Effects of O_2 Concentrations and Various Haemoglobins on Respiration and Nitrogenase Activity of Bacteroids from Stem and Root Nodules of *Sesbania rostrata* and of the Same Bacteria from Continuous Cultures

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(*Received 23 April 1986; revised 16 June 1986*)

Bacteroids from both stem and root nodules of *Sesbania rostrata* inoculated with *Rhizobium* sp. strain ORS571 were studied in the presence of the respective leghaemoglobins or soybean root nodule leghaemoglobin. Concentrations of free dissolved O_2 and rates of O_2 consumption and nitrogenase activity were measured during steady states in a special reaction chamber. The two types of bacteroids had high-affinity terminal oxidase systems. They were capable of respiration-coupled nitrogenase activity at concentrations below 10 nM free dissolved O_2; at such concentrations the homologous leghaemoglobins were more effective than soybean leghaemoglobin because of their higher affinity for O_2. *S. rostrata* bacteroids were less efficient in nitrogenase activity (consuming 4 to 11 mol O_2 per mol of C_2H_2 reduced) than soybean bacteroids (O_2/C_2H_4 = 3·6) in comparable experiments, but efficiency was not affected by the source of leghaemoglobin. Similar experiments with ORS571 grown in N_2-fixing continuous cultures showed that these bacteria also had high-affinity terminal oxidase systems coupled to nitrogenase, but those grown at <1 μM dissolved O_2 were less efficient (O_2/C_2H_4 >21) than those grown at 7 to 11 μM-O_2 (O_2/C_2H_4 <8). Nitrogenase activity of bacteria grown at the higher O_2 concentration increased when the O_2 concentration in the chamber was raised from 0·1 to 3 μM in experiments in which mammalian myoglobin replaced leghaemoglobin in the reaction solution. With bacteria grown at <1 μM-O_2, nitrogenase activity was inhibited (reversibly) by 50% after an increase from 0·1 to 1·4 μM-O_2 in the reaction chamber. After changes in rates of supply of dissolved O_2 there were oscillations in rates of O_2 consumption before establishment of new steady states. These effects were greater in bacteria grown at 7 to 11 μM-O_2 than in those grown at <1 μM-O_2, but with the latter, a sixfold increase in O_2 flux produced only very small increases in concentration of dissolved O_2.

INTRODUCTION

Amongst legume root nodule bacteria, *Rhizobium* sp. strain ORS571 is unusual in being able (i) to produce both stem and root nodules on *Sesbania rostrata*, (ii) to grow in culture with atmospheric N_2 as the principal source of nitrogen (Dreyfus *et al.*, 1983), and while doing so, (iii) to tolerate up to 12 μM dissolved O_2 (Gebhardt *et al.*, 1984; Stam *et al.*, 1984; Kush *et al.*, 1985). Since stem nodules on *S. rostrata* have chloroplasts in the cortex and in other tissues (Dreyfus & Dommergues, 1981), it seemed possible that O_2 concentrations in stem nodules may be higher than usual in legume root nodules, due to photosynthetic production of O_2 in the tissue. The leghaemoglobins from stem and root nodules have been purified (D. Bogusz & C. A. Appleby, unpublished) and their O_2-binding kinetics measured (J. B. Wittenberg, B. A. Wittenberg, Q. H. Gibson, D. Bogusz & C. A. Appleby, unpublished). Surprisingly the affinities for O_2 of proteins from these two sources were even higher than that of soybean leghaemoglobin and were not significantly different from each other.

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The experiments reported in this paper were undertaken to examine the properties of O_2 tolerance in bacteria from continuous cultures grown at contrasting concentrations of O_2, and to compare these with properties of bacteroids from stem and root nodules, when the conditions of supply of O_2 to suspensions of the bacteria were modulated by the presence of partially oxygenated haemoglobins of low (mammalian myoglobin), high (soybean leghaemoglobin) or higher (*S. rostrata* leghaemoglobins) affinity for O_2.

METHODS

Plants, growth and production of nodules. S. rostrata seeds were surface-sterilized for 25 min in conc. H_2SO_4 and then transferred to soft agar (0·9%, w/v) in Petri dishes to germinate at 25 °C. After 48 h, germinated seeds were transferred to a perlite/vermiculite (2:1, v/v) mixture in pots which were placed in a 30/25 °C controlled environment glasshouse (30 °C, 0800–1600; 25 °C, 1600–0800) receiving 13 to 14 h natural light per day. The plants received modified Hoagland's nutrient solution for 21 d, after which time this high-N solution was washed thoroughly from the pots and daily watering with N-free nutrient solution (Gibson, 1980) was commenced. At this stage of plant development (80 cm tall), a suspension of ORS571 (Gebhardt *et al.*, 1984) was sprayed on the stems or used to inoculate the roots. Nodules appeared within 5 to 7 d, and they were 4 to 6 weeks old when used to prepare bacteroids.

For production of soybean nodules, *Rhizobium japonicum* strain CB1809 was used to inoculate soybeans (*Glycine max*. Merr. *cv.* Lincoln) grown in a glasshouse as described by Bergersen & Turner (1970).

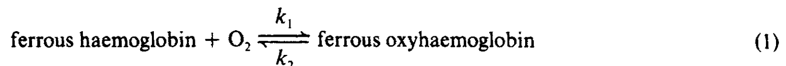
Bacteria. The *S. rostrata* stem nodule isolate (ORS571) was originally kindly supplied by B. L. Dreyfus, ORSTOM, Dakar, Sénégal. The cultures used in the present work were symbiotically tested single colony isolates from the original culture.

