Plant Physiol. (1987) 84, 25–30
0032-0889/87/84/0025/06/$01.00/0

Proton Gradients in Intact Cyanobacteria[1]

Received for publication August 27, 1986 and in revised form December 10, 1986

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ABSTRACT

The internal pH values of two unicellular cyanobacterial strains were determined with electron spin resonance probes, over an external pH range of 6 to 9, in the light and in the dark. The slow growing, thylakoid-lacking *Gloeobacter violaceus* was found to have a low capacity for maintaining a constant internal pH. The distribution pattern of weak acid and amine nitroxide spin probes across the cell membranes of this organism, in the light and in the dark, was consistent with the assumption that it contains a single intracellular compartment. At an external pH of 7.0, intracellular pH was 6.8 in the dark and 7.2 in the light. The cells of *Agmenellum quadruplicatum*, a marine species, were found to contain two separate compartments; in the dark, the pH of the cytoplasmic and the intrathylakoid spaces were calculated to be 7.2 and 5.5, respectively. Upon illumination, the former increased and the latter decreased by about 0.5 pH units.

The study of the distribution pattern of either permeable ions or weak acids and bases has become the most accepted method for the determination of the two components of the proton motive force—the electrical membrane potential ($\Delta\psi$) and the proton concentration gradient (ΔpH) (see Refs. 4, 16, 20 for reviews). Radioactively labeled or fluorescent molecules are the more commonly used probes for determination of these parameters in bacterial, membrane, organelle and cellular preparations (4, 5, 16, 20, 21, 24). Similar use has been made of nitroxide spin probes (11–13), but to a much more limited extent. Such probes are stable radicals, easily detected by ESR[3] spectroscopy, whose chemical nature can be designed to be that of a weak acid, a weak base, or a permeable ion. Unlike the radiolabeled probes, the distribution data of the spin probes are available immediately upon the introduction of the sample into the spectrometer cavity. In addition, the method allows for fast, reliable measurements of the cells' water volume, an essential parameter for the calculation of the internal concentration of any probe molecule.

The use of these and other methods has led to an accumulation of data concerning the quantification of the proton motive force in various systems. Surprisingly little information, however, exists regarding electrochemical gradients in cyanobacteria (see Ref. 17 for review), and most available results are fragmentary (5, 6, 8, 10, 15). From these, the picture emerging is that at an external pH of approximately 7.0, the pH of the cytoplasmic space is between 7 and 8, and that of the intrathylakoid space is from 4.5 to 5.5. Upon illumination, the pH in the thylakoids decreases, and that of the cytoplasm increases, similar to the pattern in chloroplasts (7). The alkalinization of the cytoplasm is apparently caused not only by uptake of protons into the thylakoid space, but also by proton extrusion into the surrounding medium (14, 22).

A detailed study of dark ΔpH and $\Delta\psi$ in the cyanobacterium *Anacystis nidulans* was recently published by Peschek *et al.* (18). Although the results are in general agreement with previously reported data, this appears to be the first attempt to characterize the parameters involved over a wide range of external pH values for both compartments of the intact cyanobacterial cell.

In the present report, we describe the pH gradients in intact cyanobacteria using ESR spin-probe techniques. We present new data regarding the internal pH values of the unique, thylakoid-less *Gloeobacter violaceus* and of the marine species *Agmenellum quadruplicatum*, and for the first time quantify dark and light values for both organisms over an external pH range of 6.0 to 9.0.

MATERIALS AND METHODS

Cyanobacterial Strains and Culture Conditions. *Gloeobacter violaceus* (PCC 7421, ATCC 29082, 19) was grown in BG-11 medium (19), at 25°C in dim light (5–10 $\mu E \cdot cm^{-2} \cdot s^{-1}$). Batches of 500 ml, in 21 flasks, were neither shaken nor aerated. Exponential growth phase lasted approximately 6 weeks, at the end of which cells were harvested and immediately used for experiments. A typical suspension of *G. violaceus* generated 140 μmol of O_2 (mg Chl)$^{-1} \cdot h^{-1}$ under saturating light conditions and consumed 60 μmol of $O_2 \cdot$(mg Chl)$^{-1} \cdot h^{-1}$ in the dark.

Agmenellum quadruplicatum (strain PR-6, PCC 7002, ATCC 27264), a generous gift from the late Professor C. Van Baalen (University of Texas, Port Arkansas, TX), was grown in ASP-2 medium (2) with 18 $g \cdot L^{-1}$ (0.3 M) NaCl. Growth temperature was 33°C, and the light intensity 50 to 100 $\mu E \cdot cm^{-2} \cdot s^{-1}$. The cultures, 500 ml in 1 L flasks, were stirred magnetically and slowly bubbled with 1% CO_2 in air. Cells at late exponential growth phase (4–5 d) were used for all experiments. A typical suspension of *A. quadruplicatum* generated 210 μmol O_2 (mg Chl)$^{-1} \cdot h^{-1}$ under saturating light conditions and consumed 26 μmol O_2 (mg Chl)$^{-1} \cdot h^{-1}$ in the dark.

