BBA 45991

OXIDATIVE PHOSPHORYLATION COUPLED TO OXYGEN UPTAKE AND NITRATE REDUCTION IN *MICROCOCCUS DENITRIFICANS*

PHILIP JOHN* AND F. R. WHATLEY

Department of Botany, King's College, University of London, 68 Half Moon Lane, London. S.E. 24. (Great Britain)

(Received March 17th, 1970)

SUMMARY

A procedure is described for preparing particles from cells of *Micrococcus denitrificans* which were broken osmotically after treatment with lysozyme.

1. The preparations catalysed ATP synthesis coupled to O_2 uptake or NO_3^- reduction. With NADH or succinate as the electron donors the P:O ratios were about 1.5 and 0.5, respectively; and the P:NO_3^- ratios were about 0.9 and 0.06, respectively.

2. Addition of ADP or P_i to the reaction mixture increased the rates of NADH-dependent O_2 uptake and NO_3^- reduction. Addition of 1 mM 2,4-dinitrophenol, which inhibited phosphorylation by 50–60%, increased the basal rates of electron transport.

3. Evidence derived from spectrophotometry and from the differential inhibition by antimycin A of O_2 and NO_3^- reduction leads to the conclusion that the nitrate reductase interacted with the respiratory chain in the region of the *b*-type cytochrome, and that the *c*-type cytochrome present was not involved in the reduction of NO_3^- to NO_2^-.

INTRODUCTION

Micrococcus denitrificans is a facultative autotroph capable of growing either anaerobically in the presence of NO_3^- or aerobically. Under anaerobic conditions NO_3^- acts instead of O_2 as a terminal acceptor of electrons from donors such as succinate, glucose and hydrogen[1,2].

Studies[3,4] made with cell-free extracts have revealed that NADH-oxidase and succinoxidase activities are associated with the cell membrane, which contains flavin, Q-10 (ref. 5), and *b*-, *c*-, *a*- and *o*-type cytochromes. Cells grown anaerobically in the presence of NO_3^- contain higher concentrations of the *b*- and *c*-type cytochromes than aerobically grown cells[4,6]. Anaerobically grown cells also possess a soluble nitrite reductase which has cytochrome *c* oxidase activity and *c*- and *a*-type haem

Abbreviation: TMPD, tetramethyl-*p*-phenylenediamine.

* Present address: Department of Biochemistry, University of Aberdeen, Aberdeen, Great Britain.

groups[7,8], as well as a membrane-bound nitrate reductase, which has been partially purified and shown to contain molybdenum[9].

The ability of a crude extract of *M. denitrificans* to catalyse the oxidation of reduced mammalian cytochrome *c* by NO_3^- led to the formulation of an electron transport pathway from NADH to NO_3^- which involved the *c*-type cytochrome[10]. However, later work[11] indicated that when the oxidising effect of NO_2^- was taken into account the addition of NO_3^- to crude extracts caused primarily the oxidation of the *b*-type cytochrome rather than the *c*-type cytochrome.

ATP synthesis in extracts of *M. denitrificans* has been observed by a number of workers[3,12–15]. IMAI *et al.*[3] have studied in detail the phosphorylation accompanying O_2 uptake by washed membrane fragments of aerobically grown cells broken by exposure to sonic oscillation. P:O ratios were 1.0 and 0.4 with NADH and succinate as the respective electron donors. The phosphorylation activity was found to be tightly bound to the membranes and, unlike preparations from many other bacteria, no components of the supernatant fractions were found to be necessary for ATP synthesis. The NO_3^--dependent ATP synthesis observed by WHATLEY[13] was revealed in subsequent experiments[14] to be due to substrate-level phosphorylation. Crude extracts of *M. denitrificans* have also been obtained from cells broken by repeated freezing and thawing[15]. These extracts brought about low rates of ATP synthesis with NADH, succinate, malate, pyruvate or glutamate as electron donors and NO_3^-, NO_2^- or O_2 as terminal acceptors.

In this paper a procedure is described for the isolation of particles from cells of *M. denitrificans* grown anaerobically with NO_3^-. The particles synthesise ATP in the presence of O_2 or NO_3^- with relatively high P:O or P:NO_3^- ratios compared with other bacterial extracts, and unlike most other bacterial preparations, the rate of electron transport is diminished when either ADP or P_i is omitted from the reaction mixture. Evidence is presented for the location of the nitrate reductase on the respiratory chain in the region of the *b*-type cytochrome present.

