

## The Microbial Metabolism of C<sub>1</sub> Compounds

### OXIDATIVE PHOSPHORYLATION IN MEMBRANE PREPARATIONS OF *PSEUDOMONAS AM1*

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A method is described for preparation of membrane vesicles (diameter 80 nm) capable of respiration-linked ATP synthesis. Vesicles prepared from succinate-grown bacteria oxidized NADH, succinate and ascorbate plus *NNN'N'*-tetramethylphenylenediamine; vesicles prepared from methanol-grown bacteria also oxidized methanol and formaldehyde, but they were otherwise identical. The uncoupling agent carbonyl cyanide chlorophenylhydrazone and the adenosine triphosphatase inhibitor dicyclohexylcarbodi-imide both inhibited ATP synthesis, whereas they had no effect on the rate of respiration. Rotenone inhibited ATP synthesis and respiration with NADH as substrate; antimycin A inhibited with succinate as substrate, and cyanide inhibited with all substrates. P/O ratios were usually 0.7–1.3 with NADH, 0.6–1.0 with succinate and 0.2–0.6 with reduced *NNN'N'*-tetramethylphenylenediamine or methanol as respiratory substrate. When 2,6-dichlorophenol-indophenol was used as an alternative electron acceptor to O<sub>2</sub> (NADH as donor) the P/2e ratio was 1.65. Although these P/O ratios are minimum values, because they do not take into account unknown amounts of uncoupled O<sub>2</sub> consumption, they are consistent with previous proposals [O'Keeffe & Anthony (1978) *Biochem. J.* 170, 561–567] based on measurements of proton translocation in whole cells. The results also confirm that methanol dehydrogenase and cytochromes *c* and *a/a<sub>3</sub>* are arranged so that the first step in methanol oxidation is coupled to synthesis of ATP.

The methylotrophic bacterium *Pseudomonas AM1* contains membrane-bound cytochromes *b* and *a/a<sub>3</sub>* and a cytochrome *c* that is to some extent readily removed from membranes. This organism is unusual in that a mutant lacking the cytochrome *c* is still able to grow on compounds such as succinate, although unable to grow on C<sub>1</sub> compounds (Anthony, 1975; Widdowson & Anthony, 1975). Furthermore, measurements of respiration-driven proton translocation in wild-type and mutant bacteria have indicated that the cytochrome *c* is not essential for energy transduction during oxidation of NADH and succinate, and that a maximum P/O ratio of 2 might occur in these methylotrophic bacteria during NADH oxidation (O'Keeffe & Anthony, 1978). This suggestion conflicts with the generalizations that may be made relating possession of cytochrome *c*, ATP synthesis and cell yields in typical heterotrophic bacteria (Jones, 1977). Methanol is oxidized in methylotrophic bacteria by way of an unusual methanol dehydrogenase that may have a pteridine moiety as the prosthetic group and that is assayed in extracts with the artificial electron acceptor phenazine metho-

sulphate (Anthony & Zatman, 1967). In whole bacteria the methanol dehydrogenase is coupled (not necessarily directly) with the cytochrome *c*, both these proteins being essential for oxidation of methanol (Anthony, 1975) (and methane; Tonge *et al.*, 1977), but it is uncertain whether this first step in the oxidation of methanol is coupled to ATP synthesis.

An alternative approach to the study of this unusual electron-transport system would be to measure directly the ATP generated during respiration, but this can only be done after removal of cell walls in a preparation with sites for substrate oxidation and in which the generated ATP can be readily measured.

The present paper describes a method for preparation and the initial characterization of membrane vesicles from *Pseudomonas AM1* that are capable of high rates of oxidative phosphorylation in the presence of methanol, NADH or succinate.

#### Materials and Methods

##### Chemicals

NAD<sup>+</sup>, NADP<sup>+</sup> and lactate dehydrogenase were from Boehringer Corp. (London) Ltd., Lewes, East Sussex, U.K. Other enzymes and coenzymes were

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from Sigma (London) Chemical Co., Kingston upon Thames, U.K., and all other chemicals were from BDH Chemicals, Poole, Dorset, U.K.

#### *Organism and growth media*

*Pseudomonas* AM1 (N.C.I.B. 9133) was maintained on methylamine agar slopes and the defined liquid medium described by MacLennan *et al.* (1971) was used. Carbon sources were used at 40mM for succinate and 0.4% (w/v) for methylamine and methanol.

