OXIDATIVE PHOSPHORYLATION IN *AZOTOBACTER VINELANDII*

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SUMMARY

1. An extract of *Azotobacter vinelandii* was fractionated and oxidative phosphorylation studied with a small-particle fraction.

2. The phosphorylating system in the particles was inactivated by suspension in dilute salt solutions. This inactivation could be partly reversed by raising the salt concentration. Bivalent cations were more effective for protection of the phosphorylating enzymes than monovalent. Phosphorylation was lost in sucrose solutions.

3. The phosphorylating enzymes were more sensitive to temperature, surface inactivation and ageing than the oxidizing enzymes. They could be protected by addition of serum albumin, or of the supernatant obtained in centrifuging the particles from the extract.

4. No significant correlation could be observed between the phosphorylating and the oxidizing activities with DPNH, malate, succinate or lactate as substrate.

5. Phosphorylation with DPNH as substrate was not sensitive to 10^{-4} *M* dinitrophenol. It was partly uncoupled by 10^{-4} *M* menadione.

6. Addition of Mg^{++} is necessary to obtain phosphorylation.

INTRODUCTION

Respiratory-chain phosphorylation in cell-free extracts of micro-organisms or in particulate fractions isolated from these extracts has been described for *Alcaligenes faecalis*[1-3], *Azotobacter vinelandii*[4-8], *Mycobacterium phlei*[9-12], *Corynebacterium creatinovorans*[9], yeast [13,14] and *Proteus vulgaris*[13,14].

PINCHOT[1] separated the extract of *Alcaligenes faecalis* into three fractions—a particulate, a soluble and a heat-stable—all of which were needed for oxidative phosphorylation. The heat-stable factor is a polynucleotide[2,3]. BRODIE AND GRAY[10] separated the system in extracts of *Mycobacterium phlei* into two fractions, both of which were necessary for oxidation and phosphorylation. They suggested that one of the components of the system is menadione (vitamin K_3) reductase[10,11].

Previous papers of the present series[5,7] have described the isolation from extracts of *Azotobacter vinelandii* of a particulate fraction which showed respiratory-chain phosphorylation with succinate and DPNH as substrates. The addition of the supernatant fraction to the reaction mixture increased the P:O ratio with succinate as

Abbreviations: DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide; ADP, ATP, adenosine di- and triphosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate.

References p. 496.

substrate[5], but not with DPNH[7]. This paper deals with the effect of several factors on the oxidative and phosphorylative activity of the particles, and on the stability of the phosphorylating system.

METHODS

Preparation of enzyme fractions

The methods of cultivation of the *Azotobacter vinelandii* (strain O Wisc.), of preparation of the cell-free extract and of isolation of the particulate fraction have been described previously[7,15]. The fractions used in this paper were:

Medium particles (*MP*). The extract was centrifuged 10 min at 12,000 × *g*. The residue was discarded and the supernatant again centrifuged 10 min at 12,000 × *g*. The supernatant obtained was centrifuged 30 min at 20,000 × *g*. The sediment (MP) was suspended in 0.05 *M* Sørensen phosphate buffer, pH 7.0.

S_1, the supernatant after the centrifugation for 30 min at 20,000 × *g*.

Washed small particles (*WSP*). S_1 was centrifuged for 1 h at 145,000 × *g*. The sediment was suspended in 0.05 *M* Sørensen phosphate buffer, pH 7.0, centrifuged again for 30 min at 145,000 × *g* and the residue, unless stated otherwise, resuspended in 0.05 *M* phosphate buffer.

S_2, the upper third of the supernatant after the centrifugation of S_1 for 1 h at 145,000 × *g*.

Throughout this paper centrifugal fields refer to the bottom of the tube.

Ammonium sulphate fractionation

The supernatant S_1 was brought to 35–40 % saturation with satd. $(NH_4)_2SO_4$, pH 7.0–7.4, and centrifuged. The sediment was suspended in 0.01 *M* Sørensen phosphate buffer, pH 7.4, or 0.05 *M* Sørensen phosphate buffer, pH 7.0, and dialysed against the same buffer overnight. The next day the contents of the dialysis bag (referred to as AB in Table 1) were centrifuged for 30 min at 20,000 × *g*. The sediment was suspended in the same buffer used for the dialysis (fraction A); the supernatant was called fraction B.

