

Photosynthetic Reactions by Lysed Protoplasts and Particle Preparations from the Blue-Green Alga, *Phormidium luridum*^{1, 2}

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Summary. Reactions of photosynthetic electron transport and photophosphorylation were studied in preparations from the blue-green alga, *Phormidium luridum*. Osmotic lysis of protoplasts proved to be a superior technique for the production of cell-free preparations with high enzymatic activity. Such lysed protoplasts sustain high rates of photophosphorylation coupled to the photo-reduction of NADP⁺ or ferricyanide. P/2e⁻ ratios close to unity were routinely observed. The same preparations, and also those prepared by grinding the cells in solutions containing sucrose or ethylene glycol, are active in cyclic photophosphorylation mediated by phenazine methosulfate or dichlorophenolindophenol. The particles prepared by grinding the cells are, however, inactive in non-cyclic photophosphorylation.

Extensive washing of the membranes with solutions containing sucrose removes the majority of the residual soluble fraction of the algal cell which includes cytochromes C₅₅₄ and C₅₄₉ and phycocyanin. Cyclic photophosphorylation activity is unimpaired by this treatment, but is abolished when the membranes are washed with very dilute buffers. This activity is restored by the addition of a soluble protein which is not a known redox constituent such as cytochrome C₅₅₄ or plastocyanin, and may be a coupling factor.

Analysis of the well-washed membranes by low temperature (77°K) difference spectrophotometry reveals the presence of cytochrome b₆ and a bound form of cytochrome C₅₅₄ in proportions similar to that found in higher plant chloroplasts. The concentration of the membrane-bound cytochrome C₅₅₄, relative to cytochrome b₆ is not altered by extensive washing, sonication or treatment with 1% digitonin. This indicates that this cytochrome is an integral component of the cytoplasmic lamellae and we suggest that it is of functional significance. The soluble form of cytochrome C₅₅₄, which is present in concentrations about 3-fold higher than the bound form, depending upon growth conditions, is not essential for cyclic photophosphorylation. The concentration of cytochrome b₆: chlorophyll a was found to be 1:500.

Under the conditions employed, we were unable to detect a bound form of the low potential cytochrome C₅₄₉.

The blue-green algae are unique in that they are prokaryotic but their photosynthetic mechanism is more typical of eucaryotic cells. As is the case in green algae and higher plants, 2 photosystems are necessary and the high potential oxygen evolution mechanism is present (30).

An important difference between the morphology of the photosynthetic apparatus of blue-green algae and the chloroplasts of higher plants is the degree of molecular organization of the constituents. In common with the red algae and cryptomonads, the accessory pigments in the blue-green algae are soluble

biliproteins and are readily removed from their location on the chlorophyll-bearing lamellae by aqueous washing (25). Certain soluble cytochromes are present in blue-green algae in high concentrations. Katoh extracted a soluble cytochrome of type c from *Tolypothrix tenuis* and noted its possible equivalence with the photosynthetic cytochrome f found in the chloroplasts of higher plants (21). This cytochrome, and also a low potential soluble cytochrome type c (549, *Anacystis*), has been purified and fully characterized by Holton and Myers (18, 19, 20). Thus, as noted by Susor, Duane, and Krogmann (30), the mechanical ease by which these components are removed from the photosynthetic lamellae renders the blue-green algal system particularly amenable to the study of photosynthesis at the biochemical level. It offers, for example, the possibility for the reconstitution of the photosynthetic apparatus using purified components. Hence, studies of the partial reactions

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of photosynthetic electron transport may lead to specific functional assignments for these soluble components.

Available reports concerning the photosynthetic mechanism in blue-green algae indeed confirm that the apparatus is quite susceptible to physical disruption by very mild procedures. For instance, in an investigation of the Hill reaction in *Anacystis nidulans*, Fredricks and Jagendorf found that aqueous extraction of lamellar fragments led to a loss in activity (9). The activity could be restored by the addition of a macromolecular component in the soluble extract. Apart from confirming that the component was not phycocyanin, the restorative factor was not characterized. Duane, Hohl, and Krogmann (7) were unable to demonstrate photophosphorylation coupled to the photoreduction of NADP⁺ or ferricyanide by preparations from *Anabaena* which were, nevertheless, very active in cyclic photophosphorylation. Extracts of *Anacystis nidulans* were found to be inactive in cyclic and non-cyclic photophosphorylation although the same preparations were capable of electron flow (5). Gerhardt and Trebst (11) have recently reported non-cyclic photophosphorylation by preparations from *Anacystis nidulans* but the observed rates and P/2e⁻ ratios are low.

The present report is concerned with photosynthetic electron transport and photophosphorylation in the blue-green alga *Phormidium luridum*. We have investigated the functional role of the soluble redox components in photophosphorylation, and, in an effort to preserve the coupling of ATP synthesis to electron flow, we have successfully utilized the rupture of metabolically active protoplasts (3) as a means of preparing cell-free extracts.

Methods

Algae and Protoplasts. *Phormidium luridum* was cultured and protoplasts were isolated by the procedure described in the accompanying report (3). Protoplasts were washed once with either ice cold-mannitol-tris-maleate pH 7.2, mannitol-tris-Cl (pH 7.5), or 0.5 M mannitol titrated to neutrality.

For large scale enzyme preparations, algae were cultured in 5 gallon carboys and rapidly flushed with air. Illumination was provided by a bank of fluorescent tubes. The algae were harvested by continuous flow centrifugation, washed once in metal-free water and frozen. Stocks were stored at -20°.

