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The Reversibility of Active Sulphate Transport in Membrane Vesicles of *Paracoccus denitrificans*

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An uncoupler-sensitive active transport of sulphate into membrane vesicles prepared from the plasma membrane of *Paracoccus denitrificans* (previously *Micrococcus denitrificans*) can be driven by respiration or by a trans-membrane pH gradient (alkaline inside) generated by the addition either of KCl (in the presence of nigericin) or of NH_4Cl. Valinomycin does not substitute for nigericin. Respiration-driven transport is observed in right-side-out vesicles but not in inside-out vesicles, whereas transport driven by the addition of KCl (in the presence of nigericin) or of NH_4Cl is observed in both types of membrane vesicle. The active transport of sulphate into these vesicles is shown to be carrier-mediated by its sensitivity to thiol-group reagents. It is proposed that the sulphate carrier in the plasma membrane of *P. denitrificans* operates by a mechanism of electroneutral proton symport, and is capable of actively transporting sulphate in either direction across the plasma membrane, but that in whole cells respiration-driven proton expulsion drives the accumulative uptake of sulphate.

The uptake of sulphate into *Paracoccus denitrificans*, previously *Micrococcus denitrificans* (Davis *et al.*, 1969), has been examined as part of a study of sulphur metabolism in this organism. In the present paper we describe the mode of operation of the sulphate carrier present in membrane vesicles prepared from the plasma membrane of *P. denitrificans*. Our studies of sulphate uptake into whole cells will be reported elsewhere.

Previously, sulphate uptake has been studied with whole cells of *Salmonella typhimurium* and *Escherichia coli* (Pardee *et al.*, 1966; Springer & Huber, 1972). Sulphate uptake into bacterial membrane vesicles has not been reported (see Kaback, 1974).

However, membrane vesicles prepared from a variety of bacteria (Kaback, 1974), including *P. denitrificans* (White *et al.*, 1974), have proved to be useful systems for analysing the mechanism of bacterial active transport (Harold, 1974). Such vesicles lack the cytoplasmic contents of intact cells (Kaback, 1974), and thus the transport of a particular solute can be studied in the absence of cell metabolism, and without the complications arising from the possible movement of other solutes normally present in the intact bacterial cell. The accumulative uptake of amino acids and β-galactosides into membrane vesicles of *Escherichia coli* has been shown to be driven by the energy released by respiration (see Kaback, 1974). Evidence that this energy is in the form of a pH gradient and membrane potential across the membrane, as postulated by the chemiosmotic theory (Mitchell, 1970), has come from the observation that an accumulative uptake of amino acids and β-galactosides can be driven in the absence of respiration by an electrogenic efflux of K^+ from the vesicles (Hirata *et al.*, 1973; Altendorf *et al.*, 1974).

In the present work two types of vesicle are used: right-side-out vesicles, in which the vesicle membrane has the same orientation as the plasma membrane of the intact cell; and inside-out vesicles, in which inversion has occurred during their preparation so that the orientation of the vesicle membrane is the reverse of that of the plasma membrane of the intact cell. In previous studies of active transport in bacterial vesicles, respiration-driven uptake has been observed with right-side-out vesicles but not with inside-out vesicles (Harold, 1972; Kaback, 1974; Hare *et al.*, 1974; Mével-Ninio & Yamamoto, 1974). However, Ca^{2+} appears to be an exception, since respiration drives the uptake of Ca^{2+} into inside-out vesicles, but not into right-side-out vesicles (Rosen & McClees, 1974). Active transport driven by artificially induced ionic gradients has been observed with preparations consisting predominantly of right-side-out membrane vesicles (Hirata *et al.*, 1973; Altendorf *et al.*, 1974) and with intact cells (Kashket & Wilson, 1973; Asgar *et al.*, 1973; Niven & Hamilton, 1974), but not with inside-out vesicles.

In the present work we show that when a pH gradient (alkaline inside) is applied across the vesicle membrane both right-side-out and inside-out membrane vesicles are equally capable of transporting

sulphate, but that respiration is effective in driving uptake of sulphate only with the right-side-out membrane vesicles.

The specificity of respiration-driven sulphate transport for right-side-out membrane vesicles of *P. denitrificans* extends the findings of other authors using different bacteria and different solutes; the demonstration of sulphate uptake driven by an artificially induced trans-membrane pH gradient in both right-side-out and inside-out membrane vesicles provides the first indication of the reversibility of active transport across a bacterial membrane. From a comparison of the relative effectiveness of electrogenic and electroneutral ionophores (see Harold, 1972; Hamilton, 1975) required in the induction of sulphate transport, it can be inferred that the sulphate carrier in the plasma membrane of *P. denitrificans* operates by a process of electroneutral proton symport (see Mitchell, 1970).