Oxidase activities

Succinic oxidase was measured in differential manometers as described by TISSIÈRES[15]. The reaction vol. was 1.0 ml, the temperature 25° and the pH 7.0. The oxidation of malate and lactate were measured similarly, using respectively 0.02 *M* *dl*-malate and 0.02 *M* *dl*-lactate as substrate.

DPNH oxidase was estimated according to SLATER[16] with a reaction vol. of 2.5 ml. The cuvette (1 cm) contained 0.05 *M* phosphate buffer, pH 7.4 or 7.0, and $8 \cdot 10^{-5}$ *M* DPNH (prepared by reduction of Sigma "Cozymase 90" with ethanol in the presence of alcohol dehydrogenase[17]). The rate of decrease in absorbance at 340 mμ was constant for 2.5–4 min under these conditions. The Q_{O_2} (μl O_2/mg protein/h) was calculated from the initial velocity.

Oxidative phosphorylation

Oxidative phosphorylation with DPNH as substrate was determined as previously described[7]. The time needed to complete the oxidation of the DPNH varied from 1 to 15 min, but usually it was less than 5 min.

References p. 496.

Oxidative phosphorylation with malate was determined as previously described for succinate[5].

Protein was estimated by the biuret method[18].

Adenosinetriphosphatase (*ATPase*) was determined as described by MYERS AND SLATER[19].

RESULTS

Ammonium sulphate fractionation

In agreement with PINCHOT's experiments with *Alcaligenes faecalis*, precipitation of the extract of *Azotobacter* with ammonium sulphate (35–40% satn.), followed by dialysis against 0.01 *M* phosphate buffer, pH 7.4, brought about almost complete loss of the phosphorylative activity in the particles obtained after dialysis (Fraction A, Expts. 1 and 3, Table I). However, in contrast with PINCHOT's experience with *Alcaligenes faecalis* the phosphorylative activity could not be restored by the addition of the soluble fraction (B) even when a "Kochsaft" of S_1 (K) was also added.

TABLE I

AMMONIUM SULPHATE FRACTIONATION OF S_1

See METHODS for preparation of fractions AB, A and B. K refers to a "Kochsaft" obtained by boiling S_1 and removing the ppt. by centrifugation. For measurements of P:O ratios, DPNH was used as substrate.

Expt.	*Concn. phosphate during dialysis (M)*	*Fraction*	*P:O*
1	—	S_1	0.61
	0.01	AB	0.05
		A	0.08
		A + B	0.08
		A + B + K	0.08
2	—	S_1	0.47
	0.05	AB	0.29
		A	0.28
		B	—
		A + B	0.29
3	—	S_1	0.63
	0.01	AB	0.18
	0.05	AB	0.38

It was found that the inactivation was much less when 0.05 *M* phosphate buffer was used in place of 0.01 *M* (Table I, Expts. 2 and 3).

An attempt to fractionate WSP by ammonium sulphate was not successful; the protein was recovered quantitatively by precipitation at 40% satn. followed by dialysis against 0.05 *M* phosphate buffer, while the P:O ratio decreased slightly from 0.37 to 0.33. In this case also, the phosphorylation was sensitive to dilute phosphate. The P:O declined to 0.06 after incubation at 0° for 60 min in 0.005 *M* buffer, followed by centrifugation and resuspension in 0.05 *M* buffer. This value was not increased by addition of the "Kochsaft".

References p. 496.

TABLE II

INFLUENCE OF SUSPENSION MEDIUM ON OXIDATIVE PHOSPHORYLATION

WSP was suspended in the medium for 120 min at 0° and oxidative phosphorylation with DPNH as substrate then studied.