Membrane Preparations. Cell-free preparations were created by adding the protoplasts to the reaction mixture of interest, thereby causing an 8-fold dilution of mannitol which resulted in cell lysis. Microscopic examination of such reaction mixtures revealed that about 95% cell lysis had occurred indicating that some cells were still protected by cell wall.

Membrane fragments were also prepared by grinding the washed cells with sand. The homogenate was extracted with 10 volumes of either 40% ethylene glycol, 0.05 M tris-Cl (pH 7.5) or 0.3 M sucrose,

0.05 M tris-Cl (pH 7.5). The homogenate was centrifuged at 5000 g to remove sand, whole cells and large fragments and then at 27,000 g for 15 minutes to sediment heavy particles. This supernatant was then centrifuged at 48,000 g for 30 minutes to sediment light particles. Residual chlorophyll in the final supernatant amounted to about 5% of the total chlorophyll in the homogenate. Chlorophyll *a* was measured spectrophotometrically after extraction with cold 80% acetone using $E_{mM}^{663nm} = 82$.

Photophosphorylation. Photophosphorylation was carried out using an illuminated Warburg apparatus at 25°. The gas phase and reaction conditions were as indicated. The incorporation of ³²P into ATP was measured in trichloroacetic acid-denatured supernatants by the method of Avron (1).

Photoreduction. As a measure of electron flow during non-cyclic photophosphorylation, NADPH was determined spectrophotometrically after the incubation period prior to denaturation of the reaction mixtures with trichloroacetic acid. Ferrocyanide was determined by the procedure of Avron and Shavit (2).

Spectrophotometry. Room temperature spectra of solutions were obtained using a Cary Model 15 automatic recording spectrophotometer. Low temperature (liquid N₂) and room temperature spectrophotometry of particles was carried out using a more modern version of the split-beam spectrophotometer described by Chance and co-workers (6, 36). A large end-on phototube (Dumont KM2433) was used. For low temperature studies, particles were suspended in 0.4 M sucrose, 0.05 M tris-Cl (pH 7.5) to enhance the band intensities (35) and the samples were rapidly frozen in precooled Plexiglass cuvettes of 2 mm light path. During measurements the samples were maintained close to liquid nitrogen temperature in an unsilvered glass Dewar.

Biochemicals. NADP⁺ and ADP were obtained from Boehringer Mannheim Corporation, New York; 2,6-dichlorophenol indophenol (DPIP) and phenazine methosulfate (PMS) from Sigma, and ³²P from Squibb, New Brunswick. Dr. R. Goldsby, Central Research Department, DuPont Chemical Company, Wilmington, Delaware, kindly provided 3-(3,4-dichlorophenyl)-1, 1-dimethylurea (DCMU).

Protein Preparations. Crude aqueous extracts of the algae were prepared by pouring a 500 ml cold suspension of cells (100 g wet wt) into 4 volumes of -20° acetone with vigorous stirring. The resulting slurry was then centrifuged at low speed and the yellow-brown supernatant discarded. The precipitate was homogenized in 1 liter 0.05 M phosphate (pH 7.5), 1.0 M ferricyanide and the plastocyanin solution was then centrifuged and the blue supernatant retained as the crude extract.

The extract was then made 55% saturated with ammonium sulfate at pH 7.5 and, after 30 minutes, was centrifuged. Usually all of the phycocyanin was removed by this step. The 55% saturated solution was then made 100% saturated with ammonium sulfate and stirred overnight in the cold. The pre-

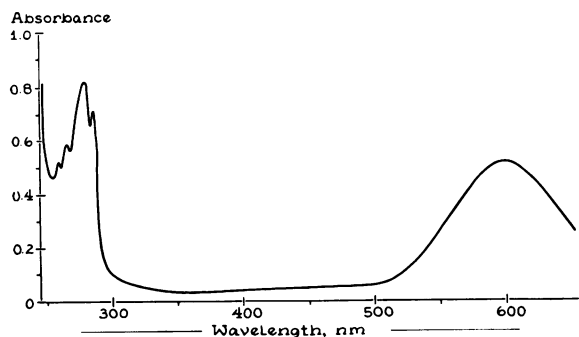


FIG. 1. Absolute absorption spectrum of the oxidized form of plastocyanin from *Phormidium luridum*.

precipitated protein was collected by high speed centrifugation at 48,000 g for 20 minutes and suspended in 10 mM phosphate (pH 7.5). The protein was dialyzed against the same buffer to remove salt. This solution contained ferredoxin, phytoflavin, ferredoxin-NADP⁺ reductase (NADP⁺:ferredoxin oxidoreductase, EC 1.6.99.4), cytochrome-C₅₅₄, cytochrome-C₅₄₉, plastocyanin and the PMS-cyclic photophosphorylation component.