Materials and Methods

Reagents

$Na_2{}^{35}SO_4$ and $[^{32}P]P_i$ were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. $[^{32}P]P_i$ was converted into $[^{32}P]PP_i$ by pyrolysis (Lee Peng, 1956) and adjusted to 0.25 Ci/mol. Bee venom (grade I), NADH (grade III), deoxyribonuclease (EC 3.1.4.5), lysozyme (EC 3.2.1.17) and Triton X-100 were obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.; naphthalene (scintillation grade) was from Koch–Light Ltd., Colnbrook, Bucks., U.K.; butyl-PBD [5-(4-biphenyl-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] was from CIBA–Geigy Ltd., Cambridge, U.K.; carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was from Boehringer Corp. (London) Ltd., London W.5, U.K.; valinomycin was from Calbiochem Ltd., London W.1, U.K. Nigericin was a gift from Professor Lester Packer, University of California, Berkely, Calif., U.S.A. All other reagents were of analytical-reagent grade and were obtained either from Sigma (London) Chemical Co. Ltd. or from BDH Chemicals Ltd., Poole, Dorset, U.K.

Culture of bacteria

Paracoccus denitrificans (N.C.I.B. 8944) was maintained and grown with succinate as the substrate and nitrate as the added terminal electron acceptor under the growth conditions of John & Whatley (1970) but in a medium which contained, per litre: 0.68 g of $(KH_4)_2PO_4$ (adjusted to pH 6.8 with KOH); 0.60 g of $(NH_4)_2HPO_4$ (adjusted to pH 6.8 with HCl); 0.1 ml of 'modified Hoagland's trace element solution' (Collins, 1969); 2 mg of EDTA (ferric monosodium salt); 54 mg of Na_2SO_4; 25 mg of $CaCl_2,2H_2O$; 25 mg of $MgCl_2,6H_2O$; 13.5 g of sodium succinate hexahydrate; 10.1 g of KNO_3. Solutions of Na_2SO_4 and $CaCl_2$ were autoclaved separately and added when cool. Cells were also grown with H_2 as the reductant, CO_2 as the carbon source and O_2 as the terminal electron acceptor in a medium of similar composition to that containing succinate, except that succinate and nitrate were omitted and 0.5% (w/v) $NaHCO_3$ was added. The 1-litre culture solutions, contained in 1.5-litre cylindrical flasks (7.5 cm diam.), were flushed with a gas mixture composed of $H_2+O_2+CO_2$ (60:30:10). The gas mixture was supplied at a rate of 660 ml·min^{-1} through a sintered-glass 'sparger' which ensured a thorough distribution of gas through the medium. The cultures were maintained at 30°C.

Cells grown with H_2 as the reductant were obtained from cultures inoculated with a 1% inoculum of cells previously adapted to growth with H_2. The mean generation time of *P. denitrificans* grown with succinate and nitrate was 2 h, and that with H_2 and O_2 was about 8 h.

Preparation of membrane vesicles

The procedure used in the preparation of the membrane vesicles was based on a method described by John & Whatley (1970). All the solutions and apparatus used were maintained at 1–4°C and all operations, except the lysozyme treatment, were carried out at these temperatures. Stock buffer solutions at a concentration of 0.4 M were adjusted to pH 7.3 at room temperature, and then diluted to 10 or 100 mM as required.

Cells from 2 litres of mid-exponential phase culture were sedimented by centrifugation at 5000*g* for 30 min and were washed in 800 ml of 50 mM-NaCl containing 10 mM-Tris–HCl buffer. The cells were then suspended in 400 ml of 0.5 M-sucrose containing 10 mM-Tris–HCl buffer, so that a 0.1 ml sample of the suspension diluted to 2.5 ml with water had an absorbance at 550 nm of 0.3 when read in a Beckman DB spectrophotometer. This was equivalent to about 25 mg wet weight of cells/ml of 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 left in a water bath at 30°C for 20–30 min until the absorbance of a sample diluted 1:25 with water decreased from 0.3 to 0.05.

After treatment with lysozyme the cells were sedimented by centrifugation at 40000*g* for 10 min and resuspended in 40 ml of 100 mM-Tris–acetate buffer by using a Potter–Elvehjam homogenizer. The suspension was then diluted with 360 ml of water to disrupt the cells. The suspension was left for 20 min, then a trace of deoxyribonuclease (EC 3.1.4.5) and 2 mM-magnesium acetate were added. The suspension

was shaken gently to distribute the deoxyribonuclease and magnesium acetate. When the viscosity of the suspension was sufficiently lowered, the suspension was centrifuged at 40000*g* for 40min to yield a double-layered pellet and a clear supernatant, which was discarded. The upper, red, layer of the pellet was resuspended in 400ml of 1mM-magnesium acetate containing 10mM-Tris–acetate buffer. The lower, white, layer of the pellet, which probably consists of poly-β-hydroxybutyrate (Scholes & Smith, 1968*a*), was discarded. The suspension was centrifuged at 40000*g* for 40min and the resulting pellet was resuspended in 1mM-magnesium acetate containing 10mM-Tris–acetate buffer to a concentration of about 5mg of membrane protein/ml.

