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Acid-Base Titration Across the Plasma Membrane of *Micrococcus denitrificans*: Factors Affecting the Effective Proton Conductance and the Respiratory Rate

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Abstract

The object of this work was to measure the effective proton conductance of the plasma membrane of *Micrococcus denitrificans* under various conditions and to investigate possible connections between respiration and proton translocation.

1. Pulsed acid-base titrations of suspensions of *M. denitrificans* in a medium containing the permeant thiocyanate ion, or when K^+ ion permeability was induced by valinomycin in a KCl medium, showed that the normal effective proton conductance of the membrane system was less than 1 μmho/cm^2.
2. A pH-overshoot artefact was suppressed by adding carbonic anhydrase.
3. The effective proton conductance was increased by the uncoupler FCCP* in the same concentration range as was required to stimulate respiration. Concentrations of FCCP above 1·5 μM inhibited respiration after an initial stimulation.
4. The effective proton conductance in presence of 2 μM FCCP was at least 17 μmho/cm^2.
5. The quantitative relationships between the respiratory rate, the stoichiometry of respiration-driven proton translocation, and the effective proton conductance of the membrane of the cells are compatible with the suggestion that stimulation of respiration by FCCP is due to a release of back-pressure exerted by a protonmotive potential on the respiratory chain system in the membrane. Only one amongst other possible explanations of the stimulation of respiration by FCCP is, however, excluded.

Introduction

Observations by Gilby and Few[1] indicated that the titration of the cytoplasmic buffers of *Micrococcus lysodeikticus* with HCl was limited by the rate of equilibration of H^+ and OH^- ions across the plasma membrane. These observations were confirmed in *M. lysodeikticus* and *Escherichia coli* by Mitchell,[2] who also found that DNP catalysed acid-base equilibration, probably by the conduction of protons across the plasma membrane, and that destruction of the membrane with *n*-butanol allowed a very rapid titration of the intracellular buffers. More recently[3,4] it has been shown that CCCP, TCS, TMPA, and dicoumarol facilitate acid-base titration across the plasma membranes of *E. coli* and *Streptococcus faecalis*. The effects of FCCP on the decay of pH differences across the plasma

* Abbreviations: DNP, 2,4-dinitrophenol; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; FCCP, carbonylcyanide *p*-trifluoromethoxy phenylhydrazone; TCS, tetrachloro-salicylanilide; TMPA, tetramethyldipicrylamine; $t_{1/2}$, time for half equilibration.

membrane of intact photosynthetic bacteria and across the corresponding chromatophore membrane of sonically prepared vesicles from the bacteria have been interpreted in terms of a proton-conducting action of this uncoupling agent.[5, 6]

In mitochondria respiring without phosphate-acceptor, the release of the controlled state of respiration by proton-conducting uncouplers may be accounted for quantitatively by the increase in the effective proton conductance (meaning the sum of the conductance to H^+ and OH^- ions) of the cristae membrane[7] as required by the chemiosmotic hypothesis of the coupling mechanism.[8] In the present paper, we describe measurements of the respiratory rate and the effective proton conductance of the membrane system of *M. denitrificans* in the presence and absence of the uncoupling agent FCCP; and we show that one possible explanation of the stimulation of the respiration of *M. denitrificans* by low concentrations of FCCP is that the effective proton conductance of the membrane is enhanced by the uncoupler, and inhibition of respiration by the back-pressure of the protonmotive force on the respiratory chain system is released.

Materials and Methods

Reagents

FCCP and valinomycin were gifts from Dr. P. G. Heytler of E. I. du Pont de Nemours and Co., Inc. (Wilmington, Delaware, U.S.A.) and Dr. J. C. MacDonald of Prairie Research Laboratory (Saskatoon, Saskatchewan, Canada), respectively. Gramicidin (mixture of A, B, and C, predominantly A) was obtained from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.).

Simple organic and inorganic reagents were of Analar grade where available, or otherwise of the highest purity obtainable commercially.

Standard acid (50 mM HCl) and alkali (50 mM KOH) for acid-base titrations of the bacteria were prepared in 100 mM KCl and were made anaerobic by degassing and flushing with oxygen-free nitrogen in Thunberg tubes. Ethanolic solutions of FCCP (2 mM), valinomycin (2 mg/ml), and gramicidin (1 mg/ml) were freed of air by bubbling with oxygen-free nitrogen. The standard acid, alkali, and the ethanolic solutions of FCCP, valinomycin, and gramicidin were dispensed from all-glass micrometer syringes (Agla micrometer syringes, Burroughs Wellcome and Co., London, N.W.1) fitted with long glass needles as previously described.[7]

Hydrogen peroxide used for introducing oxygen was dispensed as an aqueous solution approximately equivalent to 0·25 M O_2. Stock solutions of catalase were prepared by diluting 0·1 ml of C-100 catalase suspension of Sigma London Chemical Co. Ltd. (London, S.W.6) to 1 ml with 150 mM KCl. Carbonic anhydrase (Sigma) was freshly prepared (10 mg/ml) in 150 mM KCl.

