Biochimica et Biophysica Acta, 766 (1984) 222–232
Elsevier

BBA 41552

ON THE EFFECTS OF THIOCYANATE AND VENTURICIDIN ON RESPIRATION-DRIVEN PROTON TRANSLOCATION IN *PARACOCCUS DENITRIFICANS*

G. DUNCAN HITCHENS and DOUGLAS B. KELL *

Department of Botany and Microbiology, University College of Wales, Aberystwyth, Dyfed SY23 3DA (U.K.)

(Received February 27th, 1984)

Key words: Chemiosmosis; Thiocyanate; Proton translocation; Venturicidin; (P. denitrificans)

(1) A fast-responding O_2 electrode has been used to confirm and extend observations of a significant kinetic discrepancy between O_2 reduction and consequent proton translocation in 'O_2-pulse' experiments in intact cells of *P. denitrificans*. The permeant, chaotropic SCN^- ion abolishes this discrepancy, and greatly increases the observable $\rightarrow H^+/O$ ratio, to a value approaching its accepted, true, limiting stoichiometry. The observable H^+ decay rates are very slow, particularly in the absence of SCN^-. (2) The submaximal $\rightarrow H^+/O$ ratios observed in the absence of SCN^- are essentially independent of the size of the O_2 pulse, in a manner not easily explained by a delocalised chemiosmotic energy-coupling scheme. (3) Osmotically active protoplasts of *P. denitrificans* do not show a significant kinetic discrepancy between O_2 reduction and H^+ translocation, even in the the absence of SCN^-. However, the submaximal $\rightarrow H^+/O$ ratios observed in the absence of SCN^- are again essentially independent of the size of the O_2 pulse. As in intact cells, the observable H^+ decay rates are very slow. (4) The energy-transfer inhibitor venturicidin causes a significant increase in the $\rightarrow H^+/O$ ratio observed in protoplasts of *P. denitrificans* in the absence of SCN^-; the decay kinetics of the H^+ translocation process are also somewhat modified. Nevertheless, the $\rightarrow H^+/O$ ratio observed in the presence of venturicidin is also independent of the size of the O_2 pulse. This observation militates further against arguments in which (a) a non-ohmic leak of protons from the bulk aqueous phase might alone be the cause of the low $\rightarrow H^+/O$ ratios observed in the absence of SCN^-, and (b) in which there might be a Δp-dependent change ('redox slip') in the actual $\rightarrow H^+/O$ ratio. (5) It is concluded that the observable protonmotive activity of the respiratory chain of *P. denitrificans* in the absence of SCN^- is directly influenced by the state of the H^+-ATP synthase in the cytoplasmic membrane of this organism. We are unable to explain the data in terms of a model in which the putative protonmotive force may be acting to affect the $\rightarrow H^+/O$ ratio. The possibility is considered that the delocalised bulk-to-bulk phase membrane potential set up in response to protonmotive activity is energetically insignificant.

1. Introduction

It is now well established that the activity of respiratory and photosynthetic electron transport chains is more or less tightly coupled to transmembrane, (initially) electrogenic proton translocation [1]. Amongst the respiratory bacteria, the relationship between electron transport and concomitant proton translocation was first reported in suspensions of *Micrococcus* (now *Paracoccus*) *denitrificans* by Scholes and Mitchell (Ref. 2; for taxonomy see Ref. 3). These workers exploited the 'O_2-pulse' technique previously used for work in mitochondria [4], and observed that both the extent (stoichiometry) and the rate of transfer of protons to and from the aqueous phase external to

* To whom all correspondence should be addressed.

the organisms, a phase whose pH was in equilibrium with the measuring pH electrode, were markedly changed upon the addition of valinomycin or the relatively permeant SCN^- ion to the normal potassium chloride/glycylglycine reaction mixture. They also observed that in the absence of these compounds there was a marked kinetic discrepancy between the reduction of O_2 (measured as changes in the redox state of cytochrome aa_3) and the half time of observable H^+ translocation [2].

Gould and Cramer [5,6] carried out similar experiments to those of Scholes and Mitchell [2], using *Escherichia coli*. In addition to the observed effects of SCN^-, which we have confirmed [7], these workers found that the $\rightarrow H^+/O$ ratio in the absence of SCN^- was essentially independent of the size of the O_2 pulse, a result not easily reconciled with a ('delocalised') chemiosmotic model in which a large, delocalised membrane potential might be acting to inhibit proton translocation to the phase in equilibrium with the measuring electrode [5,6,8–13]. We have recently repeated some of these experiments in *P. denitrificans*, with qualitatively similar results [10], although it was necessary to infer the respiration rates during the O_2 pulse from steady-state measurements on parallel suspensions.

By using the rapidly responding O_2 electrode described by Lehninger and co-workers [14], we have now been able to make these measurements directly, confirming the kinetic discrepancies observed by those mentioned above, and showing that these kinetic discrepancies are not exhibited by *P. denitrificans* protoplasts. Finally, we have studied the suggestion of Ferguson (see Ref. 15) that significant ATP synthesis (or, by implication, any very rapid back-leakage of pumped protons from the external bulk phase) may take place during O_2 pulse experiments, by using the energy transfer inhibitor venturicidin, which, by analogy with its effects in photosynthetic bacteria [24,25], should inhibit both phosphorylative and non-phosphorylative, non-ohmic proton back-leakage. These experiments form the subject of the present report. It is concluded that phenomena of 'redox slip' and non-ohmic leaks cannot of themselves explain the data observed within the framework of a delocalised, chemiosmotic energy-coupling scheme.

2. Experimental

Organism. Paracoccus denitrificans 8944 was maintained and grown as described [10,16,17]. For work with intact organisms, cells were harvested in mid-log phase, and washed three times in 150 mM KCl containing 0.25 mM glycylglycine (pH 7.0), essentially as described [10]. Protoplasts were prepared using lysozyme, and their osmotic sensitivity assessed, as described [16]; they were resuspended in 0.5 M sucrose containing 150 mM KCl/0.25 mM glycylglycine (pH 7.0). Protoplast concentrations are given as the equivalent dry weight of cells determined turbidimetrically in 0.5 M sucrose containing 10 mM Tris Cl (pH 7.3).

Respiration-driven proton translocation was measured in the reaction vessel described [10,18] which, in addition to the combination pH microelectrode, also contained an O_2 electrode of the type described by Reynfarje et al. [14] and a combination Pt (counter-) and saturated calomel (reference-) electrode. The voltage on the O_2 electrode (-0.65 V vs. the reference electrode) was set using a PAR 174A 3-electrode potentiostat [19], and the output from the device directed to a Linseis LS23 chart recorder. The voltage developed by the combination pH electrode was amplified by a high impedance differential FET amplifier (bandwidth 15 Hz) (Dulas Engineering, Llwyngwern Quarry, Machynlleth, Powys, U.K.), and was directed to the Linseis chart recorder. O_2 pulses were added as air-saturated 150 mM KCl, which was taken to contain 235 μM O_2 at the reaction temperature of 30 °C [10]. Reaction mixtures are given in the legends to the figures.

