COUPLED OXIDATIVE PHOSPHORYLATION IN CRUDE EXTRACTS OF AZOTOBACTER¹

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Two outstanding characteristics of azotobacter, their ability to fix nitrogen and unusually high rates of oxidation, have stimulated interest in obtaining cell free extracts suitable for biochemical analysis of these processes. Many difficulties have been encountered in obtaining such extracts. Attempts to obtain cell free extracts capable of nitrogen fixation have given extremely inconsistent results (reviewed by Burris, 1956, and Magee and Burris, 1956). The methods used for demonstrating coupled oxidative phosphorylation in crude and fractionated extracts of mycobacteria and corynebacteria (Brodie and Gray, 1955a, 1955b, 1956a, 1956b, 1956c) did not yield satisfactory results with azotobacter. Recently coupled oxidative phosphorylation has been demonstrated in fractionated extracts of azotobacter (Slater, 1955; Tissières and Slater, 1955; Rose and Ochoa, 1956).

Reinvestigation of the crude azotobacter system has revealed that the initial erratic results were largely due to the extremely labile nature of the crude system. The results achieved with extracts stabilized during preparation were reproducible. The nature and properties of this labile system are described and compared with other azotobacter and mycobacterial systems.

MATERIALS AND METHODS

Azotobacter agile (*A. vinelandii*), ATCC strain 9104, was grown in 2 L shake flasks containing 600 ml of nitrogen free medium. The composition of the medium was similar to that described by

Wilson and Knight (1952), except that the content of calcium was decreased to 0.05 g $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}/\text{L}$. The carbon sources, added after autoclaving, consisted of 0.5 per cent glucose and 0.75 per cent potassium acetate. The cells were grown at 37 C for 24 hr, harvested by centrifugation at +2 C, washed by thorough stirring in cold distilled water and recentrifuged. The resulting pellet from 3 L of medium (8 to 10 g wet wt) was suspended in 5 ml of 0.06 M "tris" (hydroxymethyl) aminomethane buffer, pH 8.0, containing 0.06 M MgSO_4 and 25 μmoles of adenosine triphosphate (ATP). The thick suspension was treated for 3 to 4 min in a 10 kc Raytheon magnetostrictive oscillator and then diluted with 5 ml of mannose dialysis solution (see below). The homogenate was centrifuged for 30 min at 20,000 $\times$ G. The supernatant (crude extract) was decanted carefully and dialyzed for 15 min against 200 ml of 0.15 M KCl containing 0.005 M tris buffer (pH 8.0), 0.001 M MgSO_4 , 10 μmoles of ATP and 0.25 M mannose. This dialysis was followed by two additional 30 to 45 min dialyses. All extracts were tested immediately after preparation.

The protein content of the cell free preparations was estimated by the procedure of Stadtman *et al.* (1951). Determinations of phosphate and manometric measurements were similar to those described by Brodie and Gray (1956a).

Specimens for election microscopy were diluted in 0.15 M KCl and deposited onto collodion membranes by a rapid filtration method analogous to that described by Kellenberger (1954). They were then fixed for 1 min in the vapors of 2 per cent osmium tetroxide or for 10 min in the vapors of 10 per cent formalin. Specimens were examined in an RCA EMU-2 electron microscope in collaboration with Delbert E. Philpott, Marine Biological Laboratory, Woods Hole, Massachusetts.

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TABLE 1
*Distribution of coupled activity in cell
free components*

Fraction	Oxy- gen	ΔPi^*	P/O
	μatoms	μmoles	
"Fluffy layer".....	2.8	0	—
Fluffy layer + α -ketoglutarate..	2.8	0	0
Extract.....	3.8	2.9	0.76
Extract + α -ketoglutarate.....	8.0	6.2	0.79

The system consisted of: MgCl_2 , 15 μmoles ; KF, 25 μmoles ; α -ketoglutarate, 20 μmoles ; inorganic phosphate, 12.8 μmoles ; 0.7 ml undialyzed extract containing 20.0 mg protein; and 0.7 ml "fluffy layer" containing 14.8 mg protein. The acceptor system consisted of 3 mg of yeast hexokinase (previously dialyzed against mannose), 5 μmoles of mannose and 2.5 μmoles of adenosine-diphosphate. The reactions were carried out at 30 C for 10 min, stopped with 1 ml of 10 per cent trichloroacetic acid, and the inorganic phosphate analyzed.

* ΔPi indicates disappearance of inorganic phosphate.

TABLE 2
*Coupled oxidative phosphorylation with
various substrates*

Substrate	Oxy- gen	ΔPi^*	P/O
	μatoms	μmoles	
Succinate.....	8.6	4.4	0.51
Malate.....	9.5	4.9	0.52
Fumarate.....	8.4	5.0	0.59
Glutamate.....	4.1	3.5	0.85
α -Ketoglutarate.....	2.6	3.2	1.22
β -Hydroxybutyrate.....	3.9	2.4	0.62
Succinate (anaerobic).....		+0.3†	0

Conditions are similar to table 1 except for substrate. The flasks contained 0.8 ml of dialyzed crude extract containing 12.8 mg protein. The final concentration of all substrates was 20 μmoles . All values have been corrected for endogenous activity.

