favored working model for most current photosynthesis research (19, 20). Alternative models have been proposed for the photosynthetic electron transport pathways in green plants and bacteria (21, 22, 23). These, too, have been recently reviewed (23, 24, 25). The principle purpose of these schemes has been to explain the consistent and conflicting data that have accumulated concerning the various oxidation and reduction reactions which occur during photosynthesis. We will not concern ourselves with these reactions.

In terms of the modified Hill and Bendall scheme for photosynthesis (Figure 1), non-cyclic photophosphorylation is associated with the evolution of oxygen and the reduction of $NADP^+$. The actual coupling site(s) are thought to be somewhere along the electron transport chain that connects photosystem II to photosystem I. The number and position of the site(s) are under dispute. The fact that many investigators have observed the P/e_2 ratio for non-cyclic photophosphorylation to be one has often been accepted as evidence that only one coupling site exists on this pathway (5, 6, 7). However, many other investigators have observed higher ratios (26, 27, 28, 29, 30, 31), with at least two reports showing that isolated chloroplasts can be induced to produce different P/e_2 ratios, in excess of

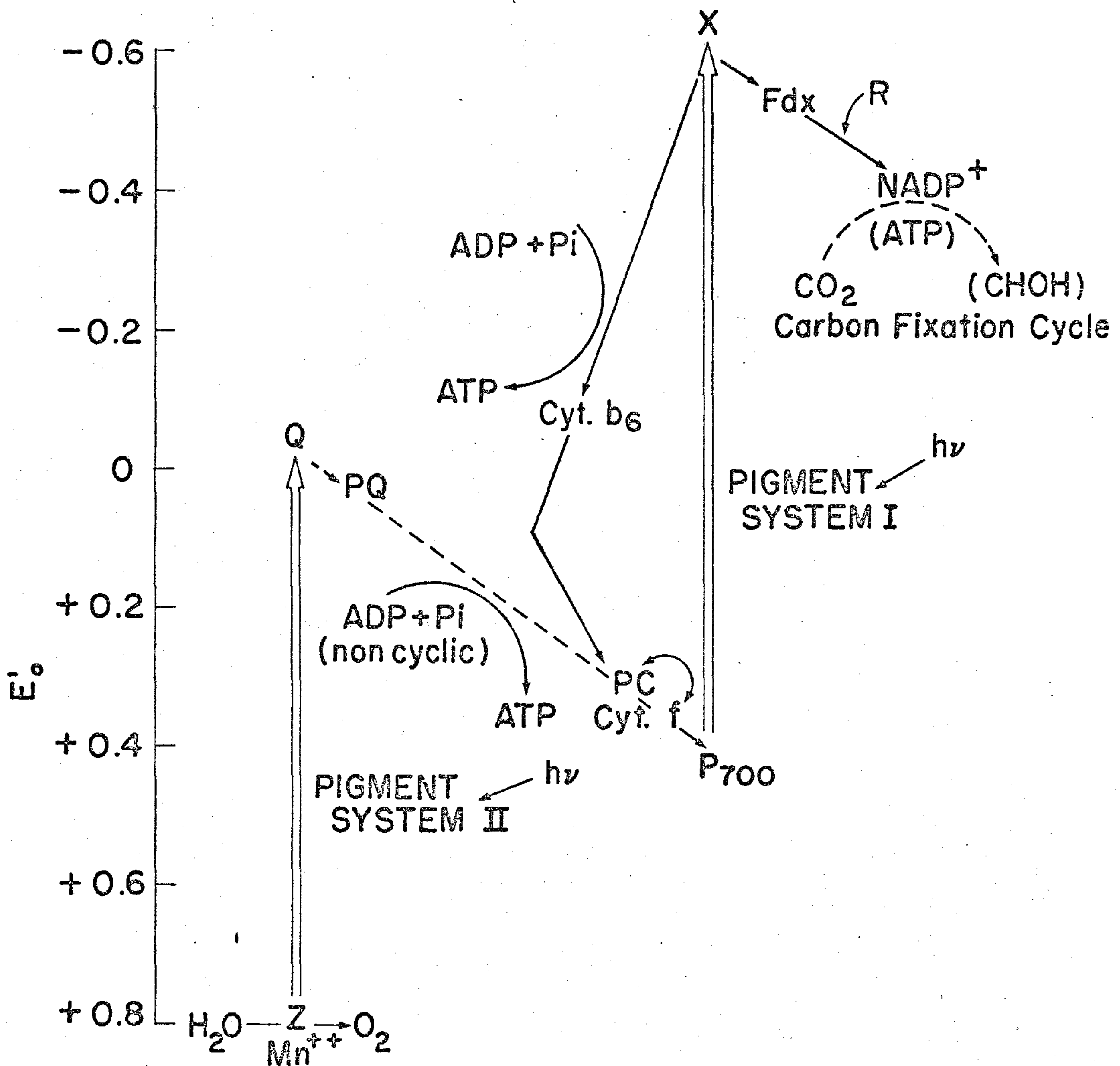


Figure 1. Modified Hill and Bendall scheme for photosynthetic electron transport in higher plants. Z and Q are the unknown primary electron donor and acceptor for photosystem II, respectively. P700 is the photochemical reaction center of photosystem I and X is its unknown primary electron acceptor. Refer to text for a description of the other pertinent symbols.

one, depending on the experimental conditions (30, 32). The only thing that seems certain about non-cyclic photophosphorylation is that the coupling site(s) must precede cytochrome f, because it was clearly shown by the classical "cross-over" approach that the addition of phosphorylation reagents or uncouplers to chloroplasts under conditions where they were limiting the electron flow in the light, allowed cytochrome f to be reduced (33).

Pseudo-cyclic photophosphorylation involves non-cyclic electron transport to ferredoxin (Fdx) and then directly to oxygen. It looks like cyclic photophosphorylation because no oxygen is set free and no $NADP^+$ is reduced (34). It is generally believed to be a special type of photophosphorylation that occurs in isolated chloroplasts, but not in whole cells (27).

Cyclic photophosphorylation produces no oxygen evolution or $NADP^+$ reduction. The only measurable product is ATP and it is usually observed in isolated chloroplasts only after the addition of a cofactor, e.g., phenazine methosulfate (PMS) or vitamin K (6). The actual number and position of coupling sites for cyclic photophosphorylation are no more clear than they are for non-cyclic photophosphorylation. On the basis of the differential effects of uncouplers (35, 36, 37), the addition of electron donors (38, 28), cytochrome f reduction (33), and work on mutants (39), there is strong evidence that there are at least two coupling sites. However, no clear picture has developed as to whether cyclic photophosphorylation has separate coupling site(s) or whether it shares one or more coupling sites with non-cyclic photophosphorylation. If the cyclic electron flow entered the non-cyclic pathway near Q (see Figure 1), say at plastoquinone (PQ)

or cytochrome 559 (6, 40), then cyclic photophosphorylation could share the non-cyclic coupling site and the non-cyclic coupling site could still meet the criterion that its coupling site must precede cytochrome f (33). The only positive evidence against a shared coupling site has been from the work on Chlamydomonas mutants which lacked specific electron transport components. Fragments isolated from the chloroplasts of a cytochrome f-less mutant still possessed PMS mediated cyclic photophosphorylation activity and the photo-induced electron transfer from ascorbate + DCPIP to $NADP^+$, while those fragments isolated from a plastocyanin-less mutant did not. In addition, neither type of fragments could catalyze non-cyclic photophosphorylation (39). This was believed to be consistent with the concept of separate coupling sites for cyclic and non-cyclic photophorylation (5). However, such data may not be consistent with the concept that plastocyanin donates electrons to cytochrome f which donates to P700 (41).

The assumption that photophosphorylation studies in isolated chloroplasts may be extrapolated to _in vivo_ conditions may be in error. For example, isolated chloroplasts may have a capacity, both for demonstrating different photophosphorylation pathways and rates, that exceed what actually occurs _in vivo_.

In this thesis, we will primarily be concerned with what occurs _in vivo_ in the green and blue-green algae. Do both cyclic and non-cyclic photophosphorylation occur in these algae? Are both cyclic and non-cyclic photophosphorylation necessary _in vivo_? Is there a distinct difference in the photophosphorylation apparatus of the two types of organisms? During the course of finding the answers to the above questions, we may obtain insight regarding the relative amounts of these two types of photophos-

phorylation and possibly even some information regarding the location of the coupling sites.

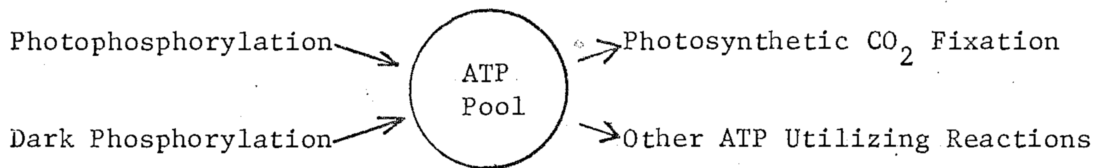
We were particularly interested in distinguishing the different types of photophosphorylation in these algae because of the current controversy regarding the relative importance of the different types of photophosphorylation. Many investigators believe that their data suggest that non-cyclic electron transport alone can supply all the ATP (as well as the $NADPH_2$) needed for CO_2 fixation to the level of a hexose, without any requirement for extra ATP (26, 27, 28, 30, 31). Angiosperms with the C_4-acid pathway of CO_2 fixation are possible exceptions (2). This view is supported by other investigators who maintain that cyclic photophosphorylation occurs at such low levels in whole cells that it cannot be obligatory for CO_2 fixation (42, 43, 44, see discussion in 45). These views are in contrast to those investigators who claim that cyclic photophosphorylation is mandatory for photosynthesis to occur _in vivo_ (34, 46), or that it occurs at very high levels _in vivo_ (47, 11).

Until this thesis, no _in vivo_ measurements have been made, involving the direct measurement of ATP levels using the luciferin-luciferase-ATP assay technique, on whole algal cells with any attempt to distinguish the different types of photophosphorylation. (During the preparation of this manuscript, three reports of this type of ATP measurement have appeared in the literature abstracts, but their detailed results were unavailable to this author (10, 11, 12). The reason the measurement of _in vivo_ ATP levels has played such a small role toward our understanding the photosynthetic processes may be due to the fact that it has been virtually impossible to obtain ATP values from extracts of illuminated algal cells that exceed the ATP values obtained from similar samples which were main-

tained in the dark (positive light minus dark ATP (ΔATP) levels (10, 48)). (The previous attempts to measure *in vivo* ATP levels using indirect assay techniques e.g., K^+ influx, ^{32}P incorporation, glucose uptake, etc., have provided valuable information and have not been ignored (40, 42, 43, 47, 49-60).) A recent investigation of the greening of bean leaves has been published in which positive ΔATP levels have been obtained using the luciferin-luciferase-ATP assay technique (15). The investigators mention that their study was feasible only because of the low drain on ATP during early stages of development. (We have not used synchronously grown algal cultures in our study, so we do not know if the same thing would be true for very young algal cells.)

B. The Problem of the Interpretation of Results

Although the luciferin-luciferase-ATP technique for the measurement of ATP levels is specific and the most sensitive of all such assays (48, 61, 62), the interpretation of the results of *in vivo* ATP levels is extremely complex. Since ATP has apparently achieved evolutionary predominance as the terminal chemical energy source for all living cells, many biochemical reactions have evolved to permit its synthesis and utilization. This implies that the measured *in vivo* ATP level can only reflect the level of the transitory ATP pool that exists as a result of the flux between the rate of synthesis and the rate of utilization. The complexity increases for photosynthetic organisms since an additional mechanism of both ATP synthesis and ATP utilization has been inserted into the cellular metabolism. As a result, the following diagram may be used to illustrate what we are measuring in photosynthetic cells:



Clearly, the size of the measured ATP pool, at the instant of extraction, will reflect the activity of the different processes and their interrelationships. To observe synthesis the utilization reactions must be slower than the synthesis reactions. If they are not naturally slower then they must be shut down or severely diminished by some other means. Furthermore, to distinguish between the two phosphorylation reactions, one of them must be shut down or diminished in such a manner as to leave the other synthetic reaction intact.

We will attempt to shut down or diminish the dark phosphorylation reactions (which we believe to be predominantly due to respiration) by the addition of respiratory poisons, anaerobiosis, or a combination of both. It is possible that the inhibition of respiration might have a temporary effect on the rate of other ATP utilizing reactions, even including photosynthetic CO_2 fixation. The reason for this is that in dark adapted cells, the ATP utilizing reactions are dependent on the ATP provided primarily by respiration and its accompanying electron flow. If respiration is inhibited in such a manner as to slow or stop electron flow, there may be a back-up of chemical intermediates and the likelihood of further inhibitions is increased due to feed-back control mechanisms. These inhibitions could possibly affect the initial turnover rate of the Calvin-Benson-Bassham cycle, at least for the first few seconds of illumination, until alternate biochemical pathways can start functioning. The question is, approximately how much illumination time is required to activate full ATP utilization in the absence of poisons and how much longer

will it take to have full ATP utilization (before alternative pathways are activated completely) in the presence of poisons? During this interval the rate of ATP synthesis in the light should exceed the utilization reactions, permitting the light minus the dark ATP levels to be positive. $^{14}CO_2$ labeling experiments in Chlorella, under high CO_2 tension, indicate that the labeling of chemical intermediates by photosynthetically fixed CO_2 is a relatively slow process. When labeled CO_2 is added in the light and the cells are killed and extracted at the end of 10 seconds, 70 percent of the fixed CO_2 can be found in 3-phosphoglyceric acid while the remaining 30 percent is mostly distributed between the hexose, heptose and sugar di-phosphates. After 40 seconds 70 percent of the label shows in sugar phosphates and 30 percent in the amino acids. The labeling rate of $^{14}CO_2$ into alanine by Chlorella exceeds the labeling rate for sucrose. Only after 1 to 2 minutes exposure to $^{14}CO_2$ in the light does 30 percent of the label show up in lipid-like substances (63). This would imply that the ATP utilization rate, in our cells and under our conditions, by processes other than the photosynthetic CO_2 fixation reactions, should be expected to be low unless there are a large number of light-triggered ATP utilization reactions (light activated ATP-ase reactions have been reported in the literature (64, 65)) and the large ATP utilization by these reactions precedes the full turnover capacity of the Calvin-Benson-Bassham cycle. However, we believe that such an immediate, large, light-activated ATP utilization is unlikely since we will have at least partially shut down respiration which, in turn, should create the back-up of biochemical intermediates, as we mentioned previously. We will, of course, make some measurements as a function of the time of illumination, but from the

forgoing discussion we can imagine that illumination periods of less than 30 seconds would be optimal. Most previous attempts at the measurement of in vivo photophosphorylation have involved at least several minutes illumination time (10, 13, 14, 15, 47, 66, 67). There is the possibility that as the time of illumination is increased the rate of oxidative phosphorylation in the light may be inhibited. It has been shown that the metabolic pathways change in the light with the respiratory precursors being decreased (68). The kinetics of these changes are slow, with the maximum change occurring after 4 to 8 minutes. This indicates that while mitochondrial respiration probably does not cease (57), the light versus the dark rate may be significantly altered. Such findings are also supported by the observations of other investigators that while the steady state levels of reduced pyridine nucleotide and ATP did not differ in the light or in the dark, there seemed to be a large shift from NAD^+ to $NADP^+$ by the chloroplast when exposed to the light (69, 70). It should be re-emphasized that the changes are slow and are believed to be mediated by the photoproduced ATP which suppresses the oxidative phosphorylation (45). We normally would not expect these latter changes to take place in cells in which oxidative phosphorylation is already inhibited.

The Calvin-Benson-Bassham cycle (and hence, photosynthetic ATP utilization will be inhibited by the addition of 10^{-5}M 3-(3,4-dichlorophenyl) -1,1-dimethylureau (DCMU) which has been shown by ourselves and others to completely block the non-cyclic electron flow between photosystem II and photosystem I at a point near Q, probably between Q and plastoquinone (see Figure 1) (71-74). As an additional check, non-cyclic electron transport will be inhibited by the removal of CO_2 which is the terminal electron acceptor of the Calvin cycle.

Corresponding oxygen measurements will be made. These will be our crucial experiments.

Supportive data will be provided by varying the light intensities, and the concentrations of various poisons, electron donors and acceptors and the addition of an uncoupler.

C. The Algae to be Used

The green alga Chlorella pyrenoidosa and the blue-green algae Anacystis nidulans and Synechococcus lividus (clone 75°C) were selected for this investigation. They are unicellular algae representing genera from both the eukaryotes and the prokaryotes. Chlorella and Anacystis will be used for most experiments. They were selected because many of their metabolic and photosynthetic processes have been extensively characterized, permitting our results to be more clearly compared and interpreted. These algae were also selected to permit the data to be compared for differences having possible evolutionary implications and to be tested for consistency regarding our efforts to determine the best working model for photosynthesis. It is possible that there is no "universal" working model for photosynthesis.

II. MATERIALS AND METHODS

A. Algal Culture and Sample Preparation

Chlorella and Anacystis were autotrophically grown in inorganic medium under continuous illumination as previously described (75). Synechococcus was also grown in an inorganic medium except for the inclusion of some nitriloacetic acid (NTA) [$N(CH_2CO_2H)$, M.W. 191.14] as a chelating agent (76). It is believed, though not actually proven, that blue-green algae do not metabolize this compound (77). Chlorella and Anacystis (Synechococcus) were continuously gassed with 5.0% and 0.5% CO_2 in air, respectively, to provide a carbon source and mixing of the cultures. The algae were grown in flasks in constant temperature water baths. Chlorella was grown at 20 ± 1°C and Anacystis at 34 ± 1°C. Chlorella and Anacystis were grown under relatively low intensity illumination with a single 60 watt and a 40 watt tungsten bulb (25 cm away from the flask), respectively. The Chlorella grown in this manner are referred to as regularly grown (RG). Cells placed in the dark for 24 hours prior to the experiment are referred to as dark adapted (DA). Chlorella cells exposed to two 100 watt tungsten bulbs, for 24 hours preceeding the experiment, are referred to as HLE (high-light exposed). Synechococcus was grown under high intensity of illumination: one 500 watt and one 150 watt tungsten bulb, 20 cm away from the flask. Synechococcus grows best under high intensity light at 65°C.

Samples for the experiments were taken from the Chlorella culture 3 to 6 days after inoculation; 5 to 7 days for Anacystis and Synechococcus. Absorption spectra were made initially to determine the percent absorption by the suspension at 678 nm. The cells were centrifuged for 10 minutes at

2000 x g to remove the growth medium, washed in buffer, and recentrifuged for 10 minutes at 2000 x g. Unless otherwise stated, the final pellet was resuspended in enough buffer to give the suspension an absorbance of 0.6 at the 678 nm band of chlorophyll a. Also, unless otherwise stated, the only suspension buffer for all experiments was 0.1 M carbonate-bicarbonate in water, pH 8.2. This buffer was selected because of its similarity to the Warburg buffer #9 used for oxygen measurements by manometry (21, 78). (Manometry measurements using this buffer compared favorably with the data obtained using the Warburg buffer #9.) This buffer also provided the optimum pH for ATP synthesis in Chlorella as has been determined by others (62, 79) and confirmed by us. Therefore, this buffer permits spectral, oxygen exchange, and ATP data to be directly compared. An absorption spectrum of the final cell suspension was taken and the suspension was placed in the dark, at room temperature (Synechococcus was maintained at 65°C) until ready for use. The cells were kept in the dark for one hour before the final dilution was made. To be certain that all cells were exposed to the light during the experimental illumination, suspensions were diluted to give 50 percent absorption at the 678 nm peak of chlorophyll a. All dilutions were made in very dim green light. After the dilutions were made, an additional 20 to 40 minutes were allowed to permit adequate penetration by the added chemicals in the dark. Anaerobic suspensions were gassed for 20 to 40 minutes in the dark with 100 percent argon.

B. Instrumentation

1. Absorption Measurements

The absorption spectra were measured with a recording spectrophotometer (Bausch and Lomb Co., Rochester, N.Y., model Spectronic 505) equipped

with an Ulbricht integrating sphere into which both the sample and the reference cuvettes are placed. The Barium sulfate (Eastman Kodak Co., Rochester, N.Y., white reflective paint #6080) coating on the interior of the sphere allows all the scattered light from the sample to reach the photodectector, eventually. The measuring beam of the instrument has a half-band width of 5 nm. Since chlorophyll a does not absorb beyond 740 nm, the baseline, determined by using the buffer alone, was raised slightly to match the sample spectrum at 740 nm. (The small apparent absorption at 740 nm is due to some residual scattering by the cell suspension despite the use of the integrating sphere.) The cuvettes had a 1 cm path length. The absorption measurements were not corrected for the "sieve effect" or for the "detour" effect (80, 81).

The total chlorophyll content was determined by extracting Chlorella with 100 percent methanol and Anacystis and Synechococcus with 80 percent acetone in water (82). The formula used to calculate the chlorophyll content of Chlorella was: Chlorophyll a + b $\mu g\ ml^{-1}$ = 25.5 x absorbance at 650 nm + 4.0 x absorbance at 665 nm. An extinction coefficient of 82 cm^2 $millimole^{-1}$ for a 1 cm pathlength measured at 663 nm was used to determine the chlorophyll concentration in Anacystis and Synechococcus. A direct proportionality between the absorbances of the cell suspensions (up to 0.6) at 678 nm and the amount of chlorophyll per ml of suspension was observed. The absorption spectra of the chlorophyll solutions were made in a recording spectrophotometer (Cary Instruments, Monrovia, Calif., model 14).