The suspension of membrane vesicles was stored at 1–4°C and could be used for experiments over a period of 3 days.

Measurement of sulphate uptake by membrane vesicles

Assays were conducted at 30°C in a small flat-bottomed cylindrical flask (1.5cm×3.5cm) which was stirred magnetically. Standard incubation mixtures contained, in a total volume of 2ml, 1μmol of $Na_2{}^{35}SO_4$ (1μCi/μmol), 0.2ml of membrane vesicles (1.0–1.5mg of protein) and 100μmol of Tris–HCl buffer, pH7.3. Membrane vesicles were added to start the experiment. Samples (0.1ml) were withdrawn at intervals and membrane vesicles collected by filtration on a Millipore filter (0.22μm diam. pores). Membrane vesicles collected on the filters were washed twice with 5ml portions of 50mM-Tris–HCl (pH7.3) at 30°C. Where uptake was driven by respiration the appropriate respiratory substrate was included in the wash solution at the same concentration as that used in the reaction medium, and a water-saturated atmosphere of O_2 was maintained in the reaction flask.

Filters with washed vesicles were individually placed in scintillation vials, dried (to minimize quenching by water), and 5ml of scintillation fluid was added. The scintillation fluid contained 7g of butyl-PBD and 80g of naphthalene made up to 1 litre with a 3:2 (v/v) mixture of toluene and 2-methoxyethanol. The vials were left for 12h before counting for radioactivity in a liquid-scintillation counter. The complete compositions of both the reaction mixtures and wash solutions are given in the legends of the appropriate Figures.

Identification of sulphate by electrophoresis

Vesicles were incubated with 0.1mM-phenazine methosulphate and 20mM-sodium ascorbate for 20min in the presence of $Na_2{}^{35}SO_4$ in a standard incubation mixture for measuring sulphate uptake with right-side-out membrane vesicles. A portion (0.5ml) was filtered and the vesicles were washed with 20ml of hot water. The washings were evaporated to dryness by rotary evaporation and the residue was dissolved in 0.5ml of water. Samples (50μl) were subjected to paper electrophoresis on Whatman 3MM chromatography paper in 0.1M-sodium citrate buffer (pH5.0) at 5.5V/cm for 3h. Paper-electrophoresis strips were cut into sections (1.0cm) and the radioactivity was counted in a scintillation counter as described above after the addition of 1ml of scintillation fluid.

Determination of NADH dehydrogenase activity

The oxidation of NADH was linked to the reduction of 2,6-dichlorophenol-indophenol and the initial rate of decrease in E_{600} monitored spectrophotometrically at 30°C in a Pye Unicam SP.8000. The reaction mixture contained in 3ml: 50mM-Tris–HCl (pH8.0), 33μM-2,6-dichlorophenol-indophenol, 2mM-KCN (pH7.5) and membrane vesicles (0.1mg of protein). The reaction was started by the addition of 1mM-NADH. In the absence of membrane vesicles a slow rate of NADH-dependent reduction of 2,6-dichlorophenol-indophenol was observed. This non-enzymic rate (12% of the maximum rate observed in the presence of membrane vesicles) was subtracted from the rates observed in the presence of membrane vesicles to give a measure of the NADH dehydrogenase activity of the membrane vesicles. The molar extinction coefficient for 2,6-dichlorophenol-indophenol was taken to be 21000 litre·mol^{-1}·cm^{-1} (King, 1963).

Protein

Protein was determined by the Folin method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Results

Orientation of the membrane vesicles

As in other bacteria (Hare *et al.*, 1974; Hampton & Freese, 1974), the plasma membrane of *P. denitrificans* (Scholes & Smith, 1968*b*) is effectively impermeable to NADH, and the NADH dehydrogenase of the respiratory chain is located on the inside of the plasma membrane so that it is accessible to NADH only from the inside of the intact cell. Thus those membrane vesicles that have retained the orientation of the plasma membrane of the intact cell (right-side-out membrane vesicles) would be expected to oxidize NADH added to the reaction mixture only slowly unless the permeability of the plasma membrane to NADH was increased. On the other hand, membrane vesicles that have the reverse orientation of the

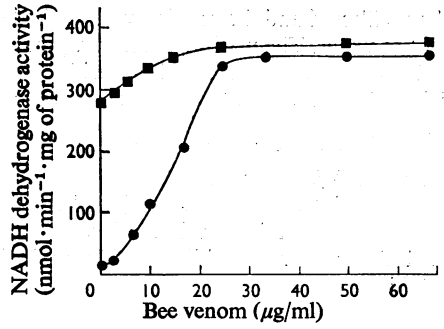

Fig. 1. *Effect of bee venom on the NADH dehydrogenase activities of membrane vesicles prepared from the plasma membrane of P. denitrificans*

Membrane vesicles were prepared from cells grown either with H_2 as the reductant (●) or with succinate as the substrate (■). NADH dehydrogenase activities were measured as described in the Materials and Methods section.

plasma membrane to that of the intact cell (inside-out membrane vesicles) would be expected to oxidize NADH added to the reaction mixture rapidly, and this rate of oxidation would not be much affected by treatments that increase the permeability of the plasma membrane to NADH.