Preparation of bacteroid suspensions. Bacteroids were prepared anaerobically from samples of nodules (about 50 g fresh wt) essentially as described by Bergersen & Turner (1973), but the homogenizing medium was 100 mM-potassium phosphate buffer (pH 7·4) containing 0·3 M-sucrose, and the bacteroids were washed twice in 25 mM-potassium phosphate buffer (pH 7·4) and suspended to give a final density of 40 to 60 mg dry wt ml^{-1}. In the case of *S. rostrata* stem nodules, initial bacteroid pellets were contaminated with chloroplast fragments. Surface layers containing these were scraped off and washed away with anaerobic buffer before the bacteroid pellet was resuspended at each washing step; all operations were done in dim light to minimize photosynthetic production of O_2 and consequent damage to nitrogenase. After the final wash there was only very light or no detectable green colour remaining in the upper layer of the final pellet.

Preparation of ferrous oxyhaemoglobins. Unfractionated soybean oxyleghaemoglobin was prepared from root nodules as described by Appleby & Bergersen (1980). Mammalian oxymyoglobin was prepared from commercial crystalline sperm whale myoglobin (Sigma), using the same procedures for oxygenation.

Unfractionated *Sesbania* root and stem nodule oxyleghaemoglobins were prepared by a procedure to be described in detail elsewhere (D. Bogusz, A. A. Kortt & C. A. Appleby, unpublished). Briefly, this involved ammonium sulphate fractionation of nodule extracts, specific adsorption of leghaemoglobin to DEAE-cellulose at low salt strength (pH 7) to separate the protein from a large amount of viscous impurity, oxidation of the eluted leghaemoglobin to the ferric species with ferricyanide at pH 5·2, and passage through Sephadex G-25 to separate the ferric leghaemoglobin from excess ferricyanide and low M_r impurities. The eluted, mixed components of ferric leghaemoglobins were converted to oxyleghaemoglobin as above (Appleby & Bergersen, 1980).

Oxygenation–deoxygenation rate constants of haemoglobins. In the reaction



$$\text{ferrous haemoglobin} + O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{ferrous oxyhaemoglobin} \quad (1)$$

the following values of k_2/k_1 were used for calculations in experiments: sperm whale myoglobin $0{\cdot}786 \times 10^{-6}$ mol^{-1} (Antonini & Brunori, 1971); soybean leghaemoglobin $0{\cdot}04 \times 10^{-6}$ mol l^{-1} [Wittenberg *et al.*, 1972; the value used previously (Bergersen & Turner, 1979, 1985)]; *S. rostrata* stem and root nodule leghaemoglobins, $0{\cdot}02 \times 10^{-6}$ mol l^{-1} (J. B. Wittenberg, B. A. Wittenberg, Q. H. Gibson, D. Bogusz & C. A. Appleby, unpublished).

Continuous cultures. Continuous cultures of the N_2-fixing strain ORS571 were grown in lactate medium with a range of O_2 solution rates and steady state dissolved O_2 concentrations as described by Gebhardt *et al.* (1984). N_2 fixation rates were derived from differences between N-analyses of influent medium and effluent culture. O_2 consumption rates were calculated as described by Gebhardt *et al.* (1984). The experiments to be described in this paper used bacterial suspensions from cultures in four steady states, two grown at low concentrations of dissolved O_2 (< 1 μM) and two at 7 to 12 μM-O_2. In addition, one experiment used bacteria taken 16 h after conditions were changed from high to low concentrations of dissolved O_2, but before a new steady state was established.

For experiments with the flow chamber, 150 ml of the culture (total volume 590 to 600 ml according to stirring rate) were collected into an evacuated flask, and then centrifuged under Ar. The pelleted bacteria were resuspended in approximately 1 ml of the same medium, to give suspensions containing 25 to 50 mg dry wt ml^{-1}.

Flow chamber methods. The measurements of relationships between rates of O_2 consumption and nitrogenase activity at various concentrations of free dissolved O_2 were made in the flow chamber assembly described by Bergersen & Turner (1985) using methods developed for an earlier version of the apparatus (Bergersen & Turner, 1979). As before, the concentration of free dissolved O_2 in the reaction solution (effluent) passing from the reaction chamber was calculated from spectrophotometric determination of the proportional oxygenation (Y) of the O_2-carrying haemoglobin. Nitrogenase activity was calculated from the concentration of C_2H_4 in the effluent, produced in the reaction chamber by reduction of C_2H_2 supplied dissolved in the reaction solution. The gas phase in the reaction solution reservoir contained (%, v/v, at 93·1 to 94·6 kPa): O_2 (21), C_2H_2 (21·5) and Ar (57·5). The concentration of O_2 in the stirred reservoir solution was calculated from tables of solubility of O_2 in H_2O at the temperatures and atmospheric pressures prevailing during the experiments. The reaction solutions were (*a*) for bacteroid experiments, 25 mM-potassium phosphate, pH 7·4, containing 2 mM-$MgSO_4$, 10 mM-sodium succinate and approximately 60 to 90 μM-oxyhaemoglobin; and (*b*) for experiments with cultured bacteria, lactate culture medium supplemented with oxyhaemoglobin (70 to 90 μM).

Rates of O_2 consumption were calculated at intervals of 1 min using the following expressions.

$$Y_t = \frac{\Delta A_t - \Delta A_{red}}{\Delta A_{oxy} - \Delta A_{red}} \qquad (2)$$

where Y_t = the proportion oxyleghaemoglobin/total leghaemoglobin, at time t; ΔA_{oxy} = absorbance of the fully oxygenated haemoglobin (in air) at the wavelength of the α peak *minus* absorbance at the trough between α and β peaks; ΔA_t and ΔA_{red} are the absorbance values at the same wavelengths at time t and for the fully deoxygenated haemoglobin ($Na_2S_2O_4$) respectively.

$$C_t = \frac{Y_t k_2/k_1}{1 - Y_t} \qquad (3)$$

where C_t is the concentration (μM) of free dissolved O_2 at time t.

$$O_2 \text{ consumption rate } [\text{nmol } O_2 \text{ min}^{-1} (\text{mg dry wt})^{-1}] = 1/B\,\{F(1 - Y_t)C_p + (C_o - C_t) + V_{ch}(C_p\, dY/dt + dC/dt)\} \qquad (4)$$

where B is the bacterial dry weight (mg) in the chamber; F is the medium flow rate (ml min^{-1}); C_p the concentration of haemoglobin (μM); C_o the concentration of free dissolved O_2 (μM) in the reservoir and C_t that in the effluent at time t; V_{ch} is the volume (ml) of the reaction chamber and connections; dY/dt and dC/dt are the rates of change of Y and C during the preceding minute.

