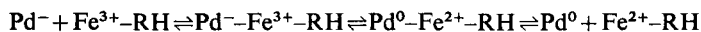


deazariboflavin (dRFI) can be progressively reduced by successive flashes by the following reaction sequence (Sligar & Gunsalus, 1976):



The reducing species is actually the unstable semi-reduced dRFIH[•] radical, which decays spontaneously to an inactive dimer (Massey & Hemmerich, 1978). Thus reduction cannot proceed beyond the stage where the second-order Pd⁰ reduction becomes slower than the second-order dimerization, and in the dark the solution contains no extra reducing equivalents.

On cooling, the Soret band of the cytochrome shifts from 410 nm (Fe²⁺-RH) toward 417 nm (Fe³⁺-RH, low spin) (Fig. 2). This reversible shift follows perfectly heating ⇌ cooling cycles, and clearly indicates the reverse electron transfer from Fe²⁺-RH to Pd⁰ as another possibility of uncoupling.

The various possibilities opened by the combination of subzero temperatures and light-activation can be tested on other sites of the cytochrome *P*-450 cycle and other multi-component electron-transport chains.

We are grateful to Dr. I. C. Gunsalus for the gift of highly purified cytochrome *P*-450 and for helpful suggestions. We thank Dr. J. P. Lhoste for providing 5-deazariboflavin, and Dr. P. Douzou for constant interest in this work.

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On the Current-Voltage Relationships of Energy-Transducing Membranes: Phosphorylating Membrane Vesicles from *Paracoccus denitrificans*

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According to the chemiosmotic hypothesis of biological membrane energy transduction the link between substrate oxidation and ATP synthesis is provided by a bulk phase, transmembrane, electrochemical gradient of protons, the proton-motive force (Δp). Although it is generally believed that proton gradients of some kind are intimately involved in these processes there is some controversy as to whether such gradients are delocalized in the chemiosmotic sense or are of a more localized, membrane-associated nature (Mitchell, 1977; Williams, 1978). The purpose of the present paper is to describe some experiments that we have carried out with phosphorylating membrane vesicles from the bacterium *Paracoccus denitrificans*, and that were designed to investigate the degree of localization of such protein gradients. In contrast to the topologically analogous submitochondrial particles these membrane vesicles exhibit a marked respiratory control by phosphate acceptor (Kell *et al.*, 1978a). Such respiratory control is widely thought to arise from a thermodynamic back-pressure exerted by the functional proton gradient

Fig. 1 shows a titration with rotenone of the rates of NADH oxidation catalysed by the inside-out vesicles from *P. denitrificans*. The rate of oxidation was measured under 3 sets

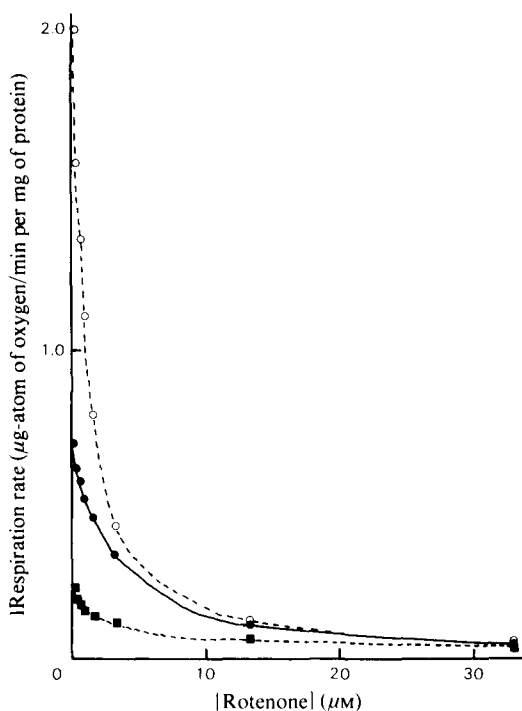


Fig. 1. Titration with rotenone of the rates of NADH oxidation in *P. denitrificans* vesicles