In the present study, bee venom (Habermann, 1972; Verma *et al.*, 1974) was used to increase the permeability of the plasma membrane to NADH. Fig. 1 shows that in the presence of increasing concentrations of bee venom there is an increase in the observed NADH dehydrogenase activity of membrane vesicles prepared from cells grown with H_2 as the reductant. On the other hand, similar concentrations of bee venom have relatively little effect on the observed NADH dehydrogenase activity of the membrane vesicles prepared from cells grown with succinate as the substrate. In the presence of saturating amounts of bee venom the observed NADH

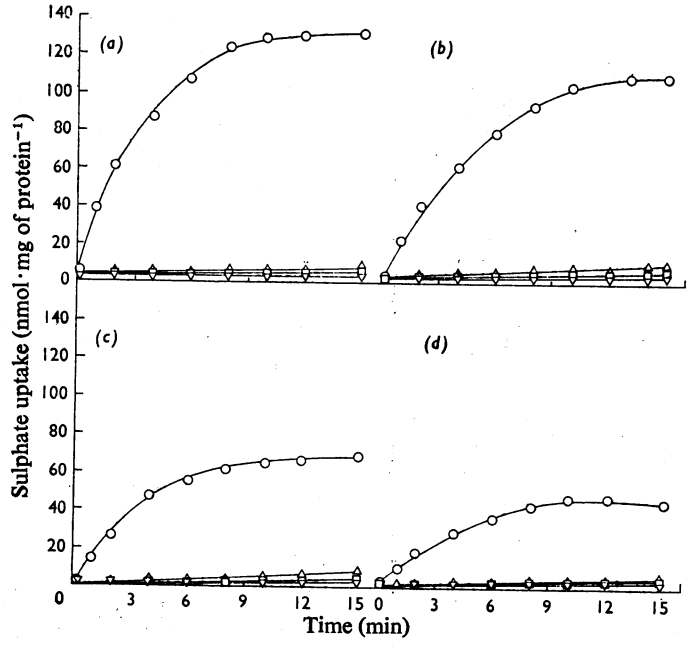

Fig. 2. *Effect of membrane orientation on respiration-driven uptake of sulphate into membrane vesicles of P. denitrificans*

Sulphate uptake into right-side-out (○, □) and inside-out (△, ▽) membrane vesicles was measured as described in the Materials and Methods section in a medium that contained, in 2ml: 1 μmol of $Na_2{}^{35}SO_4$ (1 μCi/μmol); 0.2ml of the membrane vesicle suspension (1.5mg of protein); 100μmol of Tris–HCl buffer (pH7.3). Vesicles collected on the filter were washed with 2×5ml portions of a solution that contained 50mM-Tris–HCl buffer, pH7.3, and the respective respiratory substrate at the concentration used in the reaction mixture. The respiratory substrates were: (*a*) 20mM-sodium ascorbate+0.1mM-phenazine methosulphate; (*b*) 20mM-sodium ascorbate+0.1mM-*NNN'N'*-tetramethyl-*p*-phenylenediamine; (*c*) 10mM-sodium D-lactate; (*d*) 10mM-sodium succinate. The 5μM-carbonyl cyanide trifluoromethoxyphenylhydrazone was added (□, ▽) or omitted (○, △) as indicated.

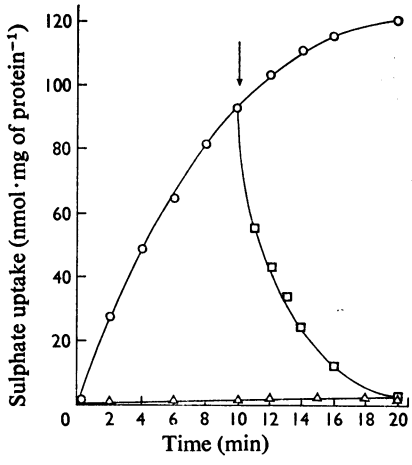

Fig. 3. *Sulphate uptake by right-side-out membrane vesicles of P. denitrificans*

Sulphate uptake was measured as described in Fig. 2(*a*) in the presence (○, □) and absence (△) of 20mM-sodium ascorbate plus 0.1mM-phenazine methosulphate. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (5μM) was added at the time indicated by the arrow to one of the reaction mixtures which contained the respiratory substrates (□).

dehydrogenase activities of the two types of membrane vesicles are essentially the same.