MATERIALS AND METHODS

Organism and culture conditions

The original culture of *M. denitrificans* was supplied by Professor Pirt of Queen Elizabeth College, University of London.

Bacteria were grown at 30° in a liquid medium which filled a flat-bottomed flask of 2 l capacity to the neck, and contained, per l: 13.5 g of hydrated sodium succinate, 10.1 g of KNO_3, 0.94 g of sodium glutamate, 12 mg of ferric monosodium EDTA, 25 mg of $MgSO_4 \cdot 7H_2O$, 0.05 ml of modified Hoagland trace element solution[16], and 1 g $(NH_4)_2HPO_4$, adjusted to pH 6.7. Cells were harvested after 24 h at which time the cultures were in the middle of the logarithmic phase. The 2 ml inoculum used was taken from the culture just before harvesting. Cultures for biochemical experiments were routinely maintained by daily subculturing in this way. At intervals of about 3 months a fresh inoculum was obtained from stock cultures maintained on agar slopes made from the liquid medium solidified with 15 g of agar per l, and stored at 1–4°.

No special precautions were taken to exclude air from the liquid cultures since continued growth was dependent upon the presence of NO_3^-, and was accom-

panied by a vigorous evolution of nitrogen and nitrous oxide, which effectively maintained anaerobic conditions.

Preparation of particles

The procedure employed in the preparation of the particles was based on a method described by SCHOLES AND SMITH[17] for isolating the cell membrane of *M. denitrificans*.

All the solutions and apparatus used were maintained at 1–4° and all operations, except the lysozyme treatment, were carried out at these temperatures. Stock buffer solutions at a concentration of 0.4 M were brought to pH 7.3 at room temperature.

Cells in the log phase were sedimented from about 1800 ml of medium by centrifugation at 5000 × *g* for 10 min and were washed in 800 ml of 50 mM NaCl containing 10 mM Tris–HCl buffer. The cells were then suspended in 0.5 M sucrose containing 10 mM Tris–HCl buffer so that an 0.1-ml aliquot of the suspension diluted to 2.5 ml with water gave an absorbance density reading at 550 nm of 0.3 unit in a Unicam SP-600 spectrophotometer. This was equivalent to about 25 mg of wet weight of cells per ml of the suspension in the 0.5 M sucrose. Lysozyme (EC 3.2.1.17) was added at a concentration of 250 μg/ml to this suspension which was then allowed to stand in a water bath at 30° for 20–30 min until the absorbance of a diluted aliquot decreased from 0.3 to about 0.06 unit.

The lysozyme-treated cells were sedimented by centrifugation at 40000 × *g* for 10 min and resuspended to 0.1 of the original volume in 100 mM Tris–acetate buffer, using a loosely fitting Teflon plunger in a 50-ml centrifuge tube. The suspension was then diluted 10-fold with water to disrupt the cells. The resulting suspension had a high viscosity, which was rapidly lowered by adding 0.5 ml of a solution containing 1 mg/ml deoxyribonuclease (EC 3.1.4.5) and 50 mM magnesium acetate to each 100 ml of the disrupted cell suspension. The suspension was then centrifuged at 40000 × *g* for 10 min to give a hard white pellet and a reddish, opaque supernatant. Any whole cells present formed a layer beneath that of the white material. The supernatant was decanted, magnesium acetate was added to bring the final concentration to 5 mM, and the supernatant was centrifuged at 40000 × *g* for 30 min. This centrifugation resulted in the formation of a double-layered pellet and a clear supernatant, which was discarded. The upper layer of the pellet was deep red in colour and was easily separated from the small amount of white material which formed a lower layer. The red particles were washed and suspended in 5 mM magnesium acetate containing 10 mM Tris–acetate buffer (pH 7.3) (approx. 10 ml suspension; 10 mg protein/ml).

