Growth Energetics of *Rhizobium leguminosarum* in Chemostat Culture

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The efficiency of oxidative phosphorylation in symbiotic nitrogen-fixing bacteria of the genus *Rhizobium* in the root nodules of leguminous plants is of fundamental importance as it influences the efficiency of ATP generation from the oxidation of plant photosynthate for the ATP-dependent process of nitrogen fixation. A poor efficiency of energy coupling was found for *Rhizobium leguminosarum* grown glucose-limited in chemostat culture as indicated by low growth yields on glucose [$Y_{\text{glucose}}^{\text{max}} = 80.3 \text{ g dry wt bacteria (mol glucose)}^{-1}$] and O_2 [$Y_{\text{oxygen}}^{\text{max}} = 25.6 \text{ g dry wt bacteria (mol O}_2\text{)}^{-1}$]. (These values are low when compared with those for aerobic heterotrophic bacteria which have two or three sites of oxidative phosphorylation.) Although H^+/O measurements suggested a potential P/O ratio of 3 there was a very rapid rate of proton decay across the cell membrane; this coupled with the low growth yields suggested that the actual P/O ratio was nearer 1. Under conditions of excess dissolved oxygen cytochromes of the *c*, *b*, *o* and *aa*₃ types were detected. Under oxygen limitation a *d* type terminal cytochrome oxidase was also produced and was associated with a further decrease in the efficiency of energy conservation. It remains to be seen whether such a low efficiency of energy conservation is expressed by *R. leguminosarum* within the root nodules of a host legume.

INTRODUCTION

An estimation of the P/O ratio in bacteroids and free-living rhizobia is important in relation to the energetics of biological nitrogen fixation in plants. The supply of oxidizable plant photosynthate, and hence energy, may be a factor which under certain conditions can limit nitrogen fixation in legumes (Ratcliffe *et al.*, 1980). The efficiency of energy coupling in the bacteroids will be a major factor which governs the amount of ATP available for nitrogen fixation from the oxidation of plant photosynthate. Studies on ATP production (Appleby *et al.*, 1975; Bergersen & Turner, 1975), terminal cytochrome oxidases (Bergersen & Turner, 1980), membrane energization (Laane *et al.*, 1978), and H^+/O measurements in bacteroids (Ratcliffe *et al.*, 1980) have indicated that the maximum potential P/O ratio may approach 3. Less is known about the efficiency of energy conservation in free-living rhizobia.

The efficiency of energy conservation in bacteria may depend on the respiratory chain composition. Measurements of whole cell H^+/O ratios and maximum growth yields with respect to the utilization of O_2 or the carbon/energy source indicate that the absence of cytochrome *c* in some species may be associated with a decrease in the potential P/O ratio from 3 to 2 (Jones *et al.*, 1977). Within a species the partial or complete replacement of cytochrome oxidases *aa*₃ by *o* had no significant effect on the potential P/O ratio (Jones *et al.*, 1977; Farmer & Jones, 1976).

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Abbreviation: TMPDH₂, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride, reduced.

However, replacement of either *o* or *aa*₃ by cytochrome oxidase *d* (e.g. under conditions of O₂ limitation) in the presence of cytochrome *c* decreases the efficiency of respiratory chain energy conservation (Jones, 1978). In addition to changes in respiratory chain composition the permeability of the cell membrane to protons may influence the efficiency of energy conservation (Ratcliffe *et al.*, 1979; McKay *et al.*, 1982); when the membrane is highly permeable to protons the organisms must respire rapidly to maintain the necessary transmembrane protonmotive force (the H⁺/ATP ratio is greater than 2) and this leads to a low P/O ratio.