Chemicals and biochemicals. Venturicidin, predominantly venturicidin A, was obtained from BDH chemicals, Poole, Dorset. Other chemicals and biochemicals were of analytical grade, and were obtained from previously disclosed sources [10,18].

3. Results

Fig. 1 shows traces of both respiration and H^+ translocation during the early stages of a typical O_2 pulse experiment with intact cells of *P. denitrificans* in the absence (A) and in the presence (B) of 60 mM KSCN. The kinetic discrepancy

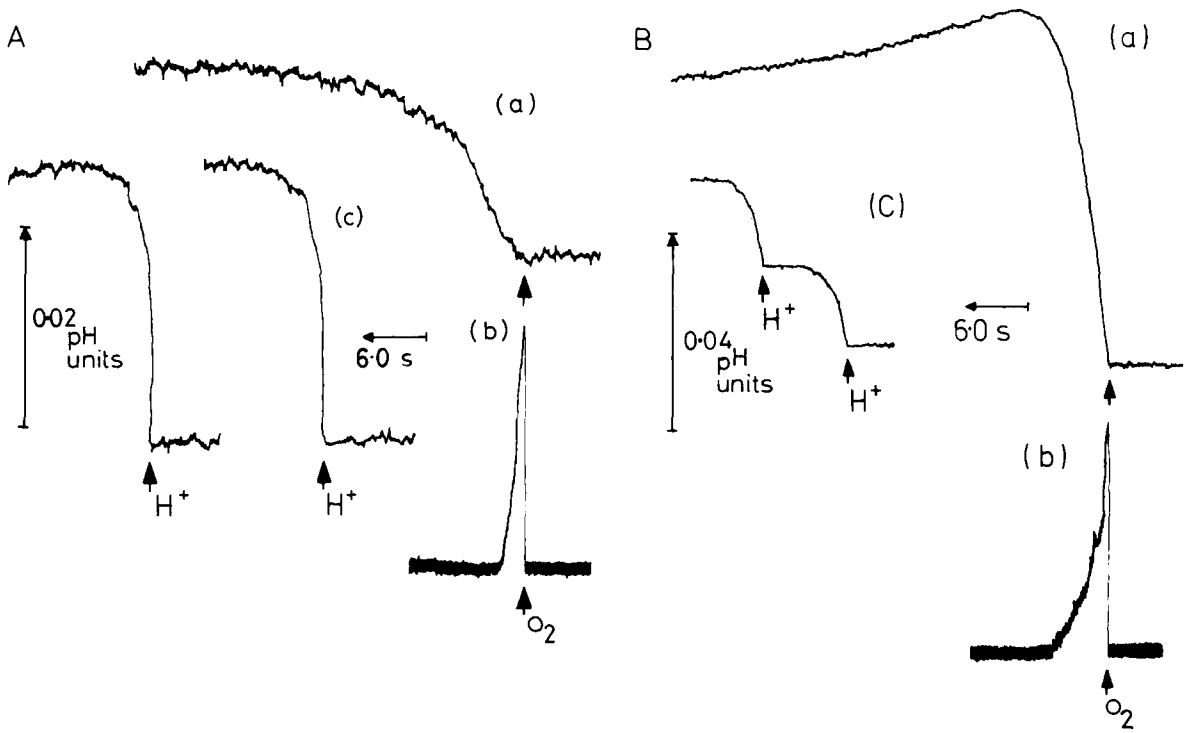

Fig. 1. Respiration and H^+ translocation in intact cells of *P. denitrificans* during an O_2 pulse. Respiration-driven H^+ translocation experiments were carried out as described in the Experimental section. The reaction mixture contained, in a final volume of 6 ml, 150 mM KCl/0.25 mM glycylglycine (pH 7.0)/480 μg carbonic anhydrase/10.1 mg dry wt. cells. The temperature was 30 °C. In (B) 60 mM KSCN was also present. In traces (a) and (b), the arrow marks the addition of 80 μl air-saturated KCl, corresponding to a total of 37.6 ng atom O. H^+ translocation (a) and respiration (b) are as indicated. At the arrow in (c), anaerobic HCl (50 ng ion) was added to the reaction mixture. It may be observed that the response of the pH electrode per se (c) was not limiting the pH changes observed in (a).

between the half-time for O_2 reduction and for H^+ translocation in the absence of SCN^- (Fig. 1A) is clearly evident. In the presence of SCN^- (Fig. 1B), however, no such kinetic discrepancy is observable, and the $\rightarrow H^+/O$ stoichiometry is significantlly increased. The pH changes observed are fully sensitive to the uncoupler carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone at a concentration of 2 μM (data not shown, but see Ref. 2, 10). The absolute values of the $\rightarrow H^+/O$ ratio are slightly lower than those observed previously [10], presumably due to the more alkaline pH used in the present work [2].

According to the interpretation of these types of data given by Scholes and Mitchell [2], it is proposed that, due to the low electrical capacitance of the bacterial cytoplasmic membrane, the transmembrane electrogenic translocation of a very small number of protons into the aqueous phase external to the organisms causes the build-up of a delocalised membrane potential sufficiently large either to inhibit further protonmotive activity or to drive back protons subsequently pumped across the coupling membrane before the pH electrode can respond. Transmembrane electrophoretic cotransport of the relatively membrane-permeant SCN^- ion with pumped H^+ would act to dissipate this membrane potential and allow the full extent of respiration-linked H^+ translocation to be determined.