Assay of electron-transport and coupled phosphorylation

The reaction was carried out at 30° in a Warburg flask of 14 ml capacity with a single side arm. The reaction mixture contained in a total volume of 2 ml: 100 μmoles of Tris–acetate buffer (pH 7.3), 10 μmoles of magnesium acetate, 40 μmoles of potassium [^{32}P]phosphate (pH 7.3) (25000 counts/min per μmole), 0.5 μmole of ADP, 10 units crystalline hexokinase (EC 2.7.1.1), 55 μmoles of glucose, and 0.2 ml of the particle suspension containing 2 mg protein. When NADH was the substrate, 250 μmoles of alcohol, 30 μmoles of semicarbazide (pH 7.0), 12 units alcohol dehydrogenase (EC 1.1.1.1) and 0.1 μmole of NAD^+ were added. When succinate was

the substrate, 30 μmoles of potassium succinate (pH 7.3) were included. When NO_3^- was the terminal electron acceptor, 30 μmoles of KNO_3 were included and the flasks were rendered anaerobic by flushing with argon.

The reaction was started by tipping the particle suspension from the side arm into the main compartment, which contained all the other constituents of the reaction mixture. The reaction was allowed to proceed for 30 min.

O_2 uptake

O_2 uptake was measured either by conventional Warburg manometry[18] or by means of a Gilson respirometer, readings being taken at intervals of 5 min during the 30-min reaction period.

NO_3^- and NO_2^-

To a 0.4-ml aliquot of the reaction mixture was added a mixture containing 0.1 ml of 1 M zinc acetate, 0.5 ml of water and 5.0 ml of absolute ethanol[15,19]. The suspension was then shaken vigorously and allowed to stand for 10 min in an ice bath before being centrifuged at 2500 × *g* for 10 min. The supernatant was assayed for NO_2^- and NO_3^-.

NO_2^- was determined by means of the Griess–Ilosvay reaction. 1 ml of supernatant was added to 5 ml of 1 % (w/v) sulphanilamide in 1 M HCl. 4 ml of 0.1 % (w/v) *N*-(1-naphthyl)-ethylenediamine·2HCl were then added and the absorbance at 540 nm read after 20 min. NO_2^- was estimated by comparison with a standard curve.

NO_3^- was determined by a modification of a method[20] which employs a cadmium–mercury column to reduce the NO_3^- quantitatively to NO_2^- in an NH_3–NH_4Cl buffer; the NO_2^- was subsequently determined colorimetrically as described above.

Cadmium amalgam was prepared as described by GRASSHOFF[20], except that a finely powdered form of cadmium was used. The columns of cadmium amalgam were prepared from a slurry of cadmium amalgam in glass tubes of the type described by HAGIHARA AND LARDY[21]. The glass tube was fitted into a rimless, light-wall test-tube. The column was then centrifuged at 25 × *g* for 10 min in a bench centrifuge with a swing-out head to compact the cadmium amalgam to a column of 1.3 cm length. The column is prevented from drying out by the accumulation of water in the jacketing test-tube (Fig. 1). 1 ml of the test solution was mixed with 9 ml of a buffer solution made by dissolving 25 g NH_4Cl in 100 ml 6 % (w/v) ammonia and diluting to 1 l. One ml of the buffered test solution was taken for the initial NO_2^- assay. Water remaining above the level of the cadmium-amalgam column was poured off, the inside of the tube was washed with about 4 ml of the buffered sample solution and the washings discarded. The tube was then filled with the remainder (approx. 5 ml). The outside of the column was dried with a paper tissue and the column was fitted into a clean test tube. After centrifuging for 10 min at 25 × *g* a 1-ml aliquot of the eluate was assayed for NO_2^-.

NO_2^- was determined as described above. The absorbances at 540 nm were read against blanks obtained by treating an equal volume of water in the same way as the samples. The relationship between absorbance and NO_2^- *plus* NO_3^- concentration was found to be linear up to a total concentration of 1.5 μmoles per 1 ml of the original sample. Passage through the columns consistently resulted in a 90 % recovery

of NO_2^- (± 3 %) and an apparent 90 % yield of NO_2^- from NO_3^- (± 3 %). This was automatically allowed for in the standard curve. The amount of NO_3^- in a mixture of NO_3^- and NO_2^- was obtained by subtracting the amount of NO_2^- present before reduction from that present after reduction on the cadmium amalgam. The columns could be re-used after washing then with an excess of water to remove the residual NO_2^-.

