Leghaemoglobin and the Supply of O$_2$

to Nitrogen-fixing Root Nodule Bacteroids: Studies of an
Experimental System with No Gas Phase

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SUMMARY

The effects of purified oxyleghaemoglobin added to suspensions of bacteroids prepared anaerobically from soybean root nodules, were studied in terms of uptake of dissolved O$_2$, nitrogenase activity and dissolved O$_2$ concentration, in the absence of a gas phase. Leghaemoglobin allowed maximum rates of O$_2$ uptake to continue to a much lower range of concentrations of free dissolved O$_2$ than in the absence of the protein. This effect was diminished when the leghaemoglobin concentration was less than about 50 $\mu$M. Nitrogenase activity at a given O$_2$ concentration was not increased by raising the leghaemoglobin concentration above about 50 $\mu$M. The fractional oxygenation of leghaemoglobin giving half the maximal O$_2$ consumption rate by bacteroids was usually about 0.2; with limiting leghaemoglobin concentrations it was higher. Rates of nitrogenase activity were invariably greater during periods when the discharge of O$_2$ from oxyleghaemoglobin was occurring at the maximum rate, than when similar maximum O$_2$ uptake rates were being supported by higher concentrations of free dissolved O$_2$. When myoglobin was used as the O$_2$ carrier protein in similar experiments, enhanced nitrogenase activity also accompanied the maximum rate of discharge from the carrier. This occurred at about four times the concentration of free dissolved O$_2$ at which it occurred when leghaemoglobin was the carrier. These results are discussed in relation to current theories about the mechanism of leghaemoglobin action.

INTRODUCTION

The presence of the myoglobin-like haemoprotein, leghaemoglobin, in the central tissue of legume root nodules is an intriguing feature of these N$_2$-fixing organs (reviewed by Bergersen, 1971, 1973; Appleby, 1974; Appleby & Dilworth, 1975). The protein is probably located in the space between the symbiotic bacteria (bacteroids) and the membranes which enclose them within the host cell cytoplasm (Truchet, 1972; Bergersen & Goodchild, 1973; Gourret & Fernandez-Arias, 1974). In soybean nodules, leghaemoglobin concentration in this location is calculated to be about 0.3 to 1.5 mM during N$_2$-fixation (Bergersen & Goodchild, 1973). Leghaemoglobin is concerned with O$_2$ supply to the bacteroids and hence, by inference, in the supply of ATP to the nitrogenase. When bacteroids are incubated with shaking in the presence of leghaemoglobin and limiting concentrations of dissolved O$_2$, nitrogenase activity is enhanced much more than would be expected from the modest increases in O$_2$ consumption which are obtained (Bergersen, Turner & Appleby, 1973). Wittenberg et al. (1974) investigated the mechanism of this effect under carefully controlled conditions in the presence or absence of leghaemoglobin with a gas phase containing various
concentrations of $O_2$. The results were explained by leghaemoglobin-facilitated enhancement of the $O_2$ flux (Wittenberg, 1970) across the unstirred layer which surrounds the bacteroids in suspensions. This conclusion has been subsequently modified (Appleby et al. 1975a). Wittenberg et al. (1974) also showed that a variety of $O_2$-binding proteins stimulated nitrogenase activity and that their efficiency was related to the kinetics of their binding of $O_2$. This made unlikely the suggestion (Bergersen et al. 1973) that a specific mechanism involving direct interaction between oxyleghaemoglobin and an oxidase was involved.

The interpretations of Wittenberg et al. (1974) have been challenged recently by Stokes (1975, and personal communication) whose theoretical evaluations of their data led him to propose that the very great effect of leghaemoglobin in stabilizing the supply concentration of $O_2$ would be a much greater factor in the shaken bacteroid assays than the facilitation of $O_2$ flux.

In the shaken experiments the degree of oxygenation of the leghaemoglobin was uncertain during the steady rates of $O_2$ consumption and nitrogenase activity by the bacteroids. The effects of leghaemoglobin on the consumption of $O_2$ and the nitrogenase activity of bacteroids have been studied in the absence of a gas phase, in the belief that this would allow us to eliminate possible confusion between effects occurring near the bacteroids and effects which may have operated near a gas–liquid interface in the shaken assays. These experiments deal with the supply of $O_2$ to the oxidase(s) of the bacteroids.

**METHODS**

**Nodules and bacteroid suspensions.** Nodules of soybeans (Glycine max Merr. cultivar Lincoln) were produced by inoculation with *Rhizobium japonicum* strain CBI 809, and suspensions of bacteroids were prepared as previously described (Bergersen & Turner, 1973) and finally washed and resuspended in 50 mM-potassium phosphate buffer pH 7-4. They were stored under argon at 25 °C until used. Dry weights of the bacteroids were based on suspension samples dried at 80 °C.

