Electron Transport and Oxidative Phosphorylation in the Blue-green Alga *Anabaena variabilis*

By C. K. LEACH AND N. G. CARR

*Department of Biochemistry, The University of Liverpool,
P.O. Box 147, Liverpool, L69 3BX*

(Accepted for publication 14 September 1970)

SUMMARY

The respiratory electron transport system involved in NADPH (2.0 nmoles/min./mg.) and NADH (1.0 nmoles/min./mg.) oxidation in the dark that was operative in extracts of light-grown *Anabaena variabilis* has been examined. NADPH oxidation was inhibited 50% by cyanide (2×10^{-2} M), rotenone (10^{-4} M); antimycin A, amytal and azide were markedly less inhibitory. NADPH:ferrocytochrome *c* oxidoreductase, NADPH:menadione oxidoreductase, ferrocytochrome *c*:oxygen oxidoreductase and succinic dehydrogenase were detected. A phosphorylation (0.4 nmoles/min./mg.), associated with NADPH oxidation, was measured, NADPH could be replaced by NADP and isocitrate. This phosphorylation was absolutely dependent upon oxygen and was inhibited 25% by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (5 μ M).

Blue-green algae, in common with other autotrophic micro-organisms, are capable of growth in an inorganic environment. However, autotrophs will utilize to some extent reduced carbon compounds (Carr & Pearce, 1966; Hoare, Hoare & Moore, 1967; Kelly, 1967; Smith, London & Stanier, 1967; Pearce & Carr, 1967, 1969) and it is their dependence upon a specific energy source (an inorganic oxidation or photosynthesis) that distinguishes them most clearly from heterotrophic bacteria. Typically, growth and respiratory rates do not increase when organic substrates are supplied to obligate autotrophs. However, when the rate of respiration of *Anabaena variabilis* is reduced by prior starvation of CO₂, oxygen uptake is partially restored by addition of glucose (Kratz & Myers, 1955; Pearce & Carr, 1967). Although respiratory rates in blue-green algae are low, Q_{O_2} values of 5 to 10 being common, some respiration does occur; its inhibition by cyanide, azide or carbon monoxide suggests an electron transport chain at least comparable to that found in aerobic bacteria (Webster & Frankel, 1953).

The observation by Smith *et al.* (1967) that obligate chemoautotrophs and blue-green algae lack an NADH oxidase led these workers to suggest that such a metabolic lesion is the characteristic and general feature of obligately autotrophic micro-organisms. However, oxidation of reduced pyridine nucleotides by extracts of autotrophic thiobacilli (Hemphling & Vishniac, 1965; Trudinger & Kelly, 1968) and blue-green algae (Horton, 1968; Leach & Carr, 1968) suggests that these organisms can oxidize organic substrates, and is consistent with their low but reproducible respiratory oxygen uptake. Furthermore, in *Anabaena variabilis* the coupling in the dark of NADPH oxidation to ATP synthesis has been shown in cell-free extracts (Leach & Carr, 1969).

and a proton translocation, outwards through the membrane, in response to oxygenation of intact cells has been found (Scholes, Mitchell & Moyle, 1969). In view of the lack of information on the respiratory activity of blue-green algae and because of the relevance of such data to the understanding of autotrophic physiology, a study of respiratory enzymes and oxidative phosphorylation in *A. variabilis* is here presented. Preliminary accounts of some of these results have been reported elsewhere (Leach & Carr, 1968, 1969).

METHODS

Growth of Anabaena variabilis. Organisms were grown autotrophically in light as previously described (Pearce & Carr, 1967) and were harvested by centrifugation in the late log phase of growth.

Preparation of cell-free extracts. Three procedures were employed. Unless otherwise stated, method (a) was used for the preparation of extracts in which electron-transport enzymes were measured, and method (b) for phosphorylation studies.

(a) A suspension of washed organisms (50 to 100 mg. dry wt./ml.) in 0.1 M-potassium phosphate buffer, pH 7.6 was subjected to ultrasonic treatment (M.S.E. 100 W 20 KHz instrument) for three 45 sec. periods at 0° under a constant stream of nitrogen. Cell debris was removed by centrifugation at 10,000 *g* for 15 min. Cell breakage under oxygen-free nitrogen yielded extracts several times more active with regard to NADH and NADPH oxidases.

(b) A suspension of washed organisms (50 to 250 mg. dry wt./ml.) in sorbitol, 330 mM; HEPES, 10 mM; MgCl₂, 1 mM; EDTA, 1 mM; pH 7.6 (hereafter called 'HEPES buffer') was incubated with lysozyme (0.1 mg./ml.) at 20° for 20 min. and then extruded through a pre-cooled French pressure cell (Aminco Instrument Co. Inc., Silver Spring, Maryland, U.S.A.) at 500 lb./in.² Debris was removed by centrifugation at 5000 *g* for 10 min. Material was further fractionated as described in Results section.

(c) A suspension of washed organisms (150 to 250 mg. dry wt./ml.) in HEPES buffer, pH 7.6, was added to an equal volume of washed sand and ground with a mortar and pestle for 15 min. at 0 to 4°. The sand was removed by centrifuging at 2000 *g* for 10 min. and the suspension of broken organisms was then centrifuged at 10,000 *g* for 15 min. to remove unbroken cells and cell-wall debris.