#### *Buffers used during disruption and washing of bacteria*

Potassium phosphate buffer (50mM, pH 7.8) containing 5mM-MgCl<sub>2</sub> was used for washing whole cells and for measurements of respiration and ATP synthesis. During disruption of bacteria this buffer also contained sodium succinate (1mM), ATP (2mM), dithiothreitol (0.2mM) and bovine serum albumin (0.5mg/ml).

#### *Growth and harvesting of bacteria*

Bacteria were grown at 30°C as 500ml batch cultures in 2-litre baffled conical flasks on a rotary shaker (200 rev./min). They were harvested by centrifugation (10000g, 30min) from the exponential phase of growth before 50% of maximum possible growth had occurred.

#### *Disruption of bacteria*

Bacteria were suspended in disruption medium (1g wet wt. of cells plus 3ml of buffer) and passed through an X-Press (LKB-Producter A.B., Stockholm, Sweden) in the frozen state at 20000lb/in<sup>2</sup> (135MPa). Undisrupted cells and large debris were removed by centrifugation at 40000g for 10min (at 2°C) and the supernatant was used for preparation of membrane vesicles. The French pressure cell (Aminco, Silver Spring, MD 20910, U.S.A.) was also found to be suitable for preparation of active vesicles.

#### *Isolation of membrane vesicles*

The cell-free supernatant was centrifuged at 400000g for 1h in the titanium head (10×10ml) of an MSE Super-Speed centrifuge. The supernatant was discarded, the pellet resuspended in the same volume of disruption buffer as above (excluding succinate and albumin) and re-centrifuged as above. The resulting pellet was suspended in a small volume of potassium phosphate buffer (50mM, pH 7.8) containing MgCl<sub>2</sub> (5mM). Protein was determined by the

method of Lowry *et al.* (1951); crystalline bovine serum albumin (fraction V) dried to constant weight was used as a standard.

#### *Measurement of absorption spectra*

Measurement of cytochromes in membrane preparations and of cytochrome reduction by substrates was done exactly as previously described (Anthony, 1975; Widdowson & Anthony, 1975).

#### *Assay of ATP synthesis and respiration*

All experiments were done at 22–24°C in a modified Cary 118C spectrophotometer. A 10mm-light-path cuvette was fused to a Rank oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.), O<sub>2</sub> consumption being recorded on a Servoscribe 1s recorder. The top of the cuvette was fitted with a Suba-Seal stopper through which passed syringe needles for addition of chemicals and for preliminary flushing of the dead space above the incubation mixture with N<sub>2</sub>. The oxygen-electrode cuvette was placed in the light-path as close to the photomultiplier as possible. The reference cuvette contained the same mixture as the reaction cuvette except for respiratory substrate. Although this apparatus was used in the experiments described in the present paper, the results were the same when rates of ATP synthesis and O<sub>2</sub> consumption were measured by using a separate spectrophotometer and oxygen electrode.

The ATP assay system contained in a 4ml final volume: 150μmol of potassium phosphate buffer, pH 7.8; 15μmol of MgCl<sub>2</sub>; 4μmol of NADP<sup>+</sup>; 20μmol of glucose; 20μmol of AMP; 4μmol of ADP; NH<sub>4</sub><sup>+</sup>-free hexokinase (type F300) (10 units); NH<sub>4</sub><sup>+</sup>-free crystalline glucose 6-phosphate dehydrogenase (type XV) (5 units) (1 enzyme unit catalyses the conversion of 1μmol of substrate/min); membrane vesicles (0.3–0.5mg of protein). Substrates for oxidation were succinate (5mM); ascorbate (5mM) plus *NNN'*-tetramethylphenylenediamine (0.5mM); methanol (1mM); NADH (1mM). For the study of oxidation of NADH coupled to ATP synthesis an NADH-generating system was used; this contained NAD<sup>+</sup> (0.4μmol), lactate (5μmol) and lactate dehydrogenase (10 units). Rates of ATP synthesis (measured as NADPH formation at 340nm) and O<sub>2</sub> consumption were measured in the first 5min after addition of respiratory substrate; during this time the rates were constant. ATP was added at the end of all experiments to calibrate the system.