**Oxyleghaemoglobin.** This protein, prepared from soybean root nodules as described by Bergersen et al. (1973), was stored in liquid N$_2$ and thawed immediately before use. Two batches of similar stability were prepared in 50 mM-potassium phosphate buffer pH 7-4. Concentrations were measured as before from the reduced-minus-oxidized difference spectra of alkaline pyridine haemochromogens. Abbreviations: (ferrous) deoxyleghaemoglobin, Lb; (ferrous) oxyleghaemoglobin, LbO$_2$.

**Oxymyoglobin.** The method of preparation was substantially that of Hugli & Gurd (1970), using bovine skeletal muscle. The final purification step was passage through a 3-5 x 60 cm Sephadex G-50 column equilibrated with tris-HCl buffer (25 mM, pH 7-4). The protein had a haem:protein ratio of 0·85 and was obtained in the oxygenated form without detectable oxidation products. Abbreviations: deoxymyoglobin, Mb; oxymyoglobin, MbO$_2$.

**Reaction medium.** All reactions were conducted in 50 mM-potassium phosphate buffer pH 7-4, containing 2 mM-MgSO$_4$ and 50 mM-sodium succinate as substrate. Oxyleghaemoglobin was added as indicated, and the solutions were stirred under large volumes of appropriate gas mixtures at a pressure of 700 mmHg, for at least 30 min at 25 °C. They were transferred to argon-flushed reaction vessels by syringe. Concentrations of gases in solution were calculated from the solubilities in water given in International Critical Tables (at 25 °C and 760 mmHg, they were: Argon, 1·45 mm; $O_2$, 1·26 mm; $C_2H_4$, 41·90 mm; $C_3H_8$, 4·87 mm).

**Measurement of Lb oxygenation.** Most assays were made in glass cuvettes of 4·5 ml volume and 10 mm light path, which contained two glass beads to promote initial mixing by shaking.
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and were closed with rubber serum stoppers. The sample cuvettes were flushed with argon before completely filling with reaction medium containing leghaemoglobin at concentrations of 4 to 200 µM. The reference cuvettes contained medium without leghaemoglobin. A double beam spectrophotometer, with sample chamber maintained at 25 °C, absorbance scale expansion and zero off-set facility, was used with a repetitive scanning accessory (Varian Techtron Pty. Ltd, North Springvale, Victoria 3171, Australia). It was set either to record absorption spectra between the limits of 585 and 525 nm at intervals of about 1·5 min, or continuously to record absorbance changes at 575 and 560 nm as a function of time. First the spectrum of the medium was recorded, and then 1 mg of bacteroids was added from a microsyringe to both of the cuvettes, the contents were mixed and timing and spectral recording commenced. The fractional oxygenation, $Y_t = \frac{LbO_2/(Lb + LbO_2)}{LbO_2}$, was calculated with reference to the $\Delta E_{575-560}$ at time $t$, and the $\Delta E$ values when the cuvette contents were fully oxygenated in air and when fully reduced by the addition of a few crystals of Na$_2$S$_2$O$_4$ at the end of the assay:

$$Y_t = \frac{\Delta E_t - \Delta E_{red}}{\Delta E_{oxy} - \Delta E_{red}}.$$  

Concentrations of O$_2$ in free solution were calculated from $Y_t$ values using $k/k' = 3·73 \times 10^{-8}$ M (Wittenberg, Appleby & Wittenberg, 1972) for the reaction,

$$LbO_2 \underset{k'}{\overset{k}{\rightleftharpoons}} Lb + O_2,$$

where $k$ and $k'$ are the rate constants for deoxygenation and oxygenation, respectively, of leghaemoglobin.

Because of the optical limitations of this method, most work was done with a Lb + LbO$_2$ concentration of about 0·1 mM. When the concentration was varied, a 5 mm path cuvette was used for the highest concentration, using the same relative proportions of assay constituents.

Myoglobin oxygenation. Experiments with myoglobin as the carrier of O$_2$ were similar to those with leghaemoglobin. The equilibrium constant used to relate free O$_2$ concentration and $Y$ was $1 \times 10^{-6}$ M (Antonini & Brunori, 1971).