2. Oxygen Measurements

a. Manometry

The differential manometers (closed type), the experimental procedures and the technique have been thoroughly described previously (83). Pressure changes were determined every minute by measuring the meniscii of the manometer fluid with the aid of cathetometers. White and monochromatic (5 to 10 nm half band width) light were obtained from the Emerson-Lewis monochromator by turning the grating to 0 order and 1st order, respectively. Intensity measurements were made with a large surface bolometer (78).

b. Platinum Rate Electrode

The stationary platinum rate electrode is similar to that designed by Bannister and Vrooman (84). The instrument consists of a 2 mm x 12 mm, flat platinum (Pt) cathode which is embedded in a well 0.3 mm deep in a lucite plastic stage and a large Ag/AgCl reference anode. A cell suspension is placed on the Pt and covered with a tightly stretched dialysis membrane. Both electrodes are then immersed in an electrolyte solution, consisting of phosphate buffer (KH_2PO_4 + K_2HPO_4, 0.1 M, pH 8.0) and 0.01 M KCl, which was constantly stirred by a stream of 5 percent CO_2 in air. The Pt cathode was polarized with reference to the Ag/AgCl anode (freshly coated) by applying a negative patential of 0.55v to the cathode. The voltage (V) applied to the cathode was empirically determined as giving the plateau in the V versus I (current) curve in the absence of the algal cells. The sample was illuminated from the top by a ribbon filament tungsten bulb with the approximate heat, interference (Baird-Atomic, Inc., Cambridge, Mass.) and Corning (Corning Glass Works, Corning, N.Y.) or Schott (Schott Optical Glass, Inc., Duryea, Penn.) glass filters. The current from the

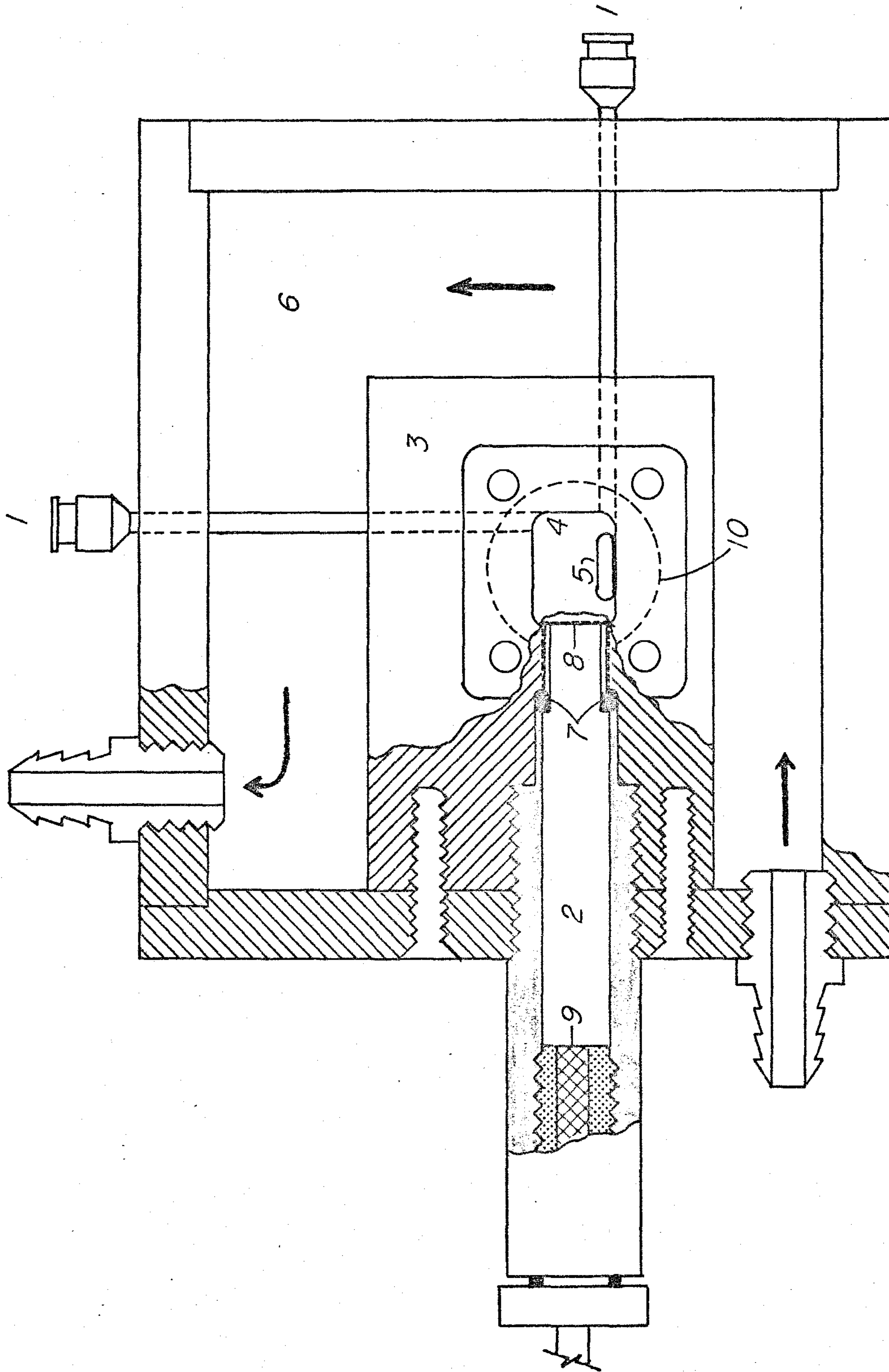
cathode is directly proportional to the rate at which oxygen is reduced to H_2O upon contact with the Pt cathode. Diffusion delays are minimal because the sample is in direct contact with the Pt. The rate of photosynthetic oxygen evolution is proportional to the difference between the electronic signals obtained in the presence and absence of light excitation, the latter being taken as the correction for respiratory oxygen uptake and the "dark current". The electrode signal is amplified by a 150A Keithley microvolt ammeter and recorded by a Heathkit Servo recorder (Heath Company, Benton Harbor, Michigan). Although the amount of current measured can be converted into μ equivalents of O_2 reduced at the anode, we present our results in relative units.

c. Clark-Type Concentration Electrode

The oxygen concentration of the algal samples was measured by using a teflon covered Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) with a model 53 monitor which contains the appropriate polarizing and amplifying circuitry. The signal from the monitor is fed directly to a Heathkit Servo recorder. The sample chamber and the electrode holder was specially designed and built by the author (Figure 2). It has the following unique features: a small stainless steel sample chamber with a total volume of 1.2 ml; two optical glass windows for simultaneously illuminating the sample with two lights; a 1 cm pathlength for the light; two #14 hypodermic needles, connected at right angles with the chamber to permit the change of sample, the addition of chemicals or the removal of air bubbles; magnetic stirring at very close proximity to the electrode; a temperature regulating water jacket; and a design that permits rapid disassembly for cleaning purposes. The electrode is separated from

Figure 2. Clark-type concentration electrode mounted in sample holder of new design, (side view). Not drawn to scale.

Key:
1. Hypodermic needles, #14 gauge.
2. Electrode.
3. Stainless steel bar.
4. Sample chamber, 1 cm pathlength.
5. Magnetic Stirring Bar.
6. Circulating water chamber, made of lucite.
7. O-Ring seal.
8. Teflon Membrane.
9. Connecting lead from electrode to electronics.
10. Two Pyrex optical glass windows over teflon seals and held down by stainless steel plates and four bolts (one window assembly on either side).


1
6
3
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4
10
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the magnetically stirred sample by the teflon membrane which covers the electrode. The instrument was calibrated with air saturated carbonate-bicarbonate buffer (0.1 M, pH 8.2) at the appropriate temperature. The sample was illuminated by an 8V Unitron Lamp (Frank E. Fryer Co., Chicago, Illinois) using the appropriate heat and Corning glass filters.

3. ATP Measurements

The adenosine triphosphate (ATP) content of the algal samples was determined with a luciferin-luciferase (firefly) enzyme assay technique that is sensitive to picogram (10^{-12} gm) amounts of ATP. The technique was similar to that devised by Lyman and DeVincenzo (61). Their technique was a refinement of the assay method originally worked out by McElroy (85) and Strehler and Totter (8). It is the most sensitive and the most specific assay for ATP known (48, 61, 62). 2.0 ml of cell suspension was given the proper actinic illumination or dark treatment for the specified time. The sample was quickly (<2 seconds) poured into 3.0 ml of boiling ($100^{o}C$) glycine buffer (2×10^{-2} M, NH_4^+ free, pH 7.7, from Calibiochem Corp., Los Angeles, Calif.) and boiled for exactly 15 minutes. It has been previously determined that 10 to 15 minutes boiling time has no appreciable effect on the ATP content of the extract at pH $\leq$ 8.0 (62). This was confirmed by comparing boiled ATP standard samples to an unboiled standard curve similar to Figure 3. It has been determined that the extraction of all soluble ATP from Chlorella is complete by that time (62, 86). To keep dilution errors to a minimum, volumetrically calibrated Pyrex (#8080, 15 ml) centrifuge tubes were used as extraction tubes. To decrease the possibility of errors due to "hanging drops" upon emptying the sample tubes, the mouth of thé sample tubes were brought into contact

with the surface of the boiling buffer contained in the large volumetric tube. This procedure greatly increased the reproducibility of results. At the end of 15 minutes the centrifuge tubes were placed in an ice bath to bring them quickly to room temperature or to $0^{o}C$, if necessary. It has been reported that nanomole amounts of ATP are stable for at least 16 hours at pH 7.4 and $0^{o}C$ (87). Extracts of ATP remained stable for at least 2 hours at room temperature. The amount of ATP in these extracts was usually in the nanomole range. Upon reaching room temperature, all the water which evaporated during the boiling was restored by adding enough glass distilled water by a syringe to bring the total volume of each tube to 5.0 ml. The tubes were then centrifuged for 10 minutes at 3000 x g to remove all cell debris. The volumes of the centrifuge tubes were checked twice and their supernatants, containing the extracted ATP, were emptied into clean test tubes. It should be noted that all glassware used in these experiments was carefully cleaned by hand to prevent any possibility of contaminating the ATP extract or adding ions which would reduce the activity of the firefly enzyme (8). (After washing and rinsing with de-ionized water, the glassware was rinsed in acid cleaning solution (concentrated H_2SO_4 plus saturated $K_2Cr_2O_7$) and finally rinsed several times with glass distilled water.)

The luciferin-luciferase enzyme was prepared by adding 5.0 ml of glass distilled water to 50 mg of buffered (0.05 M $KAsO_4$ and 0.02 M Mg SO_4, pH 7.4) firefly lantern extract (Sigma Chemical Co., St. Louis, Mo. FLE-50) at room temperature. After being allowed to dissolve and mix thoroughly for 20 minutes, the milky suspension was cleared by 30 minutes centrifugation at 10,000 x g in a cold room ($5^{o}C$).

For the ATP determination, 0.75 ml of each cell extract sample was transferred by pipette, to separate 1.0 cm pathlength glass cuvettes (Pyrocell Manufacturing Co., Westwood, N.J. Cat #6646). In turn, each cuvette was positioned in the sample holder of the measuring instrument.

The measuring instrument, which was designed and built by the author, but patterned after that described by other investigators (61), consists of sample holder which is separated from an EMI 9558 B photomultiplier (Electra Megadyne, Inc., New York, N.Y.) tube by a simple shutter with a 2.0 cm aperture. The front face of the sample cuvette is located 4.0 cm from the photomultiplier tube surface. A syringe holder is attached to the shutter housing and is adjusted to hold a gas-flushed syringe (Hamilton Co., Whittier, Calif., Cat #GF 1750 SNCH) so that the tip of the 2 inch, #18 gauge needle is 35 mm above the surface of the sample in the positioned cuvette. The gas port of the syringe barrel is located at 250 µl. The regulated high voltage power supply (Northeast Scientific Corp., Acton, Mass., model #RE 3002) for the photomultiplier is set at 1 KV. The signal from the photomultiplier is amplified by a 150 A Keithley microvoltmeter ammeter (Keithley Instrument Corp., Cleveland, Ohio) which is usually set to 100 nanoamp sensitivity. The signal was then passed through a 1 megohm potentiometer to a Heathkit Servo recorder which is operated at 250 mV sensitivity with a chart speed of 12 inches min^{-1}.

With the sample cuvette in position and the light shield in place, 0.25 ml of the cleared luciferin-luciferase enzyme mixture was drawn into the syringe (all air bubbles were eliminated) and the syringe was placed into its holder. Another light shield was placed over the syringe and the room lights were turned off. The recorder was turned on, the shutter to the photomultiplier opened and the switch to the solenoid (Skinner

Electric Valve Co., New Britain, Conn., model #C2DA1062) was turned on. The opening of the solenoid valve permits 100 percent oxygen gas, delivered at a constant pressure of 2 psi, to uniformly flush the enzyme from the syringe into the sample. The height of the initial signal is proportional to the total amount of ATP present in the sample. The amount of ATP contained in each sample was determined from an ATP standard curve which was prepared using the same technique, apparatus and buffer, but with samples containing known amounts of ATP (Figure 3). The residual ATP in 0.25 ml of firefly enzyme (50 mg ml^{-1}), upon injection into 0.75 ml of buffer, was observed to contain about 10 to 20 picomoles ml^{-1}. Since most of the data presented in this thesis was obtained by subtracting the ATP values obtained from dark maintained cells from ATP values obtained from light exposed cells, the amount of residual ATP in the firefly enzyme was neglected. In any case, it was usually 1000 times smaller than the usual signal and, thus negligible. (0.75 ml aliquots of cell extract from 2.0 ml of algal cells, having a final absorbance of 0.3 at 678 nm usually contain 10 to 100 nanomoles of ATP ml^{-1}.)

The source of illumination for the ATP measurements was a 1000 W tungsten lamp (General Electric Co., Cleveland, Ohio, Light-house type, no longer made) regulated by a Variac autotransformer (General Radio Corp., Concord, Mass., model W10MT3) that was connected to a constant voltage supply (Raytheon Manufacturing Co., Waltham, Mass., model VR5). All light was passed through two heat filters, a Corning glass C.S. 197 (90 percent transmission from 400 nm to 540 nm, dropping to 50 percent at 750 nm) and a Schott glass M978 (60 percent transmission from 380 nm to 540 nm, dropping to 0 percent at 900 nm). The light intensity was

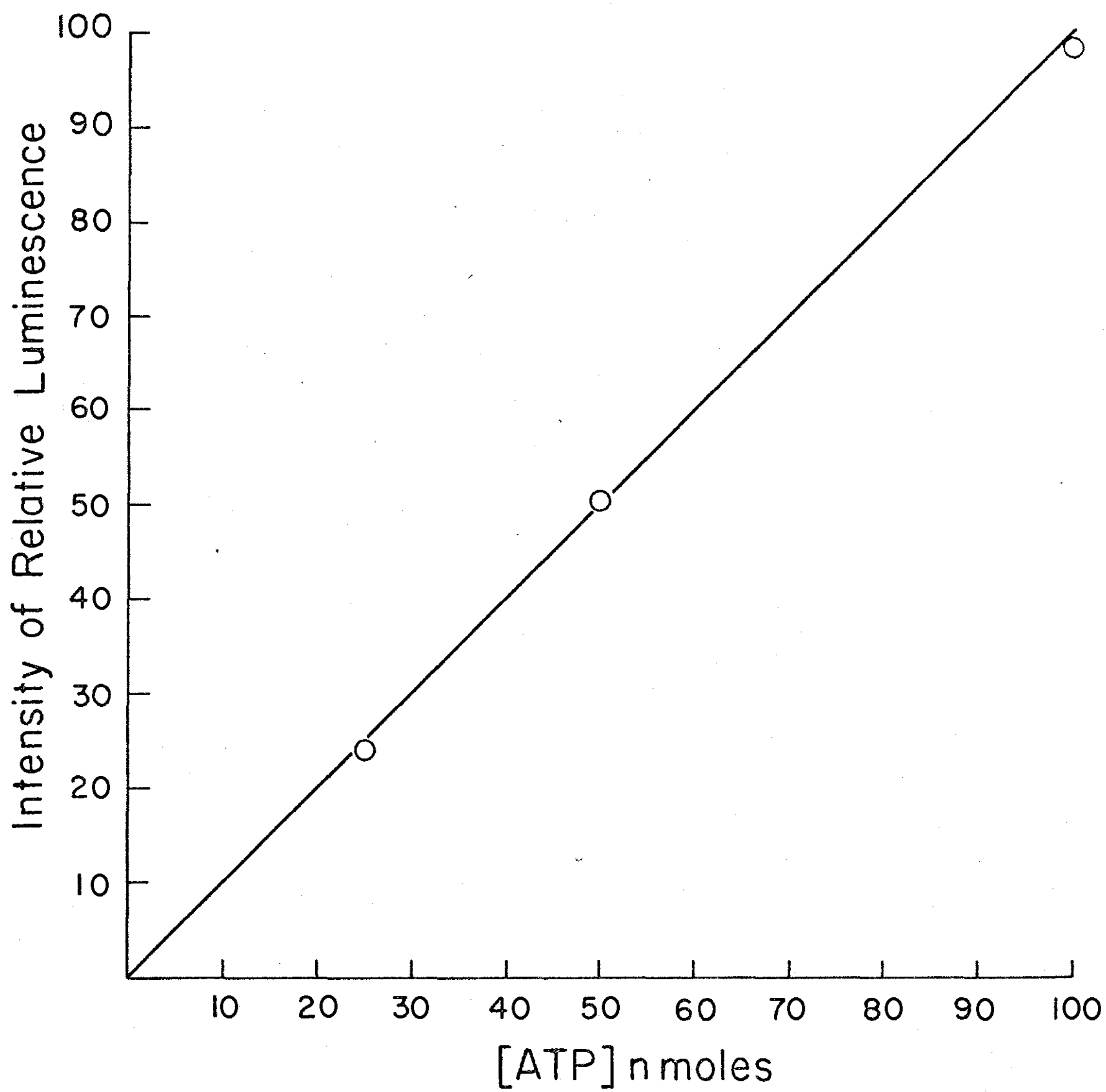


Figure 3. Standard curve for ATP.

regulated by the use of calibrated neutral density filters (Geratebauanstalt, Balzers, Furstentum, Liechtenstein). Corning glass filters (C.S. 2-64, 3-67, 4-72) and interference filters (Farrand Optical Co., New York, N.Y. or Baird-Atomic, Inc., Cambridge, Mass.) were used to obtain different wavelengths of the actinic light. The light intensities were measured with a Yellow Springs radiometer (Yellow Springs Instrument Co., Yellow Springs, Ohio, model 63). The white light emission (Figure 4) was measured with an Isco spectroradiometer (Instrumentation Specialities Co., Lincoln, Neb., model SRR).

A glass electrode pH meter (Beckman Instrument, S. Pasadena, Calif.) was used for all pH measurements.

Each sample of algal suspension was stirred vigorously in the dark prior to illumination to resuspend any cells which had settled. The sample tube (1 cm diameter) was placed in a large (6 x 9 x 18 inches) water bath. The focus of the actinic beam was at the center of the 1.0 cm diameter sample tube and the magnification was such that the filament covered the width of the tube. For measurements of ATP in Synechococcus at 65°C, the water bath was heated by a 450 W Cenco heater (Central Scientific Co., Chicago, Ill., model 16551-3) which was connected to a thermister controlled solid state constant temperature regulator designed and built by the author. The water was circulated through the bath with the use of a pulse tubing pump (Cole-Palmer Co., Chicago, Ill., model Masterflex 1017). With this system the bath temperature could be held to 65 ± 1°C. The same water bath system was used to regulate the temperature of the concentration electrode assembly for those measurements requiring 65°C temperatures by simply connecting the water jacket of the electrode to the pumped water flow circuit.

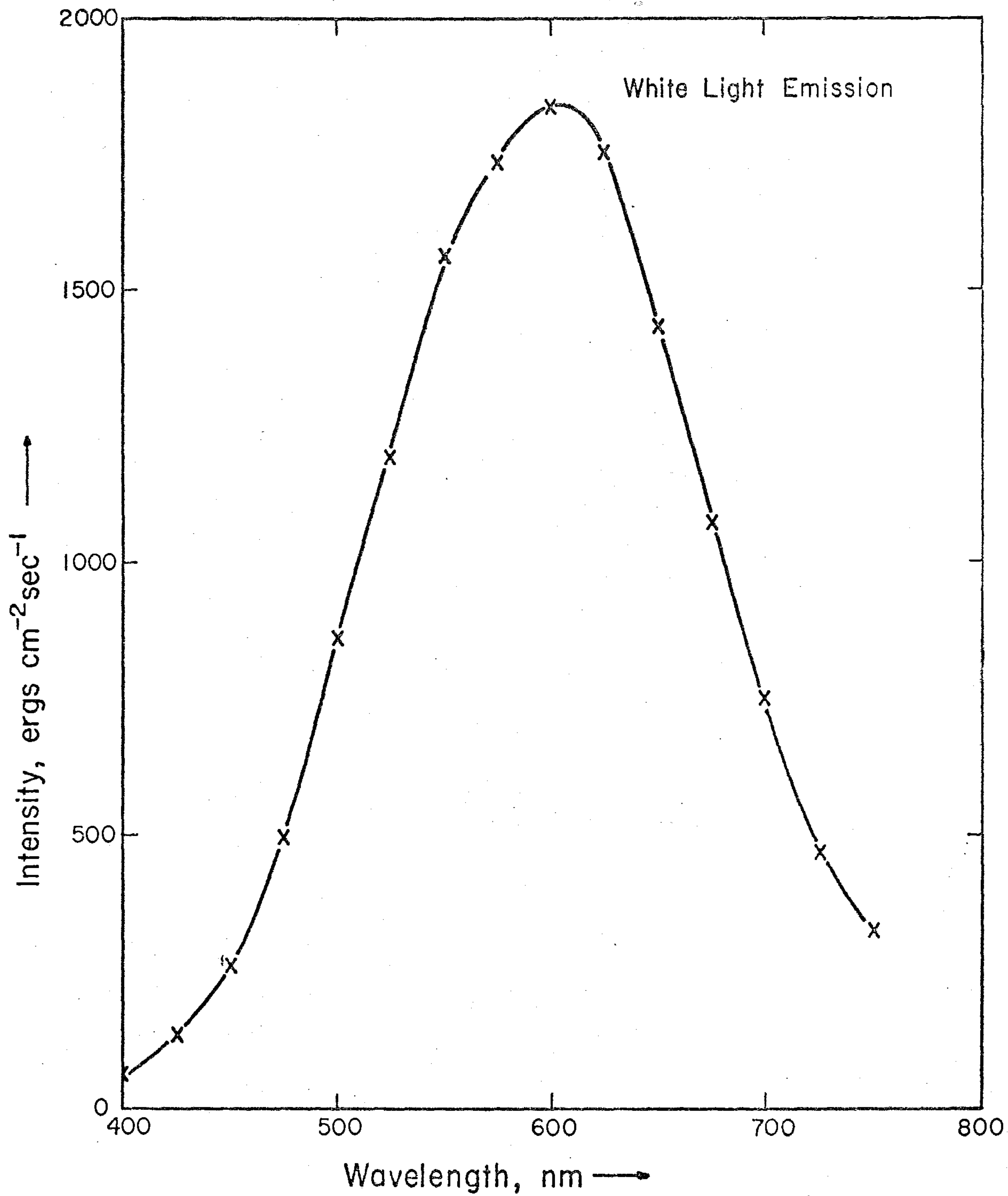


Figure 4. White light emission from a tungsten filament filtered by a Corning glass C.S. 197 and Schott glass M978 filter combination. Integrated intensity, 7 x 10^5 ergs cm^{-2} sec^{-1}.