Expt.	mg protein/ml	Suspension medium	DPNH oxidized (μmoles)	AP esterified (μmole)	P:O
1	—	0.05 *M* phosphate	1.54	0.54	0.38
	—	0.002 *M* phosphate	1.54	0.01	0.01
2	6.8	0.05 *M* phosphate*	1.44	0.69	0.48
	4.5	0.005 *M* phosphate*	1.44	0.06	0.05
	2.0	0.15 *M* sucrose*	1.44	0.05	0.04
	7.0	0.08 *M* NaCl*	1.44	0.65	0.45
	6.0	0.08 *M* KCl*	1.44	0.69	0.48
3	—	0.05 *M* phosphate	1.48	0.58	0.39
	—	0.005 *M* phosphate	1.48	0.28	0.19
4	—	0.1 *M* phosphate	1.50	0.62	0.41
	—	0.05 *M* phosphate	1.50	0.61	0.40
	—	0.005 *M* phosphate	1.50	0.11	0.08
5	—	0.05 *M* phosphate	1.82	0.39	0.27
	—	0.005 *M* phosphate	1.82	0.10	0.05
	—	0.2 *M* sucrose	1.82	0.01	0.00
		0.5 *M* sucrose	1.82	0.05	0.03
	—	1.0 *M* sucrose	1.82	0.02	0.00
	—	2.0 *M* sucrose	1.82	0.17	0.09
6	5	0.05 *M* phosphate	1.30	0.52	0.40
	1.2	0.05 *M* phosphate	1.30	0.42	0.32
	5	0.005 *M* phosphate	1.30	0.39	0.30
7	8.0	distilled water	1.78	0.30	0.17
	4.2	distilled water	1.78	0.10	0.05
	2.4	distilled water	1.78	0.08	0.05
	1.9	distilled water	1.78	0.12	0.07
8	2.0	0.05 *M* phosphate	1.34	0.67	0.50
	2.2	0.04 *M* $MgCl_2$	1.34	0.65	0.48
	2.7	0.004 *M* $MgCl_2$	1.34	0.73	0.54
	2.7	0.0008 *M* $MgCl_2$	1.34	0.56	0.42
	2.4	0.05 *M* phosphate + 0.04 *M* $MgCl_2$	1.34	0.68	0.50
9	1.2	0.04 *M* KCl	1.96	0.73	0.37
	1.2	0.008 *M* KCl	1.96	0.22	0.11
	0.9	0.004 *M* $MgCl_2$	1.96	0.68	0.35
	0.9	0.0008 *M* $MgCl_2$	1.96	0.65	0.33
	—	0.004 *M* $MnCl_2$	1.96	0.74	0.38
	—	0.0008 *M* $MnCl_2$	1.96	0.66	0.34
	1.4	0.03 *M* $CaCl_2$	1.96	0.62	0.32
	1.4	0.004 *M* $CaCl_2$	1.96	0.73	0.37
	1.4	0.0008 *M* $CaCl_2$	1.96	0.45	0.23
10	—	0.05 *M* phosphate + 0.05 *M* Tris	1.76	0.41	0.23
	—	0.05 *M* Tris	1.76	0.44	0.25
	—	0.005 *M* Tris	1.76	0.02	0.01

* In this experiment the fractions were centrifuged 30 min at 140,000 × *g* after 75-min incubation and resuspended in 0.05 *M* phosphate buffer, immediately before the measurement of the P:O ratio.

References p. 496.

Influence of suspension medium

Following these experiments, the effect of incubation of WSP for short periods in different suspension media was investigated more systematically, with the results shown in Table II. It is clear that electrolytes are necessary to retain the phosphorylation activity. Phosphate buffer may be replaced by other salts (KCl, NaCl, $MgCl_2$, $MnCl_2$, $CaCl_2$) or tris(hydroxymethyl)aminomethane buffer but not by sucrose. A lower ionic strength is required with the bivalent cations. The enzyme concentration is also important, a higher concentration favouring stability as is shown in Expts. 6 and 7.