In this work we have estimated the efficiency of energy conservation in *R. leguminosarum* grown with a minimal salts medium in chemostat culture under glucose and oxygen limitations. Growth efficiencies were estimated from measurements of growth yields on O₂ and glucose and by H⁺/O measurements and were correlated with the cytochrome content of the bacteria. A preliminary report of this work (Ratcliffe *et al.*, 1979) indicated that there was a low efficiency of energy conservation due to a high membrane permeability to protons and is supported by the low yields on oxygen for the growth of *R. trifolii* grown with a complex medium in chemostat culture (de Hollander *et al.*, 1979).

METHODS

Organism. *Rhizobium leguminosarum* TA 101, a gift from Dr J. C. Burton, Nitragin Sales Corporation, Milwaukee, Wisconsin, U.S.A., was maintained on yeast extract mannitol agar plates at 4 °C and subcultured monthly.

Medium. Bacteria were grown in a medium, designed to give glucose limitation, which contained (g l⁻¹): glucose, 8.0; (NH₄)₂SO₄, 3.3; KH₂PO₄, 0.34; Na₂HPO₄, 0.355; MgSO₄·7H₂O, 0.11; FeSO₄·7H₂O, 0.025; CaCl₂·2H₂O, 0.33; nitrilo-triacetic acid, 0.05; calcium pantothenate, 2.0 mg; thiamin·HCl, 1.5 mg; biotin, 6.0 µg and 4.5 ml trace elements solution containing (g l⁻¹): CuSO₄·5H₂O, 0.025; ZnSO₄·7H₂O, 0.16; MnSO₄·4H₂O, 0.20; CoCl₂·2H₂O, 0.002; Na₂MoO₄·2H₂O, 0.24; KI, 0.16; H₃BO₃, 0.08 and 5 ml 1.0 M-H₂SO₄.

The medium (30 l) was sterilized by autoclaving at 121 °C. Glucose, MgSO₄·7H₂O and CaCl₂·2H₂O were autoclaved separately and added aseptically when the medium was at room temperature. Solutions of vitamins, FeSO₄·7H₂O and (NH₄)₂SO₄ were filter-sterilized (Millipore 0.45 µm filter) before aseptical addition to the medium reservoir.

Chemostat. A stirred (600 r.p.m.) Biotec fermenter (LKB) with a modified head plate to take a direct drive assembly was used. The culture temperature was maintained at 28.0 °C ± 0.5 °C, the pH at 6.8 ± 0.02 by the addition of 0.75 M-KOH/0.25 M-NaOH, and the culture working volume at approximately 2.5 l. The gas supply was air (300 to 600 ml min⁻¹) except for studies on oxygen limitation where air/nitrogen mixtures were used.

Gas composition. A fully automatic computerized gas chromatograph system (for O₂, CO₂ and N₂) was used to determine *in situ* O₂ uptake or CO₂ production rates. The gases were separated by the modified gas chromatographic method of Deans *et al.* (1971). Helium was the carrier gas.

Hydrogen was assayed using the katharometer detector on a Varian model 3700 gas chromatograph. A 5 Å molecular sieve column of length 6 ft (1.8 m) and internal diameter 1/8 in (3.2 mm) was used with N₂ as the carrier gas (20 ml min⁻¹) at 60 °C. The outlet from the fermenter was passed through a 50 ml conical flask with a Suba-seal stopper for 30 min. Samples were removed from the flask for the assay.

Bacterial dry weight. Samples of the culture (25 ml) were washed twice with distilled water and dried to constant weight (Linton *et al.*, 1975).

Total bacterial carbon. Analysis of total culture carbon and supernatant carbon was made with a Beckman model 915 total organic carbon analyser (Linton *et al.*, 1975).

Glucose assay. The glucose concentrations in the medium and supernatant were measured by either the glucose oxidase system (Boehringer Glucose Oxidase Test Kit, ser. no. 124028) or a UV system (Boehringer Glucose-Fructose, UV Test Kit, ser. no. 139106).

Carbon, hydrogen and nitrogen analysis. The percentage of elemental carbon, hydrogen and nitrogen in freeze-dried samples of bacteria was determined with a Hewlett-Packard 185 C, H and N analyser according to manufacturer's instructions.