Cell Preparations. Cells were harvested by centrifugation, washed once, and resuspended in the experimental medium to a final density of approximately 0.1 and 1.0 mg Chl $\cdot ml^{-1}$ for *G. violaceus* and PR-6, respectively. The experimental medium for *G. violaceus* was BG-11, supplemented with 25 mM bis-tris propane buffer. For PR-6 the medium consisted of NaCl (0.3 M),

[1] Supported by the National Institute of Health grant AG-04818, by the Department of Energy (grants DE-ATO380-ER10637 and DE-AC03-76SF00098), and by National Aeronautics and Space Administration grant A-14563C.

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[3] Abbreviations: ESR, electron spin resonance; Bis-tris propane, 1,3-bis[tris(hydroxymethyl)-methylamino]propane; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; NiTEPA, Ni tetraethylenepentaamine sulfate.

KCl, K_2HPO_4, and $MgCl_2$ (5 mM each), and bis-tris propane (25 mM). The pH of the cell suspension was adjusted to the desired value immediately prior to the ESR assay. To ascertain that the condition of the cells did not deteriorate over the course of the experiment, photosynthetic O_2 evolution and respiratory O_2 uptake were monitored before and after each experiment with a Clark-type O_2 electrode. In no case was the difference in activity greater than 15%.

Spin Probes. Chemical structures of the nitroxide spin probes used in this study are presented in Figure 1. T-517 (2,2,5,5-tetramethylpyrrolidin-1-oxyl-3-carboxylic acid) and A-519 (3-amino-2,2,5,5-tetramethylpyrrolidin-1-oxyl) were obtained from Molecular Probes, Inc. (Junction City, OR). HO-877 (2,2,5,5,-tetramethylpyrrolidin-1-oxyl was a generous gift from K. Hideg (University of Pecs, Hungary.)

ESR Assays. All assays were conducted using a Varian model E-109E EPR spectrometer, at a power setting of 10 mW, a modulation amplitude of 1 gauss, and a time constant of 0.064 s. In all cases, the samples consisted of 50 μl of the cell suspension, to which the appropriate spin-probe was added (not more than 1 μl). When required, 3 μl of 1.7 M NiTEPA were added to quench the ESR signal emanating from probe molecules in the medium, and thereby visualize only the intracellular signal. This amount was sufficient to completely quench the external signal without causing osmotic changes in cell volume. To samples not containing NiTEPA (unquenched), 3 μl of the experimental medium were added, to allow direct comparison with signal heights of the quenched samples. NiTEPA solutions were pretitrated to the desired pH with sulfuric acid.

For determination of internal volumes or of dark pH values, the samples were inserted into the spectrometer cavity in thin (75 μl) glass capillaries, sealed at one end. Due to the high cell density and to the limited surface area of the sample in the capillary, all the available O_2 was consumed within a few seconds, and the assays were therefore conducted under anaerobic conditions. For measurements in the light, the samples were introduced in thin-walled gas permeable tubing (0.032 inch i.d., 0.005 inch wall thickness, Zeus Industrial Products, Inc., Raritan, NJ). In the latter case, N_2 gas (1.2 $L \cdot min^{-1}$) was flushed directly along the tubing to minimize light-dependent accumulation of O_2, thereby avoiding oxygen broadening of the ESR signals (3). Samples were illuminated, when required, within the spectrometer cavity, with a 1000 W Xenon Arc Lamp (model 6140, Oriel Corp., Stamford, CT). The light was passed through a 6 cm water filter, three neutral density glass filters, and a yellow filter with a cutoff below 540 mm. To further minimize sample heating, the cavity was continuously flushed with a rapid flow of air (100 $L \cdot min^{-1}$). The intensity of the light reaching the cavity was approximately $10^4 \mu E \cdot cm^{-2} \cdot s^{-1}$.

Ratios of internal to total probe concentrations were calculated from height ratios of the midfield lines of the quenched and unquenched samples. To follow changes in internal signals, the magnetic field was locked onto the peak of the same line (3390 gauss) and peak height was followed with time.