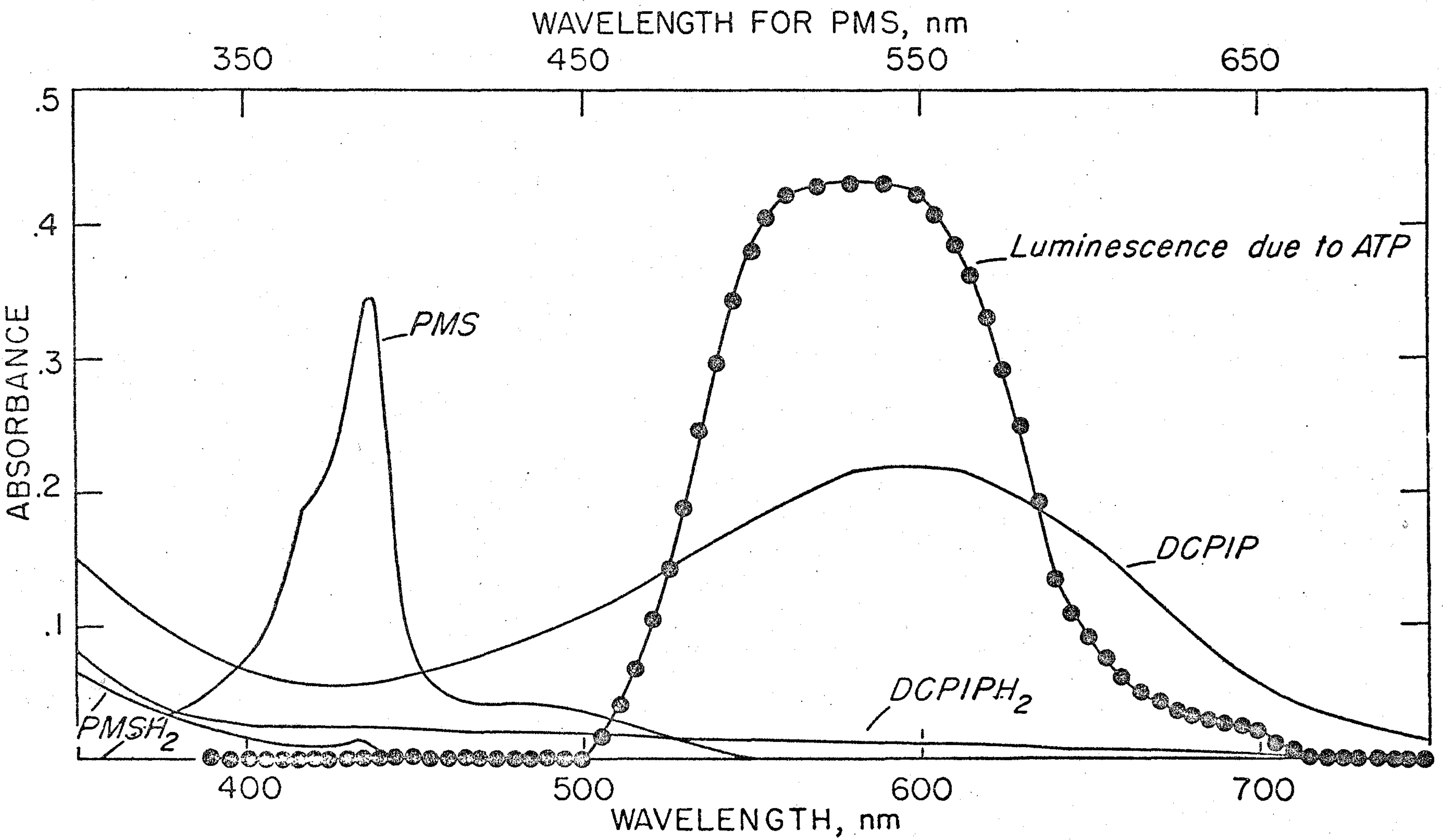
C. The Absorption Spectra for Oxidized and Reduced Dichlorophenol Indophenol and Phenazine Methosulfate and the Emission Spectrum for the Luciferin-Luciferase-ATP Reaction

The absorption spectra for oxidized and reduced dichlorophenol indophenol (DCPIP) and phenazine methosulfate (PMS) were compared to the luciferin-luciferase-ATP emission spectrum to determine if these compounds would interfere with the ATP measurements that would be done later. We wanted to examine the effect of various electron donors and acceptors on DCMU, 3-(3,4-dichlorophenol)-1.1-dimethylurea, poisoned Chlorella. This would be necessary to confirm the types and amounts of photophosphorylation *in vivo* in Chlorella. DCPIP and PMS were always in their reduced form during the ATP measurements to be presented later. Figure 5 shows that reduced DCPIP and PMS are almost colorless throughout the region of luciferin-luciferase-ATP emission (from 500 nm to 710 nm) at pH 8.2. Since the ATP values from dark maintained suspensions are subtracted from the ATP values measured after extraction from light exposed cells when the ΔATP values are determined, any absorption of the firefly enzyme-ATP emission due to these reduced compounds would be cancelled out by the subtraction. Therefore any real effect by these reduced dyes on reducing the intensity of the firefly enzyme-ATP emission would be negligible.

D. Presentation of Experimental Results

All ATP values are given as relative rather than absolute units. We could only measure the net ATP levels that existed in the cells at the time of extraction. Also, we had no way of measuring any bound ATP that remained in the cells and would not permit contact with the firefly enzyme. Of course, the measurement itself was of absolute amounts because

Figure 5. Absorption spectra of oxidized and reduced DCPIP and PMS and Emission spectrum for Luciferin-Luciferase-ATP mixture. Concentration PMS, 0.34 µmole ml^{-1}; ascorbate (PMS) 0.34 µmoles ml^{-1}; DCPIP, 1.0 µmoles ml^{-1}; ascorbate (DCPIP) 10 µmoles ml^{-1}. Luminescence due to ATP is in relative units. Sampler in carbonate-bicarbonate buffer (0.1 M, pH 8.2) at 25°C.



WAVELENGTH FOR PMS, nm
350
450
550
650
ABSORBANCE
.5
.4
.3
.2
.1
Luminescence due to ATP
PMS
DCPIP
$DCPIPH_2$
$PMSH_2$
400
500
600
700
WAVELENGTH, nm

ATP values were obtained using a standard curve as shown in Figure 3. The symbol ΔATP (light minus dark) will be used to represent the net phosphorylation values. (The reason the term ΔATP is used rather than the term photophosphorylation is simply for convenience. Also, as mentioned earlier, the ATP values we measure reflect only the ATP levels, thus, the ΔATP value is obtained with the understanding that all other ATP synthesizing or utilizing processes probably continue and may even be changing in rate in light.) However, due to the consistent differences we obtained between dark and light values, particularly the consistent dark levels, we believe that the positive ΔATP values reflect the relative increase in ATP levels due to photophosphorylation. Furthermore, because of differences between the rates of ATP formation and utilization, which were discussed in the introduction, and the changes observed as a result of our deliberate manipulation of the experimental conditions, we believe that our results will show that the ΔATP values can often be separated into two components, one representing non-cyclic photophosphorylation and the other cyclic.

For convenience, our oxygen measurements are presented in relative units. For the measurements of the Emerson enhancement effect, the Chlorella cells, grown for 3 to 6 days, (refer to section B) were transferred to the dark or high light intensity (HLE) for the 24 hours prior to the oxygen measurements. The cells from both flasks were quickly removed from their growth media, washed, and resuspended in enough buffer to give an absorbance of 0.4 at 680 nm. The dark (adapted) cells were measured last and were kept in the dark until placed into the manometer vessel. To ensure maximum enhancement (see reference 8), the intensity of the short-wave beam (650 nm) was chosen so that it alone provided five to seven times more photosynthesis

than the long-wave beam (714 nm) alone. The enhancement (E) was calculated according to the following equation, based on the assumption that it is the low efficiency of the far-red light which is enhanced,

$$E = \frac{R(S + L) - R(S)}{R(L)}$$

where R stands for the rate of oxygen evolution (Light - Dark per unit time), S for the short-wave light and L for the long-wave light.

Other details are given in the legends of the tables and figures.

III. EXPERIMENTAL RESULTS

A. Characteristics of Regularly Grown and High Light Exposed Chlorella Cell Suspensions

Several investigators, ourselves included, have reported the absence of the Emerson enhancement effect for oxygen evolution in algal cells (21, 88, 89, 90, 91). Since we particularly noticed its absence in high light exposed (HLE) Chlorella cells (the data presented here was mentioned in a preliminary report of our findings), it was suggested that the absence of the Emerson enhancement effect may be tied to a different organization of the photosynthetic electron transport pathway in the HLE cells versus the regularly grown (RG) or the dark adapted (DA) Chlorella cells(21). Such a reorganization would probably involve a difference in the ratio of cyclic to non-cyclic photophosphorylation in the HLE cells versus the RG or DA cells. Therefore, we decided to examine this possibility and to note other gross differences between the two types of cells:

1. The Emerson enhancement effect for oxygen evolution. Irrespective of the order of the presentation of the monochromatic light to the samples or the relative age (3 to 6 days after inoculation) of the non-synchronously grown Chlorella cells, cells which were regularly grown (RG) or adapted to the dark (DA) always showed enhancement, whereas cells exposed to high intensity illumination (HLE) always showed a lack of enhancement (Table 1, line 1).
2. Dark oxygen uptake. Oxygen measurements show the rate of dark oxygen uptake from the HLE suspensions to be 2 to 4 times that of the RG or DA suspensions (Table 1, line 3).

Table 1. Differences in the Emerson Enhancement Values for Oxygen Evolution and Other Characteristics of Regularly Grown (RG) or Dark Adapted (DA) and High Light Exposed (HLE) Chlorella Cells.

Characteristic	RG or DA	HLE
1. Emerson enhancement of oxygen evolution	+3.5	+0.5
2. Rate of oxygen evolution in red (> 650 nm) light, µmoles O_2 mg Chl^{-1} hr^{-1}	4.6	6.0
3. Rate of dark oxygen uptake, µmoles O_2 mg Chl^{-1} hr^{-1}	3.8	14.6
4. Ratio of absorbance at 678 nm to that at 650 nm	1.3	1.67
5. Number of cells (cells ml^{-1} of (suspension)	15.8×10^6	7.9×10^6
6. Packed cell volume (ml of cells ml^{-1} of suspension)	3.2×10^{-4}	6.2×10^{-4}

Conditions: Cells were suspended in Warburg's #9 buffer to have an absorbance of 0.3 at 678 nm. Oxygen measurements made in the linear part of photosynthesis versus light intensity curve at $9.6^o \pm 1^oC$, others at 25^oC. Data are average of 3 experiments.

3. Ratio of the absorbance at 678 nm. HLE cells usually showed about a 28 percent larger 678 nm to 650 nm ratio than the RG or DA cells (Table 1, line 4).

4. The number and volume of cells per ml of suspension. HLE suspensions having an absorbance of 0.3 at 678 nm contained about one-half the number of cells (cells ml^{-1} of suspension) as compared to the RG or DA suspensions (Table 1, line 5). The calculated volume of one HLE cell was four times larger than for the RG cell. The difference in size could be observed visually. (The counting and visual observations were made by using a microscope and a hemocytometer, and the packed cell volume was obtained by centrifuging the samples in Hopkins vaccine tubes and reading the pellet volumes with the aid of a cathetometer.)

As noted above, the HLE cells showed a total absence of the Emerson enhancement effect for oxygen evolution and actually had a dark respiration rate that was about 4 times that of the RG or DA cells on a chlorophyll basis. The HLE samples contained about one-half the amount of chlorophyll per cell volume than could be observed in RG or DA cells. However, on a cell basis, the HLE cells had twice as much chlorophyll as the RG or DA cells. The ratio of the absorbance at 678 nm to that measured at 650 nm was lower (1.30 versus 1.67) in the RG cells than in the HLE cells, suggesting a high Chl a/b ratio in HLE cells.

B. The Relative Light Minus Dark ATP (ΔATP) Values from Extracts of Unpoisoned Chlorella Cell Suspensions

In an attempt to obtain positive ΔATP (light-dark) values from unpoisoned Chlorella cells we tried to determine whether or not the negative

ΔATP levels we originally obtained during some preliminary experiments were simply due to the possibility that our suspension absorbance was too high thus placing the majority of the cells effectively in the dark. This could cause the dark values to dominate the light values which would yield the negative ΔATP values. We believed that this point could best be determined by simply measuring the ΔATP as a function of the absorbance by the suspension.

Regardless of the absorbance of the suspensions, negative ΔATP values were usually obtained from the extracts of unpoisoned Chlorella cells which had been suspended in 0.1 M carbonate-bicarbonate buffer (pH 8.2) with no added poisons or cofactors (Table 2). The illuminated suspensions were exposed for 30 seconds to high intensity (1.6×10^5 ergs cm^{-2} sec^{-1}) white light. (See emission spectrum shown in Chapter II, Figure 4.) Although measurements were not made for suspensions having an absorbance of less than 0.1 at 678 nm, the data presented in Table 2 clearly indicate that the ΔATP values become more negative as the absorbance of the suspension was increased. The relative ATP values for the dark sample extracts also increased with the increase in the absorbance of the suspension. Our first impression was that the negative values were probably due to the fact that a larger percentage of the cells remained effectively in the dark, especially at the higher absorbance, as we had predicted. However, if we plot the ratio of the negative ΔATP value to the dark ATP value (-ΔATP per dark ATP) for each absorbance of the suspensions, the values tend to be rather constant except at the higher absorbance value of 2.0 (Table 2, line 3) where Beer's law may not be valid. The relatively constant -ΔATP to dark ATP ratio suggests that the negative ΔATP values may be due to other than a simple shading effect, i.e., some photoact. If this were not so we would

Table 2. Relative Light Minus Dark (ΔATP) Values of Extracts from Unpoisoned Aerobic Chlorella Suspensions as a Function of the Absorbance of Chlorophyll a at 678 nm.

	Suspension Absorbance at 678 nm					
	0.1	0.2	0.3	0.5	1.0	2.0
1. Average relative ΔATP values	-1.5	-2.4	-4.3	-5.5	-8.9	-10.5
2. Average relative dark ATP values	5.0	7.0	9.2	16.9	31.9	65.1
3. Ratio of -ΔATP to dark ATP values	0.30	0.34	0.47	0.33	0.28	0.17

Conditions: Cells suspended in 0.1 M carbonate-bicarbonate buffer, pH 8.2; suspensions were exposed to 30 seconds of high intensity (1.6 x 10^5 ergs cm^{-2} sec^{-1}) white light filtered through a Schott M978 and Corning C.S. 1-97 glass filters; temperature 25°C. Data are averages of two experiments.

have expected the ratio to decline as the suspension absorbance is increased.

Positive ΔATP values were observed from extracts of unpoisoned aerobically maintained Chlorella suspensions only on two occasions out of, at least, 24 experiments (Table 3, line 1). For anaerobically maintained Chlorella suspensions the small positive values were more reproducible (Table 3, line 2). For the aerobic condition the small positive ΔATP values were observed only after short illumination periods (10 seconds), the values became increasingly negative as the time of illumination was increased. (The latter trend is again demonstrated in experiments reported in section C, Table 5.) In anaerobic suspensions the dark ATP values were reduced to about 70 percent of that of the aerobic cells. The illumination time over which small positive ΔATP values could be observed was extended by anaerobic treatment to 20 to 30 seconds. Although the anaerobic conditions gave reproducible positive values, we felt these values were too small for meaningful changes to be observed as we changed the experimental conditions; therefore, we abandoned this treatment. The results shown in Table 3 are, again, an average of two experiments. Furthermore, since our original intent was to simply screen several methods in a quest to obtain positive ΔATP values, no standard curve was made for these initial experiments.

C. The Effects of Various Poisons on the ΔATP Levels of Chlorella

Several poisons were added to Chlorella suspensions in an attempt to obtain larger, consistently positive ΔATP values upon illumination with white light. If we were to observe photophosphorylation *in vivo*, it would be necessary to not only reduce or eliminate respiration (respiratory ATP

Table 3. Relative Light Minus Dark (ΔATP) Values for Unpoisoned Chlorella Cell Extracts as a Function of Illumination Time Under Aerobic and Anaerobic Conditions.

	Time of Illumination (Seconds)				
	0*	10	20	30	60
1. Aerobic suspensions, ΔATP values, relative units	15.8	+3	-2	-3	-6
2. Anaerobic suspensions, ΔATP values, relative units	10.5	+2	+4	+1	-2

Conditions: Anaerobiosis was obtained by gassing the cell suspensions for 30 minutes with 100 percent argon. Suspension medium, light intensity and temperature, same as in Table 2.

*At zero time, only the dark ATP values are given, not ΔATP values.

synthesis), but to dampen ATP utilization without inhibiting photophosphorylation. Apparently, few poisons have been examined regarding their effects on respiration versus their effects on the photosynthetic apparatus of Chlorella. A study of the effects of various poisons on isolated Chloroplasts has, however, been made. All five poisons examined in Table 4 have been shown to have very little or no effect on chloroplast reactions for concentrations of less than 10^{-3} M) (35, 92). Of these five, only cyanide is known to have been specifically tested in Chlorella. Surprisingly, 10^{-4} HCN was observed to stimulate dark oxygen uptake in these cells, rather than stop it (93, 94). We decided to examine the effects of cyanide (using KCN rather than HCN) to see if the increased oxygen uptake might have been due to an uncoupling of oxidative phosphorylation which, in turn, would increase electron transport and ultimately oxygen uptake. The result is included in Table 4 and in the following description.

Very high ($\bar{>}$ 10^{-2} M) concentrations of the poisons tested permitted the observation of positive ΔATP values during 10 second illumination (Table 4). High concentrations of iodoacetate (ICH_2COOH), sodium arsenite ($NaAsO_2$), potassium cyanide (KCN) and sodium azide (NaN_3) did not produce as much positive ΔATP values as low concentrations of oligomycin (Table 4). The largest positive ΔATP values obtained after illuminating the suspensions poisoned with 10^{-2} M KCN or NaN_3 were less than one-half the values obtained upon illumination after adding 10^{-5} M oligomycin. Lower concentrations (10^{-4} M) of KCN or NaN_3 were undesirable because they caused a 10 percent stimulation in the dark ATP levels which resulted in the ΔATP values being more negative than the unpoisoned control (not shown). Although such results agree with earlier findings regarding the effect of cyanide on respira-

Table 4. Effect of Various Poisons on the Relative Light Minus Dark (ΔATP) Values from Aerobic Chlorella Cell Extracts.

Poison	Concentration	Relative Dark ATP Values*	Relative ΔATP Values**
1. (4) None		86.6	- 5.0
2. (2) ICH_2COOH	1.0×10^{-2} M	39.6	- 6.6
3. (2) $NaAsO_2$	1.0×10^{-2} M	34.8	+11.4
4. (2) KCN	1.0×10^{-2} M	22.8	+18.4
5. (3) NaN_3	2.0×10^{-2} M	22.0	+19.6
6. (2) Oligomycin	1.2×10^{-7} M	75.0	+ 7.0
7. (2) Oligomycin	1.2×10^{-6} M	54.4	+27.6
8. (2) Oligomycin	1.2×10^{-5} M	33.6	+48.4
9. (2) Oligomycin	2.4×10^{-5} M	33.4	+49.0
10. (2) Oligomycin	4.8×10^{-5} M	33.0	+48.2

Conditions: Suspension and white light intensity same as given in Table 2; illumination time, 10 seconds; temperature 25°C. Data are averages of number of experiments given within parentheses.

*1 relative dark ATP unit represents 0.074 µmoles ATP mg Chl^{-1}.
**1 relative ΔATP unit represents 26.6 µmoles ATP mg Chl^{-1} hr^{-1}.

tion (93, 94), further indicating that the stimulation is not due to an uncoupling process, they did not appear pertinent to our problem. Another observation, also not given in Table 4, which indicated that KCN or NaN_3 were not desirable for our purposes was that, in the presence of high ($\gtrsim 10^{-2}$ M) concentrations of these salts, periods of high intensity illumination for more than 15 seconds gave negative ΔATP values that exceeded the negative ΔATP values obtained from the unpoisoned control. ICH_2COOH and $NaAsO_2$ were not examined for these characteristics. (Refer to Tables 3, 4, and 5 for unpoisoned cell values.)

