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Photophosphorylation in intact algae: Effects of inhibitors, intensity of light, electron acceptor and donors

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The luciferin-luciferase method was used to determine ATP extracted from dark-maintained and light-exposed samples of the green alga *Chlorella pyrenoidosa* and of the blue-green alga *Anacystis nidulans*. A few measurements on *Synechococcus lividus* (a blue-green thermophile, clone 65°C) are also reported.

1. The light-minus-dark ATP levels (ΔATP) from aerobic cells of *Chlorella* and *Anacystis* were negative; however, ΔATP from *Synechococcus* was positive. Large positive ΔATP was obtained in regularly grown (RG: moderate light) *Chlorella* treated with oligomycin; dark levels were reduced, light levels remained essentially unaffected. In high-light exposed (HLE) *Chlorella*, oligomycin reduced both light and dark ATP levels, but positive Δ ATP was still obtained. However, in *Anacystis*, which has a different organization of thylakoid membrane, oligomycin severely reduced both the light and the dark ATP levels and the ΔATP remained negative.

2. The oligomycin (12 μM) treated *Chlorella* and the untreated *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 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), or not allowed to operate by the absence of CO_2. Cyclic photophosphorylation ranged from 10–30% of the maximum ΔATP in RG, to 40–50% in HLE *Chlorella*. In RG *Chlorella*, cyclic and non-cyclic (in the absence of DCMU) photophosphorylation (ΔATP) saturate at about 10^3 ergs cm^{-2} sec^{-1} and 10^4 ergs cm^{-2} sec^{-1} red (>640 nm) light, respectively; a lag was observed in the light curve.

3. In *Chlorella*, the addition of the photosystem I electron acceptor methyl viologen (MV; 1 mM) increased ΔATP by twofold. Further addition of DCMU (25 μM) reduced this to the level observed with DCMU alone. If 1 mM reduced dichlorophenol indophenol or phenazine methosulphate ($DCPIPH_2$ or $PMSH_2$, respectively) was added along with DCMU, the ΔATP level was 30–40% of the control. Further addition of MV increased the ΔATP to be 70–80% of that of the control. These and other results confirm the presence of both non-cyclic and cyclic photophosphorylation in vivo, the former predominating in *Chlorella*, and the latter in *Anacystis* and *Synechococcus*.

Abbreviations: DA, dark adapted cells; ΔATP, light minus dark ATP levels; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; $DCPIPH_2$, reduced dichlorophenol indophenol; HLE, high light exposed cells; MV, methyl viologen; $PMSH_2$, reduced phenazine methosulfate; RG, regularly grown cells; S_{13}, 5-chloro-3-t-butyl-2′-chloro-4′-nitrosalicylanilide.

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Most measurements of photophosphorylation have been made on isolated chloroplasts, which can be induced to yield very high rates of photophosphorylation upon the addition of ADP, inorganic phosphate and various cofactors (see review, *1*). The results from such studies in isolated chloroplasts may not reflect what occurs in whole cells. For example, pseudo-cyclic photophosphorylation, observed in isolated chloroplasts, is believed not to exist in whole cells (*2*). Also, very high rates of photophosphorylation have never been obtained from whole cells. Though the presence of ATP in photosynthetic organisms was first observed in extracts from intact cells of the green alga *Chlorella* in the early 1950's (*3*, *4*), few workers measured photophosphorylation in vivo (*5*, *6*, see ref. *7* for earlier literature). Usually, under aerobic conditions, negative light-minus-dark ATP levels (ΔATP) were observed (*8*). Recently, Oelze-karow and Butler (*5*) showed definite positive ΔATP in greening bean leaves, feasible only because of the low drain on ATP during the early stages of development of the chloroplast.

Interpretation of the results of in vivo ATP measurement is complex. The measured in vivo ATP levels reflect the transitory ATP pool that exists as a result of the flux between the rates of synthesis (by photophosphorylation and oxidative phosphorylation) and the rates of utilization (by photosynthetic CO_2 fixation and other synthetic reactions). The obvious starting point for a study of in vivo photophosphorylation is to diminish or shut down oxidative phosphorylation by the addition of specific poisons, anaerobiosis, or a combination of both. Furthermore, conditions should be chosen to diminish or shut down ATP utilization processes. This can be accomplished partially by using specific poisons and/or using short-term light exposures, as photosynthetic CO_2 fixation (utilizing ATP) is a slow process (see Bassham, *9*). (Most attempts to measure in vivo photophosphorylation have involved at least several minutes of illumination (e.g., see *5*, *6*, *10*).)

Several reports on different aspects of ATP measurement have recently appeared in the literature (*11–13*). Evidence is presented in this paper that the ΔATP, normally negative in *Chlorella*, can be made positive by adding the proper poison(s), e.g., oligomycin, and using short periods of illumination ($\sim$20 sec). However, in *Anacystis*, oligomycin decreases both dark and light-induced phosphorylation, maintaining negative ΔATP. We report parallel measurements of O_2 evolution and ΔATP in oligomycin-treated *Chlorella* with increasing concentrations of DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea). At those concentrations of DCMU when O_2 evolution was reduced to zero, positive ΔATP was measured (cyclic phosphorylation). In blue-green alga *Anacystis* (or *Synechococcus*, without oligomycin) ΔATP changed from negative (or less positive) to positive (or more positive) values as the DCMU concentration was increased, suggesting a relatively important role of cyclic phosphorylation in these algae. Experiments with added electron acceptor, methyl viologen (MV) and donors, reduced 2,6-dichlorophenol indophenol and phenazine methosulphate ($DCPIPH_2$ and $PMSH_2$), confirm the presence of both noncyclic and cyclic photophosphorylation, the former dominating in green algae and the latter in blue-green algae. Light curves of the two types of phosphorylation in vivo suggest that cyclic saturates at ten times lower intensity than noncylcic in algae.

