Bioenergetics (1970) **1**, 309–323.

Respiration-Driven Proton Translocation in Micrococcus Denitrificans

Peter Scholes and Peter Mitchell

Glynn Research Laboratories, Bodmin, Cornwall, England

Abstract

The polarity and stoichiometry of respiration-driven proton translocation was studied by electrometric and spectrophotometric techniques in *Micrococcus denitrificans* in the context of the energy transduction mechanism in bacterial oxidative phosphorylation.

1. Protons are ejected through the plasma membrane during respiratory pulses and thereafter diffuse slowly back.

2. In presence of ionic species mobile across the membrane (K^+-valinomycin, K^+-gramicidin, or SCN^-), limiting $\rightarrow H^+/O$ quotients of 8 were obtained with endogenous respiratory substrates, and the rate of translocation (14·3 μg ions of H^+/sec g cell dry weight) was commensurate with that of respiration optimally stimulated by FCCP* at an $\rightarrow H^+/O$ quotient of 8.

3. The rate of decay of the proton pulses was greatly increased by FCCP, but there was little or no effect on the $\rightarrow H^+/O$ quotient characteristic of the respiratory system.

4. Various interpretations of the observations are discussed, and it is concluded that respiration is probably coupled directly or indirectly to electrogenic proton translocation. The observations are compatible with the chemiosmotic hypothesis of coupling between respiration and phosphorylation.

Introduction

Mitchell[1] observed that during the oxidation of endogenous substrates or of ethanol by *Micrococcus lysodeikticus*, there was an outward translocation of protons. More recently, proton translocation has been observed in whole photosynthetic bacteria and blue-green algae.[2–7] It was found that these organisms, like rat liver mitochondria,[8] translocated protons outwards.

The object of the work described in this paper was to investigate the conditions required for obtaining stoichiometric proton translocation during the rapid reduction of small pulses of oxygen by endogenous substrates in *Micrococcus denitrificans*; and to obtain information that would help to shed light on the possible mechanisms by which activity of the respiratory chain is coupled to proton translocation.

As the $\rightarrow H^+/O$ quotient has been found to be equal to the product of the P/O quotient and the $\rightarrow H^+/P$ quotient in rat liver mitochondria,[8–10] and the P/O stoichiometry of bacterial oxidative phosphorylation cannot readily be measured, except in comparatively uncoupled subcellular membrane preparations,[11] the $\rightarrow H^+/O$ quotients estimated in

* Abbreviations: FCCP, carbonylcyanide *p*-trifluoromethoxy phenylhydrazone; BCP, bromocresol purple; BTB, bromothymol blue; BSA, bovine serum albumin; $t_{1/2}$, time for half equilibration or half reaction; pH_0, the pH of the outer or suspension medium.

whole bacteria may provide valuable information concerning the possible energy-transducing characteristics of the bacterial respiratory chain system.

There is an obvious disadvantage in using endogenous substrates as reductants in studies of characteristics of the respiratory chain system such as the $\rightarrow H^+/O$ quotient (or the P/O quotient), because such characteristics depend on the point or points from which reducing equivalents travel down the respiratory chain during oxygen reduction. There is, however, the advantage that the participation of substrate porter systems that may themselves be linked to proton translocation[12] is avoided. Moreover, it has been shown that the respiratory chain system of *M. denitrificans* is very similar to that of mitochondria[11, 13, 14] and includes NAD- and NADP-linked dehydrogenase systems; and observations on the absorbance difference $A_{374-340}$ have indicated oxidoreduction of nicotinamide adenine nucleotides during respiratory pulse experiments similar to those described in the present paper (P. Scholes and P. Hinkle, unpublished data). Thus, with the knowledge that the respiratory chain and the nicotinamide adenine nucleotides were reduced by the endogenous substrates under appropriate conditions, we expected to be able to obtain $\rightarrow H^+/O$ quotients characteristic of all or of most of the respiratory chain.

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.). Bromocresol purple (BCP) and glycylglycine were obtained from Hopkin and Williams Ltd. (Chadwell Heath, Essex). Crystalline bovine serum albumin and carbonic anhydrase were obtained from Sigma London Chemical Co. Ltd. (London, S.W.6).

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

Carbonic anhydrase was freshly prepared (10 mg/ml) in 150 mM KCl. Standard acid and alkali, and ethanolic solutions of FCCP, valinomycin, and gramicidin were prepared and made oxygen-free as described previously.[15]

Growth and Harvesting of Bacteria

M. denitrificans ATCC 13543 was grown and maintained as previously described.[15] The bacteria were harvested at 15,000 × *g* in a M.S.E. High Speed 18 centrifuge (Measuring and Scientific Equipment Ltd., 25–28 Buckingham Gate, London, S.W.1). After two washes in 150 mM KCl-3 mM glycylglycine buffer at pH 7·0, the cells to be used in experiments at outer pH (pH_0) 7·0–7·1 were suspended in the same medium at approximately 50 mg cell dry weight/ml, but cells to be used in experiments at pH_0 6·0–6·1 were suspended in 150 mM KCl–10 mM glycylglycine buffer at pH 7·0. All washing and suspending media were de-oxygenated by bubbling with a stream of oxygen-free nitrogen gas, and the temperature was maintained at about 4° during the preparation of the washed cell suspensions.

Measurement of Respiration-Driven Proton Translocation

The reaction cell and electrode system was as described by Mitchell and Moyle.[16] The degassed media of 150 mM KCl–3 mM glycylglycine for experiments at pH_0 7·0–7·1 or 150 mM KCl–10 mM glycylglycine for experiments at pH_0 6·0–6·1 was introduced into the reaction chamber (volume 4 ml) under a stream of nitrogen gas. About 25 mg dry weight of bacterial cells in 0·5 ml of the appropriate anaerobic medium were then added with the stirrer running, and the volume enclosed by the vessel was returned to 4 ml by lowering the piston of the reaction cell. Residual oxygen was rapidy used up, and thereafter the organisms were maintained under strictly anaerobic conditions. The cells were allowed to equilibrate for 1–2 h before experiments were started. After this time any baseline drift of the pH_0 was very slow. Carbonic anhydrase was added to suspension media as described previously.[15]

Known quantities of oxygen in air-saturated 150 mM KCl were introduced into the anaerobic suspension, and the resulting respiration-driven acidification of the medium was compared with calibrations of pH_0 displacement due to addition of standard acid or alkali to permit estimation of the quantity of protons translocated per oxygen atom reduced, referred to as the $\rightarrow H^+/O$ quotient, as described before.[8]

Corrections for Response-Time of the pH-Measuring System

The rationale for correcting respiration-driven proton pulses has been discussed previously.[8] When the $t_{1/2}$ of decay is 100 times greater than the response-time of the electrode, the error introduced by extrapolating the value of the observed outer pH change (ΔpH_0) is about 1%. In most experiments described in this paper the decay rate of ΔpH_0 was slow, and corrections have been made by the previous method[8] only in the experiments of Fig. 10 where rapid decay rates were observed.

