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Estimation with an ion-selective electrode of the membrane potential in cells of *Paracoccus denitrificans* from the uptake of the butyltriphenylphosphonium cation during aerobic and anaerobic respiration

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1. Aerobic respiration by cells of *Paracoccus denitrificans* drives the uptake of the lipophilic cation butyltriphenylphosphonium. Anaerobiosis or addition of an uncoupler of oxidative phosphorylation (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) results in efflux of the cation. Changes in the concentration of butyltriphenylphosphonium in the suspension medium were measured by using an ion-selective electrode, the construction of which is described. 2. If the uptake of butyltriphenylphosphonium is used as an indicator of membrane potential, then at pH 7.3 an estimate of about 160mV is obtained for cells of *P. denitrificans* respiring aerobically in 100mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH or 100mM-NaH_2PO_4/NaOH. This potential, however, is decreased by more than 20mV in reaction media containing a high concentration of phosphate (100mM) together with at least 1mM-K^+. 3. Anaerobic electron transport with NO_3^-, NO_2^- or N_2O as terminal electron acceptor generates a membrane potential of about 150mV in described suspension media. The presence of these species under aerobic conditions, moreover, has negligible effect upon the extent of uptake of butyltriphenylphosphonium normally driven by aerobic respiration. These data indicate that none of these molecules exert a significant uncoupling effect on the protonmotive force. 4. No $^{204}Tl^+$ uptake into respiring cells was detected. This adds to the evidence that $^{204}Tl^+$ is not a freely permeable cation in bacterial cells and therefore not an indicator of membrane potential as has been proposed. The absence of respiration-driven $^{204}Tl^+$ uptake indicates that *P. denitrificans* cells grown under the conditions specified in the present work do not possess K^+-transport systems of either the Kdp or TrkA types that have been described in *Escherichia coli*.

Amongst the bacteria *Paracoccus denitrificans* has recently become a popular species for the study of electron-transport-linked ATP synthesis and the intimately related problems of the thermodynamic efficiency of bacterial growth (John & Whatley, 1977; Stouthamer, 1978). The attributes of *P. denitrificans* include its relatively well-defined respiratory chain with a choice of several terminal electron acceptors (including O_2, NO_3^-, NO_2^- and N_2O), the resemblance of the respiratory chain in the aerobically grown bacterium to its mammalian mitochondrial counterpart, and the availability of exceptionally well-coupled phosphorylating membrane vesicles. Experiments with whole cells have mainly had the objective of elucidating the stoichiometry of respiration-driven proton translocation together with its relationship to the stoichiometry of ATP production (P/O ratio) and molar growth yields (Stouthamer, 1978). On the other hand, recent work with phosphorylating vesicles has focused on the problem of reconciling the extent of ATP synthesis with the size of the protonmotive force responsible for driving the reaction (Kell *et al.* 1978*a*). This latter work produced two main

Abbreviations used: $BTPP^+$, butyltriphenylphosphonium cation; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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findings. First, the size of the protonmotive force estimated was rather smaller than is required by the original chemiosmotic hypothesis to account for the extent of ATP synthesis (expressed as the phosphorylation potential). Second, in the presence of NO_3^- (10mM), which acts as an electron acceptor for coupled electron transport, the size of the proton-motive force fell below the limit of detection available with the methods used [in fact, improved techniques have subsequently permitted the measurement of a small protonmotive force in the presence of 10mM-NO_3^- (McCarthy *et al.* 1979)]. As the protonmotive force in the absence of nitrate comprised mainly a membrane potential, this meant that the nitrate ion was effective at substantially decreasing the membrane potential component of the protonmotive force.

The information available about the bioenergetics of *P. denitrificans* clearly requires to be supplemented with estimates of the protonmotive force in whole cells. Availability of this data would permit the energetics of proposed stoichiometries to be analysed, and also would be valuable for comparison with results from experiments with membrane vesicles. There has been but one estimate of the protonmotive force in whole cells (Deutsch & Kula, 1978), but that study gave results that were atypical of bacteria as a whole and thus provided a further need for quantitative study of the membrane potential.

In the present work we introduce the use of the butytriphenylphosphonium cation ($BTPP^+$) as a probe of membrane potential in cells of *P. denitrificans*, and follow the distribution of this ion between the cells and extracellular medium through the use of an ion-selective electrode. In contrast to the earlier report (Deutsch & Kula, 1978), which failed to show clear evidence for an energy-linked accumulation of the related cation triphenylmethylphosphonium, we find that the extent of uptake of the lipophilic cation $BTPP^+$ is commensurate with the existence of a membrane potential within the range 130–160mV, depending upon suspension conditions.

The availability of a method for estimating the membrane potential in intact cells of this organism has permitted an investigation not only of the membrane potential supported by aerobic respiration, but also of the ability of NO_3^-, NO_2^- and N_2O to act as terminal electron acceptors. The results so obtained show that anaerobic electron flow to all three of these compounds generates a membrane potential only slightly smaller than the maximal potential developed in the presence of oxygen. These results suggest that neither NO_3^- nor NO_2^- has a significant uncoupling effect on cell respiration and that N_2O is a probable intermediate in the pathway of NO_3^- conversion to N_2.

Materials and methods

Cells of *P. denitrificans* (N.C.I.B. 8944) were grown to late exponential phase in 2 litres of the succinate/nitrate medium described by Burnell *et al.* (1975) [note that weight of Fe^{3+}–EDTA should be 12mg/litre and not as misprinted in that paper]. After harvesting by centrifugation for 15min at 4°C and 5000*g* in the 6 × 1 litre rotor of an MSE Coolspin centrifuge, the cells were washed (unless indicated otherwise) with 750ml of 100mM-Hepes/NaOH, pH7.3, and finally resuspended in approx. 25ml of the same medium supplemented with 1mg of bovine serum albumin/ml.