If the foregoing explanation of these data is correct, one must assume that the inferred membrane potential (which should dominate the protonmotive force) that is built up following the reduction of an amount of O_2 significantly less than that added in the experiment of Fig. 1, is the maximum sustainable by the bacterial cytoplasmic membrane under the conditions prevailing. Otherwise

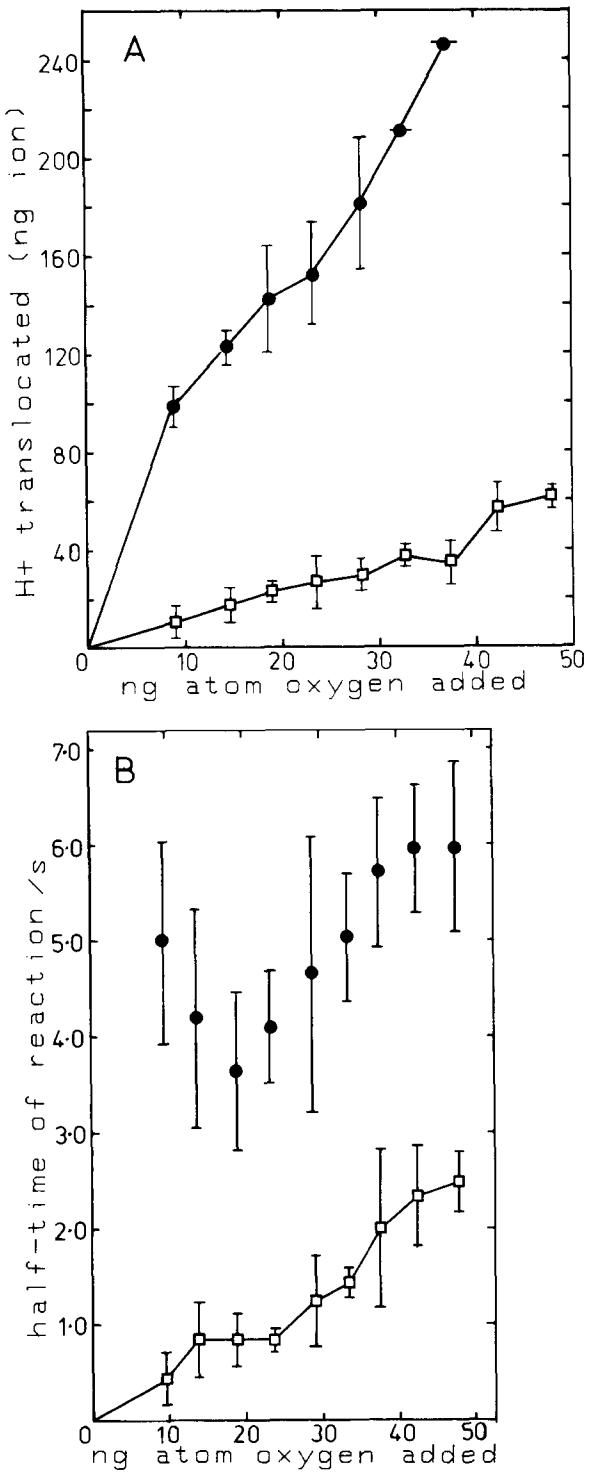

Fig. 2. The effect of the size of the O_2 pulse on the observable stoichiometry and kinetics of respiration-driven H^+ translocation in intact cells of *P. denitrificans*. (A) The quantity of H^+ translocated was measured from the furthest excursion of the pH trace as described in the legend to Fig. 1, except that the size of the O_2 pulse was varied as indicated. Thiocyanate was either absent (□) or present (●) at a concentration of 60 mM. Values are given as means ± S.E. ($n = 3$). Cell concentratioans were in the range 1.4–1.7 mg dry wt. per ml. (B) The half-times of H^+ ejection (●) and O_2 uptake (□) were calculated from traces such as those illustrated in Fig. (□) 1A, in the absence of SCN^-. Values are given as means ± S.E. ($n = 3$).

the full number of translocated protons should have been observed, as in Fig. 1B. In other words, the $\rightarrow H^+/O$ ratio observed in the absence of SCN^- should be a monotonically decreasing function of the size of the O_2 pulse, since the membrane potential caused by the O_2 pulse in Fig. 1A should, if present, act to inhibit any further proton translocation. However, Fig. 2A shows that this prediction is not fulfilled: within experimental error, the $\rightarrow H^+/O$ ratio is essentially independent of the size of the O_2 pulse over the range of O_2 pulses examined, both when SCN^- is present and, in particular, when it is absent. The half-times for O_2 reduction and for the consequent H^+ ejection in the absence of SCN^- are plotted as a function of the size of the O_2 pulse in fig. 2B. In all cases, H^+ ejection to a phase in equilibrium with the measuring electrode continued for a significant time after the reduction of O_2 (Figs. 1A and 2A). The half-time for the decay of the observable pH changes caused by the respiration-linked H^+ transfer was of the order of minutes ([2,10]; present data not shown), and thus of no significance to the present considerations. It was also essentially independent of the size of the O_2 pulse over the range examined (not shown).

The kinetic discrepancies observed above using intact cells of *P. denitrificans*, which were also mentioned by Scholes and Mitchell [2], are qualitatively similar to, though not so extensive as, those observed by Gould and Cramer [5,6] in *E. coli* and by ourselves in intact cells of *Rhodopseudomonas capsulata* [20]. However, they do not seem generally to be exhibited by the sphaeroplast preparation of *E. coli* studied by Garland and colleagues (see Ref. 21 and references therein), nor by mitochondria [22], nor by bacterial chromatophores [11,23]. This prompted us to determine the properties of respiration-driven proton translocation in protoplasts of *P. denitrificans*.