The data presented in Table 4 clearly indicates that, of the poisons tested, oligomycin (Sigma Chemical Co., St. Louis, Mo.) provided the largest positive ΔATP values for Chlorella. Oligomycin was effective at all concentrations between 1 x 10^{-7} M and 5 x 10^{-5} M, with saturation of its inhibitory effects on the dark ATP levels occurring between 1 and 2 x 10^{-5} M. Unlike the results obtained with KCN and NaN_3, long illumination periods (up to 10 minutes) in the presence of 1.2 x 10^{-5} M oligomycin yielded no negative ΔATP values, although such values did decline slightly after 20 seconds (Table 5). The dark ATP levels of suspensions containing 1 to 2 x 10^{-5} M oligomycin were reduced, but not eliminated. On the average, the dark ATP levels for suspensions having an absorbance of 0.3 at 678 nm to which 1 to 2 x 10^{-5} M oligomycin had been added were about three times lower than those of the unpoisoned control. Relatively high intensity 650 nm light (4.6 x 10^4 ergs cm^{-2} sec^{-1})* produced results, in the presence or absence of oligomycin, which were essentially identical to those presented in Table 4.

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*The red light experiment was done later, after we had improved our optics to provide higher intensities with monochromatic light.

Table 5. Relative Light Minus Dark (ΔATP) Values for Extracts from Chlorella Cells as a Function of Illumination Time in the Presence or Absence of Oligomycin (1.2×10^{-5} M).

Treatment	Time of Illumination (Seconds)						
	0*	10	20	30	45	60.....	600
1. Control (unpoisoned), ΔATP values, relative units	85	-5	-4	-10	-16	-19	-44
2. + Oligomycin, ΔATP values, relative units	32	+43	+45	+42	+40	+37	+ 4

Conditions: Suspension, white light intensity, and temperature, same as given in Table 2. Data are averages of 2 experiments.

*At zero time, only the dark ATP values are given, not ΔATP values. 1 relative dark ATP unit represents 0.074 μmoles ATP mg Chl^{-1}.

D. The Effect of Oligomycin on the Net Photosynthetic Oxygen Evolution and the Relative ATP Levels in Regularly Grown and High Light Exposed Chlorella

In order to provide comparative oxygen data for the ATP measurements that were to be made on regularly grown (RG) and high light exposed (HLE) cells, we wanted to examine the effect oligomycin would have on oxygen uptake and evolution. Manometric measurements of steady state net photosynthetic oxygen evolution in saturating (> 1×10^5 ergs cm^{-2} sec^{-1}) white light indicated differences between RG and HLE Chlorella cells (see chapter II, section A) in the presence or absence of 1.2×10^{-5} M oligomycin (Table 6). In the absence of oligomycin the cells had a dark oxygen uptake rate which was more than twice that for RG cell suspensions (Table 6, column 2, lines 1 and 2). This result is similar to that observed in RG versus HLE cells during Emerson Enhancement effect studies (Table 1, line 2). The light induced apparent oxygen evolution for HLE cells was about 36 percent less than for RG cells (Table 6, column 1, lines 1 and 2). As a result of the increased oxygen uptake and lower apparent oxygen evolution in the light, the HLE cells had about 14 percent lower net photosynthesis than the RG cells (Table 6, column 3, lines 1 and 2). If we had a way of knowing that the effect of light on oxygen uptake was negligible, then the net photosynthesis would be true photosynthesis.

The addition of oligomycin to RG and HLE cells caused about a 25 and a 35 percent decrease, respectively in the net photosynthesis values (Table 6, column 3, lines 1 and 3; 2 and 4). Thus, the HLE cells averaged 27 percent less net photosynthesis than the RG cells (Table 6, column 3, lines 3 and 4), in the presence of oligomycin. This was the result of the fact that HLE cells which were treated with oligomycin had about 41 percent lower

Table 6. Net Oxygen Evolution Obtained Manometrically during Steady State Photosynthesis Measurements on Regularly Grown and High Light Exposed Chlorella in the Presence or Absence of Oligomycin (1.2×10^{-5} M).

Treatment	Oxygen Uptake or Evolution, µmoles O_2 mg Chl^{-1} hr^{-1}				
	(1) Light	-	(2) Dark	=	(3) Net Photosynthesis
1. Control, Regularly Grown	61.1	-	(-10.8)	=	+71.9
2. Control, High Light Exposed	38.9	-	(-23.2)	=	+62.1
3. + Oligomycin, Regularly Grown	47.3	-	(- 6.9)	=	+54.2
4. + Oligomycin, High Light Exposed	27.8	-	(-11.6)	=	+39.4

Conditions: Aerobic; illumination time, 10 minutes; white light intensity, 3.5×10^5 ergs cm^{-2} sec^{-1}; suspension absorbance (at 678 nm), 0.3 in carbonate-bicarbonate buffer, 0.1 M, pH 8.2. Data are averages of 4 experiments.

apparent oxygen evolution in the light and that oxygen uptake by the suspensions was inhibited more in the HLE cells than in the RG cells (Table 6, column 1, lines 3 and 4). Oligomycin reduced by 36 to 50 percent the rate of oxygen uptake by both HLE and RG suspensions, respectively, compared to the unpoisoned controls (Table 6, column 2, lines 1 and 3; 2 and 4). The HLE cells were generally more sensitive to oligomycin than the RG cells.

ATP measurements were made to see whether it was possible that the lack of Emerson enhancement in high light exposed (HLE) Chlorella cells may be correlated to higher endogenous levels of ATP in those cells and for comparison with the oxygen data presented in the preceding section. The ATP values were obtained after illuminating the suspensions for 10 minutes with saturating ($>1 \times 10^5$ ergs cm^{-2} sec^{-1}) white light). The experimental conditions for these experiments were essentially identical to those used for the oxygen experiments reported in the preceding section (see Table 6). These results also indicate distinct differences between RG (regularly grown) and HLE cells in the presence or absence of 1.2×10^{-5} M oligomycin.

Unpoisoned Chlorella suspensions (both RG and HLE cells) always showed large negative ΔATP values (similar to those observed after 10 minutes illumination as documented in Table 5 for RG cells). However, the HLE suspensions yielded ΔATP values which were about 40 percent less negative, but both the light and the dark ATP values were higher than those obtained from RG suspensions (Table 7, lines 1 and 2). In contrast to the oxygen data described previously (Table 6, column 1), both the unpoisoned and the oligomycin poisoned illuminated HLE cells suspensions yielded rates having larger ATP values than did the RG cells (Table 7, column 1, lines 3 and 4 versus 1 and 2). While positive ΔATP values were obtained from all cell suspensions which were poisoned with oligomycin, the values were always

Table 7. Relative Light and Dark ATP Values Obtained in the Presence Or Absence of Oligomycin (1.2×10^{-5} M) From Extracts of Regularly Grown and High Light Exposed Chlorella Cells.

Treatment	Relative ATP Values				
	(1) Light	-	(2) Dark*	=	(3) ΔATP**
1. Control, Regularly Grown	39.0	-	77.2	=	-38.2
2. Control, High Light Exposed	82.1	-	105.5	=	-23.4
3. + Oligomycin, Regularly Grown	37.1	-	26.8	=	+10.3
4. + Oligomycin, High Light Exposed	55.6	-	52.2	=	+ 3.4

Conditions: White light intensity (with M978 Schott and C. S. 1-97 Corning glass filters), 3.5×10^5 ergs cm^{-2} sec^{-1}; other conditions same as in Table 6. Data are average of 4 experiments.

*1 relative dark ATP unit represents 0.074 μmoles ATP mg Chl^{-1}.
**1 relative ΔATP unit represents 0.4 μmoles ATP mg Chl^{-1} hr^{-1}.

less positive from the HLE suspensions than from the RG cell suspensions (Table 7, column 3, lines 3 and 4). The illuminated RG cells, both unpoisoned and oligomycin poisoned, yielded nearly identical ATP values (Table 7, column 1, lines 1 and 3). Similarly treated HLE cells showed that the oligomycin poisoned HLE cells had about 32 percent lower values than those obtained from unpoisoned HLE cells (Table 7, column 1, lines 2 and 4). Of all the data presented in Table 7, only the ATP values obtained from the dark samples of both the unpoisoned and poisoned RG or HLE cells appeared to parallel the oxygen data presented in Table 6, column 2. For both oxygen and ATP data, the dark HLE were larger than RG values. Also, for the HLE cells, both the oxygen and the ATP value data were reduced by 50 percent upon the addition of oligomycin (Table 6, column 2 and Table 7, column 2). Similar reductions occurred for the oxygen and ATP values from RG cells when oligomycin was added, but the percent of reduction was not as large as for the HLE cells. However, unlike the oxygen data, the dark suspensions of unpoisoned RG and HLE cells yielded ATP values which were larger than the light values (Tables 6 and 7, columns 1 and 2). Again, these ATP results were similar to what was reported for 10 minutes illumination in Table 5.

E. The Effect of Various Concentrations of DCMU on the Net Photosynthetic Oxygen Evolution and the Relative Light and Dark ATP Levels from Oligomycin Poisoned Regularly Grown and High Light Exposed Chlorella Cells

In order to distinguish between cyclic and non-cyclic photophosphorylation, it was necessary to stop non-cyclic electron transport from photosystem II to photosystem I by DCMU, 3-(3,4-dichlorophenyl)-1, 1-

dimethylurea which has been shown to be among the most effective poisons for this purpose (73, 74, 95, 96). The effects of this poison were examined as a function of concentration for both oxygen evolution and ATP levels in both regularly grown and high light exposed Chlorella cells.

Oligomycin (1.2 x 10^{-5} M) was added to regularly grown (RG) and high light exposed (HLE) Chlorella cells and the suspensions were allowed to stand in the dark for 15 to 20 minutes. A given concentration of DCMU in carbonate-bicarbonate buffer (0.1 M, pH 8.2) was added to the two tubes containing equal aliquots of cell suspension (a sample for illumination and a dark control), in preparation for the ATP determination. The dilution was done in dim green light and the tubes were returned to the dark for another 15 to 20 minutes prior to illumination. Chlorella cells which have been treated with DCMU (5 x 10^{-5} M) are not irreversibly damaged by the DCMU during the exposure times used (40, 97). The ΔATP values for each concentration of DCMU used were calculated from the measurement of the ATP extracted from each tube pair, after the illumination tube was exposed for 20 seconds to high intensity (1.1 x 10^{-5} ergs cm^{-2} sec^{-1}) red (> 640 nm) light which also passed through M978 Schott and C.S. 1-97 Corning glass filters, as described previously.

For oxygen measurements, the remaining suspension of Chlorella cells, not used in the ATP measurements, were centrifuges and the cells were washed with phosphate buffer (0.1 M, pH 8.0). After recentrifugation, the cells were resuspended in a few drops of fresh phosphate buffer and placed on the platinum electrode as previously described in chapter II, section B. (The absorbance of the cells on the electrode ranged from approximately 0.3 to 0.6 at 678 nm.) Both the Ag/AgCl and the Pt (on which the cells were deposited) electrodes were immersed in the phosphate buffer.

The gas tank with the appropriate gas mixture (5% CO_2, 2% O_2 and 93% N_2) for the aerobic conditions and 100% argon for anaerobic conditions was opened. The cells were left to equilibrate for 20 to 30 minutes in the dark under the selected gas condition. The Chlorella cells were then illuminated with red (> 640 nm) light (2×10^5 ergs cm^{-2} sec^{-1}) at 25°C. At the end of one or two measurements under that condition, either the bath was drained and fresh buffer containing the appropriate concentration of DCMU was added to replace it, or DCMU was added directly to give the proper concentration. Both methods were used and gave the same results after at least 15 minutes equilibrium period in the dark in the presence of the new buffer mixture. Because it was impossible to duplicate cell concentrations on the oxygen rate electrode, for comparative purposes all results are presented as a percent of the unpoisoned control. Also, due to the diffusion lag of the oxygen to the electrode and the apparent elimination of the oxygen induction spike in the HLE cells, the values presented were measured at the end of 1 minute of illumination, rather than 20 seconds. For RG cells, the values obtained at either 20 seconds or after 1 minute of illumination were usually quite comparable.

When compared to the samples poisoned only with oligomycin, the results show that about a 50 percent (six experiment average, ranging from 38 to 54 percent) of the light minus dark ATP (ΔATP) levels and about 60 percent of the oxygen evolution are inhibited by 2.5×10^{-7} M DCMU in RG Chlorella cells (Figures 6 and 7). In HLE cells only 25 percent of the ΔATP levels and 45 percent of the oxygen evolution were inhibited by the same concentration (Figure 8).

The inhibition of ΔATP levels in RG cells at 2.5×10^{-5} M DCMU ranged from about 70 to 95 percent and about 100 percent for oxygen evolution

(Figures 6 and 7). Our oxygen data for Chlorella, which were regularly grown, compares favorably with those obtained by other investigators regarding the addition of various concentrations of DCMU (40, 45). For HLE cells the addition of 2.5 x 10^{-5} M DCMU only inhibited the ΔATP level by about 50 percent but, as in the RG cells, completely eliminated oxygen evolution (Figure 8).

It should be reemphasized that even when the maximum inhibition of oxygen evolution occurs a positive ΔATP level remains in both cell types. This positive ΔATP level was always observed and the larger level occurred in the HLE cells. The ΔATP level remaining in the presence of DCMU varied from sample to sample with changes in experimental conditions, averaging about 12 percent of the unpoisoned control ΔATP levels with regularly grown Chlorella cells. This point will be made clear in the following sections.

F. Relative Light Minus Dark ATP (ΔATP) Levels as a Function of Light Intensity in Oligomycin Poisoned Chlorella Cells in the Presence or Absence of DCMU and under Aerobic or Anerobic Conditions.

From the preceding section (section E) it was observed that positive ΔATP levels could be observed in oligomycin (1.2 x 10^{-5} M) poisoned Chlorella cells, even in the presence of 10^{-5} M DCMU which stopped all oxygen evolution. This fact suggested that both cyclic and non-cyclic photophosphorylation were operating in Chlorella cells. The question was raised as to whether the addition of DCMU induced cyclic photophosphorylation. In other words, could cyclic and non-cyclic photophosphorylation, which could represent two separate pathways, be separated without the addition of DCMU? Multiphasic curves have been obtained for cytochrome absorbancy changes at

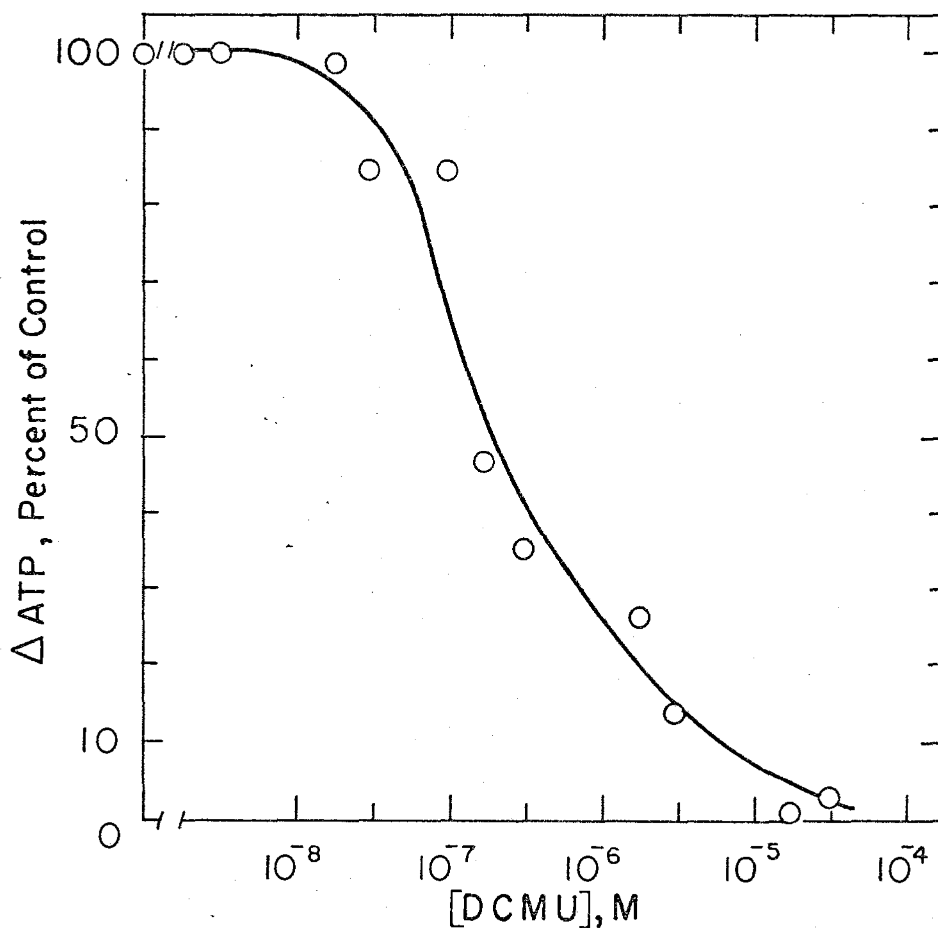

Figure 6. Effect of various DCMU concentrations on the relative light minus dark (ΔATP) levels from extracts of oligomycin (1.2×10^{-5} M) poisoned regularly grown Chlorella.

Conditions: Illumination time, 20 seconds; red (> 640) nm) light intensity, 1.1×10^{5} ergs cm^{-2} sec^{-1} (with M978 Schott C.S. 2-64 and C.S. 1-97 Corning glass filters); other conditions as in Table 6. Data are averages of 3 experiments at each concentration of DCMU.

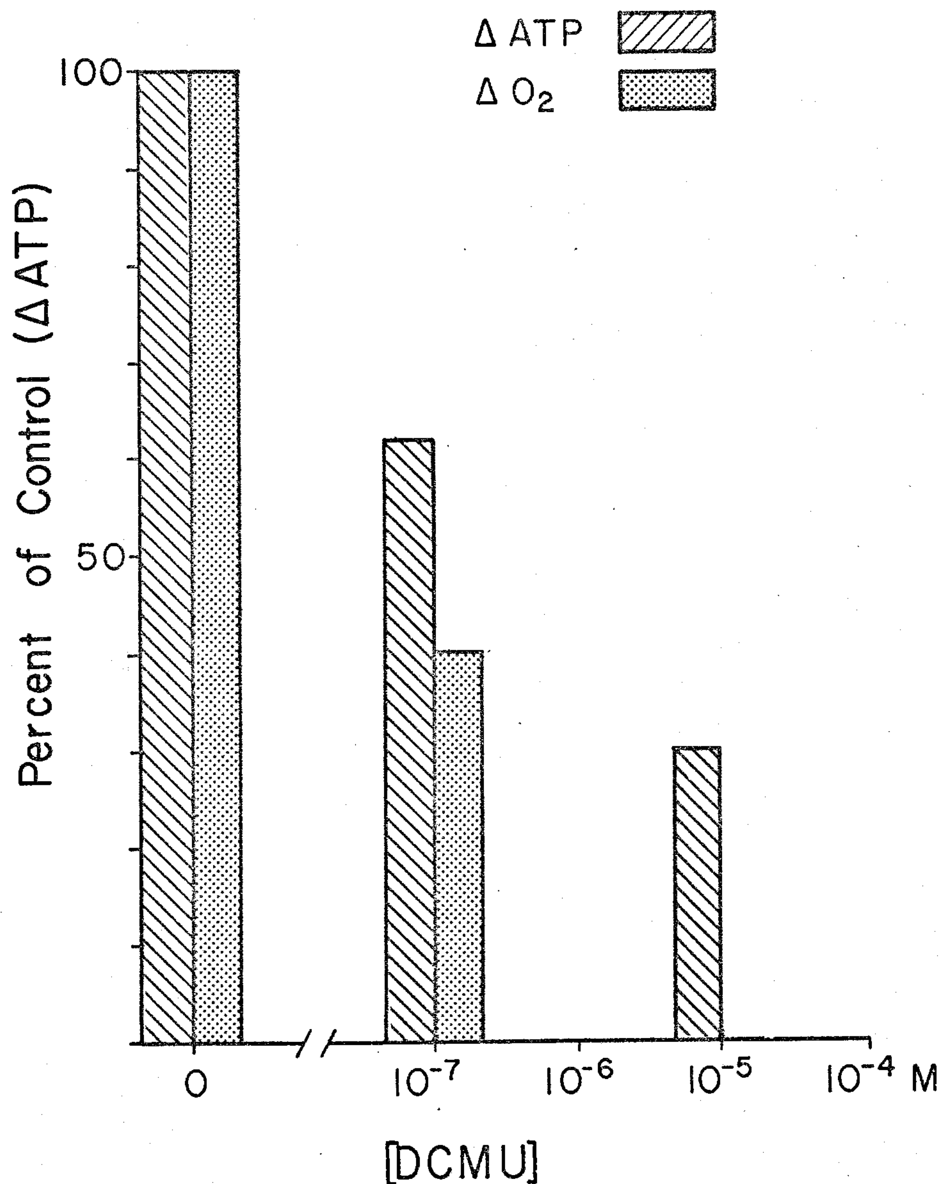

Figure 7. Effect of three different concentrations of DCMU on the light minus dark (ΔATP) levels and the photosynthetic oxygen evolution of oligomycin (1.2 x 10^{-5} M) poisoned regularly grown Chlorella cells.