The dry weight of cells was determined by filtering cells on a glass fibre filter (Whatman GF/M) and drying to constant weight. In this way the relationship between absorbance of a culture and dry weight was obtained for routine use. The internal volume of the cells was determined (cf. Kell, 1979) as the sucrose-impermeable space (2μl/mg dry wt.) which, as noted by Stock *et al.* (1977) and Booth *et al.* (1979) should not include the intermembrane or periplasmic space.

The concentration of $BTPP^+$ in suspension media was measured by using a $BTPP^+$-sensitive electrode. All the data shown were obtained by using an electrode of the type originally described by Moody & Thomas (1978), and recently adapted for use as a tetraphenylphosphonium-cation-selective electrode by Kamo *et al.* (1979) and Muratsugu *et al.* (1979). A poly(vinyl chloride) membrane was prepared as described by Kamo *et al.* (1979), and a 1.5cm diameter disc cut from the membrane preparation was glued with tetrahydrofuran to a poly(vinyl chloride) sleeve that was fitted over an open-ended, fully screened, SW74-type pH electrode (supplied by Russell pH, Auchtermuchty, Fife, Scotland, U.K.). The electrode body was filled with 1mM-$BTPP^+Br^-$ in 100mM-NaCl as the inner reference solution. Similar results were obtained with an alternative electrode constructed on the same principles as the dibenzyldimethylammonium electrode described by Muratsugu *et al.* (1977). The response of the latter type of electrode, however, was found to be more susceptible to interference from various components of solution, e.g. uncouplers of oxidative phosphorylation, and thus the poly(vinyl chloride) matrix membrane type of electrode was the instrument of choice.

The potential of either type of electrode was measured versus an Orion double junction reference electrode (92-02), with an outer bridging solution of 10% (w/v) KF (John, 1977; Kell *et al.* 1978c), by using an Orion 701A Ionalyzer. The output from the Ionalyzer was fed to a Servoscribe 1S chart recorder.

Uptake of $BTPP^+$ was measured in 50ml reaction

mixtures contained in a glass vessel which was closed with a tight-fitting rubber bung through which the $BTPP^+$-cation-selective electrode and reference electrode were inserted. Additions to the reaction mixture were made via a 18-gauge steel needle which had also been positioned in the bung. In experiments where exclusion of oxygen was required, argon was blown over the reaction mixture via a suitable entry port.

Attempts to detect $^{204}Tl^+$ uptake during aerobic respiration were made by using the flow-dialysis method. A portion (1 ml) of a cell suspension (the composition of which is described in the Results section) was stirred under a jet of water-saturated oxygen in the upper chamber of the flow-dialysis cell [built in the workshop of the Biochemistry Department, Birmingham University, according to drawings supplied by Dr. K. Feldmann; Feldmann (1978)]. The outflow from the cell was fed through a flow-through scintillation cell (constructed from a scintillating plastic in the Biochemistry Department workshop, Birmingham University, to the design of Dr. W. F. R. Pover). The scintillation pulses thus generated were detected by a Nuclear Enterprises LSF1 counter, the output of which was monitored by an SR5 Scaler–Ratemeter from which the data were displayed on a Servoscribe 1 S chart recorder.

The respiration of cells was measured with a Clark-type oxygen electrode (Ferguson *et al.*, 1979*c*); nitrate and nitrite reduction were measured with an Orion 93-07 electrode combined with the reference electrode and digital mV meter described above.

$BTPP^+Br^-$ was purchased from Koch–Light, Colnbrook, Bucks., U.K., or from Aldrich Chemical Co., Gillingham, Dorset, U.K. All other chemicals were of the highest grade commercially available.

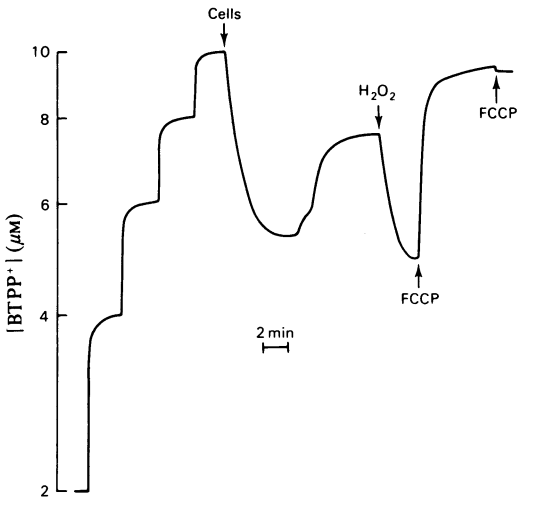

Fig. 1. *Uptake of $BTPP^+$ by P. denitrificans under aerobic conditions*

The reaction vessel contained a 50 ml suspension mixture, including 100 mM-Hepes/NaOH, pH 7.3, plus 5 mM-sodium succinate, to which $BTPP^+$ was added in increments of 2 μM for electrode calibration. Cells (equivalent to 61 mg dry wt.) were added as shown, followed by H_2O_2 to a final concentration of 500 μM, and then FCCP to a final concentration of 1 μM. The interference with the electrode by FCCP is clearly shown by the subsequent further addition of FCCP to a total of 2 μM. The measurements were performed at 19°C.

Results and discussion

Uptake of $BTPP^+$ linked to aerobic respiration

The electrode responded to increasing concentrations of $BTPP^+$ almost exactly in accordance with the Nernst equation. Addition of cells caused the concentration of free $BTPP^+$ to drop, corresponding to an uptake of the cation by the cells. In the experiments illustrated in Fig. 1, partial efflux of $BTPP^+$ began about 5.5 min after the addition of the cells. This was correlated with partial anaerobiosis, since in this experiment no attempt was made to exclude air from above the reaction mixture. Parallel experiments in the oxygen electrode indicated that, after 5.5 min, the concentration of dissolved O_2 was decreased to less than 5 μM, and thus the cells could presumably then maintain only a lowered membrane potential because respiration was limited by the very low steady-state dissolved O_2 concentration. When the suspension was fully reoxygenated by the action of the catalase activity of the cells upon added H_2O_2, the amount of $BTPP^+$ taken up by the cells was restored approximately to its initial value, and subsequent introduction of an uncoupler of oxidative phosphorylation, FCCP, led to rapid and essentially complete efflux of the $BTPP^+$ from the cells. The small displacement of the final electrode response from its original reading can almost entirely be accounted for by the dilution caused by addition of the cells and by a small artefactual shift induced by FCCP.