Thus the membrane vesicles isolated from cells grown with succinate are mainly inside-out vesicles, and those isolated from cells grown with H_2 are essentially all right-side-out vesicles.

The orientation of the vesicle membrane is discussed at length below, but for convenience in the remainder of the Results section of this paper the two types of membrane vesicle will be referred to by the inferred orientation of the membrane rather than by reference to the growth substrates of the cells from which they were prepared.

Sulphate uptake driven by respiration

Sulphate uptake into right-side-out vesicles was observed in the presence of a variety of respiratory substrates (Fig. 2). However, inside-out vesicles did not accumulate sulphate under these conditions (Fig. 2). The uptake of sulphate into the right-side-out vesicles depended on the presence of a respiratory substrate (Fig. 3), and was abolished by the inclusion of the uncoupling agent carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Fig. 2). When this uncoupler was added to right-side-out vesicles that had already accumulated sulphate, there was a rapid efflux of the accumulated sulphate from the vesicles (Fig. 3). Sulphate efflux was also observed on depletion of the respiratory substrate.

Sulphate uptake driven by additions of salts

In the absence of respiratory substrates, the addition of a pulse of 150mM-KCl to a suspension of membrane vesicles containing nigericin resulted in transient accumulation of sulphate. This accumulation occurred to a similar extent when inside-out or right-side-out membrane vesicles were used (Figs. 4*a*,*b*). No uptake was observed when nigericin was omitted and valinomycin could not substitute for nigericin (Figs. 4*c*,*d*). Further, in the presence of nigericin the inclusion of valinomycin resulted in no uptake (Figs. 4*c*,*d*). Similarly, addition of a pulse of 150mM-NH_4Cl caused uptake to a similar extent in both right-side-out and inside-out membrane vesicles even in the absence of any ionophore (Figs. 4*e*,*f*). The extent of NH_4Cl-induced sulphate uptake was similar to that observed on the addition of KCl to a suspension of membrane vesicles containing nigericin (Fig. 4).

Inclusion of the thiol-group reagents *N*-ethylmaleimide, *p*-chloromercuribenzoate and methylmercuric chloride prevented the KCl-induced sulphate uptake. When these compounds were added to a suspension of membrane vesicles that had accumulated sulphate, the rate of efflux of the accumulated sulphate was decreased (Fig. 5).

The inclusion of selenate in the reaction mixture decreased the rate and extent of sulphate uptake and also decreased the rate of efflux of sulphate (Fig. 6).

Validity of the assay of sulphate uptake

The sulphate accumulated by right-side-out membrane vesicles during a 20min incubation period (as described for Fig. 2) could be quantitatively recovered (98%) by washing the cells in hot water, and identified as sulphate by electrophoretic examination. Similarly the sulphate accumulated by both inside-out and right-side-out membrane vesicles in response to a pulse of KCl (in the presence of nigericin) was quantitatively extracted (96%) and electrophoretically identified as sulphate. Further, the initial enzyme involved in the assimilation of sulphate, ATP sulphurylase (ATP–sulphate adenylyltransferase, EC 2.7.7.4) was not detected either in inside-out or in right-side-out vesicles (Table 1). These observations show that the sulphate accumulated by membrane vesicles could not be further metabolized but remained as sulphate.

Discussion

In the Results section of the present paper it has been stated that membrane vesicles obtained from cells grown with succinate consist predominantly of inside-out vesicles, and that membrane vesicles obtained from cells grown with H_2 consist predominantly of right-side-out vesicles. The importance of

