Energy Transduction in the Photosynthetic Membranes of the Cyanobacterium (Blue-Green Alga) *Plectonema boryanum*

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Membrane preparations isolated from the photosynthetic lamellae of the cyanobacterium *Plectonema boryanum* generate upon illumination a transmembrane pH gradient of approximately 2 to 3 pH units (acid inside), as determined from the distribution of either fluorescent or radioactive amines (9 aminouacridine and 14C)methylamine, respectively. Using the distribution of permeant ions to measure the electrical potential across the membrane, it was found that the latter is practically nil under conditions in which the ΔpH is formed and photophosphorylation takes place. In agreement with the above findings cyclic photophosphorylation in this membrane preparation is inhibited by agents shown to collapse the ΔpH but not by agents which should collapse the electrical potential. It is deduced that the pattern of proton movement in the photosynthetic lamellae of intact *Plectonema* spheroplasts corresponds to that of the cell-free membrane system, as both preparations show similar light dependent accumulation of fluorescent amine. It is concluded that the pattern of energy transduction in *Plectonema* photosynthetic lamellae is similar to that of chloroplast thylakoid membranes and not to that of bacterial cytoplasmic membranes. The evolutionary implications of the findings are discussed and a model for the directionality of H+ movements in the whole cell is presented.

As predicted by Mitchell's chemiosmotic theory (1) an electrochemical gradient of protons (ΔµH) is formed across energy-transducing membranes and is involved in the function of these membranes. Nevertheless, the contribution of the chemical (ΔpH) and electrical (ΔΨ) components comprising the ΔµH seems to vary in different membranous systems (reviews in Refs. 2 to 5 and 6 to 8). Thus, in the internal membrane of intact mitochondria and in the intact bacterial cytoplasmic membrane under physiological conditions, ΔΨ forms the main component of ΔµH. On the other hand, in chloroplast thylakoid membranes ΔpH is the major component. In the former case protons are directed outward from the osmotic space formed by the membrane, whereas in the latter case, the direction is inward from the extra membranous space. In some particulate preparations derived from either chloroplasts, mitochondria, or bacteria the differences are less pronounced, which is understandable in view of the experimental manipulations of the membranes. The different patterns observed in the intact preparations may reflect different physiological as well as different evolutionary patterns.

In the evolution of photosynthetic organisms the cyanobacteria (blue-green algae) occupy a special position. They are among the most ancient organisms known, appearing in the Precambrian rock and perhaps even earlier (9). They share with bacteria their prokaryotic cellular organization, but they possess an oxygenic eukaryotic plant-type photosynthetic system with two photosystems (10). Their similarity to chloroplasts of primitive eukaryotic algae has been documented recently both in photosynthetic function as well as in pigment composition, structure, protein synthesis, and even nucleic acids (10-12). It is this combination of characteristics that led to the suggestion that the cyanobacteria may be evolutionarily related to the predecessors of chloroplasts (10-12). However, the differences between the membranes of the chloroplast thylakoids and that of the prokaryotic cytoplasm appear wider in view of the different orientation of proton movement as well as the composition of ΔµH formed upon energization. In a cyanobacterium, similar to prokaryotes, proton extrusion from the cells has been shown upon energization (13). Nevertheless, the pattern of proton movement in many cyanobacteria would appear to be a complex one. Electron micrographs of these cells show that the photosynthetic lamellae run parallel to the cytoplasmic membranes with no discernible interconnections between the two membranes (10, 14).

The study of the characteristics of energy conversion in the photosynthetic membranes of such an organism is, therefore, an intriguing challenge for the understanding of evolutionary patterns in energy conversion. In the present study energy transduction in the photosynthetic membranes of *Plectonema boryanum* has been shown to involve primarily the generation of a large chemical potential gradient of protons (ΔpH).

**EXPERIMENTAL PROCEDURES**

The cyanobacterium used was *Plectonema boryanum* (594 Gomot), obtained from the Indiana University Culture Collection (Blooming-
It was grown photoautotrophically in a modified CHU No. 3282 medium. Cells at the logarithmic phase of growth, which lasted for 5 to 6 days, were used in the experiments.