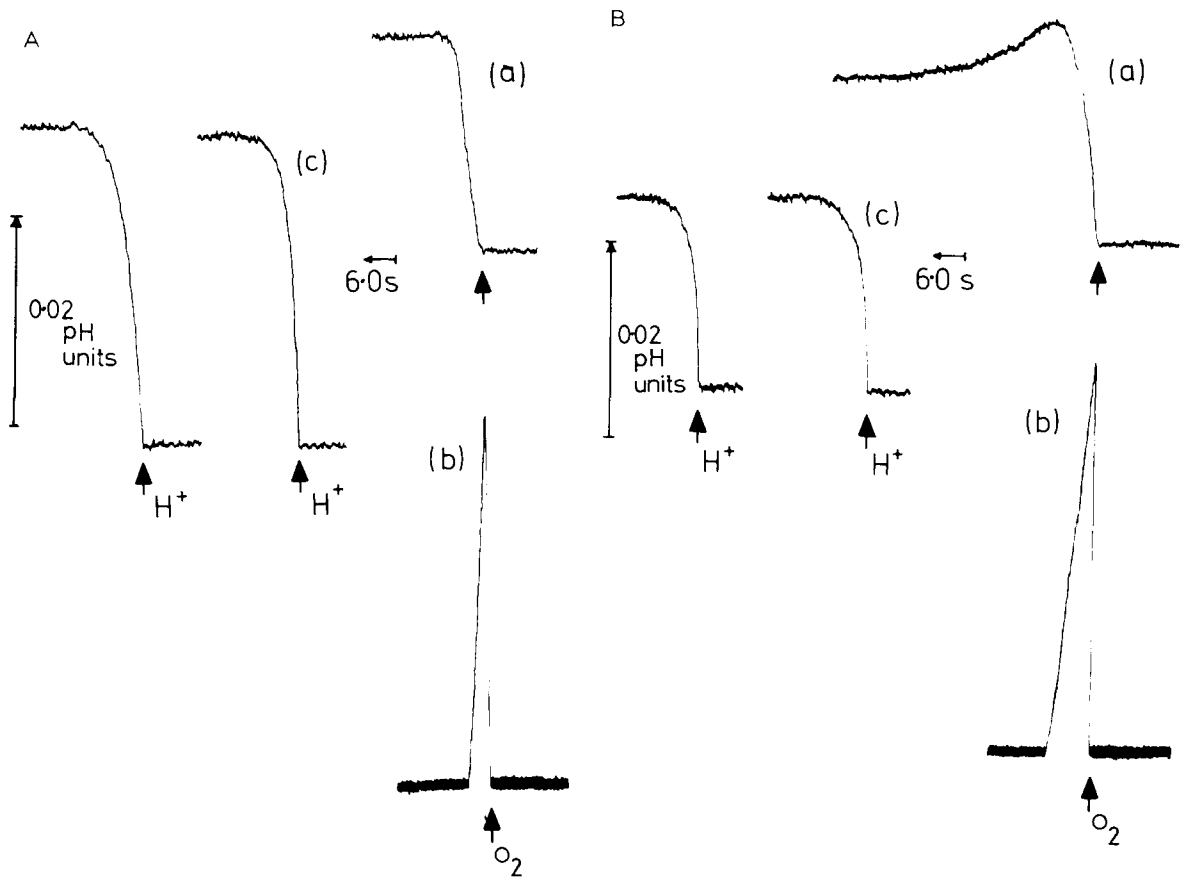

Fig. 3. Respiration and H^+ translocation in *P. denitrificans* protoplasts. Measurements were carried out as described in the Experimental section. The reaction mixture contained, in a final volume of 6 ml, 0.5 M sucrose, 150 mM KCl, 0.25 mM glycylglycine (pH 7.0), 480 μg carbonic anhydrase and protoplasts corresponding to a dry wt. of 3 mg. In (B), 60 μg venturicidin was also present. At the arrows indicated, O_2 (47 ng atom) was added as air-saturated 150 mM KCl, and the pH (a) and O_2 content (b) of the medium followed as described in the legend to Fig. 1. In (c), anaerobic HCl (50 ng ion) was added at the arrow indicated.

Fig. 3A shows typical traces of respiration-driven proton translocation in *P. denitrificans* protoplasts in the absence of KSCN. There is no significant kinetic discrepancy between the respiratory and visible protonmotive activities of the protoplasts, whether SCN^- is absent (Fig. 3A) or present (data not shown). Fig. 4A shows that, as in intact cells, the apparent $\rightarrow H^+/O$ ratio is independent of the size of the O_2 pulse. As discussed above, these observations do not seem to be consistent with a model in which the observable protons are acting to decrease the $\rightarrow H^+/O$ ratio.

Since it is evident that the effect of membrane energisation, as caused by the reduction of an amount of O_2 less than even the lowest amount added in the present work, is not to inhibit further protonmotive activity per se (Figs. 2A and 4A), one is inclined to infer that some protons that were pumped across the membrane returned back across the membrane without ever entering a phase in equilibrium with the measuring pH electrode. Work by Jackson and co-workers in photosynthetic bacteria has demonstrated that a potentially major 'leak' pathway is constituted by the F_0F_1-H^+-ATP synthase, under both phosphorylating and non-phosphorylating conditions [24,25]; we therefore chose to study the effect of the energy-transfer inhibitor venturicidin,, which is active against the F_0F_1-H^+-ATP synthase of this organism [26], on respiration-driven proton translocation. The idea behind this experiment was as follows. If the reason for the low $\rightarrow H^+/O$ ratios

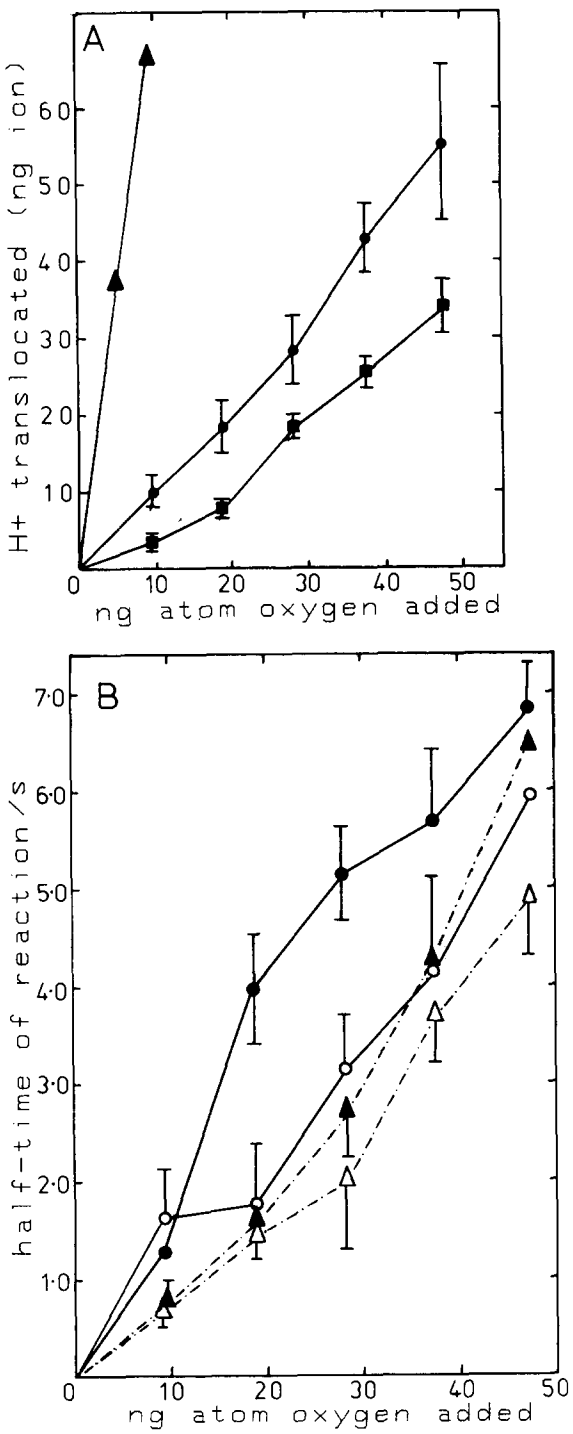

Fig. 4. The effect of venturicidin on the observable stoichiometry and kinetics of respiration-driven H^+ translocation in *P. denitrificans* protoplasts. Measurements were carried out as described in the legend to Fig. 3. The protoplast concentration varied between 0.5 and 1.6 mg/ml. (A). The quantity of H^+ translocated, as calculated from the furthest excusion of the pH trace, is plotted against the size of the O_2 pulse. Venturicidin was either absent (■) or present (●) at a concentration in the range 4–20 μg/mg dry wt. protoplasts. Where indicated (▲), KSCN was also present at a concentration of 60 mM; only two points are shown so as to allow the scale on the diagram more clealry to indicate the effect of venturicidin. Values (■, ●) are given as means ± S.D. ($n = 9$). (B). The observed half-times of H^+ ejection (○, ●) and O_2 uptake (△, ▲), obtained from traces similar to those shown in Fig. 3, are plotted against the size of the O_2 pulse. Open symbols: venturicidin absent. Closed symbols: venturicidin present in the range 4–20 μg/mg dry wt. protoplasts. Values are given as means ± S.D. ($n = 9$).