Fractionation of cell-free extracts of Anabaena variabilis. The fractionation of a suspension of disintegrated *Anabaena variabilis* by differential centrifugation is summarized in Fig. 1.

Conditions and extinction values employed in enzyme assays. The spectrophotometric determinations described below were carried out using an Optica CFR4 or a Unicam SP 800 recording spectrophotometer at 34° in silica cuvettes (*d* = 1 cm.). Owing to the relatively dense cell-free extracts employed it was sometimes necessary to use the maximum energy of incident light available in the spectrophotometer, which resulted in an increased background 'noise' level; slow chart speeds (1 cm./min.) were also necessary to measure enzymic rates. Ferrocyclochrome *c* was oxidized by ferricyanide and reduced by dithionite and dialysed against four changes of water at 4°. Quinones (menadione and vitamin K₁) were dissolved in ethanol and diluted with water to the required concentration. Brief ultrasonic treatment assisted in the even suspension of these compounds. Extinction coefficients ($E_{1\text{cm}}^{1\text{mg}}$) employed were: NADPH and NADH, 6.2 at 340 nm.; ferrocyclochrome *c* red/ox, 19 at 550 nm. (Haas, 1955); vitamin K₁,

0.258 at 245 nm. The latter value was kindly supplied by Dr P. J. Dunphy of this department. Amytal, rotenone and antimycin A were dissolved in ethanol; control assays showed that the ethanol at the concentration employed did not affect the enzyme examined. Anaerobic measurements were carried out in a spectrophotometer cuvette, modified to act as a Thunberg tube, that had been evacuated and gassed three times with oxygen-free nitrogen.

Fig. 1. Flow sheet of the subcellular fractionation of *Anabaena variabilis*.

Reduced pyridine nucleotide oxidase (NADPH: oxidoreductase)

Spectrophotometric measurement. Cuvettes contained in 3 ml.: potassium phosphate, pH 7.6, 200 μ moles; NADPH, 0.3 μ mole and cell-free extract (0.5 to 5 mg. protein). The decrease in E_{340} in the experimental cuvette was measured against a boiled enzyme control.

Measurement by O_2 uptake. Oxygen consumption was measured using a Clark electrode (Yellow Springs Instrument Co. Inc.). The reaction mixture contained in 5 ml.: potassium phosphate, pH 7.6, 200 μ moles; extract containing 40 to 60 mg. microbial protein, and 10 mg. NADPH added to start the reaction. The rate of back-diffusion of oxygen into the electrode cell was corrected for, as were blank determinations without NADPH or with boiled enzyme.

Transhydrogenase (EC 1.6.1.1)

Acetyl pyridine analogue procedure (Kaplan, 1967). Potassium phosphate pH 7.6, 66 μ moles; NADPH, 0.5 μ moles and KCN, 20 μ moles were added to cell-free extract of *Anabaena variabilis* (5 to 10 mg. protein) in 1 ml. final volume. After the addition of acetyl pyridine NAD⁺, the increase in extinction at 375 nm. was measured.

Coupling with pig heart isocitrate dehydrogenase (Kaplan, 1955). The reaction mixture containing, in 3 ml., NADP⁺, 0.1 μ mole; potassium phosphate, pH 7.6, 100 μ moles and isocitrate dehydrogenase (0.1 mg.; sp. act. 9.8 μ moles, min.⁻¹, mg.⁻¹) was allowed to proceed to completion after the addition of isocitrate (10 μ moles) with the small amount of NADP⁺ present, and then NAD⁺ (1 μ mole) was added. After incubation for a few minutes (any transhydrogenase would then be detected) extract of *Anabaena variabilis* (2 to 5 mg. protein) was added. Increase in absorption at 340 nm. was assumed to be due to the transfer of hydrogen from NADPH to NAD⁺ thus releasing NADP⁺ for further reduction. Extracts of *A. variabilis* contain no detectable NAD⁺-active isocitrate dehydrogenase (Pearce & Carr, 1967).

NADPH (NADH): Ferrocycytochrome c oxidoreductase (EC 1.6.9.3). Cuvettes contained in 3 ml.: potassium phosphate, pH 7.6, 200 μ moles; KCN, 20 μ moles; NADPH or NADH, 0.3 μ moles and cell-free extract (0.5 to 2.0 mg. protein). The reaction was started by the addition of oxidized ferrocycytochrome c (0.2 μ mole) to each cuvette and the increase in E_{550} measured against the blank lacking NADPH.

NADPH (NADH): menadione oxidoreductase (EC 1.6.99.2). The oxidation of NADPH in the presence of menadione was observed at 340 nm. Cuvettes contained in 3 ml.: KCN, 20 μ moles; NADPH, 0.3 μ moles; potassium phosphate, pH 7.6, 200 μ moles, and cell-free extract (0.5 to 2.0 mg. protein). The reaction was started by the addition of menadione (0.5 μ mole) to the experimental cuvette. The enzyme was also measured with vitamin K₁ used in place of menadione, in which case the increase of E_{245} due to vitamin K₁ reduction was measured as well as the decrease of E_{340} due to pyridine nucleotide oxidation.

Ferrocycytochrome c: oxygen oxidoreductase (EC 1.9.3.1). Oxidation of reduced ferrocycytochrome c was measured by decrease in E_{550} . Cuvettes contained in 3 ml.: potassium phosphate, pH 7.6 (200 μ moles) and 2 to 4 mg. algal protein. 50 nmoles reduced ferrocycytochrome c were added to the experimental cuvettes.