* ΔPi indicates disappearance of inorganic phosphate.

† + indicates excess phosphate due to ATPase.

RESULTS

Distribution of activity. Centrifugation of extracts after cellular disruption yielded a bacterial pellet beneath a reddish gelatinous layer. When the pH was maintained above pH 7.2 (Brodie and

Lipmann, 1955; Brodie and Gray, 1957) the pellet of undisturbed cells was overlaid by a light, buff colored, loosely sedimenting "fluffy layer," and a reddish supernatant. Although the fluffy layer had oxidative activity, no detectable oxidation of α -ketoglutarate or disappearance of orthophosphate could be demonstrated. The supernatant extract exhibited both oxidative and coupled phosphorylative activity (table 1).

Stabilization of extract. Extracts prepared in KCl-tris (absence of Mg^{++} and ATP) retained an essentially constant ability to oxidize malate, fumarate, succinate, lactate and glutamate throughout the day of preparation. In contrast, α -ketoglutarate was oxidized by only one-third of the extracts prepared in these early experiments. The extracts which oxidized α -ketoglutarate could esterify inorganic phosphate with all other substrates tested except lactate. Thus, the capacity to oxidize α -ketoglutarate provided a means of recognizing preparations capable of coupling phosphorylation to oxidation. In later work the presence of Mg^{++} and ATP during all stages of preparation of extracts helped stabilize α -ketoglutarate oxidation and aerobic phosphate esterification. These activities decline rapidly following dilution with distilled water or after the addition of either 0.01 M Ca^{++} or 0.001 M versene. Similar activation and inactivation with aged mitochondria has been demonstrated (Ernster and Low, 1955).

Oxidative phosphorylation. Using preparations which oxidized α -ketoglutarate, coupled oxidative phosphorylation could be demonstrated with other electron donors (table 2). These P/O ratios are representative of a large series of experiments. The esterification of inorganic phosphate was inhibited under anaerobic conditions, except with pyruvate which showed some phosphorylation associated with the substrate. Ratios greater than 1 were routinely obtained with pyruvate (corrected) and usually with α -ketoglutarate. The oxidations observed with succinate, malate and fumarate were approximately twice that found for other substrates tested and were found to be partially inhibited by KCN. The incomplete cyanide-inhibition was similar to that found by Brodie and Gray (1955a) and indicated that a portion of the electrons were being transferred to oxygen by a soluble, nonphosphorylative pathway.

When dialyzed extracts were used, the esterification of inorganic phosphate required the addi-

tion of a phosphate acceptor system (table 3). Undialyzed preparations esterify inorganic phosphate to a limited extent in the absence of the acceptor system. Although some preparations were obtained in which the oxidation was stimulated by the presence of an acceptor system most preparations failed to show this effect.

ATPase and myokinase were also found in the extracts. These activities were reflected by the presence of excess orthophosphate under anaerobic conditions and in experiments lacking an acceptor system. Increasing the concentration of fluoride did not selectively eliminate the activity of ATPase (table 3). The ATPase activity probably contributed to the low P/O ratios.

Effective uncoupling of phosphorylation from oxidation in the azotobacter extracts was obtained with 2,4-dinitrophenol (DNP) in several experiments. However, only partial uncoupling or extreme resistance to this agent was the more frequent observation. The effects of DNP and KCN are presented in table 4.

Cytological studies on whole cells and extracts. Penetration of whole azotobacter cells by an intense electron beam revealed the presence of intracellular electron dense granules of a wide range of sizes, shapes, and distributions, although the diameters of most of the granules measured between 30 and 100 μ . Thin sections of frozen dried, alcohol fixed cells showed the presence of a double structure about the periphery of the cell, representative of the cell wall. The cytoplasm was usually finely granular in structure and contained electron dense granules which were clearly separate from the cell periphery. The nuclear regions demonstrated approximately the same electron density and general appearance as the cytoplasm. Examination of the fluffy layer, washed once in 0.15 M KCl, revealed collapsed cell walls and cell wall fragments. Two types of particles were found in active cell free extracts fixed in osmium: small dense raised particles measuring 20 to 30 μ in diameter, and larger flattened membranous discs measuring 60 to 180 μ . Similar types of particles have been found in active particle fractions (Brodie and Gray, 1956b, 1956c, 1957) from a mycobacterium (Hartman *et al.*, 1956) and various extracts of other bacteria (reviewed in Alexander, 1956). Formalin fixation allowed the small particles to flatten out more extensively (30 to 40 μ diameter) while preserving to a greater extent a three dimensional structure of the membranous discs. This differ-