The reaction mixture contained at 30°C in a total volume of 3 ml: 10 mM-phosphate/Tris, pH 7.3, 5 mM-magnesium acetate, 0.2 mg of alcohol dehydrogenase, 30 μ l of ethanol and 0.5 mg of vesicle protein. Rotenone was added to the vesicles 2 min before adding 0.6 mM-NAD⁺ to start the reaction. Symbols: ■, State 4 rate; ●, State 3 rate with 0.2 mM-ADP present; ○, uncoupled rate with 1 μ g of gramicidin D plus 30 mM-ammonium acetate present. O₂ uptake was measured with a Clark oxygen electrode.

of conditions: (1) in the absence of ADP (State 4 in the nomenclature of Chance & Williams, 1956); (2) in the presence of ADP (State 3); (3) in the presence of sufficient gramicidin D and ammonium acetate to cause a maximal stimulation of respiration. Titration of the State 4 rate (Fig. 1) shows that the State 4 rate is immediately sensitive to rotenone, which is not necessarily the result to be expected in terms of hypotheses (Mitchell, 1966, 1977) stressing bulk phase, delocalized proton gradients as the cause of respiratory control. On such a basis, if the primary dehydrogenase is not rate-limiting, it would be expected that the State 4 rate would be insensitive to rotenone in the initial stages of the titration. That the primary dehydrogenase is not rate-limited is demonstrated by the (large) stimulation in respiration rate observed on addition of uncoupler (Fig. 1).

Comparison of the titration curve for State 4 respiration with that for the uncoupled rate shows that the extent of stimulation by the addition of an uncoupler declines as increasing amounts of rotenone are added. The data shown resemble closely the analogous curves obtained by Hinkle *et al.* (1975) for submitochondrial particles, but differ from the profiles reported (also for submitochondrial particles) by Lee *et al.* (1969). Hinkle *et al.* interpreted their data in terms of respiratory control being exerted by a bulk phase Δp , which was assumed to decline as the respiration rate was decreased and thus proton translocation inhibited. An assumption in their work was that the protic

resistance (and capacitance) of the membrane was constant, so that a linear relationship between the rate of respiration and Δp was both predicted and fitted to the titration curves.

With the membrane vesicles from *P. denitrificans*, however, inhibition of NADH oxidation by as much as 80% with rotenone has been found not to affect the magnitude of the membrane potential component ($\Delta\psi$) of the proton-motive force (Table 1). Since under the reaction conditions used $\Delta\psi$ is the sole component of the proton-motive force (Kell *et al.*, 1978a) we conclude that Δp is not measurably decreased by an inhibition of the rate of NADH oxidation of up to 80%. Thus although the titrations of Fig. 1 resemble those of Hinkle *et al.* (1975) we cannot interpret our data in the same way as these latter authors. In particular, the assumption of a constant, voltage-independent membrane capacitance or resistance is not justified for the case of *P. denitrificans*, and may indeed not be justified for bovine heart submitochondrial particles (Sorgato *et al.*, 1978).

The titration of the State 3 rate of NADH oxidation has several points of interest (Fig. 1). First, if oxidation and phosphorylation are linked by an intermediate that is delocalized over the whole membrane then it would be anticipated that the initial additions of rotenone would not have caused any inhibition. This is because the activity of the respiratory chain *per se* at low rotenone concentrations is not rate-limiting (cf. the uncoupled rate), and, as the activity of the adenosine triphosphatases should not be influenced by the addition of rotenone the State 3 respiration rate might also have been unaltered. The rate of State 3 respiration also reflects the rate of ATP synthesis since, in these vesicles, the addition of rotenone causes no significant alteration to the ADP/O ratio (Ferguson *et al.*, 1976). The form of the State 3 respiration curve could be consistent with a mechanism in which the addition of rotenone caused a decline in Δp , which in turn might drive ATP synthesis at a slower rate. This explanation is unacceptable, however, because measurements (Table 1) of SCN^- uptake during respiration under State 3 conditions indicate that rotenone addition again causes no decrease in $\Delta\psi$ (and thus in Δp). Therefore the rate of ATP synthesis is decreased at constant Δp .