seen in the absence of SCN^- was the build-up of a large, delocalised membrane potential, which might then cause a rapid (non-ohmic) back-leakage of some of the protons pumped during the O_2 pulse, then decreasing this leak should increase the membrane potential still further, and thus, if Δp-dependent redox slip is possible, presumably act to inhibit observable protonmotive activity yet more potently. This should therefore either decrease the $\rightarrow H^+/O$ ratio observed for a given size of O_2 pulse or exacerbate the expected decrease in the $\rightarrow H^+/O$ ratio as a function of the size of the O_2 pulse. Nevertheless, it is known that venturicidin stimulates the extent of light- and respiration-driven H^+ translocation in *Rps. capsulata* [24].

Fig. 3B shows the observable respiratory and protonmotive activities of *P. denitrificans* protoplasts preincubated with an appropriate concentration of venturicidin, in the absence of SCN^-; the $\rightarrow H^+/O$ ratio calculated from the furthest excursion of the pH trace is significantly greater than that observed in the absence of the energy transfer inhibitor (Fig. 3A) although still greatly submaximal. The observable rate of H^+ decay following the O_2 pulse is, following a small, relatively rapid phase (see Fig. 3B), still very long on the time-scale under consideration. (The $\rightarrow H^+/O$ ratio calculated after the relatively rapid decay phase is essentially unchanged). Fig. 4A shows that this increase in the $\rightarrow H^+/O$ ratio is, within the limits of the experimental noise, independent of the size of the O_2 pulse. A synthesis of these data gives values for the $\rightarrow H^+/O$ ratio in protoplasts (in the absence of SCN^-) of 0.57 ± 0.04 (mean ± S.D., $n = 49$) in the absence, and 1.06 ± 0.03 (mean ± S.D., $n = 44$) in the presence of

venturicidin. Fig. 4A also shows representative values of H^+ translocation in the presence of 60 mM KSCN. No systematic changes in the $\rightarrow H^+/O$ ratio were observed upon changing the venturicidin concentration in the range 4–20 μg per mg dry wt. protoplasts. That there were no large kinetic discrepancies between respiration and proton pumping in protoplasts, especially in the absence of venturicidin, is demonstrated by the data of Fig. 4B.

4. Discussion

4.1. General

We wish first to make some general remarks concerning the experimental design. Since the O_2 was added as a 'pulse', one might question the stationarity assumption [27] inherent in the use of macroscopic concepts such as $\Delta\psi$ and ΔpH. Two lines of reasoning give us confidence that, at least as far as the macroscopically observable bulk-phase phenomena are concerned [28], we may treat the system as though it attained a (quasi-)stationary and homogeneous state for a time corresponding to the overwhelming majority of that in which the cells or protoplasts were respiring, and which was sufficiently uniform to justify the use of macroscopic thermodynamic quantities: (1) the observable decay rates of pumpted H^+ were generally extremely small, and (2) in all cases, for a given set of parameters (pH, batch of cells, concentration of SCN^- and venturicidin) the observable $\rightarrow H^+/O$ ratio was independent of the size of the O_2 pulse. Further, according to chemiosmotic considerations, it may be calculated that the transmembrane electrical potential that should be built up by electrogenic H^+ transfer should exceed say 150 mV during the reduction of an amount of O_2 corresponding to less than even one tenth of the lowest amount used in the present experiments [10]. Finally, the very reasonable linearity of the half-times for both O_2 reduction and H^+ translocation with the size of the O_2 pulse (Figs. 2B and 4B) adds further justification to our view that, as far as the macroscopically observable phenomena are concerned, the stationarity assumption is, for the variables measured, an adequately close approximation to reality.

Although work with intact cells and protoplasts is perhaps inherently more prone to certain, but not all, types of interpretative difficulty than work with isolated cytoplasmic membrane vesicles, we wish to mention experimental evidence (a) that in the absence of terminal electron acceptors this organism is incapable of creating or maintaining an energised membrane [17], and (b) that deenergised intact cells of this organism do not maintain a Donnan potential of either positive [10] or negative [17] polarity inside, and there is thus no preexisting pH gradient prior to the O_2 pulse (see also [2,47]). From a heuristic standpoint, therefore, we believe that we may treat the macroscopic membrane bioenergetics of this organism as ade-

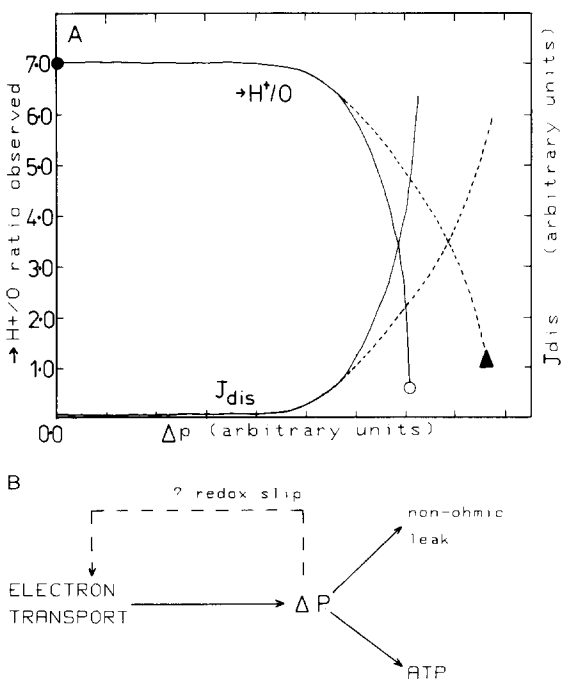

Fig. 5. (A) An attempt to relate the measured $\rightarrow H^+/O$ stoichiometries to the putative values of the protonmotive force developed during the O_2 pulse, assuming that a significant and rapid (non-ohmic) return of H^+ across the coupling membrane, from the bulk aqueous extracellular phase (J_{dis}), may occur at high values of the protonmotive force. The measured points indicate the $\rightarrow H^+/O$ ratios measured (with protoplasts) in the absence (○, ▲) and presence (●) of SCN^-, and in the presence of venturicidin (▲). It is taken that venturicidin modifies the 'native' $J_{dis}/\Delta p$ relationship (solid line) in the fashion indicated (dotted line). For fuller discussion, see the text. (B) A diagrammatic, delocalised chemiosmotic energy-coupling scheme corresponding to the plots in (A).

quately fulfilling the (quasi-)stationary state assumption both before and during the O_2 pulses. These considerations also rule out the possibility of an O_2-pulse size-dependent change in the pathway of electron transfer and its efficiency of coupling to protonmotive activity.

