Phosphorus-31 Nuclear Magnetic Resonance Analysis of Internal pH during Photosynthesis in the Cyanobacterium *Synechococcus*†

T. Kallas‡ and F. W. Dahlquist*

ABSTRACT: Phosphorus-31 nuclear magnetic resonance (^{31}P NMR) spectra were obtained from actively photosynthesizing and darkened suspensions of the unicellular cyanobacterium *Synechococcus*. These spectra show intracellular resonances belonging to inorganic phosphate (P_i), a sugar phosphate (sugar-P), nucleotide di- and triphosphates, and polyphosphates. The pH-dependent chemical shifts of P_i and sugar-P allowed the estimation of intracellular pH. When irradiated with high-intensity tungsten–halogen light (100×10^4 ergs·cm^{-2}·s^{-1}, measured in the visible range), concentrated cell suspensions in the NMR spectrometer incorporated $NaH^{14}CO_3$ at approximately two-thirds the rate shown by a dilute suspension of cells at saturating light intensity. On the basis of $NaH^{14}CO_3$ incorporation, the effective light intensity obtained under NMR conditions would support growth at approximately one-fourth the maximum rate in dilute suspensions of cells. Irradiated cells maintained a cytoplasmic pH of 7.1–7.3 when exposed to an external pH from 6.4 to 8.3. At an external pH of 6.7, a darkness to light shift caused a 0.4 pH unit alkalinization of the cytoplasm. Treatment of cell suspensions with the uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), in light or darkness, collapsed the internal pH to the level of the external pH. The results suggest a strong light- or energy-dependent buffering of the cytoplasm over a range of external pH. The study demonstrates that ^{31}P NMR can be used to investigate intracellular events in an actively photosynthesizing microorganism.

Recently phosphorus-31 nuclear magnetic resonance (^{31}P NMR)[1] has become a powerful tool for the study of diverse aspects of metabolism and bioenergetics. ^{31}P NMR analysis can simultaneously yield information on the levels of a number of intracellular phosphate metabolites [e.g., Navon et al. (1977a,b) and Shulman et al. (1979)] and on the pH within one or more intracellular compartments (Salhany et al., 1975; Navon et al., 1977b; Cohen et al., 1978). Furthermore, data can be obtained in vivo from intact cells in a nonperturbing manner. This potential has begun to be exploited in studies with microorganisms (Salhany et al., 1975; Navon et al., 1977b; Ugurbil et al., 1978; Ogawa et al., 1978b), animal cells (Moon & Richards, 1973; Navon et al., 1977a; Cohen et al., 1978), tissues (Burt et al., 1976), and organelles (Casey et al., 1977; Njus et al., 1978; Ogawa et al., 1978a).

^{31}P NMR analysis has apparently not yet been applied to either chloroplasts or photosynthetic microorganisms. This might stem from difficulty in providing uniform irradiation and maintaining cells under actively photosynthesizing conditions in the NMR spectrometer. Nonetheless, ^{31}P NMR should hold as much potential for the study of photosynthetic metabolism as it does for heterotrophic metabolism. Photosynthetic bacteria and cyanobacteria might be particularly interesting objects of investigation. Both groups are prokaryotes and are easily and rapidly cultivated. Progress is being made in the development of genetic analysis systems, especially with photosynthetic bacteria [e.g., Marrs (1978)] but also with cyanobacteria (van den Hondel et al., 1980). Some members of both groups are able not only to photosynthesize but also to fix nitrogen and/or produce hydrogen. These processes may have far-reaching practical applications, particularly in these organisms, since the reactions are driven by light energy. Cyanobacteria have "higher plant" type, O_2-evolving photosynthesis, and cyanobacterial-like organisms may have been the endosymbiotic progenitors of modern chloroplasts (Margulis, 1968). Indeed, it now appears that cyanobacteria are bioenergetically (Padan & Schuldiner, 1978), as well as, in basic respects, structurally and photochemically, analogous to chloroplasts. Salient aspects of cyanobacterial biology have recently been reviewed by Stanier & Cohen-Bazire (1977).

This study shows that ^{31}P NMR can be used to analyze cytoplasmic pH in a strain of the unicellular cyanobacterium *Synechococcus*, under actively photosynthesizing and dark conditions. Data to be presented show that the pH of the cytoplasm is maintained within a rather narrow range, especially in the light, suggesting a strong light- or energy-dependent buffering of cytoplasmic pH.

The results presented here show that ^{31}P NMR techniques can be applied to photosynthetic microorganisms and should hold considerable potential for the study of various aspects of photosynthetic metabolism and bioenergetics.

Materials and Methods

Cyanobacterial Strain and Culture Conditions. Cultures of the unicellular thermophilic cyanobacterium *Synechococcus* (strain Y-7c-s, culture collection of R. W. Castenholz, University of Oregon) were maintained axenically in D medium (Castenholz, 1969), either as liquid batch cultures at 55 °C and 15 klx or 1.5% agar plates supplemented with 0.8 g·L^{-1} glycylglycine at 45 °C and 7.5 klx. Cultures were irradiated with cool-white fluorescent lamps.

Cells for NMR experiments were grown in 0.5- or 1.0-L "bubbler" cylinders containing D medium lacking phosphate. Sterile 1.0 M phosphate (45% K_2HPO_4, 45% Na_2HPO_4, and 10% NaH_2PO_4) was added to give a final concentration of 1.0–4.0 mM. Generally, cells were grown in 2.0 mM phos-

† From the Department of Biology and Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403. *Received February 8, 1981*; *revised manuscript received May 19, 1981*. T.K. was supported by National Science Foundation Grant DEB 7809694 awarded to R. W. Castenholz.

‡ Present address: Département de Biochimie et Génétique Microbienne, Institut Pasteur, 75724 Paris, Cedex 15, France.

[1] Abbreviations used: ^{31}P NMR, phosphorus-31 nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid, Mes, 4-morpholineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; ATP, adenosine 5′-triphosphate; P_i, inorganic phosphate.

0006-2960/81/0420-5900$01.25/0

phate. Gas mixtures consisting of either air or 1.0% CO_2 in air were bubbled into the medium from the bottom of the vessel. Cells were grown under saturating light intensities at 55 °C (18 klx or greater, cool-white fluorescent) for ~2 days and harvested in mid to late log phase. OD_{750} at the time of harvest was typically 0.4–0.8 and represented (3–6) × 10^7 cells·mL^{-1} (based on counts taken with a Petroff-Hauser chamber).