Succinic dehydrogenase (EC 1.1.99.1). This assay was carried out as previously described (Pearce, Leach & Carr, 1969).

The oxidative synthesis of ATP by extracts of Anabaena variabilis

The synthesis of ATP coupled with the oxidation of reduced pyridine nucleotides and organic substrates was determined by a modification of a procedure of Avron (1960). The standard reaction mixture, in a total volume of 1 ml., contained in μ moles: sorbitol, 330; [³²P]sodium phosphate, 1 (specific radioactivity, 1.5 to 2.5 μ Ci/ μ mole); HEPES buffer pH 7.6, 10; MgCl₂, 1; EDTA, 1; ADP, 1; extract (1.5 to 4.0 mg. protein); and NADPH, NADH (1.5 μ mole) or organic substrates (20 μ moles) and 1.5 μ mole of NADP⁺ and NAD⁺. Appropriate controls were assayed, omitting the oxidized nucleotides or organic substrates. The reactions were allowed to proceed in the dark for 10 min. at 34° and were terminated by the addition of 0.1 ml. of 20 % (w/v) trichloroacetic acid. The precipitated protein was separated by centrifugation and

0.3 ml. samples of the supernatants were used for ATP measurement. Subsequent steps were carried out at 0 to 2°.

Acetone (1.2 ml.) was added to each sample, mixed, allowed to stand for 10 min., and the water content of each tube was then made up to 2.5 ml. with water saturated with isobutanol + benzene (1:1) mixture. Seven ml. of isobutanol + benzene (1:1) saturated with water was added and thoroughly mixed. After phase separation, 0.8 ml. of molybdate reagent (5 g. ammonium molybdate dissolved in 40 ml. 10N-H₂SO₄ and made up to 100 ml. with water) was run down the side of each tube and the water layer was mixed gently. After allowing to stand for 5 min., the contents were mixed thoroughly for 30 sec. and allowed to stand. After phase separation, the upper layer which contained the majority of [³²P]PO₄ molybdate complex was removed and 0.02 ml. of 0.02 M-KH₂PO₄ were added to the aqueous layer, followed by 7.0 ml. of the isobutanol:benzene mixture saturated with water. After re-extraction of the phosphate molybdate complex, the isobutanol:benzene phase was removed. Some of the aqueous phase was placed in a stainless steel planchet and the [³²P] content counted in a Nuclear Chicago gas flow counter at an efficiency of 33 % to an accuracy of at least 0.5 %. Samples of the aqueous phase were also used in chromatographic determinations.

The use of [³²P]ATP in glucose-6-phosphate formation. To link the [³²P]ATP synthesized to hexokinase activity, 15 i.u. of hexokinase (E.C. 2.7.11) and glucose (5 μmoles) were added to the standard phosphorylation mixture. After incubation, the reaction was stopped by the addition of 0.1 ml. of 20 % trichloroacetic acid and the remaining [³²P]PO₄ removed as a molybdate complex as described above. [³²P] present in the glucose-6-phosphate formed was assayed after chromatographic separation.

Chromatography of [³²P]ATP and [³²P]glucose-6-phosphate. Chromatographic separations were achieved on thin layer cellulose plates and on paper (Whatman no. 1) and radioactive peaks were measured on a Nuclear Chicago Actiograph III scanner. Solvents used for the separation of ATP were: isobutyric acid + water (7 + 5 + 3, by vol.); *n*-propanol + water + trichloroacetic acid + ammonia (75 + 20 + 5 + 0.3, by vol.) (see Block, Durrum & Zweig, 1968). Inorganic phosphate, AMP, ADP, and ATP were chromatographed as standards and were located by molybdate reagent or the unstained nucleotides were observed when viewed under u.v. light. Glucose-6-phosphate was chromatographed in methanol + ammonia + water (6 + 1 + 3, by vol.) and in methanol + formic acid + water (80 + 15 + 5, by vol.) (see Block *et al.* 1968).

Protein determination. A modified biuret reaction was used (Pearce & Carr, 1967).

Chlorophyll and phycocyanin determinations. Spectra of diluted extracts were measured between 400 nm. and 700 nm. and the extinction of chlorophyll was measured at 663 nm. and that of phycocyanin *c* at 620 nm. Extinction values were not converted to weight of material present.

Determination of ATP by a luciferin: luciferase assay system. The assay procedure was a simplification of the method of Lyman & DeVincenzo (1967). A modified single-sided Locarte fluorometer MK 11 was used in a dark room. The photomultiplier tube was dismantled from its housing and a plate with a small mirrored chamber and a 10 mm. × 80 mm. spectrophotometric tube attached was fitted. The standard reaction mixture in 1.55 ml. contained glycine buffer at pH 8.0 (100 μmoles) and MgCl₂ (2 μmoles). Commercial ATP samples at a suitable dilution were added in 0.2 ml. of solution. Readings on the instrument's galvanometer were recorded and then 0.25 ml.

of firefly lantern extract (10 mg. firefly tails homogenized in 10 ml. of glycine buffer and filtered) were rapidly injected into the tube by means of a syringe. The maximum reading on the galvanometer for each ATP sample was recorded and a standard relationship between this and the ATP supplied was constructed. This procedure could be employed only with purified ATP samples, owing to quenching by other co-factors.