Measurement of free O$_2$ concentration. Consumption of free O$_2$ from solution by bacteroids was followed by recording the output of an O$_2$ electrode with a Teflon membrane (0·01 mm thick) located at the bottom of a magnetically-stirred cylindrical chamber which was closed by a cylindrical plunger with a centrally-bored hole for injection of reactants (Rank Bros, Bottisham, Cambridge). Some of the measurements presented in this paper were made at the lower end of the useful range of an O$_2$-electrode. The methods used are therefore described in detail so that their authenticity may be readily assessed. With this platinum electrode polarized at $-0·6$ V with respect to the Ag–AgCl half cell, a current of about 1 µamp flows when the stirred chamber contains air-saturated buffer. With the manufacturer’s measuring unit, the electrode current produces a potential difference across a variable input resistance of 500 to 4000 ohms. This potential difference was measured with a strip chart recorder having variable full-scale ranges of from 0–2 V to 0–1 mV (Tohshin Electron Co., Tokyo, Japan; model T 02N1-H). Alternatively, an amplifier with a calibrated variable input resistance of 1 to 100000 ohms and a gain of $10 \times$ was used with the same recorder. This amplifier had an electrical ‘back-off’ facility to compensate for electrode background current. However, such currents were quite low and this facility was seldom used. Zero O$_2$ was recorded as a small signal in the most sensitive range of measurement to be used, when the stirred electrode chamber contained buffer which had been exhausted of dissolved O$_2$ by bubbling with argon followed by the addition of a few crystals of Na$_2$S$_2$O$_4$. The electrode was then calibrated by equilibrating samples of fresh reaction solutions (at 25 °C and
atmospheric pressure, i.e. 700 mmHg) with gas mixtures provided from cylinders of argon containing known O₂ concentrations (determined by mass spectrometer analysis), by means of a two-channel gas metering pump (H. Wosthoff oHG, Bochum, West Germany). The diluent gas contained 0·004 or 0·005 % (v/v) O₂ and solutions equilibrated with this gas produced a measurable O₂-electrode signal relative to the Na₂S₂O₄ zero (e.g. typically, with the amplifier unit, the signal with solution equilibrated with diluent gas was 2 to 3·5 mV above the Na₂S₂O₄ zero). The diluent gas produced the same signal, whether supplied to the solution in the chamber directly from the cylinder or through the metering pump, indicating absence of air leakage in the system. The electrode response was linear with O₂ concentrations between that of air-saturated water (244 μM-O₂) and 1·6 μM-O₂, but departed from linearity at lower O₂ concentrations. Calibration curves drawn as log mV versus log O₂ concentration were used. They were checked by injecting small volumes of water containing known O₂ concentrations, into a sample of reaction solution equilibrated at low PO₂. With these methods, O₂ concentrations down to 0·05 μM could be measured reliably and estimates made down to 0·02 μM.

For assays, liquids of pre-determined initial O₂ content were transferred under argon to the chamber, completely filling it to the capillary injection port. The electrode trace was then commenced and the reaction initiated by the injection of 0·25 to 1 mg bacteroids in 50 or 100 μl. Rates of O₂ consumption at various O₂ concentrations were calculated from the electrode traces during depletion of O₂ from the system.

Measurement of nitrogenase activity. This utilized the C₂H₂ reduction technique as described previously (Bergersen et al. 1973; Wittenberg et al. 1974). In these experiments without a gas phase, bacteroid nitrogenase was assayed in the electrode chamber. The reaction medium was previously equilibrated with a gas mixture containing 0·15 to 0·2 atm C₂H₂. At intervals during the depletion of O₂ from the system by bacteroids, reactions were terminated by injection of 1 ml of 20 % (w/v) trichloroacetic acid. The reaction mixture was then withdrawn slowly by syringe, avoiding the formation of bubbles, and injected into 15 ml evacuated ‘Venoject’ tubes (Jintan Terumo Co. Ltd, Tokyo). The evolved gas was displaced from these tubes into 1 ml gas-tight syringes by rapid displacement with argon-saturated water. The C₂H₄·C₂H₂ ratios were determined by gas chromatography and the amount of C₂H₄ produced was calculated with regard to the solubilities of the various gases used. The reaction volumes of 4·5 ml contained sufficient dissolved gas to allow duplicate samples of 0·4 ml of gas from each assay to be collected for analysis. There was good agreement between duplicate assays.

Experimental details. In most experiments, O₂ consumption and nitrogenase activity were assayed simultaneously. Leghaemoglobin deoxygenation was measured in parallel spectrophotometer assays. In the presence of oxyleghaemoglobin, the O₂ concentration was generally measured by O₂-electrode above 1 μM and by calculation from Y below this value. Rates of reaction were consistent in all experiments and were similar to those measured by Wittenberg et al. (1974) in shaken reactions with a gas phase and repeated in some respects as a part of the present work. In all experiments the temperature was 25 °C and atmospheric pressure was about 700 mmHg (Canberra is about 700 m above sea-level).