TABLE 3
Effects of phosphate acceptor system

	KF Add.	Oxy- gen	Δ Pi*	P/O
	μ moles	μ atoms	μ moles	
Complete system.....	50	4.5	5.7	1.28
Complete system.....	100	3.6	3.6	1.00
Complete system.....	150	2.8	4.3	1.56
Complete system.....	200	2.5	3.2	1.29
Complete system minus α -ketoglutarate.....	50	0.7	1.6	
Complete system minus acceptor system.....	50	5.5	0	0
Complete system minus acceptor system and α - ketoglutarate.....	50	0.4	+0.7†	

Conditions as in table 2 except α -ketoglutarate used as substrate. The acceptor system consisted of 2.5 μ moles of ADP, 3 mg of hexokinase, and 5 μ moles of mannose.

* Δ Pi indicates disappearance of inorganic phosphate.

† + indicates excess phosphate.

TABLE 4
Effects of DNP and KCN

Substrate	Addition	Oxy- gen	Δ Pi*	P/O
		μ atoms	μ moles	
Succinate.....	None	4.5	2.6	0.58
Succinate.....	DNP	5.5	0.5	0.09
Succinate.....	KCN	2.6	0.2	0.08
α -Ketoglutarate.....	None	4.7	4.5	0.96
α -Ketoglutarate.....	DNP	2.3	1.8	0.78
Malate.....	None	5.6	3.0	0.54
Malate.....	DNP	5.7	1.7	0.30
Malate.....	KCN	3.0	1.3	0.42
Fumarate.....	None	4.4	3.6	0.82
Fumarate.....	DNP	4.8	2.6	0.53
Fumarate.....	KCN	2.0	0	0

Conditions similar to table 2; addition of DNP, 8×10^{-5} M, and KCN 8×10^{-4} M.

* Δ Pi indicates disappearance of inorganic phosphate.

ence in behavior towards fixatives indicated that there are 2 separate and distinct classes of particles.

The extracts used contained a maximum of 50,000 intact cells per ml and usually 5,000 or less. Reconstruction experiments showed that this number of cells could not significantly affect the results obtained in the short term experiments.

DISCUSSION

The results show that essentially cell free extracts, prepared from *A. agile*, are able to esterify orthophosphate only in the presence of oxygen, and oxidizable substrate, and an acceptor system. It seems reasonable to assume that the exceedingly unstable component in the system consists of particles which carry out an important portion of the reactions necessary for oxidative phosphorylation (Brodie and Gray, 1957). Partial stabilization of this component has been achieved by the addition of ATP and Mg^{++} during preparation of the extracts. With respect to lability and greater range of activity this system appears to differ somewhat from those found by others in azotobacter extracts (Hyndman, *et al.*, 1953; Slater, 1955; Tissières and Slater, 1955; Rose and Ochoa, 1956).

In azotobacter, oxidation of α -ketoglutarate provided a reliable indicator of integrated enzyme activities. Coupled oxidative phosphorylation in azotobacter extracts, however, is far more labile than the ability to oxidize succinate (Repaske, 1954) or *p*-phenylenediamine. This is in contrast to the mycobacterial system of Brodie and Gray (1957) in which a correlation exists between succinoxidase, *p*-phenylenediamine activity, and oxidative phosphorylation. These results suggest the use of labile particulate bound oxidase activity as an index of a preparation's ability to couple phosphorylation to oxidation.

Uncoupling agents such as DNP had relatively little effect in most experiments with azotobacter extracts. However, in some experiments excellent uncoupling has been noted. The cause for this inconsistency is not known. Even greater resistance to uncoupling agents has been noted by Slater (1955), Tissières and Slater (1955), and Rose and Ochoa (1956).

The relationship between structures found in the intact cell and the elements responsible for oxidative phosphorylation in extracts remains obscure. While these extracts contain particles which exhibit some of the properties expected of mitochondrial structures, we cannot state whether they are derived from the cellular membranes or from polar bodies observed in whole cells. Such particles, however, do participate in the classical biochemical functions associated with mitochondria (Brodie and Gray, 1957).

Experience thus far indicates that the mycobacterial system (because of its greater stability,

higher P/O ratios and its sensitivity to uncoupling agents), is superior to the azotobacter system for studying mechanisms of oxidative phosphorylation. Nonetheless, the rapid oxidations and the possibility of facilitating nitrogen fixation in cell free extracts will continue to create special interest in the azotobacter systems.

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SUMMARY

Coupled oxidative phosphorylation has been demonstrated in crude cell free extracts of *Azotobacter agile*, with a large number of electron donors. This system is extremely labile but can be stabilized by the addition of ATP and Mg^{++} . The presence of particle bound oxidase in the form of α -ketoglutarate oxidase serves as a reliable indicator of the ability to couple phosphorylation to oxidation. The resistance of this system to uncoupling agents is similar to that observed with other azotobacter preparations. The properties of the azotobacter system are compared with similar systems from other bacteria.

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