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Materials and methods

Algal culture and sample preparation.

Chlorella pyrenoidosa and *Anacystis nidulans* were grown in inorganic media under continuous illumination as previously described (*14*). *Chllorella* grown in this manner is referred to as regularly grown (RG). Cells placed in the dark for 24 hr prior to the experiments are referred to as dark-adapted (DA). *Chlorella* cells exposed to two 100 watt tungsten bulbs (25 cm away from flask) for 24 hr preceeding the experiments are referred to as high light-exposed (HLE). *Synechococcus lividus* (a blue-green thermophile, 65°C clone) was also autotrophically grown, with some nitriloacetic acid added as a chelating agent (*15*). It was grown at 65°C, gassed and stirred with 0.5% CO_2 in air under high intensity tungsten illumination (one 500 watt and one 150 watt bulb 20 cm from flask).

Samples for the experiments were taken from the *Chlorella* culture 3 to 6 days after inoculation; 5 to 7 days for *Anacystis* and *Synechococcus*. The cells, after washing, were suspended in carbonate-bicarbonate buffer (0.1 M, pH 8.2); we chose this buffer because it provided optimum pH for ATP synthesis (*3*) and O_2 evolution (*16*) in *Chlorella*. The cells were kept in the dark for one hr, diluted in dim green light to give an absorbance of 0.3 (50% absorption) at the 678 nm chlorophyll *a* peak, and finally left for 20 to 40 min with any added chemicals for equilibration. Suspensions, when necessary, were made anaerobic by gassing during the latter dark waiting period with 100% argon.

Absorption measurements.

The absorption spectra were measured with a recording spectrophotometer (Bausch and Lomb Co., Rochester, N.Y., model Spectronic 505; half-band width, 5 nm) equipped with an Ulbricht integrating sphere.

The absorbance of chlorophyll, extracted from *Chlorella* with 100% methanol and from *Anacystis* with 80% acetone in water, was measured in another spectrophotometer (Cary Instruments, Monrovia, Calif., model 14), and its concentration was calculated according to McKinney (*17*). (Also, see Strain and Svec, ref. *18*).

Oxygen measurements.

The differential manometer (closed type) and experimental procedures for manometry were as previously described (*19*). Pressure changes due to oxygen exehange 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. Intensity measurements were made with a large surface bolometer. The rate of O_2 evolution was also measured by a stationary platinum/Ag-AgCl electrode similar to that of Bannister and Vrooman (*20*). The O_2 concentration of the algal samples was measured by using a teflon covered Clark-type O_2 electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) with a YSI model 53 monitor. The sample chamber and the electrode holder, built by one of the authors (*21*), had a small volume (1.2 ml) with the magnetic stirring at very close proximity to the electrode to increase the response and sensitivity.

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Illumination system for ATP measurements.

The illumination source for the ATP measurements was a 1000 watt tungsten lamp regulated by a (Variac) autotransformer connected to a constant voltage power supply. White light was passed through two filters, a Corning glass C.S. 1-97 and a Schott glass M978. The emission spectrum of this light, measured with an Isco spectroradiometer, had a peak at 600 nm with 50% emission at 500 and 700 nm. The total light intensity was 7.5×10^4 ergs cm^{-2} sec^{-1} as measured by a Yellow Springs radiometer (Yellow Springs Instrument Co., Yellow Springs, Ohio, model 63).

ATP measurements.

The ATP content of the algal samples was determined with a luciferin-lnciferase (firefly) technique sensitive to picogram amounts of ATP (*22*). Two ml of cell suspension was given the proper actinic illumination or dark treatment for the specified time and temperature (5°C for *Chlorella* and *Anacystis* and 65°C for *Synechococcus*). The sample was quicly (<2 sec) poured into 3.0 ml boiling glycine buffer (20 mM, NH_4^+ free, pH 7.7, from Calbiochem Corp., Los Angeles, Calif.), boiled for 15 min, and then placed in an ice bath to bring it quickly to room temperature. The tubes were then centrifuged for 10 min at $3000 \times g$ to remove cell debris. The total volume of each tube was adjusted to 5.0 ml (for other details, see ref. *21*).

For the ATP determination, 0.75 ml of cell extract was transferred to 1.0 cm pathlength glass cuvettes, mounted in the measuring instrument (see ref. *21*) containing an Electra Megadyne Inc. 9558 B photomultiplier, and injected with 0.25 ml of the firefly enzyme extract[1]. The height of the initial signal, measured with a Heathkit Servo recorder, was found to be proportional to the total amount of ATP present in the sample[2]. The amount of ATP contained in each sample was determined from an ATP standard curve, prepared using the above techniques; this curve was perfectly linear in the 0–100 nanomole range.

Results

Characteristics of regularly grown and high light-exposed Chlorella *cells.*

Before making ATP measurements, we measured some of the characteristics of RG and HLE *Chlorella*. These are summarized in Table 1. An interesting point to note is that RG (or DA) cells showed the usual Emerson enhancement ($=[R(S+L)-R(S)]/R(L)$, where R is the rate of O_2 evolution, S and L represent 650 nm and 710 nm light, respectively), *whereas* HLE cells showed a lack of it.