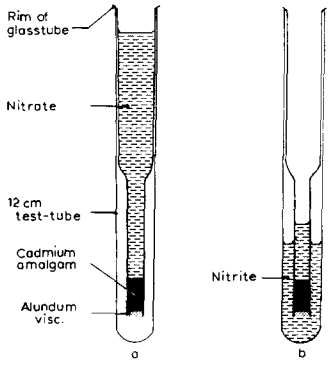

Fig. 1. Cadmium–mercury column. a. Before centrifugation. b. After centrifugation. Details are given in the text.

Phosphorylation

Esterified phosphate was estimated by the procedure of HAGIHARA AND LARDY[21], measuring the incorporation of ^{32}P-labelled orthophosphate into an esterified form.

Absorption spectra

Difference spectra were measured at room temperature in a Unicam SP-800 recording spectrophotometer with the scale expanded by coupling to a Servoscribe recorder with expanded scale. Thunberg-type cuvettes (1 cm light path) were used in a position close to the photomultiplier to avoid errors due to scattering.

Protein

Protein was determined by the biuret method in the presence of 1 % deoxycholate[22]. A blank containing 3 % (w/v) NaOH instead of the biuret reagent was subtracted to correct for the slight turbidity and absorbance of the sample at 550 nm. The assay was standardised with bovine serum albumin.

Chemicals

Crude deoxyribonuclease (*ex* beef pancreas), crystalline yeast hexokinase, antimycin A, NAD^+, succinic acid and Tris were obtained from Sigma (London) Chemical Co. Crystalline yeast alcohol dehydrogenase, crystalline lysozyme (*ex* egg white), and ADP were obtained from Boehringer Corp., Mannheim, Germany. Cadmium powder from Hopkins and Williams, Chadwell Heath, Great Britain. ^{32}P-labelled orthophosphate from the Radiochemical Centre, Amersham, Great Britain. Other chemicals were of A.R. grade and they were obtained either from

British Drug Houses, or from Hopkins and Williams. Tetramethyl-*p*-phenylenediamine (TMPD) was recrystallised from ethanol[23].

RESULTS

Stoichiometry of phosphorylation coupled to O_2 uptake and NO_3^- reduction

A series of preliminary experiments was carried out to determine the conditions of extraction and assay necessary for demonstrating *in vitro* the highest P:O ratios. From these experiments it was found that: (a) higher P:O ratios were obtained with Tris than with a variety of other buffers; (b) higher P:O ratios were obtained when acetate, rather than chloride or sulphate, was the anion accompanying the Tris; and (c) addition of EDTA, bovine serum albumin or sucrose to the reaction mixture failed to increase the P:O ratios. The optimum pH and optimum concentrations of various factors in the media were also determined.

Table I shows that in the presence of NADH or succinate the preparations catalysed the reduction of NO_3^- or O_2 coupled to the esterification of P_i. In the absence of either electron donor or electron acceptor, ATP synthesis was barely detectable.

TABLE I

OXIDATIVE PHOSPHORYLATION WITH O_2 AND NO_3^- AS THE TERMINAL ELECTRON ACCEPTORS

Conditions were as described in MATERIALS AND METHODS.

Electron acceptor	*Electron donor*	*Oxygen consumed (μatoms)*	*NO_2^- accumulated (μmoles)*	*Total NO_3^- plus NO_2^- at end of reaction (μmoles)*	*P_i esterified (μmoles)*	*P:2e⁻ ratio*
O_2	None	0.0	—	—	0.03	—
	Succinate	9.9	—	—	5.65	0.57
	NADH	12.4	—	—	19.8	1.60
NO_3^-	None	—	0.0	30.6	0.02	—
	Succinate	—	7.8	30.3	0.59	0.07
	NADH	—	13.1	30.6	13.4	1.02
None	None	—	—		0.01	—
	Succinate	—	—	—	0.05	—
	NADH	—	—	—	0.16	—

With NADH the P:O ratios were mostly in the range 1.3–1.6 (average of 22 experiments = 1.46) while the P:NO_3^- ratios were in the range 0.8–1.0 (average of 10 experiments = 0.89). With succinate the P:O ratios were in the range 0.4–0.6 (average of 18 experiments = 0.48) while the P:NO_3^- ratios were in the range 0.05–0.09 (average of 10 experiments = 0.06).