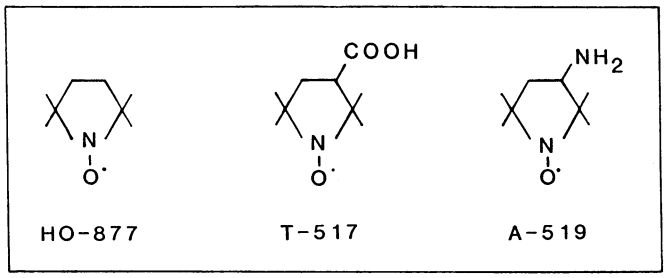

FIG. 1. Schematic structures of the three nitroxide spin-probes used in this study. HO-877, 2,2,5,5-tetramethylpyrrolidin-1-oxyl; T-517, 2,2,5,5-tetramethylpyrrolidin-1-oxyl-3-carboxylic acid; A-519, 3-amino-2,2,5,5-tetramethylpyrrolidin-1-oxyl.

Calculation of Internal pH Values. The following notation is used:

P_{in} = % of probe molecules inside the cells
P_{out} = % of probe molecules outside the cells ($100\% - P_{in}$)
C_{in} = intracellular probe concentration
C_{out} = extracellular probe concentration
V_{in} = intracellular sample volume (%)
V_{out} = extracellular sample volume ($100\% - V_{in}$)
V_{cyt} = fraction of total cell volume occupied by the cytoplasm
V_{thy} = fraction of total cell volume occupied by the intrathylakoid space ($1 - V_{cyt}$)
pH_{out} = external pH
pH_{in} = internal pH
pH_{cyt} = cytoplasmic pH
pH_{thy} = intrathylakoid pH
pH_{av} = internal pH, assuming equal distribution of the probe in the total cell volume.

Calculating Concentration Ratios (C_{in}/C_{out}). The ratio of the quenched to the unquenched signal of a given probe, as calculated from the ESR spectra, is equal to the fraction of the probe molecules which are present in the intracellular volume and are therefore unaffected by the impermeable quencher. We present this value (P_{in}) as percent of the total sample volume. For the neutral probe HO-877, this value is equal to the intracellular volume (V_{in}).

Since $C_{in} = P_{in}/V_{in}$, and $C_{out} = P_{out}/V_{out}$,

$$C_{in}/C_{out} = P_{in}/P_{out} \times V_{out}/V_{in} \quad (1)$$

Using C_{in}/C_{out} to Calculate Internal pH Values. In *G. violaceus*, only one intracellular compartment is involved; therefore, for the weak acid probe T-517 ($pK < 4.5$), according to (20).

$$\Delta pH = \log C_{in}/C_{out} \quad (2)$$

The pK_a of the amine probe A-519, however, as calculated from acid-base titrations, is 7.55; this has to be taken into account (20), and the internal pH, when calculated according to the distribution of this probe is

$$pH_{in} = -log(C_{in}/C_{out}[10^{-pK} + 10^{-pH_{out}}] - 10^{-pK}) \quad (3)$$

To calculate pH values for both intracellular compartments of *A. quadruplicatum*, their fractional volumes (V_{cyt} and V_{thy}) have to be known or assumed. We have used a V_{cyt} of 0.93 and a V_{thy} of 0.07, as previously calculated for *A. nidulans* (1). Because of the expected low pH of the thylakoid space, it is assumed that practically all the acidic T-517 molecules will be in the cytoplasmic space. C_{in}/C_{out} has therefore to be corrected accordingly, and the pH gradient between the medium and the cytoplasmic space is

$$\Delta pH_{cyt\text{-}out} = \log(C_{in}/C_{out} = 1/V_{cyt}) \quad (4)$$

and

$$pH_{cyt} = pH_{out} + \Delta pH_{cyt\text{-}out} \quad (5)$$

The amine probe, on the other hand, will tend to accumulate in the thylakoid volume; however, due to the relatively large V_{cyt}, the amount of probe present in the cytoplasm cannot be ignored. To solve that, an average pH (pH_{av}) is calculated, as if the probe molecules are equally distributed in the total cell volume, using equation 3. The relationship between pH_{cyt}, pH_{av}, and pH_{thy} is

$$10^{-pH_{av}} = 10^{-pH_{thy}} \times V_{thy} + 10^{-pH_{cyt}} \times V_{cyt} \quad (6)$$

and therefore

$$pH_{thy} = -\log([10^{-pH_{av}} - 10^{-pH_{cyt}} \times V_{cyt}]/V_{thy}) \quad (7)$$

In all cases, ΔpH is referred to in this report as $pH_{in} - pH_{out}$; *i.e.* as $pH_{cyt} - pH_{out}$ or as $pH_{cyt} - pH_{thy}$.