HARTMAN, BRODIE AND GRAY[8] found a lowered P:O ratio after addition of 0.01 *M* Ca^{++} or 0.001 *M* ethylenediaminetetraacetate (EDTA) to their *Azobacter* extracts (a different strain from the one used by us). Table II, Expt. 9, shows that Ca^{++} in low concentrations has a protective effect on our WSP, while a concentration as high as 0.03 *M* decreased the ratio only from 0.37 to 0.32. Addition of 0.03 *M* EDTA to particles, suspended in 0.05 *M* phosphate 10 min before the reaction started, also had no effect.

Table III shows that the loss of phosphorylating activity caused by suspension of the particles in a medium lacking electrolytes may be partially reversed by raising the ion concentration again. The P:O ratio was either raised or at least remained the same after one night at the higher salt concentration, compared with a further decrease in the control held at the low salt concentration. Mg^{++} seemed to be somewhat more effective than K^+ (Expt. 3). Addition of S_2, ADP and Mg^{++} gave a better stimulation than S_2 and Mg^{++} alone (Expt. 4). The enzyme polynucleotide phos-

TABLE III

REVERSIBILITY OF LOSS OF PHOSPHORYLATION ACTIVITY

WSP was suspended in the indicated medium for 2 h at 0° and oxidative phosphorylation with DPNH as substrate then studied. Various additions were then made to the enzyme suspension, which was stored at 0–5° for 18 h before measurement of oxidative phosphorylation.

Expt.	*Suspension medium*	*P:O after 2 h*	*Addition after 2 h*	*P:O after Further 18 h*
1	0.05 *M* phosphate	0.38	—	0.33
	0.005 *M* phosphate	0.21	0.05 *M* phosphate	0.27
			S_2	0.30
2	0.05 *M* phosphate	0.30	—	0.25
	0.005 *M* phosphate	0.04	0.05 *M* phosphate	0.14
3	0.08 *M* KCl	0.35	—	0.27
			—	0.06
	0.008 *M* KCl	0.15	0.08 *M* KCl	0.14
			0.0033 *M* $MgCl_2$	0.19
4	0.05 *M* phosphate	0.31	—	0.19
			—	0.00
	0.005 *M* phosphate	0.01	0.004 *M* $MgCl_2$	0.08
			0.004 *M* $MgCl_2$ + S_2	0.10
			0.004 *M* $MgCl_2$ + 0.1% serum albumin	0.20
			0.004 *M* $MgCl_2$ + S_2 + 5.10^{-4} *M* ADP	0.15

References p. 496.

phorylase[20] present in S_2 might be expected to form polynucleotide under these conditions. A polynucleotide-stimulated phosphorylation in particles derived from *Alcaligenes faecalis* has been reported by PINCHOT[2,3]. However, with our *Azotobacter* particles the stimulatory effect was not specific for S_2 + ADP, since serum albumin, in the presence of Mg^{++}, was even more effective.

Table IV shows that the inactivation brought about by suspension in 0.005 *M* phosphate is almost complete in 22 min, but the reactivation by serum albumin + Mg^{++} was slower. Here again, serum albumin + Mg^{++} had a greater effect than Mg^{++} alone, while serum albumin alone was ineffective.

TABLE IV

SPEED OF INACTIVATION AND REACTIVATION OF OXIDATIVE PHOSPHORYLATION

WSP were suspended in 0.005 *M* phosphate and oxidative phosphorylation with DPNH as substrate estimated at different times. After 195 min, $MgCl_2$, serum albumin or both were added to the inactivated enzyme and the P:O ratios again estimated at different times.

Suspension medium	*Time of incubation before reaction*	*P:O*	*Addition after 195 min*	*Time after addition*	*P:O*
0.05 *M* phosphate	45 min	0.49	—	—	—
	1 day	0.28	—	—	—
0.005 *M* phosphate	22 min	0.07			
	75 min	0.04			
	195 min	0.03	0.1 % serum albumin + 0.004 *M* $MgCl_2$	7 min	0.11
				45 min	0.13
				135 min	0.22
				1 day	0.18
			0.1 % serum albumin	135 min	0.03
			0.004 *M* $MgCl_2$	135 min	0.17

Ageing of preparations

The P:O ratios were lowered 10–50 % after keeping the WSP fraction (in 0.05 *M* phosphate) one night at 0–4°. The stability was increased by adding S_2, kochsaft or serum albumin. The P:O ratio was lowered 50 % after 10 min incubation at 30°.