The AMP included in the assay system was sufficient to decrease the low rate of ATP synthesis due to adenylate kinase activity by 80%. This inhibited rate of ATP synthesis was 5 to 10% of the rate in the presence of respiratory substrate. As a double-

beam spectrophotometer was used this rate was automatically subtracted from the rate due to oxidative phosphorylation. The validity of the assay system is confirmed by the dependence of the measured rates on the presence of respiratory substrates and by the inhibitory action of respiratory inhibitors, uncoupling agents and ATPase inhibitors (see the Results section). No energy-linked NADH-NADPH transhydrogenase activity was detected in the membrane preparations and no NADP was produced in the absence of added hexokinase or glucose 6-phosphate dehydrogenase.

For measurement of ATP synthesis coupled to anaerobic oxidation of NADH, O<sub>2</sub>-free 2,6-dichlorophenol-indophenol (dissolved in water) was added to the O<sub>2</sub>-free mixture. The final concentration of 2,6-dichlorophenol-indophenol was 0.1 mM and reduction was measured by the decrease in A<sub>600</sub>; the molar absorption coefficient was taken to be 21 litre · mmol<sup>-1</sup> · cm<sup>-1</sup> (King, 1963). The measurement of rates of dye reduction and NADPH formation were done in the same reaction cuvette by alternating the wavelength during the reaction. The reference cuvette contained all reagents except respiratory substrate and so absorption by 2,6-dichlorophenol-indophenol at 340 nm was automatically compensated for. Separate measurements of dye reduction in the absence of vesicles showed this to be negligible and the rate of dye reduction in the absence of respiratory substrate was less than 10% of that in its presence.

#### Use of inhibitors

Membrane preparations were always incubated with inhibitors for 10 min before addition of respiratory substrates. Control experiments were always done for the solvent used for each inhibitor; the

solvents were dimethylsulphoxide for rotenone; ethanol for antimycin A; acetonitrile for dicyclohexylcarbodi-imide and ethanol for carbonyl cyanide *m*-chlorophenylhydrazone. These solvents had negligible inhibitory effects on respiration or ATP synthesis. That inhibitors were not affecting the ATP-measuring system was always checked by addition of ATP at the end of each experiment.

#### Electron microscopy

This was done by Dr. J. T. R. Fitzsimons in the School of Biochemical and Physiological Sciences, University of Southampton, using negatively stained preparations with a Hitachi HU12 electron microscope.

#### Results

##### Preparation of active membrane vesicles

To obtain consistently membrane preparations containing predominantly closed vesicles (as judged by electron microscopy) with low endogenous rates of respiration and ATP synthesis it was essential to include Mg<sup>2+</sup>, ATP, succinate and bovine serum albumin in the disruption medium. It was also essential to harvest bacteria before half the maximum possible growth was reached and to complete the stage of preparation between cell breakage and the start of high-speed centrifugation as rapidly as possible. Vesicles were 80 nm in diameter and they were active (in ATP synthesis) for up to 9 h after preparation.

##### Respiratory activity of membrane vesicles

Vesicles prepared from succinate-grown bacteria oxidized NADH, succinate and ascorbate plus

Table 1. *Respiration-coupled ATP synthesis by membrane vesicles*

Preparation of vesicles and measurement of ATP and respiration is described fully in the Materials and Methods section. The respiration rate is given as ng-atoms of O<sub>2</sub> consumed/min per mg of protein; the rate of ATP synthesis is given as nmol of ATP synthesized/min per mg of protein. The P/O ratio is the rate of synthesis of ATP (nmol) divided by the rate of O<sub>2</sub> consumption (ng-atoms). Endogenous rates have been automatically subtracted from rates with added substrate. Endogenous rates when measured separately were usually less than 10% of the rates with added NADH. Abbreviation: n.d., none detected.