Probe Reduction. The 5-membered ring nitroxides used in this study (Fig. 1) have all been shown to be relatively resistant to chemical or biological reduction (S Belkin, RJ Mehlhorn, K Hideg, O Hankovszky, L Packer, unpublished data). In cases where probe reduction or destruction did occur to a significant extent, changes in peak height were followed with time, and the value at time-zero (probe addition) was calculated and subsequently used.

Chl was determined according to Mackinney (9).

Light intensity was measured with a LI-188B Integrating Quantum/Radiometer/Photometer (Li-Cor, Inc., Lincoln, NE) equipped with a Li-190SB Quantum Sensor.

RESULTS

The internal pH of two unicellular cyanobacterial strains were studied: *G. violaceus* and *A. quadruplicatum* (PR-6). The first is a unique microorganism which, unlike all other known cyanobacteria, apparently lacks thylakoids (19). It was therefore assumed to have only a single intracellular compartment.

The partitioning of neutral (HO-877), carboxylic (A-517), and amine (A-519) probes between the intracellular space and the surrounding medium was followed at a variety of external pH values. In each case, the signal intensity of the total amount of probe present (unquenched sample) was compared with that generated only by the probe molecules present inside the cell (quenched sample). The latter values were obtained by addition of an impermeable paramagnetic broadening agent, NiTEPA, 95 mM. In the case of a neutral probe, the ratio of the internal to total signal provides an immediate and direct measurement of the fraction of the total sample volume which is inaccessible to the impermeable NiTEPA, *i.e.* the intracellular water volume. This value was later used to determine the concentration ratios of the charged probes. An example of the calculations and the results obtained for *G. violaceus* under dark anaerobic conditions, at an external pH of 7.1, is presented in Table I. These results were independent of probe concentration at least up to 100 μM for both the acidic and the amine probe.

The resulting pH values, as calculated from the distribution of the two charged probes, are in excellent agreement. Whereas the acid probe (T-517) is concentrated inside by a factor of 1.23, the amine probe (A-519) is excluded from the internal space with almost the reciprocal ratio, *i.e.* 0.81. ΔpH values, calculated independently for each probe as described under "Materials and Methods," were 0.09 and 0.13 for T-517 and A-519, respectively; the calculated internal pH was either 7.19 or 7.23.

This complementarity of the distribution of the two oppositely charged probes was consistent at other pH values, and was further augmented by the changes affected by illuminating the suspension. This was carried out with the cells not in a glass capillary as before, but in thin-walled, N_2-flushed, gas permeable tubing to avoid O_2-dependent signal broadening (3). The response of the different probe types to light is presented in Figure 2. Except for a slight dip immediately upon illumination, possibly due to an O_2 burst (3), the intensity of the intracellular signal of the neutral probe (HO-877) did not change in the light (Fig. 2b), indicating that no significant volume changes occur. The internal concentrations of the charged probes, however, changed markedly. That of the amine probe decreased by 23% (Fig. 2a),

Table I. *Internal pH of G. violaceus*

Cell preparations (77 μg Chl·ml^{-1}, V_{in} = 0.51%), including the appropriate probe, were scanned in the ESR spectrometer in the presence or absence of the quenching agent (95 mM NiTEPA [Q]). Power was 10 mW, modulation amplitude 1 gauss, time constant 0.064 s and scan speed 4 min. Peak heights of the midfield lines were measured, the values corrected for reduction and normalized to a single gain setting (10^4). The various parameters were calculated as described under "Materials and Methods." pH_{out} was 7.10.

Probe	Peak Height (arbitrary units)		P_{in}	C_{in}/C_{out}	ΔpH	pH_{in}
	−Q	+Q				
HO-877 (200 μM)	2100	10.8	0.51	1.0		
T-517 (100 μM)	720	4.5	0.63	1.23	0.09	7.19
A-519 (100 μM)	724	3.0	0.41	0.81	0.13	7.23