Growth and Harvesting of Bacteria

M. denitrificans ATCC 13543 was grown and maintained on a medium similar to that described previously,[9] except that glucose was replaced by succinate. The medium contained 10 g K_2HPO_4, 2 g K_2SO_4, 2 g yeast extract (Difco Laboratories, Detroit 1, Michigan, U.S.A.), 4 g Bacto-peptone (Difco), 6 g succinic acid, adjusted to pH 6·8 with KOH and made up to a final volume of 1 l. Bacteria were maintained on the glucose

medium[9] solidified with 1% agar. Subcultures were grown on glucose medium to approximately 1 mg dry weight/ml and a 2 ml inoculum was used for each litre of culture.[9] The cultures (1 l in 10-l flasks) were aerated as described by Mitchell[10] and were grown to 0·5–0·7 mg cell dry weight/ml. The bacteria were harvested at 15,000 × *g* in an MSE High Speed 18 centrifuge (Measuring and Scientific Equipment Ltd., 25–28 Buckingham Gate, London, S.W.1), and for use in membrane conductance measurements the cells were washed twice in 150 mM KCl (which had been previously bubbled with N_2 gas) and suspended at approximately 50 mg cell dry weight/ml in the same medium. Bacteria used in oxygen uptake experiments were washed in 150 mM KCl–3 mM glycylglycine, and 150 mM KCl–10 mM glycylglycine was used for the suspending medium.

Dry Weight

An aliquot of the final suspension was dried at 100° for 24 h and corrected for KCl and glycylglycine content.

Reaction Chamber and Measurement of pH and Oxygen Tension

The reaction chamber and electrode systems were similar to that described by Mitchell and Moyle.[7] For experiments involving measurement of the effective proton conductance of the plasma membrane, the medium (150 mM KCl) was degassed and introduced into the reaction chamber (volume 4 ml) under a stream of N_2 gas. The bacterial suspension (0·5 ml containing approximately 25 mg cell dry weight) was then introduced with the stirrer running and the volume enclosed in the vessel was returned to 4 ml by lowering the piston. Residual oxygen was rapidly utilized, and thereafter the cells were allowed to equilibrate under strictly anaerobic conditions for 1 h before experiments were started. After this time any pH drift was either very small or absent.

Oxygen uptake was measured with a Clark oxygen electrode as described previously[7] in 150 mM KCl–10 mM glycylglycine (except where stated otherwise), introduced into the reaction chamber after equilibrating with air at 25°; and 0·2 ml of stock cell suspension, containing approximately 10 mg cell dry weight, was used. In some experiments the oxygen content of the medium was replenished by introducing small volumes of stock hydrogen peroxide solution. When this was done, catalase (50 μl of stock solution) was previously added to the suspension medium.

Suppression of Bicarbonate/CO_2 Equilibrium Artefact by Carbonic Anhydrase

The suspension media used in these experiments contained small amounts of HCO_3^-, H_2CO_3, and CO_2, even after degassing. In presence or absence of bacteria, the pH electrode response, following the introduction of a pulse of acid or alkali into the reaction vessel, exhibited an overshoot with a time of half decay of about 20 sec, especially at pH values on the acid side of 7. This was attributable to a time-dependent change of buffering power due to the sluggish reaction $CO_2 + H_2O \leftrightharpoons H_2CO_3$. The catalysis of the bicarbonate/CO_2 equilibration with carbonic anhydrase at a final concentration of 40 μg/ml was found to suppress the pH overshoot artefact; and carbonic anhydrase was therefore added at this concentration when a rapid pH electrode response was required.

Explanation of Results

Membrane Conductance to Protons

The characteristics of pulsed acid titrations of anaerobic suspensions of *M. denitrificans* (Fig. 1A) were similar to those observed in rat liver mitochondria.[7] The initial rapid decrease in the pH of the medium (pH_0) involving the titration of the buffers of the outer phase occurred faster than the rate of response of the pH electrode, but was followed by a relatively slow rise towards an equilibrium pH_0 which was accurately recorded by the electrode system. This paper is concerned only with the slow stage of the titration.

Figure 1A shows the time-course of pH_0 following a pulse of acid (1·6 μl of 50 mM HCl in 100 mM KCl) to an anaerobic suspension of *M. denitrificans* in unbuffered 150 mM KCl in the pH range 7·0 to 7·1, with and without the addition of FCCP (0·5 μM). After the initial very fast stage of the titration, the pH_0 decayed exponentially from an extrapolated (zero time) value pH_0^α towards a final equilibrium value pH_0^ω, so that the plot of $\ln(pH_0^\omega - pH_0)$ against time gave a straight line. In Fig. 1B the data of Fig. 1A have been replotted as $-\ln(pH_0^\omega - pH_0)$ against time. In this typical experiment the addition of FCCP decreased the time for half equilibration ($t_{1/2}$) from 270 to 120 sec.

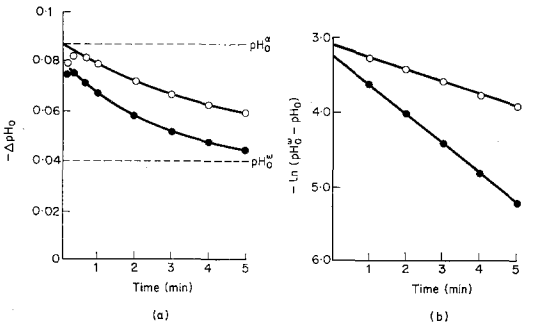

Figure 1. Time course of acid-base equilibration following pulsed acid titration of an anaerobic suspension of *M. denitrificans* in the presence and absence of FCCP. A. The initial pH was 7·098, and 1·6 μl of oxygen-free 50 mM HCl in 100 mM KCl was injected at zero time into 4 ml of anaerobic suspension (6·05 mg cell dry weight/ml) of *M. denitrificans* in the 150 mM KCl medium. Additions: none (○); 0·5 μM FCCP (●). B. The data of A plotted as $-\ln(pH_0^\omega - pH_0)$ against time.