Photosynthetic membrane preparations of *Plectonema* were prepared, as in other cyanobacterial systems (16, 17), by conversion of the cells into osmotically sensitive spheroplasts followed by lysis of the spheroplasts by dilution in a hypotonic reaction mixture. Spheroplasts were obtained essentially as described previously (16). Cells were washed in a medium containing 0.5 M mannitol, 10 mM MgCl₂, 5 mM sodium phosphate, and 10 mM Tricine/NaOH (pH 7.8) and resuspended to a final chlorophyll concentration of 40 to 50 µg ml⁻¹. Lysozyme (Sigma) was added to give a final concentration of 100 µg ml⁻¹ and the suspension was incubated at 35°C in the dark, during which time the suspension was transferred into small microfuge tubes (0.4 ml) in triplicate and placed in the Beckman microfuge (model 159) precooled by CO₂ flushing. At zero time membrane preparation was provided by two (1000 watts each) Fluorco halogen lamps, filtered through a 5-cm water column. Incident light intensity was 1.3 × 10⁸ ergs cm⁻² s⁻¹. After 3-min incubation in the light, the suspensions were centrifuged at 1 min in the light. Samples of 50 µl and a slice of the pellet (avoiding the supernatant pellet interface) were each mixed with 0.5 ml of perchloric acid (0.6 N). The samples were kept at 4°C for approximately 12 h and centrifuged (8000 × g, 2 min, room temperature). Aliquots of 0.2 ml of each supernatant were mixed with 10 ml of Insta-Gel scintillation fluid (Packard) and counted for both ³²P and [³°Cl content in Packard's liquid scintillation spectrometer (model 2650); [²H₄O served as a marker for the pellet water content.

The data presented in Table I show typical results obtained by the functioning of a cyclic photosystem I-driven reactions. The phosphorylation activity is dependent on light and PMS, is insensitive to DCMU (not shown), and drastically inhibited by DCCD, the specific inhibitor of the mitochondrial and bacterial reversible ATPases (for review see Ref. 22). The uncoupler FCCP which allows electrogenic transport of protons across biological membranes (for review see Refs. 3, 5, and 23) is also a potent inhibitor. This is expected from the already established involvement of the proton electrochemical gradient (ΔpH) across biological membranes in energy transduction as suggested by Mitchell (1).

The ΔpH may be comprised of either ΔpH or Δψ components or both according to the equation

\[ ΔpH_{(in.m)} = Δψ - ZApH \]

For the calculation of ΔpH from the distribution of methylamine between the intramembranous space and the medium the reaction mixture (1.2 ml) contained 10 mM MgCl₂, 5 mM sodium phosphate, 10 mM Tricine/NaOH (pH 7.8), 50 µM PMS, [³°Cl]methylamine (100 µCi ml⁻¹), and [³°Cl]methylamine (10, 1 to 2 × 10⁻⁴ M). Spheroplasts were lysed by dilution (1:40) in the reaction mixture and preincubated for 1 min in the dark, during which time the suspension was transferred into small microfuge tubes (0.4 ml) in triplicate and placed in the Beckman microfuge (model 159) precooled by CO₂ flushing. At zero time membrane preparation was provided by two (1000 watts each) Fluorco halogen lamps, filtered through a 5-cm water column. Incident light intensity was 1.3 × 10⁸ ergs cm⁻² s⁻¹. After 3-min incubation in the light, the suspensions were centrifuged at 1 min in the light. Samples of 50 µl and a slice of the pellet (avoiding the supernatant pellet interface) were each mixed with 0.5 ml of perchloric acid (0.6 N). The samples were kept at 4°C for approximately 12 h and centrifuged (8000 × g, 2 min, room temperature). Aliquots of 0.2 ml of each supernatant were mixed with 10 ml of Insta-Gel scintillation fluid (Packard) and counted for both ³²P and [³°Cl content in Packard's liquid scintillation spectrometer (model 2650); [²H₄O served as a marker for the pellet water content.

**RESULTS AND DISCUSSION**

The present study of the pattern of energy conversion in the photosynthetic membranes of the cyanobacterium *Plectonema boryanum* concentrated on photosystem I-driven reactions. The data presented in Table I show typical results obtained by the functioning of a cyclic photosystem I-driven reactions. The phosphorylation activity is dependent on light and PMS, is insensitive to DCMU (not shown), and drastically inhibited by DCCD, the specific inhibitor of the mitochondrial and bacterial reversible ATPases (for review see Ref. 22). The uncoupler FCCP which allows electrogenic transport of protons across biological membranes (for review see Refs. 2, 3, 5, and 23) is also a potent inhibitor. This is expected from the already established involvement of the proton electrochemical gradient (ΔpH) across biological membranes in energy transduction as suggested by Mitchell (1).