Extrapolation of ΔpH_0 to Give Initial Quantity of Protons Translocated

In mitochondria, it has been shown that the rate of proton translocation at the beginning of a pulse of respiration is faster than the State 3 rate of respiration under conditions where the membrane potential is neutralized.[17] The $\rightarrow H^+/O$ quotients were measured by extrapolation of the ΔpH_0 decay curve, which may be scaled to read as an $\rightarrow H^+/O$ curve, back to a time corresponding to 50% reduction of the oxygen in the pulse.[8,18] In *M. denitrificans*, the rate of respiration, stimulated by FCCP (corresponding to the State 3 rate), is 1·39 μg atoms of O/sec g cell dry weight,[15] while the rate of respiration estimated from the rate of proton translocation during a respiratory pulse in presence of valinomycin, measured using BCP (Fig. 11) and assuming an $\rightarrow H^+/O$ quotient of 8, was 1·79 μg atoms of O/sec g cell dry weight. We have accordingly estimated the time for half reduction ($t_{1/2}$) of 1 μg atom of O/g cell dry weight in the pulsed respiration experiments as 0·3 sec, and have extrapolated the decay of the proton pulses back to this time. In practice extrapolation to zero time did not differ significantly from extrapolation to 0·3 sec except when the $t_{1/2}$ of decay of the proton pulses was less than 30 sec.

Measurement of the Rate of Proton Translocation

Changes of pH_0 occurring within 2 sec cannot be closely followed using the glass electrode system described here, because the observed changes are dominated by the

response-time of the electrode, which corresponded to a $t_{1/2}$ of 0·33 sec. We have therefore followed the pH_0 change spectrophotometrically, using BCP (ref. 19).

The problems associated with the use of BTB (refs. 20–23) for measuring the pH of a particular phase of three-phase systems, such as mitochondrial or bacterial suspensions, apply also to the structurally similar, but rather less lipid-soluble, BCP. We found that some 15% of BCP (20 mM) added to a bacterial suspension (5 mg cell dry weight/ml) records the pH of the inner phase. However, when BCP was adsorbed on bovine serum albumin (BSA), present as a 1% solution in the suspension medium, the equilibrium pH_0 changes measured with a glass electrode were identical to those measured by the change of absorbance of BCP, but the apparent rate of pH_0 change recorded by the latter technique during respiratory pulses was the more rapid, as expected.

The absorbance changes of BCP were measured using a dual wavelength spectrophotometer of conventional design, fitted with a toothed light-chopping wheel giving alternation between the reference and measuring beams at 5000 cycles/sec. A measuring wavelength of 580 nm and reference wavelength of 620 nm were used. The cuvette and the arrangement of the pH electrode and stirrer were as described previously.[20] Recordings of signals from the pH electrode and spectrophotometer were fed into a multichannel strip-chart recorder (Oscillograph type 5-127 of Bell and Howell Ltd., Basingstoke, Hants.). The measurements were done in the pH_0 range 6·0–6·1 in bacterial suspensions (5 mg cell dry weight/ml) in 150 mM KCl–10 mM glycylglycine (total volume 5·5 ml) with addition of 1% BSA, 450 μg of carbonic anhydrase and a final concentration of 20 μM BCP.

The concentration of BCP was maintained constant by incorporating 20 μM BCP in the calibrating acid, alkali and air-saturated KCl solutions which were otherwise as described previously.[8]

Rationale of the Measurement of $\rightarrow H^+/O$ Quotients in Bacteria

The conclusions drawn from the measurements of $\rightarrow H^+/O$ quotients in mitochondria,[8] that it is important to use the smallest oxygen pulses consistent with the accurate measurement of the corresponding pH_0 displacement, and that conditions should be arranged so as to minimize the build up of an opposing pH difference or electric potential difference across the coupling membrane, have been used in the measurements on the bacterial suspensions described in this paper.

Results

The Displacement of pH_0 During Pulses of Respiratory Activity

Figure 1 shows the time-course of the observed outer pH change (ΔpH_0) when anaerobic suspensions of *M. denitrificans* in a medium containing 150 mM KCl buffered with 3 mM glycylglycine at pH_0 7·0–7·1 or 10 mM glycylglycine at pH_0 6·0–6·1 were pulsed with small amounts of oxygen (about 1 μg atom/g cell dry weight, as indicated in legend). The medium was slowly acidified, and the subsequent decay of ΔpH_0 was extremely slow in both pH_0 ranges. When the effective proton conductance of the plasma membrane was increased by the presence of 2·5 μM FCCP (ref. 15), the respiration-driven changes in pH_0 were much decreased. These observations indicate that the changes in pH_0 are the result of proton translocation.

The change of pH_0 is sluggish compared with respiratory activity, for, under similar experimental conditions, the cycle of oxidation and reduction of cytochrome $(a + a_3)$, estimated by the absorbance difference $A_{607-630}$, occurs within 1 sec (P. Hinkle and P. Scholes, unpublished data).

When the size of the injected oxygen pulse was decreased from about 1 μg atom/g cell dry weight, as in Fig. 1, to 0·1 μg atom/g cell dry weight, there was an increase in the $\rightarrow H^+/O$ quotient, and in some cases values greater than 4 were recorded.

Figure 2 shows that when the oxygen was added at different constant rates to anaerobic cell suspensions at pH_0 7·0–7·1, the $\rightarrow H^+/O$ quotient was higher at the slower rates, but the decay rate of ΔpH_0 was apparently unaffected. At pH_0 6·0–6·1, there was a relatively greater increase in the $\rightarrow H^+/O$ quotient at slower rates of oxygen utilization, and the decay of ΔpH_0 was more rapid. The $\rightarrow H^+/O$ quotients obtained by extrapolation of the time-course of ΔpH_0 (see Materials and Methods) are given under the curves of Fig. 2.

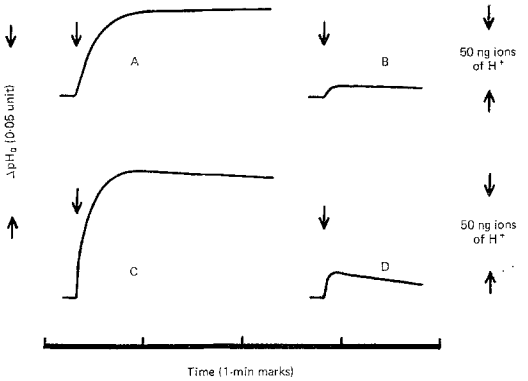

Figure 1. Time-course of respiration-driven ΔpH_0 in presence and absence of FCCP. Bacteria (5·25 mg cell dry weight/ml) were equilibrated under anaerobic conditions at 25° in 150 mM KCl-3 mM glycylglycine for measurements (A and B) in the pH_0 range 7·0–7·1, and in 150 mM KCl-10 mM glycylglycine for measurements (C and D) in the pH_0 range 6·0–6·1. Oxygen (23·5 ng atoms) was added as air-saturated 150 mM KCl at the arrows. FCCP (2·5 μM) was present in B and D. The quantity of H^+ ions equivalent to the recorded pH_0 changes is given for the 4 ml of suspension. Increase in acidity of the outer phase is shown upwards.