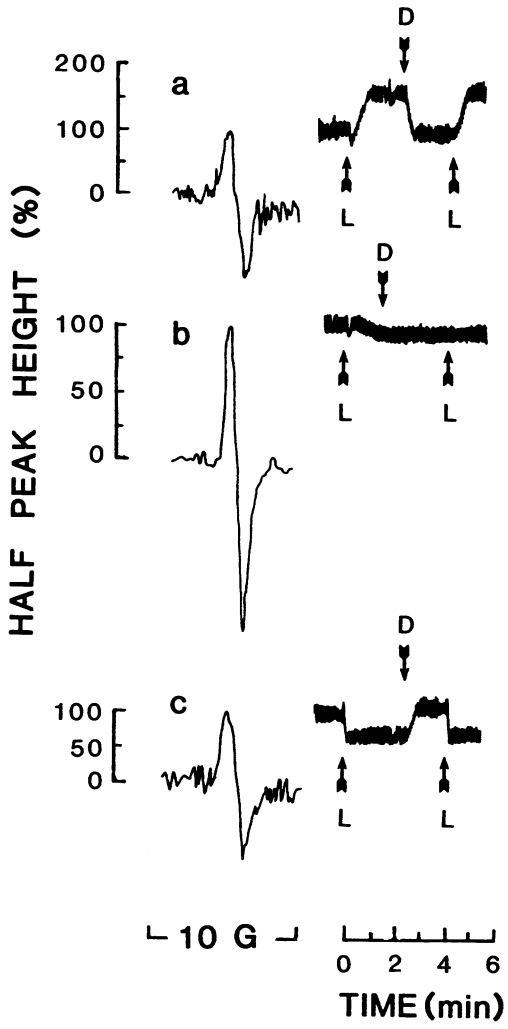

FIG. 2. Light dependent pH shifts in *G. violaceus*. Cell suspensions (77 μg Chl·ml^{-1}, V_{in} = 0.51%), in the presence of the appropriate probe and 95 mM NiTEPA, were incubated in the ESR spectrometer cavity in gas permeable tubing under a flow of N_2 gas. For each probe, the midfield line was traced, and the magnetic field was then locked onto the peak of the line. The line height was then followed with time before, during, and after illumination, where marked by arrows. L, light on; D, light off. a, T-517 (weak acid probe), 100 μM. b, HO-877 (neutral probe), 200 μM; c, A-519 (amine probe), 100 μM. In all cases the power was 10 mW, and the modulation amplitude 1 gauss. The scan range was 100 gauss, centered at 3390 gauss. Time course scans were conducted at 40 cm·h^{-1}, with the field set at zero width and locked on the peak. pH_{out} was 7.5.

whereas that of the carboxyl probe increased by 67% (Fig. 2c). These shifts indicate a light dependent increase in internal pH by 0.24 or 0.22 units, respectively.

Employing the same types of measurements and calculations as described in Table I and Figure 1, the internal pH of *G. violaceus* was determined for an external pH range of 6.0 to 9.0 (Fig. 3). It appears that at least under the experimental conditions described, this organism is not adequately equipped for maintaining a relatively constant internal pH. With a change of 3 pH units, pH_{in} increases by almost 2 units, with the ΔpH values constantly low. Nevertheless, the light-induced changes in pH were in the direction expected of a single-compartment photosynthetic organism, pumping out protons upon illumination.

In similar experiments conducted with *A. quadruplicatum*, the emerging pattern was different and more complex than in *G. violaceus*. In a typical cyanobacterial cell, two distinct compartments are supposed to exist—the thylakoid space and the cytoplasmic space (17); measurements of probe distribution must take this into account and, ideally, provide data for calculating pH values for both compartments. The mathematical approach facilitating such calculations has been presented for chloroplasts (7) as well as for cyanobacteria (5), and has been adapted by us for the ESR assay as presented in "Materials and Methods." The V_{cyt} and V_{thy} used in our calculations, 0.93 and 0.07, were those calculated for *A. nidulans* from EM measurements of thin sections (1). We have no direct proof that this value is identical for *A. quadruplicatum*; however, the use of different V_{thy} values, 0.05 or 0.1, had a negligible effect on the calculated pH_{cyt}, and modified pH_{thy} by not more than 0.1 pH units.

An example of data and calculations leading to determination of the internal pH values of this organism is presented in Table II, and the response to light is shown in Figure 4. Upon illumination, intracellular concentrations of both probes increased. This can only be explained by a decrease in pH_{thy}, leading to a greater accumulation of the amine probe in the thylakoid space, and an accompanying rise in pH_{cyt}, which accounts for the increase in the carboxyl probe.

Studies over a wider pH range yielded the data summarized in Figure 5. In contrast to *G. violaceus*, *A. quadruplicatum* seems to have a much better control over its internal pH, and greater pH gradients are maintained in the dark and/or generated upon illumination.

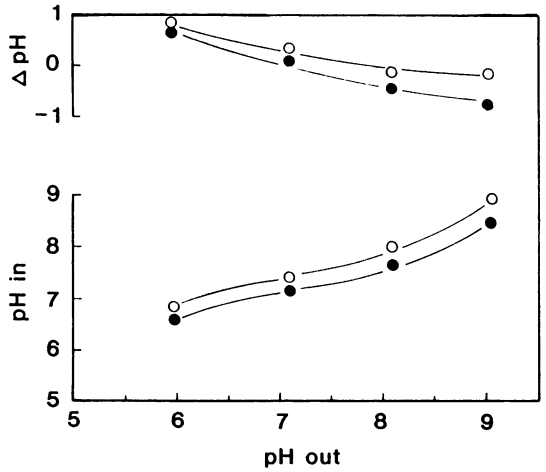

FIG. 3. Proton gradients in *G. violaceus*. Internal pH values were calculated from line height ratios of either the T-517 or the A-519 probes (100 μM), as described in Table I, Figure 2, and in "Materials and Methods." Samples (50 μl, 77 μg Chl·ml^{-1}, V_{in} = 0.51%) were incubated either in glass capillaries (dark values) or in gas permeable tubing under a flow of nitrogen gas in the light (light values). The pH of the preparation was adjusted to the desired value before each assay. (○), light; (●), dark. $\Delta\alpha pH = pH_{in} - pH_{out}$.