Chemicals and enzymes. Nucleotides, pig heart isocitrate dehydrogenase and hexokinase were purchased from Boehringer und Soehne G.m.b.H., Mannheim, Germany; horse ferrocytochrome *c* from Seravac Ltd, Maidenhead, Berkshire, [³²P]phosphate from The Radiochemical Centre, Amersham, Buckinghamshire; firefly tails and acetyl pyridine nucleotide from Sigma (London) Ltd; amytal and antimycin A from Koch-Light Ltd, Colnbrook, Buckinghamshire. Vitamin K₁ was kindly given by Dr P. J. Dunphy (of this department), and FCCP was a gift from Dr P. Scholes (Glyn Research Laboratories, Bodmin, Cornwall).

Abbreviations. Non-standard abbreviations used: EDTA, ethylene-diaminetetra-acetic acid disodium salt; HEPES, *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulphonic acid; FCCP, carbonylcyanide *p*-tri-fluoromethoxyphenylhydrazone.

RESULTS

Oxidative activities of cell-free extracts. NADPH and NADH oxidases were detected in cell-free extracts of *Anabaena variabilis*, and after fractionation as described in Fig. 1 these activities were distributed throughout the supernatant fractions and were present at highest specific activity in fraction VII. After breaking *A. variabilis* by grinding with sand in HEPES buffer, the partial localization of NADPH oxidase in the particulate fractions II and IV was observed (Table 1). The supernatant fraction VII prepared from ultrasonic treated cells contained some NADPH oxidase activity but no chlorophyll, demonstrating a complete separation of chlorophyll from part of the oxidase activity. Most of the oxidase activity, however, sedimented with the chlorophyll-containing fractions and with extracts prepared from cells ground in sand the oxidase was confined to the heavy fractions along with the chlorophyll. The sedimentation characteristics of NADPH and NADH oxidase were similar, and the activity with both coenzymes present was not greater than with NADPH alone. NADH oxidase activity varied between 40 % and 65 % of the NADPH oxidase in all extracts assayed. Transhydrogenase activity could not be detected by the acetyl pyridine analogue procedure of Kaplan (1967) but a low rate (0.31 nmole/min./mg.) was detected using this method coupled with pig heart isocitrate dehydrogenase (Kaplan, 1955). In subcellular fractions prepared from ultrasonic treated extracts succinic dehydrogenase showed a broad distribution of activity, similar to that of NADPH oxidase (see Table 1). Confirmation that the oxidations of reduced pyridine nucleotides were by true oxidases was given by lack of activity under anaerobic conditions (Fig. 2*a*). After oxidation the NADP⁺ was re-reduced by the action of isocitrate dehydrogenase when isocitrate (10 μmoles) was added to the cuvette (Fig. 2*b*), which indicated that the initial decrease in $E_{340\text{ nm}}$ could be attributed to an oxidase activity and not to the destruction of the nucleotide. NADPH oxidase had a broad pH optimum around 7.6, although a limited activity could be detected at pH 9.0 (Fig. 3*a*). NADPH oxidation by cell-free extracts showed marked dependence on temperature, the rate at 34° being

Table 1. *Distribution of NADPH oxidase compared with that of succinic dehydrogenase in subcellular fractions of Anabaena variabilis*

Fraction (see Fig. 1)	NADPH oxidase extracts prepared by						Succinic dehydrogenase extract prepared by ultrasonic disintegration	
	Ultrasonic disintegration		French pressure cell		Ground with sand			
	Total act.		Total act.		Total act.			
	Sp. act.		Sp. act.		Sp. act.		Sp. act.	
I. Supernatant	2.0	210	9.4	1000	8.1	720	0.2	20
II. Precipitate	0.8	13	3.1	4.4	13	290	0	0
III. Supernatant	2.6	200	10	880	7.1	450	0.22	17
IV. Precipitate	1.1	44	4.2	130	10	330	0.09	3.7
V. Supernatant	3.5	120	13	650	2.1	61	0.34	12
VI. Precipitate	1.9	55	8.4	320	0.23	38	0.18	5.1
VII. Supernatant	9.7	21	21	110	0	0	0.89	4.8

Total activities = nmoles/min. Specific activities = nmoles/min./mg. protein.

several times faster than at room temperature. Maximal rates were obtained at 37 to 39° (Fig. 3*b*).

The electron-transfer pathway of NADPH oxidase was examined in extracts of *Anabaena variabilis* by the assay of component enzymes and by the effect of inhibitors

Fig. 2*a*. Spectrophotometric traces showing the aerobic oxidation of NADPH and NADH by ultrasonicated extracts of *Anabaena variabilis*. Cuvettes contained 2.8 mg. protein (for further details see Methods).

Fig. 2*b*. The regeneration of NADPH after its aerobic oxidation by an extract of *Anabaena variabilis* following the addition of isocitrate (10 μ moles). The cuvette contained 3.4 mg. protein (for further details see Methods). NADPH was excluded from the blank cuvette.

Fig. 3*a*. pH dependence of NADPH oxidase activity in ultrasonicated cell-free extracts of *Anabaena variabilis*. Cuvettes contained 2 mg. protein and buffer (100 μ moles). O, Phosphate buffer; ●, tris + HCl buffer; and ■, borate + NaOH buffer (for further details see Methods).