Nitrogenase activity was measured during steady O_2 concentration/consumption conditions by using the expression

$$\text{nmol } C_2H_4 \text{ min}^{-1} (\text{mg dry wt})^{-1} = 1/B\,(V_{ch}\, dC_a/dt + C_a F) \qquad (5)$$

where C_a is the concentration of C_2H_4 (nmol ml^{-1}) in the effluent solution in three or four samples collected at close intervals of t min. This calculation is necessary because C_a may only slowly reach equilibrium values although the rate of production is constant. This calculation replaces the double reciprocal plot method advocated previously (Bergersen & Turner, 1979).

Analytical. Dry weight of bacteria in samples of continuous cultures was determined from a calibration relating dry weight of water-washed bacteria to the OD_{700} (10 mm pathlength) of culture samples, or by drying (at 80 °C) water-washed samples of residual bacterial suspensions used in flow chamber experiments. Total N in cultures and suspensions was measured by Kjeldahl digestion, distillation and titration (Bergersen, 1980). Concentration of dissolved C_2H_4 was measured by the method of Turner & Gibson (1980). Concentrations of haemoproteins in reaction solutions were measured by the pyridine haemochrome method (Appleby & Bergersen, 1980).

RESULTS

Bacteroids from nodules on S. rostrata

Fig. 1 presents data from two similar flow chamber experiments with stem nodule bacteroids in the presence of oxyleghaemoglobin from either soybean root nodules (Fig. 1*a*) or *S. rostrata* stem nodules (Fig. 1*b*). In both, the following features (common to all experiments with bacteroids) are seen. (i) Following initiation of flow of reaction solution (O_2 supply) the concentration of free dissolved O_2 declined slowly, approaching equilibrium between 10 and 20 min later, at which time there were fluctuations in rates of consumption of O_2 as the bacteroids adjusted to a steady state condition; this effect is more marked in Fig. 1*b*. During the initial phase, rates of activities were irregularly related to concentrations of free dissolved O_2,

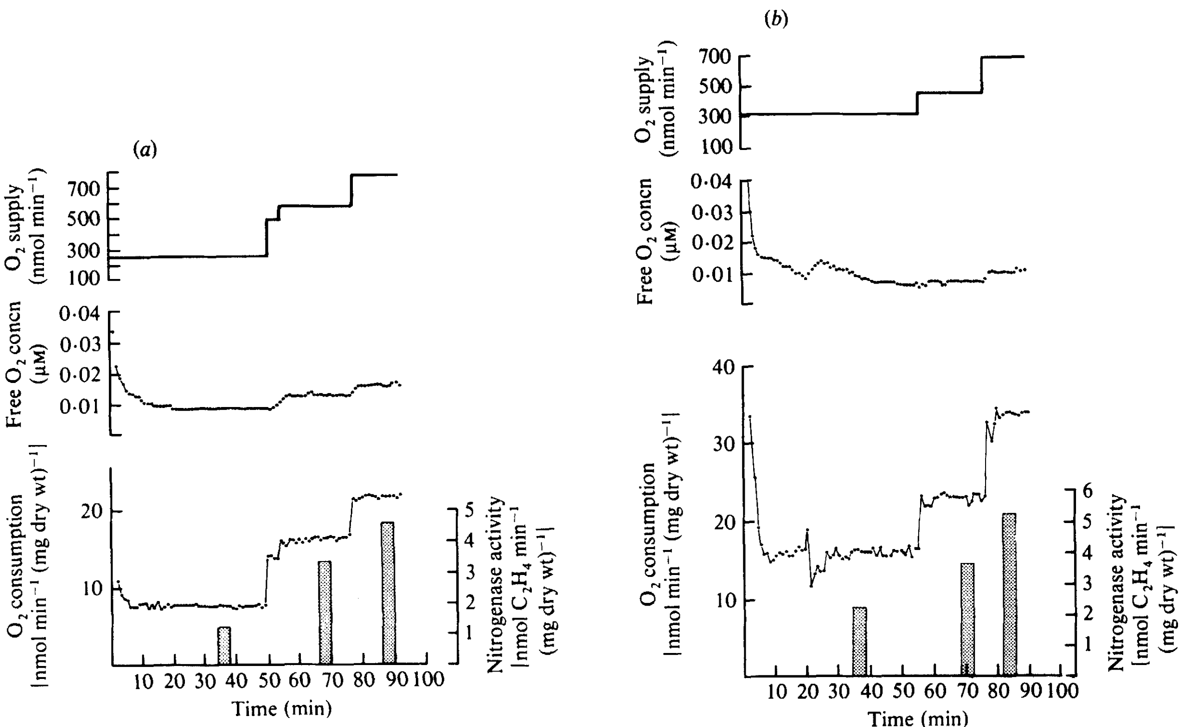


Fig. 1. Time course data for two similar flow chamber experiments with bacteroids from *S. rostrata* stem nodules. Reaction solutions supplied to the chamber contained (*a*) soybean oxyleghaemoglobin (87·2 µM) or (*b*) *S. rostrata* stem nodule oxyleghaemoglobin (76·6 µM) plus 262 µM free dissolved O_2. The histograms represent mean values for nitrogenase activity measured in four samples of effluent collected during each steady state: the maximum SEM value was 0·05 nmol C_2H_4 min^{-1} (mg dry wt)$^{-1}$. Changes in O_2 supply were effected by increased rates of flow of reaction solution. Concentrations of free O_2 were calculated from the relative oxygenation of leghaemoglobin in the effluent solution.