[1] The firefly enzyme extract 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 $MgSO_4$, pH 7.4) firefly lantern extract (Sigma Chemical Co., St. Louis, Mo., FLE-50) at room temperature. After 20 min, the milky suspension was cleared by 30 min centrifugation at $10{,}000 \times g$ at 5°C.

[2] The residual ATP in 0.25 ml of firefly enzyme (10 mg ml^{-1}), upon injection into 0.75 ml of buffer, contained 10 to 20 picomoles per ml. This is negligible, as 0.75 ml extracts from 2.0 ml of algal cells usually contain 10 to 100 nanomoles of ATP.

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Table 1 *Characteristics of regularly grown (RG) or dark adapted (DA) and high light exposed (HLE)* Chlorella *cells*

Characteristic	RG or DA	HLE
1. Number of cells (cells ml^{-1} of suspension)	15.8×10^6	7.9×10^6
2. Packed cell volume (μl of cells ml^{-1} of suspension)	0.32	0.62
3. Ratio of absorbance at 678 nm to that at 650 nm	1.3	1.67
4. Rate of dark oxygen uptake, μmoles O_2 mg Chl^{-1} hr^{-1}	3.8	14.6
5. Rate of oxygen evolution in red (>650 nm) light, μmoles O_2 mg Chl^{-1} hr^{-1}	4.6	6.0
6. Emerson enhancement of oxygen evolution	3.5	0.5

Conditions: Cells suspended in Warburg's buffer #9; absorbance of Chl, 0.3 at 678 nm. Oxygen measurements, by manometry, made in the linear portion of photosynthesis versus light intensity curve at 9.6°±1°C, others at 25°C. Average of 3 experiments.

ΔATP levels in Chlorella *and* Anacystis.

Since research with aerobic algal cells (*8*) indicate the ATP values from extracts of illuminated cells not to exceed those from "dark" cells, we first checked these observations with our preparations. Experiments with aerobic *Chlorella* and *Anacystis* cells gave negative ΔATP (Tables 2, 3, and 4).

Small positive ΔATP could be obtained from *Chlorella* suspensions made anaerobic, provided illumination was less than 30 sec. However, these values became increasingly negative as the time of illumination was increased; the latter may have caused aerobic conditions due to O_2 evolution from photosynthesis. The dark ATP values from "anaerobic" cells were about 70% of those from aerobic

Table 2 *Effect of various inhibitors on the relative light minus dark (ΔATP) values from aerobic regularly grown* Chlorella

Inhibitors	Concentration	Relative ATP values [a]		Relative ΔATP values [b]
		Light	Dark	
1. (4) None		81.6	86.6	− 5.0
2. (2) ICH_2COOH	10 mM	33.0	39.6	− 6.6
3. (2) $NaAsO_2$	10 mM	46.2	34.8	+11.4
4. (2) KCN	10 mM	41.2	22.8	+18.4
5. (3) NaN_3	20 mM	41.6	22.0	+19.6
6. (2) Oligomycin	0.12 μM	82.0	75.0	+ 7.0
7. (2) Oligomycin	1.2 μM	82.0	54.4	+27.6
8. (2) Oligomycin	12 μM	82.0	33.6	+48.4
9. (2) Oligomycin	24 μM	82.4	33.4	+49.0
10. (2) Oligomycin	48 μM	81.2	33.0	+48.2

Conditions: Cells suspended in 0.1 M carbonate-bicarbonate buffer, pH 8.2; light intensity, 1.6×10^5 ergs cm^{-2} sec^{-1}; white light, filtered through Schott M978 and Corning C.S. 1-97 glass filters; illumination time, 10 sec; temperature 25°C. Data are averages of number of experiments given within parentheses.

[a] 1 relative dark ATP unit represents 0.074 μmoles ATP mg Chl^{-1}.

[b] 1 relative ΔATP unit represents 26.6 μmoles ATP mg Chl^{-1} hr^{-1}.

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Table 3 *Relative light minus dark (ΔATP) values for extracts from regularly grown* Chlorella *cells as a function of illumination time in the presence or absence of 12 μM of oligomycin*

Treatment	Time of illumination (sec)						
	0 [a]	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: Same as in Table 2, except that time of illumination was varied.
[a] 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}.

cells. Although the "anaerobic" conditions gave reproducible positive ΔATP values upon short (<30 sec) illumination periods, the values were too small to permit meaningful interpretations regarding photophosphorylation; this method was, therefore, abandoned.

Efect of various inhibitors, including oligomycin, on ΔATP in Chlorella *and* Anacystis.

Several inhibitors were added to aerobic, RG *Chlorella* suspensions in an attempt to obtain larger, consistently positive ΔATP. High (10 mM) concentrations of potassium cyanide (KCN; inhibiting cytochrome oxidase in organisms other than *Chlorella* and retarding the Calvin cycle) and sodium azide (NaN_3; uncoupling oxidative phosphorylation and retarding the Calvin cycle) were found undesirable for our research because they caused inhibition of both the dark and light ATP levels. However, with the exception of iodoacetate (ICH_2COOH; an inhibitor of triose phosphate dehydrogenase), high (10 mM) concentrations of KCN, NaN_3 and sodium arsenite ($NaASO_2$; an inhibitor of pyruvate and keto acid oxidation) gave positive ΔATP levels after 10 sec illumination (Table 2), although these values were half as much positive as with low (10 μM) concentrations of oligomycin. In the latter case, the light ATP level is not inhibited.