The enzymes for oxidation of DPNH are more stable than the phosphorylation enzymes. The rate of DPNH oxidation was not decreased by keeping a suspension containing 30 μg protein/ml 10 min at 15°, or a suspension containing 2 mg protein/ml one night at room temperature (20–24°), but the oxidase activity was lost after warming the more dilute suspension to 60° for 10 min. This difference in stability between oxidation and phosphorylation enzymes may cause a lowered P:O ratio if the reaction occurs under conditions which are unfavourable for the phosphorylation enzymes. Thus, when DPNH was oxidized manometrically at 25°, with a reaction time of 30 min, the P:O ratios were lowered 50–100 %. This inactivation could be prevented by adding serum albumin or S_2 to the reaction mixture, though not always completely. For this reason, P:O ratios obtained by the manometric method with succinate or malate as substrate cannot very well be compared with the values found for DPNH oxidation by the spectrophotometric procedure described in this paper.

The rapid inactivation of the oxidative phosphorylation and the protection afforded by S_2 or serum albumin makes it difficult to distinguish between truly specific stimulatory effects on oxidative phosphorylation and a non-specific protective

References p. 496.

action, when the experiments are carried out manometrically. For example, the apparent stimulation of oxidative phosphorylation by the addition of S_2 to WSP with succinate as substrate, reported by TISSIÈRES AND SLATER[5], was very likely due to protection of the system from inactivation.

Influence of changes in reaction mixture

Table V shows that even 10^{-3} *M* 2,4-dinitrophenol caused little uncoupling of oxidative phosphorylation. Addition of vitamin K_1 or α-tocopherol, either to phosphorylating or non-phosphorylating particles, had no effect. Addition of menadione (vitamin K_3) uncoupled the phosphorylation (Table VI). Uncoupling by menadione was also observed by PINCHOT[3] in *Alcaligenes faecalis*, and by MARTIUS AND NITZ-LITZOW[21] in mitochondria. BRODIE, WEBER AND GRAY[11] found that vitamin K_1 could restore both oxidation and phosphorylation in particles of *Mycobacterium phlei*, after inactivation by ultraviolet radiation.

TABLE V

EFFECT OF 2:4-DINITROPHENOL ON OXIDATIVE PHOSPHORYLATION

Expt.	*Fraction*	*DNP (M)*	*DNPH oxidized (μmoles)*	*AP esterified (μmole)*	*P:O*
1	S_1	—	1.46	0.80	0.55
		10^{-4}	1.46	0.72	0.49
	MP	—	1.46	0.80	0.55
		10^{-4}	1.46	0.74	0.49
2	WSP	—	1.85	0.71	0.38
		10^{-4}	1.85	0.70	0.38
		10^{-3}	1.85	0.56	0.31

TABLE VI

INFLUENCE OF MENADIONE ON OXIDATIVE PHOSPHORYLATION

Oxidative phosphorylation by WSP with DPNH as substrate was measured.

Ethanol (%)	*Menadione (M)*	*P:O*
—	—	0.33
10	—	0.22
10	10^{-4}	0.00
1.2	—	0.33
1.2	$1.2 \cdot 10^{-5}$	0.18

Variation of the pH between 6.5 and 8.0 had little effect on the phosphorylation; some lowering of the P:O ratio was observed at pH's 8.5–9.5 (Table VII). The effect of variation of the pH on the oxidase activities is more pronounced, as is shown in Fig. 1.

Table VIII shows that no phosphorylation occurred in the absence of added Mg^{++}. Maximal P:O ratios were obtained with a total magnesium concentration of 0.0048 *M* (0.0038 *M* Mg^{++} allowing for magnesium bound by the EDTA present in the reaction mixture). Higher concentrations lowered the P:O ratio. The effect of varying the Mg^{++} concentration is similar to that found by KIELLEY AND BRONK[22] and PURVIS AND SLATER[23] with particles derived from rat-liver mitochondria.

