Short Communications

Oxidative Phosphorylation in an Extract of *Anabaena variabilis*

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Autotrophic micro-organisms are characterized by their ability to grow in an inorganic environment. Recently it has been shown that species of 'obligate' chemosynthetic and photosynthetic microbes will utilize, to some extent, reduced carbon material for growth (Hoare, Hoare & Moore, 1967; Kelly, 1967; Pearce & Carr, 1967; Smith, London & Stanier, 1967). Their dependence on a specific inorganic oxidation or on photosynthesis separates autotrophs most clearly from heterotrophic bacteria. It has been suggested, as a general explanation for obligate autotrophy, that such organisms lack an NADH oxidase and hence are incapable of linking the oxidation of organic substrates to ATP synthesis (Smith *et al.* 1967). However, the oxidation of reduced nicotinamide nucleotides by extracts of blue-green algae (Horton, 1968; Leach & Carr, 1968) and *Thiobacillus* sp. (Trudinger & Kelly, 1968) have been reported. The present communication describes the coupling of NADPH oxidation in the dark with ATP synthesis by extracts of an obligate autotrophic blue-green alga, *Anabaena variabilis*.

Methods. *A. variabilis* was grown autotrophically in light as described by Pearce & Carr (1967), harvested by centrifugation and resuspended in 0.33 M-sorbitol-0.01 M-N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid-mM-MgCl₂-mM-EDTA (disodium salt), at pH 7.6, to give 0.5 g. wet wt./ml. The suspension of organisms was incubated with lysozyme (0.1 mg./ml.) at 20° for 20 min. and then extruded through a French pressure cell at 5000 lb./in.² at 0-4°. The broken organisms were centrifuged at 5000 g_{av.} at 0° for 15 min. and the supernatant was used for phosphorylation studies.

The standard reaction mixture contained in a total volume of 1.0 ml.: sorbitol, 330 μ moles; N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid, pH 7.6, 10 μ moles; MgCl₂, 1 μ mole; EDTA, 1 μ mole; ADP, 1 μ mole; sodium [³²P]phosphate (specific radioactivity, 1.5-2.5 μ C/ μ mole), 1 μ mole; NADPH (or NADH), 1.5 μ moles; extract containing 1.5-4.0 mg. of protein. The reaction proceeded for 10 min. at 34° and was ended by the addition 0.1 ml.

of 20% (w/v) trichloroacetic acid. The precipitated protein was separated by centrifugation and a 0.3 ml. sample of the supernatant used for ATP determination by the procedure of Nielson & Lehninger (1958) as modified by Avron (1960). This involved the extraction of the remaining [³²P]phosphate as a molybdate complex into 2-methylpropan-1-ol-benzene (1:1, v/v). The addition of unlabelled phosphate to the supernatant ensured complete removal of [³²P]phosphate. Total phosphate esters remained in the aqueous layer and were counted at infinite thinness in a Nuclear-Chicago gas-flow planchet counter at an efficiency of 34% to an accuracy of 1%. T.l.c. on cellulose confirmed the complete removal of the [³²P]phosphate and showed that ATP was the major phosphate ester formed (see under 'Results and Discussion'). The production of ATP over a period of time required that the standard incubation conditions were scaled up fivefold, with 0.5 ml. samples removed at various times. Commercial sodium [³²P]phosphate was freed of pyrophosphate by heating at 100° in 50 mM-HCl for 1 hr. and standing (5 min.) over acid-washed charcoal (Avron, 1960).

Chemicals. Sodium [³²P]phosphate was purchased from The Radiochemical Centre, Amersham, Bucks.; AMP, ADP, ATP, NADH and NADPH were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; firefly lanterns were from Sigma (London) Chemical Co., London S.W.6; N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid was from British Drug Houses Ltd., Poole, Dorset. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was kindly given by Dr P. J. Scholes, Glyn Research Laboratories, Bodmin, Cornwall.