Oligomycin was effective at all concentrations between 0.1 μM and 50 μM, with saturation of its inhibitory effect (~70%) on the dark ATP level occurring between 1 and 20 μM. Unlike the results obtained with KCN and NaN_3, long illumination periods (up to 10 min) in the presence of 12 μM oligomycin did not yield negative ΔATP. The values declined slightly after 20 sec illumination and continued to decline to a very small positive value at the end of 10 min illumination (Table 3). Oligomycin did not affect the light ATP values of the RG cells, but it did reduce the values from the HLE cells.

From experiments with *Anacystis* (Table 4), it was apparent that the ΔATP from cells suspended in 0.1 M carbonate-bicarbonate buffer at pH 8.2 was negative, just as observed in *Chlorella*. However, the negative values were much smaller. The addition of various concentrations of oligomycin to *Anacystis* suspensions not only lowered the dark ATP levels, but, in contrast to *Chlorella*, also the light levels (Table 4). Furthermore, the ΔATP values remained negative for all concentrations examined. The data also show that *Anacystis* is much more sensitive to oligomycin than *Chlorella*. A positive ΔATP could be obtained from *Anacystis*, only by further addition of DCMU.

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Table 4 *Relative light minus dark ATP (ΔATP) levels in* Anacystis *as a function of the concentration of oligomycin*

Concentrations	ATP levels, relative units		
	Light	Dark [a]	ΔATP [b]
1. Control	28.4	33.0	−4.6
2. +0.21 μM Oligomycin	14.8	15.9	−1.1
3. +0.6 μM Oligomycin	13.7	14.8	−1.1
4. +2.4 μM Oligomycin	12.8	14.6	−1.8
5. +6.0 μM Oligomycin	12.6	14.5	−1.9
6. +12 μM Oligomycin	12.6	14.8	−2.2
7. +12 μM Oligomycin +25 μM 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 sec, orange (>540) light (4×10^5 ergs cm^{-2} sec^{-1}) at 25°C. Average of 2 experiments.

[a] 1 relative dark ATP unit represents 0.074 μmoles ATP mg Chl^{-1}.

[b] 1 relative ΔATP unit represents 13.3 μmoles ATP mg Chl^{-1} hr^{-1}.

Effect of oligomycin on the steady state (10 min) oxygen evolution and the ATP levels in RG and HLE Chlorella *cells.*

Since the addition of oligomycin to *Chlorella* cells permitted a positive ΔATP, indicating photophosphorylation activity, it was necessary to make comparative measurements on oxygen exchange (see Table 5). Two correlations can be observed in untreated cells in these long term (10 min) experiments: (1) the dark ATP levels increase as the rate of dark O_2 uptake increases, as ATP production is coupled to electron flow; (2) as the rate of photosynthesis increases, the ΔATP levels decrease because ATP produced in light is being more fully utilized in CO_2 fixation reactions. Superimposed on this effect is the oligomycin effect that allows us to observe positive ΔATP. Furthermore, we note that HLE cells had higher light and dark ATP levels than in RG cells (see below).

Table 5 *Oxygen exchange and light and dark ATP values during steady state in regularly grown and high light exposed* Chlorella

	Oxygen uptake (−) or evolution, μmoles O_2 mg Chl^{-1} hr^{-1} (by manometry)			Relative ATP values		
	(1) Light	(2) Dark	(3) Photosynthesis	(1) Light	(2) Dark [a]	(3) ΔATP [b]
1. Control, regularly grown	61.1	−10.8	+71.9	39.0	77.2	−38.2
2. Control, high light exposed	38.9	−23.2	+62.1	82.1	105.5	−23.4
3. +Oligomycin, regularly grown	47.3	−6.9	+54.2	37.1	26.8	+10.3
4. +Oligomycin, high light exposed	27.8	−11.6	+39.4	55.6	52.2	+3.4

Conditions: Aerobic; illumination time, 10 min; 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. Average of 4 experiments.

[a] 1 relative dark ATP unit represents 0.074 μmoles ATP mg Chl^{-1}.

[b] 1 relative ΔATP unit represents 0.4 μmoles ATP mg Chl^{-1} hr^{-1}.

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Effect of DCMU on oxygen evolution and ATP levels in Chlorella.

In order to distinguish cyclic from non-cyclic photophosphorylation, electron transport from photosystem II to photosystem I was blocked by DCMU (*23*). The effects of this poison were examined as a function of concentration for both O_2 evolution and ATP levels in both RG and HLE oligomycin-treated *Chlorella*. Fig. 1A shows that 0.25 μM DCMU suppresses 40% of the ΔATP levels, and 60% of the O_2 evolution in RG *Chlorella*. 25 μM DCMU, however, completely inhibits O_2 evolution but only 70% of ΔATP, indicating the presence of cyclic photophosphorylation.

Since HLE *Chlorella* cells were shown to have no Emerson enhancement effect (Table 1) and it has been suggested that its absence may be correlated to the higher ATP levels occurring in these cells (*24*), a series of experiments identical to the preceeding was done on these HLE cells. Fig. 1B shows that 0.25 μM DCMU suppresses 25% of the ΔATP levels and only 45% of the oxygen evolution. However, 25 μM DCMU inhibited the ATP level by only 50%, although oxygen evolution was completely eliminated, again indicating, but more clearly, the presence of cyclic photophosphorylation. The cylcic photophosphorylation levels were always higher in the HLE than in the RG cells. Thus, it appears that the absence of Emerson enhancement effect in HLE cells parallels the higher "cyclic" ATP (see ref. *24* for a possible explanation).