References p. 496.

TABLE VII

EFFECT OF pH ON OXIDATIVE PHOSPHORYLATION

Oxidative phosphorylation was measured as described in ref. (7), except that 0.025 *M* tris(hydroxymethyl)aminomethane buffer of the desired pH was added, and the pH of the phosphate buffer was made the same as that of the tris(hydroxymethyl)aminomethane.

Expt.	*Fraction*	*pH*	*DPNH oxidized (μmoles)*	*AP esterified (μmole)*	*P:O*
1	WSP	6.5	1.66	0.800	0.48
		7.0	1.66	0.760	0.46
		7.5	1.66	0.845	0.51
		8.0	1.66	0.740	0.40
		8.5	1.66	0.670	0.40
		9.0	1.66	0.705	0.42
		9.5	1.66	0.630	0.38
2	S_1	6.0	1.46	0.97	0.66
		7.4	1.46	1.04	0.71
	MP	6.0	1.46	0.98	0.67
		7.4	1.46	1.01	0.69

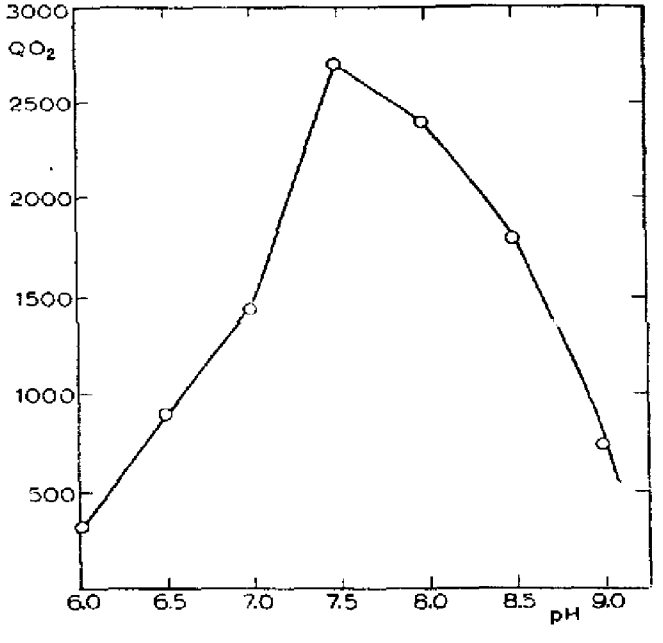

Fig. 1. DPNH oxidase activity of WSP was estimated as described in METHODS except that phosphate buffer of the desired pH was used.

Oxidation of succinate, malate and lactate

The oxidase activities for these substrates are given in Table IX. There was no clear difference in activity between particles suspended in 0.05 *M* phosphate or in 0.005 *M* phosphate. Addition of DPN had no influence on malate or lactate oxidation.

References p. 496.

TABLE VIII

INFLUENCE OF MAGNESIUM ON PHOSPHORYLATION

WSP used in both experiments.

Expt.	Mg^{++} (M)	DPNH oxidized (μmoles)	ΔP esterified (μmoles)	P:O
1	0.0012	1.09	0.54	0.32
	0.0024	1.60	0.71	0.42
	0.0048	1.60	0.76	0.45
	0.0096	1.60	0.68	0.40
	0.0192	1.60	0.56	0.32
2	0	1.85	0.045*	0.02
	0.0048	1.85	0.73*	0.40

* In this experiment, 1.14 μmoles ADP served as the phosphate acceptor in place of glucose.

TABLE IX

OXIDATION OF MALATE, LACTATE AND SUCCINATE BY WSP

Expt.	Suspension medium*	Protein (mg)	Substrate	Q_{O_2}
1	0.05 M phosphate	0.125	succinate	610
		0.125	malate	1600
		0.125	lactate	590
	0.005 M phosphate	0.10	succinate	465
		0.10	malate	1940
		0.10	lactate	630
2	0.05 M phosphate	0.10	lactate	505
		0.10	lactate + DPN**	520
		0.10	malate	1485
		0.10	malate + DPN**	1280

* in which WSP was suspended before assay.
** $1.5 \cdot 10^{-3}$ M.