To further verify the validity of the data above, the effects of several inhibitors on the pH gradients maintained by *A. quadruplicatum* were studied (Fig. 6). In the presence of the ionophore nigericin, which exchanges K^+ ions for protons, the pH gradients were almost fully collapsible; 38 μM of this compound sufficed to bring the cytoplasmic pH to within 0.2 units of that of the external medium, with no requirement for additional potassium beyond the 5 mM already present in the medium. In the presence of 75 μM nigericin, the calculated ΔpH was 0.07; higher concentrations of the ionophore had no further effect. This residual pH gradient probably represent the maximal error that binding of probe molecules to cellular components may introduce into the calculations.

The use of the uncoupler CCCP, on the other hand, did not result in complete removal of the gradient. Even in the presence of 0.5 mM, a residual ΔpH of about 0.6 units remained (Fig. 6).

DISCUSSION

The use of nitroxide spin probes for the determination of internal pH has been previously shown to be highly effective and accurate (11–13). Nevertheless, the ESR methodology used here has very rarely been applied before to intact cells. One of the main reasons was that nitroxide spin probes are susceptible to signal loss in a reducing environment, usually by reduction to the hydroxylamine. We have recently carried out a comprehensive survey of spin probes, potentially useful for determination of bioenergetic parameters, in various chemical and biological systems (S Belkin, RJ Mehlhorn, K Hideg, O Hankovszky, L Packer, unpublished data). By examining the results of that study it was possible to pick a few probes which were much more resistant to reduction, and therefore more reliable in biological environments. The probes we have used in the present study, HO-877, A-519, and T-517, were among those selected. Their stability was superior to other probes more commonly used, like the neutral Tempone, the weak acid Tempacid or the weak base Tempamine (11–13).

Once the stability problem of the probe is solved, there are many advantages of the ESR method over other assays for ΔpH: (1) the results are immediately available; the internal ESR signal can be visualized as soon as the sample is introduced into the spectrometer cavity; (b) cell volumes are also immediately apparent, and actual probe concentrations can therefore be calculated with no lag period; (c) cell volumes are determined under identical conditions to those employed for assaying the distribution of the charged probes; the effects of osmotic changes in cell volume, or of bound water inaccessible to the probe molecules, are therefore cancelled out; (d) the precision is high, and even minor differences in probe concentrations inside and outside the cell can be reproducibly discerned. Relatively small pH gradients can therefore be reliably determined. (e) For the same reason, one can use spin probes even at the wrong side of their pK without significant loss of sensitivity. (f) Since very small internal probe concentrations can be detected, negative probe accumulation can be measured, up to a certain limit, as easily as a positive one. A pH gradient of one unit, for example, acid inside, can be measured using either a weak base probe, with its inside concentration 10-fold higher than that outside, or a weak base probe with an inside concentration 10 times lower, arriving at the same results. (g) Probe binding may still be a problem in correct interpretation of the experimental results, as in other methods; the easily demonstrated reciprocity of oppositely charged probes, however, as shown here, may serve as a convincing control.

In view of these points, and having selected relatively stable probes, we have applied the ESR method to the determination

Table II. *Internal pH Values of A. quadruplicatum*

Data collected and the various parameters calculated as in Table I and in "Materials and Methods." The cell suspension contained 0.82 mg Chl·ml^{-1}, and pH_{out} was 7.20.