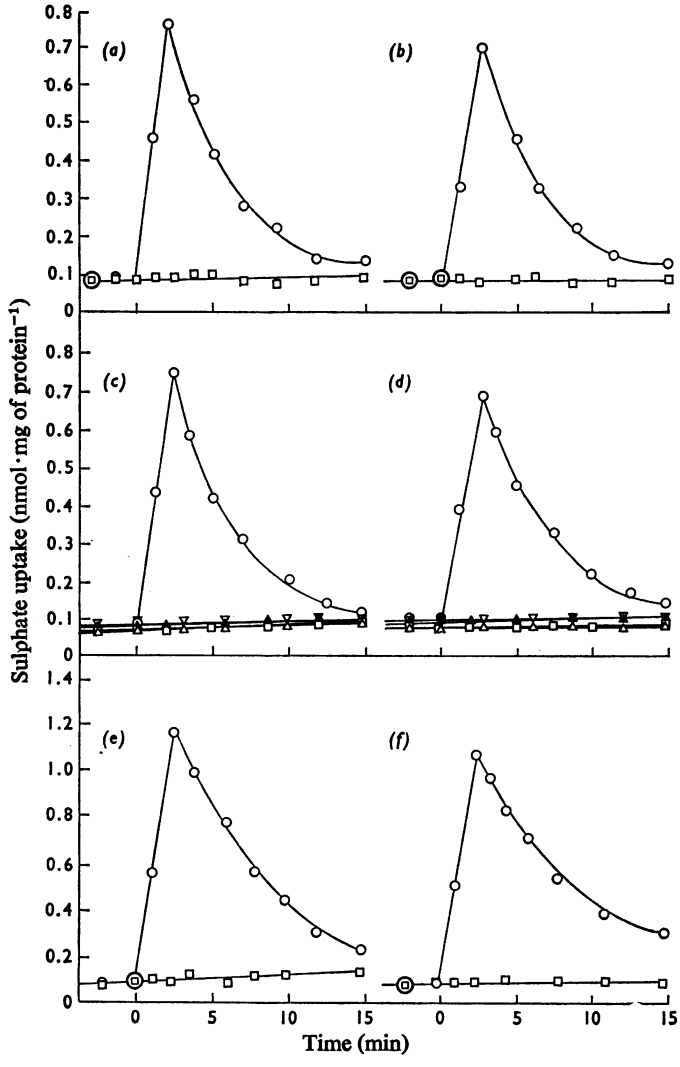

Fig. 4. *Transient uptake of sulphate into membrane vesicles of P. denitrificans driven by the addition of salts*

Sulphate uptake into right-side-out (*a*, *c*, *e*) and inside-out (*b*, *d*, *f*) membrane vesicles was measured as described in the Materials and Methods section in a reaction mixture (2ml) that contained 1μmol of $Na_2{}^{35}SO_4$ (1μCi/μmol), 0.2ml of membrane vesicles suspension (1.5mg of protein) and 100μmol of Tris–HCl (pH7.3) (○). At 0min either 0.15M-KCl (*a*, *b*, *c*, *d*) or 0.15M-NH_4Cl (*e*, *f*) was added. In (*a*), (*b*), (*c*) and (*d*), 1μg of nigericin was included. Additions and subtractions to the reaction mixture were as follows: □, plus 5μg of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; △, plus 1μg of valinomycin (added in the presence of 1μg of nigericin); ▲, plus 1μg of valinomycin (minus 1μg of nigericin); ▽, minus 1μg of nigericin.

this conclusion to the interpretation of the rest of the data presented in this paper justifies further comment.

There is sufficient evidence from previous work with a variety of bacteria for the following two generalizations to be made. (1) NADH, supplied at a concentration of approximately 1mM in the suspending medium, is oxidized at an appreciable rate by inside-out membrane vesicles (or by non-vesicular membranes), but not by right-side-out membrane vesicles (Eisenberg *et al.*, 1970; Harold, 1972; Hare *et al.*, 1974; Hampton & Freese, 1974; Futai, 1974*a*,*b*). (2) Respiration drives the active uptake of substances normally accumulated within the bacterial cell into right-side-out vesicles, but not into inside-out vesicles (Hirata

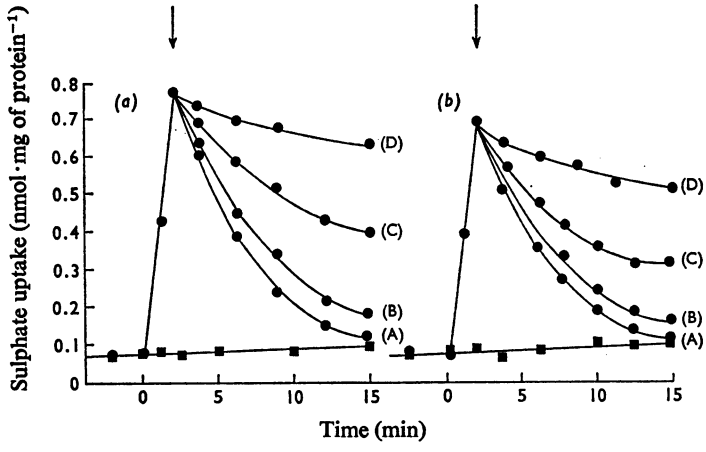

Fig. 5. *Effect of thiol-group reagents on the efflux of sulphate accumulated by membrane vesicles*

Transient uptake into right-side-out (*a*) and inside-out (*b*) membrane vesicles was driven by the addition of 0.15 M-KCl in the presence of 1 μg of nigericin (curved lines) as in Figs. 4(*a*) and 4(*b*) in the presence (■) or the absence (●) of 5 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. Further additions were made at time 2 min as indicated: curves (A) none; (B) 8 μM-*p*-chloromercuribenzoate; (C) 10 mM-*N*-ethylmaleimide; (D) 10 mM-methylmercuric chloride.