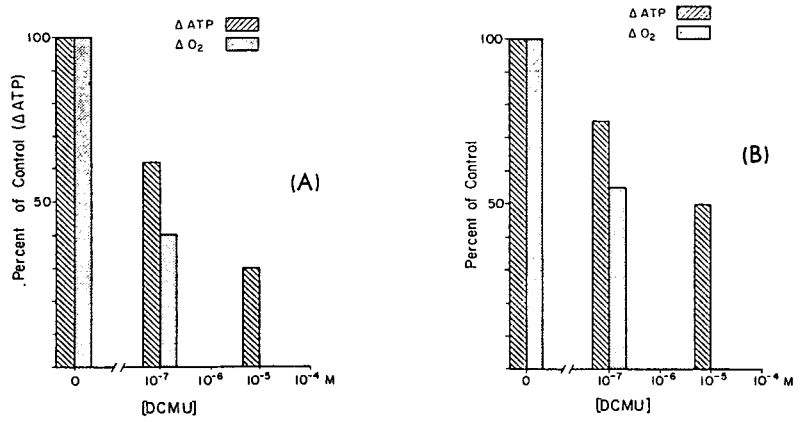

Fig. 1. *Effect of two different concentrations (2.5×10^{-7} M or 0.25 μM and 2.5×10^{-5} M or 25 μM) of DCMU on the light minus dark (ΔATP) levels and oxygen evolution in oligomycin (12 μM) treated regularly grown (A) and high light exposed* Chlorella *cells (B).* OD at 678 nm, 0.3. Oxygen measured on platinum rate electrode; phosphate buffer, 0.1 M, pH 8.0; gassed with 5% CO_2, 2% O_2 and 93% N_2 mixture; illumination time, 4 min; values given, measured at 20 sec (A) or 1 min (B); red light (>640 nm; M978 Schott, C.S. 2-64 and C.S. 1-97 Corning glass filters); light intensity, 2×10^5 ergs cm^{-2} sec^{-1}; temperature, 25°C. Aaverage of 3 experiments.

Effect of DCMU on ΔATP and oxygen evolution in Anacystis.

For comparative purposes the techniques and buffers used were nearly the same as those used in the *Chlorella* studies, minus oligomycin. As the concentration of DCMU is increased, the O_2 level is lowered and the ΔATP level becomes less

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Table 6 *Relative net oxygen evolution and light minus dark ATP (ΔATP) levels as a function of DCMU concentration from* Anacystis

Conditions	ATP levels, relative units			Rate of O_2 evloution, relative units	Percent control
	Light	Dark [a]	ΔATP [b]		
1. Control	(1) 35.1	39.2	−4.1	(1) 253	100
	(2) 36.7	38.1	−1.4	(2) 240	100
2. +0.25 μM DCMU	36.7	37.9	−1.2	158	63
3. +25 μM DCMU	(1) 42.0	37.1	+4.9	(1) 12	5
	(2) 46.7	38.1	+8.7	(2) 15	6
4. +25 μM DCMU +1 μM S_{13}	24.6	28.2	−3.6	—	—

Conditions: Cells suspended in carbonate-bicarbonate buffer, 0.1 M, pH 8.2; absorbance, 0.3 at 678 nm; illumination, 20 sec, orange (>540) light (4×10^5 ergs cm^{-2} sec^{-1}) at 25°C. Oxygen levels measured under similar conditions on the platinum rate electrode. Average of 2 experiments. (1) and (2) experiments with separate cultures.

[a] 1 relative dark ATP unit represents 0.074 μmoles ATP mg Chl^{-1}.

[b] 1 relative ΔATP unit represents 13.3 μmoles ATP mg Chl^{-1} hr^{-1}.

Table 7 *Relative net oxygen evolution and light minus dark ATP (ΔATP) levels in* Synechococcus

	ATP values, relative units			Rate of O_2 evolution, relative units
	Light	Dark [a]	ATP [b]	
1. Control	33.5	22.2	+11.3	92
2. +25 μM DCMU	34.8	22.0	+12.8	32
3. +1 mM MV	24.1	21.7	+2.4	—

Conditions: Same as for Table 6, except that the temperature was 65°C. Oxygen measurements were made under similar conditions on the concentration electrode (Clark-type). Average of 2 experiments.

[a] 1 relative dark ATP unit represents 0.074 μmoles ATP mg Chl^{-1}.

[b] 1 relative ΔATP unit represents 13.3 μmoles ATP mg Chl^{-1} hr^{-1}.

negative, changing to positive at about 25 μM DCMU (Table 6). Even though, there appears to be residual O_2 evolution in the presence of 25 μM DCMU, this is probably due to light induced decrease of O_2 uptake (*25*). The initial O_2 gush, a true index of O_2 evolution, was completely abolished by 25 μM DCMU. Chlorophyll fluorescence studies on *Anacystis* have also been interpreted to suggest that non-cyclic electron transport is completely inhibited by this concentration of DCMU (*26*).

The uncoupler of phosphorylation S13 (1 μM) not only eliminated the positive ΔATP, but caused it to be negative.

ΔATP and oxygen evolution in Synechococcus.

In an effort to see whether the effects of DCMU may somehow be unique to *Anacystis* we examined the effects of 25 μM DCMU on the thermophile *Synechococcus*. To our surprise, the ΔATP level from *Synechococcus* was positive even in the absence of DCMU (Table 7). (We may speculate that the constantly positive ΔATP of *Synechococcus* is due to a low CO_2 fixation activity). As in *Anacystis*, the presence of 25 μM DCMU did not decrease the ΔATP level but

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permitted it to become slightly more positive. When 1 mM MV was added in the absence of DCMU to *Synechococcus*, the ΔATP level was strongly reduced, not stimulated as in the case of *Chlorella* (see below). This difference may be related to the higher rate of cyclic than non-cyclic photophosphorylation in blue-green algae. (The MV had little or no effect on the dark ATP level.).