4.2. The $\rightarrow H^+/O$ ratio and membrane ionic conductance as a function of the putative protonmotive foce

To facilitate the discussion, we wish to consider the reasonably sophisticated chemiosmotic scheme shown in Fig. 5. This assumes (see also Ref. 29) that under steady-state conditions there exists a monotonic relationship between the $\rightarrow H^+/O$ ratio, the protonmotive force and the return of bulk-phase protons across the coupling membrane (J_{dis}), which latter may or may not be coupled to phosphorylation. Fig. 5A indicates a non-ohmic relationship between the membrane ionic current density, J_{dis}, and the putative protonmotive force, similar in form to that implicated by Jackson and co-workers [25,30,31] in *Rps. capsulata* and widely observed in 'acid-bath' phosphorylation experiments [32,33]. If we assume that SCN^- acts to decrease the membrane potential component of the protonmotive force, we may plot two points on the graph representing the relationship between the $\rightarrow H^+/O$ ratio and the protonmotive force. The independence between the $\rightarrow H^+/O$ ratio and the size of the O_2 pulse indicates that the 'low protonmotive force' value ((●), Fig. 5A) might be placed essentially anywhere (but see Ref. 29) on the horizontal section of this curve. The stationarity condition means (i) that the assumed protonmotive force generated by the O_2 pulses in the present work iin the absence of SCN^- ((○), Fig. 5A) must be the maximum sustainable by the membranes under these conditions, and (ii) that the $\rightarrow H^+/O$ versus Δp curve must be a mirror image (in shape) of that of J_{dis} versus Δp. The data in the absence of venturicidin might therefore be construed broadly as being consistent with the chemiosmotic scheme of Fig. 5B, although we find it difficult to understand how the number of protons translocated into the bulk, measuring phase (the pH change they cause corresponding roughly to half the ΔpH generated [2]), might continue to increase with the size of the O_2 pulse with no change in the protonmotive force, if the protonmotive force constitutes a 'primary macroerg' [34] in this system.

Parenthetically, it is worth remarking here that for very high O_2/cell ratios (or, for photosynthetic systems, extended periods of illumination), the $\rightarrow H^+/e^-$ ratio does of course decrease; under static head conditions it attains a net value of zero. These conditions are not relevant to the present considerations (which were chosen to fulfil the stationarity condition), and it is to be assumed that under static head conditions the observable proton transport may be limited, particularly in ion-leaky membranes, by the unfavourable intravesicular pH per se.

4.3. The effect of venturicidin

Venturicidin possesses the classical properties of an F_0-type energy-transfer inhibitor: it acts to induce respiratory control in 'leaky' membrane vesicles [26] and to inhibit ADP-stimulated respiration [35] in well-coupled everted membrane vesicles derived from this organism. In the present experiments, venturicidin increased the (submaximal) $\rightarrow H^+/O$ ratio in a fashion independent of the size of the O_2 pulse.

If we are to fit this value of $\rightarrow H^+/O$ to the graph in Fig. 5A, we cannot do so simply by modifying the (bulk) J_{dis} versus Δp graph so as to increase the maximum Δp attainable (Fig. 5A) [25]. We cannot change self-consistently the (bulk) J_{dis} vs. Δp relationship, since the removal (or signifcant decrease of) the non-ohmicity should mean, in a chemiosmotic model, that, whatever the maximum Δp attainable by the membranes under a given set of conditions, the magnitude of J_{dis} at this value of Δp should be in inverse proportion to the $\rightarrow H^+/O$ ratio measured, given the stationarity condition. In the absence of venturicidin (and of SCN^-), the value of the observable J_{dis} is demonstrably of negligible magnitude (Fig. 1A; [2,20]). Any 'non-ohmicity' model must therefore invoke a very rapid back-leakage (whether through the H^+-ATP synthase or otherwise) that occurs before the pH electrode can respond. However, a non-linear back-decay of H^+ was observable in the venturicidin experiments (Fig. 3), and may be taken as a quantitative measurement of the value of J_{dis}; thus the relative magnitudes of the $\rightarrow$

H^+/O ratio observed in protoplasts with and without added venturicidin (Fig. 4) indicate that the non-ohmicity required (Fig. 5A) to account for the $\rightarrow H^+/O$ values observed in the absence of venturicidin (more than 50% of those in its presence; Fig. 4), would have been easily measurable in our experimental arrangement (Fig. 3). Further, (a) venturicidin does not act to increase the maximum state 4 Δp attainable by coupled membrane vesicles of this organism whether added ADP is present or not [16], and (b) the difference in the apparent Δp generated by such vesicles in state 3 and state 4 conditions is independent of the rate of electron transport (Ref. 36, and see Ref. 13). Nevertheless, venturicidin consistently, and in a fashion that is independent of the size of the O_2 pulses examined, acts to increase the observable $\rightarrow H^+/O$ ratio in the absence of SCN^-. If we wished to fit this finding to the graph of Fig. 5A (solid line) we might assume that venturicidin acts to decrease the protonmotive force generated in its absence; as discussed elsewhere [11,37], this is a degree of freedom that is not permitted for orthodox energy transfer inhibitors in delocalised coupling models. A Δp-dependent change in the actual $\rightarrow H^+/O$ ratio, caused, perhaps, by variable 'redox-slip' reactions [38,40] is also ruled out by the observation that venturicidin raises the $\rightarrow H^+/O$ ratio. We may also mention that the submaximal $\rightarrow H^+/O$ ratios observed in the absence of SCN^- are found even when the calculated membrane potential (at very low O_2/cell ratios) is energetically insignificant [5,10], conditions in which the putative J_{dis} value is of negligible magnitude. Thus we are forced to conclude that the low $\rightarrow H^+/O$ ratios observed in the absence of SCN^-, relative to those seen in its presence, are not caused by the build-up of a delocalised protonmotive force.