Fig. 3*b*. The effect of temperature on the rate of NADPH oxidase from ultrasonicated cell-free extracts of *Anabaena variabilis*. Cuvettes contained 1.2 mg. protein and were pre-equilibrated for 5 to 6 min. before the addition of reduced pyridine nucleotide. (For further details see Methods.)

Fig. 4. The effect of inhibitors on electron transport activities of cell-free extracts of *Anabaena variabilis*. Reaction mixtures as described in Methods and the inhibitors were added in a small volume (0.2 ml.). The figures show the % of electron transport activities remaining against -log of molar concentration of the inhibitor; O, NADPH oxidase; ■, NADH oxidase; ▲, NADPH + cytochrome *c* reductase.

Table 2. *Electron transport activities present in extracts of Anabaena variabilis prepared by ultrasonic treatment*

	Electron donor	
	NADPH (nmole/min./ mg. protein)	NADH (nmole/min./ mg. protein)
Reduced pyridine nucleotide:oxidase (by $E_{340\text{nm.}}$)	2.1	1.2
Reduced pyridine nucleotide:oxidase (by O_2 uptake)	2.3	1.3
*Reduced pyridine nucleotide:cytochrome <i>c</i> reductase (by $E_{340\text{nm.}}$)	4.5	1.2
*Reduced pyridine nucleotide:cytochrome <i>c</i> reductase (by $E_{550\text{nm.}}$)	4.7	1.6
Reduced pyridine nucleotide:menadione reductase (by $E_{340\text{nm.}}$)	23	1.1
Reduced pyridine nucleotide:vitamin K_1 reductase (by $E_{340\text{nm.}}$)	16	1.0
Reduced pyridine nucleotide:vitamin K_1 reductase (by $E_{340\text{nm.}}$)	15	1.1
Transhydrogenase		0.31
*Cytochrome <i>c</i> oxidase (by $E_{550\text{nm.}}$)		2.3
Succinic dehydrogenase		0.2

Enzyme activities were measured as in Methods, the particular procedure employed being indicated in parentheses. Enzyme activities are expressed as nmole/min./mg. protein and are the mean of at least three determinations.

* These rates are from extracts that had been dialysed against phosphate buffer (see Results).

on oxidation and reduction rates. The activities of respiratory enzymes were consistently higher with NADPH than with NADH, and only the former values will be discussed in detail.

Electron-transfer reactions that were detected in cell-free fractions are summarized in Table 2. The rates of NADPH-menadione reductase and NADPH-vitamin K_1

reductase showed a dependence on the concentration of quinone. Maximal rates were obtained with quinone concentrations greater than 2 mM. When oxidized ferrocytochrome *c* was added to extracts that had been stored at -18° for 3 days, it was immediately reduced without the addition of an electron donor; after dialysis of such extracts, the reduction of ferrocytochrome *c* required an electron donor. Thus dialysis permitted the measurement of both NADPH:ferrocytochrome *c* reductase and ferrocytochrome *c* oxidase in stored extracts.

The rates of reduction of ferrocytochrome *c*, menadione and vitamin K_1 by NADPH were all greater than the overall NADPH oxidase activity. The rate of activity of cytochrome *c* oxidase was similar to that of NADPH oxidase. With NADH as the

Fig. 5. The release of cyanide inhibition of NADPH oxidation in extracts of *Anabaena variabilis* following the addition of ferrocytochrome *c* ($0.2 \mu\text{moles}$). The cuvette contained 3.4 mg. protein (further details as in Methods); NADPH was excluded from the blank cuvette.

electron donor, the rate of reduction of the quinones and ferrocytochrome *c* were of the same order as the NADH oxidase activity, which was about half that of NADPH oxidase.

Effects of inhibitors on electron transport activities (Fig. 4). Relatively high levels of inhibitors were necessary to demonstrate inhibition. Thus total inhibition of NADPH oxidase was obtained with $5 \times 10^{-2} \text{ M-cyanide}$; NADH oxidase was completely inhibited by $2 \times 10^{-2} \text{ M-cyanide}$. NADH oxidase activity was ten times more sensitive to inhibition by antimycin A than was NADPH oxidase; rotenone was several times more effective against NADH oxidase than NADPH oxidase. Amytal (10^{-3} M) inhibited the NADH oxidase activity by approximately 50%; sodium azide 10^{-3} M had little effect on either oxidase. NADPH:ferrocytochrome reductase was not affected by cyanide, but ferrocytochrome *c* oxidase was as sensitive to cyanide as was the reduced pyridine nucleotide oxidase. The release of the cyanide inhibition of NADPH oxidation by ferrocytochrome *c* is shown in Fig. 5.

When extracts were incubated with oxidized ferrocytochrome *c* and NADH, in the absence of cyanide, no net accumulation of reduced ferrocytochrome *c* was detected spectrophotometrically. However, with NADPH as electron donor, there was an accumulation of reduced cytochrome *c* which was detected as an increase in optical density at 550 nm. The redox dye, phenolindo-2,6-dichlorophenol was also rapidly reduced by NADPH (90 nmoles/min./mg. protein) but the rate with NADH was substantially less (1.02 nmoles/min./mg.).

Fig. 6. The separation of [^{32}P]ATP and [^{32}P]inorganic phosphate by paper chromatography.