Effect of absence of CO_2 on ΔATP and oxygen evolution in Chlorella.

ΔATP data in Fig. 1 show that both cyclic (in the presence of DCMU) and non-cyclic photophosphorylation are present in *Chlorella*. However, one could ask whether the addition of DCMU induced the cyclic photophosphorylation. A further check, therefore, would be to observe the effect of the absence of CO_2 on the ΔATP levels and on O_2 evolution; a deprivation of CO_2 should ultimately shut down O_2 evolution and concomitant non-cyclic photophosphorylation but leave cyclic photophosphorylation unaffected. The latter may be expected to be higher than we normally observe in the presence of CO_2 because the rate of ATP utilization would 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 (25 μM). Our results were in agreement with these expectations (data not shown). The absence of CO_2 lowered the oligomycin-treated (control) value by about 65% and this level did not change upon the addition of 25 μM DCMU, the suspending medium being phosphate buffer (0.1 M, pH 8.2).

ΔATP levels as a function of light intensityin Chlorella.

The preceeding experiments showed that positive ΔATP levels could be observed in oligomycin (12 μM) treated *Chlorella* cells, even in the presence of 25 μM DCMU which stopped all O_2 evolution; this suggested that both cyclic and non-cyclic photophosphorylation operate in *Chlorella*. The question was raised: could cyclic and non-cyclic photophosphorylation, which represent two separate pathways, be further separated without the addition of DCMU? PMS supported cyclic photophosphorylation in chloroplasts has been reported to require about 200,000 lux of white light for saturation (*27*), whereas non-cyclic photophosphorylation saturated at about 20,000 lux (*28*). Hence, non-cyclic and cyclic photophosphorylation might be separated into two phases by measuring ΔATP as a function of light intensity.

The oligomycin-treated *Chlorella* suspensions showed an initial lag in the light-curves for ΔATP. This may correspond to the low intensity lag observed in chloroplasts (*1*, *29*). Under white light (not shown), positive ΔATP values appeared at about 10^3 ergs cm^{-2} sec^{-1}, slowly increasing and then rising rapidly until saturation was reached at about 2×10^4 ergs cm^{-2} sec^{-1}. 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 (at close intervals) showed a multiphasic curve in the absence of DCMU (Fig. 2) under aerobic conditions. After a "lag" to an intensity of about 400 ergs cm^{-2} sec^{-1}, ATP photoproduction rises and saturates at about 1×10^3 ergs cm^{-2} sec^{-1}, and again at 1×10^4 ergs cm^{-2} sec^{-1} as noted above. The average ΔATP levels obtained under anaerobic conditions for two intensities gave results similar to those observed in aerobic conditions.

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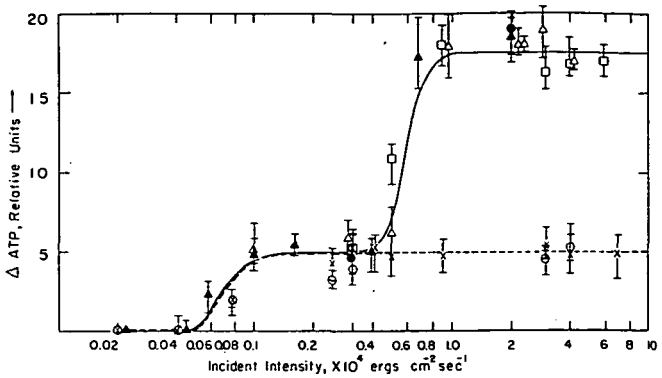

Fig. 2. *Relative Δ ATP values as a function of intensity of red (>640 nm) light in oligomycin (12 μM) poisoned regularly grown* Chlorella *cells in the presence or absence of DCMU (25 μM) and under aerobic or anaerobic conditions.* Symbols: -▲-, 10 sec illumination at 685 nm (8 nm half-bandwidth), average of 5 experiments (aerobic); -△-, 10 sec illumination at 657 nm (10 nm half-bandwidth), average of 6 experiments (aerobic); -□-, 20 sec illumination at >640 nm (aerobic); -×-, 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 -○-, 20 sec illumination at >640 nm with DCMU, average of 4 experiments (anaerobic). (Note the logarithmic scale of the abscissa.)

In the presence of 25 μM DCMU, and under aerobic or anaerobic conditions, 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. For intensities above 4×10^3 ergs cm^{-2} sec^{-1} the values clearly appear to be at the same level as at the first saturation level. The ratio of the saturation level for ΔATP in the presence of DCMU to that in the absence of DCMU was higher (0.28) here than we observed (0.12) in some other samples of RG *Chlorella* cells. 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 ~0.37. For HLE cells under aerobic conditions and in the presence of 25 μM DCMU this ratio was the highest (0.50) we ever observed in *Chlorella* (see Fig. 1).

Effect of various electron donors and acceptors on ΔATP in Chlorella.