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The ΔpH may be comprised of either ΔpH or Δψ components or both according to the equation

\[ ΔpH_{(in.m)} = Δψ - ZApH \]
Tables II and III show the effect of conditions which affect differentially one or both components of $\Delta \mu_i$. Whereas in the absence of KCl, nigericin (20 to 50 nmol·mg$^{-1}$ of chlorophyll) does not affect phosphorylation, and its addition to nigericin does not enhance the inhibitory effect of the latter. Rendering biological membranes freely permeable to K$^+$, valinomycin in the presence of K$^+$ should collapse any existing $\Delta \phi$ (2, 3, 5, 23). The absence of an appreciable effect by valinomycin suggests the absence of a substantial contribution of $\Delta \phi$ to $\Delta \mu_i$ in the photosynthetic membranes of *Plectonema* under these conditions.

The data presented in Table III show the effect of NH$_4$Cl on photophosphorylation of *Plectonema* membranes. Photophosphorylation is inhibited in the presence of NH$_4$Cl (1 to 10 mM), and the presence of valinomycin (with or without KCl) enhances significantly this inhibitory effect. NH$_4$Cl is a potent uncoupler in chloroplast preparations in which proton movement is directed inward during energization and $\Delta \mu_i$ is comprised almost exclusively by the $\Delta \phi$ (5). A widely accepted mechanism suggested (5, 24) to explain amine uncoupling implies that the membrane is freely permeable only to undissociated amines (in this case NH$_4^+$). Therefore, the cation of an amine with the proper $pK$ will accumulate upon energization in the acidified intrathylakoid space. At adequately high internal concentration of NH$_4^+$, the ion may leak out, short-circuiting the proton current. Valinomycin, which renders biological membranes permeable to NH$_4^+$ (in addition to K$^+$), may enhance the uncoupling effect of ammonia (5, 25, 26). The effect of NH$_4$Cl on *Plectonema* membranes thus further substantiates our conclusions that the $\Delta \phi$ is a major factor in *Plectonema* membrane energy conversion and also implies that proton movement during energization is directed into the membrane-bound space.

The proposed mechanism of amine uncoupling (24) is the basis for calculating $\Delta \phi$ from amine distribution across the membrane (19, 20). Fig. 1 shows that when *Plectonema* membranes are incubated in the light in the presence of the fluorescent amine 9-aminoacridine, the fluorescence is rapidly quenched. After approximately 60 s the fluorescence reaches a new steady state. The reaction is completely reversible in the dark and abolished in the presence of the uncoupler, FCCP (Fig. 1, B and C). Based on the extensive work done in other systems (19, 27, 28), the fraction of the total fluorescence that was quenched ($Q$) is interpreted to indicate that fraction of the amine which is taken up into the internal space formed by the photosynthetic membranes driven by the $\Delta \phi$ across the membrane. The remaining fluorescence (1 - $Q$) is proportional to the external amine concentration. These assumptions are borne out in the present experimental systems since $Q/(1 - Q)$, at saturating light intensity, was independent of the amount of amine added in the tested concentration range (1 to 10 $\mu M$) when the test was performed with a fixed amount of membranes. Furthermore, it increased linearly with the amount of membranes added (2 to 10 $\mu M$ of chlorophyll·mg$^{-1}$) at a fixed concentration of total amine (not shown).

The $\Delta \phi$ was calculated from the following equations (19) for 9-aminoacridine (Fig. 2). Based on the extensive work done in other systems (19, 27, 28), when the test was performed with a fixed amount of membranes:

$$-\Delta \phi = \log \left( \frac{Q}{1 - Q} \right) \times \frac{1}{V}$$

where $V$ is the fraction of the total volume occupied by the osmotic volume of the photosynthetic membranes. (It is assumed that in the dark the internal amine concentration is very low.)

Thus, $\Delta \phi$ plotted against $\log \left( \frac{Q}{1 - Q} \right)$ should provide a linear relationship of slope = 1.0 with an intercept equal to $1/V$. These predictions were tested in an experiment in which $\Delta \phi$ values were artificially imposed across the photosynthetic membranes (Fig. 2). In this experiment different $\Delta \phi$ values were artificially set up across the photosynthetic membranes by addition, in the dark, of varying amounts of NaOH. The intercept allows for the calculation of $V$ which equals 0.006 $\mu L$, yielding an osmotic volume of 450 $\mu L$·mg$^{-1}$ of chlorophyll or 5 $\mu L$·mg$^{-1}$ of protein. Similar results were obtained when different membrane concentrations were used. This value of the photosynthetic membrane osmotic volume...
the calculated osmotic volume of the membranes (see above) the concentration ratio of $[\text{CH}_3\text{NH}_2^+]_\text{in}$/$[\text{CH}_3\text{NH}_2^+]_\text{out}$ was determined. The concentration ratio was independent of the external amine concentration from 1 to 100 $\mu$m, and photophosphorylation was unaffected. The $\Delta pH$ was calculated according to Rottenberg et al. (20).