Respiratory substrate	Vesicles prepared from methanol-grown bacteria			Vesicles prepared from succinate-grown bacteria		
	Respiration rate	Rate of ATP synthesis	P/O ratio	Respiration rate	Rate of ATP synthesis	P/O ratio
NADH-generating system	46.6	55.9	1.2	67.5	58.1	0.86
Succinate	34.2	33.2	0.97	38.6	28.1	0.73
Methanol	12.8	5.6	0.44	n.d.	n.d.	—
Formaldehyde	34.6	18.0	0.52	n.d.	n.d.	—
Ascorbate plus <i>NNN'</i> N'-tetramethylphenylene-diamine	44.8	14.3	0.32	57.3	26.4	0.46

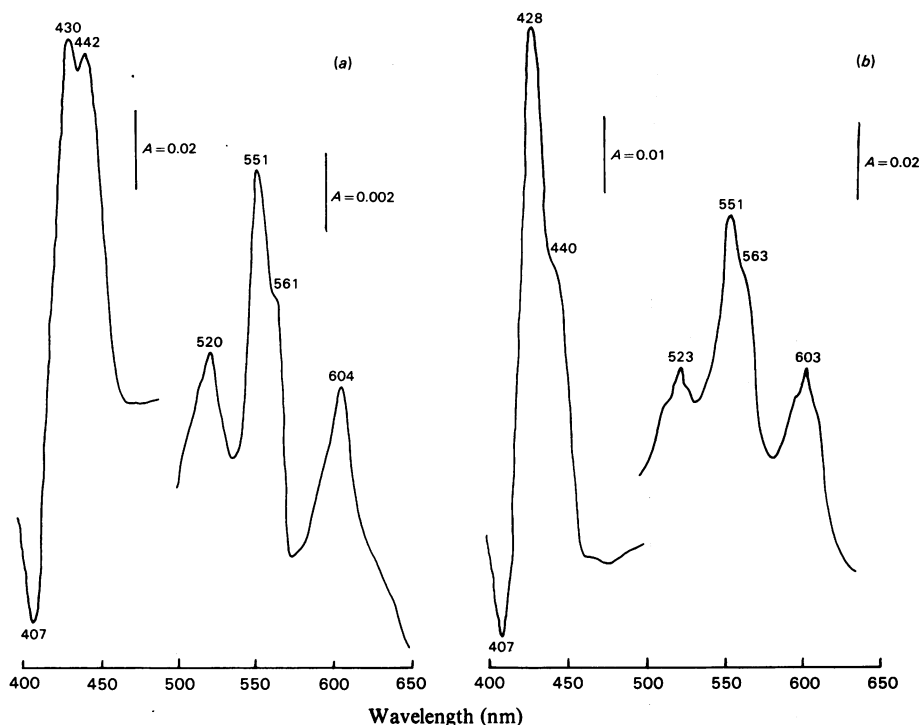


Fig. 1. Reduced-minus-oxidized difference spectra of membrane vesicle preparations

Details of methods for preparation of membrane vesicles and for recording spectra are given in the Materials and Methods section. Membranes were reduced with a little solid sodium dithionite and oxidized in the reference cuvette with 0.1 ml of 3% (v/v)  $\text{H}_2\text{O}_2$ . (a) Membranes (1.2 mg of protein/ml) prepared from methanol-grown bacteria; (b) membranes (1.2 mg of protein/ml) prepared from succinate-grown bacteria.

*NNN'*-tetramethylphenylenediamine; in addition to these substrates vesicles prepared from methanol-grown bacteria also oxidized methanol and formaldehyde (see Table 1). Less than 5% of the respiratory activity of broken cells with NADH as substrate remained in the supernatant after centrifugation at 400000g for 1 h. Presumably methanol was oxidized by way of the low proportion of phenazine methosulphate-linked methanol dehydrogenase that remained in the membrane preparations. Formaldehyde may be oxidized by this enzyme and by other dye-linked dehydrogenases; because of the resulting difficulties of interpretation no further experiments were done with this substrate.

The vesicle preparations contained cytochromes *a/a*<sub>3</sub>, *b* and *c* in approximately equal amounts; most of the cytochrome *c* remained in the supernatant after high-speed centrifugation (Fig. 1). Cytochrome *b* was usually masked by the slight excess of cytochrome *c* and so its reduction by added respiratory substrates could not be demonstrated with certainty. Cytochromes *c* and *a/a*<sub>3</sub> were reduced by NADH and by

succinate in all vesicles, whereas methanol was also able to reduce these cytochromes in vesicles prepared from methanol-grown bacteria. Rotenone (10  $\mu\text{M}$ ) completely inhibited cytochrome reduction by NADH, but had no effect on cytochrome reduction by other substrates.