The Effect of Valinomycin on ΔpH_0 During Pulses of Respiration

Figure 3 shows the quantity of protons translocated outwards per oxygen atom reduced when a cell suspension in the range pH_0 6·0–6·1 was pulsed with air-saturated saline at several time intervals after the addition of valinomycin (0·75 mg/g cell dry weight). Under these conditions, proton translocation occurred very rapidly and, as shown in the semi-logarithmic plots of Fig. 4A to C, the subsequent decay of the pulses was exponential. Over a period of 1–3 h incubation with valinomycin, an increase was observed both in the extrapolated $\rightarrow H^+/O$ quotient and in the subsequent rate of ΔpH_0 decay (Fig. 4). The extrapolated $\rightarrow H^+/O$ quotients obtained after 2 h preincubation with valinomycin in the range pH_0 6·0–6·1 gave an average of $7·5 \pm 0·3$ in 12 experiments. The $t_{1/2}$ of decay was between 120 and 150 sec, after preincubation with about 0·75 mg valinomycin/g cell dry weight. However, after preincubation of 2 h with larger amounts of valinomycin (about 2·0 mg/g cell dry weight), the $t_{1/2}$ of decay fell to 75 sec.

Experiments in the pH_0 range 7·0–7·1 gave qualitatively similar results to those of Fig. 3, although longer preincubation in the presence of valinomycin was required in the higher pH_0 range to obtain the transition from the type of proton pulse shown in Fig. 1 to that shown in Fig. 3. The limiting $\rightarrow H^+/O$ quotient was usually lower after preincubation at neutral than at acid pH_0. The average value for six experiments was $6·0 \pm 1·2$, but values between 7 and 8 were recorded in some experiments. An important difference between experiments carried out in the two pH_0 ranges is seen in the decay

of ΔpH_0, which was not exponential (Fig. 4D) at pH_0 7·0–7·1. A non-exponential decay of ΔpH_0 was obtained in the range pH_0 7·0–7·1, when preincubation of the anaerobic suspension with valinomycin was carried out at either acid or neutral pH_0 ranges.

Under the conditions where high stoichiometries were obtained in the presence of valinomycin, the extrapolated $\rightarrow H^+/O$ quotient remained constant over a wide range of oxygen quantities added. In one series of experiments at pH_0 6·0–6·1, the value of

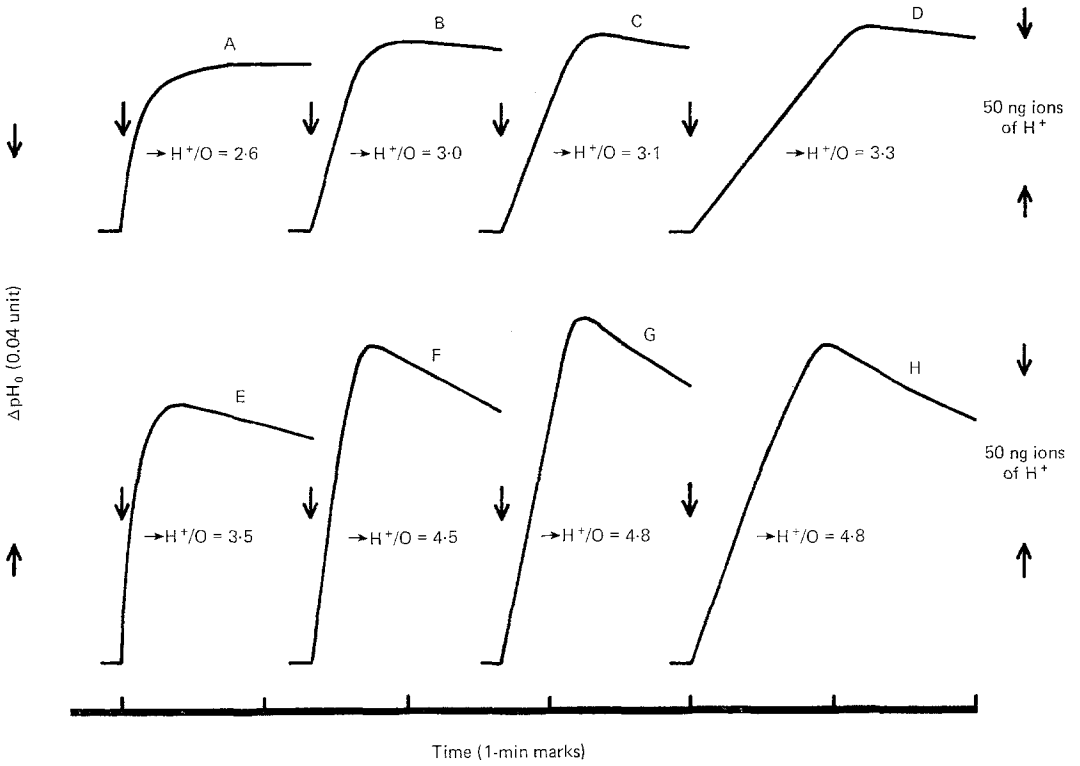

Figure 2. The effect of respiration rate on the time-course of respiration-driven ΔpH_0. Experiments were done at pH_0 7·0–7·1 (A, B, C, D) with a cell suspension containing 5·1 mg cell dry weight/ml; and at pH_0 6·0–6·1 (E, F, G, H) with a cell suspension containing 5·9 mg cell dry weight/ml under the same conditions as for Fig. 1. The oxygen (23·5 ng atoms O) as air-saturated 150 mM KCl was injected (commencing at the arrows) during the following times: A, about 0·2 sec; B, 18 sec; C, 29 sec; D, 68 sec; E, about 0·2 sec; F, 16 sec; G, 32 sec; H, 50 sec. The quantity of H^+ ions equivalent to the recorded pH_0 changes is given for the 4 ml of suspension. The values of $\rightarrow H^+/0$ given for each trace are calculated by extrapolating the decay of ΔpH_0 to the $t_{1/2}$ of oxygen reduction. Outward proton translocation is represented upwards.

the extrapolated $\rightarrow H^+/O$ quotient was 7·5 when the amount of oxygen reduced was between 0·4 and 2·0 μg atoms/g cell dry weight.