Cytochrome C₅₅₄ and Plastocyanin. The dialyzed extract was passed through a column, 3 × 20 cm, packed with DEAE-cellulose equilibrated with 10 mM phosphate (pH 7.5). Cytochrome C₅₅₄ and plastocyanin were not absorbed and this effluent was then made 10 μM in ferricyanide. This solution was then drained through a column, 1 × 10 cm packed with CM-cellulose. The ferricytochrome C₅₅₄ was not absorbed whereas the oxidized plastocyanin was retained as a blue band near the top of the column. The column was washed with 10 mM phosphate (pH 7.5), 1.0 M ferricyanide and the plastocyanin

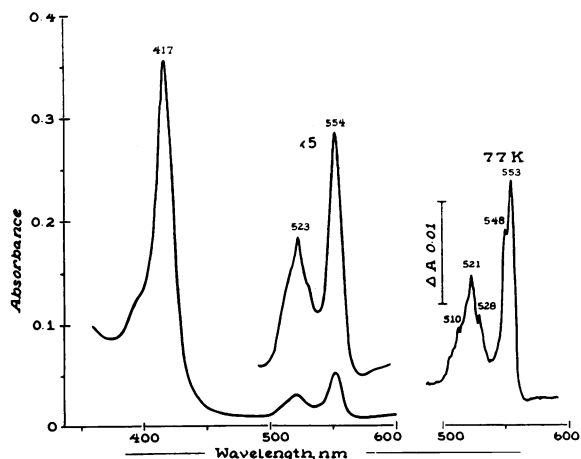


FIG. 2. Room temperature and liquid nitrogen absolute spectra of the reduced form of cytochrome type-c (554, *Phormidium*). The liquid nitrogen spectrum was obtained using a 2 mm light path and the solvent was 0.4 M sucrose. The room temperature spectrum was obtained using a 4 mm light path.

was then eluted with 25 mM phosphate (pH 7.5). The protein was dialyzed and then stored as a frozen solution at -20°.

Figure 1 shows an absolute absorption spectrum of *Phormidium* plastocyanin after oxidation with ferricyanide. It is similar to the plastocyanin of higher plants (23), green algae (12) and *Anabaena* (24) in that there is a broad absorption band in the red with a maximum at 597 nm and complex bands in the ultraviolet.

Cytochrome C₅₅₄ was precipitated from the eluate by ammonium sulfate and stored in saturated ammonium sulfate solution at 4°. Figure 2 shows room temperature and liquid nitrogen absolute spectra of the reduced form of this cytochrome. At room temperature our observed absorption maxima agree very well with those reported for the *Anacystis* cytochrome by Holton and Myers (18, 19, 20) and the *Anabaena* cytochrome by Susor and Krogmann (31). At liquid nitrogen temperature the splitting of the α band is accompanied by a blue shift of the absorption maximum. This behavior is similar to that described by Hill and Bonner (16) for parsley cytochrome f and most c-type cytochromes (8, 35).

Cytochrome C₅₄₉. This component was absorbed on the DEAE-cellulose column and was eluted with 50 mM NaCl, 10 mM phosphate (pH 7.5) and concentrated by rechromatography on DEAE-cellulose. This cytochrome was occasionally contaminated by residual phycocyanin but this pigment could be removed by a further salt fractionation.

Ferredoxin-NADP⁺ Reductase and Ferredoxin. These proteins were eluted from the DEAE column by 0.1 M and 0.4 M NaCl respectively. They were not separated for the photoreduction experiments.

When the proteins were isolated from Swiss chard the procedure of San Pietro and Lang (28) was employed through the 75% acetone precipitation step. After dialysis, the proteins were further purified by the first DEAE-cellulose column treatment of Hill and Bendall's method (15).

Results

Reactions of photosynthetic electron transport and associated photophosphorylation were measured in *Phormidium luridum*. Cell-free preparations of the alga were prepared by either protoplast lysis or, alternatively, particles were isolated after grinding the cells with sand in solutions containing high concentrations of sucrose or ethylene glycol.

Photoreactions of Lysed Protoplasts. In an attempt to retain as much enzymic activity as possible, metabolically active algal protoplasts were prepared and added to the reaction mixtures of interest at the start of the reaction periods. The ensuing 8-fold decrease in stabilizing osmoticum caused the protoplasts to rupture thus instantaneously creating a cell-free preparation in the reaction vessel at the start of the reaction period. This technique eliminated the prolonged exposure of membranes to buffers and

consequent loss of activity during the work-up of homogenates as prepared by conventional methods.

Table I illustrates the capability of the algal preparations to sustain high rates of photophosphorylation coupled to the photoreduction of NADP⁺ or ferricyanide. When NADP⁺ is electron acceptor, the P/2e⁻ is close to unity indicating a considerable preservation of activity in these preparations. This ratio is, however, most likely slightly overestimated because of the incomplete recovery of NADPH in these determinations. Hoch and Martin (17) and Sauer and Biggins (29) showed the back-reaction to be significantly high during photoreduction by chloroplasts. It is possible that some oxidation of the NADPH occurred during the experiments reported here and work-up of the reaction mixtures. Nevertheless, the P/2e⁻ ratio for photophosphorylation coupled to ferricyanide reduction is high and comparable with the better measurements made with higher plant chloroplasts.

The reaction rates for NADP reduction are quite high for the open system with water as ultimate electron donor. The inhibition by the herbicide, DCMU, implicates the involvement of photosystem 2

and the recovery of activity in the presence of added electron donor is similar to that for higher plant chloroplasts and algae, the reconstituted *Anabaena* system (31) and *Anacystis nidulans* (5, 11).