To provide an additional check on whether we were able to distinguish between cyclic and non-cyclic photophosphorylation in *Chlorella* we examined the effects of various electron donors, acceptors, inhibitors, and an uncoupler on the ΔATP levels of oligomycin poisoned *Chlorella.*

The addition of methyl viologen (MV) to oligomycin (12 μM) treated RG *Chlorella* affects the ΔATP values in two ways depending on the concentration (Fig. 3). Low concentrations of MV (1 μM) caused an inhibition of 25 percent, whereas high concentrations (1 mM) caused a stimulation of about 100 percent when compared to the ΔATP values of the control (also see Table 8). [The illumination time was 20 sec with red (640 nm) light (1.1×10^5 ergs cm^{-2} sec^{-1}).] The addition of various concentrations of MV to DCMU (25 μM) and oligomycin

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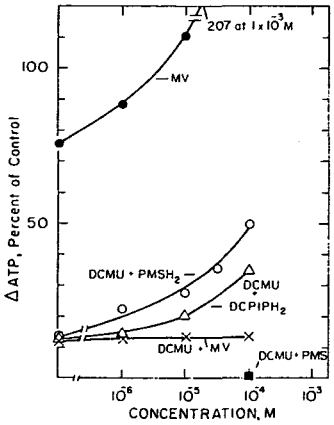

Fig. 3. *Effect of various concentrations of different electron acceptors or donors in the presence or absence of 25 μM DCMU on the light minus dark ATP (ΔATP) values from oligomycin treated regularly grown* Chlorella. (In the text, we have used μM for 10^{-6} M, and mM for 10^{-3} M for convenience.) 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. Cells were exposed for 20 sec to an intensity of 1.1×10^5 ergs cm^{-2} sec^{-1} of red (>640 nm) light. DCMU plus MV data are average of 2 experiments, the other points of at least 3 experiments.

(12 μM) treated *Chlorella* cells usually left the ΔATP values essentially unchanged (Fig. 3, ×—×).

ΔATP levels of *Chlorella* which were treated with 12 μM oligomycin and 25 μM DCMU were stimulated by the addition of either $DCPIPH_2$ or $PMSH_2$ to the reaction medium (Fig. 3, △—△ and ○—○, respectively). (It should be pointed out that 10 mM sodium ascorbate, in the presence of 12 μM oligomycin, has no effect on ATP in *Chlorella* cells.) For all concentrations used, $PMSH_2$ was slightly more effective than $DCPIPH_2$ in increasing the ΔATP level in the presence of DCMU. 1 mM $PMSH_2$, the highest concentration used, restored about 50

Table 8 *Effect of electron acceptors, donors, inhibitors and an uncoupler on oligomycin (12 μM) treated* Chlorella cells

Treatment	Relative ATP values			Percent control
	Light	Dark[a]	Δ ATP[b]	
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 Fig. 3. Concentrations: Oxidized or reduced DCPIP, 1 mM; MV, 1 mM; DCMU, 25 μM; $PMSH_2$, 1 mM; cycloheximide, 17 μM; and S_{13} (5-Cl, 3-t-butyl, 2′-Cl, 4′-NO_2-salicylanilide), 1 μM; 5 μM DCPIP and PMS were reduced by 10 mM sodium ascorbate. Average of at least 3 experiments. For comparison, cycloheximide and S_{13} data were normalized to DCMU values in dark.

[a] 1 relative dark ATP unit represents 0.074 μmoles mg Chl^{-1}.

[b] 1 relative ΔATP unit represents 13.3 μmoles mg Chl^{-1} hr^{-1}.

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percent of the control ΔATP level that had been reduced by the addition of DCMU; $DCPIPH_2$ restored only 30 percent of the control value. Subsequent addition of 1 mM MV further increased the ΔATP value by twofold (Table 8): 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 of the control.

The solid black square plotted in Fig. 3 represents the average ΔATP level obtained as a result of adding 1 mM oxidized PMS in the presence of DCMU under anaerobic as well as aerobic conditions. In the presence of oxidized PMS or partially reduced PMS (50 μM PMS plus 25 μM sodium ascorbate) the ΔATP level in the presence of DCMU was always reduced to zero. The same result was observed with about 100 times lower concentration of PMS. The above results may suggest that PMS does not induce cyclic photophosphorylation in intact *Chlorella*.

Since reactions other than CO_2 fixation, such as cytoplasmic protein synthesis, utilize ATP, we checked if cycloheximide (an inhibitor of cytoplasmic protein synthesis) would further reduce ATP utilization and create an increase in the apparent cyclic photophosphorylation level: 17 μM cycloheximide did induce an increase in the ΔATP level which had been established in the presence of 25 μM DCMU and oligomycin. The 12% ΔATP level increased to 21%.

The test of any photophosphorylation is whether or not it can be uncoupled. The addition of 1 μM of the powerful uncoupler S_{13} completely eliminated the ΔATP level which had been present in the presence of oligomycin and DCMU only (Table 8). The decrease, as a percentage of the control, was from 12 to zero.

Data obtained upon blue (340 nm to 620 nm) light (incident intensity, 1.1×10^5 ergs cm^{-2} sec^{-1}) illumination showed results (not shown) similar to those obtained with red (>640 nm) light reported in Table 8.

Discussion

Sensitivity to oligomycin: positive ΔATP.

Our studies on the effect of oligomycin on the ATP levels from aerobic cells of the eukaryotic green alga *Chlorella* (RG) and the prokaryotic blue-green alga *Anacystis* clearly show (Tables 2–4) that this inhibitor of oxidative phosphorylation (*30*, *31*) has different in vivo effects on the ATP levels in the two organisms. While low concentrations (12 μM) of oligomycin were observed to have little effect on the light ATP levels obtained from RG *Chlorella* cells, the dark ATP levels were always suppressed by at least 50%, thus giving positive ΔATP. In contrast, similar or smaller amounts of oligomycin added to the *Anacystis* cells always severely suppressed both the light and the dark ATP values. Furthermore, oligomycin treatment always permitted the ΔATP of *Chlorella* cells to become positive with short illumination periods, whereas the ΔATP of *Anacystis* cells always remained negative in the presence or absence of this treatment.