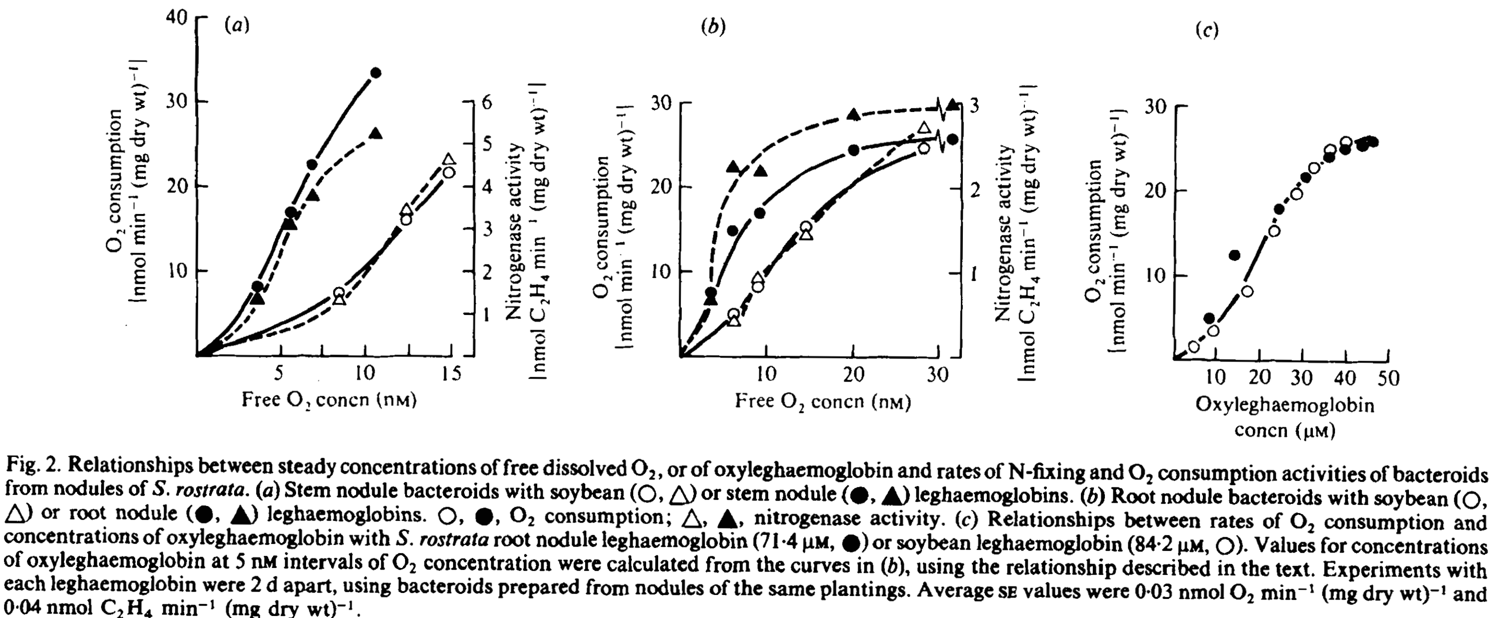


Fig. 2. Relationships between steady concentrations of free dissolved O_2, or of oxyleghaemoglobin and rates of N-fixing and O_2 consumption activities of bacteroids from nodules of *S. rostrata*. (*a*) Stem nodule bacteroids with soybean (○, △) or stem nodule (●, ▲) leghaemoglobins. (*b*) Root nodule bacteroids with soybean (○, △) or root nodule (●, ▲) leghaemoglobins. ○, ●, O_2 consumption; △, ▲, nitrogenase activity. (*c*) Relationships between rates of O_2 consumption and concentrations of oxyleghaemoglobin with *S. rostrata* root nodule leghaemoglobin (71·4 μM, ●) or soybean leghaemoglobin (84·2 μM, ○). Values for concentrations of oxyleghaemoglobin at 5 nM intervals of O_2 concentration were calculated from the curves in (*b*), using the relationship described in the text. Experiments with each leghaemoglobin were 2 d apart, using bacteroids prepared from nodules of the same plantings. Average SE values were 0·03 nmol O_2 min^{-1} (mg dry wt)$^{-1}$ and 0·04 nmol C_2H_4 min^{-1} (mg dry wt)$^{-1}$.

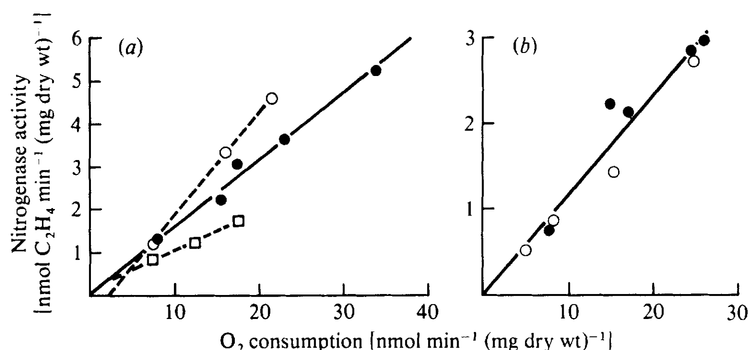


Fig. 3. The efficiency of coupling of O_2 consumption to nitrogenase activity in bacteroids from *S. rostrata* nodules. (*a*) Stem nodule bacteroids with soybean leghaemoglobin (○, □) or *S. rostrata* stem nodule leghaemoglobin (●). Correlation coefficients and slopes [nmol O_2 (nmol C_2H_4)$^{-1}$] were respectively (●) 0·992, 6·54; (○) 0·999, 4·16; (□) 0·996, 11·6. (*b*) Root nodule bacteroids with soybean (○) or *S. rostrata* root nodule leghaemoglobin (●), r = 0·967, slope 8·60.

but in spite of irregularities, nitrogenase activity followed closely rates of O_2 consumption. (ii) Following each successive change in rate of O_2 supply, there were similar fluctuations but equilibrium conditions were reached more quickly. (iii) Large differences in rates of consumption of O_2 were seen with only small associated changes in concentration of free dissolved O_2. (iv) Differences in rates of nitrogenase activity during steady states were associated with differences in rates of O_2 consumption.