It is interesting to note that the inclusion of supplementary ferredoxin and ferredoxin: NADP⁺-reductase, prepared from either *Phormidium* or Swiss chard, does not lead to any improvement of the rate of NADP⁺ photoreduction or the associated photophosphorylation by these lysed protoplasts (table I). This suggests that the endogenous enzymes are saturating for the reaction conditions employed. For this reason we were hopeful of demonstrating a cyclic photophosphorylation mediated by such endogenous components. We were, however, unable to achieve a significant synthesis of ATP via a cyclic process, even in the presence of DCMU and under anaerobic conditions, unless a conventional cyclic-cofactor was added (table II). We would suggest, therefore, that the ferredoxin-mediated cyclic photophosphorylation described by Tagawa, Tsujimoto and Arnon (33) is operating under what we would consider to be abnormally high concentrations of ferredoxin.

The effectiveness of PMS and DPIP as media-

Table I. *Non-cyclic Photophosphorylation by Lysed Protoplasts*

Measurements were for 6 minutes, saturating white light, 25°. The reaction mixture (1.5 ml) contained the following in μ moles: tris-maleate (pH 7.5), 40; MgCl₂, 30; ADP, 5; K₂H³²PO₄, 5; and NADP⁺, 3 or potassium ferricyanide, 5. Where indicated, the mixtures also contained DCMU, 0.2; DPIP, 0.1 and ascorbate, 10. In treatment number 4 ferredoxin and ferredoxin:NADP⁺ - reductase were added in quantities sufficient to saturate in a Swiss-chard chloroplast assay. Protoplasts were equivalent to 50 μ g chlorophyll *a*.

Additions	Photophosphorylation		Photoreduction	P/2e ⁻
	μ moles	ATP/mg Chl/hr	μ moles oxidant/mg Chl/hr	
1. NADP ⁺		132	149	0.855
2. NADP ⁺ , DCMU		5	8	...
3. NADP ⁺ , DCMU, DPIP, ascorbate		121	163	...
4. NADP ⁺ , ferredoxin, reductase		125	156	0.8
5. Ferricyanide		181	193	0.94
6. Ferricyanide, DCMU		15	24	...

Table II. *Cyclic Photophosphorylation by Lysed Protoplasts*

Measurements were for 6 minutes, saturating white light at 25°. The reaction mixtures (1.5 ml) contained the following in μ moles: tris-maleate (pH 7.2), 60; MgCl₂, 10; ADP, 5; K₂H³²PO₄, 5 and protoplasts equivalent to 25 μ g chlorophyll *a*. The following compounds were included as indicated in μ moles: PMS, 0.05; DPIP, 0.1; ascorbate, 50 and DCMU, 0.45.

Conditions			Photophosphorylation	
			μ moles	ATP/mg Chl/hr
PMS		N ₂		201
PMS	DCMU	N ₂		188
PMS		N ₂		238
PMS		air		202
PMS	Ascorbate	N ₂		357
PMS	Ascorbate	air		354
PMS	Ascorbate	N ₂		431
DPIP	Ascorbate	air		187
DPIP	Ascorbate	N ₂		10
No cofactor		N ₂		1.3
No cofactor	DCMU	N ₂		1.1
No cofactor		air		0.04

Table III. *Cyclic Photophosphorylation by Particles Prepared by Grinding with Sand*

Conditions were as in table II but the cofactor concentration as indicated. Particles contained 25 μg chlorophyll *a*.

Preparation	Buffer system	Photophosphorylation	
		100 μM PMS	33 μM PMS
		$\mu\text{moles ATP/mg}$	Chl/hr
Light particles	Ethylene glycol	166	90
	Sucrose	132	88
Heavy particles	Ethylene glycol	86	50
	Sucrose	22	19

tors of cyclic photophosphorylation by lysed protoplasts is shown in table II. Ascorbate relieves the inhibitory action of air in the PMS system, but air is necessary for full activity when DPIP is cofactor. The activity is not appreciably diminished by relatively high concentrations of DCMU indicating that the reaction is a true cyclic process proceeding in the absence of photosystem 2. Thus, the blue-green algal mechanism is essentially similar to that of higher plant chloroplasts and green algae. We do, however, confirm the observation of Duane, Hohl and Krogmann (7) that the pH response for cyclic photophosphorylations mediated by PMS is anomalous with a major peak at pH 7.0 and a minor peak in the vicinity of pH 8.0.

Photoreactions of Particles Obtained by Grinding.

In agreement with reports on blue-green algae by previous investigators (7, 27), we found that particles prepared from *Phormidium luridum* by conventional methods of cell disruption in the presence of a high concentration of sucrose or ethylene glycol were active in cyclic photophosphorylation. High yields of broken cells were obtained from *Phormidium* by grinding a wet cell paste in a mortar with pestle and sand, and lamellar fragments were isolated from the homogenate by differential centrifugation. Table III compares the activity of particles obtained by this method as isolated in either buffered sucrose or ethylene glycol as grinding medium. The light particles obtained by high speed centrifugation were much more active than the heavy particles, irrespective

of the grinding buffer used. In general, it was found that the ethylene glycol medium was more suitable than sucrose and rates from 250 to 450 $\mu\text{moles/mg}$ Chl/hr for PMS cyclic photophosphorylation were typically observed.

The optimum concentration of PMS necessary for cyclic photophosphorylation by the light particles was found to be 0.1 mM rather than 33 μM which is optimal for lysed algal protoplasts and higher plant chloroplasts.

We were unable to demonstrate non-cyclic photophosphorylation coupled to the photoreduction of NADP⁺ or ferricyanide by these particles. Duane, Hohl and Krogmann were also unable to demonstrate non-cyclic photophosphorylation by similarly prepared particles from *Anabaena* (7). It appears, therefore, that non-cyclic photophosphorylation is much more susceptible to physical disruption and can only be demonstrated when the algae are broken by relatively mild techniques.