It can also be seen from Table I that the total amount of NO_3^- *plus* NO_2^- remaining at the end of the experiment was, within experimental error, equivalent to the amount of NO_3^- added. Although a reduction of NO_3^- to NO_2^- was easily measurable it is clear that no further reduction of NO_2^- had occurred. Similarly, reduction of added NO_2^- (30 μmoles of $NaNO_2$ per flask) by the preparations described in this paper was never observed. The extent of NO_3^- reduction was therefore accurately determined by measuring the amount of NO_2^- which accumulated.

When NADH was the electron donor, the rates of O_2 uptake, NO_3^- reduction and phosphorylation were linear over the whole reaction period. When succinate was used linear rates were preceded by initial lag periods of about 8 min duration. With either NADH or succinate the P:O and P:NO_3^- ratios were constant during the whole of the experiment.

When 10 μmoles of ascorbate was used as an electron donor in the presence of 25 μM TMPD, O_2 uptake was not accompanied by a significant rate of ATP synthesis. Ascorbate–TMPD was shown by difference spectrophotometry to reduce the *c*-type but not the *b*-type cytochromes present in the preparations.

Effect of the phosphorylation system on the rate of electron transport

Table II shows that when NADH was the electron donor the omission of ADP and P_i, either alone or in combination, from the reaction mixture resulted in lower rates of O_2 uptake and NO_3^- reduction. The addition of 1 mM 2,4-dinitrophenol,

TABLE II

THE EFFECT OF ADP, P_i AND DINITROPHENOL ON O_2 UPTAKE, NO_3^- REDUCTION AND PHOSPHORYLATION

Conditions were as described in MATERIALS AND METHODS except that ADP (0.5 μmole), potassium phosphate (pH 7.3) (40 μmoles) and 2,4-dinitrophenol (2 μmoles) were added as indicated.

Electron acceptor	*Electron donor*	*System*	*Oxygen consumed (μatoms)*	*NO_2^- accumulated (μmoles)*	*P_i esterified (μmoles)*	*P:2e$^-$ ratio*
O_2	NADH	Complete	9.3	—	13.0	1.40
		plus dinitrophenol	9.4	—	5.8	0.62
		minus ADP	6.2	—	0.1	—
		minus ADP, *plus* dinitrophenol	7.9	—	0.1	—
		minus P_i	5.1	—	—	—
		minus P_i, *plus* dinitrophenol	7.3	—	—	—
		minus ADP, P_i	5.2	—	—	—
		minus ADP, P_i, *plus* dinitrophenol	7.3	—	—	—
	Succinate	Complete	7.1	—	3.2	0.45
		plus dinitrophenol	6.1	—	0.3	0.05
		minus ADP, P_i	7.8	—	—	—
		minus ADP, P_i., *plus* dinitrophenol	6.3	—	—	—
NO_3^-	NADH	Complete	—	10.8	9.3	0.86
		plus dinitrophenol	—	11.2	3.9	0.35
		minus ADP	—	7.0	0.1	—
		minus ADP, *plus* dinitrophenol	—	9.2	0.1	—
		minus P_i	—	7.1	—	—
		minus P_i, *plus* dinitrophenol	—	9.5	—	—
		minus ADP, P_i	—	6.7	—	—
		minus ADP, P_i, *plus* dinitrophenol	—	9.2	—	—

a high concentration which partially uncoupled phosphorylation in the complete system, caused an increase in the rate of electron transport in the absence of ADP and P_i. When succinate was the electron donor the omission of ADP and/or P_i caused no significant decrease in the rate of O_2 uptake; the addition of 1 mM 2,4-dinitrophenol uncoupled phosphorylation by about 90 % and slightly diminished the O_2 uptake.