Cells used for NMR experiments were harvested from growth medium by centrifugation and washed 1–2 times in 30–40 mL of NKR solution (New Kreb's Ringer) containing 100 mM buffer and 2.0 mM EDTA. NKR solution contains the following: NaCl, 69.3 mM; KCl, 6.5 mM; $CaCl_2$, 0.28 mM; $MgSO_4$, 0.77 mM. Buffers were Mes at pH 6.0–6.5, Mops at pH 6.6–7.2, or Bicine at pH 8.0 or greater. The final suspension consisted of cells in NKR solution containing 100–250 mM buffer and 2.0 mM EDTA. This suspension also contained 20% D_2O (Bio-Rad) and sometimes an additional 2.0 mM EDTA. Typically, 2.0 mL was used for an NMR experiment, with a cell density of ~2.0 × 10^{10} cells·mL^{-1} (~50 mg dry weight·mL^{-1}). Cell density was estimated on the basis of 1.0 OD_{750} = 8 × 10^7 cells·mL^{-1}.

Measurements of pH were made at 55 °C with a Radiometer PHM 63 digital pH meter calibrated against Radiometer S1500 precision buffer (pH 6.83 at 55 °C). Since cell suspensions contained ~20% D_2O, pH measurements reflected both pH and pD. However, since the effect of D_2O in solution is to shift the pK_a in one direction and glass electrode response in the opposite direction, these two effects tend to cancel each other out, and fractional ionizations at given pD values are virtually indistinguishable from those at the same pH values.

^{31}P NMR spectra were obtained with a Varian XL-100 NMR spectrometer operating in the pulsed, Fourier-transform mode at a magnetic field strength of 23 kG. Temperature was maintained at 54 ± 2 °C. Field homogeneity was adjusted and the field locked on 20% added D_2O. Proton decoupling was generally used, although it probably did not enhance spectral quality significantly. Typically 0.5 s of free-induction decay was accumulated for 12000 pulses, representing 1.8 h. This produced acceptable signal to noise levels in spectra from intact *Synechococcus* cells. A computer-controlled weighting function was used to enhance sensitivity and reduce noise. Peak positions are expressed as parts per million (ppm) relative to external 85% H_3PO_4.

Irradiation and Light Measurement. Irradiation was provided by a 1000 W DXN tungsten–halogen projector lamp in an air-cooled housing. The light was passed through a series of focusing lenses and reflected into the NMR tube from the top, by a mirror suspended on the spectrometer. A stationary, 2.5 mm diameter glass rod was anchored to the suspending apparatus and reached to the bottom of the NMR tube. When the NMR tube was spun by air flow, this rod mixed cell suspensions in the tube. The NMR tubes have dimensions of 12-mm diameter by 23-cm depth. Spinning rates were approximately 4–6 revolutions per second.

Light intensities were measured with an EEl photometer (Evans Electroselenium Ltd., England), a Model 65 YSI Kettering radiometer (Yellow Springs Instrument Co., Yellow Springs, OH), and a Lambda Instruments Li-185 quantum/radiometer/photometer equipped with an Li-192a quantum sensor. The photometer has a 4.5 cm diameter probe, and readings are expressed in kilolux (1.0 klx = 10^3 lumens·m^{-2}). The YSI radiometer has a 3 mm diameter probe and measures total radiation expressed in ergs·cm^{-2}·s^{-1}. All radiometer measurements were taken through a Corning I-69 filter (visible transmitting, infrared absorbing) and therefore are estimates of photosynthetically active radiation. The quantum meter has a 1.0 cm diameter probe and responds to radiation in the 400–700-nm region, which is the photosynthetically active range for cyanobacterial photosynthesis. Quantum measurements are expressed in μEinsteins = 6.02 × 10^{17} photons).

A light intensity of 20 klx (cool-white fluorescent) saturates growth rate (at normal cell density) and is equivalent to ~2.0 × 10^4 ergs·cm^{-2}·s^{-1}, or 240 μEinsteins·m^{-2}s^{-1}. The incident irradiance during NMR experiments was estimated by reconstructing the apparatus on the laboratory bench and measuring the light that passed through the bottom of an empty NMR tube. NMR tubes were shielded so that only light passing down the tube and through the bottom was measured. The maximum attainable irradiance, measured under these conditions, corresponded to 44 klx, 125 × 10^4 ergs·cm^{-2}·s^{-1}, or 5000 μEinsteins·m^{-2}·s^{-1}. Most NMR experiments were conducted at an incident irradiance corresponding to 34 klx, 100 × 10^4 ergs·cm^{-2}·s^{-1}, or 4000 μEinsteins·m^{-2}·s^{-1}. Since the area of the photometer probe is much larger than the area of the spot of light escaping from the bottom of the NMR tube, the kilolux values for irradiance under NMR conditions are gross underestimates.

Photosynthetic Rate of Synechococcus Cells under NMR Conditions. Cells from the same inoculum were grown in unbuffered D medium (1% CO_2, air) at 20 klx. For the experiment at low cell density, 200 mL of cells was harvested, washed in buffer (NKR, 1.0 mM K_2HPO_4, 10 mM Bicine at pH 8.5), and distributed to 3 cm diameter tubes containing either 100 mL of buffer or D medium. These tubes have caps equipped with two ports (consisting of 20-gauge syringe needles) which allow sampling and gassing of the cell suspension. After equilibration at 54 °C under experimental light intensities, 1.0 μCi of $H^{14}CO_3^-$ was added to each 100 mL of suspension (New England Nuclear, 20 μCi in 200 μg of $NaH^{14}CO_3$ in 1.0 mL). The initial samples were taken 2 min after label addition and then at 10 min intervals for 40 min. At ~10-min intervals, suspensions were bubbled with 20 mL of air. Metabolic activity was stopped by injecting samples into formalin (final concentration 5% v/v) and storing in darkness.

For incorporation under NMR conditions, 950 mL of cells was harvested, washed in buffer (NKR, 250 mM Bicine, 2 mM EDTA, pH 8.5), and resuspended in 1.0 mL of distilled H_2O containing 20 μCi of ^{14}C in 200 μg of $NaHCO_3$ (New England Nuclear). Concentrated buffer was added to give NKR with 250 mM Bicine at pH 8.5 in the final suspension. EDTA, K_2HPO_4, and D_2O were added to give final concentrations of 2 mM, 0.9 mM, and 20%, respectively. The final volume was 2.1 mL. This suspension was placed in the NMR spectrometer and allowed to equilibrate at 53 °C. The initial sample was taken in darkness. The suspension was then irradiated, and samples were taken at ~10-min intervals for 40 min. Samples (~0.1 mL) were removed from the spectrometer by means of narrow gauge polyethylene tubing attached to a needle and syringe. Metabolic activity was stopped and samples diluted by injection into 20 mL of buffer (NKR, 10 mM Bicine, pH 8.5) containing 5% v/v formalin.