Effect of Washing the Algal Particles. If the light particles prepared in ethylene glycol medium are washed in the grinding buffer, the majority of the soluble fraction of the cell is removed. The soluble fraction contains the accessory pigment, phycocyanin, the soluble cytochromes type c (554, *Phormidium*) and type c (549, *Phormidium*), ferredoxin and ferredoxin: NADP⁺ reductase. As shown in table IV, the cyclic photophosphorylation activity was unaltered by this treatment. However, a substantial loss in activity (90%) occurred when the

Table IV. *Cyclic Photophosphorylation: Effect of Washing the Particles or Protoplasts; Reconstitution by a Soluble Protein*

Conditions were as in table II using PMS as a cofactor. Protoplasts or particles contained 25 μg chlorophyll *a*. The protein added back was the 50 to 100% saturated ammonium sulfate precipitate from the soluble fraction of the cell.

Treatment	Rate of photophosphorylation	
	$\mu\text{moles ATP/mg}$	Chl/hr
<i>Light particles:</i>		
Control	291	221
Once washed, ethylene glycol buffer	289	218
Twice washed, ethylene glycol buffer	280	215
Once washed, 50 mM tris	20	15
<i>Protoplasts:</i>		
Control	230	218
Once washed, 50 mM tris	21	15
Twice washed, 50 mM tris	9	5
Twice washed plus active component	94	73

particles were washed with dilute buffer. A similar loss in activity was also observed when the algal protoplasts were lysed in a 40-fold excess of dilute buffer. Furthermore, the membranes obtained from protoplasts lysed in this fashion simultaneously lost their capacity for non-cyclic photophosphorylation when either ferricyanide or NADP⁺ was used as electron acceptor. The phosphorylation coupled to NADP⁺ photoreduction was not restored by the addition of supplementary ferredoxin and ferredoxin: NADP⁺ reductase.

Reconstitution and Fractionation of an Active Component. In the case of the PMS-mediated cyclic photophosphorylation it was possible to restore some 15% of the activity lost on aqueous washing by addition of the entire soluble fraction of the cell. The component of interest was partially purified by fractionation of an aqueous extract from 100 g wet weight of acetone-treated cells prepared as described in Methods.

The 50 to 100% saturated ammonium sulfate sediment was dialyzed and then subjected to bulk fractionation on DEAE cellulose. The protein was loaded on a column equilibrated with 10 mM phosphate (pH 7.5). After washing with 25 mM buffer to remove cytochrome C₅₅₄ and plastocyanin, the column was eluted with 0.3 M NaCl, 50 mM phosphate (pH 7.5) and the eluate retained and dialyzed against 10 mM phosphate (pH 7.5). In addition to the active component of interest, this fraction contained cytochrome C₅₄₉, phytoflavin and ferredoxin.

The dialyzed protein was then re-chromatographed using DEAE-cellulose. A column, 2 × 25 cm equilibrated with 10 mM phosphate (pH 7.5) was charged with the protein and was eluted with a gradient of NaCl increasing linearly from 0 to 0.6 M,

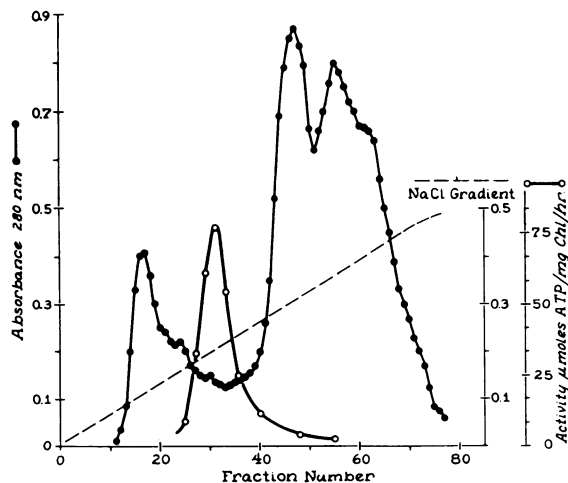


FIG. 3. Chromatography of the component active in the reconstitution of cyclic photophosphorylation. Fractions were 4.5 ml and the salt gradient was determined from refractive index measurements. The photophosphorylation assay was performed as described in table II using once-washed protoplast membranes and PMS as cofactor.

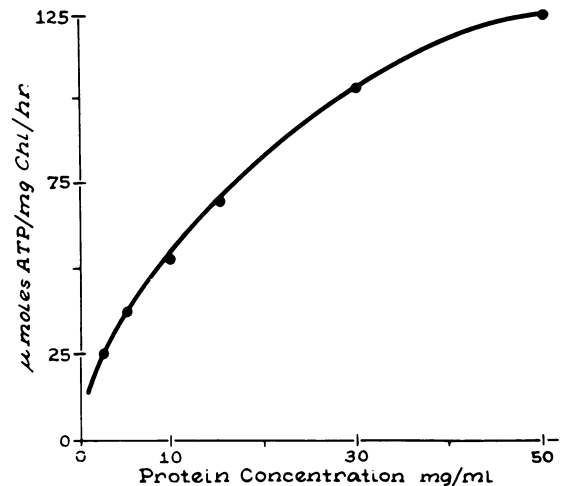


FIG. 4. Dependence of cyclic photophosphorylation on the active component. The photophosphorylation assay was performed as described in table II using once-washed protoplast membranes and PMS as cofactor. The protein added was that after salt gradient chromatography on DEAE-cellulose as shown in figure 3.