Sesbania leghaemoglobin compared with soybean leghaemoglobins. Bacteroids prepared from both stem and root nodules had high-affinity terminal oxidase systems, which permitted sustained respiration-dependent nitrogenase activity at concentrations of free O_2 as low as 3 nM, when the high-affinity leghaemoglobin from *S. rostrata* was present (Fig. 2). In the presence of soybean leghaemoglobin, activity was much less than with *S. rostrata* leghaemoglobin in this very low range of O_2 concentration, although at about 30 nM-O_2, root nodule bacteroids were equally active in the presence of either leghaemoglobin (Fig. 2*b*). When data for bacteroid respiration from these experiments were plotted against concentration of the oxyleghaemoglobins (Fig. 2*c*), the differences between the leghaemoglobins were narrowed greatly (cf. Figs 2*b* and 2*c*), suggesting that the different rates were largely a consequence of rate-limiting differences in the concentrations of the oxygenated carriers at concentrations of free O_2 less than 20 nM.

Efficiency of respiration coupled to nitrogenase. With all the leghaemoglobins, rates of nitrogenase activity of bacteroids from *S. rostrata* nodules were positively correlated with rates of O_2 consumption (Fig. 3) in the range of concentrations of free O_2 used (0 to 30 nM). The slopes of the regression lines were a measure of efficiency of the coupling between respiration and nitrogenase. Values were variable, 4·2 to 11·6 nmol O_2 being consumed per nmol C_2H_2 reduced to C_2H_4 (Fig. 3). In similar experiments spanning 3 years (data not shown), soybean bacteroids supplied with 10 mM-sodium succinate were less active but more efficient [the slope of the regression (r = 0·99) line indicating 3·6 nmol O_2 consumed per nmol C_2H_2 reduced]. Greater efficiencies (2 to 2·3 nmol O_2 per nmol C_2H_2) were obtained with soybean bacteroids with no exogenous substrate.

There was no evidence for any effect of source of leghaemoglobin on efficiency. With *S. rostrata* stem nodule bacteroids, differences in slope were attributable to differences between bacteroid preparations from nodules of different ages (Fig. 3*a*), whilst for *S. rostrata* root nodule bacteroids with soybean leghaemoglobin and *S. rostrata* root nodule leghaemoglobin, a single regression line could be drawn through the points obtained 2 d apart (Fig. 3*b*).

Table 1. *Continuous culture of Rhizobium strain ORS571*

Continuous cultures were grown in lactate medium as described in Methods. Bacteria were collected for the five flow chamber experiments described. Except where indicated, steady states had been established for at least 36 h before collection.

Dissolved O_2 (μM)	Dilution rate (h^{-1})	O_2 consumption [nmol min^{-1} (mg dry wt)$^{-1}$]	N_2 fixation [nmol N_2 min^{-1} (mg dry wt)$^{-1}$]
Low			
0·2–0·8*	0·049	48·6	2·2
0·1–0·2*	0·052	63·5	1·7
High			
7·2–11·4*	0·086	132·9	4·3
8·8–11·7*	0·086	100·0	3·4
High to low			
5·0–7·9*†	0·068	113·9†	5·6
0·2	0·093	148·8	ND

ND, Not determined because the culture was not in steady state, and cell density was declining.
* Limits shown are those between which dissolved O_2 was controlled.
† The conditions prevailing until 16 h before establishing the conditions at the time of collection, shown on the following line.

Bacteria from continuous cultures

The properties of the continuous cultures for which results are presented are shown in Table 1. The cultures appeared to be O_2-limited, since yields broadly followed O_2 solution rates (data not shown). However, they may have been N-limited by reason of limitations to N_2 fixation imposed by limited O_2 supply. Over all steady states, rates of N_2 fixation were positively correlated ($r = 0{\cdot}95$; $P < 0{\cdot}01$) with rates of O_2 consumption. In the limited data of Table 1, the correlation was not so strong ($r = 0{\cdot}85$; $P < 0{\cdot}05$). We were not able to distinguish between the two possible limitations in these experiments.

Time courses of flow chamber experiments. Fig. 4 presents data from two representative flow chamber experiments using soybean oxyleghaemoglobin and bacteria grown at low (0·2 to 0·8 μM-O_2; Fig. 4*a*) or higher (7 to 11 μM-O_2; Fig. 4*b*) concentrations of dissolved O_2. The former shows lower rates of nitrogenase activity and a great capacity to consume increasing fluxes of O_2 with minimum fluctuations whilst maintaining very low concentrations of dissolved O_2. Bacteria grown at high concentrations of dissolved O_2 had higher rates of nitrogenase activity in the cultures (Table 1) and also in the flow chamber (Fig. 4), but changes in rates of supply of O_2 to the chamber produced a greater range of concentrations of dissolved O_2 and greater perturbations following changes (Fig. 4*b*), particularly at higher concentrations of dissolved O_2. This effect was examined in experiments in which myoglobin (which acts as an O_2 carrier at 0·1 to 10 μM-O_2) replaced leghaemoglobin in the flow reaction chamber. Fig. 5 compares the oscillations in the concentration of O_2 and in rates of O_2 consumption elicited by an increase in the O_2 supplied. The amplitude of the perturbation was much greater for bacteria grown at 9 to 12 μM-O_2 than for those grown at 0·07 to 0·2 μM-O_2.