The Effect of Gramicidin on ΔpH_0 During Pulses of Respiration

The change in the characteristics of the time-course of ΔpH_0, and in the observed maximum values of $\rightarrow H^+/O$, after preincubation with gramicidin in the pH_0 ranges 6·0–6·1 and 7·0–7·1, are illustrated in Fig. 5. In both cases there was a considerable increase in the observed maximum $\rightarrow H^+/O$ values compared with corresponding

respiratory pulses in the absence of gramicidin (Fig. 1). In the pH_0 range 6·0–6·1 the average maximum extrapolated $\rightarrow H^+/O$ quotient was 7·3 ± 0·6 (two experiments) but the value obtained at pH_0 7·0–7·1 was only 5·0 ± 1·1 (four experiments). In both pH_0 ranges the decay of ΔpH_0 was not exponential (Fig. 6), but the initial decay rate was dependent on the quantity of gramicidin present. Maximum extrapolated $\rightarrow H^+/O$ quotients were observed with a lower concentration of gramicidin in experiments at pH_0 7·0–7·1 than at pH_0 6·0–6·1, and the initial $t_{1/2}$ of decay was near 30 sec in either pH_0 range. However, for a given amount of gramicidin, the initial decay rate was faster in the pH_0 range 7·0–7·1 than in the range 6·0–6·1.

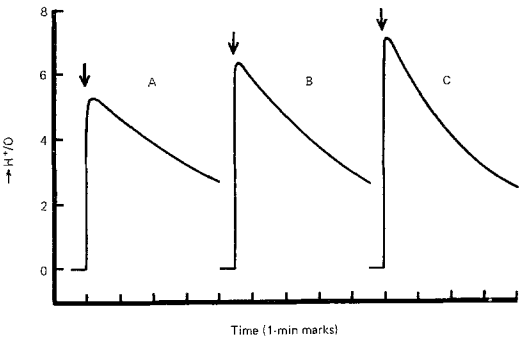

Figure 3. The effect of valinomycin on the time-course of respiration-driven proton translocation. Oxygen (23·5 ng atoms) in air-saturated 150 mM KCl was injected (at the arrows) into the anaerobic bacterial suspension under the conditions used in Fig. 1 for the pH_0 range 6·0–6·1. The bacterial suspension (4 ml) containing 5·03 mg cell dry weight/ml was preincubated at pH_0 6·0–6·1 with valinomycin (0·75 mg/g cell dry weight) for: A, 45 min; B, 90 min; C, 120 min. The traces represent ΔpH_0 scaled to read as $\rightarrow H^+/O$, outward proton translocation being represented upwards.

The Effect of Potassium Thiocyanate on ΔpH_0 During Pulses of Respiration

After cells of *M. denitrificans* were equilibrated for 15 min with potassium thiocyanate, respiratory pulses were accompanied by rapid outward proton translocation, followed by a relatively slow decay towards equilibrium (Fig. 7). Maximum values of the extrapolated $\rightarrow H^+/O$ quotients were 7·4 ± 0·3 (three experiments) and 8·0 ± 0·1 (three experiments) at pH_0 6·0–6·1 and pH_0 7·0–7·1 respectively. The maximum extrapolated $\rightarrow H^+/O$ quotient at pH_0 6·0–6·1 required 17·5 mM thiocyanate, while the maximum value at pH_0 7·0–7·1 required 100 mM thiocyanate. As shown in Fig. 8, the decay of the ΔpH_0 was initially exponential at pH_0 6·0–6·1 when the SCN^- concentration was at least 17·5 mM; but at pH_0 7·0–7·1 the decay was not exponential even at 100 mM SCN^-. When the concentration of thiocyanate was increased above the level giving maximum extrapolated $\rightarrow H^+/O$ quotients, the $t_{1/2}$ of decay was more rapid. This effect was more pronounced in the range pH_0 6·0–6·1; and at thiocyanate levels of 100 mM (corresponding to that required for maximum extrapolated $\rightarrow H^+/O$ quotients at pH_0 7·0–7·1) the $t_{1/2}$ of decay was about 20 sec, but the extrapolated $\rightarrow H^+/O$ quotient was only slightly depressed.

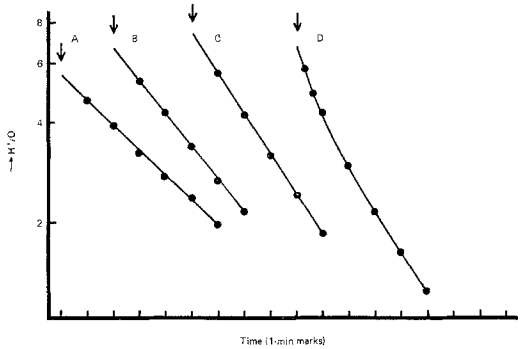

Figure 4. Semi-logarithmic plot of the decay of respiration-driven proton translocation. Data of Fig. 3 are plotted as curves A, B and C. For comparison, D is plotted from an identical experiment carried out in the pH_0 range 7·0–7·1. In D, the bacterial suspension (4 ml) containing 4·3 mg cell dry weight/ml was preincubated at pH_0 6·0–6·1 for 3 h with valinomycin (0·9 mg/g cell dry weight) and was reequilibrated at pH_0 7·0–7·1 prior to injection of 23·5 ng atoms of oxygen as air-saturated 150 mM KCl. The arrows indicate the time to which the observed $\rightarrow H^+/O$ value (obtained by scaling ΔpH_0) should be extrapolated to obtain the $\rightarrow H^+/O$ quotient corrected for decay.

In rat liver mitochondria, Ca^{2+} ions release respiration-driven proton translocation when pulses of oxygen are added in excess of the normal backlash.[8] In contrast, the addition of $CaCl_2$ (60 μmoles/g cell dry weight) had no effect on respiration-driven proton translocation in *M. denitrificans*.

The Effect of FCCP on ΔpH_0 During Pulses of Respiration

Figure 9 shows the effect of the proton-conducting uncoupler FCCP on the decay of respiratory pulses in the normal suspension medium or with the addition of valinomycin, potassium thiocyanate or gramicidin.

The addition of 2·5 μM FCCP to untreated cells after a respiratory pulse increased the decay rate of ΔpH_0. In an experiment (A) where oxygen was added rapidly (0·2 sec) or (B) at a slow constant rate, the $t_{1/2}$ of decay was about 100 sec. Subsequent respiratory pulses were very small, as described in Fig. 1.