Conditions: For ATP measurements, same as Figure 6; oxygen measured on platinum rate electrode; phosphate buffer, 0.1 M, pH 8.0; gassed with 5% CO_2, 2% O_2 and 93% mixture; illumination time, 4 minutes, values given, measured at 20 seconds; red (> 640 nm) light intensity, 2 x 10^5 ergs cm^{-2} sec^{-1} (with M978 Schott, C.S. 2-64 and C.S. 1-97 Corning glass filters); temperature, 25°C. Data are averages of 3 experiments

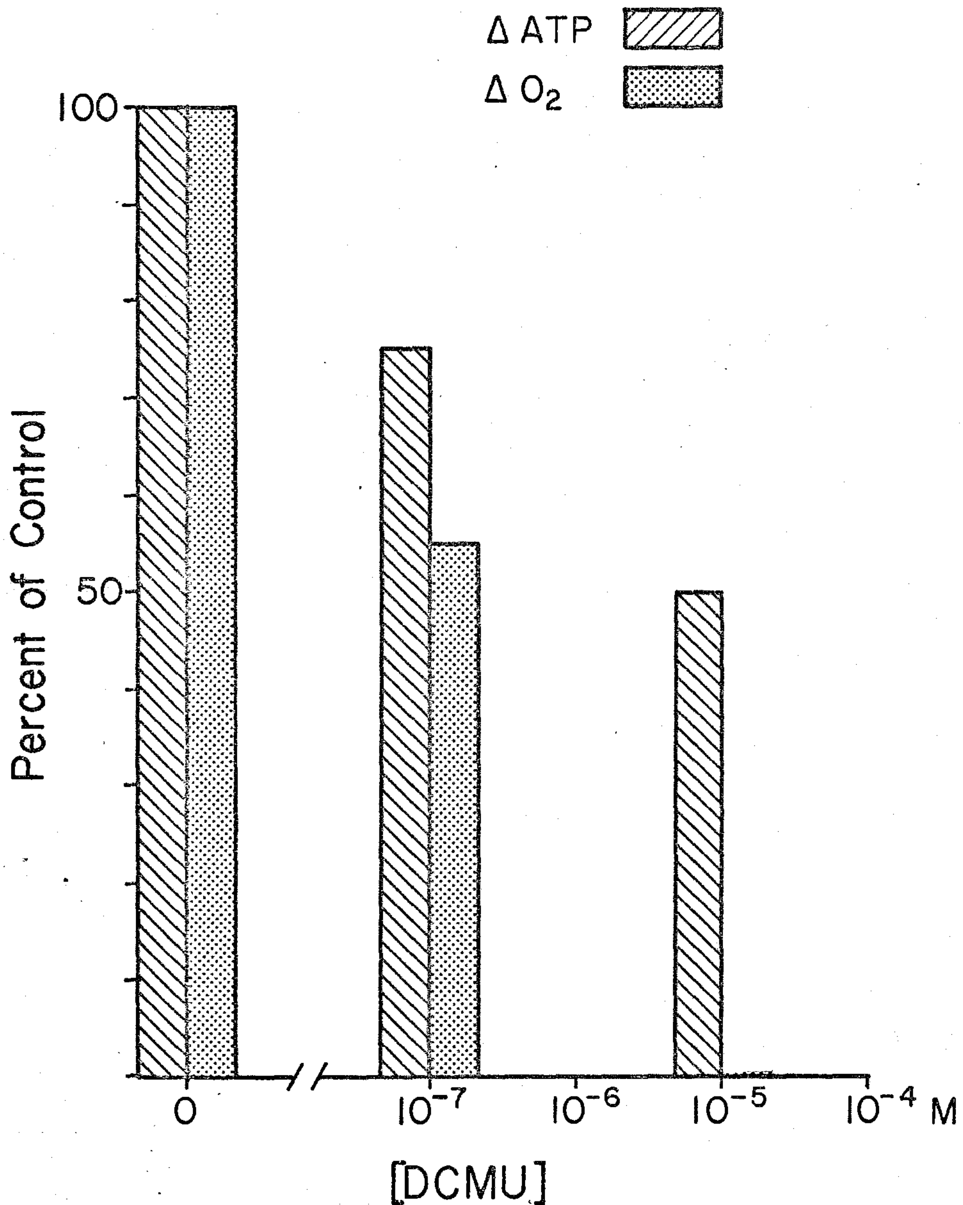

Figure 8. Effect of three different concentrations of DCMU on the light minus dark (ΔATP) levels and the photosynthetic oxygen evolution of oligomycin (1.2×10^{-5} M) poisoned high light exposed Chlorella cells.

Conditions: Same as in Figure 7, except oxygen values were measured at 1 minute. Data are averages of 3 experiments.

425 nm as a function of light intensity in Rhodospirillum rubrum; the occurrence of the different phases were used to support the hypothesis of the presence of two light reactions in the photosynthesis of these bacteria (98). Furthermore, data from the literature suggested that photophosphorylation should also show a multiphasic light curve. PMS supported cyclic photophosphorylation has been reported to require of the order of 200,000 lux of white light for saturation (99), whereas non-cyclic photophosphorylation saturated at about 20,000 lux (100-102). Hence, we reasoned that non-cyclic and cyclic photophosphorylation might be separated into similar phases as the result of varying the light intensity on Chlorella cell suspensions.

Regularly grown Chlorella cells were treated with oligomycin (1.2 x 10^{-5} M) and were left in the dark for about 30 to 40 minutes prior to illumination. The absorbance of all samples was adjusted to 0.3 at 678 nm. 2.0 ml aliquots of the suspension were exposed from 10 to 20 seconds (see figure legends) to various intensities of white (see emission spectrum, chapter II, section B) and red (> 640 nm, note legend of Figure 10 for wavelengths) light passed through two heat filters as described in chapter II, section B). The ATP was extracted and measured from both the light exposed sample and the dark control. The light minus dark (ΔATP) levels were calculated, and the results were plotted as shown in Figures 9 and 10. (For measurements made under anaerobic conditions, the samples were gassed with 100 percent argon for 25 to 30 mintues prior to illumination.)

Instead of obtaining a multiphasic light curve as the result of white light illumination, we obtained an S-shaped curve (Figure 9). The oligomycin treated Chlorella suspensions yielded no positive ΔATP levels for intensities

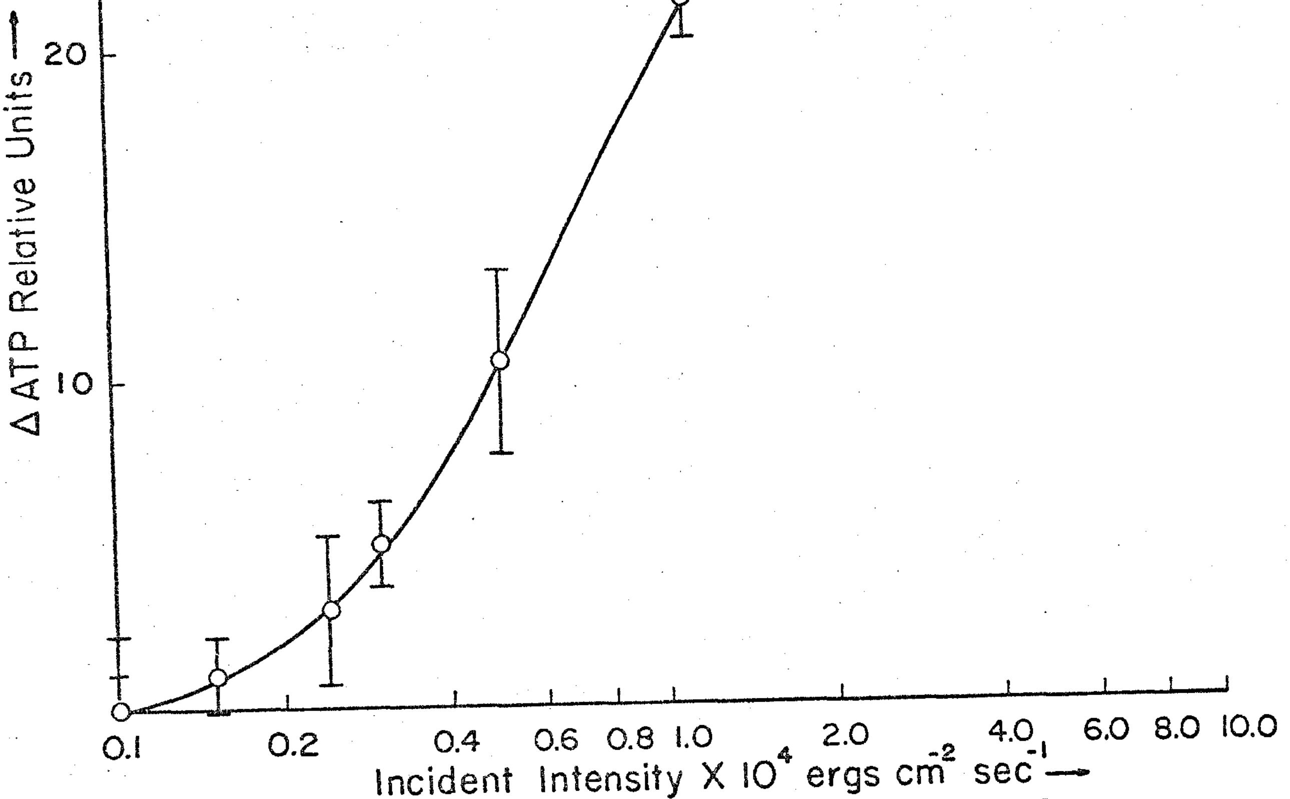

Figure 9. Relative light minus dark (ΔATP) levels as a function of white light intensity from oligomycin (1.2×10^{-5} M) poisoned Chlorella cells. Conditions: Same as Table 2, except that the white light (see emission spectrum chapter KK, section B, Figure 4) illumination periods were 20 seconds. Intensities were varied by the use of neutral density filters.

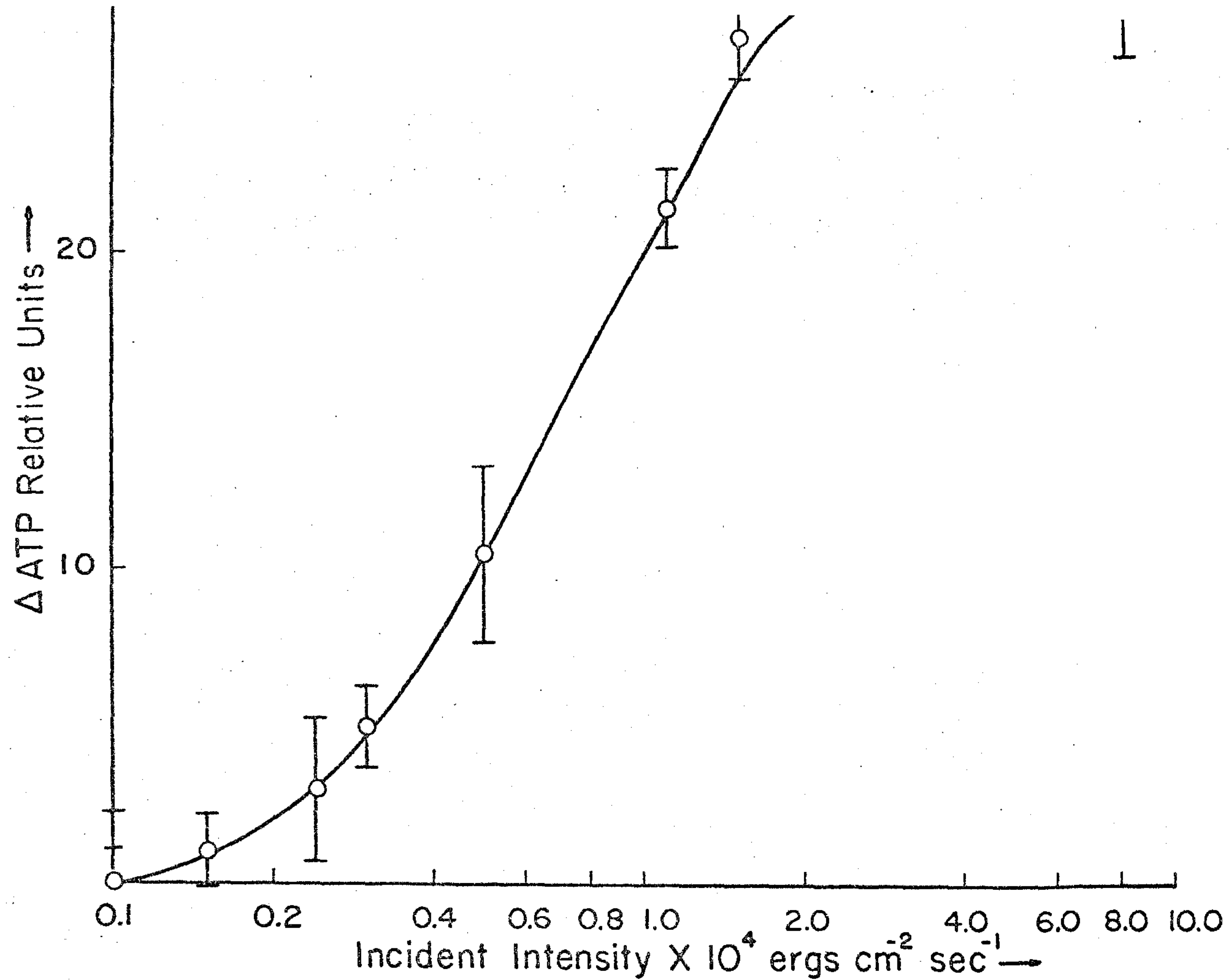

Figure 9. Relative light minus dark (ΔATP) levels as a function of white light intensity from oligomycin (1.2×10^{-5} M) poisoned Chlorella cells. Conditions: Same as Table 2, except that the white light (see emission spectrum chapter KK, section B, Figure 4) illumination periods were 20 seconds. Intensities were varied by the use of neutral density filters.

Figure 10. Relative ATP values as a function of intensity of red (> 640 nm) light in oligomycin (1.2×10^{-5} M) poisoned Chlorella cells in the presence or absence of DCMU (2.5×10^{-5} M) and under aerobic or anaerobic conditions.

Conditions: Same as described in Figure 5. Anaerobic cells, gassed 30 min with 100% argon. Symbols: -▲-▲-, 10 sec illumination at 685 nm (8 nm half-bandwidth), average of 5 experiments (aerobic); -Δ-Δ-, 10 sec illumination at 657 nm (10 half-bandwid[illegible] average of 6 experiments (aerobic); -□-□- 20 sec illumination at >640 (aerobic); -x-x-, 20 sec illumination at > 640 nm with DCMU average of 4 experiments (aerobic); -●-●-, 20 sec illumination at > 640 nm, average of 2 experiments (4 tube-pairs, anaerobic); and -o-o-, 20 sec illumination at > 640 nm with DCMU, each point the average of 4 experiments (anaerobic). (Note the logarithmic scale of the absicissa.)

below 1 x 10^3 ergs cm^{-2} sec^{-1} and there was an initial lag as the intensity was increased which may correspond to the low intensity lag observed in Chloroplasts (5, 129). Positive ΔATP values started to appear at about 1 x 10^3 ergs cm^{-2} sec^{-1}, slowly increasing and then rising rapidly until saturation was reached at about 2 x 10^4 ergs cm^{-2} sec^{-1}. (The absolute values we report for white light may be a little high due to problems inherent in measuring white light intensities, such as residual heat integrated over all wavelengths, etc., but the shape of the curve is believed to be correct. The points in Figure 9 represent an average of 5 ΔATP values at each intensity (note error bars). It should be mentioned, however, that at much higher white light intensities than those shown in the graph (> 2 x 10^5 ergs cm^{-2} sec^{-1}) large variations in the ΔATP values occurred. These large variations differed from sample to sample, showing no consistent trend, hence, no averaging was attempted. We do not know the cause of those fluctuations; perhaps the lamp became unstable at high operating voltages. These values were omitted from the graph.

A plot of the relative light minus dark (ΔATP) values calculated from the measurement of ATP extracted from Chlorella cells exposed to red light of varied intensities appears to show a multiphasic curve in the absence of DCMU (Figure 10, solid and open triangles and open squares) under aerobic conditions. After a "lag" to an intensity of about 4 x 10^2 ergs cm^{-2} sec^{-1}, ATP photoproduction rises and appears to saturate at about 1 x 10^3 ergs cm^{-2} sec^{-1} (see error bars). No matter how the curve is drawn through the error bars, the curve remains multiphasic with two saturation regions.

As observed with white light (Figure 9), the shape of the curve at low intensities is apparently in agreement with earlier chloroplast work

(129). It suggests that at low intensities either there is a threshold intensity prior to ATP synthesis (5), or ATP utilization equals ATP synthesis allowing no net ATP to be observed.

A complete intensity curve with red (> 640 nm) light was not measured under anaerobic conditions since the results of two experiments involving several pairs of tubes at each of two intensities under these conditions (100 percent argon, carbonate-bicarbonate buffer) gave similar results to those observed at the same intensities under aerobic conditions. The average ΔATP levels obtained under anaerobic conditions for the two intensities are included in the graph in Figure 10 (solid black circles).

In the presence of 2.5×10^{-5} M DCMU, aerobically or anaerobically, the ΔATP levels between 4×10^{2} ergs cm^{-2} sec^{-1} and 4×10^{3} ergs cm^{-2} sec^{-1} appeared to be slightly depressed although the error bars were so close to our original intensity curve in this region that we decided to leave the curve unchanged. For intensities above 4×10^{3} ergs cm^{-2} sec^{-1} the values clearly appear to be at the same level as the first saturation level (Figure 10, dashed line; values denoted by X's aerobic and open circles, anaerobic). It should be emphasized that the ratio of the saturation levels for the ΔATP values obtained in the presence of DCMU to the saturation level of the second saturation level in the absence of DCMU was about as high ($\sim$ 0.28) in this series of experiments as we ever observed for Chlorella under our normal experimental conditions (in Figure 7, the ratio was 0.30). In the majority of our experiments, the ratio of oligomycin plus DCMU to oligomycin only ΔATP levels for regularly grown cells averages about 0.12 (Tables 8 and 9). However, under anaerobic conditions and in the absence of CO_2 (in the presence or absence of DCMU) we have observed as high a ratio as $\sim$0.37

(Table 8B). The data in Tables 8 and 9, including oxygen data, will be described in the following sections. For high light exposed (HLE) cells under aerobic conditions and in the presence of DCMU (2.5×10^{-5} M) the ratio was the highest we ever observed in Chlorella cells. The HLE cells showed a ratio of 0.50 (Figure 8). Clearly, cell age or growth conditions and/or experimental conditions influence this ratio.

G. The Effect of CO_2 and DCMU on the Relative Light Minus Dark ATP (ΔATP) and Oxygen Evolution Levels from Oligomycin Poisoned Chlorella Cells

The results of previously obtained light minus dark (ΔATP) level data in the presence or absence of DCMU (Figures 6, 7, and 8) and as a function of light intensity (Figure 10) suggest that both cyclic (in the presence of DCMU) and non-cyclic photophosphorylation may be present in Chlorella cells and that their ratio may be variable. As mentioned in the previous section, we were concerned with whether the addition of DCMU induced cyclic photophosphorylation. Although we did observe a multiphasic light intensity curve and possibly the lower intensity saturating phase may be correlated to cyclic photophosphorylation, we reasoned that a further check would be to observe the effect of an absence of CO_2 (carbonate-bicarbonate buffer, 0.1 M, pH 8.2) on the ΔATP levels and on oxygen evolution. In terms of the modified Hill and Bendall scheme for photosynthesis (19, 20), a deprivation of the Calvin cycle electron acceptor (CO_2) should shut down oxygen evolution and its concomitant non-cyclic photophosphorylation but leave cyclic photophosphorylation unaffected. In fact the cyclic photophosphorylation levels may be expected to be higher than we normally observe in the presence of CO_2 because the rate of ATP utilization may also be reduced. Furthermore, since the non-cyclic electron transport pathway is effectively

blocked by the absence of CO_2, cyclic levels should remain unaffected by the further addition of DCMU (2.5×10^{-5} M). Essentially, the results shown in Table 8 are in agreement with our expectations.

In the presence of CO_2 (carbonate-bicarbonate buffer, 0.1 M, pH 8.2) the results were very similar to those we obtained earlier (Figure 10) under aerobic and anaerobic conditions in the presence and absence of DCMU with saturating red (> 640 nm) light. DCMU lowered the unpoisoned (oligomycin only) control level by about 90 percent (Table 8A, lines 1-4).

In the absence of CO_2 (Table 8B, line 6) the oligomycin only poisoned control value was lowered by about 65 percent and this level was not further reduced by the addition of DCMU (2.5×10^{-5} M), thus fulfilling our predictions.

To obtain comparable oxygen data, the experiments reported in Table 8B were repeated and the oxygen values were measured, immediately after the ATP measurements were made, on the oxygen rate electrode. The procedure was almost identical to that described in section E, except that the anaerobic samples were gassed for at least 30 minutes with 100 percent argon. In this set of experiments (Table 8C) the size of the values obtained as a percent of the control were smaller but the trend regarding the effect of the absence of CO_2, unpoisoned (Table 8C, line 9; 15.2 percent) and poisoned (Table 8C, line 10; 16.5 percent) with DCMU, on the ΔATP levels were consistent. The absence of CO_2 lowered the oligomycin only poisoned control oxygen level by 97.8 percent (Table 8C, line 9) and the further addition of DCMU completely eliminated even this residual oxygen evolution (Table 8C, line 10) as we had previously observed in section E. Thus, in the absence of CO_2 we were able to abserve a positive ΔATP level and an essential absence of oxygen evolution that remained unaffected by the further addition of DCMU.

Table 8. Relative Light Minus Dark (ΔATP) and Oxygen Levels From Oligomycin (1.2×10^{-5} M) Poisoned Chlorella Under Aerobic and Anaerobic Conditions, in the Presence or Absence of DCMU (2.5×10^{-5} M) CO_2.