#### *Measurement of respiration-linked ATP synthesis by vesicle preparations*

ATP was measured continuously by using a trapping system involving hexokinase (glucose as co-substrate with ATP) and glucose 6-phosphate dehydrogenase. The rate of ATP synthesis was therefore measured as a rate of reduction of  $\text{NADP}^+$ . Because large amounts of NADH (as respiratory substrate) would prevent this measurement an NADH-generating system was used; this consisted of lactate, lactate dehydrogenase and  $\text{NAD}^+$ . The rate of  $\text{O}_2$  consumption was the same with this system as with NADH, and inhibitors acted similarly on respiration with NADH as with the NADH-generat-

ing system. Lactate was not oxidized by vesicle preparations.

In no case did the ATP-measuring system affect the rate of respiration with any substrate, and rates of respiration and ATP synthesis were shown to be proportional to the concentration of membrane protein in the reaction mixture.

The results in Table 1 show that all substrates that were oxidized also supported synthesis of ATP. Except for the range of substrates oxidized, vesicles from methanol-grown cells and from succinate-grown cells were similar. P/O ratios were usually in the range 0.7–1.3 for NADH; for succinate ratios were between 0.6 and 1.0 and always lower than for NADH; with methanol, formaldehyde and ascorbate plus *NNN'*-tetramethylphenylenediamine the ratios were usually 0.2–0.6 and usually lower than the value obtained with succinate using the same batch of vesicles.

#### *Effect of inhibitors on rates of respiration and ATP synthesis*

The results in Table 2 obtained with NADH as respiratory substrate show that the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone completely prevented ATP synthesis, but had no effect on respiration; in a few experiments carbonyl cyanide *m*-chlorophenylhydrazone (1  $\mu$ M) stimulated the rate of respiration by up to 20%. Dicyclohexylcarbodi-imide, an ATPase inhibitor (Garland, 1977), inhibited ATP synthesis, but had no effect on respiration (below 1  $\mu$ M). KCN inhibited both respiration and ATP synthesis. That about 40% of respiration continued with NADH as substrate when ATP synthesis was completely abolished suggests that up to 40% of the measured O<sub>2</sub> consumption in the absence of KCN may not have been by way of the normal electron-transport chain. If this O<sub>2</sub> consumption is

Table 2. *Inhibition of NADH oxidation and ATP synthesis in membrane vesicles*

Experimental details are given in the Materials and Methods section and in the legend to Table 1. The uninhibited rates are as given in Table 1.

Inhibitor	Inhibition (%) of respiration and ATP synthesis			
	Vesicles prepared from methanol-grown bacteria		Vesicles prepared from succinate-grown bacteria	
	Inhibition of O <sub>2</sub> consumption	Inhibition of ATP synthesis	Inhibition of O <sub>2</sub> consumption	Inhibition of ATP synthesis
<b>Carbonyl cyanide <i>m</i>-chlorophenylhydrazone</b>				
0.01 $\mu$ M	0	3	0	0
0.05 $\mu$ M	0	30	0	21
0.1 $\mu$ M	0	52	0	49
0.5 $\mu$ M	0	71	0	64
1.0 $\mu$ M	0	92	0	96
5.0 $\mu$ M	0	100	0	100
<b>Dicyclohexylcarbodi-imide</b>				
0.01 $\mu$ M	0	0	0	4
0.05 $\mu$ M	0	15	0	12
0.1 $\mu$ M	0	29	0	31
0.5 $\mu$ M	0	76	0	75
1.0 $\mu$ M	0	100	0	100
5.0 $\mu$ M	10	100	18	100
<b>KCN</b>				
0.01 mM	2	5	3	7
0.05 mM	28	44	31	41
0.1 mM	44	74	49	68
0.5 mM	63	100	68	100
1.0 mM	78	100	81	100
5.0 mM	93	100	97	100
<b>Rotenone</b>				
0.1 $\mu$ M	13	18	12	9
1.0 $\mu$ M	74	77	61	52
10.0 $\mu$ M	98	99	99	100

Table 3. *Respiration-linked ATP generation in the presence of various substrates and inhibitors*  
 Experimental details are given in the Materials and Methods section and in the legend to Table 1.