RESULTS

The effects of O₂ concentration in shaken assays

The effects of PO₂ on O₂ uptake and nitrogenase activity of CB1809 bacteroids in shaken assays were repeated on a more extensive basis than previously reported (Wittenberg et al.
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Fig. 1. Effects of $pO_2$ on (a) $O_2$ uptake from a gas phase and (b) nitrogenase activity of bacteroids in standard shaken assays (Wittenberg et al. 1974). Means, plus and minus the standard deviations are shown for pooled data from measurements on three successive days, on each of which the full $pO_2$ range was covered. ●, Leghaemoglobin (0.5 mM); ○, no leghaemoglobin.

For nitrogenase activity there was good agreement with the previous results, but the effects of $pO_2$ on $O_2$ uptake now showed that leghaemoglobin brought about an increment of $O_2$ uptake which increased with $pO_2$ (Fig. 1). Previous conclusions that leghaemoglobin caused an increment of $O_2$ uptake which was independent of $pO_2$ over the measured range, were due to inadequate replication and the use of too few $pO_2$ values, as the individual values recorded (cf. Fig. 3 of Wittenberg et al. 1974) fell within the ranges we observed (Fig. 1). These results bear significantly on the interpretations offered previously and it became necessary that the relationship between the concentration of $O_2$ in solution and the activity of bacteroid oxidases be more closely defined.

Effects of $O_2$-carrying proteins on $O_2$ electrode calibrations and agreement between electrode and spectral measurement of $O_2$ concentration

Figure 2 shows that Lb$O_2$ produced an increment of current from the $O_2$-electrode when the solution in the chamber was in equilibrium with a gas mixture of low $O_2$ pressure. The magnitude of the increment was affected by the leghaemoglobin concentration and by the stirring rate in the chamber (not illustrated). A similar effect occurred at higher free-$O_2$ concentration with myoglobin. To minimize these effects, the electrode was always calibrated with solutions of the same composition as those used in the experiments and the stirrer was connected to a stabilized power supply. Under these conditions, the time course of $O_2$ consumption by bacteroids measured by the $O_2$-electrode agreed well with the time course of $O_2$ consumption calculated from $Y_t$ values measured in the optical cuvette (Fig. 3), and values of $Y$ and $O_2$ concentration agreed well (Table 1). This finding permitted results from the two types of measurements to be directly compared in each experiment.
Fig. 2. The effect of 0.1 mM-leghaemoglobin on the calibration of the oxygen electrode. Two ml of the medium described in the text, with (○) or without (○) the protein, were equilibrated with gas mixtures of various O_2 contents and the solution O_2 concentration was calculated from the solubility of O_2 (1.26 mM at 25 °C and atmospheric pressure; see text).

Fig. 3. The time course of O_2 consumption by bacteroids measured by O_2-electrode (○, O), and from Y values for 90 μM-leghaemoglobin (●). The shapes of the curves for the electrode data were traced from a single typical recorder trace and the mean values for up to six traces have been superimposed as points with standard deviations, at 1 min intervals. ○, With LbO_2; ○, no LbO_2.

Table 1. Comparison of measurements of O_2 concentration made by O_2-electrode and by measurement of leghaemoglobin oxygenation

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>O_2 concn (μM)</th>
<th>From electrode μM-O_2</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>By electrode</td>
<td>Calculated from Y observed</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.25 ± 0.06</td>
<td>0.17</td>
<td>0.836-0.919</td>
</tr>
<tr>
<td>6</td>
<td>0.16 ± 0.05</td>
<td>0.12</td>
<td>0.747-0.849</td>
</tr>
<tr>
<td>7</td>
<td>0.08 ± 0.02</td>
<td>0.088</td>
<td>0.617-0.728</td>
</tr>
<tr>
<td>8</td>
<td>0.05 ± 0.01</td>
<td>0.063</td>
<td>0.478-0.640</td>
</tr>
</tbody>
</table>

Means and standard deviations are given for a series of O_2-electrode readings made during deoxygenation of 88 μM-LbO_2 by 1 mg bacteroids in a 4.6 ml assay. Values of Y were calculated from the extremes of the standard deviations of O_2 concentration at each time. These values are compared with Y observed in a single cuvette, during the course of a parallel experiment. The data come from experiments illustrated in Figs. 3 and 8.

of tests with the injection of small volumes of O_2 dissolved in water demonstrated that the response of the electrode was exactly as calibrated and was at least 20 times faster than the rate of O_2 depletion in these experiments. The rates of change in leghaemoglobin absorption spectra were also much slower than the response of the spectrophotometer to a change in absorbance.