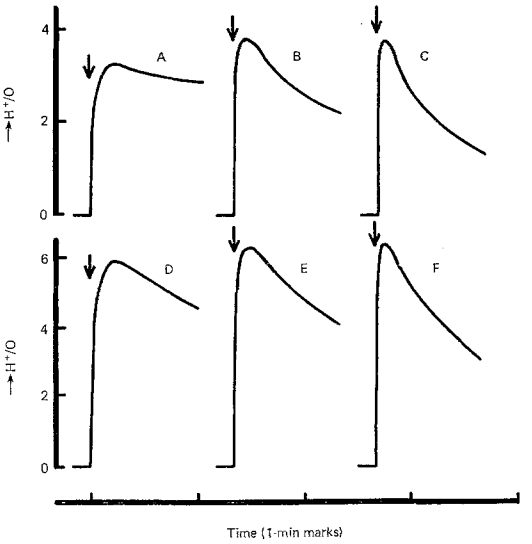

Figure 5. The effect of gramicidin on the time-course of respiration-driven proton translocation. Oxygen (23·5 ng atoms) was injected as air-saturated 150 mM KCl at the arrows. The conditions in the pH_0 range 6·0–6·1 or 7·0–7·1 were as described in Fig. 1. At pH_0 7·0–7·1 (A, B, C) the cell suspension (4 ml) contained 5·3 mg cell dry weight/ml with addition of gramicidin: A, 2·5 μg; B, 5·0 μg; C, 10 μg. At pH_0 6·0–6·1 (D, E, F) the cell suspension (4 ml) contained 5·2 mg cell dry weight/ml with the addition of gramicidin: D, 4 μg; E, 8 μg; F, 15 μg. The traces are shown as $\rightarrow H^+/O$ as in Fig. 3.

In the presence of valinomycin, SCN^- or gramicidin at pH_0 6·0–6·1, the large values of ΔpH_0 induced by pulses of respiration were collapsed rapidly on adding FCCP. Fig. 9C shows the collapse of a respiratory pulse produced in the presence of valinomycin, by adding 1 μM FCCP. The results observed with respiratory pulses produced in the presence of SCN^- and gramicidin were qualitatively similar. In experiments on the same batch of cells used for the experiment of Fig. 9C, the $t_{1/2}$ of decay of ΔpH_0 in the pH_0 range 6·0–6·1 after equilibration with 17·5 mM SCN^-, or after preincubation with gramicidin (0·8 mg/g cell dry weight) or valinomycin (2·0 mg/g cell dry weight) was 55 sec, 25 sec, and 75 sec respectively; and the addition of 1 μM FCCP decreased the $t_{1/2}$ to 20 sec, 12 sec and 3·5 sec respectively. When subsequent pulses were done in the presence of these reagents and FCCP, a considerable pH_0 displacement was still observed with a similar short $t_{1/2}$ of decay.

When respiratory pulses were done in the presence of 100 mM SCN^- at pH_0 7·0–7·1, ΔpH_0 was completely collapsed by 1 μM FCCP, and the $t_{1/2}$ decreased from 70 sec to 12 sec (Fig. 9D). On the other hand, in the presence of gramicidin at pH_0 7·0–7·1, ΔpH_0 did not decay to the baseline after the addition of 1 μM FCCP (Fig. 9E), and in some instances this effect was also observed with valinomycin at pH_0 7·0–7·1. However, subsequent respiratory pulses in the presence of FCCP and gramicidin, SCN^- or valinomycin gave large values of ΔpH_0 that decayed rapidly to the baseline.

Figure 10 shows the effect of increasing concentrations of FCCP on the decay rate and stoichiometry of respiratory pulses in the presence of KSCN at pH_0 7·0–7·1. In these experiments the points have been corrected for the response-time of the electrode. The values extrapolated back to the time of half reduction of the oxygen pulse show that the stoichiometry remains high until the $t_{1/2}$ of decay becomes less than 5 sec.

The Rate of Proton Translocation in the Presence of Valinomycin

Figure 11 illustrates a typical experiment in which the pH_0 was followed by measuring the change in absorbance of BCP and compared with measurements using the glass electrode system. Control injections of HCl and KOH under the conditions used in these experiments showed that the injection and mixing times did not exceed 0·1 sec. The rate of pH_0 change and the corresponding rate of proton translocation given by the spectrophotometric method in the type of experiment described by Fig. 11 was constant for the first 4/5 of the pH_0 excursion or for about 0·4 sec. The rate of proton translocation calculated for this part of the pulse was 14·3 μg ions of H^+/sec g cell dry weight.

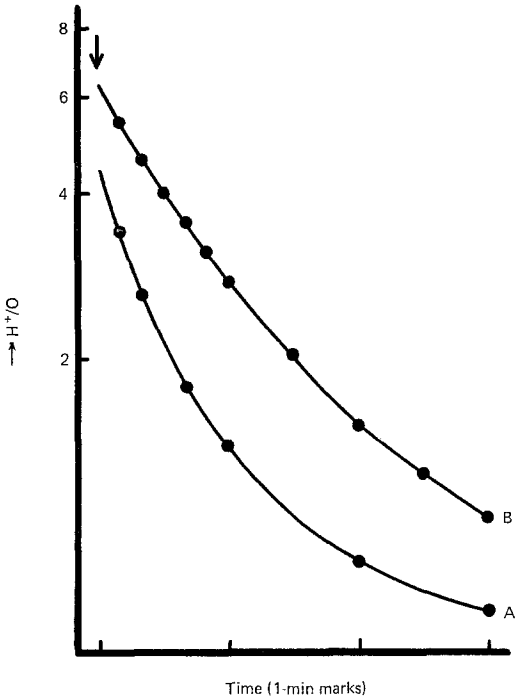

Figure 6. Semi-logarithmic plot of the decay of respiration-driven proton translocation. Data of Fig. 5C and F, are plotted as A and B respectively. Arrow convention as in Fig. 4.

Discussion and Conclusions

The results show that when transitory respiratory activity is induced in anaerobic suspensions of *M. denitrificans* with pulses of oxygen, protons are translocated outwards. The polarity of proton translocation during activity of the respiratory chain is thus the same as in mitochondria,[8] prokaryotic photosynthetic microorganisms,[2,3] and other aerobic bacteria.[1]

In contrast to rat liver mitochondria, in absence of ion-conducting agents such as valinomycin or permeant ions such as SCN^-, the observed change of pH_0 during pulses of respiration-driven proton translocation in *M. denitrificans* was sluggish and did not give a stoichiometric value of $\rightarrow H^+/O$. When the size of the oxygen pulse was decreased from 1·0 μg atom of oxygen to 0·1 μg atom of oxygen/g cell dry weight, the stoichiometry of proton translocation increased and values greater than 4 were recorded in some cases. Under similar conditions in mitochondria the $\rightarrow H^+/O$ quotient during the oxidation of β-hydroxybutyrate and succinate is very close to 6 and 4 respectively.[8]

As discussed previously for the case of mitochondria,[18] if no charge leakage occurred across the M phase, the translocation of a small quantity of H^+ would produce a large membrane potential. Mitochondria and bacteria appear to be alike in that the conductance

of the coupling membrane to the major ions usually present is low[15,16,24,25] so that a membrane potential is rapidly produced by the chemically coupled translocation of ions, and ATP synthesis (resulting in net alkalinization) may occur. The fact that in *M. denitrificans* low $\rightarrow H^+/O$ quotients are observed in the normal KCl media, and the stoichiometry increases at lower respiratory rates, when the extent of reversal of the ATPase reaction should be less, is thus to be expected. The contrast between the bacteria and rat liver mitochondria in this respect is explained (see ref. 26) by the fact that in the mitochondria endogenous Ca^{2+} ions are mobile across the membrane and can electrically neutralize the translocation of up to some 10 μg ions of H^+/g of protein. The specific translocation of Ca^{2+} ions across the membrane of mammalian mitochondria evidently does not apply to the plasma membrane of *M. denitrificans*; for although proton translocation is released by the ion-conducting reagents valinomycin and gramicidin or the permeant anion SCN^- in the bacteria as in mitochondria, it is not released by Ca^{2+} ions.