Substrates and inhibitors	Vesicles prepared from methanol-grown bacteria			Vesicles prepared from succinate-grown bacteria		
	Respiration rate	Rate of ATP synthesis	P/O ratio	Respiration rate	Rate of ATP synthesis	P/O ratio
NADH-generating system	49.8	38.4	0.76	49.4	36.5	0.75
Plus rotenone (0.01 mM)	0.6	0.1	—	0.3	0.0	—
Plus succinate	44.2	28.8	0.65	29.5	7.7	0.26
Plus antimycin A (10 µg/mg of protein)	5.8	1.2	—	8.4	0.0	—
Plus ascorbate/ <i>NNN'</i> -tetramethyl- phenylenediamine	45.4	12.3	0.27	43.3	17.3	0.04
Plus KCN (1 mM)	5.9	0.0	—	6.4	0.0	—

Table 4. *Anaerobic ATP generation coupled to reduction of 2,6-dichlorophenol-indophenol by NADH in membrane vesicle preparations*

The reaction mixture in spectrophotometer cuvettes was made anaerobic by bubbling with O<sub>2</sub>-free N<sub>2</sub>. Anaerobic solutions of substrates and inhibitors were added to the reaction mixture with syringes through a Suba-Seal stopper. The reference cuvette lacked the NADH-generating system. Details are given in the Materials and Methods section. The rates of reduction of 2,6-dichlorophenol-indophenol and the rates of ATP synthesis are expressed as nmol/min per mg of protein. The rate of ATP synthesis divided by the rate of dye reduction is given as the P/2e ratio.

Inhibitor	Vesicles prepared from methanol-grown bacteria			Vesicles prepared from succinate-grown bacteria		
	Rate of 2,6-dichlorophenol- indophenol reduction	Rate of ATP synthesis	P/2e ratio	Rate of 2,6-dichlorophenol- indophenol reduction	Rate of ATP synthesis	P/2e ratio
None	16.1	26.5	1.65	25.4	41.1	1.62
Rotenone (10 µM)	0.2	0.4	—	0.4	0.0	—
Antimycin A (10 µg/mg of protein)	0.3	0.05	—	0.2	0.0	—
Carbonyl cyanide <i>m</i> - chlorophenylhydrazone	16.5	0.0	—	27.8	0.0	—

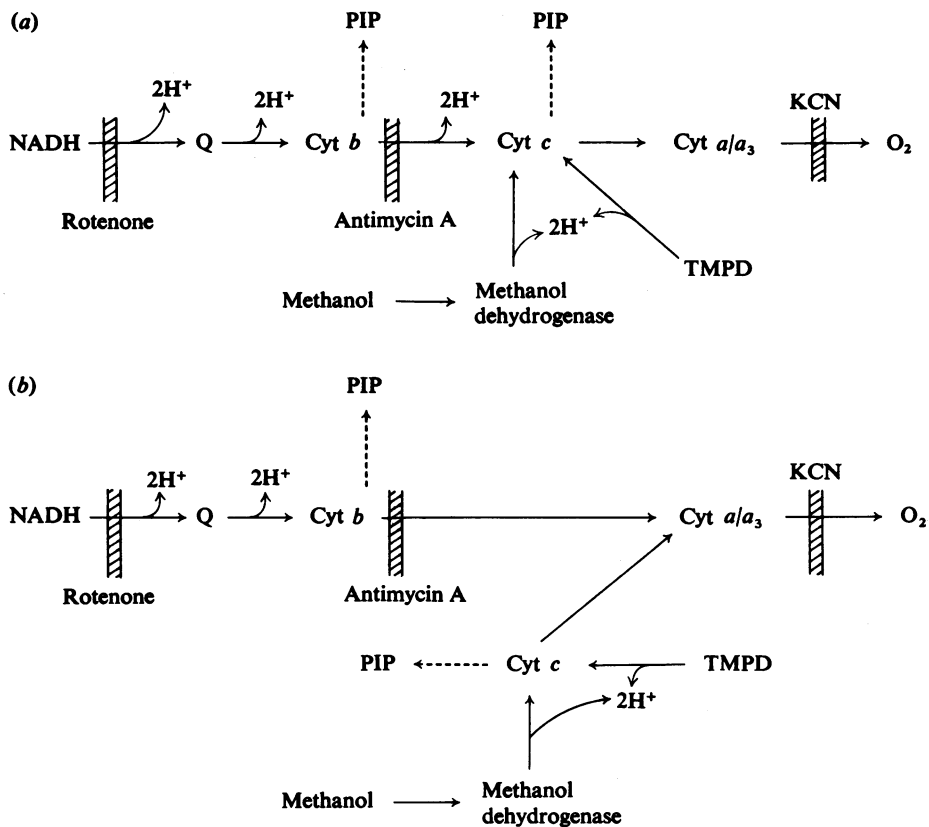
ignored then the P/O ratio for NADH oxidation would be raised from the usually measured 1.2 to 2.0.