The consumption of O_2 from solution by bacteroids

The use of the O_2-electrode permitted the measurement of the relationship between the rate of O_2 consumption and the O_2 concentration, as O_2 was being depleted from experi-
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Fig. 4. Deoxygenation of leghaemoglobin by bacteroids. Representative spectra from a single cuvette experiment. (a) Spectrum before addition of bacteroids (10 μM-O₂); (b) immediately after adding 1 mg bacteroids; (c) after 6-6 min; (d) after 1-4 h; (e) with added Na₂S₂O₅. The leghaemoglobin concentration was 88 μM, liquid volume 4-6 ml and the light path was 10 mm.

mental systems by bacteroid respiration. In most experiments with 1 mg of bacteroids in 4.5 to 4.6 ml, rates of O₂ consumption without added leghaemoglobin were 18 to 25 nmol/min/mg until the O₂ concentration fell below 2 to 3 μM. In the standard type of experiment (Fig. 3), the maximum rate of O₂ consumption lasted only about 2 min.

When LbO₂ was present, the bacteroids received significant amounts of O₂ from this source when the concentration of free O₂ fell below about 6 μM; hence the rate of consumption of free dissolved O₂ was slowed between 6 and 1.5 μM. However, the rate of decline of free dissolved O₂ in the presence of LbO₂ was faster than in the absence of LbO₂ (Fig. 3) by a factor of 4 at 0.65 μM-O₂.

Deoxygenation of LbO₂ by bacteroids

It was possible to use the fractional oxygenation (Y) of leghaemoglobin to follow the consumption of bound O₂ and to calculate the decline in concentration of free O₂ in solution, using the well-established kinetic constants for the Lb + O₂ ⇌ LbO₂ equilibrium. Figure 4 shows representative spectra recorded during a cuvette experiment. It can be seen that 1 mg bacteroids in a reaction volume of 4.6 ml does not perturb the peak-minus-trough extinction in the fully oxygenated state. Further, bacteroids are capable of completely exhausting the O₂ from LbO₂. The small sharp peak at 552 nm, which can be seen superimposed upon the broader Lb peak, has been noted before and is believed to be due to reduced bacteroid cytochrome c (Appleby, 1969). The O₂ consumption of bacteroids was measured from solution and from LbO₂ by recording the change in Y with time at several leghaemoglobin concentrations (Fig. 5). It is seen that increasing the leghaemoglobin concentration from 63
Fig. 5. Effects of leghaemoglobin concentration on O₂ consumption by bacteroids. (a) The time course and (b) effects of Y on rates for leghaemoglobin concentrations of: ●, 63 μM; ▲, 121 μM; ○, 229 μM. (c), (d) The reactions were extended by using dilute bacteroids with low carrier concentrations: □, 41 μM-LbO₂, 1 mg bacteroids; △, 25 μM-LbO₂, 0·5 mg bacteroids; ▶, 9 μM-LbO₂, 0·25 mg bacteroids; ■, 4·3 μM-LbO₂, 0·125 mg bacteroids.

Fig. 6. The time course of (a, c) O₂ consumption and (b, d) nitrogenase activity of bacteroids when the initial O₂ concentration was 11 μM and leghaemoglobin was absent. The bars indicate plus and minus one standard deviation in (a) and (b). In (c) and (d), either 0·5 mg (●) or 1 mg (○) of bacteroids were used in each assay. In (b) the free dissolved O₂ concentration (μM) is shown at the times indicated by arrows.

to 229 μM did not affect the maximum O₂ consumption rate but merely prolonged it (Fig. 5a). When the concentration was reduced below about 40 μM the maximum rate of O₂ consumption from the carrier at low free O₂ concentration was progressively diminished (Fig. 5c). The Y value giving half the maximum rate at any one leghaemoglobin concentration increased with decreasing Lb concentrations below about 40 μM (Fig. 5b, d). However, it should be observed that, in the lower range of Lb concentrations, the use of different weights of bacteroids per assay may have influenced the results (Fig. 5a, c).

The relationship between O₂ consumption and nitrogenase activity in the absence of leghaemoglobin

Oxygen is essential for the support of bacteroid nitrogenase activity (for review see Bergersen, 1971) but the relationships between O₂ consumption rates and nitrogenase activity have not been studied in detail hitherto. When the initial free O₂ concentration was 11 μM, nitrogenase activity was maintained at 1·15 nmol C₃H₄/min/mg for almost 2 min
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but declined to a very low value when \( \text{O}_2 \) became limiting (Fig. 6a). In another experiment in which the initial \( \text{O}_2 \) was 19 \( \mu \text{M} \), the \( \text{O}_2 \) consumption rate was the same but \( \text{O}_2 \) did not become limiting until between 3 and 4 min. There was a lag of almost 1 min before nitrogenase activity reached its maximum rate of 2.5 nmol/min/mg; again it declined to almost zero when \( \text{O}_2 \) became limiting. Values for all experiments fell between these limits and nitrogenase activities always fell coincident with the onset of \( \text{O}_2 \) limitation.