Portions of samples (~50 μg) were collected on cellulose filters (Schleicher & Schuell AE 91, 24-mm diameter, 0.8-μm pore size) and washed with 2% (w/v) HCl and water to remove externally bound $NaHCO_3$. Filters were placed in scintillation vials, 9 mL of PCS fluor–solvent mixture (Amersham) was added to each vial, and radioactivity was determined with a

Table I: Relative Photosynthetic Rates of *Synechococcus* at Low Cell Density and at High Cell Density under NMR Conditions[a]

condition	cell density (mg dry weight·mL^{-1})	light intensity and source (10^4 ergs·cm^{-2}·s^{-1})	[^{14}C]HCO_3^- incorporation [10^3 cpm·(mg dry weight)$^{-1}$·min of exposure)$^{-1}$]
dilute cell suspension in			
D medium	0.075	2.0, fluorescent	4.9
buffer	0.075	2.0, fluorescent	3.3
buffer	0.075	0.6, fluorescent	3.1
buffer	0.075	darkness	0.3
concd cell suspension in NMR spectrometer	55.0	100, tungsten-halogen	2.0

[a] Cells were grown, harvested, prepared for the experiment, and assayed as described under Materials and Methods. Dilute cells were suspended in NKR, 10 mM Bicine, and 1.0 mM K_2HPO_4 buffer, pH 8.5, or in D medium and irradiated with cool-white fluorescent light. Concentrated cells were suspended in NKR, 0.25 M Bicine, and 2 mM EDTA buffer, at pH 8.2, containing added 0.9 mM K_2HPO_4 and 20% D_2O. Specific activities were 0.033 and 0.040 μCi·mL^{-1}·OD_{750}^{-1} in dilute and concentrated cell suspensions, respectively. Label incorporation under NMR conditions was linear for ~30 min.

Packard Tri-carb scintillation counter (gain 20%; discriminator settings, 40 and 1000). Dry weights were estimated by measuring optical densities in the diluted samples (for *Synechococcus*, 1.0 OD_{750} = 250 μg dry weight·mL^{-1}).

Results

With current spectrometers, ^{31}P NMR spectra of intracellular resonances can only be obtained from highly concentrated cell suspensions. The 23-kG spectrometer used in this study requires a cell density of ~2 × 10^{10} cells·mL^{-1}. This represents a packed cell volume that is 30–40% of the total volume. Do cells receive sufficient light under these conditions to support photosynthesis at a rate that approximates the rate under growth conditions? To test this, we compared the rate of $H^{14}CO_3^-$ incorporation by cells suspended at a low density (at light intensities which support growth) with the rate of incorporation in a concentrated suspension, under NMR conditions.

The results of these experiments are shown in Table I. At low cell density in buffer, high and low light intensities (2.0 and 0.6 × 10^4 ergs·cm^{-2}·s^{-1}) supported essentially equal rates of $H^{14}CO_3^-$ incorporation. Hence, photosynthesis under these conditions was saturated at 0.6 × 10^4 ergs·cm^{-2}·s^{-1}, which is approximately one-third saturating for growth under ordinary conditions. Cells in D medium incorporated label somewhat more rapidly than did cells in buffer. Cells under NMR conditions incorporated [^{14}C]HCO_3 at a rate that was only slightly lower than the rate shown by dilute cell suspensions in buffer. A conservative interpretation of these data is that cells under NMR conditions were light limited and that the effective light absorption supported photosynthesis at two-thirds the rate shown by a dilute suspension at 0.6 × 10^4 ergs·cm^{-2}·s^{-1}. This level of effective light absorption would still support growth at one-fourth the maximum rate. These data demonstrate that *Synechococcus* does maintain a reasonably high rate of photosynthesis at the high cell density required for NMR analysis.

Data Accumulated during 2-h Time Blocks. With the Varian XL-100 spectrometer used in this study, 1.8 h was required for data accumulation from *Synechococcus*. Obviously, shorter times would have been preferable. However, cells photosynthesize actively under NMR conditions (Table I), and while data accumulated over 1.8 h may not be ideal, it appears to represent a valid estimate of the steady-state, intracellular pH levels. That concentrated *Synechococcus* cells may be kept in an NMR spectrometer for many hours, without apparent ill effects, is suggested by the following observations: spectra accumulated from irradiated cell suspensions are similar and indicate similar values of internal pH whether they were accumulated immediately or after cells had already been in the spectrometer for several hours. A spectrum accumulated from irradiated cells, after the accumulation of an initial spectrum from darkened cells, is shown in Figure 1.

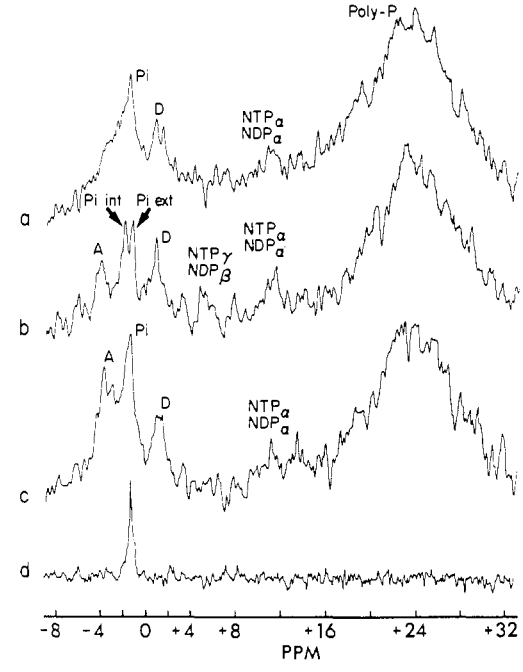

FIGURE 1: ^{31}P NMR spectra of *Synechococcus* cells suspended in NKR medium containing 0.25 M Mops and 2 mM EDTA, with added 20% D_2O, 0.9 mM K_2HPO_4, and 2 mM EDTA. Each spectrum represents accumulation of 12000 pulses, of 0.5 s, over 1.8 h. Conditions during data accumulation: (a) darkened cell suspension; (b) irradiated cell suspension, 100 × 10^4 ergs·cm^{-2}·s^{-1} incident light intensity; (c) cells from suspension b pelleted and resuspended in fresh medium to the original conditions, but with added 200 μM CCCP, and then irradiated during data accumulation, as in b; (d) supernatant from suspension b. Values of pH in the medium before and after each run were 6.80–6.65, 6.65–6.60, 6.71–6.76, and 6.63–6.66 for spectra a–d, respectively. Values of pH in the medium, based on the chemical shifts of external P_i, were 6.7, 6.6, 6.75, and 6.7 for spectra a–d, respectively. Peak identities and chemical shift ranges are listed in Table II.

Since each spectrum represents the accumulation of at least 12000 scans, it is conceivable that the environment of a phosphate compound, and hence its location on the spectrum, might change during data accumulation. In some cases, this can be ruled out. In spectrum d of Figure 1 (accumulated from a supernatant solution), the narrow, intense peak at −1.30 ppm clearly indicates a single phosphate compound whose environment did not change during data accumulation. In spectra obtained from intact cell suspensions, intense peaks that have a narrow apex likewise suggest data accumulated

from a relatively unchanging environment. For example, the pH of the medium measured before and after the accumulation of spectrum b of Figure 1 was 6.65 and 6.60, respectively. Spectrum b shows a relatively narrow an intense peak corresponding to external phosphate.