4.4. Might some protons 'hide' in the periplasmic space of intact cells?

One possible means to explain the kinetic discrepancy between H^+ ejection and O_2 reduction seen in intact cells in the absence of SCN^- is to suggest that the cell wall might act as a significant permeability barrier to protons [5]. Although this idea could provide a simple explanation for the lack of kinetic discrepancy seen in protoplasts, it would conflict markedly with the well-estblished finding that the cell wall does not provide a permeability barrier of any significance either to H^+ in this organism [41]; Fig. 1, calibrating acid pulses) or to low molecular weight substances generally [42]. We are therefore inclined to implicate some as yet poorly characterised interactions which must exist between the cell wall and the cytoplasmic membrane, interactions that can affect the ability of the cytoplasmic membrane itself to retain energised protons in a state inaccessible even to the periplasmic space. Nevertheless, the finding of a consonance between the P/O ratios implicated in vivo and those observed in vitro using cytoplasmic membrane vesicles of this organism [15,43,44], indicates that the kinetic discrepancies observed, although of interest, are of relatively little significance to the process of oxidative phosphorylation.

4.5. How much phosphorylation takes place during O_2 pulses in the absence of SCN?

Although, for technical reasons, we were unable to measure the extent to which ATP synthesis took place during the O_2 pulses, the data of Hanselmann [45], obtained with this organism, indicate that, upon initiation of aerobic respiration in the absence of SCN^-, ATP synthesis commences after a lag of between 0 and 2 s. Thus it is overwhelmingly likely that, in the absence of venturicidin and of SCN^-, some H^+ return through the H^+-ATP synthase was indeed occurring. However, the quantitative considerations raised in subsection 4.3 indicate quite clearly that those protons which returned across the membrane during the O_2 pulses in the absence of SCN^-, and which remained invisible to the pH electrode, remained invisible because they never ever entered a phase with which the measuring electrode was in equilibrium (and see Ref.46). However, the question of whether any of the H^+ backflow through the H^+-ATP synthase was coupled to phosphorylation or not does not of itself materially affect the thrust of the argument presented herein.

Parenthetically, we may mention that any scalar pH changes associated with phosphorylation [18] would have taken place intracellularly, and thus would not have served to obfuscate the extracellular pH measurements on the timescale under consideration. In this regard, one might remark that experiments of the present type in bacterial proto-

plasts possess a useful advantage over those with either mitochondria (which contain an adenine nucleotide translocase) or with phosphorylating membrane vesicles in which the F_1 portion of the ATP synthase faces the external phase.

4.6. The membrane potential and the role of 'permeant' ions

It is surely established that respiration can change the pH both internal and external to intact cells of this organism [2,10,15,47,48]. What is far less clear is to what extent the protonmotive activities of the respiratory chain are coupled to the formation of a bulk-to-bulk phase transmembrane electrical potential difference. We find several lines of evidence which are more than consistent with the view that this extent is of a lower magnitude than that of an artificially applied membrane potential demonstrably required for, and capable of [32], driving ATP synthesis. Such an idea, albeit drastic, would serve usefully to reconcile the demonstrable respiration-linked proton translocatioan into the bulk phase with the anomalies discussed above.

Thus, in brief: (1) observable protonmotive activity causing proton movements to the bulk, measuring phase, seems absolutely dependent upon, and correlated with, the ion permeability of the vesicles of interest [10,11,22,49]; the small number (Ref. 50) of electrogenically pumped protons required maximally to charge the membrane capacitance, though sufficient to be measurable, seem not to be observed [5,10]; (2) in the cases where non-proton ion movements have also been followed, the data are consistent with the view that each observably pumped proton is accompanied by the co- or counter-transport of another ion [51], so that there is no significant net charge imbalance between the bulk phases that the coupling membrane separates; (3) the effect of membrane energisation on the kinetics of unidirectional fluxes of cations often used in estimating $\Delta\psi$ (in mitochondria) is not consistent with the view that the driving force stimulating their uptake and inhibiting their efflux is a delocalised, bulk-to-bulk phase membrane potential [51,52]; (4) in all but one case [53], direct measurements with microelectrodes indicate that the delocalised membrane potential across membranes capable of carrying out electron transport phosphorylation is energetically insignificant (less than 50 mV), even under transient conditions in the absence of any significant pH gradient [52,54]; (5) electroneutral ionophores are capable of fully stimulating the decay of the observable electron transport linked pH changes [23]; in *Paracoccus* protoplasts neither nigericin, acetate nor propionate, which would be expected to collapse the pH gradient, acted rapidly enough to do so persuasively (before the pH electrode could respond) under the conditions tested herein (Hitchens, G.D. and Kell, D.B., unpublished data).

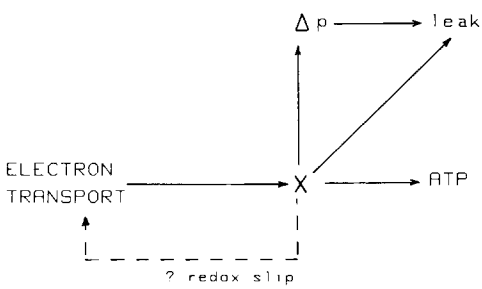

Fig. 6. An energy-coupling scheme in which the observable proton movements are responsible neither for inhibiting further protonmotive activity nor for driving ATP synthesis. X represents a 'high-energy intermediate' whose detailed nature remains unspecified, but which is taken to include both osmotically-inactive protons and 'high-energy' conformational states of membrane proteins, inter alia [11–13]. 'Leak' represents any return of protonic charge across the coupling membrane which is not coupled to phosphorylation. It is taken that the true value of Δp attained in response to electron transport does not exceed that threshold value demonstrably required for, and capable of, driving phosphorylation.

A gross energy-coupling scheme compatible with the above findings, with those in the present article, and with many others in the literature (e.g., Refs. 8–13), is given in Fig. 6. Studies of the present type in photosynthetic systems, exploiting the inherent time resolution possible with trains of single turnover flashes coupled with sensitive spectroscopic methods, might prove of value in further testing such a scheme.

Acknowledgements

We are indebted to Professor A.L. Lehninger for the use of the fast-responding O_2 electrode, and to the Science and Engineering Research

Council, U.K. for financial support. We also wish to thank Miss S.W. Evans for secretarial assistance.