10 mM phosphate (pH 7.5). The total volume of the gradient was 400 ml, and 1.5 ml fractions were collected. Figure 3 shows the distribution of protein in the eluted fractions and their activity in the restoration of cyclic photophosphorylation. The active fraction appears to be a single component and is eluted by about 0.2 M NaCl. Difference spectrophotometry did not reveal the presence of cytochrome C₅₄₉ which is present in the first peak chromatographing at about 0.1 M NaCl. Ferredoxin was eluted by 0.3 M NaCl and the protein which is eluted by 0.25 M NaCl is thought to be phytoflavin.

Figure 4 shows the dependence of cyclic photophosphorylation on the active component, and the restorative activity is compared with that of the known soluble redox intermediates of the algal photosynthetic apparatus in table V. It is apparent that both cytochrome C₅₅₄ and plastocyanin are inactive and they do not appear to improve the activity of the component when added simultaneously.

The active component is a protein and the purest fractions available so far represent a 150-fold increase in purity capable of restoring some 40 to 50% of the PMS cyclic photophosphorylation. Difference spectrophotometry confirms the absence of a haemoprotein, but we have observed the presence of some flavin. The flavin content was variable and it is possible that this could be contamination from the adjacent flavoprotein in the chromatogram which is thought to be phytoflavin.

Membrane-bound Components. From the data presented above we can conclude that the washed algal membranes require a soluble protein for activity in cyclic photophosphorylation. The protein does not appear to be a known intermediate of the photosynthetic electron transfer chain and the question arises

Table V. *Cyclic Photophosphorylation, Reconstitution by the Active Component and Comparison with Known Redox Intermediates*

Conditions were as in table II using PMS as cofactor. Protoplasts or once-washed (50 mM tris) membranes contained 25 μg chlorophyll *a*. The active component was 30 μg protein purified by salt gradient chromatography as in figure 3.

Components added		Rate of photophosphorylation	
		$\mu\text{moles ATP/mg Chl/hr}$	
Protoplasts	Control	151	183
Washed membranes	Control	23	19
Washed membranes	Active component	89	93
Washed membranes	Cytochrome c_{554}	25	18
Washed membranes	Plastocyanin	31	25
Washed membranes	Cytochrome c_{549}	28	32
Washed membranes	Cytochrome c_{554} + plastocyanin	...	27
Washed membranes	Active component + cytochrome c_{554}	...	97
Washed membranes	Active component + plastocyanin	...	89
Washed membranes	Active component + plastocyanin + cytochrome c_{554}	...	91

whether photophosphorylation by these washed membranes is truly proceeding in the complete absence of components such as cytochrome C_{554} , plastocyanin, and ferredoxin. These proteins are assumed to be removed from the membranes by the washing treatments.

Estimates based upon the recovery of cytochrome in the washings indicate that the aqueous treatment does lead to a complete removal. However, such measurements are probably erroneous because of the uncertainty of measuring low concentrations of haemoproteins in the presence of high concentrations of phycocyanin. Furthermore, the cytochrome:chlorophyll ratio was found to vary considerably depending upon the age of the algal culture. We, therefore, analyzed the washed membranes directly by low temperature difference spectrophotometry. The membranes were extracted with 80% acetone at -20° to remove interfering pigments such as chlorophyll. They were finally resuspended in sucrose to enhance the intensity of the absorption bands at liquid nitrogen temperature (35).

Representative difference spectra of the membranes obtained at temperatures close to that of liquid nitrogen are shown in figure 5. Spectrum C is a reduced minus untreated difference spectrum measuring all components which are oxidized in the untreated condition i.e., as extracted. This spectrum is that of cytochrome b_6 . The small shoulder at 562 nm is suggestive of another component and the asymmetry to the blue of the main α band is thought to be a contribution by a small fraction of cytochrome C_{554} which is oxidized in the untreated condition.

All components in the reduced form as extracted are shown in figure 5B, which is an untreated minus oxidized difference spectrum. The spectrum is of a bound form of cytochrome C_{554} with a well-defined α_2 band at 548 nm due to splitting of the α band at low temperature. The possibility that this 548 nm band is the α band of cytochrome C_{549} , and not the α_2 band of cytochrome C_{554} , is ruled out on the

grounds that cytochrome C_{549} is very autooxidizable (18, 19, 20) and, therefore, would not appear in an untreated minus oxidized difference spectrum. Unless the acetone treatment selectively destroyed a membrane-bound form, we can also conclude that cytochrome C_{549} is a totally soluble protein.

The relative intensities for the α_1 and α_2 bands of the soluble cytochrome C_{554} (fig 2) and the membrane bound form (fig 5B) are different. The α_2 band of the membrane bound form is considerably more intense than that of the soluble form and this may be a direct reflection of the differing physical environments of the 2 forms.

Figure 5A is a difference spectrum, reduced minus oxidized, showing all cytochromes present. Both cytochrome b_6 and cytochrome C_{554} can be seen. A similar difference spectrum at room temperature does not resolve cytochrome C_{554} as is also the case for spinach chloroplast lamellae (4).