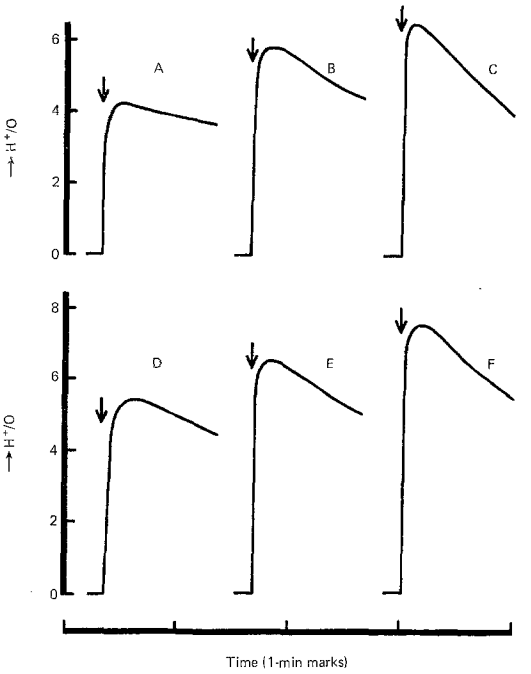

Figure 7. The effect of thiocyanate on the time-course of respiration-driven proton translocation. Oxygen (23·5 ng atoms) was injected as air-saturated 150 mM KCl at the arrows. The conditions of the experiments in the pH_0 range 6·0–6·1 and 7·0–7·1 were as described in Fig. 1. At pH_0 6·0–6·1 (A, B, C), the cell suspension (4 ml) contained 5·1 mg cell dry weight/ml with addition of: A, 2·5 mM KSCN; B, 7·5 mM KSCN; C, 17·5 mM KSCN. At pH_0 7·0–7·1 (D, E, F) the cell suspension (4 ml), contained 4·7 mg cell dry weight/ml with addition of: D, 25 mM KSCN; E, 55 mM KSCN; F, 100 mM KSCN. The traces are shown as $\rightarrow H^+/O$, as in Fig. 3.

Some comment is required on the relative ineffectiveness of valinomycin as an ion-conducting reagent near pH_0 7, and on the long preincubation times required to release proton translocation near either pH_0 6 or 7. When the preincubation with the antibiotic was done near pH_0 6 it was more effective in releasing respiration-driven proton translocation tested near pH_0 7 than when the preincubation was done near pH_0 7. Our earlier observations on the effect of valinomycin on acid-base titration across the M phase of *M. denitrificans*[15] indicated that the K^+ ion conductance of the membrane was increased by valinomycin on incubation at acid pH_0, but that incubation with valinomycin at neutral or alkaline pH_0 had relatively much less effect. The slowness of development of K^+ ion permeability in *M. denitrificans* on incubation with valinomycin, and the pH-dependence of this process probably reflect the resistance of the cell wall and plasma membrane structure to the penetration and mobility of the valinomycin molecules, as in the case of other reagents studied in bacterial suspensions.[27–29] It is noteworthy, incidentally, that the release of respiration-driven proton translocation by SCN^- did not require preincubation beyond the time (15 min) permitting equilibration of the permeant SCN^- ion across the plasma membrane of the anaerobic cell suspensions. This was consistent with osmotic swelling

experiments, using spheroplasts of *M. denitrificans* (P. Scholes, unpublished data) under conditions corresponding to those employed with mitochondrial suspensions,[30] which showed that the plasma membrane of *M. denitrificans* like that of staphylococci,[24] has a relatively high permeability to SCN^- ions. The increase in the rate of decay of the respiration-driven proton pulses at SCN^- concentrations approaching 100 mM, particularly at acid pH_0, are probably attributable to changes in the charge distribution in the lipid phase of the plasma membrane of the cells by the lipid-soluble SCN^- ion.

The effects of gramicidin in releasing proton translocation were qualitatively similar to those of valinomycin, but the maximum $\rightarrow H^+/O$ quotients obtained were lower than in the case of valinomycin. This, and other differences in the effects of gramicidin are probably attributable to the fact that gramicidin conducts not only alkali metal ions but also protons across lipid membranes.[15,31]

The extrapolated $\rightarrow H^+/O$ quotients obtained under different conditions in the pH_0 ranges 6·0–6·1 and 7·0–7·1 are summarized in Table I. The highest stoichiometries were obtained with valinomycin at pH_0 6·0–6·1 and with thiocyanate at pH_0 7·0–7·1. The lower stoichiometries observed can be ascribed to a decreased efficiency of the electrical potential-collapsing agent or to an increased conductance of the membrane to protons, induced by the reagent used, or to both causes.

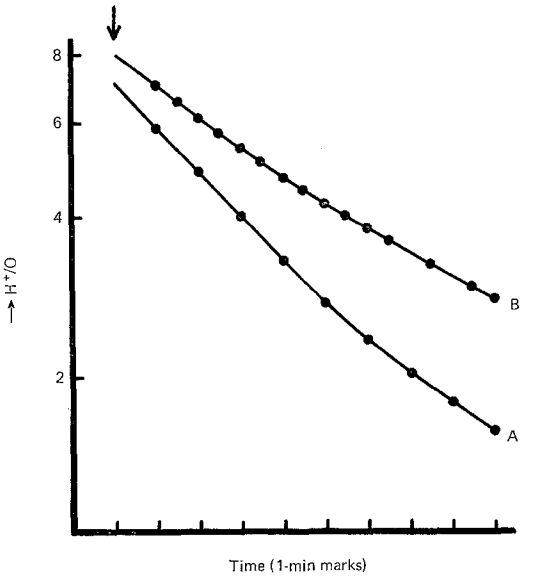

Figure 8. Semi-logarithmic plot of the decay of respiration-driven proton translocation. Data of Fig. 7C and F are plotted as A and B respectively. Arrow convention as in Fig. 4.