Treatment	ATP Levels, Relative Units (1) Light	-	(2) Dark*	=	(3) ATP**	Percent Control
A. Carbonate-Bicarbonate Buffer (0.1 M, pH 8.2)						
1. Control, Aerobic	31.0	-	15.4	=	+15.6	100
2. Control, Anaerobic	30.2	-	13.4	=	+16.6	100
3. +DCMU, Aerobic	16.7	-	15.3	=	+ 1.4	8.9
4. +DCMU, Anaerobic	15.2	-	13.3	=	+ 1.9	11.4
B. Phosphate (Monobasic plus Dibasic) Buffer (0.1 M, pH 8.2)						
5. Control, Aerobic + CO_2	34.6	-	16.0	=	+18.6	100
6. Control, Anaerobic - CO_2	16.5	-	10.0	=	+ 6.5	34.9
7. +DCMU, Anaerobic - CO_2	16.8	-	9.9	=	+ 6.9	37.0

Treatment	ATP Levels, Relative Units	%C	O_2 Evol	%C
C. Phosphate Buffer, Experiment #2				
8. Control, Aerobic + CO_2	52.5 - 30.1* = 22.4**	100	540	100
9. Control, Anaerobic - CO_2	16.6 - 13.2 = 3.4	15.2	12	2.2
10. +DCMU, Anaerobic - CO_2	17.4 - 13.7 = 3.7	16.5	0	0

Conditions: Sample absorbance, 0.3 at 678 nm. Aerobic samples in phosphate buffer, gassed with 5 percent CO_2 plus 2 percent O_2 in 93 percent N_2; anaerobic suspensions, gassed for 30 minutes in dark with 100 percent argon. Illumination periods for A and B, 20 seconds red (> 640 nm) light (5×10^4 ergs cm^{-2} sec^{-1}); for C, 15 seconds red (> 640 nm) light (1.1×10^5 ergs cm^{-2} sec^{-1}) at 25°C. The values are an average of 2 experiments each.

*1 relative dark unit represents 0.074 μmoles ATP mg Chl^{-1}.
**1 relative ΔATP unit represents 13.3 μmoles ATP mg Chl^{-1} hr^{-1}, for A and B.
1 relative ΔATP unit represents 18.7 μmoles ATP mg Chl^{-1} hr^{-1}, for C.

H. The Effect of Various Electron Donors and Acceptors in the Presence or Absence of Various Poisons and an Uncoupler on the Relative Light Minus Dark ATP (ΔATP) Levels from Oligomycin Poisoned Chlorella Cells

To provide an additional check on whether we were indeed able to distinguish between cyclic and non-cyclic photophosphorylation in Chlorella we decided to examine the effects of various electron donors, acceptors, poisons, uncouplers, etc. on the ΔATP levels of oligomycin poisoned Chlorella.

Methyl viologen (MV, paraquat dichloride, i.e., 1,1'-dimethyl-4,4'-dipyridilum dichloride) which has a redox potential E_o' of -0.445 eV at pH 7.0 has been shown to undergo a one electron photoreduction upon the illumination of the chloroplasts (103, 104). While 10^{-4} M MV has been shown to have a saturating effect on the absorption change of P700 in chloroplasts (saturating the P700 oxidation), almost no work has been done with this compound in whole cells (104). Although no measurements apparently have been made as a function of concentration, 10^{-4} M MV has been shown to enter Chlorella cells (97, 105).

The purpose of using MV was two-fold: to stimulate non-cyclic photophosphorylation from water to MV and to possibly inhibit cyclic electron transport. Non-cyclic photophosphorylation levels in the presence of MV may be expected to be higher than in its absence, since MV is known to compete very effectively at X for the electrons that would normally enter the $NADP^+$ reduction pathway at X (106). The reason the ΔATP would be expected to be higher is that in the presence of MV, $NADP^+$ could not be reduced and thus ATP could not be utilized by the CO_2 fixation reactions due to the lack of reducing power; ATP would build up. If MV competes for electrons at X it might also prevent the cycling of electrons through X,

which might inhibit cyclic photophosphorylation as has been suggested by other investigators (29). (Refer to Figure 1.)

The addition of MV to oligomycin (1.2×10^{-5} M) poisoned Chlorella affects the ΔATP values in two ways depending on the concentration (Figure 11, -o-o-). Low concentrations of MV (1×10^{-6} M) caused an inhibition of 25 percent, whereas high concentrations (1×10^{-3} M) caused a stimulation of about 100 percent when compared to the ΔATP values of the control. MV appeared to have no effect on Chlorella cells at concentration of 5×10^{-5} M. (The illumination time was 20 seconds with red (> 640 nm) light (1.1×10^{5} ergs cm^{-2} sec^{-1}).)

The addition of various concentrations of MV to DCMU (2.5×10^{-5} M) and oligomycin (1.2×10^{-5} M) poisoned Chlorella cells usually left the ΔATP values essentially unchanged (Figure 11, -x-x-). (However, in a few cases the ΔATP level in the presence of DCMU was depressed slightly by 1×10^{-3} M MV. The depression was occasionally observed after using either broad band blue (340 nm to 620 nm) or red (> 640 nm) light (intensity, 1.1×10^{5} ergs cm^{-2} sec^{-1}).) The data obtained with red light are shown in Figure 11.

We were not only interested in providing a competition for electron acceptance at X, but in providing electron donors that would feed electrons beyond the DCMU inhibition site. Such a donation might not only provide information regarding cyclic and non-cyclic photophosphorylation _in vivo_ but might provide information concerning the coupling site(s) themselves. Data obtained from subchloroplast particles have suggested that reduced 2, 6-dichlorophenol indophenol ($DCPIPH_2$) and phenazine methosulfate ($PMSH_2$) will donate electrons at different sites along the non-cyclic electron transport pathway after the DCMU inhibition site (18, 28, 107). Furthermore,

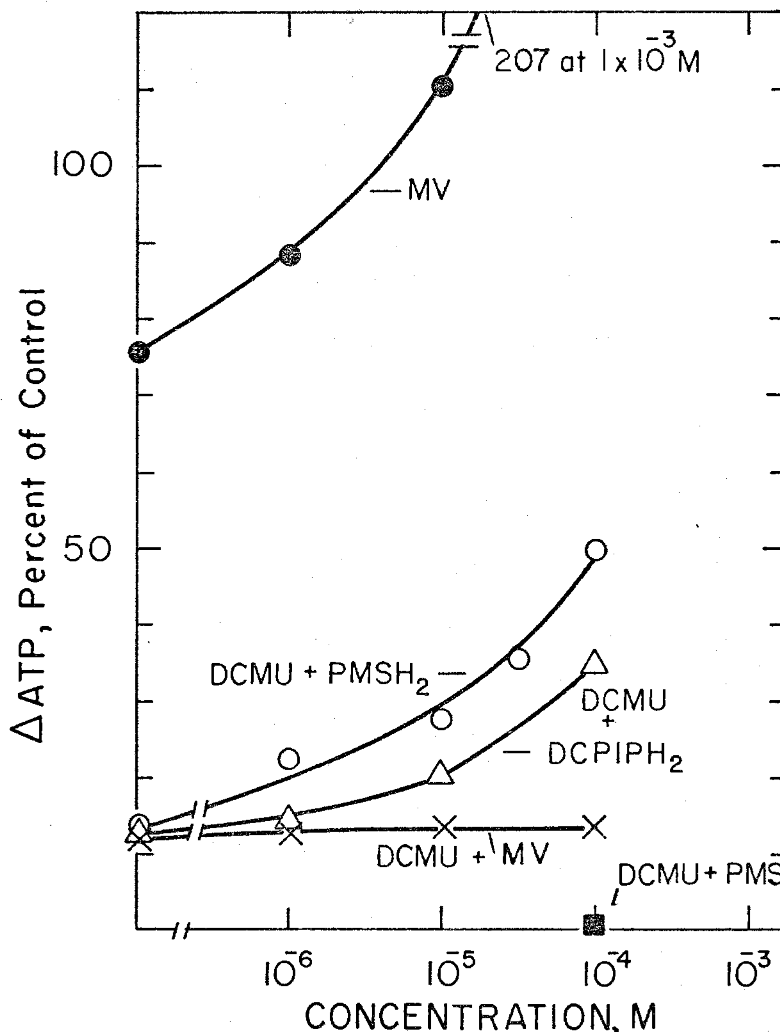

Figure 11. Effect of various concentrations of different electron acceptors or donors in the presence or absence of DCMU (2.5×10^{-5} M) on the light minus dark ATP (ΔATP) values obtained from oligomycin (1.2×10^{-5} M) poisoned Chlorella cells. Values are expressed as a percent of the oligomycin poisoned control.

Conditions: Cells suspended in carbonate-bicarbonate buffer (0.1 M, pH 8.2); suspension absorbance, 0.3 at 678 nm, under aerobic conditions at 25°C. Illuminated cells were exposed for 20 seconds to an intensity of 1.1×10^5 ergs cm^{-2} sec^{-1} of red (> 640 nm) light. DCMU plus MV data is the average of 2 experiments, the other points are an average of at least 3 experiments.

$DCPIPH_2$ has been shown to donate electrons at two sites on the non-cyclic electron transport pathway, one of which is just beyond the coupling site(s) (28, 108). However, Neumann et al.(28) believe that there are two non-cyclic coupling sites before P700 and that $DCPIPH_2$ donates to both whereas $PMSH_2$ only donates to one of them. This would suggest that the addition of $DCPIPH_2$ may produce twice as much ATP as the addition of $PMSH_2$ in the presence of DCMU.

Ascorbate-reduced (added in a ratio of 10 to 1) 2,6-dichlorophenol indophenol ($DCPIPH_2$) and phenazine methosulfate ($PMSH_2$) have been shown to enter and to affect the photoreactions of Chlorella (109). However, few measurements have been made on their in vivo effects as a function of concentration. 4.6×10^{-4} M $DCPIPH_2$ lowered the fluorescence yield at 687 nm by 71 percent in DCMU (4×10^{-5} M) poisoned Chlorella cells as compared to 29 percent by 2.3×10^{-4} M $DCPIPH_2$ (100). (Concentration studies with $PMSH_2$ have, apparently, been limited to Chloroplast preparations.) Therefore, it was necessary to establish the best concentrations for our system.

Control suspensions of Chlorella which were poisoned with oligomycin (1.2×10^{-5} M) and DCMU (2.5×10^{-5} M) were very slightly stimulated by the addition of low concentrations (1×10^{-6} M) of either $DCPIPH_2$ or $PMSH_2$ to the reaction medium (Figure 11, -Δ-Δ- and -o-o-, respectively). At this concentration, $DCPIPH_2$ increased the control value in the presence of DCMU about 0.5 percent, whereas $PMSH_2$ caused a 1.5 percent increase. For all concentrations used, $PMSH_2$ was slightly more effective than $DCPIPH_2$ in increasing the control ΔATP level in the presence of DCMU. 1×10^{-3} M $PMSH_2$, the highest concentration used, overcame about 50 percent of the control ΔATP level that had been reduced by the addition of DCMU. $DCPIPH_2$ was less

effective at the same concentration; it restored only 35 percent of the control value. Even though whole cells are apparently less permeable to most chemicals than cell organelles, e.g., cholorplasts, higher concentrations of $DCPIPH_2$ or $PMSH_2$ were not used in order to reduce the possibility of non-specific effects. Therefore saturation or inhibition levels which have been observed to be caused by these compounds were not attained in Chlorella (110).

Solid black squares plotted in Figure 11 represent the average ΔATP level obtained as a result of adding 1×10^{-3} M oxidized PMS in the presence of DCMU under anaerobic as well as aerobic conditions. In the presence of oxidized PMS or partially reduced PMS (5×10^{-5} M PMS plus 2.5×10^{-5} M sodium ascorbate) the ΔATP level in the presence of DCMU was always reduced to zero. This result was not an artifact due to a high concentration of oxidized PMS because the same result was observed with about 100 times lower concentration and partial reduction.

After establishing adequate working concentrations for the electron donors and acceptor, we proceeded to examine their effects alone and in combination and to examine several poisons and an uncoupler.

1×10^{-3} M MV and oxidized DCPIP greatly stimulated the light minus dark (ΔATP) levels when compared to the oligomycin poisoned control. Their addition stimulated ΔATP production above that of the control by 100 percent and 33 percent for MV and oxidized DCPIP, respectively (Table 9, lines 1, 2 and 3). (Oxidized DCPIP was not studied as a function of concentration. It absorbs light throughout the chlorophyll absorption region which would make calculations too difficult and also there is much controversy regarding the location of its acceptance of electrons (28, 111).) MV is known to

Table 9. Effect of Electron Acceptors, Donors, Poisons and an Uncoupler on Oligomycin (1.2×10^{-5} M) poisoned Chlorella Cells.

	Treatment	Relative ATP Values						Percent
		Light	-	Dark*	=	ΔATP**		Control
1.	Control	51.6	-	19.3	=	+32.3		100.0
2.	DCPIP (Oxidized)	61.5	-	18.4	=	+43.1		133.4
3.	MV	86.2	-	19.1	=	+67.1		207.7
4.	DCMU	23.3	-	19.4	=	+ 3.8		12.0
5.	DCMU + MV	23.6	-	18.9	=	+ 4.7		14.5
6.	DCMU + $DCPIPH_2$	27.6	-	17.8	=	+ 9.8		30.3
7.	DCMU + $PMSH_2$	32.4	-	19.0	=	+13.4		41.7
8.	DCMU + $DCPIPH_2$ + MV	41.2	-	18.1	=	+23.1		71.5
9.	DCMU + $PMSH_2$ + MV	45.4	-	18.8	=	+26.6		82.5
10.	DCMU + cycloheximide	26.1	-	19.4	=	+ 6.7		20.9
11.	DCMU + S_{13}	18.6	-	19.4	=	- 0.8		0.0

Conditions: Same as in Figure 11. Concentrations: Oxidized or reduced DCPIP, 1×10^{-3} M; MV, 1×10^{-3} M; DCMU, 2.5×10^{-5} M; $PMSH_2$, 1×10^{-3} M; cycloheximide, 1.7×10^{-5} M; and S_{13} (5-Cl, 3-*t*-butyl, 2'-Cl, 4'-NO_2-Salicylanide, 10^{-6} M), 5×10^{-6} M. DCPIP and PMS were reduced by 1×10^{-2} M sodium ascorbate. All data are an average of at least 3 experiments, except cycloheximide and S_{13} data which are an average of 2 each. For comparison, cycloheximide and S_{13} data were normalized to DCMU values.

*1 relative dark ATP unit represents 0.074 µmoles mg Chl^{-1}.
**1 relative ΔATP unit represents 13.3 µmoles mg Chl^{-1} hr^{-1}.

compete for the electrons from the complete photosynthetic electron transport pathway at X with the $NADP^+$ reduction pathway from X and, possibly, the cyclic photophosphorylation pathway (29, 106) whereas oxidized DCPIP is known to be primarily a photosystem II electron acceptor (112). Probably, oxidized DCPIP accepts electrons beyond the non-cyclic photophosphorylation coupling site(s) somewhere near plastocyanin and cytochrome f.) The fact that oxidized DCPIP did not stimulate the control value as much as MV may be due to its absorption of light which was, in turn, unavailable to the photosystems.

In the presence of 2.5×10^{-5} M DCMU plus 1.2×10^{-5} M oligomycin, the ΔATP value was reduced by 88 percent (Table 9, line 4). This low value was typical for most (more than 19) experiments using DCMU, as mentioned in this chapter, section E. However, this value has been observed to range from a low of about 3 percent to a high of about 37 percent in non-synchronously grown Chlorella cells under regular light conditions in measurements separated by days or months. The reason for the variation is not known. (It must be remembered that only the net ATP level was being measured, so the ΔATP level would probably reflect the difference between synthesis and utilization, chapter I, section B.) The addition of 1×10^{-3} M MV had no effect on the DCMU plus oligomycin treated Chlorella suspensions regarding the ΔATP levels (Table 9, lines 4 and 5), refer to description of Figure 11.

The addition of 10^{-3} M $DCPIPH_2$ or $PMSH_2$ in the presence of DCMU restored a significant portion of the control ΔATP value. The added $DCPIPH_2$ increased the ΔATP level to about 30 percent (line 6) and $PMSH_2$ increased it to about 42 percent of the control (line 7). Subsequent addition of 1×10^{-3} M MV further increased the ΔATP value by two fold (lines 8 and 9): the $DCPIPH_2$ value rose from about 30 percent to about 72 percent and the

$PMSH_2$ value rose from about 42 percent to about 83 percent (lines 8 and 9, respectively) of the control.

Although DCMU was completely blocking the electron transport pathway from photosystem II to photosystem I (see absence of oxygen evolution, Figure 7), stopping the Calvin-Benson-Bassham CO_2 fixation cycle reactions through the loss of reducing power, we were convinced by the preceding experiments that some endogenously supported cyclic photophosphorylation was occurring (see discussion, next chapter). Since reactions other than CO_2 fixation, such as cytoplasmic protein synthesis, utilize ATP, it occurred to us that the use of the specific inhibitor of cytoplasmic protein synthesis, cycloheximide, might further reduce ATP utilization and create an increase in the apparent cyclic photophosphorylation level. On two consecutive occasions, 1.7×10^{-5} M cycloheximide induced an increase in the ΔATP level which had been established in the presence of DCMU (2.5×10^{-5} M) and oligomycin. This increase was only 12 to 21 percent (Table 9, lines 4 and 10). Whether the values are significant or not, the trend appeared to be clear and consistent.

A further test of any photophosphorylation is whether or not it can be uncoupled. The addition of the powerful uncoupler S_{13} (5-Cl, 3-<u>t</u>-butyl, 2'-Cl, 4'-NO_2-salicylanilide) completely eliminated the ΔATP level which had been present in the presence of oligomycin and DCMU only (Table 9, lines 4 and 11). The decrease, as a percentage of the control, was from 12 to zero.

It should be pointed out that 1×10^{-2} M sodium ascorbate, in the presence of 1.2×10^{-5} M oligomycin, had no apparent effect on the Chlorella cells. MV appears to cause a slight depression of the dark ATP level, but the ΔATP changes are so large it is probably insignificant.

Data obtained after blue (340 nm to 620 nm) light (incident intensity, 1.1×10^5 ergs cm^{-2} sec^{-1}) illumination showed results similar to those obtained with red (> 640 nm) light. These data are not included in Table because all the treatments were not examined. But of those examined, all were consistent with the data for red light. The ΔATP levels, obtained by blue light exposure, were generally slightly larger than the red light levels, probably due to the increased number of absorbed quanta in the blue region. The blue light intensity was equal to the red light intensity.

I. The Effect of Various Poisons on the Relative Light Minus Dark ATP (ΔATP) and Net Oxygen Evolution Levels from the Blue-Green Algae Anacystis nidulans and Synechococcus lividus

The measurement of ATP or oxygen evolution in the thermophile Synechococcus at 65°C has not been reported before. At least one attempt has been made to measure *in vivo* ATP levels from Anacystis in the presence or absence of DCMU (11). Oxygen evolution measurements, of course, have been reported for Anacystis many times (97, 113, 114). For comparative purposes the techniques and buffers used were nearly the same as those used in the Chlorella studies. Thus, the values reported for the blue-green algae may be less than what could be obtained, but our results valid for comparison with the Chlorella data. The purpose of these experiments was to see if there were any gross differences between the prokaryotic blue-green algae and the eukaryotic green algae regarding their light and dark ATP levels that might suggest differences in their photosynthetic apparatus, especially photophosphorylation.

From preliminary experiments on Anacystis it was apparent that the light minus dark (ΔATP) levels from cells suspended only in carbonate-

bicarbonate buffer, 0.1 M, pH 8.2, are negative, just as observed in Chlorella (this chapter, section B). We therefore attempted to obtain positive ΔATP values by adding oligomycin, as we were able to do with Chlorella (section C). Much to our surprise, the addition of various concentrations of oligomycin not only lowered the dark ATP level, but also the light level. Furthermore, the light minus dark ATP (ΔATP) level remained negative (Table 10, lines 1 through 6). The ΔATP level in Anacystis only became positive when DCMU (2.5×10^{-5} M) was added in addition to oligomycin (Table 10, line 7). We abandoned the use of oligomycin.

Since the addition of 2.5×10^{-5} M DCMU permitted us to obtain positive ΔATP values, the obvious experiment was to compate the effect of various concentrations of DCMU on both the net oxygen evolution and the ΔATP levels. As the concentration of DCMU is increased from zero to 2.5×10^{-5} M, the net oxygen level is lowered and the ΔATP level becomes less negative, changing to positive at 2.5×10^{-5} M DCMU (Table 11, lines 1 to 3 and Figure 12). Even though from the table and the figure, there appears to be oxygen evolution (at 20 sec. illumination) in the presence of 2.5×10^{-5} M DCMU, this is probably false due to the effect of light on O_2 uptake (55, 66, 123). If we take the oxygen spike as the true index of photosynthetic electron transport from photosystem II to photosystem I, then electron transport is shown to be inhibited by this concentration of DCMU because the spike is completely abolished. Fluorescence studies on Anacystis have also been interpreted to suggest that non-cyclic electron transport is completely inhibited by this concentration of DCMU (97). We conclude that in Anacystis, positive ΔATP values can be obtained in unpoisoned cells only when the non-cyclic electron transport from photosystem II to photosystem I is completely blocked by the presence of 10^{-5} M DCMU.

Table 10. Relative Light Minus Dark ATP (ΔATP) Levels in Anacystis as a Function of the Concentration of Oligomycin.