Writing ΔpH_0^α for the initial pH_0 change resulting from the titration of the buffers outside the osmotic barrier or M phase of the plasma membrane, the buffering power of the outer aqueous phase per gramme dry weight of bacteria (B_0) is given by

$$B_0 = -\frac{\Delta H_0^+}{\Delta pH_0^\alpha} \qquad (1)$$

where ΔH_0^+ is the quantity of acid added per gramme dry weight of bacteria, and given that B_0 is virtually constant over the small pH_0 range corresponding to ΔpH_0^α. The value of ΔpH_0^α required to calculate B_0 is obtained from the extrapolation to zero time of the semilogarithmic plots of the titrations, as illustrated by Fig. 1B.

The value of the buffering power of the inner aqueous phase of the bacteria (B_I) is likewise obtained approximately[11] from the relationships

$$B_T = B_0 + B_I \qquad (2)$$

and

$$B_T = -\frac{\Delta H_0^+}{\Delta pH_0^\omega} \qquad (3)$$

where B_T stands for the total buffering power and ΔpH_0^ω means the difference between the initial and final equilibrium values of pH_0, either when the titration goes to completion in intact bacteria, or when the bacteria are lysed with *n*-butanol.[2] As shown in Fig. 2, the

value of B_T obtained after lysis by butanol was greater than that obtained when acid-base equilibration was hastened by the presence of valinomycin, SCN^-, and FCCP. As previously discussed[11] B_I would be expected to be underestimated in the intact bacterial suspensions, but overestimated in the lysed suspensions. For this reason we used the mean of the values of B_T (Fig. 2, curve C) obtained by the two methods to calculate the value of B_I (equation 2).

From the knowledge of the slope of the time-course of the acid-base titration and the values of B_0 and B_I, the effective proton conductance (C_M) of the M phase was calculated, using the relationship

$$C_M = \frac{B}{t_{1/2}} \ln 2 \qquad (4)$$

where, as previously discussed,[7] the differential buffering power B is defined by

$$\frac{1}{B} = \frac{1}{B_I} + \frac{1}{B_0} \qquad (5)$$

and the value of ln 2 is 0·69. This treatment of the data has been used to calculate values for C_M under various conditions for *M. denitrificans* in the pH_0 range 7·0 to 7·1. The normal cells give a value of 0·071 ± 0·004 μg ion of H^+/sec pH unit gramme cell dry weight (nine measurements). Other typical values, given in the same units, are: 0·075 for bacteria treated with 400 μg valinomycin per gramme cell dry weight; 0·086 for bacteria treated with 2 mg valinomycin per gramme cell dry weight; 0·082 in the presence of 7·5 mM KSCN; 0·133 in the presence of 28 mM KSCN; 0·150 in the presence of 2 μM FCCP; 0·167 for bacteria treated with 0·9 mg gramicidin per gramme cell dry weight. These values show that the effective proton conductance of the membrane of *M. denitrificans* is apparently only doubled by adding 2 μM FCCP alone. This is predictable in a system where the conductance to the major ions present is low, because the diffusion of protons (and OH^- ions) across the M phase is an electrogenic process which is limited by the development of a membrane potential unless a compensating movement of some other ion species can occur.

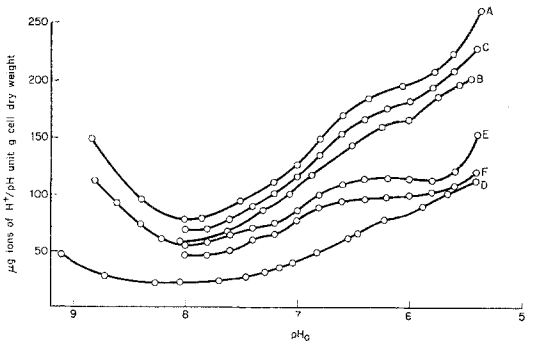

Figure 2. Buffering power of *M. denitrificans* as a function of pH_0 under various conditions. The measurements were done at 25° in 150 mM KCl with pulses of acid (oxygen-free 50 mM HCl in 100 mM KCl) as in the experiments of Fig. 1. Curve A shows the total buffering power in the presence of *n*-butanol (4%), and curve B shows the total buffering power in the presence of 2 mg valinomycin/g cell dry weight, 50 mM KSCN, and 5 μM FCCP. Curve C shows the average buffering power from curves A and B. Curve D shows the outer buffering power. Curve E shows the inner buffering power computed from curve A and curve D, while curve F shows the inner buffering power computed from curve C and curve D.