A short time course such as that shown in Fig. 6(b), with only one observation between zero time and exhaustion of \( \text{O}_2 \), would allow for higher rates of nitrogenase activity if there were a significant lag. Therefore either the nitrogenase activity and \( \text{O}_2 \) consumption were measured at 0.5 min intervals, or the reactions were prolonged by using 0.5 mg bacteroids instead of 1.0 mg. There was some evidence of a lag of about 30 s in nitrogenase activity, but there was no evidence of the rates subsequently exceeding 1 to 2 nmol \( \text{C}_2\text{H}_4/\text{min/mg} \) at any time during depletion of \( \text{O}_2 \) from the system (Fig. 6d).

The relationship between \( \text{O}_2 \) consumption and nitrogenase activity in the presence of leghaemoglobin

In the presence of leghaemoglobin, \( \text{O}_2 \) consumption by bacteroids leads to progressive deoxygenation of the protein and to an altered relationship between \( \text{O}_2 \) consumption rate and nitrogenase activity. When bacteroids obtained their \( \text{O}_2 \) from \( \text{LbO}_2 \), the rates of nitrogenase activity closely paralleled the rates of \( \text{O}_2 \) consumption (Fig. 7) as in experiments without \( \text{LbO}_2 \) (Fig. 6). However, with \( \text{LbO}_2 \) the reactions were prolonged, because of the
greater store of O₂. The maximum rates of O₂ consumption were very similar in the two systems, but in the presence of LbO₂, nitrogenase activity continued to a much lower free O₂ concentration (0.01 μM-O₂ or Y = 0.24, compared with about 1 μM-O₂ without LbO₂).

Increasing leghaemoglobin concentration above 50 μM did not result in increased nitrogenase activity. This contrasts with the shaken assays (Wittenberg et al. 1974) in which nitrogenase activity increased up to about 1 mM-leghaemoglobin. In one experiment in the absence of a gas phase, using 81, 152 and 216 μM-LbO₂, a single line described the time course at all three Lb concentrations, with a maximum rate of 6 nmol C₂H₄/min/mg. It was not possible to measure nitrogenase activity at leghaemoglobin concentrations below about 50 μM (Fig. 5c, d) because the necessity of using dilute bacteroid concentrations to prolong the reaction made the resultant C₂H₄ concentrations too low for measurement. However, it seems logical to suppose that nitrogenase activity would decline with declining O₂ consumption rates (cf. Figs. 6 and 7).

In the experiments being described, in which the initial free O₂ concentration was usually about 10 μM, the source of O₂ being consumed by bacteroids was first of all free dissolved O₂ whose concentration quickly fell to values which led to increasing deoxygenation of LbO₂. Events occurring during this phase were examined in experiments such as that illustrated in Fig. 8. Here the initial free O₂ was 10 μM (Y₀ = 0.996). This experiment was done concurrently with that illustrated in Fig. 6(a), (b). Nitrogenase activity was initially 1 nmol C₂H₄/min/mg, as in Fig. 6(b), in the absence of LbO₂, and then increased to a maximum of 8.4 nmol C₂H₄/min/mg as the LbO₂ discharged its O₂ at the maximum rate at low O₂ concentrations.

The initial low rate of nitrogenase activity, as the free O₂ concentration fell from about 10 μM might represent a lag or a true rate. Therefore experiments were conducted using two bacteroid concentrations (0.5 and 1.0 mg/assay). An initial rate of about 1 nmol C₂H₄/min/mg was sustained, and an enhanced rate coincided with the onset of the maximum
rate of net discharge of O\textsubscript{2} from LbO\textsubscript{2}; these events occurred without relation to the time from initiation of the reactions (Fig. 9). The low initial rate was not therefore due to a time lag before the bacteroids reached maximum nitrogenase activity.

The enhanced rate of nitrogenase activity which accompanies discharge of O\textsubscript{2} from the leghaemoglobin may result from the supply of O\textsubscript{2} being stabilized at about 1 \mu M or less because of possible inhibition of nitrogenase at higher O\textsubscript{2} concentrations. Bovine myoglobin was therefore used to replace leghaemoglobin as the macromolecular carrier (see Wittenberg et al. 1974). The equilibrium constant for oxygenation of this protein is almost 30-fold higher than for leghaemoglobin – 1 \times 10^{-6} M (Antonini & Brunori, 1971) compared with 4 \times 10^{-8} M (Wittenberg et al. 1972). The net discharge of O\textsubscript{2} therefore occurs at much higher free dissolved O\textsubscript{2} concentrations. Essentially the same pattern of nitrogenase activity was obtained as with leghaemoglobin (Fig. 11) although the maximum rate was less than with leghaemoglobin. However, the maximum rate of discharge of O\textsubscript{2} from myoglobin and enhanced nitrogenase activity occurred in the range of concentrations of free dissolved O\textsubscript{2} which was associated with only low rates of activity in the absence of a carrier or when the carrier was leghaemoglobin (compare the O\textsubscript{2} concentration scales below Figs. 6b, 8 and 10). With myoglobin also, the enhanced nitrogenase rate commenced at lower calculated Y values (about 0.8) than when the carrier was leghaemoglobin (Y > 0.95, Fig. 9).
DISCUSSION