$$-\Delta pH = \log([\text{CH}_3\text{NH}_2^+]_\text{in})/(\text{CH}_3\text{NH}_2^+)_{\text{out}}$$

Table IV shows that upon energization a large pH gradient of 2.82 units is established across *Plectonema* photosynthetic membranes as measured by the aminoacridine method; the $[^{14}]$methylamine distribution technique gave lower values. As already discussed (30) this method yields an underestimation of the $\Delta pH$. It involves higher membrane concentrations and a centrifugation step, both of which decrease the $\Delta pH$ due to light limitation. The fluorescent amine method, on the other hand, was considered to yield an overestimation of $\Delta pH$ under several circumstances (31). In any event, a large $\Delta pH$ is established across *Plectonema* membranes upon energization. It is collapsed by FCCP and is insensitive to DCCD. In the presence of phosphorylation (addition of ADP) the $\Delta pH$ is decreased.

Tables II to IV show the effect on the $\Delta pH$ of conditions which affect either both $\Delta pH$ and $\Delta \phi$ or only one component contributing to the $\Delta \mu H$. It is observed that these conditions affect $\Delta pH$ in a similar pattern to their effect on photophosphorylation. Thus, the $\Delta pH$ is collapsed by nigericin in the presence of KCl and by ammonia; valinomycin enhances the ammonia effect. Valinomycin in the presence or absence of KCl hardly affects the magnitude of $\Delta pH$. It only accelerates the rate of proton movement across the membrane as observed.

### Table IV

<table>
<thead>
<tr>
<th>Modifications</th>
<th>9-Aminoacridine method$^a$</th>
<th>$[^{14}]$methylamine method$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$Q(1 - Q)$ $\Delta pH$</td>
<td>$\text{CH}_3\text{NH}_2^+$ pel-leat/$\text{CH}_3\text{NH}_2^+$ supernatant</td>
</tr>
<tr>
<td>Dark</td>
<td>2.11</td>
<td>2.92</td>
</tr>
<tr>
<td>DCCD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 $\mu$m</td>
<td>1.57</td>
<td>2.82</td>
</tr>
<tr>
<td>30 $\mu$m</td>
<td>1.57</td>
<td>2.82</td>
</tr>
<tr>
<td>FCCP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.8 $\mu$m</td>
<td>0.182</td>
<td>1.87</td>
</tr>
<tr>
<td>10 $\mu$m</td>
<td>0.0</td>
<td>&lt;1</td>
</tr>
<tr>
<td>ADP</td>
<td>0.8 mm</td>
<td>0.746</td>
</tr>
</tbody>
</table>

$^a$ For the determination of $\Delta pH$ by the 9-aminoacridine method the reaction mixture (2.5 ml) contained 10 mm MgCl$_2$, 5 mm sodium phosphate, 10 mm Tricine/NaOH (pH 7.8), 30 mm PMS, and 15 $\mu$g of chlorophyll. When $Q = \text{fraction of fluorescence quenched and } V = \text{fraction of the osmotic volume calculated from an osmotic volume of 450 $\mu$g of H$_2$O mg}^{-1}$ of chlorophyll (see Fig. 1 and text) the $\Delta pH$ was calculated as previously described (19) (and see text).

$^b$ For the determination of $\Delta pH$ by the $[^{14}]$methylamine method a similar reaction mixture (1.2 ml) was used except that the chlorophyll content was 35 $\mu$m and $\text{H}_2\text{O}$ (83 $\mu$Ci ml$^{-1}$) and $[^{14}]$methylamine (10 $\mu$m, 1 $\mu$Ci $\text{ml}^{-1}$) were added. The osmotic space of the pellet 0.18 was calculated from the membrane osmotic volume and the pellet total water determined from $\text{H}_2\text{O}$-specific activity and the pellet chlorophyll content. The $\Delta pH$ was calculated from the ratio of $\text{CH}_3\text{NH}_2^+$ pellet/$\text{CH}_3\text{NH}_2^+$ supernatant as previously shown (20) (and see text).
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by the increase in the rate of 9-aminoacridine quenching (not shown). The membranes did not take up either $^{14}$C]SCN$^-$ or $^{86}$Rb$^+$ (in the presence of valinomycin) upon illumination. It is concluded, therefore, that $\Delta \psi$ is not involved in energy conversion in Plectonema photosynthesis, and that the major energetic component is $\Delta p$H.