Other factors such as paramagnetic ions can led to severe broadening and even to the disappearance of certain peaks. This will be discussed further. Consequently, in this study, the locations of peaks (chemical shifts) are more significant than their absolute intensities (heights) or widths. Unless otherwise specified, the location of the apparent maximum of a peak is used to define the chemical shift.

Effect of EDTA. The inclusion of 2–4 mM EDTA in *Synechococcus* suspensions was essential to prevent line broadening apparently due to paramagnetic metal ions. Spectra from cell suspensions without EDTA totally lacked resolution and had unacceptable noise levels even after the accumulation of 60 000 scans or more. Such spectra exhibited only a single ill-defined peak in the P_i and sugar-P region with a width at half-height of approximately 200–400 Hz. Mn^{2+} is a likely paramagnetic ion that could be exerting an effect in *Synechococcus*. Paramagnetic ions can have a pronounced effect on line broadening even at concentrations 3 or 4 orders of magnitude below that of the resonating species (Cohn & Hughes, 1962; Ogawa et al., 1978a).

^{31}P NMR Spectra from Synechococcus Suspensions. Figure 1 shows ^{31}P NMR spectra accumulated from a *Synechococcus* suspension under conditions of darkness and light and after treatment with the uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Spectrum d was accumulated from the supernatant of the irradiated cell suspension (b). The only resonance seen in spectrum d can be attributed to 0.9 mM P_i that was added externally to the cell suspension. This demonstrates that metabolites containing phosphorus do not escape into the medium during NMR analysis and shows that resonances not belonging to external P_i originate intracellularly. In spectrum a from darkended cells, the single, intense P_i resonance at −1.36 ppm suggests that both intra- and extracellular pH values were 6.7. Spectrum b was accumulated from irradiated cells. Spectrum b shows two P_i resonances at −1.92 and 1.20 ppm corresponding to intra- and extracellular pH values of 7.1 and 6.6, respectively. Two new peaks also appear above the level of noise. These are peak A (sugar phosphate, −4.34 and −4.02 ppm) and a peak belonging to the γ-phosphate of nucleotide triphosphates or the β-phosphate of nucleotide diphosphates (+4.88, +5.27 ppm). These sugar-P and NTP_γ (or NDP_β) resonances also titrate with pH, and their chemical shifts in spectrum b correspond to pH in the 7.1–7.5 range. Therefore the darkness to light shift resulted minimally in an 0.4 pH unit alkalinization of the cytoplasm.

After treatment with the proton conducting uncoupler CCCP (Hopfer et al., 1968), spectrum c was accumulated. Spectrum c shows a single, intense P_i resonance at −1.43 ppm and sugar-P resonances (A) that have shifted to −3.74 and −3.00 ppm. An NTP_γ (NDP_β) resonance can no longer be seen above noise levels. The chemical shifts of sugar-P and P_i correspond to pH 6.75, the pH of the medium. These data show that the internal pH has become equal to the external pH and that internal pH maintenance is dependent on membrane impermeability to protons or possibly on the maintenance of a high energy level. The latter explanation cannot be eliminated since CCCP not only makes membranes permeable to protons but also collapses the ATP pool.

CCCP treatment converts ATP largely to ADP, but the ATP + ADP pool also drops to ~60% of its original value

Table II: Identities and Chemical Shifts of Peaks from ^{31}P NMR Spectra of *Synechococcus* Cell Suspensions[a]

peak	notation	assignment	chemical shift (ppm)
A	sugar-P[b]	sugar phosphate	−4.55, −0.30
B	P_i int[c]	internal inorganic phosphate	−2.85, −0.10
C	P_i ext[c]	external inorganic phosphate	−2.85, −0.10
D		unidentified	+0.72, +1.10
E	NTP_γ,[d] NDP_β	γ-phosphate, nucleotide triphosphates, or β-phosphate, nucleotide diphosphates	+4.93, +8.60
F	NTP_α,[e] NDP_α	α-phosphate, nucleotide tri- or diphosphates	+10.28, +11.38
G	NTP_β	β-phosphate, nucleotide triphosphates	+18.41, +19.9
H	poly-P[f]	polyphosphates	+22.5, +28.8

[a] Phosphorus resonances were tentatively identified on the basis of known chemical shifts and their pH dependence. Chemical shifts are expressed as parts per million (ppm) of the field strength (40.5 MHz relative to 85% H_3PO_4 at 0 ppm). [b] Often resolved as a twin set of peaks separated by 0.5–0.6 ppm at pH 7.0; titrated by pH with an apparent pK_a value of ~6.2 at −2.1 ppm. [c] Titrated by pH with an apparent pK_a value of 6.75 at −1.4 ppm. [d] Often resolved as a twin set of peaks separated by 0.3 ppm at pH 7.0; titrated by pH and divalent cations (Cohn & Hughes, 1960, 1962; Gupta & Yushok, 1980). The chemical shift +8.60 ppm probably does not represent the limit of titration. The pK_a value in presence of divalent cation appears to be 5.0–5.5 at +7.5–7.0 ppm. [e] Sometimes resolved as twin peaks. The upfield portion of the resonance at approximately +11.0 ppm may belong to NAD^+ (Navon et al., 1977b; Barton et al., 1980). [f] Resonances in this region probably derive from a heterogeneous array of polyphosphate compounds. In the presence of uncoupler, CCCP, a single, intense resonance remains at +3.7 ppm.

(T. Kallas, unpublished results). However, in spectrum c, the disappearance of only the NTP_γ (NDP_β) peak and not the NDP_α (NTP_α) peak is puzzling and requires comment. One possible explanation is that the resonance at +11.0 ppm belongs to NAD (see footnote *e* to Table II). Another is that paramagnetic ions were released during CCCP treatment. Cohn & Hughes (1962), for example, have shown that the addition of Cu^{2+} to a solution of ATP largely obliterates the γ and β peaks, while the α peak remains unaffected.

The identities and chemical shift ranges of other resonances are listed in Table II. These were tentatively identified by comparison with published results [e.g., Navon et al (1977a,b)]. The chemical shifts and titration of peak A resemble those of the dominant peaks of fructose 1,6-diphosphate (Navon et al., 1977a). Peak G, which corresponds to β-phosphate of nucleotide triphosphates, has been identified from spectra that are not shown here. Internal pH maintenance is summarized in Table III.

Standards for Internal pH Determination. Intracellular pH can be estimated on the basis of the chemical shifts of known phosphate metabolites as a function of pH (Moon & Richards, 1973). However, in addition to pH, the chemical shifts of ionizable moieties such as sugar-P and P_i are affected by other factors, most notably by ionic strength and divalent cations such as Mg^{2+} (Navon et al., 1977a). These factors must be taken into account when constructing calibration curves. Burt et al. (1976) have shown that P_i chemical shifts are insensitive to changes in Mg^{2+} concentration and suggested that P_i should be the most reliable indicator of internal pH.