Efflux of $BTPP^+$ also occurred after addition of Triton X-100 (0.02%) which has previously been shown to remove a permeability barrier between external ClO_3^- and the active site of nitrate reductase in intact cells (John, 1977), and also to permit aerobic reduction of nitrate (Alefounder & Ferguson, 1980). This effect of Triton X-100 on $BTPP^+$ uptake thus strengthens the interpretation (Alefounder & Ferguson, 1980) that this amount of Triton renders the cell membrane permeable to small ions, and thus the membrane potential is dissipated.

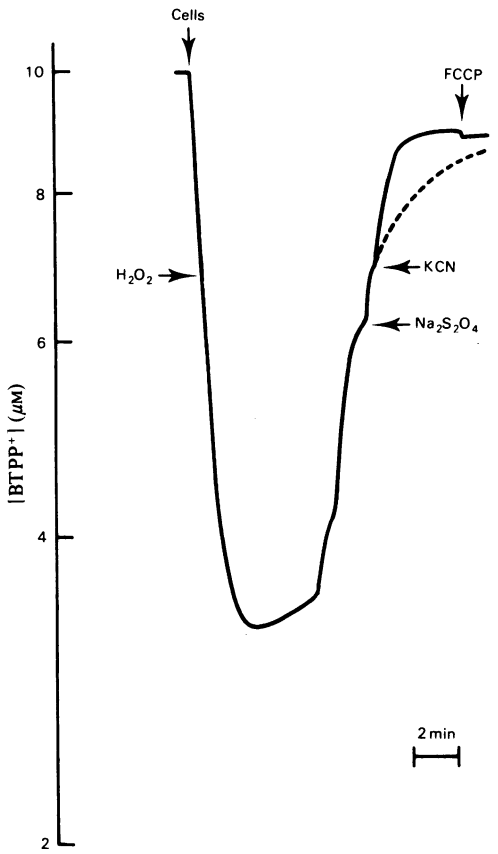

Fig. 2. *Efflux of BTTP$^+$ from cells at onset of anaerobiosis*

The contents of the reaction chamber and the calibration of the electrode were as described in the legend to Fig. 1, but argon was blown over the reaction mixture. Cells (71 mg dry wt.) were added as indicated, together with H_2O_2 (final concentration 500 μM) to ensure that BTPP$^+$ uptake reached its aerobic maximum. The subsequent additions of $Na_2S_2O_4$ (added as a fresh solution in 100 mM Hepes/NaOH to a final concentration of 500 μM) and KCN [added as a 1.5 M solution (pH adjusted to 7.3 with HCl) to a final concentration of 3 mM] respectively, induced full efflux of BTPP$^+$, and thus addition of FCCP (1 μM) induced no further release of the cation. The broken line shows the efflux of BTPP$^+$ in the absence of KCN.

Fig. 2 shows an experiment during which argon was used to displace air from the reaction chamber and sodium dithionite was added to accelerate the rate of approach to complete anaerobiosis. The further addition of 3 mM-KCN (final concentration) as a terminal oxidase inhibitor ensured cessation of respiration and complete release of BTPP$^+$, which indicates that under these conditions respiration is the sole generator of membrane potential. It was also possible to reverse fully the electrophoretically driven uptake of BTPP$^+$ by gassing the reaction vessel with argon which had been freed of contaminating traces of oxygen by bubbling it through a concentrated alkaline solution of sodium dithionite and an anthraquinone catalyst (Fieser's solution), although total efflux took 20 min. In separate experiments with the oxygen electrode, it was shown that 500 μM-sodium dithionite had no inhibitory effect upon respiration. It was possible, furthermore, to restore full aerobic uptake of BTPP$^+$ subsequent to the addition of sodium dithionite by introducing excess H_2O_2 into the reaction medium.

Before quantitative interpretations of the presented data can be made, it is necessary to consider whether the extent of uptake of a cation such as BTPP$^+$ can be used to estimate the membrane potential across bacterial cell membranes. The following criteria should ideally be fulfilled: the cation must (i) reach a Nernstian equilibrium distribution; (ii) not bind in significant amounts to cellular components; (iii) not interfere with cellular energy metabolism; and (iv) there must be a reliable estimate for the volume of the internal aqueous phase of the cell.

We found that BTPP$^+$ stimulated respiration in cells of *P. denitrificans* to an extent that was dependent upon the concentration of this cation and the medium in which the cells were suspended. In media including 100 mM-Hepes/NaOH (pH 7.3) or 100 mM-NaH_2PO_4/NaOH (pH 7.3), the addition of BTPP$^+$ to a final concentration of 40 μM stimulated respiration by 10–15%, whereas 10 μM-BTPP$^+$ gave rise to a stimulation of only 7–8%. Cells suspended in 100 mM-KH_2PO_4/KOH (pH 7.3) respired at a rate more than twice as great as that observed with cells which were suspended in the above Na^+ buffers, and the stimulatory effect of BTPP$^+$ was much less pronounced. It was also found that the apparent accumulation ratio (i.e. the uptake of BTPP$^+$ by cells as a percentage of the initial BTPP$^+$ concentration) was a function of BTPP$^+$ concentration. This effect, however, was small: in 100 mM-NaH_2PO_4/NaOH, where the BTPP$^+$ concentration-dependence was most significant, estimates of membrane potential at 40 μM-BTPP$^+$ were approx. 10 mV greater, and estimates at 10 μM-BTPP$^+$ were only 2–3 mV greater, than those based on uptake ratios at 4 μM-BTPP$^+$. It therefore seems unlikely that the values of membrane potential listed in Table 1 are significantly biased by the inability of BTPP$^+$ to comply absolutely with requirement (iii) stated above, and since a very similar accumulation ratio for BTPP$^+$ was observed over a range of concentrations in the given suspension media, it also seems probable that the first criterion listed has been satisfied. The apparent ratio of internal cellular