Constraints imposed by the experimental system

There are many difficulties in establishing the relationships between the terminal oxidases of bacteria, the $O_2$ concentration which prevails in their immediate environment, and consequent effects on other cellular metabolism. Firstly, there is the instrumental difficulty associated with the use of an $O_2$-electrode which is relatively insensitive in the range of concentrations which was of greatest interest. However, the ability to use spectrophotometry to measure the state of oxygenation of the carrier proteins to 'report on' the $O_2$ concentration prevailing during these experiments, provided indispensable confirmation. One result was the verification of electrode measurements made in critical ranges (Table 1, Fig. 3) when leghaemoglobin was present. From a common starting $O_2$ concentration, identical plots of $O_2$ consumption by bacteroids were always obtained, whether measurements were made by $O_2$-electrode or by measurement of $Y$ for leghaemoglobin and myoglobin. If this accuracy was obtained in the presence of an $O_2$ carrier, there is no reason to doubt that measurements made with the electrode in the absence of the carriers were equally accurate. Further, the results established that conditions in the stirred electrode chamber were not detectably different from those in the optical cuvette assays, which were initially shaken to disperse the bacteroids and subsequently depleted in $O_2$ by bacteroids without stirring. Both methods measure only the average $O_2$ concentration in the bulk of the solutions. Possible inhomogeneities in $O_2$ distribution over short distances within the solution would not be detected, nor would the $O_2$ concentration close to the bacteroid surfaces necessarily be the same as in the bulk solution.

In the liquid-phase experiments reported in this paper, the dissolved $O_2$ concentration declined with time. In case there are mechanisms in bacteroids which respond only slowly to changes in free $O_2$ concentration, it would be best to study steady-state $O_2$ uptake and nitrogenase activity. Only short periods of steady $O_2$ consumption occurred in the absence of $O_2$-carrying proteins (1 to 2 min with 1 mg and 2 to 4 min with 0.5 mg bacteroids/assay, Fig. 6). However, this seemed to be sufficient, since the maximum rate of $O_2$ consumption began within a few seconds of initiation of the reactions at about 10 $\mu$M free $O_2$, and was the same as achieved with leghaemoglobin present, within the limits of experimental error (Figs. 6a and 8). Steady $O_2$ consumption rates occurred in the presence of 50 $\mu$M or more leghaemoglobin between $Y$ values of 0.94 and 0.45, and with 100 $\mu$M myoglobin between $Y = 0.8$ and 0.5. With the highest leghaemoglobin concentration used (229 $\mu$M), the steady state lasted about 40 min (Fig. 5a). We consider that measurements made during steady $O_2$ consumption in the absence of a carrier or during steady rates of discharge of $O_2$ from oxyleghaemoglobin or oxymyoglobin are reliable.

Oxygen consumption in these experiments was measured continuously, but rates of nitrogenase activity could only be measured as increments of $C_2H_4$ in reactions terminated at intervals of time. These intervals had to be long enough to produce reliable measurement of $\Delta C_2H_4$. This meant that at certain stages of each experiment, free $O_2$ concentration changed substantially during measurement of nitrogenase rates. Errors in the relationships between free $O_2$ concentrations and bacteroid activities were inevitable at such periods. The use of more dilute bacteroids to extend the time of observation enabled more frequent sampling in some experiments, and indicated that this constraint was not serious (Figs. 6 and 9). However, even with dilute bacteroids it was not possible to measure the effects of very low leghaemoglobin concentrations on nitrogenase activity because total activity was insufficient to permit reliable measurements of $\Delta C_2H_4$ at any sampling intervals.
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**Effects of $O_2$-carrier proteins on bacteroid activities**

It has been suggested previously that the overall function of leghaemoglobin in $N_2$ fixation by legume root nodules is to permit the maintenance of high $O_2$ flux to the bacteroids at very low free $O_2$ concentrations (Bergersen, 1971, 1973; Wittenberg et al. 1972). This was supported by the results of recent experiments (Tjepkema, 1971; Bergersen et al. 1973; Wittenberg et al. 1974). However, as far as we are aware, this is the first report of measured $O_2$ concentration in solution during measured nitrogenase activity and of measured $O_2$ consumption by washed bacteroid suspensions.