Figure 2 shows the effect of ionic strength on the titration of the P_i chemical shift as a function of pH. Increased ionic strength allows increased ionization of P_i. This results in a

Table III: Internal pH Maintenance in *Synechococcus*[a]

assay condition	external pH (electrode)	external pH (NMR)	internal, cytoplasmic pH (NMR)	ΔpH (cytoplasmic − external)
darkness	6.3–6.4	6.1	6.7	+0.6
light	6.3–6.4	6.4	7.1	+0.7
darkness	6.8–6.7	6.7	6.7	0
light	6.7–6.6	6.6	7.1	+0.4
light + 200 μM CCCP	6.7–6.8	6.75	6.75	0
light	8.4–7.9	8.3	7.3	−1.0

[a] Data shown in this table are taken from the experiments shown in Figure 5 (external pH 6.1–6.4) and Figure 1 (external pH 6.6–6.75) and from an experiment that is not shown (external pH 8.3). Conditions during data accumulation are described in the legends to Figures 1 and 5. Data at pH 8.3 were accumulated for 20 000 pulses. External pH measurements by electrode were made before and after each run and were also estimated from the chemical shifts of external P_i. Internal pH estimates are based on the chemical shifts of internal P_i. Light intensities were 100 × 10^4 ergs·cm^{-2}·s^{-1} or greater.

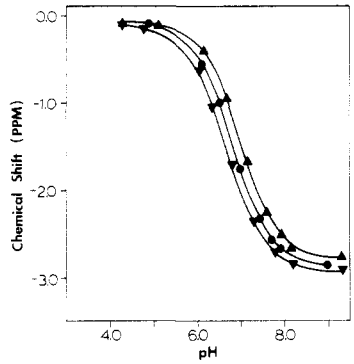

FIGURE 2: Chemical shifts of known 10 mM P_i solutions at three different ionic strengths, plotted as a function of pH. Ionic strengths were adjusted with NaCl. Symbols: (▲) I = 20 mM; (●) I = 110 mM; (▼) I = 260 mM.

lowering of the pK_a and a downfield shift of the P_i resonance at any particular pH. Figure 2 shows that ionic strengths within the 20–260 mM range have an effect on P_i chemical shift that could result in an error of up to ±0.2 pH units in pH values estimated from the center of the titration curve. Much larger errors could result in pH values estimated from the extreme ends of the curve. The appearance of only a single P_i peak in spectrum c of Figure 1 (uncoupler-treated cells) suggests that the intra- and extracellular environments of *Synechococcus* had similar ionic strengths. This is based on the assumption that the internal pH became equal to the external pH in the presence of CCCP.

Figure 3 shows ^{31}P NMR spectra from a suspension of *Synechococcus* cells that had been treated with 200 μM CCCP and stored at 4 °C for several days prior to data accumulation. Each spectrum was accumulated for ~2 h (10 000–12 000 scans). Under these conditions, all peaks except A_1, A_2, and B disappeared. Peaks A_1 and A_2, which correspond to sugar-P, and peak B, corresponding to P_i, remain. As pH increases, all three peaks shift downfield. At low values around 5.35, the three resonances begin to merge and are virtually coincident at pH 4.50. Peaks belonging to sugar-P and P_i also appear on spectra from perchloric acid extracts of cell suspensions and exhibit similar titration behavior (data not shown).

The chemical shifts of the peaks from Figure 3 are plotted as a function of pH in Figure 4. On the same graph are

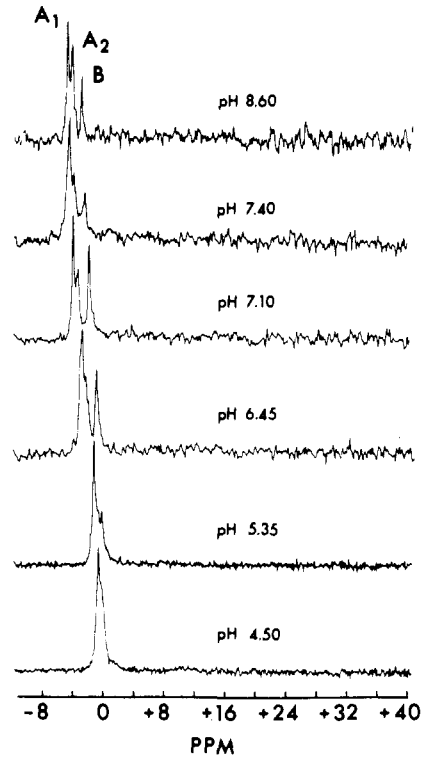

FIGURE 3: ^{31}P NMR spectra showing the effect of pH on phosphorus resonances from a CCCP-treated suspension of *Synechococcus* cells. Cells were suspended in NKR medium, 0.1 M Bicine, and 2 mM EDTA, with 20% D_2O and 2 mM additional EDTA added. 200 μM CCCP had been added and the cells stored for several days at 4 °C prior to NMR analysis. Each spectrum represents the accumulation of 10 000–12 000 pulses of 0.5-s free-induction decay. The pH was adjusted with 0.1 M HCl or 0.1 M NaOH. Peaks A_1 and A_2 represent sugar-P resonance, and peak B represents the resonance of inorganic phosphate (P_i).

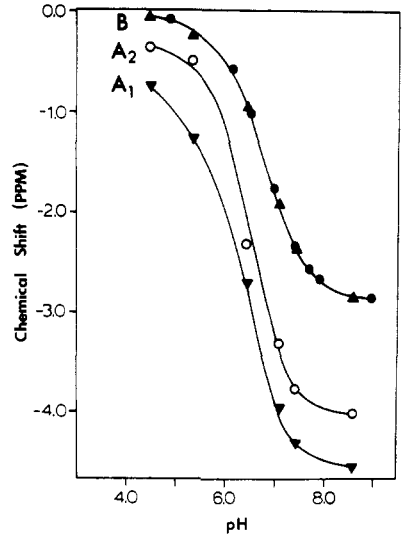

FIGURE 4: Chemical shifts of the peaks from Figure 3 plotted as a function of pH. The curves are labeled A_1, A_2, and B to correspond to the peaks in Figure 3. Symbols: (▼) peak A_1 from Figure 3; (⊙) peak A_2 from Figure 3; (▲) peak B from Figure 3. Plotted on the same graph are the chemical shifts of a known 10 mM P_i solution at an ionic strength of 110 mM (●).

plotted the chemical shifts vs. pH of a known solution of 10 mM P_i at an ionic strength of 110 mM. The titration of known P_i is identical with peak B in Figure 3. This strongly suggests that peak B in Figures 1 and 3 is indeed P_i. The identity of the two curves suggests that the ionic strength of the cell suspension under NMR conditions was ~110 mM.