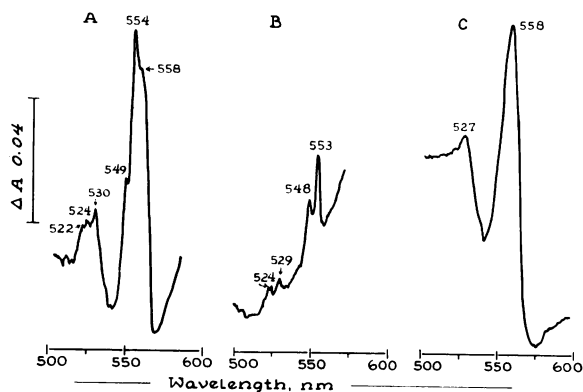


FIG. 5. Low temperature (liquid nitrogen) difference spectra of well-washed *Phormidium* membranes extracted with -20° , 80% acetone. Samples were oxidized with ferricyanide and reduced with dithionite. The solvent was 0.4 M sucrose. A) Reduced minus oxidized; B) untreated minus oxidized and C) reduced minus untreated.

Repeated washing of the membranes with dilute buffer, sonication or treatment with 1% digitonin for 1 hour did not alter the α band absorbancy ratio b_6/C_{554} which was generally 3.5 to 4. This implies that although a considerable fraction of the complement of cytochrome C_{554} is soluble, a constant amount relative to cytochrome b_6 is a lamellar component and is firmly bound to the membrane. The soluble form of this cytochrome constitutes in the order of 75% of the total depending upon growth conditions and it is not necessary for cyclic photophosphorylation. We would suggest that the membrane-bound form is probably of functional significance in the photoreactions.

Discussion

Our data indicate that the osmotic lysis of protoplasts is an excellent method for the creation of cell-free preparations from the blue-green alga, *Phormidium luridum*. The lysed protoplasts are active in cyclic photophosphorylation and photophosphorylation coupled to the Hill reaction and the photoreduction of $NADP^+$.

Previous investigators have reported that preparations of certain blue-green algae are incapable of non-cyclic photophosphorylation. Duane, Hohl, and Krogmann reported that extracts of *Anabaena variabilis* were inactive in non-cyclic photophosphorylation (7). The same preparations were, however, very active in the Hill reaction (31), $NADP^+$ photoreduction (32) and cyclic photophosphorylation (7). Similarly, Black, Fewson, and Gibbs (5) were unable to demonstrate a coupling of phosphorylation to $NADP^+$ reduction by *Anacystis* preparations (5).

More recently, Gerhardt and Trebst have prepared cell-free preparations from lyophilized *Anacystis nidulans* (11). Aqueous extracts of these cells were found to be active in non-cyclic photophosphorylation coupled to ferricyanide reduction and $NADP^+$ reduction. The rates and coupling ratios observed were low, however.

It would appear, therefore, that the enzymatic apparatus for non-cyclic photophosphorylation in blue-green algae is very susceptible to uncoupling. The high rates we obtain for this activity by the *Phormidium* system are a measure of the suitability of protoplast lysis for the preparation of algal extracts. The $P/2e^-$ ratios we observe for non-cyclic photophosphorylation are comparable to those the majority of investigators obtain using chloroplasts from higher plants.

Lysed protoplasts of *Phormidium* are unusual in that supplementary ferredoxin and the associated flavoprotein necessary for $NADP^+$ photoreduction do not improve electron flow. This would suggest that the endogenous enzymes are not detached from their functional sites on the photosynthetic lamellae during the reaction period under the conditions we have employed. This is a further indication that at

least a temporary preservation of intracellular integrity is maintained upon lysis of the algal protoplasts. When the protoplasts are disrupted in a large volume of dilute buffer and gently homogenized, however, these enzymes are removed from the lamellae and can be purified from the washings.

At this juncture, we are uncertain of the morphology of the chlorophyll-bearing cytoplasmic lamellae released upon lysis of the algal protoplasts. In the preparative centrifuge all the lamellae are sedimented in very low gravitational fields, suggesting that they are large membranes. Osmotic lysis may, indeed, liberate the complete unfragmented cytoplasmic lamellar system, and it is possible that the only membrane to undergo extensive damage is the constraining cell envelope.

The mechanism of cyclic photophosphorylation in *Phormidium luridum* appears to be similar to that in *Anabaena variabilis* investigated by Petrack and Lipmann (27) and described in detail by Duane, et al. (7). The response to cofactors, inhibitors and gas phase is the same and comparable to the higher plant mechanism. The apparatus for cyclic photophosphorylation is much more resistant to physical disruption than is the apparatus for non-cyclic photophosphorylation in blue-green algae.

The PMS and DPIP mediated photophosphorylation is catalyzed by algal particles prepared by conventional methods of cell disruption such as grinding and blending. In agreement with previous investigators (7, 10), we observe that the cyclic photophosphorylation activity is not impaired by extensive washing of the particles. The washing removes the bulk of the soluble fraction of the cell which includes the accessory pigment, phycocyanin, the soluble cytochromes type C_{549} and C_{554} , and ferredoxin. Susor and Krogmann (31) and Gerhardt and Santo (10) have shown that phycocyanin is also not essential for the Hill reaction. It is, therefore, possible that this pigment is not an integral component of quantum conversion. It is most likely functional solely in the harvesting of radiation, the energy of which is then transferred to chlorophyll.

Although cyclic photophosphorylation activity is resistant to extensive washing with solutions containing sucrose or ethylene glycol (7, 10) we observe that it is diminished when the membranes are washed with dilute buffers. The loss in activity is due to the removal of a soluble protein which, when added back to the washed membranes, quantitatively restores the photophosphorylation. This component is not removed from the membrane by washing with sucrose or ethylene glycol solutions. It could be an intermediate of the electron transfer chain or a factor necessary for the coupling of ATP synthesis to electron flow.