$BTPP^+$ concentration (assuming the given internal volume for the cells) to external $BTPP^+$ concentration was also invariant (to within 3–4 mV) over a range of cell concentrations (0.85–3.5 mg/ml). This provides not only further corroborative evidence for the applicability of criterion (i), but also suggests that any binding of $BTPP^+$ to cellular components is minimal [(ii)]. Another way to test whether there is non-energy linked binding or uptake of $BTPP^+$ is to examine the effect of heat-killed cells on the free concentration of $BTPP^+$. The addition of heat-treated cells to 100 mM-NaH_2PO_4/NaOH (pH 7.3) containing 10 μM-$BTPP^+$ gave a deflection in the reading of the $BTPP^+$-sensitive electrode of magnitude only equivalent to the extent to which the solution of $BTPP^+$ was correspondingly diluted. Such an experiment does not however, in itself, necessarily constitute an adequate control because significant binding may only occur inside the cell, where the concentration of accumulated $BTPP^+$ reaches the mM level.

Important evidence that the uptake of lipophilic phosphonium cations may be used as quantitative measurements of membrane potentials across bacterial membranes has come from the recent work of Felle *et al.* (1978, 1980) who have shown that in giant cells of *Escherichia coli* the uptake of tetraphenylphosphonium and direct measurements with microelectrodes give very similar estimates, approx. 140 mV, for the membrane potential. On the other hand, it is fair to recognize that e.s.r. studies with spin-labelled analogues of phosphonium cations (Cafiso & Hubbel, 1978) and resonance Raman measurements of the Quinaldine Red cation (Koyama *et al.*, 1979), have shown that these latter cations can partition into phospholipid phases and form strong complexes with anionic groups. Thus it may be fortuitous that direct microelectrode measurements are in such close agreement with measurements with the ion distribution method.

Table 1 shows that, if the total uptake of $BTPP^+$ into respiring cells of *P. denitrificans* is taken as the indicator of membrane potential, then values between 130 and 160 mV are found, the precise values quoted depending upon the individual suspension medium employed. These values of membrane potential are derived from calculations which use the initial electrode response to 10 μM-$BTPP^+$ as a reference point for measurement of the extent of uptake. In order to be rigorous, however, we should make some allowance for the uncertainty as to whether $BTPP^+$ uptake is fully reversible. Yet consideration of the small, apparently irreversible, component of $BTPP^+$ uptake observed in the majority of our experiments leads to the conclusion that the values of membrane potential in Table 1 can only be overestimated by at most 4–5 mV as a result of any irreversible accumulation of the cation.

Effect of NO_3^-, NO_2^- and N_2O on the aerobic uptake of $BTPP^+$

We can confirm that in cells of *P. denitrificans* there is at least 95% inhibition of NO_3^- reduction by O_2, whereas NO_3^- exerts no control over O_2 reduction, as originally demonstrated by John (1977). Thus any change in the extent of aerobic $BTPP^+$ uptake that might be caused by NO_3^- can be studied without any complication from the simultaneous reduction of both NO_3^- and O_2. The presence of 10 mM-$NaNO_3$ was found to have no effect upon the uptake of $BTPP^+$ observed under aerobic conditions (see Fig. 3 and Table 1). There is thus a difference between intact cells and inside-out vesicles of *P. denitrificans*, since 10 mM-NO_3^- decreases substantially the membrane potential of the vesicles (Kell *et al.*, 1978*a*; McCarthy *et al.* 1979), possibly as a result of electrophoretic movement of NO_3^- into the lumen of the vesicles.

Meijer *et al.* (1979) have suggested that the low apparent efficiency of respiratory-chain-linked energy conservation observed during NO_3^- respiration is caused by NO_2^- acting as an uncoupler. Inhibition of the growth rate of *P. denitrificans* by as little as 80 μM-NO_2^- has been observed together with a stimulation by 20 nmol (in 3.1 ml) of NO_2^- of the decay of the acidification of the extracellular medium which follows transient electron flow (Meijer *et al.*, 1979). However, if NO_2^- causes true

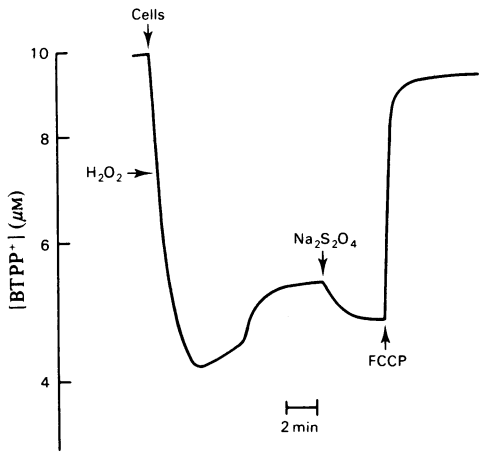

Fig. 3. *$BTPP^+$ uptake during aerobic respiration in the presence of 10 mM-$NaNO_3$ and subsequently during anaerobic electron transport to NO_3^-*

The conditions were as specified in the legend to Fig. 2, except that 10 mM-$NaNO_3$ was also present. Cells (71 mg dry wt.) were added as shown, followed by H_2O_2 (500 μM). The uptake of $BTPP^+$ at partial anaerobiosis was boosted by the addition of $Na_2S_2O_4$ (500 μM), and then FCCP (1 μM) induced $BTPP^+$ efflux.

uncoupling it should, like FCCP, prevent, or at least decrease, the energy-linked uptake of $BTPP^+$. As illustrated in Fig. 4, this was not the case; aerobic uptake of $BTPP^+$ in the presence of 10mM-$NaNO_2$ was always equivalent to a membrane potential at least as great as in control experiments where $NaNO_2$ was absent (see Table 1). We have observed with the oxygen electrode that cellular respiration is inhibited by approx. 20% in the presence of 10mM-NO_2^-, whereas experiments with the nitrate electrode [which can also detect NO_2^- (John, 1977)] have shown that NO_2^- reduction (with 2mM-NO_2^-) is inhibited about 75% by O_2. Thus the aerobic $BTPP^+$ uptake illustrated in Fig. 4 is the result of simultaneous reduction by the cells of both O_2 and NO_2^-, with the majority of the reducing equivalents moving through the electron transport chains being passed to O_2. These data are not consistent with any short term uncoupling effects being induced by NO_2^-, because even after incubation of the cells with 10mM-$NaNO_2$ for 18min (during which time the NO_2^- concentration decreased to approx. 9mM), the addition of H_2O_2 to restore full aerobiosis allowed the cells to regain their maximal membrane potential of about 160mV.