The results of these and previous experiments on *M. denitrificans*[15] show clearly that FCCP enhances the proton conductance of the membrane. When the FCCP is added after termination of the respiration-driven proton translocation, the rapid collapse of the pulse requires the presence of the ion-conducting reagents valinomycin or gramicidin, or the permeant ion SCN^-, presumably because the respiration-driven proton translocation must be accompanied by the passage of an equivalent quantity of ionic charge across the membrane under the influence of the membrane potential. When conditions are such that the neutralizing ions do not permeate readily, the displacement of ions (such as Cl^-) across the membrane, under the influence of the high membrane potential developed during the respiratory pulse, would be expected to subside relatively slowly, even when the proton conductance of the membrane was moderately high. Thus, pH equilibration should be retarded in absence of ion-conducting reagents or permeant ions as in acid-base titrations with HCl or KOH (ref. 15).

As demonstrated in Fig. 10, increasing amounts of FCCP have little effect on the $\rightarrow H^+/O$ quotient until the $t_{1/2}$ of decay is as low as 5 sec. Similar observations in mitochondria have been discussed previously.[8] One may conclude that the membrane

conductance to SCN^- is much greater than that to H^+, induced by FCCP, under these conditions.

When experiments similar to those of Fig. 10 were carried out in presence of valinomycin or gramicidin, qualitatively similar results were obtained, although for a given amount of FCCP the decay of the proton pulses was more rapid and was not exponential. These findings are difficult to interpret in detail, but we suggest that the positively charged valinomycin-K^+ complex may interact with the anionic form of FCCP to facilitate the movement of the latter across the membrane. Conversely one might expect that an increase of negative space-charge in the membrane in the presence of SCN^- might inhibit the movement of the anionic form of FCCP (refs. 32 and 33).

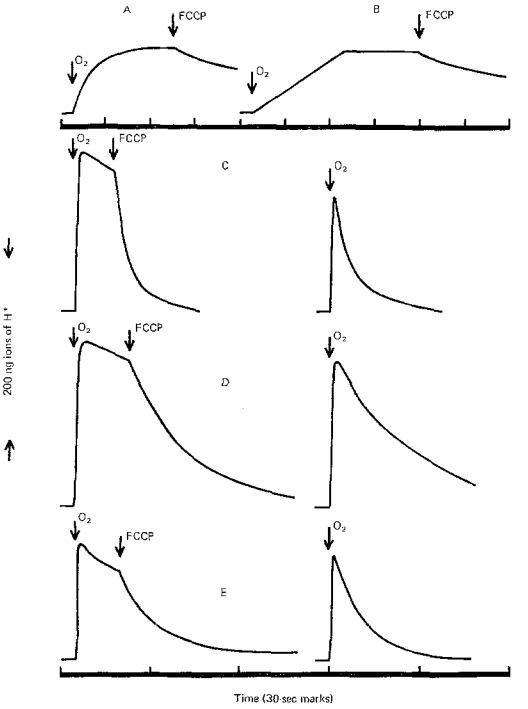

Figure 9. The effect of FCCP on the time-course of respiration-driven ΔpH_0 under various conditions. Oxygen (23·5 ng atoms) was injected as air-saturated 150 mM KCl, commencing at arrows, and taking 0·2 sec except in (B), when it was injected at a slow constant rate. The media were as described for Fig. 1. A, preincubation was at pH_0 7·0–7·1 with no addition, and 2·5 μM FCCP was added at arrow. B, was as A, but the duration of the oxygen pulse was 60 sec. In C, D, and E, the cells were preincubated respectively: with valinomycin (2·0 mg/g cell dry weight) at pH_0 6·0–6·1; with KSCN (100 mM) at pH_0 7·0–7·1; and with gramicidin (0·29 mg/g cell dry weight) at pH_0 7·0–7·1. After preincubation, an initial oxygen pulse, FCCP (1 μM), and a final oxygen pulse were added at arrows. In all experiments the cell suspension contained about 5 mg dry weight/ml. Upward deflection of traces indicates acidification of the medium. The quantity of H^+ equivalent to the recorded pH_0 changes is given for the 4 ml of suspension.

According to Harris and co-workers,[34] the electrically equivalent translocation of Ca^{2+} ions which accompanies the translocation of H^+ ions in respiration-driven proton pulses in mitochondria (in the absence of EDTA) or the electrically equivalent translocation of K^+ ions in mitochondria treated with valinomycin in presence of EDTA suggest that electrogenic proton translocation is not the primary process, but that "the primary event in proton pulse production is an energy-driven cation exchange." Evidence that is incompatible with this interpretation in rat liver mitochondria has been discussed.[35] It might be suggested that the proton pulses observed in suspensions of *M. denitrificans* should be similarly interpreted. This interpretation is not, however, acceptable because proton pulses with an $\rightarrow H^+/O$ quotient approaching 8 have been observed under conditions in which the anion SCN^- is the charge-neutralizing ion species and there is no evidence to support the suggestion that proton/cation exchange occurs under these conditions. Moreover, if non-electrogenic proton/cation exchange were the primary process, since the data of Fig. 10 show that 3 μM FCCP in presence of SCN^- does not prevent proton translocation (e.g. by hydrolysing an energy-rich intermediate) but only facilitates conduction of H^+ ions back across the mem-

brane (as discussed below), FCCP alone at this or at a lower concentration should not largely prevent the appearance of the respiration-driven proton pulses, as observed (Fig. 1).

The $\rightarrow H^+/O$ quotients approaching 8, observed in both acid and neutral pH_0 ranges (Table I), are especially interesting, since the highest $\rightarrow H^+/O$ quotient obtained from respiratory pulses in rat liver mitochondria (oxidizing β-hydroxybutyrate) was 6. Two main alternative mechanisms of primary proton translocation coupled to oxidoreduction have been suggested. According to the chemiosmotic view of the coupling mechanism, protons are produced at the outer surface and taken up at the inner surface of the M phase by the oxidation of H atoms and reduction of H^+ ions respectively at junctions between hydrogen-carrying and electron-carrying members of the respiratory chain, supposed to be arranged in a "looped" configuration in the membrane.[9] According to a version of the chemical hypothesis of the coupling mechanism,[36] the protons are translocated outwards across the membrane by a squiggle-actuated pump which drives $2H^+$ outwards per squiggle derived from the hypothetical coupling sites in the respiratory chain. The chemical or the chemiosmotic mechanism respectively could also utilize ATP, derived from substrate-level phosphorylation, either by driving the squiggle-actuated proton pump (via the ATPase system) or by driving the proton-translocating ATPase. Thus, an $\rightarrow H^+/O$ quotient of 8 demands either four oxido-reduction loops, or the generation of four squiggle bonds (including ATP generated by a substrate-level mechanism), or some combination. It appears to be unlikely that substrate-level phosphorylation could be involved because the translocation of only $2H^+$ of the observed $8H^+$ in the pulses would require some 1 μmole of ATP/g cell dry weight, and it is known that, at this ATP level in *M. denitrificans*, hydrolysis is slow (P. Scholes and F. Welsch, unpublished data) compared with the rate of appearance of the H^+ ions observed in the respiration-driven pulses. The observations described here do not permit a decision between the chemiosmotic and chemical types of proton-translocation mechanisms; but the fact that FCCP causes an increase in the rate of decay of the respiration-driven proton pulses, but does