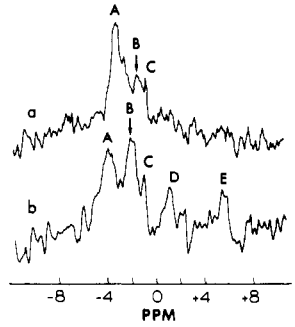

FIGURE 5: ^{31}P NMR spectra of *Synechococcus* cells in NKR medium, 0.1 M Mes, 2 mM EDTA, 0.7 mM KH_2PO_4, 2 mM additional EDTA, and 20% D_2O. Spectrum a was accumulated in the dark for ~2 h (134 000 pulses). The cell suspension was then irradiated at an incident light intensity of 125×10^4 ergs·cm^{-2}·s^{-1}, and spectrum b was accumulated for ~2.5 h (153 000 pulses). The pH of the medium was 6.29 before the start of the dark run and 6.37 after the end of the light run. Peak identities are listed in Table II. Intracellular P_i resonances are designated by arrows and correspond to pH 6.7 in spectrum a and pH 7.1 in spectrum b.

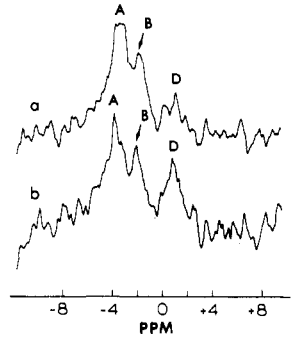

FIGURE 6: ^{31}P NMR spectra of *Synechococcus* cells in NKR medium, 0.1 Mes, 2 mM EDTA, 2 mM additional EDTA, and 20% D_2O. No external P_i was added. Spectrum a was accumulated under low light for 145 000 pulses, and then spectrum b was accumulated under high light for 132 000 pulses. Respective incident light intensities were 8×10^4 and 100×10^4 ergs·cm^{-2}·s^{-1}. The pH in the medium was 6.49 before the start of run a and 6.70 after the end of run b. Peak identities are listed in Table II. Intracellular P_i resonances are designated by arrows and correspond to pH 6.8–7.0 in spectrum a and pH 7.1 in spectrum b.

The spectra shown in Figure 3 were accumulated from CCCP-treated cells that had been stored at 4 °C for several days before NMR analysis. Storage did not affect the chemical shifts of sugar-P or P_i since these were identical in recently CCCP treated cell suspensions (data not shown). In the determination of unknown pH values, P_i resonances were used as the primary standard.

Internal pH Maintenance. Figures 5 and 6 show the effect of a shift from darkness to high-intensity light and the effect of increased light intensity on internal pH maintenance in *Synechococcus*. Spectrum a of Figure 5 was accumulated in the dark. Spectrum b of Figure 5 was accumulated from the same cell suspension during irradiation at high light intensity (125×10^4 ergs·cm^{-2}·s^{-1}). Spectrum a of Figure 6 was accumulated under low light intensity (8×10^4 ergs·cm^{-2}·s^{-1}); then the light intensity was increased to 100×10^4 ergs·cm^{-2}·s^{-1}, and spectrum b was accumulated. External P_i (0.7 mM) was added to the cell suspension in Figure 5, and the external P_i peaks C appear in both spectra. The chemical shifts to external P_i in spectra a and b of Figure 5 correspond to pH 6.1 and 6.4, respectively. The external pH of 6.1 was somewhat lower than the pH of 6.3 measured in the medium before the start of the experiment. External pH could have dropped slightly during darkness because of respiratory CO_2 production. External P_i was not added to the cells in Figure 6, an no external P_i peak is seen. Internal pH values based on P_i resonances are pH 6.7 (dark) and 7.1 (light) in spectra a and b of Figure 5, at external pH values of 6.3 and 6.4, respectively, and pH 6.8–7.0 (dim light) and 7.1 (bright light) in spectra a and b of Figure 6, at external pH values of 6.4 and 6.7, respectively.

Spectrum a of Figure 5 (from darkened cells) shows an intense sugar-P resonance (peak A), while spectrum a of Figure 1 (also from darkened cells) shows no apparent sugar-P peak. This difference may have arisen because the cells used for the experiments shown in Figures 5 and 6 were grown in 1.0-L vessels. Hence, the effective light intensities were different. Other data (not shown) indicate that slight differences in growth conditions and cell concentrations lead to different relative intensities of sugar-P and P_i peaks. Generally, spectra accumulated from cells at higher densities show intensified sugar-P peaks.

Discussion

For all known cyanobacteria except *Gloeobacter violaceous* (Rippka et al., 1974), there are two metabolic compartments separated by the photosynthetic, or thylakoid, membrane. These are the cytoplasmic and the intrathylakoidal compartments. The thylakoid membrane may be topologically complex, and the intrathylakoidal compartment may not be a continuous space.

The intracellular P_i resonances of Figures 1, 5, and 6 were assigned to the cytoplasmic space of *Synechococcus* for the following reasons. First, the volume of the intrathylakoidal space of cyanobacteria has been estimated to be only 7–10% of the volume of the intracellular space (Allen, 1968). Thus, an intrathylakoidal resonance would not be seen unless the intrathylakoidal P_i concentration were at least several times greater than the cytoplasmic P_i concentration. At the signal to noise levels attainable at 40.5 MHz, it is unlikely than an intrathylakoidal resonance could be detected. Second, the intracellular pH values shown here are consistent with the neutral to slightly alkaline cytoplasmic pH values reported for other cyanobacteria (Falkner et al., 1976; Padan & Schuldiner, 1978). The results of Falkner et al. (1976) and Padan & Schuldiner (1978) were obtained with non-NMR methods. Intracellular sugar-P resonances (peaks A of Figures 5 and 6) were assigned to the cytoplasmic compartment for the same reasons.

Figures 5 and 6 demonstrate that the pH of the cytoplasmic compartment in *Synechococcus* was maintained at a higher level than that of the medium when the external pH was in the 6.3–6.7 range. At least over exposure times of several hours, this was true regardless of whether the cells were exposed to high- or low-intensity light or whether they were kept in darkness. However, there was a slight further alkalinization of the cytoplasm when cells were shifted both from darkness to high-intensity light (Figure 5) and from a low-intensity light to a higher light intensity (Figure 6). In Figures 5 and 6 this effect is seen as a downfield shift of peaks A and C in spectrum b, relative to their positions in spectrum a. The value of ΔpH of 0.4 ± 0.1 pH unit appears to be constant within the error limits in the two experiments. The alkalinization effect of light appears to be saturated at a light intensity of 100×10^4 ergs·cm^{-2}·s^{-1}. Alkalinization of the cytoplasm by light is consistent with the earlier estimates of Falkner et al. (1976).