Relationships between concentrations of free dissolved O_2 and rates of respiration and nitrogenase activity in flow chamber experiments. Fig. 6 presents data from five experiments with bacteria from the cultures described in Table 1. The main features are as follows. (i) Bacteria grown at both ranges of concentrations of dissolved O_2 showed the presence of high affinity terminal oxidase systems in flow chamber reactions, but those grown at low concentrations had greater rates of O_2 consumption and lower rates of nitrogenase activity in the range 10^{-8} to 10^{-7} M free dissolved O_2 in the presence of soybean leghaemoglobin. (ii) in the presence of myoglobin in the range 10^{-7} to 3×10^{-6} M free dissolved O_2, there were no differences in O_2 consumption rates, but for bacteria grown at low concentrations of O_2 nitrogenase activities declined with increasing concentration of O_2 in the reaction chamber; nitrogenase activities of bacteria grown

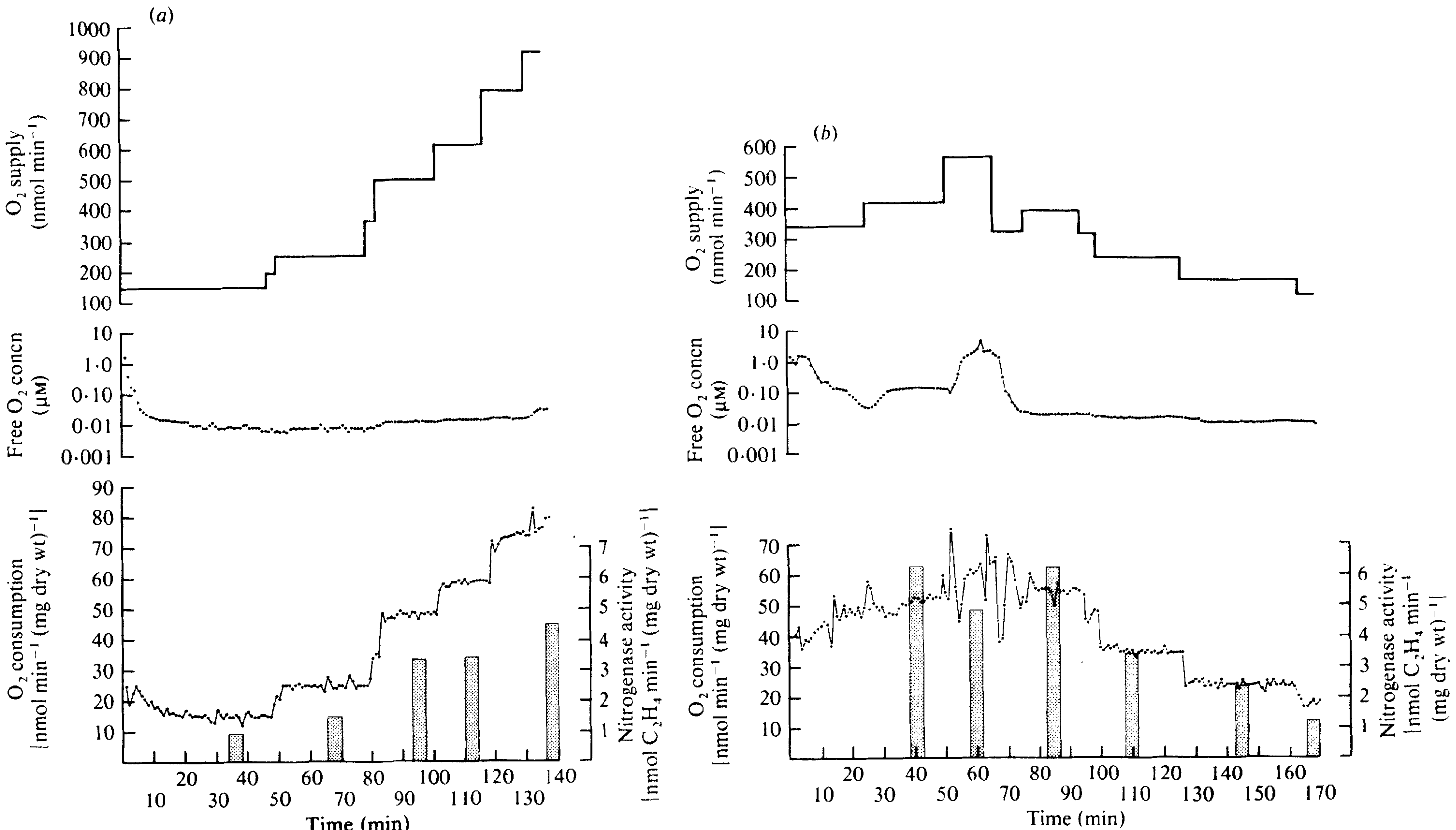


Fig. 4. Time course data for representative flow chamber experiments with ORS571 grown at low and high concentrations of dissolved O_2 (*a*). Bacteria (9·9 mg dry wt) grown at 0·2 to 0·8 μM dissolved O_2. (*b*) Bacteria (6·85 mg dry wt) grown at 7 to 11 μM-O_2. Reaction solutions supplied to the chamber contained 90·6 and 75·7 μM soybean oxyleghaemoglobin respectively, plus 221 and 233 μM free dissolved O_2. The graphs show reaction rates and conditions in the chamber. Other details as in the legend to Fig. 1.