Osmotic swelling experiments with lysozyme-treated cells (P. B. Scholes, unpublished observations) have shown that the plasma membrane of *M. denitrificans*, like the cristae membrane of rat liver mitochondria[12] and the plasma membrane of *Staphylococcus aureus*,[13] has a high conductance to SCN^- ions. Thus, in the presence of SCN^- ions, the electrogenic movement of H^+ ions catalysed by FCCP should proceed rapidly with a compensating movement of SCN^- ions across the membrane. Both valinomycin in K^+ ion-containing media, and media containing SCN^- ions, have been used to demonstrate the catalysis of acid-base equilibration across the membrane of *M. denitrificans* by FCCP.

Figure 3 shows the effect of FCCP on acid-base equilibration, plotted as percentage equilibration on a logarithmic scale against time, following displacement of pH_0 to 7·0 by the addition of HCl to the bacterial suspension, previously equilibrated for 30 min at pH_0 7·4 with different concentrations of KSCN in the 150 mM KCl medium. After equilibration in the presence of 27 mM KSCN, addition of FCCP (1 μM) caused an exponential pH_0 decay with a $t_{1/2}$ of 10 sec, as shown by curve A. When a higher concentration of FCCP (7 μM) was used, the pH equilibration rate was increased ($t_{1/2} = 3{\cdot}5$ sec), as shown by curve B. When the concentration of KSCN used for the preincubation was only 7 mM (curve C), the time course of the equilibration did not correspond to a simple exponential process, but showed a first-order rate coefficient declining towards that characteristic of the cells that had not been preincubated with KSCN (curve D). Similar results were obtained when the bacteria were equilibrated with KSCN at pH_0 7·0–7·1 and pH_0 was displaced to 6·6–6·7.

In order to observe a rapid acid-base equilibration across the plasma membrane of *M. denitrificans* in the presence of valinomycin, it was necessary to preincubate the cells with valinomycin at neutral or acid pH_0. Figure 4 shows the effect of FCCP on acid-base equilibration, plotted as percentage equilibration on a logarithmic scale against time after the displacement of pH_0 to 7·1 by the addition of KOH to the bacterial suspension, previously equilibrated for 30 min with valinomycin at pH_0 6·7 in the 150 mM KCl medium. The equilibration initially showed a rapid stage, followed by a slower stage, superficially similar to that observed with 7 mM KSCN. The initial rates of equilibration for a given amount of FCCP were much faster in the presence of valino-

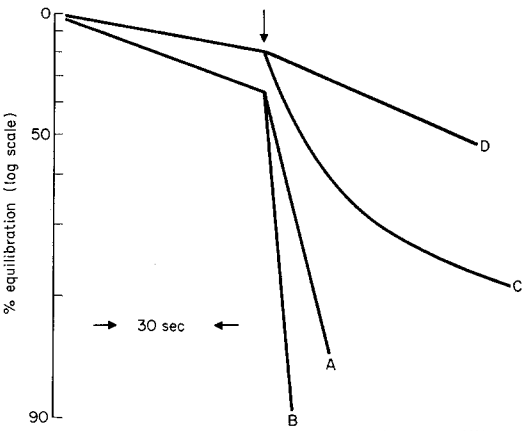

Figure 3. The effect of FCCP on acid-base equilibration in an anaerobic suspension of *M. denitrificans* previously incubated in the presence of SCN^-. The anaerobic suspension (7·2 mg cell dry weight/ml) was equilibrated with or without KSCN for 30 min at pH_0 7·4 in the 150 mM KCl medium at 25°. Oxygen-free 50 mM HCl in 100 mM KCl (8 μl) was used to displace pH_0 to about 7. Additions for preincubation: A and B, 27 mM KSCN; C, 7 mM KSCN; D, none. Addition of FCCP at arrow: A and D, 1 μM; B and C, 7 μM.

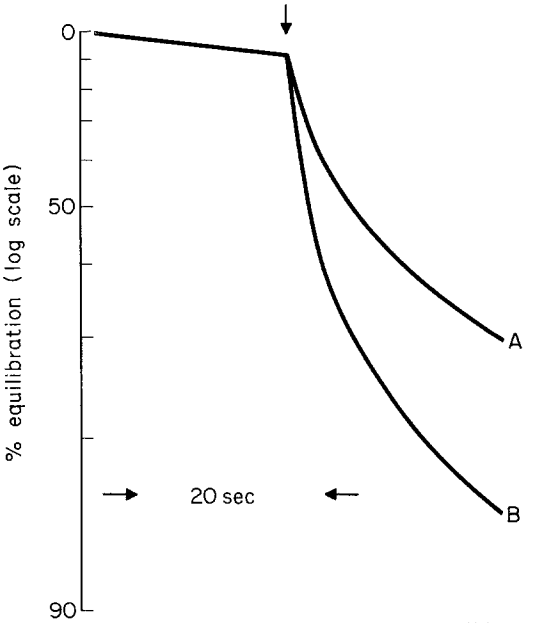

Figure 4. The effect of FCCP on acid-base equilibration in an anaerobic suspension of *M. denitrificans* previously incubated in the presence of valinomycin. The anaerobic suspension (8·2 mg cell dry weight/ml) was equilibrated for 30 min in the presence of valinomycin (1·5 mg/g cell dry weight) at pH_0 6·7 in the 150 mM KCl medium at 25°. The pH_0 was displaced to about 7·1 with 14 μl of KOH (oxygen-free 50 mM KOH in 100 mM KCl). Addition of FCCP at arrow: A, 1 μM; B, 2 μM.

mycin (2 μM FCCP, 2·8 sec) than in the presence of SCN^- (see Fig. 3); but the initial rates in presence of valinomycin declined to lower values than the constant rates observed at the higher SCN^- concentrations.