Conditions	ATP Levels, Relative Units				
	Light	-	Dark*	=	ΔATP**
1. Control	28.4	-	33.0	=	- 4.6
2. + Oligomycin (1.2×10^{-7} M)	14.8	-	15.9	=	- 1.1
3. + Oligomycin (6.0×10^{-7} M)	13.7	-	14.8	=	- 1.1
4. + Oligomycin (2.4×10^{-6} M)	12.8	-	14.6	=	- 1.8
5. + Oligomycin (6.0×10^{-6} M)	12.6	-	14.5	=	- 1.9
6. + Oligomycin (1.2×10^{-5} M)	12.6	-	14.8	=	- 2.2
7. + Oligomycin (1.2×10^{-5} M)+DCMU	14.2	-	12.0	=	+ 2.2

Conditions: Cells suspended in carbonate-bicarbonate buffer, 0.1 M, pH 8.2; absorbance, 0.3 at 678 nm; illumination, 20 seconds, orange (> 540) light (4×10^5 ergs cm^{-2} sec^{-1}) at 25°C; concentration of DCMU, 2.5×10^{-5} M. Data are an average of 2 experiments.

*1 relative dark ATP unit represents 0.074 μmoles ATP mg Chl^{-1}.
**1 relative ΔATP unit represents 13.3 μmoles ATP mg Chl^{-1} hr^{-1}.

Table 11. Relative Net Oxygen Evolution and Light Minus Dark ATP (ΔATP) Levels as a Function of DCMU Concentration from Anacystis.

Conditions	ATP Levels, Relative Units: Light - Dark* = ΔATP**	Oxygen Level, Relative Units: Net O_2 Evolution	Percent Control
1. Control	35.1 - 39.2 = - 4.1	253	100.0
2. + DCMU, (2.5×10^{-7}M)	36.7 - 37.9 = - 1.2	158	62.5
3. + DCMU, (2.5×10^{-5}M)	42.0 - 37.1 = + 4.9	12	4.7

Conditions: Same as Table 10. Oxygen levels measured under similar conditions on the platinum rate electrode. Data are averages of 2 experiments.

*1 relative dark ATP unit represents 0.074 μmoles ATP mg^{-1} Chl^{-1}.
**1 relative ΔATP unit represents 13.3 μmoles ATP mg Chl^{-1} hr^{-1}.

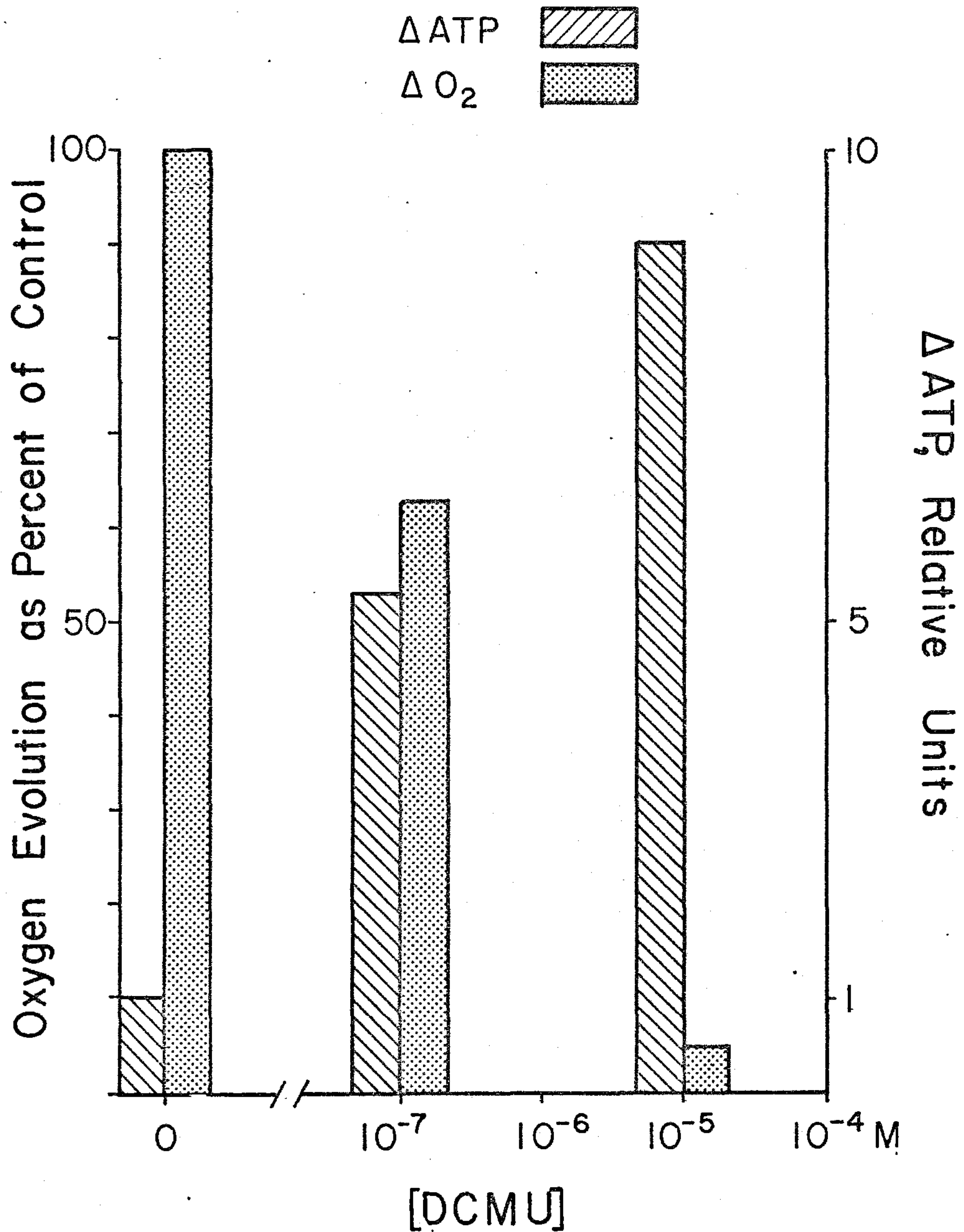

Figure 12. Relative net oxygen evolution and light minus dark ATP (ΔATP) levels as a function of DCMU concentration from Anacystis.

Conditions: Same as Table 10. Oxygen measured under similar conditions on the platinum rate electrode. Data are averages of 2 experiments. For convenience ΔATP; -4.1 = 1, -1.2 = 5.3, and +4.9 = 9.0.

To be certain the experiments were being done in saturating light, since Chlorella experiments were usually done in saturating light, we did some experiments in the presence or absence of 10^{-5} M DCMU as a function of light intensity. In the absence of DCMU (carbonate-bicarbonate buffer only) the ΔATP values remained negative and constant until an intensity of about 5×10^4 ergs cm^{-2} sec^{-1} was reached. As the intensity was increased, the values tended to become more negative and saturate at about 4×10^5 ergs cm^{-2} sec^{-1} (Figure 13, dashed line). In the presence of 2.5×10^{-5} M DCMU, the results were similar but the values remained positive at all intensities and the saturation of positive ΔATP levels occurred at about 4×10^5 ergs cm^{-2} sec^{-1}. (Refer to Figure 13, solid line.) Saturation was verified by further illumination with white light (see emission spectrum, chapter II) (7.0×10^5 ergs sm^{-2} sec^{-1}, solid circles). Therefore, in contrast to Chlorella, the ΔATP levels as a function of light intensity appears to have a single saturation level in Anacystis, rather than two. The lag in ATP synthesis is confirmed. (See chapter IV, section D for discussion.) Since it is the number of absorbed quanta which are important for photoreactions, not the incident intensity, the apparent saturation level intensity for the two algal types in the presence of 10^{-5} M DCMU may not be comparable. Furthermore, the ΔATP level reflects the rate of utilization as well as synthesis of ATP in Chlorella and Anacystis and these, apparently, are very different in these algae. Thus, the two light curves may not be comparable.

To see whether the positive ΔATP values obtained in the presence of DCMU from Anacystis could be uncoupled as they were in Chlorella (see Table 9), we examined the effect of the uncoupler S_{13} (5-Cl,3-t-butyl,2'-Cl,4'-NO_2-sali-

Figure 13. Relative light minus dark ATP (ΔATP) levels from Anacystis as a function of light intensity in the presence or absence of DCMU.

Condition: Same as in Table 10, except varied intensity of orange (> 540 nm) light with neutral density filters. White light intensity, (7×10^5 ergs cm^{-2} sec^{-1}) (see emission spectrum, Chapter II). Data are averages of 2 experiments.

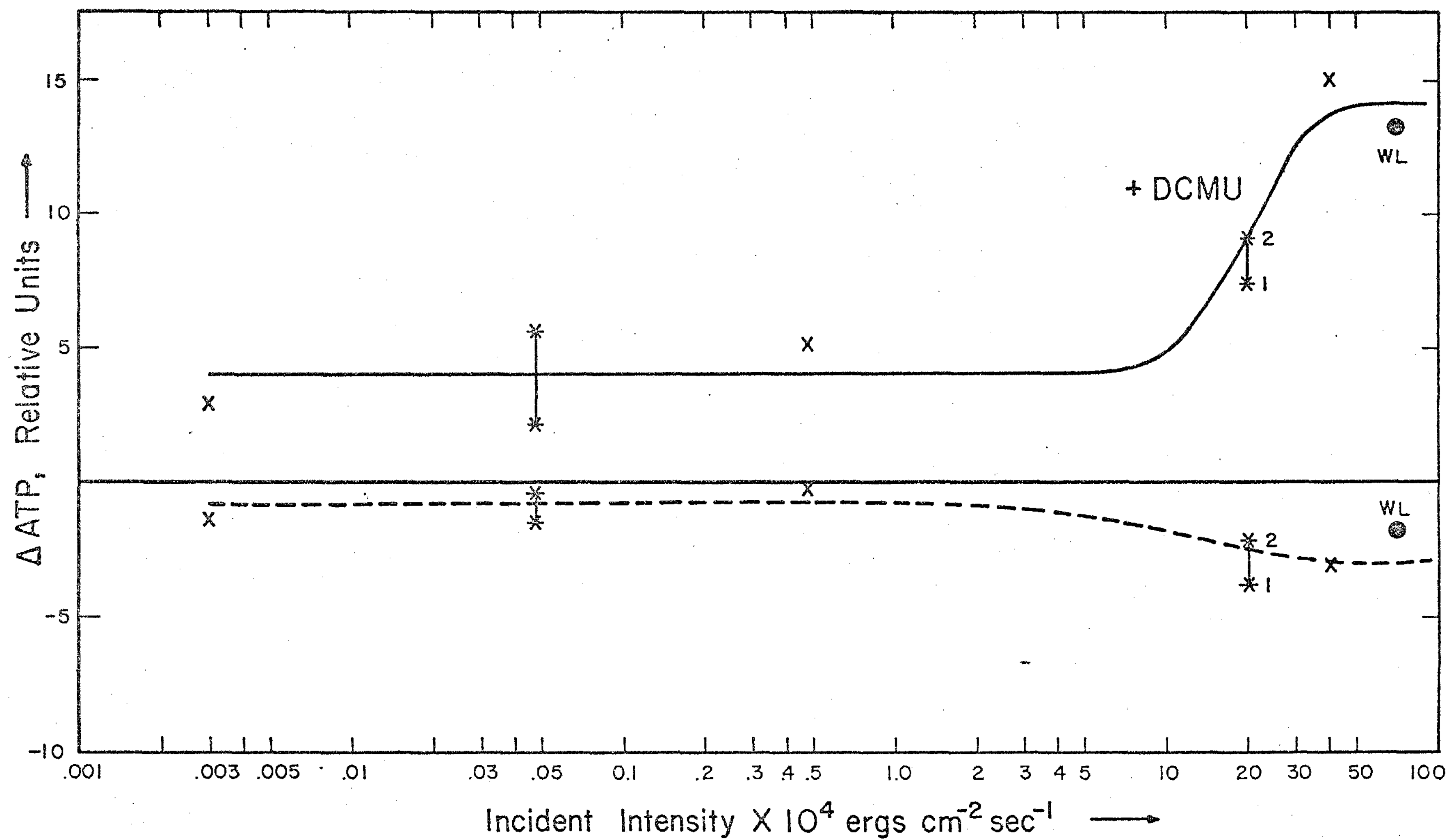
15
10
5
-5
-10
ΔATP, Relative Units
+ DCMU
WL
WL
2
1
2
1
.001
.003
.005
.01
.03
.05
0.1
.2
.3
.4
.5
1.0
2
3
4
5
10
20
30
50
100
Incident Intensity X 10⁴ ergs cm⁻² sec⁻¹

Table 12. Relative Net Oxygen Evolution and Light Minus Dark ATP (ΔATP) Levels for Anacystis and Synechococcus in the Presence or Absence of DCMU, S_{13} or MV.

A. Net Oxygen Evolution, Relative Units	Anacystis+	Synechococcus
1. Control	80	92
2. + DCMU	5	32

B. ATP Values, Relative Units	Anacystis: Light - Dark* = ΔATP**	Synechococcus: Light - Dark* = ΔATP**
1. Control	36.7 - 38.1 = -1.4	33.5 - 22.2 = +11.3
2. + DCMU	46.7 - 38.0 = +8.7	34.8 - 22.0 = +12.8
3. + DCMU + S_{13}	24.6 - 28.2 = -3.6	-- -- --
4. + MV	-- -- --	24.1 21.7 = + 2.4

Conditions: Same as for Table 10, except that the temperature for Anacystis and Synechococcus experiments were 25°C and 65°C, respectively. Concentration of: DCMU, 2.5×10^{-5} M; S_{13}, 5×10^{-6} M; MV, 1×10^{-3} M. Oxygen measurements were made under similar conditions on the concentration electrode (Clark-type). Data are an average of 2 experiments.

+Anacystis measurements were done in collaboration with P. Mohanty.
*1 relative dark ATP unit represents 0.074 μmoles ATP mg Chl^{-1}.
**1 relative ΔATP unit represents 13.3 μmoles ATP mg Chl^{-1} hr^{-1}.

cylanilide) on this alga. As in Chlorella, this uncoupler not only eliminated the positive ΔATP level that was present in the presence of 10^{-5} M DCMU, but caused the ΔATP level to become negative (Table 12B, lines 1-3).

In an effort to see whether the effects of DCMU may somehow be unique to Anacystis we decided to examine the effects of 10^{-5} M DCMU on the thermophile Synechococcus which grows at 65°C. To our surprise, the ΔATP level from Synechococcus was positive in the absence of DCMU. As in Anacystis, the presence of 10^{-5} M DCMU did not decrease the ΔATP level but permitted it to become more positive, however, to a lesser degree. When 1×10^{-3} M MV was added in the absence of DCMU to ΔATP level was strongly reduced, not stimulated as was the case in Chlorella. The MV had little or no effect on the dark ATP level. It should be pointed out that the reason for adding MV was the same as that that we discussed earlier in section H. MV should compete for electrons at X for the cyclic flow, just as it does for the electrons which would enter the $NADP^+$ reduction pathway. Thus, MV data suggests that in Synechococcus we may be seeing "cyclic" ATP.

In the presence of DCMU, the effect on oxygen evolution appeared to be similar to that reported and discussed earlier (see Figure 12 and Table 11). If the DCMU blocks all electron transport between photosystem II and photosystem I and the residual oxygen observed after 20 seconds is due to a photoinhibition of respiration, then Synechococcus appears to be more strongly inhibited than Anacystis (Table 12, lines 1 and 2).

We did not do a complete concentration study on Synechococcus as we did on Anacystis regarding the effect of oligomycin, and thus, we do not show a table of this data; however, the addition of 1.2×10^{-5} M oligomycin did lower both the light and the dark ATP level by about 50 percent as it

did in Anacystis (Table 10). Therefore, the effect of oligomycin on blue-green algae may be a universal one.

IV. DISCUSSION

A. Introduction

As mentioned previously (chapter II, section A), several investigators, including ourselves, have reported the absence of the Emerson enhancement effect for oxygen evolution in algal cells (21, 88, 89, 90, 91). We observed it repeatedly in high light exposed (HLE) Chlorella cells and always observed enhancement in regularly grown (RG) Chlorella cells (Table 1). (See chapter II, section A for the growth conditions and section C, same chapter, for method of calculating enhancement.) At the time these observations were made, and in conjunction with other work that was being done in our laboratory, a model was proposed to explain the lack of enhancement by the HLE cells (21). Without going into detail, the model predicted that HLE cells should have a larger amount of cyclic photophosphorylation when compared to RG cells and/or that they should have much larger dark ATP levels than RG cells. (We assumed that the high energy intermediate HEI of their model could be substituted for the ATP.) Most investigators now believe that ATP can and does cross the chloroplast membrane in either direction (49, 115-122).

Before proceeding it was necessary to be certain we could observe photophosphorylation *in vivo*. As mentioned previously, apparently one of the big problems with *in vivo* ATP measurements in algae was that the ATP values obtained from cells exposed to the light were usually less than those from cells which had been maintained in the dark; in other words, the light minus dark ATP (ΔATP) levels were usually negative (see chapter I, section A (10, 48).) Apparently, in Chlorella, the total ATP utilization in the light

exceeds the initial ATP level obtained from cells which are maintained in the dark for at least the first 10 minutes of illumination (Tables 3 and 5). Since the ratio of the negative ΔATP level to the dark ATP level remains constant as the absorbance of chlorophyll *a* (678 nm) is increased to a value of about 1.0 (Table 2), the negative ΔATP levels appear to be due to more than just a simple shading effect (up to at least an absorbance of 1.0 at 678 nm, the absorbance of the suspension is almost directly proportional to the number of cells present, see chapter II, section B).

B. Effects of Oligomycin

Because we were measuring the net flux of ATP between the synthesis and utilization reactions, we wanted to limit synthesis by other than photophosphorylation and to at least dampen the utilization reactions so that we could observe positive ΔATP levels (see Introduction, chapter I, section B). Only when positive light minus dark ATP (ΔATP) levels were obtained did we believe that we were truly observing photophosphorylation *in vivo*. After trying anaerobiosis (Table 3) and various poisons we found that 1.2×10^{-5} M oligomycin produced the optimum effect (Table 4), permitting relatively constant positive ΔATP levels to be obtained between 10 and 30 seconds of white light illumination (Table 5). 1.2×10^{-5} M oligomycin apparently had little effect on the ATP values obtained after exposure to light, but always suppressed the dark ATP levels by at least 50% (Tables 4 and 7). Although we know of no other reports of the effect of oligomycin on whole algal cells, the phosphorylation data is in general agreement with results obtained from various organelles (35). However, unlike the work reported for chloroplasts and mitochondria, this concentration of oligomycin suppressed the oxygen evolution of RG cells by about 14

percent and only reduced the dark oxygen uptake by about 50 percent (Table 6). Oxygen evolution in isolated chloroplasts was unaffected and the oxygen uptake by isolated mitochondria was reduced to approximately zero (35). Obviously the effects on whole cells is more complex, perhaps leading to a partial shutdown of the Calvin-Benson-Bessham cycle due to a back-up of intermediates (see Introduction, chapter I, section B). Therefore, we can suggest that the effect of oligomycin in vivo is not only to suppress respiration but to have an indirect suppressive effect on ATP utilization, possibly including, at least partially, the Calvin-Benson-Bassham cycle. (In this connection, we note that the addition of oligomycin to guinea pig brain tissue has been observed to stimulate lactic acid production (124).) Furthermore, the addition of oligomycin to Chlorella might be expected to have an even stronger, though similar, effect than would occur in most other plants. As the result of photosynthetic $C^{14}O_2$ fixation studies, it has been shown in Chlorella that 5 percent or less of the photosynthetically fixed CO_2 can be accounted for in sucrose after 3 minutes of illumination, whereas in most higher plants nearly all the fixed CO_2 ends up in sucrose (63). Instead, Chlorella seems to favor an increase in the reductive syntheses in the light (125). Therefore, the addition of oligomycin to Chlorella could be expected to not only back up the glycolytic and the four carbon acid pathways, but to ultimately suppress the Calvin-Benson-Bassham cycle (1). This appears to be what happens and to be what allowed us to obtain positive ΔATP values in Chlorella. It should be pointed out that the addition of oligomycin to the blue-green algae Anacystis and Synechococcus severely suppressed both the light and the dark ATP levels (Table 10), at all concentrations used for Chlorella. This would be in agreement with the idea that the photosynthetic apparatus in blue-green algae should be called the

photosynthetic-respiratory thylakoid because it suggests that the ATP uses for photophosphorylation and oxidative phosphorylation are identical in those algae (126). As we have pointed out, this is in contrast to what we have observed in Chlorella and what has been observed between isolated spinach chloroplasts and mitochondria. The Chlorella and spinach chloroplasts appear to have oligomycin insensitive ATP-ases whereas mitochondrial ATP-ases are extremely sensitive (35). Our finding may raise one further question for the evolutionists. Why would it be necessary for chloroplasts to develop a completely different ATP-ase from the mitochondrial ATP-ase if both these organelles were originally prokaryotic symbionts to the eukaryotic organisms (23)?

C. Inhibition of Non-Cyclic Electron Transport

As mentioned in the Introduction (chapter I, section B) the crucial experiment for this thesis would be to separate non-cyclic and cyclic photophosphorylation *in vivo*. To separate non-cyclic and cyclic transport (and hence, photophosphorylation) it is necessary to completely block the non-cyclic electron flow and yet leave the cyclic electron flow intact. This should be possible since cyclic photophosphorylation is independent of the non-cyclic electron flow in which oxygen is evolved and $NADP^+$ is reduced (chapter I, section A, Figure 1, and section B).