Malate was oxidized with a P:O ratio of 0.41 by one-day-old particles, suspended in 0.05 M phosphate buffer and with a ratio of 0.05 by particles, suspended in 0.005 M phosphate. The malate was quantitatively converted into oxaloacetate and pyruvate. Because of the correction which has to be made for these substances in the estimation[17] of hexose monophosphate and ATP, the determination of the P:O ratio was less accurate than in the case of DPNH oxidation.

No difference was found between the rate of oxidation of succinate in an atmosphere of air or oxygen, if the enzyme concentrations were kept below 0.4 mg protein/ml. With higher concentrations of protein the activity in air was about 15–20 % less than that in oxygen. Probably the diffusion rate of oxygen into the liquid in the manometer flasks became limiting in this case. These results contrast with those of BRUEMMER, WILSON, GLENN AND CRANE[24], who found with a fraction of *Azotobacter* that the rate of oxidation of succinate in air was only 60 % of that in oxygen.

ATPase

Table X shows ATPase values at different pH's in the presence and absence of

References p. 496.

2,4-dinitrophenol which had little effect. The ATPase values were of the same order when the particles were suspended in distilled water instead of in 0.08 *M* KCl. The ATPase is very low compared with mitochondria, measured under these conditions[19].

TABLE X

ATPASE ACTIVITY OF WSP

The WSP were suspended in 0.08 *M* KCl. Protein concentration, 1.26 mg/ml.

pH	*μatoms P/mg protein/h*	
	*with DNP**	*without DNP*
6.5	0.76	0.93
7.0	0.99	1.00
7.5	1.43	1.48
8.5	1.99	2.11
9.0	1.35	1.63

* 10^{-4} *M* 2,4-dinitrophenol.

DISCUSSION

The exceptionally high respiratory activity of *Azotobacter*, the highest of all known cells, gives a special interest to studies of its respiratory-enzyme systems. The present paper is a continuation of those initiated by TISSIÈRES into the nature and enzymic activity of small particles which can be isolated from extracts of *Azotobacter vinelandii*[5,7,15]. These particles (called WSP), which have been separated from larger particles also present in the extract, have a higher respiratory activity and a higher P:O ratio than the larger particles[7]. However, it is clear from the work of MARR AND COTA-ROBLES[25] and of TISSIÈRES AND WATSON[26] that the WSP fraction is not pure, but consists of at least two types of particles, one the respiratory granule, and the other with a high content of ribonucleic acid. The preparation of the "electron-transport particle (ETP)", obtained from *Azotobacter vinelandii* by BRUEMMER *et al.*[24], appears to be rather similar to our WSP preparation. The DPNH and succinate oxidase activities are of the same order of magnitude, but the malate and lactate oxidase activities of ETP are much less than of WSP. The cytochrome spectrum of ETP given by BRUEMMER *et al.*[24] is also similar to that of our preparation. Oxidative phosphorylation in the *Azotobacter* ETP preparation has not yet been reported.

The present studies have shown that electrolytes are required for the maintenance of the phosphorylative activity of the particulate fraction of *Azotobacter vinelandii*, with DPNH, malate or succinate as substrate. Oxidative phosphorylation disappears rapidly and almost completely at low salt concentrations. Sucrose even in high concentration (2.0 *M*) cannot replace the electrolytes. Bivalent cations are much more effective than monovalent in preserving the phosphorylation. This is illustrated by the following concentrations of salts which lead to almost complete disappearance of phosphorylative activity when a suspension (2–2.5 mg protein/ml) is kept 2 h at 0°: KCl, 0.01–0.02 *M*; Sørensen phosphate buffer, pH 7.0, 0.005–0.01 *M*; $CaCl_2$, about 0.0008 *M*; $MgCl_2$ or $MnCl_2$, $<$ 0.0008 *M*. It seems likely that the role of the cation is more important than that of the anion.