When *M. denitrificans* was incubated at pH_0 7·4 with valinomycin, and a pulse of HCl was added to bring pH_0 into the range 7·0 to 7·1, the effect of adding 1 μM FCCP on acid-base equilibration across the M phase was identical to that observed in the absence of valinomycin as in Fig. 3D. If, however, the bacteria were first incubated at pH_0 6·6 with valinomycin for 30 min and subsequently equilibrated at pH_0 7·4, a fast initial rate of equilibration was observed on adding 1 μM FCCP after pH_0 displacement into the range 7·0 to 7·1. Furthermore, if the bacteria were equilibrated at pH_0 7·0–7·1 with valinomycin and pH_0 was displaced to 6·6–6·7, a fast initial rate of equilibration was observed on adding 1 μM FCCP, and the $t_{1/2}$ of decay was 8 sec in a typical experiment.

In Fig. 5 the effective proton conductance of the membrane is plotted against FCCP concentration, A in 150 mM KCl after preincubation with valinomycin, and B and C in media containing 30 mM KSCN + 150 mM KCl and 50 mM KSCN + 150 mM KCl, respectively. Values for the buffering power differential B were computed from the average inner and outer buffering powers over the appropriate pH_0 range (Fig. 2), using equation (5). The $t_{1/2}$ values were obtained from experiments of the type described in Figs. 3 and 4, and the C_M values were calculated by substitution in equation (4).

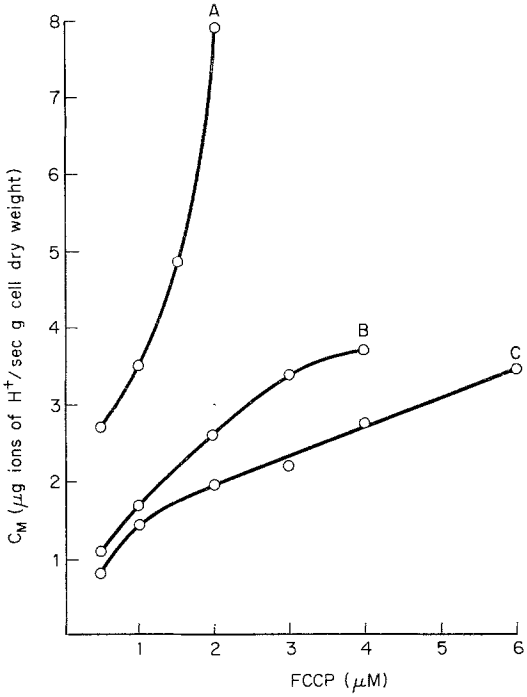

Figure 5. The effective proton conductance (C_M) of the membrane of *M. denitrificans* as a function of FCCP concentration. C_M was calculated using equation (4). The buffering power differential (B) was obtained by substituting in equation (5) the average values for B_0 and B_I obtained from curves D and F of Fig. 2 over the appropriate pH ranges. Curve A, $t_{1/2}$ was obtained as in the experiments of Fig. 4 in the presence of 1·5 mg valinomycin/g cell dry weight, FCCP being added after displacement of pH_0 from 6·7 to 7·1. Curve B, $t_{1/2}$ was obtained as described in the experiments of Fig. 3 but after preincubation with the addition of 50 mM KSCN, the FCCP being added after displacement of pH_0 from 7·4 to 7·0. Curve C, $t_{1/2}$ was obtained as for curve B but after preincubation with 30 mM KSCN.

The Effect of FCCP on the Respiratory Rate

After anaerobic cells were transferred to the aerobic medium, the rate of oxygen uptake (initial fast respiration) decreased (Fig. 6A) from a high initial value towards a lower rate which became constant after 5 min (slow steady-state respiration), the oxygen content of the medium being replenished by the addition of hydrogen peroxide in the presence of catalase. When, after a brief period of anaerobiosis, the cell suspension was given a pulse of oxygen (H_2O_2 in the presence of catalase), the initial fast respiration slowed to the same slow steady-state respiration rate obtained before anaerobiosis (Fig. 6B). In either

case, respiration could be stimulated by the addition of 0·5 μM FCCP. The maximum stimulation of the respiratory rate by FCCP, expressed as a respiratory control ratio (defined as the ratio of rate with uncoupler to rate in the absence of uncoupler), by analogy with respiratory control ratios in mitochondria, was obtained with suspensions giving slow steady-state respiration (Fig. 6C). When, after a brief period of anaerobiosis, cells in the presence of 0·5 μM FCCP were given a pulse of oxygen, the respiratory rate returned promptly to, and remained at, the stimulated rate.