Cytochrome content. The cytochrome content of bacteria was determined as previously described (Ratcliffe *et al.*, 1980).

H⁺/O ratios. H⁺/O ratios [g equiv. H⁺ translocated (g equiv. O consumed)⁻¹] were measured as previously described (Mitchell & Moyle, 1967; Jones *et al.*, 1975). The *t*₁ for collapse of the pulses was calculated from a plot of the log of peak height against time (Mitchell & Moyle, 1967).

Potential respiration rates of bacteria. Bacteria were harvested from the chemostat, washed and diluted in complete mineral salts medium minus the carbon and nitrogen sources. The respiration rates [*q*_{O₂}; mmol O₂

Table 1. *In situ* rates of substrate uptake by *R. leguminosarum* grown glucose-limited in continuous culture, pH 6.8, 28 °C

The correlation coefficient for q_{glucose} is 0.996 and for q_{O_2} is 0.975. Data are based on least square fit analysis. Values for q were determined as described in Methods. Organic carbon in the supernatant was at or below the limits of detection.

Dilution rate (h ⁻¹)	q_{glucose} [mmol glucose consumed (g bacteria) ⁻¹ h ⁻¹]	q_{O_2} [mmol O ₂ consumed (g bacteria) ⁻¹ h ⁻¹]	Carbon recovery (%)
0.05	0.66	2.10	115
0.075	0.97	2.68	104
0.10	1.31	4.27	98
0.13	1.63	4.98	106

Table 2. Elemental carbon, hydrogen and nitrogen analysis of *R. leguminosarum* organisms grown under glucose limitation, pH 6.8, 28 °C

Bacteria were harvested from the chemostat, centrifuged, washed twice with distilled water (5 °C) and freeze-dried before analysis (see Methods). Percentages are expressed on a dry weight basis.

Dilution rate (h ⁻¹)	Carbon (%)	Hydrogen (%)	Nitrogen (%)
0.05	46.7	7.3	12.7
0.075	45.6	7.0	12.4
0.10	44.8	6.7	10.9
0.13	45.0	6.9	11.1
0.16	45.1	6.9	10.9

consumed (g bacterial dry wt)⁻¹ h⁻¹] of intact bacteria were measured on addition of known quantities of substrate by following O₂ uptake as measured polarographically at 28 °C in an oxygen electrode respirometer (Rank Bros, Bottisham, Cambridge).

Dissolved oxygen. The dissolved oxygen tension (DOT) was measured with a galvanic oxygen electrode (L. H. Engineering Co. Ltd, Stoke Poges, Buckinghamshire). The electrode was calibrated against air and nitrogen. The DOT was converted into concentration assuming air saturated medium at 28 °C, 760 mmHg pressure to contain 240 µM oxygen.

Calculation of bacterial yields on glucose and oxygen. All these yields were calculated from the *in situ* substrate uptake rates in the chemostat (Linton *et al.*, 1975).

RESULTS

Determination of biomass yields on glucose and oxygen

The yield values (corrected for maintenance substrate consumption) calculated from the *in situ* substrate uptake data (Table 1) for *R. leguminosarum* grown aerobically under glucose limitation were: $Y_{\text{glucose}}^{\text{max}}$, 80.3 g dry wt bacteria (mol glucose)⁻¹; $Y_{\text{O}_2}^{\text{max}}$, 25.6 g dry wt bacteria (mol O₂)⁻¹. An equation adapted from Herbert (1976) was used to check the *R. leguminosarum* yields even though the experimental results showed good carbon recovery values (Table 1) and the correlation coefficient for the data points of q_{O_2} or q_{glucose} versus D (Table 1) was near unity. The equation was adapted to include the nitrogen source:

If a value for Y_{glucose} is assumed then a corresponding value for Y_{O_2} can be calculated when the equation is balanced with respect to a , b , c , d , e and x . The amount of carbon, hydrogen and nitrogen in c , i.e. the dry bacteria, can be calculated from elemental analysis of freeze-dried bacteria (Table 2). The effect of the nitrogen balance on the relationship between Y_{glucose} and Y_{O_2} is shown in Fig. 1.