The chromatographic separation of adenosine nucleotides and inorganic phosphate using *n*-propanol + H_2O + trichloroacetic acid + ammonia 22 (75 + 20 + 5 + 0.3) as the developing solvent. The R_F value for inorganic phosphate was 0.65. After developing the chromatogram (a) shows the spots seen under u.v. light and (b) shows the spots that develop with molybdate spray for phosphates. The radioactive traces were obtained, using an Actiograph III strip scanner (see Methods). Trace (c) shows the position of label before extraction of inorganic phosphate as a molybdate complex and trace (d) after extraction of inorganic phosphate.

Oxidative phosphorylation. When cell-free preparations of *Anabaena variabilis* were incubated in the dark under the conditions described in Methods for oxidative phosphorylation studies, there was a net accumulation of esterified [32 P]phosphate which co-chromatographed with ATP (Fig. 6). The initial rate of ATP synthesis, when extracts were incubated with NADPH, was linear with time (Fig. 7) and corresponded to a rate of 0.43 ± 0.06 nmoles of ATP formed/min./mg. of protein. NADPH was twice as effective as NADH as a source of electrons whose oxidation was linked to phosphorylation. When the potent uncoupling agent FCCP (5μ moles) was included in the reac-

Fig. 7. The time course of synthesis of [32 P]ATP concomitant with the oxidation of reduced pyridine nucleotides by a cell-free extract of *Anabaena variabilis*. Reaction mixtures as described in Methods. $\circ$, NADPH; $\square$, NADH; $\bullet$, reduced pyridine nucleotides omitted.

tion mixture, phosphorylation was reduced by 25 %. Omission of reduced pyridine nucleotide, or its replacement with oxidized nucleotide, caused a marked decrease in the amount of ATP synthesized, as did the omission of ADP or its replacement with AMP. Anaerobic conditions reduced phosphorylation to less than 4 % of the amount obtained by incubating under aerobic conditions (Leach & Carr, 1969).

When hexokinase and glucose were added to the standard phosphorylation mixture, the total amount of esterified [32 P]phosphate remained the same, but chromatographically the radioactive material was recovered in glucose-6-phosphate although some remained as unconverted ATP.

When NADPH was replaced by the oxidized nucleotide and a substrate such as malate, which is known to lead to its reduction in extracts of *Anabaena variabilis*, ATP synthesis could again be demonstrated. However, the levels of phosphorylation were then substantially lower than those obtained with the reduced nucleotides (Table 3). As would be expected, succinate served as a poor source of electrons to support ATP synthesis and was independent of the addition of pyridine nucleotides. Isocitrate or malate, in the presence of NADP^+ , was about half as effective as the commercial reduced coenzyme. The results recorded in Table 3 are the highest and lowest

of at least three determinations. They show quite a wide difference and this probably reflects the low rate of activity and fragility of the phosphorylating system.

The assay for ATP gave a linear response of light emitted to ATP added between the range of 2×10^{-6} and 10^{-3} μ moles of ATP. Unfortunately, the assay was found unsuitable for ATP estimations in supernatant obtained after arresting phosphorylation by adding trichloroacetic acid or perchlorate (see Methods).

The specific radioactivity of the biosynthesized ATP was 1.28×10^6 c.p.m. μ mole, compared to the specific activity of 1.66×10^6 c.p.m. μ mole for the inorganic [32 P]-phosphate added; the difference between these two values is probably a reflexion of the presence of a small amount of phosphate in the extract.

Table 3. *Phosphorylation supported by the oxidation of organic substrates in extracts of Anabaena variabilis*

Incubation additions	ATP formed (nmole/min./mg. protein)
Standard (see Methods)	0.35 to 0.48
NADPH replaced by:	
NADH	0.13 to 0.29
NADP	0.006 to 0.01
NADP + succinate	0.06 to 0.08
NAD + succinate	0.07 to 0.09
NADP + malate	0.16 to 0.26
NAD + malate	0.09 to 0.15
NADP + isocitrate	0.16 to 0.33
NAD + isocitrate	0.09 to 0.18
succinate	0.06 to 0.11
malate	0.08 to 0.1
isocitrate	0.05 to 0.09

The highest and lowest results of at least three separate determinations are given.

DISCUSSION

This communication describes some stages in the dark aerobic oxidation of NADPH and NADH and an associated phosphorylation in extracts of *Anabaena variabilis*. The sedimentation properties of NADH oxidase described here and the effects of various inhibitors are comparable to those of another blue-green alga, *Anacystis nidulans* (Horton, 1968). In extracts of the five species of blue-green algae tested, NADPH was oxidized more rapidly than NADH (Leach & Carr, 1968), and NADPH oxidation by extracts of *Anab. variabilis* was less sensitive to inhibitors acting prior to the terminal oxidase. In whole cells of *Anac. nidulans* in the dark, anaerobic conditions lead to an accumulation of NADPH and not NADH (Biggins, 1969).