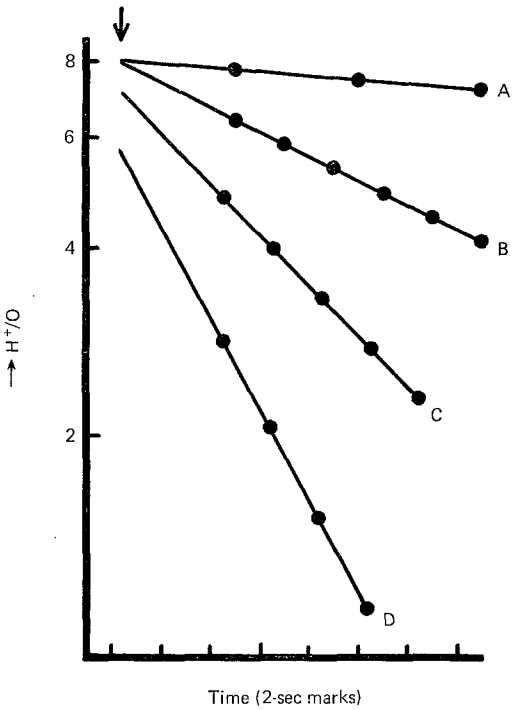

Figure 10. The effect of increasing concentrations of FCCP on the $\rightarrow H^+/O$ quotient and the rate of decay of respiration-driven proton translocation. Oxygen (23·5 ng atoms) was injected as air-saturated 150 mM KCl. The conditions were as described in Fig. 1 for experiments at pH_0 7·0–7·1. Additional reagents present were: A, 100 mM KSCN; B, 100 mM KSCN + 1 μM FCCP; C, 100 mM KSCN + 3 μM FCCP; D, 100 mM KSCN + 7 μM FCCP. The cell suspension (4 ml) contained 6·8 mg cell dry weight/ml. The $\rightarrow H^+/O$ values plotted on the vertical axis represent appropriately scaled ΔpH_0 values obtained by correcting the observed ΔpH_0 values for the response-time of the electrode (see Materials and Methods). Arrow convention as in Fig. 4.

not cause a decrease in the quantity of protons initially translocated during the pulse of respiration shows that, if a squiggle-intermediate is involved, it is relatively insensitive to destruction by the uncoupling agent. *M. denitrificans* behaves like rat liver mitochondria in this respect.[8]

The circumstances under which the $\rightarrow H^+/O$ quotients approaching 8 have been observed in *M. denitrificans* were arranged so as to minimize thermodynamic back-pressure on the proton-translocation reaction. This high stoichiometry may not therefore be characteristic of the system in the near-equilibrium state during ATP synthesis. Assuming, however, that the $\rightarrow H^+/O$ quotient of 8 may be physiologically normal, the probable implication would be that the P/O quotient is 4, whether by chemiosmotic or by chemical coupling. This is thermodynamically feasible, since total P/O quotients of 4 (including the substrate-level phosphorylation) are characteristic of α-oxoglutarate oxidation in mitochondria.[37]

The characteristics of the transhydrogenase reaction, described by Asano *et al.*[13], suggest that the respiratory chain of *M. denitrificans* may possibly consist of four oxidoreduction loops similar to those previously postulated for mitochondria;[9] but that, unlike mitochondria, Loop 0, corresponding to the transhydrogenase reaction system, may catalyse the oxidation of NADPH, at least under the special conditions of the experiments described here.

Figure 11 shows that the rate of proton translocation in presence of valinomycin

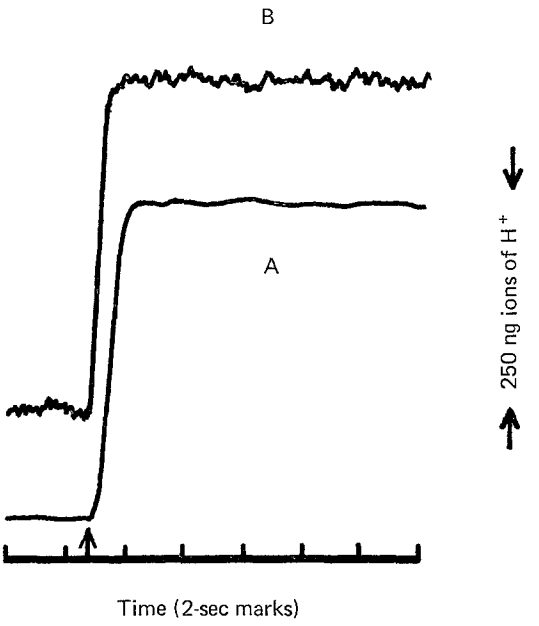

Figure 11. Time-course of respiration-driven proton translocation in the presence of valinomycin. Traces A and B respectively show the observed proton translocation measured simultaneously with the pH electrode system, and by observing the absorbance changes of BCP in the pH_0 range 6·0–6·1. For experimental details see Materials and Methods. Oxygen (47 ng atoms) was injected as air-saturated 150 mM KCl at the arrow. Upward deflection of traces represents acidification of medium. The quantity of H^+ ions equivalent to the recorded pH_0 changes is given for the 5·5 ml of suspension.

TABLE I. Summary of the extrapolated $\rightarrow H^+/O$ quotients obtained in presence of valinomycin, gramicidin, and SCN^-.

Membrane potential-collapsing agent	Extrapolated $\rightarrow H^+/O$ quotient	
	pH_0 6·0–6·1	pH_0 7·0–7·1
Valinomycin	7·5 ± 0·3 (12)	6·0 ± 1·2 (6)
Gramicidin	7·3 ± 0·6 (2)	5·0 ± 1·1 (4)
Potassium thiocyanate	7·4 ± 0·3 (3)	8·0 ± 0·1 (3)

The conditions were as described for Figs. 3, 5, and 7.

was 14·3 μg ions of H^+/sec g cell dry weight. At an $\rightarrow H^+/O$ quotient of 8 this would correspond to a respiration rate of 1·79 μg atoms of O/sec g cell dry weight. The steady-state rate of respiration optimally stimulated by FCCP is 1·39 μg atoms of O/sec g cell dry weight,[15] and it follows that the proton flux rate is high enough to account for coupling between respiration and phosphorylation by an intermediary current of protons.[9]

Acknowledgments

We are grateful to Dr. Jennifer Moyle for helpful discussion and criticism. We thank Glynn Research Ltd. for general financial support, and record our indebtedness to Mr. Roy Mitchell, Mr. Robert Harper, Mr. Michael Pearse, and Miss Stephanie Phillips for expert assistance. One of us (Peter Scholes) holds the Royal Society Horace le Marquand and Dudley Bigg Fellowship.

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