The protein has been partially purified and does not appear to be a known redox intermediate such as plastocyanin, ferredoxin, cytochrome C_{554} , or cytochrome C_{549} . Its possible functional equivalence to the *Anacystis* factor (9) will be resolved by our

current studies on the complete purification and characterization of the component.

Our spectrophotometric studies of the well-washed membranes show the presence of cytochrome b_6 and a bound form of the high potential cytochrome C_{554} . The difference spectra are virtually identical to those obtained from the lamellae of higher plant chloroplasts (4, 16). The α band absorbancy ratio b_6/C_{554} and the peak maxima are the same. We were unable to remove the bound form of cytochrome C_{554} from the algal particles by sonication or treatment with digitonin. Wessels has recently reported that spinach cytochrome f is removed from chloroplast lamellae by digitonin treatment (34). This may represent an important difference in the molecular architecture of chloroplast lamellae and algal membranes.

The bound form of cytochrome C_{554} is spectrophotometrically different from the soluble form at liquid nitrogen temperature. The α_2 band of the bound form is more intense than that of the soluble form and this may be due to the difference in physical environment. The splitting of the α band of cytochrome C_{554} at low temperature is typical of most C-type cytochromes including those from mammalian sources (8, 35) and photosynthetic tissues of higher plants and algae. Hill and Bonner (16) showed that the α band of purified parsley cytochrome f and membrane bound spinach cytochrome f split at liquid nitrogen temperature. We have shown (unpublished observations) that the α band of cytochrome type c (552, *Euglena*) also is resolved into α_1 and α_2 bands at low temperature. The cytochrome C_2 from *Rhodospirillum rubrum* does not behave in this fashion (8). Thus, the cytochrome C_{554} from *Phormidium luridum* resembles the photosynthetic cytochromes of type c from eucaryotes rather than those from procaryotic cells.

We do not have a precise chlorophyll:cytochrome ratio for the *Phormidium* membranes because of the uncertainty of the extinction coefficients of the cytochromes at liquid nitrogen temperature. This uncertainty is a combination of the contribution of band sharpening, solvent enhancement, and the slight increase in optical path length due to freezing (8, 35). From the room temperature difference spectra we obtained, which are poor in that cytochrome C_{554} is not resolved, we estimate that the cytochrome b_6 :chlorophyll ratio is 1:500.

It is highly probable that the membrane-bound form of cytochrome C_{554} in *Phormidium* is of functional significance in the photosynthetic mechanism and is the equivalent of cytochrome f which is present in higher plant chloroplast lamellae. The physiological function of the soluble form of cytochrome C_{554} is less predictable. Our studies indicate that it is not necessary for cyclic photophosphorylation and, therefore, the operation of photosystem I.

Holton and Myers found that the relative concentrations of soluble cytochrome C_{554} and chlorophyll a was constant and independent of growth conditions

in *Anacystis nidulans* (18, 20). In contrast to this finding, we observed that the bound-cytochrome:chlorophyll a ratio was constant and the soluble cytochrome:chlorophyll a ratio was variable. In rapidly growing exponential phase cells, the soluble cytochrome C_{554} concentration was relatively low compared to stationary phase cells. In this respect, *Phormidium luridum* is somewhat similar to *Chlamydomonas reinhardi* (13).

In that the blue-green algae have a soluble form of cytochrome C_{554} , they are similar to other algal divisions. *Porphyra tenera* (21, 22), *Euglena gracilis* (26) and *Chlamydomonas reinhardi* (13) have readily extractable high potential cytochromes of type c which are associated with the photosynthetic apparatus. Gorman and Levine (14) report that some fraction of the *Chlamydomonas* cytochrome C_{553} is membrane bound, and, at room temperature, is spectrophotometrically different from the soluble form. It would be of interest to know if *Euglena* and *Porphyra* also have a membrane-bound cytochrome c associated with the photosynthetic lamellae. The available difference spectrum (reduced minus oxidized) of *Euglena* membranes does not reveal a bound form of cytochrome type c (552, *Euglena*), but only cytochrome b_{561} and cytochrome a_{605} (26). As is the case for spinach chloroplast lamellae (4) and *Phormidium luridum* membranes, it is possible that the cytochrome of type c is not resolved in such a room temperature difference spectrum. In our experience, this cytochrome is only resolved in room temperature difference spectra of the type ferricyanide-oxidized minus untreated, or oxidized minus ascorbate-reduced. In both of these treatments, cytochrome b_6 , which is usually in the oxidized form on extraction, is not seen and therefore facilitates the resolution of the cytochrome c .

Under the conditions employed we have not detected a membrane-bound form of the low potential cytochrome type c (549, *Phormidium*) first discovered in *Anacystis nidulans* by Holton and Myers (18, 19, 20). The physiological function of this soluble cytochrome and also the soluble form of cytochrome C_{554} awaits further investigation.

In conclusion, our studies show that the mechanism of photosynthesis in blue-green algae is fundamentally similar to that of higher plants. Certain components related to the photosynthetic apparatus are soluble and readily removed from the chlorophyll-bearing lamellae. However, with the exception of the phosphorylation factor, they do not appear to be intrinsic or mandatory components in the structure or function of quantum conversion and reactions of electron transfer.

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