Although there are no other reports of the effects of oligomycin on intact algal cells, results obtained by other investigators (*30*), using isolated spinach organelles, have indicated that photophosphorylation is insensitive to these concentrations of oligomycin, whereas mitochondrial phosphorylation is completely

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inhibited: this explains the differential effect of oligomycin on the dark and light ATP levels in the RG eukaryotic green alga *Chlorella*. In contrast, one may speculate from some of our results that the blue-green algal photophosphorylation and dark phosphorylation have identical or similar steps and therefore oligomycin causes a decrease in both the dark and light ATP levels. This would be consistent with the findings that respiratory and photosynthetic processes of blue-green algae share certain components (*32*, *33*). Since blue-green algae are prokaryotic, resembling photosynthetic bacteria in organization, it is important to point out that our results would also be consistent with the observation that the coupling factors for photophosphorylation and "respiration" in membrane preparations from *Rhodopseudomonas capsulata* (*34*) are interchangeable. (For a discussion on evolution of eukaryotes from prokaryotes, see ref *35*.)

In HLE *Chlorella* cells oligomycin also decreases both light and dark ATP levels, although ΔATP is positive. Perhaps, other factors, e.g., exposure of "coupling factor" to different environment, and the permeability of oligomycin to thylakoids of algae examined, must also be considered.

Negative ΔATP.

Regardless of the experimental conditions, aerobic *Chlorella* cells, suspended in carbonate-bicarbonate buffer (0.1 M, pH 8.2), always produced a negative ΔATP (cf. ref. *8*; Tables 2–4). We did not expect to find the ATP utilization reactions in the light to exceed the combined synthesis reactions during 15 sec illumination periods; this time should have been short enough to insure that most fixed CO_2 would be in the form of 3-phosphoglyceric acid, as Bassham (*9*) has observed. After an extended dark period, perhaps, the combined synthetic reactions in the light are unable to meet the utilization requirements during the first few minutes of illumination. The net result is that initially the organism must draw on the ATP pool built up in the dark. It is clear that an equilibrium must be reached at some point since algae do grow well in continuous light. [We also note that Lewenstein and Bachofen (*13*) reported positive ΔATP in their *Chlorella* cells; we do not know the reasons for these different results.]

Cyclic versus non-cyclic; intensity dependence.

The occurrence of cyclic phosphorylation in vivo was shown by Forti and Parisi (*36*). Parallel measurements on O_2 evolution and photophosphorylation as a function of increasing DCMU concentration (Fig. 1; Table 6) show greater ratio of cyclic to non-cyclic phosphorylation in *Anacystis* than in *Chlorella*. HLE *Chlorella* had greater ratio of cyclic to non-cyclic than RG *Chlorella*.

Curves for ΔATP as a function of light intensity begin with a lag, which may be the same low intensity lag observed in chloroplast preparations (Fig. 2) (see review, *1*), followed by an increase that saturates between 1 and 4×10^3 ergs cm^{-2} sec^{-1}. Most hypotheses advanced to explain this lag involve the filling of a pool, i.e., hydrogen ion gradients across the chloroplast membrane (*37*) or high energy intermediates (*38*). Supposedly, photophosphorylation proceeds only when this pool is "filled" (*1*).

The low-intensity saturation curve was almost insensitive to DCMU at a concentration (25 μM) which completely stopped all oxygen evolution by the

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regularly grown (RG) and high light exposed (HLE) *Chlorella* cells, whereas the second level saturation at a 10 times higher intensity was sensitive to the same concentration of DCMU. The DCMU lowered the second level to that of the first. Therefore, it can be concluded that in *Chlorella* cells cyclic photophosphorylation (that goes on in the presence of DCMU) saturates at 10 times lower intensity than the DCMU sensitive non-cyclic photophosphorylation. This conclusion would be in agreement with previous data (*2, 7, 39, 40*) in algae. Also, the second DCMU sensitive ΔATP level saturated at the same intensity of red light at which oxygen evolution saturated in *Chlorella* (*41*). These results are opposite to the results obtained from isolated chloroplasts that had been exposed to white light (*1, 28*) that show a higher saturation for cyclic than for non-cyclic photophosphorylation.

Effect of electron acceptor and donors.

The addition of high concentration of methyl viologen to *Chlorella* causes a dramatic increase in ΔATP (Table 8; Fig. 3)—this is most likely due to the decrease in ATP utilization by the Calvin cycle as the latter is shut off due to electrons from system I being shunted to methyl viologen instead of to $NADP^+$ (*42*). Further addition of DCMU stops the non-cyclic flow of electrons and reduces the ΔATP to the lower level of cyclic photophosphorylation. Addition of $DCPIPH_2$ or $PMSH_2$ to DCMU-treated cells increased ΔATP because electron flow begins to operate. Further addition of methyl viologen causes an efficient non-cyclic flow of electrons from $DCPIPH_2$ (or $PMSH_2$) to methyl viologen causing an increase in ΔATP. Thus, these results with electron acceptors and donors confirm that in *Chlorella* non-cyclic photophosphorylation predominates over cyclic photophosphorylation. In contrast, in blue-green algae (*Anacystis* and *Synechococcus*) ΔATP is more positive in the presence of DCMU than in its absence suggesting that cyclic photophosphorylation may predominate non-cyclic in these algae.

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