Carbonyl cyanide *m*-chlorophenylhydrazone and dicyclohexylcarbodi-imide had similar effects on respiration and ATP synthesis when succinate, reduced *NNN'*-tetramethylphenylenediamine and methanol were used as respiratory substrates. Rotenone inhibited both respiration and ATP synthesis (to a similar extent) with NADH, but 10 µM-rotenone had no effect on either respiration or ATP synthesis with other respiratory substrates. Antimycin A inhibited ATP synthesis to a greater extent than respiration with either NADH or succinate as substrate, but antimycin A had no effect with methanol or reduced *NNN'*-tetramethylphenylenediamine. KCN (0.5 mM) completely abolished ATP synthesis and respiration with methanol, succinate and reduced tetramethylenediamine as respiratory substrates.

The results in Table 2 were from a number of experiments with different batches of vesicles; Table 3 summarizes results of experiments in which substrates and inhibitors were added consecutively to a single reaction mixture. These results support the conclusion that a total of three possible separate sites of inhibition of ATP synthesis are present in these membrane vesicles.

#### *Anaerobic ATP generation*

Table 4 summarizes experiments in which the electron acceptor during oxidation of NADH was 2,6-dichlorophenol-indophenol instead of O<sub>2</sub>. The rate of 2,6-dichlorophenol-indophenol reduction was about half the rate of O<sub>2</sub> consumption when measured in the same batch of vesicles, whereas the rate of ATP synthesis was not markedly different. Thus relatively more ATP synthesis is uncoupled from O<sub>2</sub> reduction



Scheme 1. *Alternative schemes for electron transport and proton translocation in Pseudomonas AM1*

The arrows indicate flow of electrons or protons; they do not necessarily imply a direct reaction between components. The hatched bars indicate the site of action of inhibitors. The broken line indicates possible sites of reaction with 2,6-dichlorophenol-indophenol. Routes (unknown) for uncoupled respiration are not indicated. Abbreviations: Q, ubiquinone/ubiquinol; Cyt, cytochrome; TMPD, reduced *NNN'*-tetramethylphenylenediamine; PIP, 2,6-dichlorophenol-indophenol.

than from reduction of 2,6-dichlorophenol-indophenol. Rotenone and antimycin A inhibited both dye reduction and ATP synthesis, whereas the uncoupler carbonyl cyanide *m*-chlorophenylhydrazine inhibited only ATP synthesis. Although the site of action of 2,6-dichlorophenol-indophenol is not certain, it is likely to be a component of the electron-transport chain with a fairly high redox potential, i.e. probably cytochrome *b* or *c* (see Scheme 1). Phosphorylation coupled to succinate oxidation by 2,6-dichlorophenol-indophenol could not be measured because of the very high rate of reduction of 2,6-dichlorophenol-indophenol by succinate dehydrogenase.

#### Discussion

It has been shown in the present work that a high proportion of closed membrane vesicles was essential

for a high rate of ATP synthesis and that this synthesis was inhibited (regardless of respiratory substrate) by the uncoupling agent carbonyl cyanide *m*-chlorophenylhydrazine and by the ATPase inhibitor dicyclohexylcarbodi-imide. ATP synthesis was also always inhibited by inhibitors of respiration; cyanide inhibited respiration with all substrates (NADH, succinate, methanol and reduced *NNN'*-tetramethylphenylenediamine); antimycin A inhibited with NADH and succinate, whereas rotenone was specific for NADH oxidation. In all these respects oxidative phosphorylation was similar to that observed in membrane vesicles prepared from mitochondria and from other bacteria. Vesicles prepared from *Pseudomonas AM1* grown on succinate were similar to those prepared from methanol-grown bacteria, except for the range of substrates supporting respiration.