For the NO_2^-/HNO_2 pair to act as an uncoupler, NO_2^- would need to undergo rapid electrophoretic movement outwards through the cell membrane in order to dissipate the $\Delta\psi$ component of the protonmotive force. Evidence that NO_2^- does not meet this requirement is that even 10mM-$NaNO_2$ has only a limited effect on the steady-state uptake of $S^{14}CN^-$ into inside-out vesicles prepared from *P. denitrificans* (J. E. G. McCarthy & S. J. Ferguson, unpublished results).

Finally, the presence of N_2O in any of the suspension media had no effect either on respiration or on $BTPP^+$ uptake under aerobic conditions.

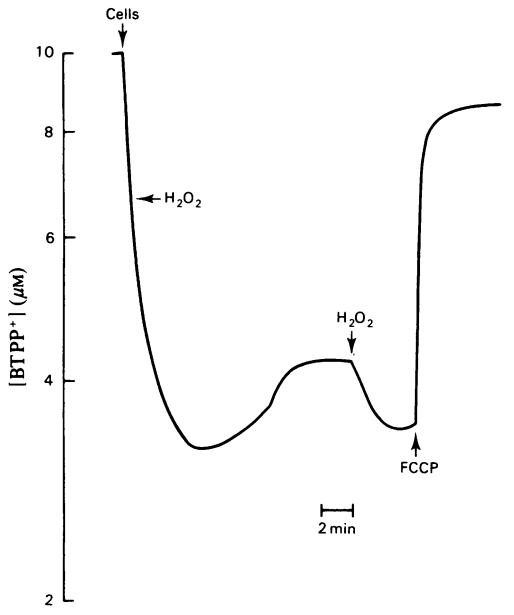

Fig. 4. *$BTPP^+$ uptake during aerobic respiration in the presence of 10mM-$NaNO_2$ and subsequently during electron transport to NO_2^-*

The conditions were identical with those described in the legend to Fig. 2, except for the presence of 10mM-$NaNO_2$ in the suspension medium. Cells (71mg dry wt.) and H_2O_2 (to 500μM) were added as indicated, followed finally by FCCP (1μM).

$BTPP^+$ uptake with NO_3^-, NO_2^- or N_2O as terminal electron acceptor

When 10mM-$NaNO_3$ was present in the initially aerobic suspension of cells the extent of $BTPP^+$ uptake decreased slightly at the point at which partial anaerobiosis was reached (Fig. 3), but the decrease was less than that seen in the absence of NO_3^- (cf. Fig. 1). Under the condition of partial anaerobiosis it was found, by using a nitrate electrode under identical conditions (including gassing with argon) to those of Fig. 3, that nitrate reduction was occurring. Thus the $BTPP^+$ uptake under these conditions is supported by electron flow to both nitrate and oxygen. When the reaction mixture was brought close to total anaerobiosis by addition of 500μM-$Na_2S_2O_4$, which does not inhibit nitrate reductase (Chance, 1955), the rate of nitrate reduction was stimulated approx. 2-fold, and an increased uptake of $BTPP^+$ was observed (Fig. 3) corresponding to a membrane potential of approx. 150mV. Addition of higher concentrations of $Na_2S_2O_4$ did not increase either the rate of nitrate reduction or the uptake of $BTPP^+$, and thus the latter value of membrane potential has been adopted as that supported by reduction of NO_3^- as sole added electron acceptor (Table 1). It appears that the conditions of partial anaerobiosis were such that the control exercised by oxygen on nitrate reduction (John, 1977; Alefounder & Ferguson, 1980) was not fully effective. The total combined rate of proton translocation linked to electron flow to oxygen plus nitrate (and perhaps to NO_2^- and N_2O formed from NO_3^-) during the state of partial anaerobiosis may be less than when conditions permit electron flow to oxygen or nitrate alone. This could explain why the extent of $BTPP^+$ uptake passed through a minimum as the concentration of oxygen decreased towards zero (Fig. 3).

The extent of uptake of $BTPP^+$ maintained by NO_2^- reduction was apparently not sensitive to the presence of low concentrations of O_2 in the reaction medium, in contrast to the high sensitivity to O_2 displayed by the activity of the reductase. Thus the level of $BTPP^+$ uptake (equivalent to about 150mV, see Table 1) attributable to NO_2^- reduction during partial anaerobiosis (Fig. 4) could not be sig-

nificantly enhanced by complete purging of O_2 from the suspension medium with O_2-free argon ($Na_2S_2O_4$ could not be used to remove the last traces of O_2 because it inhibited the nitrite reductase activity).

Thus our overall results from experiments performed under both aerobic and anaerobic conditions show that NO_2^- does not exert its retarding effect on cell growth via its action, with its conjugate acid HNO_2, as a protonophoric uncoupler, at least in the short term. The reason why the membrane potential is lower during anaerobic respiration is not known, but could be due to the rate of proton pumping being sufficiently low that the maximal membrane potential cannot be maintained. This behaviour is for instance observed at high extents of inhibition of mitochondrial respiration (Nicholls, 1974; Sorgato & Ferguson, 1979). A further point to consider is that the retention of a high (anaerobic) membrane potential in the presence of NO_3^- or NO_2^- does not mean that other key energy-linked reactions will be unperturbed by a changeover from aerobic to anaerobic respiration, as it has been shown elsewhere (e.g. Kell *et al.*, 1978*b* and Sorgato *et al.*, 1980) that, for example, the rate of ATP synthesis can vary at constant membrane potential.