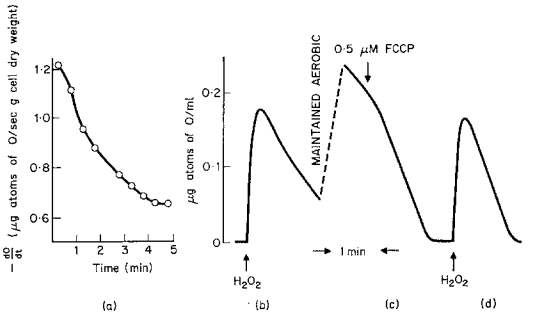

Figure 6. Different respiratory states in suspensions of *M. denitrificans* in the absence and presence of FCCP. A. Time course of respiratory rate in a typical experiment when anaerobic cells of *M. denitrificans* were transferred to the aerobic medium (10 mM glycylglycine–150 mM KCl) at pH_0 7·0–7·1 and 25°. The suspension was maintained aerobic by the addition of H_2O_2 in the presence of catalase. B. Cells giving slow steady-state respiration were allowed to go anaerobic and the respiratory rate was observed after a pulse of oxygen (H_2O_2 at arrow in presence of catalase). C. Cells were maintained in the slow steady-state of respiration by the addition of H_2O_2, and 0·5 μM FCCP was added at arrow. D. Following a brief anaerobic period, the suspension was made aerobic at arrow by addition of H_2O_2. The pH_0 was maintained in the range 7·0 to 7·1 by adding 5 mM KOH in 100 mM KCl.

The effect of a range of concentrations of FCCP on the slow steady-state respiration of the bacterial suspensions is shown in Fig. 7. The initial respiratory stimulation increased with FCCP concentration to a maximum at 1·5 μM FCCP, and remained constant with larger concentrations up to 3 μM FCCP. When bacterial suspensions were maintained aerobic in the presence of 1·5 μM FCCP or more, the stimulated respiration was followed by an inhibited respiration which developed more rapidly at the higher FCCP concentrations.

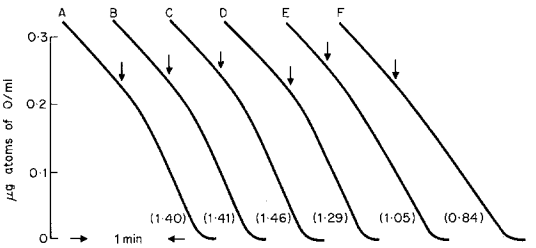

Figure 7. The effect of different FCCP concentrations on the transition from slow steady-state respiration to FCCP-stimulated respiration in *M. denitrificans*. Cell suspensions in the 10 mM glycylglycine–150 mM KCl medium were maintained aerobic at 25° for 5 min prior to addition of FCCP, added at the following final concentrations, as indicated by the arrow: A, 0·2 μM; B, 0·5 μM; C, 1 μM; D, 1·5 μM; E, 2 μM; F, 3 μM. The rate of the slow steady-state respiration was 0·7 μg atoms of O/sec gramme cell dry weight and the rate of FCCP stimulated steady-state respiration is given for each trace. The pH_0 was maintained in the range 7·0 to 7·1 by adding 5 mM KOH in 100 mM KCl.

The four states of respiration—initial fast, slow steady-state, FCCP-stimulated, and FCCP-inhibited—have been observed with cells respiring in media with pH_0 between 6 and 8. The respiratory rates corresponding to the different types of respiration in absence (A) and presence (B) of 5 mM succinate in the pH_0 range 7·0 to 7·1 are summarized in Table I.

When succinate was added to cells with slow steady-state respiration, an increase in respiratory rate was observed (Table I) and this rate could be stimulated by the addition of FCCP. With large amounts of FCCP (7 μM), an inhibited rate was also obtained. Furthermore, when succinate was added to cells respiring in presence of 7 μM FCCP, no increase in respiratory rate was observed.

One possible explanation of the increase in respiratory rate observed on adding FCCP

TABLE I. The rates of respiration of *M. denitrificans* observed under different conditions

Experimental conditions	Oxygen uptake (μg atoms of O/sec gramme cell dry weight) A	B
Initial fast rate of respiration	1·51 ± 0·18 (24)	1·44
Slow steady-state respiration after 5 min	0·72 ± 0·07 (26)	0·75
Rate after adding succinate (5 mM)		1·35
Stimulated rate after FCCP (a) 1 μM	1·30 ± 0·1 (8)	2·11
(b) 2 μM	1·39 ± 0·04 (3)	2·58
Inhibited rate after 5 μM FCCP	0·57 ± 0·05 (3)	0·73
Respiratory stimulation by 1 μM FCCP	1·78 ± 0·14 (8)	1·56

A. Average rates for four states of respiration are listed together with average respiratory stimulation ratio obtained after addition of 1 μM FCCP. The measurements were done in the pH_0 range 7·0 to 7·1 in 150 mM KCl–10 mM glycylglycine medium at 25°. Suspensions were maintained aerobic with pulses of H_2O_2 in the presence of catalase, and the pH_0 was adjusted with pulses of 50 mM KOH in 100 mM KCl. B. Rates of respiration and respiratory stimulation ratio are given for a typical experiment in which succinate was added during slow steady-state respiration.