It is probable that membrane preparations contained some vesicles with the same topological arrangement as whole bacteria (right-side-out vesicles) and some arranged inside out; some non-vesicular membrane fragments were also probably present. If the electron-transport components and ATPase were arranged in the conventional way, then only ATP synthesized by inside-out vesicles would be measured in the experiments described above. O<sub>2</sub> consumption might have occurred, however, with all vesicles thus leading to lower measured P/O ratios. If the inside-out vesicles (phosphorylating) were arranged conventionally then the binding sites for oxidation of NADH and succinate would be on the outside, and 'uncoupled' O<sub>2</sub> consumption with these substrates would be by way of any right-side-out vesicles that might be substrate-permeable, or by membrane fragments. With methanol and ascorbate plus *NNN'N'*-tetramethylphenylenediamine, phosphorylation would depend on the permeability of inside-out vesicles to reduced *NNN'N'*-tetramethylphenylenediamine and to methanol, whereas 'uncoupled' O<sub>2</sub> consumption would be by way of right-side-out vesicles and membrane fragments.

In the present discussion it is assumed that one proton-translocating segment provides the capacity for synthesizing one molecule of ATP per two electrons passing through it. The most straightforward interpretation of the measured P/O ratios is that cytochromes *c* and *a/a<sub>3</sub>* are part of one proton-translocating segment (leading to a measured P/O ratio of usually about 0.4 with methanol or reduced *NNN'N'*-tetramethylphenylenediamine as a reductant). The measured P/O ratio with NADH of about 1.2 and P/2e ratio (with 2,6-dichlorophenol-indophenol as acceptor) of 1.65 indicates that at least two proton-translocating segments are involved in NADH oxidation; one of these is sensitive to rotenone, whereas the other is sensitive to antimycin A. The P/O ratio of usually about 0.8 with succinate as substrate indicates that at least one proton-translocating segment (antimycin A-sensitive) is involved in succinate oxidation.

These P/O ratios are similar to those measured in *Pseudomonas* sp2 (Netrusov *et al.*, 1977) with methanol or ascorbate plus *NNN'N'*-tetramethylphenylenediamine as substrate, but they are lower than those measured with NADH or succinate as substrate. It should be noted, however, that the rates of ATP synthesis were higher in *Pseudomonas* AM1 than in *Pseudomonas* sp2.

The three proton-translocating segments suggested above may operate in the conventional sequence as in typical heterotrophs containing cytochrome *c*; this arrangement (Scheme 1a) should give a maximum expected P/O ratio of 3 for NADH, 2 for succinate and 1 for reduced *NNN'N'*-tetramethylphenylene-

diamine. If the components are arranged in this way then there must be considerable 'uncoupled' O<sub>2</sub> or dye reduction to account for the P/O ratios measured. The ratios measured in the present work are equally (or more) consistent with the unconventional arrangement of electron-transport components summarized in Scheme 1(b). In this scheme, originally based on measurements of proton translocation in whole cells of *Pseudomonas* AM1 (O'Keeffe & Anthony, 1978), it is proposed that there are two separate electron-transport chains with cytochrome *a/a<sub>3</sub>* as a common component, one chain also involving cytochrome *c* and the other cytochrome *b*. The NADH-ubiquinone oxidoreductase (rotenone-sensitive in vesicles) forms one proton-translocating segment in this Scheme, whereas the second, also involved in succinate oxidation, consists of ubiquinol and cytochromes *b* and *a/a<sub>3</sub>*. It was proposed that cytochrome *c* is not essential for mediating between cytochromes *b* and *a/a<sub>3</sub>*, but that it can form part of a separate (third) proton-translocating segment involved in oxidation of methanol or reduced *NNN'N'*-tetramethylphenylenediamine.

To confirm this conclusion further, it will be necessary to purify vesicles of a single orientation (inside out) and to measure ATP synthesis in vesicles prepared from mutant bacteria lacking cytochrome *c*. Regardless of the arrangement of cytochrome *c* with respect to cytochrome *b*, the results in the present paper confirm that methanol dehydrogenase, cytochrome *c* and cytochrome *a/a<sub>3</sub>* are arranged such that the first step in methanol oxidation is coupled to synthesis of ATP.

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