Fig. 1.

Fig. 1. Theoretical relationship between Y_{glucose} and Y_{O_2} for *R. leguminosarum* grown aerobically on glucose. The method for determining the values plotted was based on an adapted equation of Herbert (1976) and is described in the text. ●, NH_4^+ not included in calculation, cell empirical formula = $\text{CH}_2\text{N}_{0.25}\text{O}_{0.55}$; ▲, NH_4^+ included in calculation, cell empirical formula = $\text{CH}_2\text{N}_{0.25}\text{O}_{0.55}$; ■, NH_4^+ included in calculation, cell empirical formula = $\text{CH}_{1.84}\text{N}_{0.22}\text{O}_{0.55}$.

Fig. 2.

Fig. 2. Relationship between the steady-state DOT, residual glucose and ammonia concentrations, and bacterial dry wt in a glucose- or O_2 -limited chemostat culture of *R. leguminosarum*. Bacteria were grown at $D = 0.1 \text{ h}^{-1}$ under glucose or O_2 limitation with $(\text{NH}_4)_2\text{SO}_4$ as the nitrogen source. The DOT in the culture supernatant was adjusted by altering the ratio of air to nitrogen in the inlet gas stream: ■, glucose in supernatant (g l^{-1}); ●, ammonia in supernatant (g l^{-1}); ▲, bacterial dry wt (g l^{-1}). Biomass determinations were based on carbon analysis assuming bacteria contained approximately 45% carbon (see Table 2).

The experimental values obtained for $Y_{\text{glucose}}^{\text{max}}$ or $Y_{\text{O}_2}^{\text{max}}$ agree closely with the theoretically calculated values, e.g. an experimental $Y_{\text{glucose}}^{\text{max}}$ value gave the expected $Y_{\text{O}_2}^{\text{max}}$ result when checked against the theoretical $Y_{\text{O}_2}^{\text{max}}$ value. A $Y_{\text{glucose}}^{\text{max}}$ value of $84.2 \text{ g bacteria (mol glucose)}^{-1}$ and $Y_{\text{O}_2}^{\text{max}}$ value of $23.8 \text{ g bacteria (mol O}_2\text{)}^{-1}$ were obtained from data based on bacterial dry weights instead of on carbon analysis. The maintenance energy requirements were $0.031 \text{ mmol glucose g}^{-1} \text{ h}^{-1}$ and $0.023 \text{ mmol O}_2 \text{ g}^{-1} \text{ h}^{-1}$.

Biomass yield and oxygen limitation

The culture was grown as described in Methods (except that the glucose and $(\text{NH}_4)_2\text{SO}_4$ concentrations were approximately 5.0 g l^{-1} and 10 mM , respectively) at a constant dilution rate ($D = 0.1 \text{ h}^{-1}$). The steady state dissolved O_2 concentration in the culture vessel was maintained at values which ranged from undetectable dissolved O_2 to approximately $205 \mu\text{M O}_2$ by alteration of the inflowing air and nitrogen gas flow rates to the fermenter.

A decrease in the steady state biomass concentration was observed with decrease in the steady state DOT (Fig. 2). At undetectable dissolved O_2 (i.e. concentrations of dissolved O_2 below approximately $2 \mu\text{M}$, the minimum which can be reliably detected with the oxygen electrode), the culture was no longer glucose-limited as there was approximately $1.63 \text{ g glucose l}^{-1}$ in the culture supernatant. The concentration of ammonia in the supernatant also increased (Fig. 2). As all other substrates were in excess, the substrate limiting the growth of *R. leguminosarum* was presumed to be oxygen.