Site of interaction between NO_3^- and the cytochrome chain

The location of the nitrate reductase on the cytochrome chain was determined primarily by spectrophotometry. When a cuvette containing a particle preparation reduced by the addition of succinate was read against a cuvette containing the preparation in the oxidised state, the resulting difference spectrum (Fig. 2A) indicated the presence of a *c*-type cytochrome (absorption maxima at 555 and 525 nm) and a *b*-type cytochrome (absorption maxima at 560 and 530 nm). The absorption maxima in the Soret region were fused into a single absorption peak at 425–430 nm. The absorption maxima characteristic of cytochromes $a + a_3$ were only rarely detectable in difference spectra of preparations derived from cells grown anaerobically in the presence of NO_3^-, although difference spectra of similarly prepared particles derived from aerobically grown cells did indeed show absorption maxima where expected for cytochromes $a + a_3$.

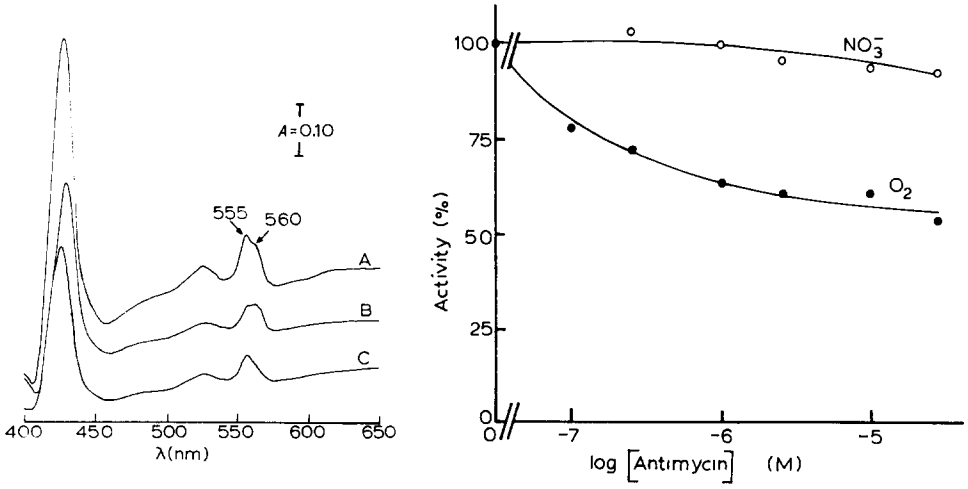

Fig. 2. The effect of NO_3^- on the difference spectra. 2.5 ml of the particle preparation containing 12.5 mg protein were added to each cuvette. Preparations reduced with succinate contained 10 μmoles of sodium succinate crystals. 20 μmoles of solid KNO_3 were added to the hollow stopper. Air was removed by repeated evacuation and flushing with argon. NO_3^- was added to the reduced suspensions by tipping the anaerobic cuvettes. In the absence of any additions to the preparations the cytochromes appeared to be in a completely oxidized state, since adding a crystal of ferricyanide or stirring in air caused no changes in the absorption spectra of the cytochromes. A, succinate-reduced *minus* oxidised; B, succinate-reduced *minus* succinate-reduced *plus* excess NO_3^-; C, succinate-reduced *plus* excess NO_3^- *minus* oxidised.

Fig. 3. The effect of antimycin A on NADH-dependent O_2 uptake and NO_3^- reduction. The conditions employed were as described in MATERIALS AND METHODS except that 1 mg particle protein was used in each reaction mixture, and antimycin A was added in small volumes (10 or 30 μl) of 95 % ethanol solution to the main compartment of the reaction flasks. Similar volumes of 95 % ethanol had no effect on the activities tested. 100 % corresponds to the uptake of 5.9 μmoles of O_2 or the reduction of 6.5 μmoles of NO_3^- in 30 min.

When a preparation reduced by the addition of succinate under anaerobic conditions was read against a similarly reduced anaerobic preparation subsequently treated with an excess of KNO_3 an absorption maximum at 560 nm, with a shoulder at 555 nm (Fig. 2B) appeared in the α region of the resulting cytochrome spectrum. Finally, when excess NO_3^- was added to the 'reduced' cuvette of a succinate reduced *minus* oxidised difference spectrum the absorption maximum in the α region at 560 nm disappeared leaving a single absorption maximum in the α region at 555 nm (Fig. 2C). This absorption maximum at 555 nm disappeared when the cuvette was made aerobic by shaking with air.