is that a change in pH_I (the inner pH) might increase the activity of the dehydrogenases of the respiratory chain, which have optima in the pH range 7·6 to 8.0.[14] When in the pH_0 range 7·0 to 7·1, butanol was used to disrupt cells during slow steady-state respiration, a pH_0 displacement was observed; and making use of the appropriate buffering power curves (Fig. 2) it was possible to compute the approximate pH_I[11] at the moment of lysis. This type of experiment showed that no matter whether the pH_0 was at 6 or 8, the value of pH_I was between 7·2 and 7·4 (i.e., acid to the pH optimum of the dehydrogenases). We were therefore able to measure the release of respiration by FCCP in suspensions with pH_0 between 7·2 and 7·4, when the pH_0 and pH_I were nearly equal. Figure 8A shows that at pH_0 7·39 the addition of 0·5 μM FCCP was not accompanied by a pH_0 change, although respiratory stimulation by a factor of 1·6 was observed. A similar result was obtained at pH_0 7·34 on the addition of 1 μM

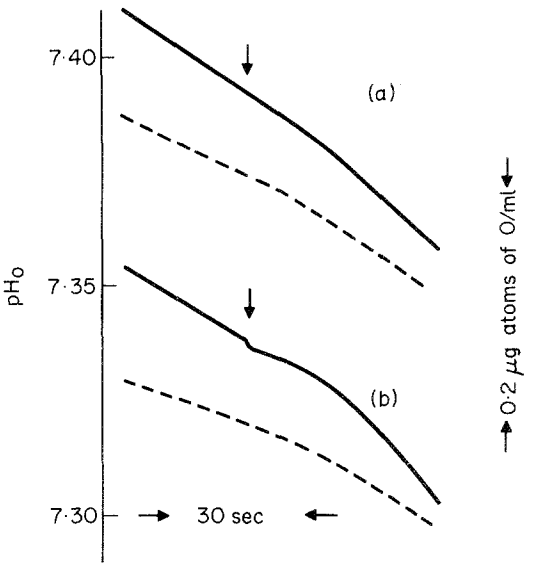

Figure 8. Changes of pH_0 and respiratory activity following the addition of FCCP to a suspension of *M. denitrificans*. The bacterial suspensions in 150 mM KCl–3 mM glycylglycine medium were maintained aerobic at 25° by addition of H_2O_2 and in the range pH_0 7·3 to 7·4 by the addition of 50 mM KOH in 100 mM KCl. A. Addition of FCCP (0·5 μM) at arrow to a cell suspension containing (2·3 mg cell dry weight/ml) at pH_0 7·39. B. Addition of FCCP (1 μM) at arrow to a cell suspension (2·0 mg cell dry weight/ml) at pH_0 7·34. Continuous curves, pH_0; broken curves, oxygen uptake.

FCCP. Since, under these conditions, pH_I is acid to the pH optimum of the dehydrogenases, the addition of FCCP to cells in slow steady-state respiration in a medium in the pH_0 range 7·0 to 7·1 would be expected to slow the respiratory rate, and not stimulate it as observed, if the rate was controlled by a pH effect on the dehydrogenases.

The FCCP-inhibited rate of respiration observed when the pH of the outer medium was acid was lower than at neutral or alkaline pH. It is possible that this is caused by a direct pH effect on the dehydrogenases since the addition of KOH to bring the outer pH near 7·6 resulted in a gradual increase of the FCCP-inhibited rate of respiration.

Discussion and Conclusions

In order to compare the effective proton conductance of the plasma membrane of *M. denitrificans* with that of other membranes it is necessary to express the values estimated as μg ion of H^+/sec pH unit gramme cell dry weight as values per unit area of membrane; and it is also convenient to convert from chemical to electrical units[7] by multiplying by the factor $F^2/2{\cdot}3RT$, where F stands for the Faraday and R is the gas constant. The membrane area of *M. denitrificans* is estimated to be approximately 25 m^2/g cell dry weight.[9,15] Thus, we calculate that the effective proton conductance of the normal plasma membrane of *M. denitrificans* (0·071 $\pm$ 0·004 μg ion of H^+/sec pH unit gramme cell dry weight) corresponds to 0·47 μmho/cm^2 of plasma membrane. This is close to the value of about 0·45 μmho/cm^2 of cristae membrane, estimated for rat liver mitochondria.[7] Since FCCP alone in the usual KCl medium stimulates the rate of acid-base equilibration across the M phase of *M. denitrificans* by a factor of about 2, it appears that the normal effective proton conductance of the M phase is about half the total conductance to other ions present. It follows that the conductance to either K^+ or Cl^-, which are the major ion species present, cannot be greater than some 1 μmho/cm^2 at pH 7·0–8·1, similar to that observed for rat liver mitochondria.[7] The low effective proton conductance of the membrane of *M. denitrificans* corresponds to that of other bacteria.[1–6]

In the presence of thiocyanate ions a rapid acid-base equilibration was observed on adding FCCP, presumably because the entry of H^+ can be compensated by an equivalent permeation of SCN^-, keeping the system near electrical neutrality. It is obvious that the extent of this process should be limited by the rise in internal concentration of the penetrating SCN^- ion. When the back-pressure of the osmotic force on the SCN^- ion approaches the forward-pressure due to the membrane potential generated by the diffusion of H^+ ions (or of OH^- ions the other way) across the membrane, the diffusion of SCN^- ions (and of H^+ and OH^- ions) across the membrane should slow; and thus the extent of the SCN^- ion effect should depend on the initial SCN^- ion concentration, as observed. The later slow acid-base equilibration observed with low SCN^- ion concentrations presumably depends on the exit of K^+ ions or entry of Cl^- ions which redistribute across the membrane relatively slowly because the conductance of the membrane to these ions is relatively low.