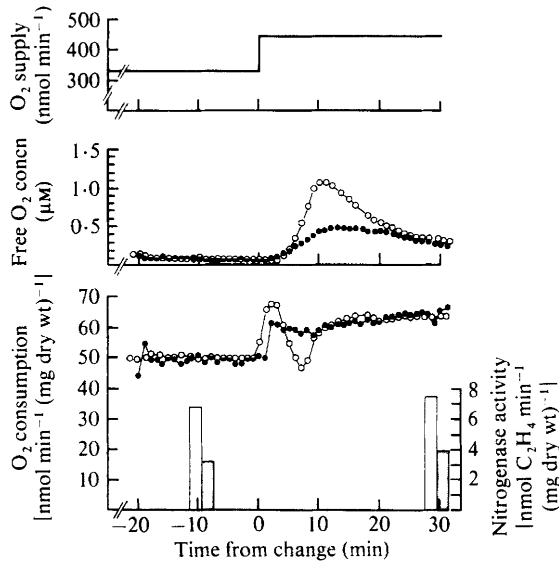


Fig. 5. The effects of increased O_2 supply in the presence of myoglobin in the reaction solution. Bacteria were grown at 9 to 12 μM-O_2 (○) or 0·1 to 0·2 μM-O_2 (●). The histograms represent nitrogenase activity: those for bacteria grown at 0·1 to 0·2 μM-O_2 are shaded.

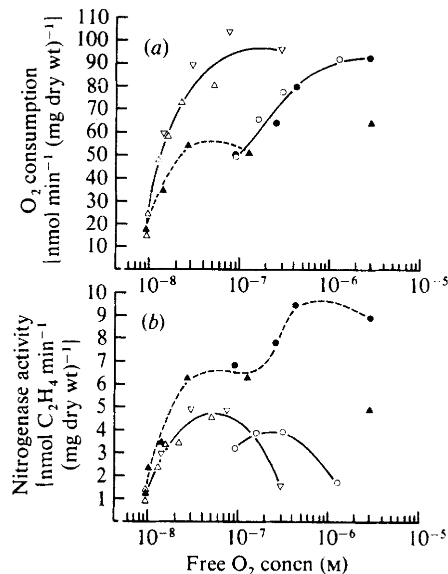


Fig. 6. The relationship between concentration of free dissolved O_2 during steady states in flow chamber experiments and rates of O_2 consumption (*a*) and nitrogenase activity (*b*) by ORS571 which had been grown at different concentrations of dissolved O_2. Reaction solutions contained soybean oxyleghaemoglobin (△, ▽, ▲) or myoglobin (○, ●). Growth was at 7 to 12 μM (●, ▲) or 0·1 to 0·8 μM-O_2 (○, △), or for 16 h at 0·2 μM-O_2 after several days at 5·8 μM-O_2 (▽). SE values for rates during each steady state were less than the dimensions of the symbols. Data are from the five continuous cultures described in Table 1.

at higher concentrations of O_2 increased over the same range. (iii) Within 16 h of a change from 5 to 7·9 μM dissolved O_2 to 0·2 μM, bacteria from continuous cultures had assumed the characteristics of bacteria grown for prolonged periods at low concentrations of O_2 (Figs 6 and 7).

Efficiency of respiration-coupled nitrogenase activity. Rates of nitrogenase activity were directly correlated with rates of O_2 consumption up to saturating or inhibitory concentrations of free dissolved O_2 (values obtained above 1 μM free dissolved O_2 in the chamber did not lie on the line), irrespective of whether soybean leghaemoglobin or myoglobin were the O_2-carrying proteins present, as was found for bacteroids of the same strain (Fig. 3). However, bacteria grown at low concentrations of O_2 were less efficient ($r = 0·96$; $O_2/C_2H_4 > 21$) than those grown at 7 to 12 μM-O_2 ($r = 0·99$; $O_2/C_2H_4 < 8$). Again bacteria subjected to 0·2 μM-O_2 for only 16 h were indistinguishable from those grown at low O_2 for several days.

DISCUSSION

The flow chamber experiments described in this paper utilized the presence of O_2-carrying haemoproteins to facilitate the fluxes of O_2 to bacteria in suspensions containing low concentrations of free, dissolved O_2. At the same time, monitoring of the relative oxygenation of the carriers by spectrophotometry provided a means of accurately determining these concentrations. The mechanisms concerned in this O_2 supply system, and the algebraic expressions describing them, have been provided by Wittenberg *et al.* (1974), Bergersen (1982), Bergersen & Trinchant (1985) and Bergersen & Turner (1985), and will not be discussed further.

The presence of high-affinity terminal oxidase systems in bacteroids from both stem and root nodules of *S. rostrata* is consistent with the presence in these nodules of leghaemoglobins of very high affinity for O_2 (J. B. Wittenberg, B. A. Wittenberg, Q. H. Gibson, D. Bogusz & C. A. Appleby, unpublished). It suggests that the concentrations of O_2 to which the bacteroids had been exposed *in vivo* were also very low, as is the case with soybean root nodules (Appleby, 1969). In some respects, the behaviour of bacteroids in the flow chamber experiments resembled that of bacteria of the same strain grown in continuous culture at low concentrations of dissolved O_2. They responded smoothly to increases in O_2 availability by increased O_2 consumption with only small changes in concentration of free dissolved O_2. The ranges of nitrogenase activity observed were similar and the efficiencies with which O_2 consumption was coupled to nitrogenase were also relatively low. In contrast, soybean bacteroids had lower nitrogenase activity than bacteroids from *S. rostrata* stem nodules, but were consistently more efficient than bacteroids from stem or root nodules. In all of these experiments the source of leghaemoglobins had no effect on efficiency. The *S. rostrata* leghaemoglobins permitted higher rates of activity than soybean leghaemoglobin at less than 10 nM free dissolved O_2. In this very low range of concentration of free O_2, the flux facilitated by *S. rostrata* leghaemoglobins is greater because the concentrations of the oxygenated form are higher as a result of their greater affinity for O_2. The enhancement of activity did not change efficiency. Similarly, the use of soybean leghaemoglobin or myoglobin did not affect the efficiency of cultured bacteria until the limit of tolerance of the nitrogenase system to O_2 was exceeded.