When NO_2^- was used instead of NO_3^- in these experiments no effect on the difference spectra obtained with freshly prepared particles was observed, although a slight irreversible oxidation of all the cytochromes of a preparation aged overnight at 1–4° was seen.

The differential effect of antimycin A was also examined in the presence of NADH or succinate as electron donors and O_2 or NO_3^- as electron acceptors. Fig. 3 shows that O_2 uptake in the presence of NADH was inhibited by concentrations of antimycin A which had little effect on the rate of NO_3^- reduction. However, when succinate was the electron donor antimycin A was much less effective and at a concentration of 10 μM inhibited the rates of O_2 uptake and NO_3^- reduction by only 15 % and 5 %, respectively.

DISCUSSION

The membrane preparations of *M. denitrificans* described in this paper catalyse a coupling between the oxidation–reduction and phosphorylation reactions of respiration which, compared with other bacterial systems, is interesting in three respects. Firstly, the $P:2e^-$ ratios are among the highest which have been observed with bacterial preparations (see ref. 24, Table 21). Secondly, significant $^{32}P_i$ incorporation is dependent upon the presence of both the electron acceptor and the electron donor, in contrast to the results obtained with extracts of *M. denitrificans* studied by previous authors[13,15]. Thirdly, the basal rates of NADH-dependent O_2 uptake and NO_3^- reduction were increased when ADP and P_i were added to the reaction mixtures. The basal rates were also increased by the addition of 1 mM dinitrophenol although the dinitrophenol-stimulated rates did not attain those observed in the presence of ADP and P_i (*cf.* refs. 3 and 15).

Our results provide clear evidence in a bacterial system for the kind of stimulation of electron transport by the inclusion of ADP and P_i which is observed with mitochondria (see ref. 25) and chloroplasts (see ref. 26), and is suggestive of respiratory control in *M. denitrificans*.

The preparations described in this paper are unable to catalyse the reduction of NO_2^-, apparently due to the loss of the nitrite reductase from the particles during the isolation procedure. NEWTON[7,8] has already shown that nitrite reductase (an enzyme containing cytochrome *c* and *d*) is released into the soluble phase when lysozyme-treated cells are osmotically broken. We find that the characteristic absorption maxima of the reduced cytochromes[7] associated with nitrite reductase could not be detected in reduced *minus* oxidised difference spectra of our preparations. The inability of these preparations to reduce NO_2^- has enabled us to study NO_3^-

reduction without incurring the complications arising from the further reduction of the NO_2^- (see refs. 10 and 11).

Addition of NO_3^- to a suspension reduced by succinate caused the oxidation principally of the *b*-type cytochrome, and only to a lesser extent of a *c*-type cytochrome present in the particles. From this it is inferred that the nitrate reductase accepts electrons from the *b*-type cytochrome of the respiratory chain and that the *c*-type cytochrome is not involved in the reduction of NO_3^- to NO_2^-. The location of the nitrate reductase adjacent to the *b*-type cytochrome is in agreement with the much smaller inhibition by antimycin A of NADH-dependent NO_3^- reduction at antimycin A concentrations (10 μM; see Fig. 3) which inhibit the rate of NADH-dependent O_2 uptake significantly. These observations confirm the tentative proposals of LAM AND NICHOLAS[11], which were based on experiments made with crude cell-free extracts which had both nitrite reductase and nitrate reductase activity.

The difference between the P:O ratios obtained with succinate and NADH was about 1.0. The P:NO_3^- ratio observed with NADH as the electron donor approached 1.0. These two values probably represent the P:$2e^-$ ratio of the phosphorylation accompanying electron transport between NADH and the *b*-type cytochrome.

The preparations described in the paper, like the membrane fragments studied by IMAI *et al.*[3], failed to synthesise ATP when ascorbate–TMPD was the electron donor, although in both cases the *c*-type cytochrome was shown spectrophotometrically to be reduced by the electron donor. VERNON AND WHITE[12] have already observed a P:O ratio of 0.39 when reduced mammalian cytochrome *c* was oxidised aerobically by their preparations of *M. denitrificans*.