This inactivation of oxidative phosphorylation can be reversed by subsequent

References p. 496.

addition of electrolytes, or of the supernatant (S_2) obtained in the isolation of the particles from the *Azotobacter* extract. The reactivation by Mg^{++} was potentiated by the simultaneous addition of serum albumin, or of S_2 + ADP.

BRODIE AND GRAY[12] found that phosphorylating particles of *Mycobacterium phlei* behaved like reversible osmotic systems. Although it is not excluded that the particles used in the present study swell and contract like osmotic systems, this is not sufficient to explain the effect of electrolytes discussed above. It appears rather that electrostatic bonds must play a role.

In contrast to the marked effect on the P:O ratio, lowering the electrolyte concentration had little effect on the rate of oxidation of DPNH, succinate, malate or lactate. HARTMAN, BRODIE AND GRAY[8] also found no correlation between the rate of oxidation and the P:O ratios in their *Azotobacter* extracts, with succinate, malate or fumarate as substrate, although there was a correlation between oxidative and phosphorylative activities in particles isolated from *Mycobacterium phlei*[12].

The reversible effect of electrolytes on oxidative phosphorylation might possibly be related to the requirement for polynucleotide, found by PINCHOT for *Alcaligenes faecalis*[2,3]. It would be expected that the properties of such a polynucleotide, containing many groups with a negative charge, would be influenced by the concentration and charge of cations present[27,28].

The mechanism of oxidative phosphorylation in *Azotobacter* particles differs from that in mitochondria in the following respects: (i), the cytochrome system consists of cytochromes b_1, c_4, c_5, a_1 and a_2, in place of cytochromes b, c, c_1, a and a_3 found in mitochondria[15]; (ii), the *Azotobacter* system is relatively very insensitive to dinitrophenol; (iii), the ATPase activity of the *Azotobacter* particles is much less than found in mitochondrial preparations, where it is believed to be intimately associated with oxidative phosphorylation; (iv), the DPNH oxidase and succinic oxidase of the *Azotobacter* particles are very much less sensitive to antimycin[21,29].

There are several possible reasons for the low P:O ratios found with *Azotobacter* particles, and with bacterial extracts in general.

1. Fewer steps in the bacterial respiratory chain than in mitochondria are associated with phosphorylation. The suggestion of PINCHOT[3] that the respiratory chain in *Alcaligenes faecalis* is shorter than in mitochondria, because only one cytochrome was detected in the extracts, appears rather unlikely and, in any case, is not applicable to the *Azotobacter* particles, which contain four cytochromes[15].

2. In view of the fact that fragments isolated from mammalian mitochondria also have low P:O ratios[22,30], the possibility must be considered that high ratios can be found only in larger units, and that fragmentation of the bacteria inevitably results in lowering of the P:O ratio. If the respiratory chain is located in the cell membrane, as many believe (*cf.* ref. 7), the isolation of an undamaged phosphorylative particle is very difficult, if not impossible.

3. The WSP preparation is a mixture of phosphorylating particles with a high P:O ratio and non-phosphorylating but respiring particles.

4. The preparation contains an inhibitor, liberated by fragmentation of larger units. NOSSAL *et al.*[13] found that an inhibitor was formed after prolonged sonic oscillation of *P. vulgaris*.

Although the P:O ratios obtained with the *Azotobacter* particles oxidizing DPNH are relatively low, the rate of oxidation of DPNH is so high[7], that the rate of esterifi-

References p. 496.

cation of phosphate (about 100 μmoles P/mg protein/h) is much greater than found in mitochondria or mitochondrial fragments (cf. 38 μmoles P/mg protein/h obtained with rat-heart sarcosomes oxidizing α-ketoglutarate, the highest rate given for rat-heart sarcosomes and rat-liver mitochondria by HOLTON *et al.*[31], and 6.4 μmoles P/mg protein/h for the mitochondrial sub-unit isolated with digitonin by COOPER AND LEHNINGER[30]). Since the WSP preparation contains as many ribonucleic acid particles as respiratory granules, the respiratory and phosphorylative activity of the latter must be much greater than the values calculated for the WSP preparation. The study of the special features of these granules which makes possible such high rates of oxidation and phosphorylation promises to be rewarding.

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