Probe	Peak Height (arbitrary units)		P_{in}	C_{in}/C_{out}	ΔpH (out-cyt)	pH_{cyt}	pH_{av}	pH_{thy}
	−Q	+Q						
HO-877 (1 mM)	7560	190	2.51	1.0				
T-517 (100 μM)	820	24	2.93	1.17	0.10	7.30		
A-519 (20 μM)	85	5.3	6.23	2.59			6.68	5.64

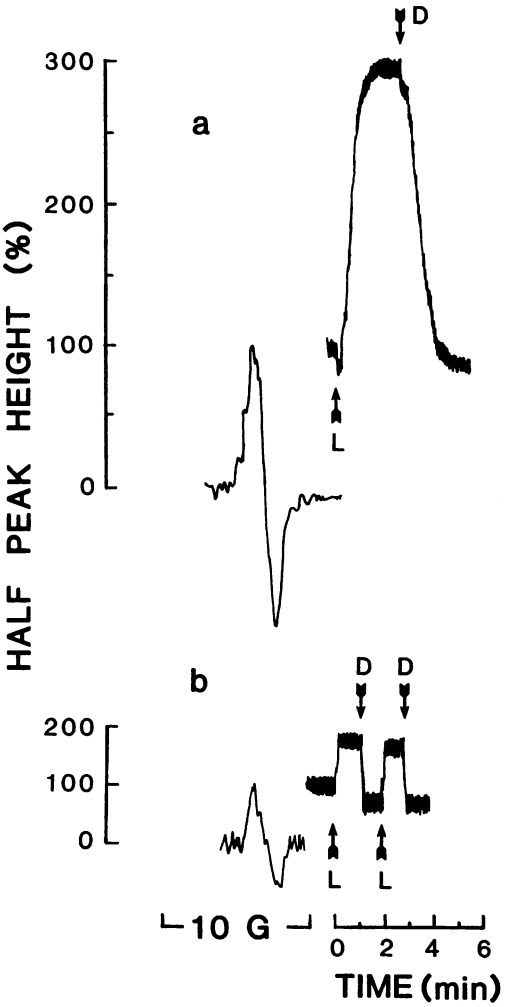

FIG. 4. Light-dependent pH shifts in *A. quadruplicatum*. Measurements and calculations as described in "Materials and Methods," under the experimental conditions described in Figure 2, but with a cell suspension of *A. quadruplicatum* containing 0.82 mg Chl·ml^{-1} (V_{in} = 2.5%) and either T-517 at 100 μM or A-519 at 20 μM. External pH was 7.5.

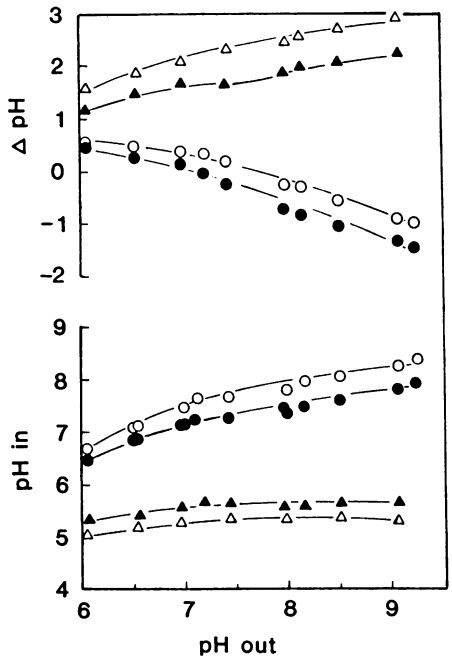

FIG. 5. Proton gradients in *A. quadruplicatum*. Experimental conditions as in Figure 3, using the cell suspension and spin probes described in Figure 4. (○), pH_{cyt} (bottom) or $\Delta pH_{cyt\text{-}out}$ (top); (△), pH_{thy} (bottom) or $\Delta pH_{cyt\text{-}thy}$ (top). Open symbols, light; closed symbols, dark.

of pH gradients across membranes of intact cyanobacteria. Until recently, available information regarding intracellular pH in these organisms was, at best, fragmentary. Data compiled from several reports (5, 6, 8, 10, 15, 22), however, present a rather consistent picture, at least regarding the cytoplasmic pH in the strains tested. The reported values range around pH_{cyt} of 7.0 for external pH values of 6.0 to 8.0, increasing by about 0.5 units in the light. pH_{thy} has been reported once for *A. nidulans* to be 5.2 in the dark and 4.6 in the light, at an external pH range of 6.5 to 8.0 (5). Determination of pH gradients across thylakoid membrane vesicles prepared from *Plectonema boryanum* (15) provided similar data. Peschek *et al.* (18) recently reported on the bioenergetic parameters of *A. nidulans*, confirming the previous reports of a generally neutral pH_{cyt}, and providing for the first time pH_{thy} values for a wide pH range (3.0–11.0). No data were provided for either of the cellular compartments in the light.