Denitrification of NO_3^- to N_2 is generally regarded as proceeding via NO_2^-, N_2O and possibly NO. The evidence that N_2O in this pathway is actually formed as a 'free' intermediate in *P. denitrificans* has been equivocal. Thus Stouthamer and coworkers (Meijer *et al.*, 1979) have reported that no proton extrusion from cells was detected upon adding N_2O to anaerobic suspensions of cells, whereas in direct contradiction Kristjansson *et al.* (1978) reported that H^+ movement out of cells was seen in experiments of very similar design. Meijer *et al.* (1979) were also led to conclude that N_2O is not a free intermediate of denitrification in *P. denitrificans* and thus not a competent terminal electron acceptor, as they were unable to detect significant growth on N_2O, although more recently the same laboratory (Boogerd *et al.*, 1980) has detected limited growth on N_2O. Yoshinari & Knowles (1976) found that acetylene, which is thought to be a competitive inhibitor of nitrous oxide reductase, was an effective inhibitor of N_2 production from NO_3^- and NO_2^- in *P. denitrificans* and accumulation of N_2O was observed. The evidence for an N_2O-reductase has been strengthened recently by the evidence for a partial purification of the enzyme (Kristjansson & Hollocher, 1980).

In view of the doubt over the role of N_2O in anaerobic respiration it was of interest to find that addition of N_2O to cells, after anaerobiosis had been reached, resulted in uptake of $BTTP^+$ to an almost equal extent to that observed when oxygen acted as terminal electron acceptor (Fig. 5). Hence N_2O-respiration generates a membrane potential of about

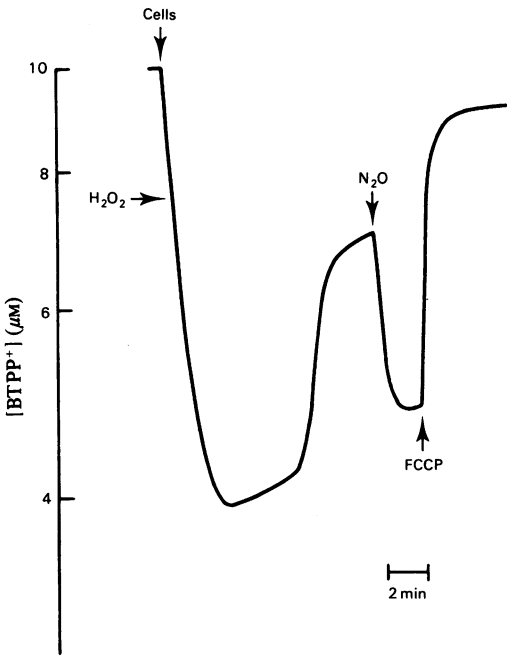

Fig. 5. *$BTPP^+$ uptake during electron transport to N_2O*
The conditions were as described in the legend to Fig. 2, except that argon bubbled through Fieser's solution (see text) was blown over the suspension medium. Cells (61mg dry wt.) and H_2O_2 (500μM) were added as shown. N_2O-saturated 100mM-Hepes/NaOH (pH7.3) was introduced during the approach to anaerobiosis (giving about 500μM-N_2O in solution) followed by FCCP (1μM).

150mV (Table 1) and the relatively poor growth of *P. denitrificans* with this gas as electron acceptor might therefore be related to a longer term toxic effect of N_2O. The observation of a membrane potential shows that electron flow to N_2O must be linked to proton translocation, as was observed by Kristjansson *et al.* (1978) (cf. Meijer *et al.*, 1979), and as would be expected if electrons destined for reduction of N_2O or NO_2^- share a common respiratory chain as far as a *c*-type cytochrome (Boogerd *et al.*, 1980).

The above interpretation that electron flow to each of NO_3^-, NO_2^- and N_2O generates a membrane potential should strictly be supported by evidence that it is not solely reduction of N_2O, produced from NO_3^- or NO_2^-, that is responsible for the membrane potentials. The latter explanation is very improbable as a membrane potential is maintained immediately after anaerobiosis with either NO_3^- or NO_2^- (Figs. 2 and 3), and also because in general the order of reduction of oxides of nitrogen is NO_3^-, NO_2^-, N_2O (Payne, 1976).

Influence of K^+ and Na^+ on $BTPP^+$ uptake

Skulachev (1978) has suggested that the prime function of Na^+ and K^+ gradients across bacterial membranes is to act as a buffer to fluctuations in the magnitude of the protonmotive force. This could be of critical importance to the viability of bacterial cells under circumstances in which they are temporarily unable to maintain a protonmotive force by respiration- or ATP-driven proton translocation, in that the collapse of the protonmotive force could be significantly delayed by K^+ efflux and Na^+ influx (via a Na^+/H^+ antiporter) down their respective concentration gradients. The need for such a buffering system could be particularly acute in a bacterium like *P. denitrificans*, in which fermentation is not possible and the apparent unidirectional properties of the ATPase enzyme (Ferguson *et al.*, 1976), at least *in vitro*, suggest that any ATP formed from cellular reserves [e.g. polyphosphate (Ferguson *et al.*, 1979*a*)], would not be able to drive proton translocation.