The biomass yield on glucose and CO_2 decreased with a decrease in DOT in the culture (Table 3). Unfortunately yields on O_2 were not measured because of faulty equipment.

Table 3. Specific rates of glucose utilization and CO₂ production in a glucose/oxygen-limited chemostat culture of *R. leguminosarum*

Bacteria were grown at $D = 0.1 \text{ h}^{-1}$, 28°C and pH 6.8. The dissolved oxygen concentration in the culture supernatant was adjusted by altering the ratio of air to nitrogen in the inlet gas stream. The carbon recovery values were calculated from carbon recovered as [(total outlet carbon)/(total inlet carbon)] $\times 100$. Yield values were calculated from the equation $Y = D/q_{\text{substrate}}$.

Dissolved oxygen concn in chemostat (μM)	q_{glucose} [mmol glucose consumed (g dry wt) ⁻¹ h ⁻¹]	Y_{glucose} [g dry wt bacteria (mol glucose) ⁻¹]	q_{CO_2} [mmol CO ₂ produced (g dry wt) ⁻¹ h ⁻¹]	Y_{CO_2} [g dry wt bacteria (mol CO ₂) ⁻¹]	Carbon recovery (%)
205.5	1.425	70.2	3.16	31.6	93
143.1	1.37	73.0	3.34	29.9	101
110.3	1.47	68.0	3.54	28.3	94
94.6	1.56	64.1	3.74	26.7	93
0	1.72	58.0	4.77	21.0	99

H⁺/O ratios in R. leguminosarum grown at different dissolved oxygen concentrations in a glucose-limited chemostat

The effect of DOT on the H⁺/O ratios in free-living *R. leguminosarum* was examined as described in Methods. In this series of experiments it was often impossible to obtain reproducible results when the organisms were maintained in a state of preparation for the experiment, i.e. when the organisms were washed, centrifuged and resuspended in buffer (140 mM-KCl, 1 mM-Tris at pH 7.0). The results (Table 4) deviated from the expected H⁺/O ratios of 6, 4 and 2 for glucose, succinate and ascorbate-TMPDH₂ (NADH, FADH and cytochrome *c* linked substrates), respectively (Mitchell & Moyle, 1979). H⁺/O ratios were obtained in all cases with endogenous substrate even though the organisms were grown in a glucose-limited (i.e. carbon-limited) chemostat. A feature of the proton pulse experiments was the rapid collapse of the pulse, with a $t_{\frac{1}{2}}$ (time for pulse height to decrease by 50%) of between 12 and 15 s.

The reason for the irreproducibility of the results was difficult to determine. The ability of the organisms to oxidize glucose (5 mM) and succinate (5 mM), when washed and resuspended in 15 mM-phosphate buffer (pH 6.8), after removal from the chemostat, was examined and found to remain almost constant over a 6 h period. The K_s of *R. leguminosarum* for succinate-dependent O₂ uptake, grown glucose-limited at $D = 0.1 \text{ h}^{-1}$, was approximately 1.2 mM and that for glucose-dependent O₂ uptake was 10 to 60 μM .

Cytochrome content of R. leguminosarum grown under O₂/glucose limitation

The quantity of cytochromes c_{550} , c_{555} , b_{560} and cytochrome oxidase aa_3 as a percentage of the total peak heights in *R. leguminosarum* grown at different values of DOT is given in Table 5. The unequivocal appearance of an adsorption maximum at 630 nm (possibly cytochrome *d*) at zero detectable dissolved oxygen is apparent (Fig. 3c). This peak was also present at 95 μM DOT in the chemostat. CO-reduced minus reduced difference spectra indicated that cytochrome *o* was present at all dissolved oxygen concentrations.