Table 1. *Effect of rotenone on the respiratory rate and proton-motive force of P. denitrificans vesicles*

O_2 uptake was determined polarographically and SCN^- uptake potentiometrically in parallel 5 ml reaction mixtures that contained (State 4) 10 mM-phosphate/Tris, pH 7.3, 5 mM-magnesium acetate, 1% (v/v) ethanol, 0.2 mg of alcohol dehydrogenase, 0.6 mM-NAD⁺, 2.65 mg of vesicle protein and 20 μM -KSCN. For State 3 measurements 10 mM-glucose, 10 units of hexokinase and 0.5 mM-ADP were also present. Portions of rotenone were added from a stock solution in ethanol in the amounts indicated. The membrane potential was calculated from the extent of SCN^- uptake measured with a SCN^- ion-selective electrode as described (Kell *et al.*, 1978b), assuming an internal vesicle volume of 4 μl /mg of protein (Kell *et al.*, 1978a). Similar results were obtained when a number of experiments were carried out, each preincubated with the appropriate amount of rotenone before initiation of respiration with NAD⁺. Control rates of respiration were 228 and 410 ng-atoms of oxygen/min per mg of protein for State 4 and State 3 respectively.

| Rotenone added (nmol/mg of protein) | Respiration rate (% of control) | | Membrane potential (mV) | |
|---|------------------------------------|---------|-------------------------|---------|
| | State 4 | State 3 | State 4 | State 3 |
| 0 | 100 | 100 | 150 | 135 |
| 10 | 42 | 53 | 150 | 135 |
| 20 | 27 | 37 | 150 | 135 |
| 30 | 22 | 28 | 150 | 135 |
| 40 | 20 | 20 | 150 | 135 |
| 50 | 17 | 17 | 150 | 135 |

It is also noteworthy that the maximum phosphorylation potential that may be generated by the vesicles (approx. 53.8 kJ/mol) (Kell *et al.*, 1978a) was not altered when the rate of NADH oxidation was inhibited by up to 60%. The corollary of this result is that if Δp is poised at equilibrium with the phosphorylation potential then, as the phosphorylation potential does not decline when NADH oxidation is inhibited, our finding that Δp is not decreased on addition of rotenone is to be expected.

The data presented above show that the resistance (or, more likely, the capacitance) of the *P. denitrificans* membrane must be variable. Both these data, and those of other workers (Casadio *et al.*, 1978; Kupriyanov & Pobochin, 1978) would seem to point to the need to consider more localized factors in biomembrane energy-transduction processes. In this regard it may be profitable to utilize membrane models containing 3 capacitors in series (e.g. Andersen *et al.*, 1978; cf. Bockris & Reddy, 1970).

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The Respiratory Chain and Proton Electrochemical Gradient in the Alkalophile *Bacillus pasteurii*

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Bacillus pasteurii requires rather unusual growth conditions. It grows at alkaline pH (up to 10.5) and requires the presence of relatively high concentrations of ammonium salts. Although the growth of cells is optimal at pH 9.0, substrate oxidation by electron-transport particles is ammonia-independent and maximal at pH 7.5; this has been interpreted to mean that the matrix pH is maintained near neutrality during growth in alkaline medium (Wiley & Stokes, 1963). Therein lies a chemiosmotic dilemma. In the equation

$$\Delta\mu_{H^+} = \Delta\psi - Z\Delta pH$$

using standard notation, the ΔpH is positive, thus for $\Delta\mu_{H^+}$ to be, say, 240 mV the $\Delta\psi$ will have to be greater than that value by an amount equal and opposite to $Z\Delta pH$. Haddock & Cobley (1976) have demonstrated that in terms of vectorial H^+ movement there is nothing unusual about this bacterium; in oxygen-pulse experiments H^+ ions are pumped out from the matrix.

The respiratory chain of *B. pasteurii* contains cytochromes of the *b*, *c* and *a* types. The cytochrome *a* is spectrally similar to the aa_3 terminal oxidase of mammalian systems