We performed experiments in which conditions were optimal for the formation of the appropriate K^+ and Na^+ gradients in *P. denitrificans* in order to determine whether such gradients could provide a significant energy storage capacity in this organism. Cells were washed and finally resuspended in media containing a high concentration of K^+ [100mM-KH_2PO_4/KOH (pH 7.3) or 100mM-Hepes/KOH (pH 7.3)]. The final resuspension was then preincubated with vigorous aeration in the presence of 5mM-sodium succinate to ensure that the intracellular K^+ concentration could be maximized, and $BTPP^+$ uptake into these cells was subsequently monitored when aliquots of the preincubated suspension were added to either 100mM-NaH_2PO_4/NaOH or 100mM-Hepes/NaOH (both at pH 7.3). It was observed that upon anaerobiosis, the characteristics of $BTPP^+$ release from the cells were exactly the same as those seen when the 'K^+-loaded' cells were added to one of the high-K^+ buffers (in fact identical to the characteristics of $BTPP^+$ release exemplified in Fig. 2). Had there been electrogenic efflux of K^+ down a concentration gradient, the collapse of the membrane potential should have been noticeably retarded, unless the K^+-driven membrane potential was extremely short-lived. Thus, whereas in *E. coli* evidence for the role of a K^+ gradient in maintaining the membrane potential in the absence of metabolism (an ATPase-deficient mutant was used) has been presented (Broun *et al.*, 1979; Skulachev, 1980), *P. denitrificans* cannot be shown to behave similarly with comparable techniques, and no respiration-independent membrane potential can be detected in this bacterium. The generality of applicability of the Na^+/K^+ gradient as a buffer for the protonmotive force thus remains to be established, although evidence for its (at least partial) operation in *Halobacterium halobium* has been reported (Wagner *et al.*, 1978).

The membrane potential at pH 7.3 estimated for cells under aerobic conditions in 100mM-KH_2PO_4/KOH was considerably lower (by 19–24mV) than that estimated for cells suspended in 100mM-Hepes/KOH or either of the high-Na^+ media (Table 1). It is of interest that, whereas cell respiration in 100mM-Hepes/KOH was 25% faster than in the high-Na^+

Table 1. *Estimates of membrane potential in P. denitrificans from $BTPP^+$ uptake under various conditions*

Each set of estimates is itself an average of results from repetitions of a particular experiment with a single preparation, and the values for membrane potential are overall averages. The cells were able to reduce O_2 and NO_2^- (but not O_2 and NO_3^-) simultaneously (see the text). The values of membrane potential given for NO_3^-, NO_2^- and N_2O as added electron acceptors relate to anaerobic conditions, although the presence of very low concentrations of O_2 in the medium ('partial anaerobiosis'; see the text) did not affect the values recorded for reduction of NO_2^- or N_2O. N_2O was added to the suspension medium in the form of N_2O-saturated 100mM-Hepes/NaOH (pH 7.3). All experiments were performed at pH 7.3 and 19°C. For estimation of membrane potentials it was assumed that $BTPP^+$ is distributed between the interior of the cells and the external aqueous phase according to the Nernst equation (e.g. Nicholls, 1974). Values of membrane potential were therefore calculated from the concentration of $BTPP^+$ remaining external to the cells together with the extent of $BTPP^+$ uptake and the measured internal volume (given in the Materials and methods section).

Medium (100mM, pH 7.3)	Addition (10mM)	Electron acceptor	$\Delta\psi$ (mV)	No. of sets of estimates	S.E.M. (mV)
Hepes/NaOH		O_2	157	7	±3
	$NaNO_3$	O_2	157	5	±3
	$NaNO_2$	O_2	157	5	±3
	$NaNO_3$	NO_3^-	149	5	±3
	$NaNO_2$	NO_2^-	152	5	±3
		N_2O	151	4	±3
Hepes/KOH		O_2	152	4	±2
NaH_2PO_4/NaOH		O_2	157	6	±3
KH_2PO_4/KOH		O_2	133	4	±2

media (respiration rates in the two buffers containing Na^+ but no K^+ were identical), cells suspended in 100mM-KH_2PO_4/KOH respired at a rate 2.3-fold faster than cells in either of the high-Na^+ media. One explanation of these results is that effective electrophoretic dissipation of the membrane potential by influx of K^+ (and thus stimulation of respiration) is dependent upon the presence of P_i. However, we found that cellular respiration was much stimulated in the presence of a high concentration of P_i combined with a much lower concentration of K^+. For example, when cells were respiring in 100mM-NaH_2PO_4/NaOH the maximal stimulation of respiration (2.3-fold relative to the rate in either of the two high-Na^+ media alone) was achieved by addition of K^+ (as 100mM-KH_2PO_4/KOH) to a final concentration of only 1mM. This stimulation was not observed when the same addition was made to cells respiring in 100mM-Hepes/NaOH or 100mM-Hepes/KOH. Similarly, the uptake of $BTPP^+$ into cells in 100mM-NaH_2PO_4/NaOH plus 1mM-KH_2PO_4/KOH, together with 10μM-$BTPP^+$, was equivalent to less than 140mV. In the light of the demonstration of a degree of mutual interdependence between the transport of K^+ and P_i into *E. coli* (Russell & Rosenberg, 1979, 1980), it is evident from our results that the movements of K^+ and P_i in *P. denitrificans* merit further investigation.

Relationship of the magnitude of $BTPP^+$ uptake to the bioenergetics of the cell

Under the conditions of measurement of the membrane potential the respiring cells will be continuously synthesizing ATP, from which the transfer of the terminal phosphate group to polyphosphate in a type of 'slip' reaction (Ferguson *et al.*, 1979*a*) can provide a route for the regeneration of ADP. Although the intracellular concentrations of ATP, ADP and P_i were not measured in the present work, it is probable that they would have been similar to the values reported by Erecinska *et al.* (1978) for *P. denitrificans*, corresponding to a phosphorylation potential of approx. 12kcal·mol^{-1} (50.4kJ·mol^{-1}), and an [ATP]/[ADP] = 8, which is within the range generally found in bacteria (Knowles, 1977). If $2H^+$ are translocated through the ATPase per ATP synthesized (Mitchell, 1976), and we assume a state of near-equilibrium for oxidative phosphorylation, then to poise the ATP synthesis reaction at 12kcal·mol^{-1} (50.4kJ·mol^{-1}) a protonmotive force of approx. 260mV would be needed. This would be compatible with our data only if the pH gradient across the cell membrane was equivalent to 100mV; i.e. the intracellular pH would be approx. 9. An internal pH of 9 is improbable, and would be at variance with the earlier work of Scholes & Mitchell (1970*a*) who concluded that the intracellular pH of a suspension of respiring cells of *P. denitrificans* was relatively insensitive to the external pH, and was buffered between 7.2 and 7.4, close to the optimal pH for the dehydrogenases of the respiratory chain. In a proper consideration of the thermodynamic status of oxidative phosphorylation, however, we should have to make allowance for the fact that energy transduction in living cells consists of imperfect coupling between irreversible processes. Nevertheless, it seems fair to conclude from the above discussion that unless the extent of $BTPP^+$ uptake gives a drastic underestimate of the membrane potential, the value of H^+/ATP for the ATP synthase in *P. denitrificans* is likely to be greater than 2, as was also indicated from comparison of the protonmotive force with the phosphorylation potential in vesicles (Kell *et al.*, 1978*a*; Ferguson *et al.*, 1979*b*).