DISCUSSION

The values of $Y_{\text{glucose}}^{\text{max}}$ and $Y_{\text{oxygen}}^{\text{max}}$ for *R. leguminosarum* grown glucose-limited were much lower than values reported for other similar cultures of heterotrophic bacteria having two or three sites of oxidative phosphorylation with O₂ as the terminal electron acceptor. For example, Jones *et al.* (1977) found $Y_{\text{O}_2}^{\text{max}}$ values of 91.0 and 61.9 g bacteria (mol O₂)⁻¹ for glucose-limited bacterial cultures which respectively contained or lacked cytochrome *c*. Also, a low $Y_{\text{O}_2}^{\text{max}}$ value

Table 4. *Respiration driven proton translocation in R. leguminosarum from a glucose/oxygen-limited chemostat culture, 28 °C, pH 6.8*

Bacteria were obtained from a chemostat ($D = 0.1 \text{ h}^{-1}$), washed and resuspended in 2 mM-Tris, 140 mM-KCl (pH 7.0), 60 mM-KSCN, 5 ml reaction volume. Not reproducible, i.e. peaks obtained bore little relationship to quantity of electron donor injected. At least 10 separate determinations were made to obtain each value. The half-life for the decay of the pulses was approximately 12 to 15 s.

Dissolved oxygen concn in chemostat (μM)	H^+/O ratios [g atom H^+ translocated (g atom O consumed) $^{-1}$]							
	Endogenous		Glucose (5 mM)		Na-succinate (10 mM)		Ascorbate-TMPDH ₂	
	NaOH for calibration	HCl for calibration	NaOH	HCl	NaOH	HCl	NaOH	HCl
205.6	5.41 $\pm$ 0.46	5.96 $\pm$ 0.02	6.47 $\pm$ 0.18	5.48 $\pm$ 0.01	4.17 $\pm$ 0.57	4.04 $\pm$ 0.04	3.97 $\pm$ 0.46	3.94 $\pm$ 0.77
144.0	3.29	4.90	Not reproducible	Not reproducible	Not reproducible	Not reproducible	2.61	3.96
110.3	5.8 $\pm$ 0.8	5.8 $\pm$ 0.8	4.12 $\pm$ 0.02	4.2 $\pm$ 0.18	5.34	3.85	3.82 $\pm$ 0.18	3.93 $\pm$ 0.19
94.6	4.83 $\pm$ 0.07	4.4 $\pm$ 0.04	3.69 $\pm$ 0.11	3.37 $\pm$ 0.05	ND	ND	ND	0.62
0	4.16	3.55	3.97 $\pm$ 1.41	3.06 $\pm$ 1.32	1.35	1.93	3.14 $\pm$ 0.07	2.65 $\pm$ 0.19

ND, Not determined.

Table 5. *Relative contents of cytochromes in R. leguminosarum grown at different dissolved oxygen concentrations in a glucose-limited chemostat*

Bacteria were grown at $D = 0.1 \text{ h}^{-1}$ and 28°C , pH 6.8 and prepared using the procedure described in Methods. Reduced *minus* oxidized spectra were recorded at 77 K. Values shown are percentages of the total cytochromes present in each case as measured by peak heights. Cytochrome *o* was present in each case in CO-reduced *minus* reduced spectra. The presence of cytochrome c_{550} was not definite although there was a slight shoulder on the c_{555} peak. Two values for the cytochrome concentrations at zero detectable dissolved oxygen are given because it was difficult to establish conclusively whether cytochrome c_{550} was present (see Fig. 3c). Bacterial concentration was 12.0 g l^{-1} .