The results of the present work regarding *A. quadruplicatum* are in good agreement with those of Peschek *et al.* (18) for both dark pH_{cyt} and pH_{thy} in *A. nidulans*, though it seems as if the former organism has a somewhat lower capacity for maintaining a constant internal pH. The fact that the examined cyanobacterium is of marine origin, and grows at a high NaCl concentration (0.3 M), does not appear to significantly change the pattern emerging from freshwater species such as *A. nidulans*. The shift in pH values for both compartments in the light indicates the expected proton movement from the cytoplasmic space into the thylakoids; our results, however, are not by themselves sufficient to indicate proton translocation in the other direction—across the cytoplasmic membrane into the medium. For that purpose one would either have to calculate the buffering capacity of the intracellular matrix, or directly assay the protons released into the medium, as previously reported (14, 22). The presented results, however, supply for the first time a complete picture of both light and dark intracellular pH values in an intact cyanobacterium.

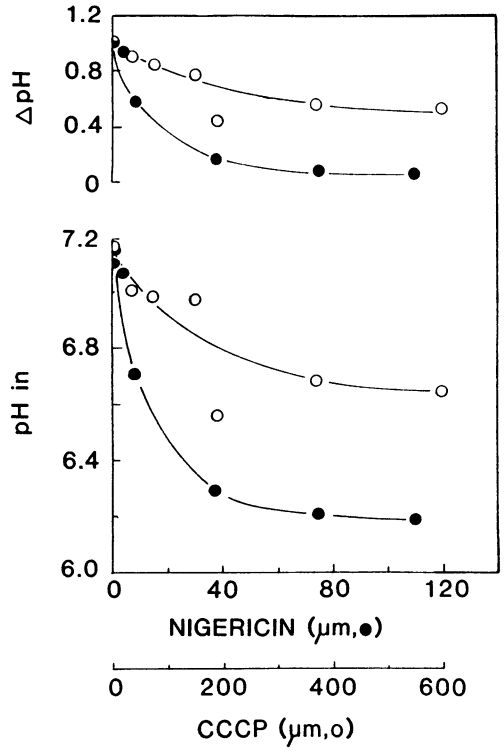

FIG. 6. Effects of nigericin and CCCP on the proton gradients in *A. quadruplicatum*. pH_{cyt} was calculated from the distribution of T-517 (50 μM) in the dark, in glass capillaries, with a cell suspension containing 1.2 mg Chl·ml^{-1} (V_{in} = 3.55%). pH_{out} was 6.1. (●), Nigericin; (O), CCCP. $\Delta pH = pH_{cyt} - pH_{out}$.

Another interesting aspect of the results of this study is the information gained as to the pH gradients of *G. violaceus*. The notion that this unique cyanobacterium possesses only a single cellular compartment was previously based mainly on EM observations (19). Here we present a bioenergetic demonstration that this is indeed the case. In spite of its obvious applicability as a simple model organism for bioenergetic/photosynthetic research, it has been rarely used in such studies; this was probably due to its very low growth rates. No other data is therefore available to compare with ours.

It seems from our results that in *G. violaceus*, components of both photosynthetic and respiratory electron transport are located on the cytoplasmic membrane, and that upon illumination protons are extruded from the cell. As mentioned above, in cyanobacteria as in chloroplasts, proton movement from the cytoplasm upon illumination is both outward, into the medium, and inward, into the thylakoid space. In this thylakoid-less organism, the latter activity is obviously impossible; like photosynthetic bacteria, therefore, the cytoplasmic membrane has to be the only barrier across which the light induced pH gradients are generated. The outward flow of protons is, indeed, similar in direction to that reported for members of this group (13, 23, 25).

Our data also indicate a low capacity, in *Gloeobacter*, for maintenance of a constant internal pH. This may be a result of the anaerobic conditions within the ESR capillary or due to the physiological state of the cells, harvested after approximately 6 weeks of growth. Nevertheless, the reproducibility of our results indicates that the above observation is indeed true. Thus, it is possible that the very low growth rates of this organism may be at least partially accounted for by its inability to create and sustain sufficiently high ΔpH values. On the other hand, it is just as possible that these low values are due to very slow electron flow rates, governed, for example, by the peculiar pigment composition of this cyanobacterium. It should be noted, however, that while light-dependent O_2 evolution rates in *Gloeobacter* are very low, its respiratory rates are comparable to other cyanobacteria (I Fry, personal communication). This may indicate an increased importance for respiratory electron transport, an aspect of *Gloeobacter*'s physiology presently under investigation. Another point that remains to be addressed is the measurement of $\Delta\psi$ in both organisms studied. Although this parameter has been studied in a few cases (18), a complete picture is yet to be presented. In *Gloeobacter*, at least, in view of the low ΔpH values, $\Delta\psi$ may assume an increased significance in the organism's bioenergetic balance.

Acknowledgment—S. Belkin is a recipient of the Dr. Chaim Weizmann Postdoctoral Fellowship for Scientific Research. Department of Energy (DFEAT 0380ER10637).

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