Just as for mitochondria, the original estimates of respiratory chain-linked H^+ translocation (Scholes & Mitchell, 1970*b*) of $2H^+$ per site in *P. denitrificans* have recently been suggested to be underestimates (Lawford, 1978; van Verseveld & Stouthamer, 1979). It is unlikely that the redox energy available from proton translocating segments of the respiratory chain would be sufficient to drive the translocation of as many as $4H^+$ per site against a protonmotive force much larger than the 160mV membrane potential component reported here.

The value of the membrane potential estimated in the present work may not be the maximum that can be developed by the respiratory chain, as the experiments were done under conditions where both ATP synthesis and possibly other energy-dependent reactions were taking place. By analogy with membrane vesicles from *P. denitrificans* (Kell *et al.*, 1978*a*) and mitochondria (e.g. Nicholls, 1974), it is probable that the maximum value of the membrane potential can only be attained when net ATP synthesis is blocked. Such an effect, accompanied by an inhibition of respiration that was reversed by an uncoupler, was recently observed in cells of *Rhodopseudomonas capsulata* (Cotton *et al.*, 1981), but despite the high respiratory control in membrane vesicles, attempts to demonstrate inhibition of cellular respiration in *P. denitrificans* with ATPase inhibitors such as venturicidin (Ferguson & John, 1978) have so far been unsuccessful. However, the effect of these compounds on the membrane potential and ATP synthesis in intact cells of *P. denitrificans* has yet to be studied.

Investigation of the possibility that $^{204}Tl^+$ might act as a probe of membrane potential

Bakker (1978) suggested that $^{204}Tl^+$ acts as a freely permeable cation towards several types of bacterial cell, including *E. coli*, and may therefore be

used as an indicator of membrane potential. Under identical reaction conditions, either 100mM-NaH_2PO_4/NaOH or 100mM-Hepes/NaOH (Bakker, 1978) plus 5μM-Te^+, with those used to study uptake of $BTPP^+$, a flow dialysis assay showed no detectable $^{204}Tl^+$ uptake by cells of *P. denitrificans*. The uptake of less than 5% of the total $^{204}Tl^+$ present would have been detected. Subsequently it has been shown that $^{204}Tl^+$ accumulation by both *E. coli* (Damper *et al.*, 1979) and *Streptococcus lactis* (Kashket, 1979) is mediated by K^+-transport systems, and thus the suggestion that $^{204}Tl^+$ uptake can be used as a probe of membrane potential has been retracted (Bakker & Harold, 1980). The lack of detectable uptake of $^{204}Tl^+$ by *P. denitrificans* implies that systems analogous to those which can transport Tl^+ in *E. coli* (Kdp and TrkA) and *S. lactis* are absent from *P. denitrificans* grown under our conditions.

Comparison with previous work

In an earlier investigation of the membrane potential in *P. denitrificans* cells, Deutsch & Kula (1978) studied the uptake of the triphenylmethylphosphonium cation or the electrophoretic exclusion of SCN^- as an indicator of membrane potential. They estimated that respiring cells of *P. denitrificans* maintained a membrane potential of only 40mV (negative inside) and did not report whether the uptake of triphenylmethylphosphonium was reversed by addition of an uncoupler of oxidative phosphorylation. A very curious feature of their results was that the uptake of triphenylmethylphosphonium, and thus the magnitude of the membrane potential, increased upon anaerobiosis, in complete contrast to the results with the related compound, $BTPP^+$, that was used in the present work. It is improbable that the use of the methyl rather than the butyl derivative could account for this discrepancy. Another unusual feature of the experiments of Deutsch & Kula (1978) was the use of a Krebs–Henseleit medium for the suspension of the bacterial cells. This type of medium is not usually employed in studies on bacteria, but as in the present work no systematic study has been done on the effects of the various components of the Krebs–Henseleit medium it cannot be excluded that the small membrane potential reported by Deutsch & Kula (1978) was caused by a component which interfered with triphenylmethylphosphonium uptake. A final point of difference between the measurements of Deutsch & Kula (1978) and the present work is that, in the former, glucose was used as the carbon source for growth and as the substrate for the determination of membrane potential, but it seems unlikely that this change would lead to such a large decrease of the membrane potential.

P. denitrificans is an example of a Gram-negative organism that can take up lipophilic cations

In general *E. coli* takes up lipophilic cations only after its cell wall has been disrupted by addition of Tris and EDTA (e.g. Muratsugu *et al.*, 1979). Even after this treatment it is often still necessary to include a lipophilic anion such as tetraphenylboron to facilitate the movement of the cation across the membrane. It is therefore of interest that *P. denitrificans*, another gram-negative organism (Scholes & Smith, 1968), is readily able to take up a lipophilic cation without any requirement for a catalytic anion such as tetraphenylboron. This is true not only in the logarithmic phase of growth, but also, we find, after the cells have reached stationary phase. Presumably *P. denitrificans* has features in the outer membrane which permit the uptake of lipophilic cations, including $BTPP^+$, which are not taken up by *E. coli* (D. J. Clarke, personal communication) but generally taken up by Gram-positive organisms.

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