Table III summarizes internal pH maintenance in *Synechococcus*. Data were taken from experiments shown in Figures 1 and 5 and from an experiment not shown. Irradiated cells maintained a constant level of cytoplasmic pH (7.1–7.3)

over external pH values of 6.4–8.3. Cells in darkness, at external pH values of 6.1 and 6.7, also maintained cytoplasmic pH, but at a somewhat lower value of pH 6.7. A shift from darkness to light caused a slight alkalinization of the cytoplasm. This is best shown at pH 6.6–6.7, where external pH remained essentially constant during a dark to light shift but the internal pH increased by 0.4 pH unit. Addition of the uncoupler CCCP abolished internal pH control in the light (Figure 1, Table III) or in darkness (not shown).

Table III shows that irradiated *Synechococcus* maintained cytoplasmic pH (at pH 7.1–7.3) over a range of external pH extending from 6.4 to 8.3. These results, obtained with ^{31}P NMR, are in good agreement with the findings of Falkner et al. (1976) obtained with the closely related, nonthermophilic cyanobacterium *Anacystis nidulans* (designated *Synechococcus AN* according to the classification of Rippka et al. (1979)]. Falkner et al. (1976) used the distribution of the permeant weak acid 5,5-dimethyloxazolidine-2,4-dione (DMO) to estimate the pH of the cytoplasm and the distribution of methylamine to estimate the pH of the intrathylakoidal space of *A. nidulans*.

Falkner et al. (1976) found that irradiated *Anacystis* maintain cytoplasmic pH at 7.5 over an external pH range of 6.5–8.0. Over the same external pH range, cytoplasmic pH is maintained at 6.9 in darkness. Data shown here indicate that *Synechococcus* also maintained cytoplasmic pH in darkness. The data of Falkner et al. (1976) were obtained after short exposures to various, external pH values. The data shown here were typically accumulated after several hours of exposure to experimental pH values. Falkner and co-workers indicate that their pH values varied "considerably" and that the values presented represent maximum values. Internal pH values presented here may be somewhat low because of the possibly less than optimal conditions in concentrated NMR cell suspensions. Considering these radical differences in methodology, the data presented here, and those of Falkner et al. (1976), are in remarkably good agreement.

The DMO and methylamine techniques as well as other non-NMR measures of both electrical and chemical components of transmembrane electrochemical proton gradients have been reviewed by Rottenberg (1975). DMO and amine techniques are based on the premise that membranes are freely permeable to the uncharged species. pH values are calculated from the distribution of radioactively labeled DMO or methylamine between the compartment of interest and the medium or from the quenching of fluorescent amines. The results of fluorescent and radioactive amine techniques have been comparable when both methods have been applied to the same system (Padan & Schuldiner, 1978; Rottenberg et al., 1972; Schuldiner et al., 1972). NMR and permeant-ion results from similar systems have also been comparable [the present study and Falkner et al. (1976); Navon et al., 1977b, Padan et al., 1976; Casey et al., 1977].

A principal difficulty, however, of the permeant-ion techniques is that the osmotic volume of the compartment of interest must be estimated and enters into the pH calculation. This is a particular problem with two-compartment systems such as chloroplasts or cyanobacteria, because the volumes of both compartments apparently cannot be estimated by quantitative, chemical means (Heldt et al., 1973). An additional difficulty with radioactive permeant ions is that the cells or organelles must be rapidly separated from the medium before reequilibration of the ion takes place. Chloroplasts and cyanobacteria therefore must be actively photosynthesizing and illuminated during the separating centrifugation.

Estimation of pH and ^{31}P NMR has the advantage of being independent of compartmental volume and ion-equilibration considerations. ^{31}P NMR allows in vivo measurements to be made on intact organisms without the use of exogenous probes. The main disadvantages of ^{31}P NMR are that a very dense cell suspension must be used and, with the spectrometer used in this study, a long 2-h period is needed for data accumulation. However, it appears that one or both of these problems can be solved with high-sensitivity spectrometers [e.g., Ugurbil et al. (1978)] and special data accumulation techniques (Ogawa et al., 1978b). These points will be further discussed below.

The present experiments with *Synechococcus* and those of Falkner et al. (1976) with Anacystis demonstrate that at least in these cyanobacteria there exits a strong light- or energy-dependent buffering of the cytoplasm at external pH values in the 6.5–8.0 range. Cytoplasmic pH buffering has also been shown with ^{31}P NMR studies of yeast (Salhany et al., 1975) and *Escherichia coli* (Navon et al., 1977b). The NMR study of *E. coli* confirmed the earlier work of Padan et al. (1976) using DMO and methylamine distribution, which showed that the cytoplasmic pH level in *E. coli* remains relatively constant at external pH values from 6 to 9. It is known that many cytoplasmic enzymes have very narrow pH optima and that many metabolically active enzymes act in concert as enzyme complexes. In view of this, the maintenance of a relatively constant cytoplasmic pH over a range of external pH values, by representatives of diverse groups of microorganisms, should not be surprising. In cyanobacteria, however, there might be an additional energetic reason for cytoplasmic pH buffering. Padan & Schuldiner (1978) have investigated transmembrane electrochemical gradients in thylakoid membrane vesicles prepared from spheroplasts of the filamentous cyanobacterium *Plectonema boryanum*. Such vesicles have the same orientation as photosynthetic membranes in intact cells. Using permeant-amine techniques to measure ΔpH, they found that ΔpH was the main component of the transmembrane electrochemical proton gradient and that the optimum for ΔpH occurred when the pH of the medium was between 7.4 and 8.0. These results suggest that *Plectonema*, and perhaps cyanobacteria in general, must maintain a high ΔpH between the cytoplasm and intrathylakoidal space in order to maintain the membrane-energized state at an optimal level. It is possible that cytoplasmic pH buffering in cyanobacteria is a requisite for optimal photosynthetic energy transduction.

The necessity of including a relatively high concentration of EDTA in *Synechococcus* suspensions raises two questions. EDTA (10 mM) is commonly used to strip *E. coli* membranes of the F1 segment of Mg^{2+}-ATPase, thus rendering them incapable of ATP hydrolysis or synthesis (Tsuchiya & Rosen, 1975a,b). The question is whether the 2–4 mM EDTA used in *Synechococcus* (at higher cell density) might have a deleterious effect on *Synechococcus* ATPase. This question can be answered by observing the chemical shifts of the intracellular γ- and β-phosphates of ATP from *Synechococcus*. In the presence of a divalent nonparamagnetic ion such as Mg^{2+}, these resonances undergo a profound downfield shift (Cohn & Hughes, 1962). The chemical shifts at neutral pH in the presence of divalent ion are approximately +5 and +19 ppm vs. +8 and +22 ppm in the absence of divalent ions for the γ- and β-phosphates, respectively. The NTP_γ peaks of spectrum b of Figure 1 and spectrum b of Figure 5, accumulated in the presence of 4 mM EDTA, occur at +4.88 and +5.27 ppm for the former and +5.40 ppm for the latter. The NTP_β peaks of spectrum b of Figure 5 occur at +18.8 and +19.2 ppm (not shown). These data indicate that the intra-

cellular concentration of divalent cations exceeds the concentration of EDTA. EDTA, in the amounts used, should not affect ATPase function.