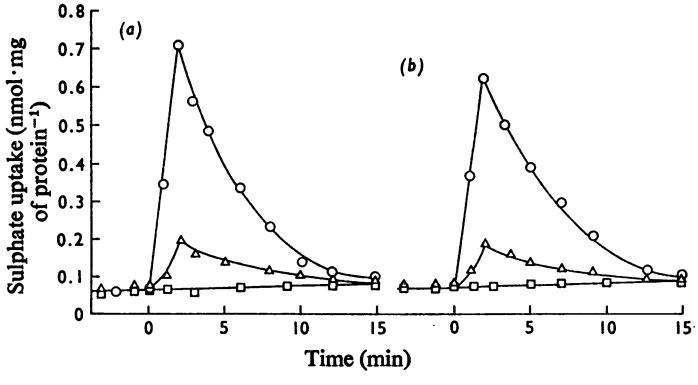

Fig. 6. *Effect of selenate on KCl-induced transient sulphate uptake into membrane vesicles*

Uptake was driven by the addition of 0.15 M-KCl in the presence of 1 μg of nigericin as in Figs. 4(*a*) and 4(*b*) (○) except that 1 mM-Na_2SeO_4 (△) or 5 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (□) was added where indicated to right-side-out (*a*) or inside-out (*b*) membrane vesicles.

& Brodie, 1972; Harold, 1972; Hampton & Freese, 1974; Hare *et al.*, 1974; Mével-Ninio & Yamamoto, 1974). With the membrane vesicles isolated from *P. denitrificans* described in the present paper the following observations were made. First, the rate of NADH oxidation observed in the absence of bee venom with the vesicles isolated from cells grown with H_2 is only 5% of the maximum rate observed in the presence of bee venom, whereas the vesicles isolated from cells grown with succinate give 80% of the maximum rate without treatment with bee venom. Secondly, the rate and extent of respiration-driven sulphate uptake by the vesicles isolated from cells grown with succinate are only 10% of the rate and extent of the respiration-driven sulphate uptake by the vesicles isolated from cells grown with H_2. Thus it is concluded that the vesicles isolated from cells grown with succinate are predominantly inside-out, whereas the vesicles isolated from cells grown with H_2 are predominantly right-side-out. From the rates and extent

Table 1. *Content of ATP sulphurylase in whole cells and membrane vesicles of P. denitrificans*

Cells were grown with either H_2 as the reductant or with succinate as substrate, and membrane vesicles were prepared from these cells as described in the Materials and Methods section. The ATP sulphurylase of cells (22 mg of protein/ml) and membrane vesicles (7 mg/ml) was monitored by sulphate-dependent ATP–PP_i exchange as described by Shaw & Anderson (1971) in the presence of Triton X-100 (0.3%, v/v).

	ATP sulphurylase activity (nmol of ATP–PP_i exchange/min per ng of protein)	
Substrate for cell growth ...	H_2	Succinate
Whole cells	4.23	4.47
Membrane vesicles	0.01	0.01

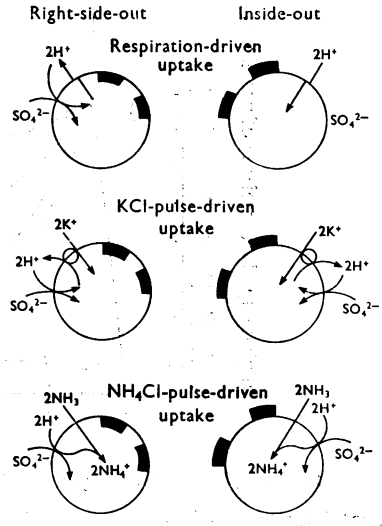

Fig. 7. *Sulphate uptake into inside-out and right-side-out membrane vesicles of P. denitrificans*

The orientation of the NADH dehydrogenase on the inner face of the right-side-out membrane vesicles and on the outer face of inside-out membrane vesicles is indicated. During respiration (upper row) in right-side-out membrane vesicles protons are pumped outwards and their return is coupled to sulphate uptake. In inside-out membrane vesicles protons are pumped inwards and sulphate accumulation cannot occur, but a coupled sulphate expulsion would be possible. Influx of K^+ via nigericin (middle row) results in an efflux of an equivalent number of protons and their return is coupled to sulphate uptake. This is true for both inside-out and right-side-out membrane vesicles. Influx of NH_3 (bottom row) causes a proton deficit inside the membrane vesicles and the consequential influx of protons is coupled to sulphate uptake. This is true for both inside-out and right-side-out membrane vesicles.

of sulphate uptake, and from the degree to which bee venom stimulates NADH oxidation, it can be inferred that right-side-out vesicles contribute no more than 10–20% of the total vesicle population in the vesicles isolated from cells grown with succinate. It is unlikely that non-vesicular membranes constitute a significant proportion of the membrane material of the preparation obtained from cells grown with succinate since these preparations show a high degree of respiratory control (John & Whatley, 1975). Bee venom contains mellitin, a haemolytic agent with mild detergent properties (Habermann, 1972; Verma *et al.*, 1974; Mollay & Kreil, 1974), and presumably this component is responsible for destroying the integrity of the plasma membrane of *P. denitrificans*, thus enabling NADH to gain access to its dehydrogenase, even when the NADH dehydrogenase is orientated towards the inner face of right-side-out vesicles. Other authors, using a variety of techniques, have detected the presence of both inside-out and right-side-out vesicles in membrane preparations obtained by osmotic lysis of *Escherichia coli* (Hare *et al.*, 1974), *Bacillus subtilis* (Hampton & Freese, 1974) and *Micrococcus lysodeikticus* (Gorneva & Ryabova, 1974).