These experiments did not consistently support or exclude the possibility that bacteroids in stem nodules on *S. rostrata* were exposed to higher concentrations of O_2 than in root nodules, because of the activities of chloroplasts in stem nodule tissues. Some preparations from stem nodules resembled continuous cultures grown at high concentrations of O_2 in having greater nitrogenase activities and higher respiratory efficiency than those from root nodules. However, there was variation between preparations, perhaps due to nodule age differences, which prevented a firm conclusion.

In the first continuous culture study of ORS571, Gebhardt *et al.* (1984) did not detect any effect of added oxyleghaemoglobin in assays of shaken culture samples, concluding that this was due to the relatively high (10 to 12 μM) concentrations of free dissolved O_2 needed for optimum nitrogenase activity. The data of Fig. 6 show that, in spite of the tolerance of this strain to such concentrations of dissolved O_2 for growth, a high-affinity terminal oxidase system is present. Its

activity is enhanced when the concentration of O_2 for growth had been lower, but the tolerance of nitrogenase activity to concentrations of free O_2 above 1 μM is lost. This loss did not involve destruction of nitrogenase since activity was quickly restored when the O_2 concentration was lowered.

Ludwig (1986) has proposed an interesting mechanism linking, synergistically, the oxidation of nicotinate with N_2 fixation in ORS571. This mechanism, in part, may have a role in maintaining low intracellular concentrations of O_2 when these bacteria are grown aerobically. It also has a bearing on our present results because nicotinate was supplied in the medium for continuous cultures and in the medium for flow chamber reactions with cultured bacteria. Gebhardt *et al.* (1984) [whose paper was not taken into account by Ludwig (1986)] described N_2 fixation by ORS571 in continuous cultures by means of a nitrogen balance of influent medium and effluent culture. Nicotinate was consumed completely and the importance of the mechanism proposed by Ludwig (1986) can be assessed. The best rate of N_2-fixation achieved in the experiments of Gebhardt *et al.* (1984) was 1398 μg N_2 fixed h^{-1} in a 590 ml culture with a dilution rate of 0·096 h^{-1} (i.e. 56·6 ml of medium flow h^{-1}). The medium contained 20 mg nicotinate l^{-1} (i.e. 2·276 mg nicotinate-N l^{-1}, or 9·2% of that fixed from N_2, if all of the nicotinate-N was incorporated into the bacteria. Lowering the concentration of nicotinate from 20 to 2 mg l^{-1}, was associated with diminution of the mean concentration of fixed nitrogen from 20·3 to 13·1 μg N (ml culture)$^{-1}$. At a dilution rate of 0·054 h^{-1}, the maximum contributions of nicotinate-N would have been, respectively, 11·1% and 1·7% of N_2-fixation when 20 or 2 mg nicotinate l^{-1} were supplied. We conclude that the mechanism suggested by Ludwig (1986) must make only a relatively minor contribution to the nitrogen nutrition of ORS571 in the conditions used for the continuous cultures in the present paper. Further, the rate of N_2 fixation was not directly related to the rate of nicotinate consumption. In the reaction chamber experiments we are unable to assess the contribution of nicotinate because differences in the concentrations in medium and effluent were not monitored. In the light of the above considerations, it seems unlikely that nicotinate consumption (small in relation to N_2 fixation, which in turn was small in relation to O_2 consumption) would have any great effect on the kinetics of O_2 consumption by cultured cells of ORS571 in the flow reaction chamber.

An intriguing feature of the flow chamber experiments with bacteroids and with bacteria from continuous culture, was the consistent ability to respond to increased flux of O_2 with a rapid increase in rate of O_2 consumption, thus minimizing the rise in concentration of free O_2 in the suspension. Thus, in Fig. 4*a*, bacteria grown at low concentrations of dissolved O_2 consumed a sixfold increase in flux of O_2 supplied and experienced only a very small increase in concentration of free dissolved O_2. Fig. 1 documents a similar effect with bacteroids from stem nodules. This feature is in part due to the very steep relationship between steady state values for concentration of free dissolved O_2 and O_2 consumption rate. In addition, there is the oscillation that follows each change in O_2 supply and precedes establishment of a new steady state. Quantitative interpretation of this feature is complicated by the delay between changes in the chamber itself and their detection as a spectral change. This arises from the volume of the collecting annulus and the capillary connection to the optical cuvette (Bergersen & Turner, 1985). The volume of these spaces is small relative to the flow rates used and all spaces are narrow to minimize mixing. The apparent delays of 1 to 2 min before effects are seen (Fig. 5) seem to reflect this. Subsequent changes should follow in sequence with the same delay and then reflect the true time course of these events. Initially, the bacteria almost completely consume the increased O_2 flux and there is only a slow increase in concentration of O_2 as the initial consumption rate declines slightly in the case of the bacteria grown with low O_2 and more steeply with bacteria grown at higher O_2. Stabilization of O_2 concentration is then achieved with a slow adjustment to consumption rate. Values for O_2 consumption and O_2 concentration during these adjustments do not fall on the curves for steady state values of these relationships. Thus, rate of O_2 consumption is not simply a function of concentration of free O_2, but is modulated by other metabolic events in the bacteria. Perhaps there is initial depletion of an internal substrate pool when O_2 supply is increased, thus causing the subsequent decline in rate of O_2 consumption.

D. B. was on study leave at Canberra, but some nodules used to produce *S. rostrata* leghaemoglobins were grown in Senégal. The role of both laboratories in facilitating this exchange is acknowledged. Y.-Q. W. was an Exchange Scholar under the Australian Academy of Science–Academia Sinica Bilateral Exchange Agreement.

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