Biggins (1969) has observed oxidation of reduced cytochrome *c* by extracts of *Anacystis nidulans*, although Horton (1968) did not observe any activity in extracts of *Anac. nidulans* nor *Anabaena variabilis*. Because of its relative insensitivity to cyanide and azide, the terminal oxidase in the respiratory electron chain of *Anab. variabilis* is probably of the cytochrome *o* type, as has been observed in the colourless Cyanophyta (Webster & Hackett, 1965; Biggins & Dietrich, 1968) and in the photosynthetic bacterium *Rhodospirillum rubrum* (Horio & Kamen, 1962). NADH oxidase is more sensitive to inhibitors of electron flow from pyridine nucleotides to quinones or cytochrome *c* than is NADPH oxidase. This suggests that NADH and NADPH are

oxidized by separate pathways up to the cytochrome *c* stage. However, the fact that the rates of the oxidation of NADPH and NADH are not additive indicates that they share some stages of their dark electron transport pathway. The pyridine nucleotide: quinone oxidoreductase was readily detected with NADPH, with either vitamin K₁ or menadione as electron acceptor. *Anab. variabilis* does not contain vitamin K₂ or ubiquinone (Carr *et al.* 1967), which are commonly involved in bacterial respiration. It may be that both photosynthetic electron flow and dark respiratory electron transfer in *Anab. variabilis* share some common components and that this may account for the photo-inhibition of respiration observed in the blue-green algae (Brown & Webster, 1953; Jones & Myers, 1963). This explanation has been advanced by Horio & Kamen (1962) to account for this phenomenon in *Rhodospirillum rubrum*. In an examination of the link between respiration and photosynthetic electron flow in *Anac. nidulans*, Jones & Myers (1963) concluded that competition for electrons exists between chlorophyll *a* and oxygen. The cytological association of the photosynthetic lamellae with reducing activity, detected by potassium tellurite and tetranitro-blue tetrazolium reduction, has been noted in *Nostoc sphaericum* (Bisalputra, Brown & Weier, 1969). Most of the NADPH oxidase activity in our preparations sedimented with the chlorophyll-bearing fragments, but a small portion was soluble. The latter activity, measured as a decrease in extinction at 340 nm., may be due to NADP:ferredoxin diaphorase (E.C. 1.6.99.4) which was easily separated from the photosynthetic lamellae of *Anab. variabilis* (Susor & Krogmann, 1966).

Perhaps the more important question is not whether dark NADH or NADPH oxidation occurs in blue-green algae, but whether this process is linked to phosphorylation. The aerobic formation of ATP in the dark by whole cells of *Anacystis nidulans* was sensitive to the uncoupling agent 2,4-dinitrophenol and to the electron-flow inhibitors amytal and cyanide, but was insensitive to fluorocitrate (Batterton & Van Baalen, 1968). These authors suggest that their observations exclude a fermentative synthesis of ATP in *Anac. nidulans*. Recently Biggins (1969) has shown a reduction in the cellular ATP level in *Anac. nidulans* in the dark when anaerobic conditions were introduced, and he has demonstrated an increase in respiratory rate when the uncoupling agents, FCCP and 2,4-dinitrophenol, were added to intact cells. In a brief communication (Leach & Carr, 1969) the formation of ATP by extracts of *Anabaena variabilis*, concomitant with reduced pyridine nucleotide oxidation, was reported and in this paper these results are extended and include the aerobic formation of ATP in the presence of several substrates and oxidized nucleotides, together with a report of inhibition by the uncoupling agent FCCP. The development of a proton gradient (as occurs in micro-organisms known to effect oxidative phosphorylation) has been observed to follow the oxygenation of a dark suspension of *Anab. variabilis* (Scholes, Mitchell & Moyle, 1969).

The data presented in this paper, together with the whole-cell studies cited above, show that a process of oxidative phosphorylation occurred in the obligately autotrophic *Anabaena variabilis* and suggests its operation in other species of blue-green algae. This phosphorylation was based on a respiratory activity which was low compared with other aerobic procaryotes (e.g. *Bacillus megaterium*, Q_{O_2} of 120 in the presence of glucose; Weibull, 1953) and could not be increased by either exogenous substrates or by growth in the presence of oxidizable substrates (Pearce & Carr, 1967). Possibly this low respiration rate limits the amount of ATP which could be formed.

We suggest that this low level of phosphorylation may have a role in providing the 'energy of maintenance' (Dawes & Ribbons, 1962) under non-photosynthetic conditions, but its contribution to a culture in the light is possibly insignificant if only because of the photoinhibition of respiration demonstrated in this organism.

The support of the Science Research Council for this work is gratefully acknowledged, and C.K.L. was in receipt of an S.R.C. Research Studentship.

We thank Miss A. Davies for help in the enzymic measurements.