Dissolved oxygen concn in fermenter (μM)	c_{550}	c_{555}	b_{560}	aa_{3596}	d_{630}
205.6	33.6	28.6	26.1	11.8	0
144.0	29.9	29.0	27.6	13.0	0
110.3	31.6	29.1	27.2	12.1	0
94.6	28.1	26.9	26.6	11.8	6.6
0	17.6	27.4	26.0	16.0	12.9
0	0	33.3	31.6	19.5	15.6

Fig. 3. Cytochrome content of *R. leguminosarum* grown at different DOT values in glucose- or O_2 -limited chemostat cultures. Cytochrome contents of intact bacteria (approximately 12.0 g l^{-1}), from a glucose-limited or O_2 -limited chemostat ($D = 0.1 \text{ h}^{-1}$) measured in cuvettes of 1 mm light path length, at 77 K. Reduced (dithionite) *minus* oxidized (H_2O_2) difference spectra. (a) Dissolved oxygen concentration in chemostat = $205.6 \mu\text{M}$; (b) dissolved oxygen concentration in chemostat = $110.3 \mu\text{M}$; (c) dissolved oxygen concentration in chemostat = undetectable.

[approximately 33.3 g dry wt (mol oxygen)⁻¹] can be calculated for *R. trifolii*, from the data of de Hollander *et al.* (1979), when grown carbon-limited on mannitol with asparagine as the nitrogen source. There could be several explanations for these low yields in rhizobia.

Simpson *et al.* (1979) and Peterson & Evans (1979) indicate that considerable quantities of CO₂ can be fixed by *R. japonicum*. The low $Y_{O_2}^{\max}$ and $Y_{\text{glucose}}^{\max}$ values we observed for *R. leguminosarum* could be explained if the bacteria fixed considerable quantities of CO₂; this is an energy- and reductant-dependent process and would cause a decrease in the biomass yields on oxygen and glucose. The carbon recovery values obtained of approximately 100 ± 6% indicate, on the assumption that the input of carbon as CO₂ was negligible, that substantial CO₂ fixation was unlikely. This conclusion is supported by the fact that the observed values for $Y_{\text{glucose}}^{\max}$ and $Y_{O_2}^{\max}$ agreed closely with the theoretical values in which no CO₂ fixation (or H₂ evolution) was assumed.

The low yields of *R. leguminosarum* on glucose and O₂ could also be due to the production of 'overflow' metabolites, e.g. acetate or polysaccharide production, a low P/O ratio, nitrogen fixation or H₂ evolution. The absence of H₂ in the influent and effluent gas streams indicated that the organisms could neither be oxidizing H₂ to obtain energy, nor producing hydrogen. Nitrogen fixation can be discounted as the rhizobia were growing at a DOT of approximately 200 µM, well above the O₂ concentration inhibitory to nitrogenase activity in rhizobia (Bergersen *et al.*, 1976). The production of large amounts of overflow metabolites, although not directly measured, would have been apparent in the carbon recovery values and in the supernatant carbon values which were always very low for the glucose-limited cultures. Carbon recovery values near 100% were obtained for such cultures.

A P/O ratio of 3 is expected for bacteria grown aerobically on the assumption of a normal rate of H⁺ decay (t_1 of 40 to 60 s), the presence of cytochrome *c* and absence of cytochrome *d* in the respiratory chain. Jones *et al.* (1977) reported a $Y_{\text{glucose}}^{\max}$ value of 100 g dry wt (mol glucose)⁻¹ for *Arthrobacter globiformis* and 110.0 g dry wt (mol glucose)⁻¹ for *Paracoccus denitrificans* grown aerobically on glucose with corresponding $Y_{O_2}^{\max}$ values of 92.0 and 90.0 g dry wt (mol O₂)⁻¹, respectively. Therefore, from the relationship $Y_{O_2}^{\max} = Y_{\text{ATP}}^{\max} (2 \times \text{P/O})$, a $Y_{\text{ATP}}^{\max}$ value of 15.0 g dry wt (mol ATP)⁻¹ would be obtained if a $Y_{O_2}^{\max}$ value of 90 g dry wt (mol O₂)⁻¹ was used. There is no reason to assume that the $Y_{\text{ATP}}^{\max}$ value for non-nitrogen fixing rhizobia is less than approximately 15.0 g dry wt (mol)⁻¹ when grown aerobically with glucose and minimal salts. Consequently, the $Y_{O_2}^{\max}$ value of 26.0 g dry wt (mol oxygen)⁻¹ for *R. leguminosarum* with a Y_{ATP} of 15 results in a P/O ratio of approximately 1. However, the →H⁺/O ratios (Table 4) indicated a potential of 3 proton translocating loops, discounting transhydrogenase activity which is unlikely to give ATP production under normal physiological conditions. Proton translocation studies revealed that the t_1 for the decay of the proton pulse was extremely rapid, between 12 and 15 s, approximately three times faster than commonly found. This increased rate of proton decay may mean that the effective P/O ratio was only one, i.e. the overall H⁺/ATP ratio [g atom H⁺ (mol ATP synthesized)⁻¹] was 6 and that the three times increased rate of decay reflected this. At present it is not known how this fast rate of decay is mediated although it may be due to a general leakiness of the bacterial membrane to protons, a 'gate' in the ATPase, an antiport or symport mechanism for transport of protons across the cell membrane, or the action of some unknown uncoupler. Recently McKay *et al.* (1982) observed similar low yields on glucose and O₂ and a very high permeability of the cytoplasmic membrane to protons in the extreme thermophile *Thermus thermophilus* HB8.