Synechococcus, as a cyanobacterium, possesses higher plant type, O_2-evolving photosynthesis which requires Mn^{2+} as a cofactor in the O_2-evolving protein complex of photosystem II. Since EDTA was present in sufficient concentration to relieve a paramagnetic effect probably caused by Mn^{2+}, could it also have bound Mn^{2+} to an extent that would inhibit the rate of photosynthetic, noncyclic electron transport? Table I shows that *Synechococcus*, under NMR conditions in the presence of 2 mM EDTA, incorporated $H^{14}CO_3^-$ at a high rate. These data suggest that EDTA did not affect photosynthesis. The effect of EDTA was also assessed by measurements of photosynthetic O_2 evolution. The rate of O_2 evolution in the presence of 1.0 mM EDTA represented a 50–100% increase over the rate at zero EDTA. At 10 mM EDTA, the rate of O_2 evolution dropped to ~70% of the zero EDTA rate. These results indicate that while EDTA has an effect on the rate of noncyclic electron transport, the effect at low EDTA concentrations is not the inhibitory one that would result from removal of Mn^{2+}. On a per cell basis, 2–4 mM EDTA at NMR cell densities should have less effect than 1.0 mM EDTA in the control experiment.

The present study demonstrates that ^{31}P NMR can be used to study intracellular events in a photosynthetic microorganism under actively photosynthesizing conditions. Among the kinds of analyses that should be possible in photosynthetic systems, is the simultaneous, in vivo monitoring of intrathylakoidal and cytoplasmic pH changes, assuming that the intrathylakoidal concentration of phosphate or of some other titratable phosphorus moiety is sufficiently high to be detectable. The feasibility of simultaneous pH measurements from two intracellular compartments has recently been demonstrated for rat liver cells (Cohen et al., 1978), where P_i resonances from both cytoplasmic and intramitochondrial compartments could be distinguished. Such techniques should be applicable to the study of various aspects of photosynthetic metabolism, not only in cyanobacteria but also in photosynthetic bacteria, eukaryotic algae, and chloroplasts.

References

Allen, M. M. (1968) *J. Bacteriol. 96*, 836–841.

Barton, J. K., den Hollander, J. A., Lee, T. M. MacLaughlin, A., & Shulman, R. G. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2470–2473.

Burt, C. T., Glonek, T., & Barany, M. (1976) *J. Biol. Chem. 251*, 2584–2591.

Casey, R. P., Njus, D., Radda, G. K., & Sehr, P. A. (1977) *Biochemistry 16*, 972–977.

Castenholz, R. W. (1969) *Bacteriol. Rev. 33*, 476–504.

Cohen, S. M., Ogawa, S., Rottenberg, H., Glynn, P., Yamane, T., Brown, T. R., & Shulman, R. G. (1978) *Nature (London) 273*, 554–556.

Cohn, M., & Hughes, T. R., Jr. (1960) *J. Biol. Chem. 235*, 3250–3253.

Cohn, M., & Hughes, T. R., Jr. (1962) *J. Biol. Chem. 237*, 176–181.

Falkner, G., Horner, G., Werdan, K., & Heldt, H. W. (1976) *Plant Physiol. 58*, 717–718.

Gupta, P. K., & Yushok, W. D. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2487–2491.

Heldt, H. W., Werdan, K., Milovancev, M., & Geller, G. (1973) *Biochim. Biophys. Acta 314*, 224–241.

Hopfer, W., Lehninger, A. L., & Thompson, T. E. (1968) *Proc. Natl. Acad. Sci. U.S.A. 59*, 484–490.

Margulis, L. (1968) *Science (Washington, D.C.) 161*, 1020–1022.

Marrs, B. L. (1978) in *The Photosynthetic Bacteria* (Clayton, R. K., & Sistrom, W. R., Eds.) pp 873–883, Plenum Press, New York.

Moon, R. B., & Richards, J. H. (1973) *J. Biol. Chem. 248*, 7276–7278.

Navon, G., Ogawa, S., Shulman, R. G., & Yamane, T. (1977a) *Proc. Natl. Acad. Sci. U.S.A. 74*, 87–91.

Navon, G., Ogawa, S., Shulman, R. G., & Yamane, T. (1977b) *Proc. Natl. Acad. Sci. U.S.A. 74*, 888–891.

Njus, D., Sehr, P. A., Radda, G. K., Ritchie, G. A., & Seeley, P. J. (1978) *Biochemistry 17*, 4337–4343.

Ogawa, S., Rottenberg, H., Brown, T. R., Shulman, R. G., Castillo, C. L., & Glynn, P. (1978a) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1796–1800.

Ogawa, S., Shulman, R. G., Glynn, P., Yamane, T., & Navon, G. (1978b) *Biochim. Biophys. Acta 502*, s45–50.

Padan, E., & Schuldiner, S. (1978) *J. Biol. Chem. 253*, 3281–3286.

Padan, E., Zilberstein, D., & Rottenberg, H. (1976) *Eur. J. Biochem. 63*, 553–541.

Rippka, R., Waterbury, J., & Cohen-Bazire, G. (1974) *Arch. Mikrobiol. 100*, 419–436.

Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., & Stanier, R. Y. (1979) *J. Gen. Microbiol. 111*, 1–61.

Rottenberg, H. (1975) *Bioenergetics 7*, 61–74.

Rottenberg, H., Grunwald, T., & Auron, M. (1972) *Eur. J. Biochem. 25*, 54–63.

Salhany, J. M., Yamane, T., Shulman, R. G., & Ogawa, S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4966–4970.

Schuldiner, S., Rottenberg, H., & Avnon, M. (1972) *Eur. J. Biochem. 25*, 64–70.

Shulman, R. G., Brown, T. R., Ugurbil, D., Ogawa, S., Cohen, S. M., & den Hollander, J. A. (1979) *Science (Washington, D.C.) 205*, 160–166.

Stanier, R. Y., & Cohen-Bazire, G. (1977) *Annu. Rev. Microbiol. 31*, 225–274.

Tsuchiya, T., & Rosen, B. P. (1975a) *Biochem. Biophys. Res. Commun. 63*, 832–838.

Tsuchiya, T., & Rosen, B. P. (1975b) *J. Biol. Chem. 250*, 7687–7692.

Ugurbil, K., Rottenberg, H., Glynn, P., & Shulman, R. G. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2244–2248.

van den Hondel, C. A. M. J. J., Verbeek, S., van der Ende, A., Weisbeek, P. J., Borrias, W. E., & van Arkel, G. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1570–1574.