The two-stage acid-base titration induced by FCCP in the 150 mM KCl medium after treating the cells with valinomycin (Fig. 4) could not be accounted for simply by the classical cyclic K^+ ion-conducting action of valinomycin, because the high concentration of K^+ ions present should have been sufficient to permit the conduction of H^+ ions across the membrane to go almost to equilibrium without the development of an appreciable

opposing membrane potential, as was the case in 27 mM KSCN (Fig. 3). The initial extent of the fast equilibration in presence of valinomycin was about the same as the amount of valinomycin present; suggesting that the K^+–valinomycin complex in the membrane gave rise to a limited electrical neutralization, but that the valinomycin did not recycle rapidly across the membrane, as in the usual K^+ ion-ferrying action. The fact that the initial rate of acid-base equilibration induced by FCCP in presence of valinomycin was about twice as fast as in the presence of the higher concentration of SCN^- suggests that the proton-conducting effect of a given concentration of FCCP may be enhanced by the presence of valinomycin, as appears to be the case in chloroplasts.[16] This might be accounted for by a space-charge effect as previously proposed.[17-19] The presence of the comparatively lipid-soluble SCN^- ion may conversely tend to antagonize the action of FCCP by depleting the concentration of the anionic form of FCCP in the M phase. Relevant factors affecting the mobility and K^+-carrying action of valinomycin in artificial lipid membranes have been considered by Johnson and Bangham.[20,21] The failure of other agents to act in the membranes of certain microorganisms under certain conditions has previously been attributed to their failure to penetrate or to be mobile in the membrane.[22-24]

The data of Fig. 5 show that in presence of 2 μM FCCP, when the movements of ions other than H^+ and OH^- are probably not rate limiting, the effective proton conductance C_M is at least 2·6 μg ion of H^+/sec pH unit gramme cell dry weight (curve B) because, as discussed above, the presence of SCN^- may decrease the effectiveness of proton conduction by FCCP. Using the same method as before, we calculate that this effective proton conductance in presence of 2 μM FCCP corresponds to 17·4 μmho/cm^2.

We have shown elsewhere[25] that the respiration of *M. denitrificans*, using endogenous substrates, is linked to the outward translocation of H^+ ions and that the $\rightarrow H^+/O$ quotient is at least 6 and may be 8 under normal respiratory conditions. Thus, the measurements of respiratory rate described in this paper (Table I) show that normal steady-state respiration, using endogenous substrates, corresponds to an effective proton flux of 4·3 or 5·8 μg ion of H^+/sec gramme cell dry weight, using $\rightarrow H^+/O$ quotients of 6 and 8, respectively. Since the effective proton flux is given by the product of the effective proton conductance and the total protonmotive potential,[7] the observed proton conductance of 0·071 μg ion of H^+/sec pH unit gramme cell dry weight would correspond to a flux of only 0·32 μg ion of H^+/sec gramme cell dry weight at a total protonmotive potential of 270 mV. We conclude that the effective proton conductance of the plasma membrane of *M. denitrificans* is low enough (by a factor of 10) to enable coupling between respiration and phosphorylation to be mediated by a current of H^+ ions at a protonmotive potential of some 250 mV, as required by the chemiosmotic coupling hypothesis.[8]

In presence of 2 μM FCCP, the effective proton conductance of the membrane of *M. denitrificans* would correspond to a proton flux of 9·6 μg ion of H^+/sec gramme cell dry weight at a total protonmotive potential of 220 mV.[8] The FCCP-stimulated respiratory rate, using endogenous substrates, in presence of 2 μM FCCP was 1·39 μg atoms of O/sec gramme cell dry weight, and this corresponds to effective proton fluxes of 8·3 or 11·1 μg ion of H^+/sec gramme cell dry weight at a total protonmotive potential of 220 mV, using $\rightarrow H^+/O$ values of 6 and 8, respectively. Thus, in the presence of 2 μM FCCP, the proton flux that would be dissipated across the membrane at a protonmotive potential of 220

mV would be about equal to the whole of the proton flux that would be generated by respiration, if there were no other dissipatory reactions. The fact that, in absence of FCCP, the effective proton conductance of the membrane accounts for only about one tenth of the dissipation of the proton current shows that a considerable additional dissipation or utilization of the free energy of oxidoreduction must normally occur. It follows that, taking into account the existence of other reactions utilizing some of the free energy of oxidoreduction, the membrane potential should fall considerably below 220 mV when the effective proton conductance of the membrane was raised to the value observed in presence of 2 μM FCCP.

We conclude that the stimulation of respiration by concentrations of FCCP in the range 0·5 to 3 μM (Fig. 1) may possibly be due to the release of a respiratory control effect exerted at the level of the respiratory chain, as in mitochondria.[8] We have excluded the possibility that release of respiration by FCCP is due to a change of intracellular pH. But our observations do not exclude other possibilities, such as that the respiratory rate may be controlled by substrate availability and that this availability may be affected by FCCP. Further work will be required to show whether the transition from slow steady-state respiration to the stimulated steady-state induced by FCCP in *M. denitrificans* is characterized by the classical transitions of the redox states of the respiratory carriers diagnostic of respiratory control at the level of the respiratory chain.

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