The results from the experiments with *R. leguminosarum* grown at a dilution rate of 0.1 h⁻¹ with various DOT values showed a decrease in the observed Y_{glucose} and Y_{O_2} values with decrease in the DOT. This could be due to the production of cytochrome oxidase *d* with an associated decrease in the efficiency of respiratory chain energy conservation (Jones, 1978) or factors already discussed. Bergersen & Turner (1980) found that rhizobia which infect cowpea and soybean when grown in continuous culture under O₂ limitation produced cytochrome oxidases with a high affinity for O₂; these oxidases were also found in the corresponding bacteroids (Bergersen & Turner, 1975, 1980). A decrease in the efficiency of respiratory chain energy conservation in *R. leguminosarum* was confirmed by the H⁺/O ratios (Table 4). The H⁺/O ratios

for bacteria grown at DOT values above those limiting growth indicated a 'potential' P/O ratio of approximately 3 for endogenous substrate and glucose, and approximately 2 for succinate and ascorbate-TMPDH₂. The high H⁺/O ratios obtained with ascorbate-TMPDH₂ as electron donor were probably due to the presence of intracellular NADH and FADH, as it was not possible to starve the cells. However, the H⁺/O ratio with either endogenous substrate or glucose for bacteria grown oxygen-limited indicated a 'potential' P/O ratio of approximately 2.

The results reported in this paper indicate that the effective P/O ratio in free-living *R. leguminosarum* was near 1 although there were three proton-translocating loops present, i.e. the overall H⁺/ATP ratio was near 6 and not 2 (although the catalytic H⁺/ATP ratio may still be 2, but protons are leaking through the cytoplasmic membrane rather than the ATPase). Similar rapid rates of decay in respiration induced proton pulses were also seen in batch culture grown *R. japonicum* 705 and *R. japonicum* 32H1 (H. D. Ratcliffe & J. W. Drozd, unpublished observations). If rhizobia exhibited this same low efficiency of energy conservation when fixing dinitrogen in the root nodule of host legume it would provide a low efficiency of N₂ fixation with respect to the amount of carbon (plant photosynthate) oxidized. However, the bacteroids from soybean root nodules exhibited respiration-driven proton translocation with no such rapid rate of proton decay (Ratcliffe *et al.*, 1980). The low Y_{O₂} values and high respiration rates for axenic cultures of *R. leguminosarum* might indicate that free-living rhizobia have some form of respiratory protection mechanism, analogous to, but much less well developed than that in *Azotobacter* (Drozd, 1978). Such a mechanism would not be required when in symbiotic association within the root nodule of a host legume when the maintenance substrate consumption would also presumably be minimized.

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