Previous investigators have observed that the addition of 10^{-5} M DCMU will completely block all non-cyclic electron transport from photosystem II to photosystem I, (73, 74, 96) and will permit the PMS mediated cyclic photophosphorylation in chloroplasts to remain unaffected (95). Our results show that 10^{-5} M DCMU does completely eliminate all oxygen evolution by RG and HLE Chlorella cells (Figures 7 and 8, respectively) and by Anacystis

(Figure 12). The answer may not be as clear in the case of Synechococcus (Table 12). For the blue-green algae (Tables 11 and 12; Figure 12) it should be noted that a residual steady-state oxygen level (measured at the end of 20 seconds illumination) appears to remain. If the oxygen spike (observed on the platinum rate electrode) is taken as the true index of photosynthetic electron transport from photosystem II to photosystem I, the inhibition by 10^{-5} M DCMU was complete because at that concentration the spike is abolished. This suggests a complete inhibition of non-cyclic electron transport by this DCMU concentration. Fluorescence studies on Anacystis support this result, also (97). Previous investigators have attributed the residual steady-state oxygen level to a photoinhibition of respiration in the blue-green algae (55, 66, 123). It does seem that the residual amount of oxygen evolution by Synechococcus may be a little high for such an explanation or argument (Table 12); however, they are high temperature organisms and may simply be different in this respect.

1. The Effects of DCMU

The measurement of ATP as a function of the DCMU concentration provided the following results. At 10^{-5} M DCMU when all non-cyclic electron transport was blocked, cyclic photophosphorylation was observed to vary in RG Chlorella cells from about 4 to 30 percent of the control value, averaging about 12 percent for most experiments (Figures 6, 7, and 10; Tables 8 and 12). Only in the absence of CO_2 did we observe a maximum of 37 percent (Table 8). (It should be mentioned that this is when we would expect to observe a maximum because the utilization reactions would be blocked by the elimination of the non-cyclic electron flow. Therefore, ATP could build up as the result of the continued cyclic electron flow. It might

even occur at a higher rate if it had been competing with the non-cyclic electron flow.) Our results suggest that cyclic photophosphorylation occurs at relatively low levels in regularly grown (RG) Chlorella and that the level varies with growth conditions, cell age, and possible minor variations in experimental procedure (although we were very careful about the latter possibility).

That the level of cyclic photophosphorylation in Chlorella may vary with the growth conditions is strongly supported by the results from high light exposed (HLE) Chlorella cells. The level of cyclic photophosphorylation in the presence of 10^{-5} M DCMU was much higher (about 50 percent of the non-DCMU poisoned control, Figure 8) than the levels we observed for RG Chlorella cells (less than 37 percent, Figures 6, 7, and 10; Tables [illegible] and 12). In addition to having more cyclic photophosphorylation than the RG cells, it should also be pointed out that HLE cells have a much higher rate of oxygen uptake, even in the presence of oligomycin (Tables [illegible] and [illegible] on a chlorophyll basis. Also, they have higher initial dark ATP levels than the RG cells (Table 7). Thus, the HLE cells meet both of the expectations of Govindjee-Munday-Papageorgiou model for photosynthesis by these cells (21) having a higher cyclic photophosphorylation and respiration rate. However, we have no evidence that the higher respiration and cyclic photophosphorylation rates necessarily have anything to do with the scheme presented and the absence of the Emerson enhancement effect in these cells. The Govindjee *et al*. model was presented as an alternative to the Hill and Bendell model, not to replace it but to suggest a switching mechanism in HLE cells to an electron flow that may not permit non-cyclic photophosphorylation to occur and yet could explain the absence of the Emerson enhancement effect. It could be inferred from their model that all ATP is provided

cyclically or from respiration and that non-cyclic photophosphorylation does not occur in vivo. If this is indeed the case then the model may have to be re-evaluated. Our data clearly shows that HLE cells do have non-cyclic photophosphorylation. When oxygen evolution is completely inhibited, the ΔATP level has been lowered to about 50 percent of the non-DCMU poisoned control (Figure 8).

For the blue-green alga Anacystis, comparable data is even more exciting. In the presence of 10^{-5} M DCMU, we actually observe a stimulation in the ATP level over that of the control (Table 11 and Figure 12, also Table 12). In the absence of oligomycin, the ΔATP level is negative but becomes positive when 10^{-5} M DCMU is added. Furthermore, even though oligomycin strongly inhibits both the light and the dark ATP levels, the ΔATP level remains negative, but when 10^{-5} M DCMU is added it becomes positive in the same manner as it does in the absence of oligomycin. Since the oxygen data show that non-cyclic photophosphorylation is completely blocked, we can only conclude that the shutdown of the Calvin-Benson-Bassham cycle by DCMU prevents the utilization of cyclically produced ATP and the ATP level, in turn, builds up. Synechococcus yielded similar results but the high residual oxygen level in the presence of 10^{-5} DCMU was disturbing. We do not know for certain that the non-cyclic electron transport was stopped and we have no literature values to go to for support. Another interesting characteristic of Synechococcus was that its ΔATP levels were always positive and were not diminished, but increased, upon the addition of DCMU (Table 12). This result would suggest either that this organism is not as sensitive to this concentration of DCMU or that it has a very high rate of cyclic photophosphorylation.

2. The Effect of the Absence of Carbon Dioxide

Another method of inhibiting non-cyclic transport is to remove the terminal electron acceptor for higher plant photosynthesis, CO_2. In the absence of CO_2 (gassed cells with 100 percent argon in phosphate buffer for 30 minutes, chapter III, section G) the ΔATP level declined to an average of 36 and 15 percent (for two different series of experiments, respectively) of the control value in the presence of CO_2 (Table 8B and C). That non-cyclic photophosphorylation was stopped by this technique was corroborated by the fact that the further addition of 10^{-5} DCMU to the cells in the absence of CO_2 had no further effect. As mentioned previously, even under these conditions, in which we would expect to observe the largest amount of cyclic photophosphorylation, we never observed the cyclic rate to exceed more than 37 percent of the control rate. (Refer to discussion, page 90.)

D. Photophosphorylation as a Function of Light Intensity

Although we have been saying that we were observing photophosphorylation *in vivo*, the question arises, were we observing a artifact or a true photo-process? If we were truly observing the latter, we should observe some variation in our ΔATP levels as a function of light intensity. We observed changes in the ΔATP levels as a function both of white (Figure 9) and of various wavelengths of red (> 640 nm) light respectively in the case of Chlorella and orange (> 540 nm) light in the case of Anacystis.

For Chlorella, we will only discuss the red light curve. More experiments were involved in it and the various phases were more distinct. The red light curve was multiphasic, beginning with an initial lag, a small increase occurs that saturates between 1 and 4 x 10^3 ergs cm^{-2} sec^{-1},

this saturation is followed by a second rise that reaches saturation at about 1 x 10^4 ergs cm^{-2} sec^{-1} (Figure 10). The lag observed below [illegible] x [illegible] ergs cm^{-2} sec^{-1} with red (> 640 nm) light may be the same low intensity lag that has been observed for chloroplast photophosphorylation ([illegible]-129). Most of the hypotheses advanced to explain this lag involve the filling of a pool, i.e., high energy intermediates (128), hydrogen ion gradients across the chloroplast membrane (67), conformational changes (130), etc. Supposedly photophosphorylation proceeds only when the pool is "ready" (5). We made no attempt to study this low intensity lag.

The low intensity saturating curve appeared to be DCMU insensitive and saturated at about a 10 times lower intensity than the second saturation level which was DCMU sensitive. The addition of 10^{-5} M DCMU at higher intensities lowered the ΔATP level to the level of the lower intensity saturating curve. Therefore, we believe our data suggests that cyclic photophosphorylation saturates at 10 times lower intensity than the non-cyclic, DCMU sensitive saturation level in Chlorella. This conclusion would be in agreement with a large number of investigators who have used indirect methods for measuring photophosphorylation _in vivo_ (26, 27, 40, 56, 60). This may be a case where "Ontogeny recapitulates phylogeny" since recent developmental work has shown that cyclic photophosphorylation preceeds non-cyclic photophosphorylation and oxygen evolution by about 12 hours (15). These results are in direct opposition to the white light supported PMS mediated saturation curves in isolated chloroplasts (99-102). It was interesting to note that the second saturation intensity for our _in vivo_ ATP measurements with red light occurred at about the same intensity at which oxygen evolution was observed to saturate

in Chlorella (131). It was also interesting to note that the values we obtained under anaerobic conditions, at the higher saturating intensity and in the presence of CO_2, support the concept that pseudo-cyclic photophosphorylation must be virtually non-existent in whole algal cells otherwise we would expect a smaller, not an unchanged value, under these conditions (Figure 10, solid black circles).

Our conclusion as to the result of our intensity experiments with Chlorella was that we are indeed observing photophosphorylation and that cyclic photophosphorylation saturates at about 10 times lower intensity than non-cyclic photophosphorylation.

For the blue-green alga Anacystis the meaning of the intensity curve is not as clear. In the absence of DCMU the curve was always negative down to 20 ergs cm^{-2} sec^{-1}. At very high intensities (2 x 10^5 ergs cm^{-2} sec^{-1}) the curve saturated negatively (Figure 13). Of course the measurement was done with orange (> 540 nm) light and there is no way to directly compare it to what we observed in Chlorella. In the presence of 10^{-5} M DCMU we obtained the virtual mirror image, with all the values remaining positive. Again we only clearly observed a single saturation level near 2 x 10^5 ergs cm^{-2} sec^{-1}. It may be possible that only cyclic photophosphorylation occurs in Anacystis, as has been previously asserted (11), and that it saturates at much higher intensities than it does in Chlorella (note the point obtained in the presence of white light, Figure 13).

E. <u>The Effect of Electron Donors, Acceptors, Poisons and an Uncoupler</u>

It was felt that the addition of electron donors and acceptors, poisons and an uncoupler to the whole cells would not only provide more information concerning the role of cyclic photophosphorylation *in vivo*, but might provide

information concerning the number and location of the coupling sites for photophosphorylation in vivo in the algae.

The two electron donors, reduced DCPIP and PMS, and the electron acceptor MV, were selected because their effects on chloroplasts were well established (28, 29, 38, 99, 100, 103, 104, 106, 132, 133). Furthermore, they were known to, at least, enter Chlorella cells (105, 109).

Since MV is known to compete with oxidized $NADP^+$ for the electrons that are mediated by the non-cyclic electron transport pathway (28, 29, 106) and that recent evidence has also suggested that it will compete for the cyclic electron flow from X (see Figure 1) (29), it was reasoned that the addition of this compound should stimulate the amount of non-cyclic photophosphorylation over that of the control, or increase the total amount of ATP due to the fact that a simple shutdown of the Calvin-Benson-Bassham cycle would prevent utilization, creating an apparent stimulation due to the lack of utilization. The data obtained from Chlorella (Table 9, lines 1 and 3) confirmed our reasoning, *although the stimulation by MV seems* surprisingly high. The data suggested that MV may be behaving as more than just an electron acceptor in vivo. Since it has been observed that MV-type compounds are reduced preferentially to the natural oxidants, i.e., Fdx or $NADP^+$ (106), one would expect a possible build up of ATP due to an inhibition of ATP utilization by the CO_2 fixation reactions (as a result of the lack of $NADPH_2$). However, if MV also inhibits the cyclic electron flow (29), unless the relative amount of this component was very small to begin with, the contribution of ATP by this flow would be lost. Therefore, we wonder if we really should observe a 100 percent increase in the ATP level over that of the control. MV does seem to have more than one effect in vivo. Low concentrations of MV caused an inhibition of ATP production,

an effect that apparently has not been reported previously in the literature (Figure 11). Until much more work is done, we do not wish to speculate on the cause(s) of the inhibition.

We also made one other observation that seems contradictory in the case of Chlorella but consistent in the case of the blue-green alga Synechococcus. It was mentioned that MV has been observed to compete for the cyclic electron flow of spinach chloroplasts (29). Furthermore, we are fairly certain from the previous data presented in this thesis that Chlorella has some cyclic photophosphorylation and that the blue-green algae have, relatively, much more of it. The addition of MV to the blue-green alga Synechococcus inhibited its ΔATP level as expected (Table 12). However, when MV was added in the presence of DCMU in Chlorella, the level remained essentially unchanged (Table 9, lines 4 and 5), although we would have expected a lowering of that level also. We have no explanation yet.

Although oxidized DCPIP is colored and absorbs light throughout the region in which the algae absorbed light (Figure 5), we believed that it might behave as an electron acceptor like MV because various investigators have suggested that it too should compete for the non-cyclic electron flow to $NADP^+$ by accepting electrons either prior to P700 (110), or at X (see Figure 1) (28). In either case, non-cyclic photophosphorylation should be stimulated by its addition or the Calvin cycle utilization reactions should be blocked creating an apparent stimulation due to the lack of utilization. We did observe such a stimulation (Table 9, lines 1 and 2). The magnitude of the change is much lower than that observed for MV, probably due to the competition for absorbed light quanta (see Figure 5), or it may not accept electrons as efficiently _in vivo_ as MV.

The data observed as a result of these added electron acceptors also suggests that the relative levels of ATP we observe in vivo are low for reasons other than just energy charge limitations (i.e., the lack of ADP (134)). They are apparently low due to the rate of utilization of ATP because in the presence of a non-cyclic electron acceptor (MV or oxidized DCPIP), under conditions when the CO_2 fixation cycle is inhibited (by the lack of $NADPH_2$), we can observe ΔATP levels that exceed those of the control (Figure 11 and Table 9). Thus, the level of ADP or P_i does not seem to be limiting in these cells.

The addition of electron donors did not clarify the location of the cyclic and non-cyclic coupling site(s). In the presence of DCMU, the addition of $DCPIPH_2$ or $PMSH_2$ increased ΔATP levels in the presence of DCMU by about 3 fold, but these donors could not restore the ΔATP levels of the DCMU free control samples (Table 9, lines 6 and 7). The results confirm the findings that $DCPIPH_2$ and $PMSH_2$ donate electrons to the electron transport pathway of photosynthesis after the DCMU inhibition site (28, 38, 100, 107, 135) because the donation apparently causes a partial restoration of electron transport. $DCPIPH_2$ is usually less effective than $PMSH_2$ for mediating cyclic photophosphorylation (5), therefore the $DCPIPH_2$ value was expected to be a little lower than the $PMSH_2$ value, as observed. Surprisingly, results from the further addition of MV did not support the concept that $DCPIPH_2$ or $PMSH_2$ were mediating cyclic photophosphorylation in the presence of DCMU, unless MV accepts electrons just after the cyclic coupling site. (This would be contrary to the finding that MV competes for the cyclic electron flow at X (29). However, at least one investigator places the cyclic coupling site very close to X (136).) The addition of MV to $DCPIPH_2$ or $PMSH_2$ plus DCMU and oligomycin treated cells doubled the ΔATP

levels obtained with the MV free control (Table 9, lines 8 and 9). An alternative explanation may be that $DCPIPH_2$ or $PMSH_2$ are donating prior to the non-cyclic coupling site in the presence of DCMU and because oxidized NADP is present due to our in vivo conditions, "non-cyclic" electron transport to $NADP^+$ is partially restored. The amount of transport is lower than that obtained from the control in the absence of DCMU because the ability of these donors to donate electrons may be rate limiting in vivo.

We mentioned previously that many investigators believe that the P/e_2 ratio for non-cyclic photophosphorylation is greater than one (28, 30, 31, 92). This would suggest that at least two coupling sites are present on the non-cyclic electron pathway. One group, which favors two such sites prior to P700, has obtained evidence from subchloroplasts particles which suggests that $DCPIPH_2$ donates to both sites whereas $PMSH_2$ only donates to one of the two sites (28). This would mean that the addition of $DCPIPH_2$ should produce twice as much ATP as $PMSH_2$ in the presence of DCMU. Since we find the ratio for $DCPIPH_2$ to $PMSH_2$ mediated photophosphorylation in Chlorella to be about 1:1 (Table 9, lines 7 and 8; 9 and 10), rather than 2:1 as they predicted, our results may indicate that in vivo both $DCPIPH_2$ and $PMSH_2$ donate electrons to the same site. Therefore, we believe that our data strongly suggests that all the ATP formed in the presence of DCMU upon the addition of either $DCPIPH_2$ or $PMSH_2$ in the presence or absence of MV, is formed by "non-cyclic" photophosphorylation in Chlorella. In the absence of MV, some cyclic photophosphorylation may be occurring but the amount must be so low that the values are masked by the larger "non-cyclic" photophosphorylation values. However, if cyclic and "non-cyclic" photophosphorylation were occurring together we wonder why the ΔATP values in

the presence of $DCPIPH_2$ or $PMSH_2$ should be so much lower than the control values, unless they are poor donors in vivo.

An additional result involving PMS was observed that was not expected. Oxidized PMS (1×10^{-3} M) caused the small positive ΔATP values obtained in the presence of DCMU to be essentially eliminated (Figure 11). The result was not due to the use of a relatively high concentration of PMS because lower concentrations of partially reduced PMS (5×10^{-5} M PMS and 2.5×10^{-5} M sodium ascorbate) produced identical results. Furthermore, the latter results could be obtained under anaerobic as well as aerobic conditions. (These results are not shown.) It should further be stated that neither the partially reduced PMS not the higher concentrations of oxidized PMS showed any effect on the dark obtained ATP levels. Whatever we were observing seemed to be a light mediated phenomenon.

The results mentioned above were not expected because both oxidized and reduced PMS have been observed to mediate cyclic photophosphorylation in light in the presence of DCMU in isolated chloroplasts (99, 100). Furthermore, both oxidized and reduced PMS showed a similar quenching of fluorescence in the presence of DCMU in Chlorella cells (97). However, the latter result was explained in terms of a membrane conformational change, rather than as an effect on photophosphorylation. The possibility of a chemically induced artifact was ruled out because the reduction of PMS with a 10 times larger concentration of sodium ascorbate produced results which were identical to those obtained for reduced PMS (Table 9, line 7 and Figure 11). Furthermore, the absorption spectrum of the oxidized PMS was identical to that published previously (Figure 5 (112)). The results suggest that oxidized or partially reduced PMS behave as an electron "sink" in vivo, operating prior to the coupling site, preventing even the endogenous donation

to the coupling site(s) for cyclic photophosphorylation in the presence of DCMU in Chlorella cells. To our knowledge, such a finding has not been reported in the literature.

Although DCMU stops non-cyclic photophosphorylation (137), in whole Chlorella cells, other ATP utilization reactions could still be partially supplied by endogenous cyclic photophosphorylation. Therefore, it was reasoned that by inhibiting a cytoplasmic utilization process, the net, cyclically produced, ATP level should be increased in the presence of DCMU. When the well-known inhibitor of cytoplasmic protein synthesis, cycloheximide, was added in the presence of DCMU, a significant increase in the ΔATP level was observed over that obtained in the presence of DCMU alone (Table 9, lines 4 and 6) (138). Not only did this result add support to the fact that we were observing low levels of cyclic photophosphorylation *in vivo* but it also adds support to the hypothesis that molecular ATP can cross the chloroplast membrane (49, 115-122).

The final piece of evidence in support of the fact that photophosphorylation occurs *in vivo* in both the green and the blue-green algae comes from the addition of the uncoupler S_{13} (10^{-6} M). The level of cyclic photophosphorylation in the presence of 10^{-5} M DCMU for both Chlorella and Anacystis was eliminated upon the addition of this uncoupler (Tables 9 and 12, respectively).

V. SUMMARY

It can be concluded on the basis of the data presented in this thesis that both the oligomycin treated (1.2×10^{-5} M) green alga Chlorella and the untreated blue-green algae Anacystis and Synechococcus show the presence of cyclic photophosphorylation under conditions in which the non-cyclic electron flow from photosystem II to photosystem I is blocked by the presence of 10^{-5} M DCMU. In Chlorella this was additionally supported by the results of similar experiments done in the absence of the terminal electron acceptor for the Calvin Benson-Bassham cycle, carbon dioxide, with or without DCMU. That we were indeed observing cyclic photophosphorylation comes from the fact that a light curve obtained in the presence of DCMU showed saturation levels for the process as a function of intensity. In Chlorella, cyclic photophosphorylation saturates at about 1×10^{3} ergs cm^{-2} sec^{-1} and non-cyclic photophosphorylation (in the absence of DCMU) at about 1×10^{4} ergs cm^{-2} sec^{-1} red light (> 640 nm).

The level of cyclic photophosphorylation *in vivo* in Chlorella seems to be primarily dependent on the previous growth conditions, varying from an average of less than 20 percent in regularly grown (moderate light) to 50 percent in cells exposed to very high white light intensities for the 24 hour period immediately preceeding the experiments. These high light exposed cells, also characteristically show a large dark respiration (oxygen uptake) level and an absence of the Emerson enhancement effect.

The additions of the photosystem I electron acceptor MV to unpoisoned (treated only with oligomycin) Chlorella cells and to (untreated) Synechococcus cells indicates that non-cyclic photophosphorylation is the favored

pathway in Chlorella, whereas cyclic photophosphorylation appears to be favored in the blue-green algae. In Chlorella this fact was further supported by the addition of the electron donors $DCPIPH_2$ and $PMSH_2$ in the presence of DCMU. In the presence or absence of MV the results showed that these two donors probably feed non-cyclically in vivo. Also in Chlorella, the addition of the cytoplasmic protein inhibitor cycloheximide in the presence of DCMU slightly stimulated the cyclic photophosphorylation level observed in the presence of DCMU alone. This would support the idea that photosynthetically produced ATP can be used for other than just carbon fixation reactions and that it can pass through the chloroplast membrane. That the control of the ATP levels in Chlorella cells is mediated primarily by the utilization processes rather than the limitation of the availability of ADP is supported by the fact that the control ΔATP (light minus dark ATP) level could be stimulated by more than 100 percent by the addition of MV. Completely untreated (no oligomycin or other poisons) Chlorella always showed negative ΔATP levels for up to 10 minutes illumination regardless of the suspension absorbance (678 nm).

In the presence of 10^{-5} M DCMU, cyclic photophosphorylation in both Chlorella and Anacystis could be completely uncoupled by the addition of the powerful uncoupler S_{13} (5-Cl, 3-t-butyl, 2'-Cl, 4'-NO_2-Salicylanide, 10^{-6} M).

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