M. denitrificans is able to grow anaerobically in the presence of succinate as the oxidisable substrate and NO_3^- as the terminal electron acceptor. The evidence presented in this paper suggests that most of the ATP synthesis which occurs during the reduction of NO_3^- under these conditions is coupled to the oxidation of NADH that is produced during the metabolism of succinate by the tricarboxylic acid cycle (*cf.* ref. 27). This conclusion is consistent with the observation[14] that growth on [^{14}C]glucose, [^{14}C]malate and [^{14}C]succinate leads to the complete oxidation of part of the substrate to $^{14}CO_2$ and the incorporation of the remainder into new bacterial substance, and that there is at no time an accumulation of fermentation products in the medium.

ACKNOWLEDGMENT

One of us (P.J.) wishes to thank the Science Research Council for a Postgraduate Research Studentship.

REFERENCES

1 W. VERHOEVEN, A. L. KOSTER AND M. C. A. VAN NIEVELT, *Antonie van Leeuwenhoek J. Microbiol. Serol.*, 20 (1954) 273
2 A. J. KLUYVER AND W. VERHOEVEN, *Antonie van Leeuwenhoek J. Microbiol. Serol.*, 20 (1954) 337.
3 K. IMAI, A. ASANO AND R. SATO, *Biochim. Biophys. Acta*, 143 (1967) 462.
4 P. B. SCHOLES AND L. SMITH, *Biochim. Biophys. Acta*, 153 (1968) 363.
5 K. IMAI, A. ASANO AND R. SATO, *J. Biochem. Tokyo*, 63 (1968) 207.
6 R. J. PORRA AND J. LASCELLES, *Biochem. J.*, 94 (1965) 120.

7 N. NEWTON, *Biochem. J.*, 105 (1964) 21c.
8 N. NEWTON, *Biochim. Biophys. Acta*, 185 (1969) 316.
9 Y. LAM AND D. J. D. NICHOLAS, *Biochim. Biophys. Acta*, 178 (1969) 225.
10 C. A. FEWSON AND D. J. D. NICHOLAS, *Biochim. Biophys. Acta*, 48 (1961) 208.
11 Y. LAM AND D. J. D. NICHOLAS, *Biochim. Biophys. Acta*, 172 (1969) 450.
12 L. P. VERNON AND F. G. WHITE, *Biochim. Biophys. Acta*, 25 (1957) 321.
13 F. R. WHATLEY, *Plant Physiol.*, 37 (1962) viii.
14 F. R. WHATLEY AND B. R. GRANT, *Abstr. 7th Intern. Congr. Biochem., Tokyo, 1967*, Part V, p. 901.
15 M. S. NAIK AND D. J. D. NICHOLAS, *Biochim. Biophys. Acta*, 113 (1966) 490.
16 H. G. SCHLEGEL AND N. PFENNIG, *Arch. Mikrobiol.*, 38 (1961) 1.
17 P. B. SCHOLES AND L. SMITH, *Biochim. Biophys. Acta*, 153 (1968) 350.
18 W. W. UMBREIT, R. H. BURRIS AND J. F. STAUFFER, *Manometric Techniques*, Burgess, Minneapolis, 4th ed., 1964.
19 E. J. HEWITT AND D. J. D. NICHOLAS, in H. F. LINSKENS, B. D. SANWAL AND M. V. TRACEY, *Modern Methods of Plant Analysis*, Vol. 8, Springer-Verlag, Berlin, 1964, p. 65.
20 K. GRASSHOFF, *Kiel. Meeresforsch.*, 20 (1964) 5.
21 B. HAGIHARA AND H. A. LARDY, *J. Biol. Chem.*, 235 (1960) 889.
22 A. G. GORNALL, C. J. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
23 D. R. SANADI AND E. E. JACOBS, in R. W. ESTABROOK AND M. E. PULLMAN, *Methods in Enzymology*, Vol. 10, Academic Press, New York, 1967, p. 38.
24 N. S. GEL'MAN, M. A. LUKOYANOVA AND D. N. OSTROVSKII, *Respiration and Phosphorylation of Bacteria*, Plenum Press, New York, 1967.
25 B. CHANCE AND G. R. WILLIAMS, *Advan. Enzymol.*, 17 (1956) 65.
26 K. R. WEST AND J. T. WISKICH, *Biochem. J.*, 109 (1968) 527.
27 P. FORGET AND F. PICHINOTY, *Ann. Inst. Pasteur*, 108 (1967) 364.