Results and discussion. When either NADPH or NADH was oxidized by an extract of *A. variabilis* under the conditions described, concomitant ATP synthesis was demonstrated (Table 1). The initial rate of ATP synthesis was linear with time and corresponded to 0.43 ± 0.06 nmole of ATP formed/min./mg. of protein. Omission of ADP, and its replacement with AMP, caused a marked decrease in ATP synthesis, as did the omission of reduced

Table 1. *Formation of ATP by extracts of A. variabilis associated with oxidation of reduced nicotinamide nucleotides*

Assays were carried out as described under 'Methods' except where indicated. The cell-free extract contained 3.6 mg. of protein in Expt. 1, 2.65 mg. in Expt. 2 and 1.9 mg. in Expt. 3. Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Expt. no.	Incubation conditions	Time (min.)	ATP formed (nmoles)	Rate of ATP synthesis (nmoles/min./mg.)
1	Standard	0	0.09	
	Standard	4	6.0	0.40
	Standard	8	13.2	0.46
	Standard	10	16.4	0.45
	Standard	12	19.4	0.45
2	Standard	10	11.4	0.43
	ADP omitted	10	0.20	0.0075
	ADP replaced by AMP	10	0.21	0.0075
	NADPH omitted	10	0.13	0.0049
	NADPH replaced by NADH	10	5.8	0.218
3	Standard	10	8.2	0.43
	Anaerobic	10	0.3	0.016
	5 μ M-FCCP	10	6.2	0.325

nicotinamide nucleotide; NADH was about 50% as effective as NADPH in the oxidative phosphorylation described. When the standard procedure was carried out under anaerobic conditions, phosphorylation was decreased to less than 3% that of the aerobic control.

A portion of the total esterified 32 P-labelled fraction was chromatographed on thin-layer cellulose plates in isobutyric acid-water-aq. 25% (w/v) NH_3 (100:50:0.1, by vol.) and complete coincidence was obtained between carrier ATP at R_F 0.7, identified by molybdate reagent (Boulanger & Biserte, 1951), and the radioactive peak measured on a Nuclear-Chicago Actigraph III instrument. The ATP area contained 98% of the total 32 P, the remainder of the label running close to the solvent front. When hexokinase (15 international units) and glucose (5 μ moles) were added to the standard incubation mixture, chromatographic separation of the esterified [32 P]phosphate showed a major peak at the R_F of authentic glucose 6-phosphate.

The specific radioactivity of the biosynthesized ATP was determined by elution of chromatographically separated ATP from the cellulose plate by 50 mM-HClO₄. After neutralization with KOH, the ATP was estimated by the luciferin-luciferase procedure, by a modification of the method of

Lyman & DeVincerzo (1967); this permitted estimations of ATP at 0.01 nM level with an accuracy of $\pm 5\%$. The 32 P present in eluted ATP was measured as in the standard procedure. The specific radioactivity of the ATP formed in the process of oxidative phosphorylation was 1.28×10^6 c.p.m./ μ mole, and the specific radioactivity of the inorganic [32 P]phosphate added was 1.66×10^6 c.p.m./ μ mole. This discrepancy is probably due to the presence of endogenous phosphate in the cell-free extract.

The specific activity of the NADPH oxidase in the incubation mixture described above was 4.1 ± 0.5 nmoles/min./mg. of protein, somewhat higher than previously reported (Leach & Carr, 1968). This indicated a coupling factor 0.1 between the oxidation of reduced nicotinamide nucleotide and ATP synthesis by extracts of *A. variabilis*.

The results presented show that extracts of *A. variabilis* were capable of effecting an oxidative phosphorylation analogous to that found in heterotrophic bacteria. The rate of ATP formation described is low, although the coupling is of the same order as that reported for some heterotrophic bacteria (see Gel'man, Lukyanova & Ostrovskii, 1967). The metabolic significance of this example of oxidative phosphorylation, and its relation to photophosphorylation, the major energy-yielding process of *A. variabilis*, remains to be examined. It is now evident, however, that the species of blue-green alga examined, although an 'obligate' autotroph, can obtain ATP from the oxidation of organic substrates via a nicotinamide nucleotide intermediate.

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