References

1 Nicholls, D.G. (1982) Bioenergetics, Academic Press, New York
2 Scholes, P. and Mitchell, P. (1970) J. Bioenerg. 1, 309–323
3 Davis, D.H., Doudoroff, M., Stanier, R.Y. and Mandel, M. (1969) Int. J. Syst. Bacteriol. 19, 375–390
4 Mitchell, P. and Moyle, J. (1967) Biochem.. J. 105, 1147–1162
5 Gould, J.M. and Cramer, W.A. (1977) J. Biol. Chem. 252, 5875–5882
6 Gould, J.M. (1979) J. Bacteriol. 138, 176–184
7 Hitchens, G.D., Kell, D.B. and Morris, J.G. (1982) J. Gen. Microbiol. 128, 2207–2209
8 Kell, D.B. (1979) Biochim. Biophys. Acta 549, 55–99
9 Kell, D.B. and Morris, J.G. (1981) in Electron and Ion Transport in Mitochondria and Bacteria (Palmieri, F., Quagliarello, E., Siliprandi, N. and Slater, E.C., eds.), pp. 339–347, Elsevier/North-Holland, Amsterdam
10 Kell, D.B. and Hitchens, G.D. (1982) Faraday Discuss. Chem. Soc. 74, 377–388
11 Kell, D.B. and Hitchens, G.D. (1983) in Coherent Excitations in Biological Systems (Fröhlich, H. and Kremer, F., eds.), pp. 178–198, Springer-Verlag, Heidelberg.
12 Kell, D.B. and Westerhoff, H.V. (1984) in Catalytic Facilitation in Organised Multienzyme Systems (Welch, G.R., ed.), Academic Press, New York, in the press
13 Westerhoff, H.V., Melandri, B.A., Venturoli, G., Azzone, G.F. and Kell, D.B. (1984) FEBS Lett. 165, 1–5
14 Reynfarje, B., Alexandre, A., Davies, P. and Lehninger, A.L. (1982) Proc. Natl. Acad. Sci. USA 79, 7218–7222
15 Vignais, P.M., Henry, M-F., Sim, E. and Kell, D.B. (1981) Curr. Top. Bioenerg. 12, 115–196
16 Kell, D.B., John, P. and Ferguson, S.J. (1978) Biochem. J. 174, 257–266
17 McCarthy, J.E.G., Ferguson, S.J. and Kell, D.B. (1981) Biochem. J. 196, 311–321
18 Hitchens, G.D. and Kell, D.B. (1982) Biochem. J. 206, 351–357
19 Kell, D.B. and Morris, J.G. (1979) FEBS Lett. 108, 481–484
20 Kell, D.B. and Hitchens, G.D. (1984) Biochem. Soc. Trans. 12, 413–414
21 Jones, R.W., Lamont, A. and Garland, P.B. (1980) Biochem. J. 190, 79–94
22 Conover, T.E. and Azzone, G.F. in Mitochondria and Microsomes (Lee, C-P., Schatz, G. and Dallner, G., eds.), pp. 481–518, Addison-Wesley, New York
23 Cogdell, R.J. and Crofts, A.R. (1974) Biochim. Biophys Acta 347, 264–272
24 Cotton, N.P.J., Clark, A.J. and Jackson, J.B. (1981) Arch. Microbiol. 129, 94–99
25 Clark, A.J., Cotton, N.P.J. and Jackson, J.B. (1983) Biochim. Biophys. Acta 723, 440–453
26 Ferguson, S.J. and John, P. (1977) Biochem. Soc. Trans. 5, 1525–1527
27 Bendat, J.S. and Piersol, A.G. (1971) Random data. Analysis and Measurement Procedures, Wiley-Interscience, New York
28 Welch, G.R. and Kell, D.B. (1984) in The Fluctuating Enzyme (Welch, G.R., ed.), Academic Press, New York, in the press
29 Heinz, E., Westerhoff, H.V. and van Dam, K. (1981) Eur. J. Biochem. 115, 107–113
30 Jackson, J.B. (1982) FEBS Lett. 139, 139–143
31 Clark, A.J., Cotton, N.P.J. and Jackson, J.B. (1983) Eur. J. Biochem. 130, 575–580
32 Maloney, P.C. (1982) J. Membr. Biol. 67, 1–12
33 Schlodder, E., Gräber, P. and Witt, H.T. (1982) in Electron Transport and Photophosphorylation (Barber, J., ed.), pp. 105–175, Elsevier/North-Holland, Amsterdam
34 Blumenfeld, L.A. (1983) Physics of Bioenergetic Processes, Springer-Verlag, Heidelberg
35 John, P. and Whatley, F.R. (1975) Nature 254, 495–498
36 Kell, D.B., John, P. and Ferguson, S.J. (1978) Biochem. Soc. Trans. 6, 1292–1295
37 Hitchens, G.D. and Kell, D.B. (1983) Biochim. Biophys. Acta 723, 308–316
38 Pietrobon, D., Azzone, G.F. and Walz, D. (1981) Eur. J. Biochem. 117, 389–394
39 Pietrobon, D., Zoratti, M., Azzone, G.F., Stucki, J.W. and Walz, D. (1982) Eur. J. Biochem. 127, 483–494
40 Pietrobon, D., Zoratti, M. and Azzone, G.F. (1983) Biochim. Biophys. Acta 723, 317–321
41 Scholes, P. and Mitchell, P. (1970) J. Bioenerg. 1, 61–72
42 Lugtenberg, B. and van Alphen, L. (1983) Biochim. Biophys. Acta 737, 51–115
43 John, P. and Whatley, F.R. (1977) Biochim. Biophys. Acta 463, 129–153
44 Stouthamer, A.H. (1980) Trends Biochem. Sci. 5, 164–166
45 Hanselmann, K.W. (1974) Efficiency of Energy Conversion in Whole Cells of *Paracoccus denitrificans*. A Kinetic Approach, Ph.D. Thesis, University of Zürich, Juris Druck Verlag, Zürich
46 Graan, T., Flores, S. and Ort, D.R. (1981) in Energy Coupling in Photosynthesis (Selman, B. and Selman-Reimer, S., eds.), pp. 25–34, Elsevier/North-Holland, Amsterdam
47 Ferguson, S.J., Gadian, D.G. and Kell, D.B. (1979) Biochem. Soc. Trans. 7, 176–179
48 Van Verseveld, H.W., Krab, K. and Stouthamer, A.H. (1981) Biochim. Biophys. Acta 635, 525–534
49 Archbold, G.P.R., Farrington, C.L., Gill, D.J.O. and Malpress, F.M. (1976) Biochem. Soc. Trans. 2, 751–754
50 Mitchell, P. (1968) Chemiosmotic coupling and energy transduction, Glynn Research, Bodmin
51 Tedeschi, H. (1982) Biochim. Biophys. Acta 639, 157–196
52 Skulskii, I.A., Saris, N-E.L. and Glasunov, V.V. (1983) Arch. Biochem. Biophys. 226, 337–346
53 Felle, H. Stetson, D.L., Long, W.S. and Slayman, C.L. (1980) Biochemistry 19, 3585–3590
54 Vredenberg, W.J. (1976) in The Intact Chloroplast (Barber, J., ed.), pp. 53–88, Elsevier/North-Holland, Amsterdam