The reason for the different orientation of the vesicles in the preparations obtained from cells grown under the two conditions is unknown. An autotrophic mode of growth or a slower growth rate do not appear to be directly related to the production of right-side-out vesicles, since other authors (using different conditions of lysozyme treatment and of osmotic lysis) have isolated right-side-out vesicles from cells of *P. denitrificans* grown heterotrophically with high growth rates in rich media (Scholes & Smith, 1968*a*,*b*; White *et al.*, 1974). Membrane vesicles isolated by the procedure described here from cells grown in rich media are predominantly of the right-side-out variety (P. John, unpublished work), and resemble the membrane vesicles isolated from cells grown with H_2 in the latency of their NADH dehydrogenase, but such vesicles could not be used in the present study because of the absence of a sulphate carrier in cells grown in the presence of S-containing amino acids (J. N. Burnell, unpublished work).

It is also important for the further interpretation of the present data to emphasize that, although the two types of membrane preparation used in the present study differ in the orientation of the membrane-vesicles, other differences between the two types of preparation are unlikely to be significant for the active transport of sulphate. Thus both types of preparation contain a fully functional constitutive respiratory chain (John & Whatley, 1970; Knobloch *et al.*, 1971), which closely resembles the mitochondrial respiratory chain (John & Whatley, 1975).

The transient accumulation of sulphate driven by the addition of KCl (in the presence of nigericin) or of NH_4Cl can be explained most readily by assuming that additions of the appropriate salts generate an electroneutral pH gradient (alkaline inside) across the vesicle membrane, and that this gradient drives sulphate uptake as implied by the chemiosmotic theory (Mitchell, 1970), discussed in detail for the uptake of other substrates by Harold (1974) and Hamilton (1975), and represented diagrammatically in Fig. 7. Since valinomycin did not substitute for nigericin in these experiments it may be concluded that sulphate is not taken up electrophoretically. The effectiveness of valinomycin as a conductor of K^+ across the vesicle membrane under the conditions of these experiments is demonstrated by its ability to prevent the sulphate uptake driven by the addition of KCl (in the presence of nigericin).

The extent of sulphate uptake observed in response to the addition of KCl (in the presence of nigericin) or of NH_4Cl is only 1% of that observed with respiration as the driving force. The sensitivity of the sulphate uptake driven by KCl (in the presence of nigericin) to carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, to thiol-group reagents and to selenate can be readily explained only if this sulphate uptake involves a carrier.

It is possible, however, that the relatively limited transient uptake of sulphate observed on the addition of salts is due solely to an energy-dependent binding of sulphate to the sulphate carrier, without the sulphate entering the internal aqueous phase of the membrane vesicle. An analogous energy-dependent uncoupler-sensitive binding of non-transportable dansyl β-galactosides to the β-galactoside carrier of membrane vesicles of *E. coli* has already been described (Reeves *et al.*, 1973; Schuldiner *et al.*, 1975). However, by comparing our results with the sulphate carrier of *P. denitrificans* with those obtained with the β-galactoside carrier of *E. coli* we may identify the sulphate uptake, driven by the addition of salts, as sulphate transport rather than sulphate binding as follows. Addition of thiol-group reagents causes a retention of transported lactose (Kaback & Barnes, 1971) but causes an apparent release of bound dansyl β-galactoside (Reeves *et al.*, 1973). The sulphate accumulated in response to an addition of KCl (in the presence of nigericin) is normally rapidly lost from the vesicles, but an appropriately timed addition of a thiol-group reagent results in the accumulated sulphate being retained (Fig. 5). We therefore interpret our results as showing sulphate transport rather than sulphate binding, in response to an imposed pH gradient.

The observation of an energized uptake of sulphate into both inside-out and right-side-out membrane vesicles demonstrates the reversibility of the sulphate carrier present in the plasma membrane of *P. denitrificans*. Presumably the direction in which this carrier operates in whole cells is determined by the polarity of the pH gradient imposed across the plasma membrane. In whole cells of *P. denitrificans* respiration is associated with an outward movement of protons (Scholes & Mitchell, 1970). The return of these protons to the cell interior via the sulphate carrier would drive sulphate accumulation by the whole cells.

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