REFERENCES

- AVRON, M. (1960). Photophosphorylation by Swiss-Chard chloroplasts. *Biochimica et biophysica acta* **40**, 257-272.
- BATTERTON, J. C. & VAN BAALen, C. (1968). Phosphorus deficiency and phosphate uptake in the blue-green alga *Anacystis nidulans*. *Canadian Journal of Microbiology* **14**, 341-348.
- BIGGINS, J. & DIETRICH, W. E. (1968). Respiratory mechanisms in the Flexibacteraceae. 1. Studies on the terminal oxidase system of *Leucothinx mucor*. *Archives of Biochemistry and Biophysics* **128**, 40-50.
- BIGGINS, J. (1969). Respiration in blue-green algae. *Journal of Bacteriology* **99**, 570-575.
- BISALPUTRA, T., BROWN, D. L. & WEIER, T. E. (1969). Possible respiratory sites in a blue-green alga *Nostoc sphaericum* as demonstrated by potassium tellurite and tetranitro-blue tetrazolium reduction. *Journal of Ultrastructure Research* **27**, 182-197.
- BLOCK, R. J., DURRUM, E. L. & ZWEIG, G. (1958). *A Manual of Paper Chromatography and Paper Electrophoresis*. New York: Academic Press.
- BROWN, A. H. & WEBSTER, G. C. (1953). The influence of light on the rate of respiration of the blue-green alga *Anabaena*. *American Journal of Botany* **40**, 753-758.
- CARR, N. G. & PEARCE, J. (1966). Photoheterotrophism in blue-green algae. *Biochemical Journal* **99**, 28P-29P.
- CARR, N. G., EXELL, G., FLYNN, V., HALLAWAY, M. & TALUKDAR, S. (1967). Minor quinones of some Myxophyceae. *Archives of Biochemistry and Biophysics* **120**, 503-507.
- DAWES, E. A. & RIBBONS, D. W. (1962). The endogenous metabolism of micro-organisms. *Annual Review of Microbiology* **16**, 241-264.
- HAAS, E. (1955). TPNH cytochrome *c* reductase in yeast. In *Methods in Enzymology*, vol. 2, pp. 699-703. Edited by S. P. Colowick & N. O. Kaplan. New York: Academic Press.
- HEMPFLING, W. P. & VISHNIAC, W. (1965). Oxidative phosphorylation in extracts of *Thiobacillus*. *Biochemische Zeitschrift* **342**, 272-287.
- HOARE, D. S., HOARE, S. L. & MOORE, R. B. (1967). The photoassimilation of organic compounds by autotrophic blue-green algae. *Journal of General Microbiology* **49**, 351-370.
- HORIO, T. & KAMEN, M. D. (1962). Observations on the respiratory system of *Rhodospirillum rubrum*. *Biochemistry* **1**, 1141-1157.
- HORTON, A. A. (1968). NADH oxidase in blue-green algae. *Biochemical and Biophysical Research Communications* **32**, 839-845.
- JONES, L. W. & MYERS, J. (1963). A common link between photosynthesis and respiration on a blue-green alga. *Nature, London* **199**, 670-672.
- KAPLAN, N. O. (1955). Pyridine nucleotide transhydrogenase. In *Methods in Enzymology*, vol. 2, pp. 681-687. Edited by S. P. Colowick & N. O. Kaplan. New York: Academic Press.
- KAPLAN, N. O. (1967). Beef heart TPNH-DPN pyridine nucleotide transhydrogenase. In *Methods in Enzymology*, vol. 10, pp. 317-322. Edited by S. P. Colowick & N. O. Kaplan. New York: Academic Press.
- KELLY, D. P. (1967). The incorporation of acetate by the chemoautotroph *Thiobacillus neapolitanus* strain c. *Archiv für Mikrobiologie* **58**, 99-116.
- KRATZ, W. A. & MYERS, J. (1955). Photosynthesis and respiration of three blue-green algae. *Plant Physiology* **30**, 275-280.
- LEACH, C. K. & CARR, N. G. (1968). Reduced nicotinamide adenine dinucleotide phosphate oxidase in the autotrophic blue-green alga *Anabaena variabilis*. *Biochemical Journal* **109**, 4P.

- LEACH, C. K. & CARR, N. G. (1969). Oxidative phosphorylation in an extract of *Anabaena variabilis*. *Biochemical Journal* **112**, 125-126.
- LYMAN, G. E. & DEVINCENZO, J. P. (1967). Determination of picogram amounts of ATP using the luciferin-luciferase enzyme system. *Analytical Biochemistry* **21**, 435-443.
- PEARCE, J. & CARR, N. G. (1967). The metabolism of acetate by the blue-green algae *Anabaena variabilis* and *Anacystis nidulans*. *Journal of General Microbiology* **49**, 301-313.
- PEARCE, J. & CARR, N. G. (1969). The incorporation and metabolism of glucose by *Anabaena variabilis*. *Journal of General Microbiology* **54**, 451-462.
- PEARCE, J., LEACH, C. K. & CARR, N. G. (1969). The incomplete tricarboxylic acid cycle in the blue-green alga *Anabaena variabilis*. *Journal of General Microbiology* **55**, 371-378.
- SCHOLES, P., MITCHELL, P. & MOYLE, J. (1969). The polarity of proton translocation in some photosynthetic micro-organisms. *European Journal of Biochemistry* **8**, 450-454.
- SMITH, A. J., LONDON, J. & STANIER, R. Y. (1967). Biochemical bases of obligate autotrophy in the blue-green algae and thiobacilli. *Journal of Bacteriology* **94**, 972-983.
- SUSOR, W. A. & KROGMANN, D. W. (1966). Triphosphopyridine nucleotide photoreduction with cell-free preparations of *Anabaena variabilis*. *Biochimica et biophysica acta* **120**, 65-72.
- TRUDINGER, P. A. & KELLY, D. P. (1968). Reduced nicotinamide adenine dinucleotide oxidation by *Thiobacillus neapolitanus* and *Thiobacillus* strain C. *Journal of Bacteriology* **95**, 1962-1963.
- WEBSTER, G. C. & FRANKEL, A. W. (1953). Some respiratory characteristics of the blue-green alga *Anabaena*. *Plant Physiology* **28**, 63-69.
- WEBSTER, D. A. & HACKETT, D. P. (1965). Respiratory chain of colourless algae. II. Cyanophyta. *Plant Physiology* **41**, 599-605.
- WEIBULL, C. (1953). The isolation of protoplasts from *Bacillus megaterium* by controlled treatment with lysozyme. *Journal of Bacteriology* **66**, 688-695.