Fig. 3 shows the dependence of the $\Delta p$H and the internal pH on external pH. Within the entire range of external pH values tested (pH 6 to 9) the orientation of proton movement is directed inward, and the highest values of $\Delta p$H are observed within the alkaline range (pH 7.2 to 8.2). Whereas chloroplast preparations show a similar pattern of dependence on external pH (20), this is in marked contrast to the cytoplasmic membrane of E. coli (6). In the latter, the orientation of proton movement changes at an extracellular pH of 7.6, and the optimum $\Delta p$H (inside more alkaline) is observed in the acidic range (pH 5.5 to 6.5). Hence, the pH of the space delimited by the cyanobacterium membranous system drastically changes with external pH (pH 4.5 to 6.5) while that of E. coli remains constant.

The present preparation of cell free photosynthetic membranes of Plectonema is obtained by osmotic lysis of spheroplasts in the reaction mixtures with no further experimental manipulations. Using this technique with other bacteria, Kaback (32) showed that it does not yield inverted structures even when additional purification steps are included. The Plectonema membranes phosphorylate only upon addition of ADP yielding very high specific rates (Table I). The $\Delta p$H which they form upon energization is high and similar in magnitude to that obtained in energized chloroplasts (4). Phosphorylation conditions decrease the $\Delta p$H of both membranes to a similar extent (by 20 mV; see Table IV and Ref. 33). These characteristics make unlikely the possibility that the preparation of Plectonema membranes is composed of a mixed population of variously oriented membranes. Hence, we suggest that these cell-free membranes have the same orientation and characteristics as they have in the intact cell.

In this case intact spheroplasts should accumulate 9-aminoacridine in a similar pattern to that observed with the membranous preparation. This is indeed observed as shown by the data in Fig. 4. Both preparations show a light induced fluorescence quenching of 9-aminoacridine which is completely inhibited in the presence of FCCP (not shown) and NH$_4$Cl (Fig. 4). Obviously the cell-free system is dependent on exogenous electron carrier (PMS), whereas spheroplast activity is endogenous (Fig. 4, A and B). Endogenous photosynthetic activity of intact spheroplasts has previously been shown (16) with Phormidium luridum. Also, in contrast to the membranous preparation, spheroplasts show NH$_4$Cl-sensitive activity in the dark (Fig. 4, A and B). This is in agreement with the observation of respiratory energy conversion in Plectonema cells (34).

Scholes et al. (13) showed that upon energization, cells of Anabaena variabilis extrude protons into the medium similar to other prokaryotic cells. In view of our results, it is suggested that in the cyanobacterial cell of the Plectonema type two types of energy-transducing membranes exist, at least with...
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38x455 basic range of 7.4 to 8.0 (Fig. 3), may hint at the cytoplasmic materials cell membranes.

38x570 photosynthetic lamellae; respect to the orientation of proton movement (Fig. 5). One is the cytoplasmic membrane. Across this membrane, upon energization, proton movement is directed outward, from the cytoplasm to the medium. The other membrane is that of the photosynthetic lamellae which form an intralamellar osmotic compartment separated from the cytoplasm. Upon energization of this membrane protons are pumped from the cytoplasm into the intralamellar space. Under these conditions the cytoplasmic space becomes more basic, whereas the intralamellar space becomes more acidic. In this case the optimum external pH for the ΔpH of the cells. The corresponding very low intralamellar pH (4.5 to 5.0) is separated from the cytoplasm by compartmentalization.

The suggestion (10, 14) that the photosynthetic lamellae of many cyanobacteria, including Plectonema, are separated from the cytoplasmic membrane is compatible with the suggested working hypothesis. These intracellular membranes should be inverted with respect to the cytoplasmic one if they differentiate, as has been implied (14) by invagination from the latter. Furthermore, drastic inhibition of CO₂ photosynthesis by NH₄Cl (35) and uncoupling of α-methyl glucoside-active transport by NH₄Cl in the presence of valinomycin have been recently shown in Plectonema cells.² An energy-dependent, FCCP-sensitive, uptake of methylamine into intact cell upon energization, similar in magnitude to that observed in the membranes and spheroplasts, has also been shown.³ All of these features indicate the existence of an intracytoplasmic space which becomes acidic upon energization and is involved in the energy conversion mechanism of the cyanobacterial cells.

It is apparent that the pattern of energization in the photosynthetic lamellae of Plectonema is very similar to that of the chloroplast thylakoid membranes. Upon energization in both membranes an inward proton movement creates a proton chemical potential gradient (ΔpH) by which energy is conserved. This similarity between the organelle and the cyanobacterial cell thus further emphasizes their suggested, evolutionary interrelationship.

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