Oxyleghaemoglobin appeared to exert its main effect through the maintenance of $O_2$ consumption at low free dissolved $O_2$ concentration. It allowed maximum rates of $O_2$ consumption to be sustained down to free $O_2$ concentrations which were only 4% of those which were limiting in the absence of this carrier. Restriction of the rate of $O_2$ consumption, when free $O_2$ fell below about 2 $\mu$M in the absence of leghaemoglobin or when $Y$ fell below about 0.45 in its presence, also restricted nitrogenase activity. Enhanced nitrogenase activity ensued when conditions allowed the maximum rates of net discharge of $O_2$ from $LbO_2$ or $MbO_2$ with a minimum change in the concentration of free dissolved $O_2$. The highest rates of nitrogenase activity were observed in the presence of at least 50 $\mu$M-leghaemoglobin when the free $O_2$ concentration was between 1 $\mu$M ($Y = 0.96$) and 0.025 $\mu$M ($Y = 0.45$). These effects are summarized in Fig. 11.

The maximum nitrogenase rates in the presence of $LbO_2$ occurred in a range of $O_2$ concentrations which supported no activity in the absence of this carrier (Fig. 11). Substitution of $MbO_2$ for $LbO_2$ produced enhanced nitrogenase rates and the form of the data superficially resembled those from experiments with the latter carrier (cf. Figs. 8 and 10). However, the effects occurred at higher $O_2$ concentrations (1 to 4 $\mu$M) and the rates of nitrogenase activity were less than with leghaemoglobin. Thus, enhanced nitrogenase activity supported by $MbO_2$ occurred in a range of free $O_2$ concentrations which supported low but significant nitrogenase activity in the absence of a carrier. Further, at 1 to 4 $\mu$M free $O_2$, nitrogenase rates were higher with $MbO_2$ than with $LbO_2$ (Fig. 11).

Model experiments with haemoglobin and myoglobin (for review see Wittenberg, 1970) showed that facilitated fluxes of $O_2$ across thin layers of solution supported by filter membranes, increased with protein concentration up to about 7 mM. In the shaken experiments with bacteroids and a gas phase (Bergersen et al. 1973; Wittenberg et al. 1974), the maximum rates of $O_2$ uptake and nitrogenase activity were reached with about 1 mM-leghaemoglobin. The rates of activity in the liquid-phase experiments were much less sensitive to leghaemoglobin concentration. No increases in $O_2$ consumption or nitrogenase activity were recorded above about 50 $\mu$M-leghaemoglobin. A concentration-dependent effect on $O_2$ consumption was observed below about 40 $\mu$M during steady deoxygenation of $LbO_2$ but it is possible that the use of fewer bacteroids in these assays may have influenced this finding. This point could not be resolved in this experimental system because dilute bacteroids had to be used to slow the rate of change of free $O_2$ concentration at low carrier concentrations.

**The mechanism of leghaemoglobin action**

In soybean root nodule tissue the leghaemoglobin appears to be located as a layer of solution between the bacteroids and the membrane envelopes which enclose them in the host cytoplasm (Bergersen & Goodchild, 1973). The bacteroids consume $O_2$ which must pass through the leghaemoglobin solution. In the experiments described here, $O_2$ supply to the bacteroids distributed through the leghaemoglobin solution takes place in essentially
Fig. 11. The relationship between free dissolved O₂ concentration and the O₂ consumption and nitrogenase activity of soybean nodule bacteroids, as affected by leghaemoglobin and myoglobin. Observations from five experiments without carrier (a, b), five with 60 to 100 µM-leghaemoglobin (c, d) and two with 40 to 100 µM-myoglobin (e, f) in which 1 mg of bacteroids was used in 4·55 ml assays. Each bar represents rate of nitrogenase activity or O₂ consumption measured over sampling intervals of 0·5 to 2 min. Because of difficulties of labelling, individual experiments are not identified. The µM-O₂ values assigned are the means of the concentrations prevailing at the beginning and end of each sampling period. A logarithmic scale is used below 1 µM-O₂ for convenience of presentation.
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the same way, except that only $O_2$ present (as $LbO_2$ plus free dissolved $O_2$) in the bulk solution at the beginning of each experiment is consumed; in the nodule it is being continually replenished from without. In the previous experiments in which bacteroids were shaken with leghaemoglobin and $O_2$ was supplied from a gas phase (Bergersen et al. 1973; Wittenberg et al. 1974) there was opportunity for the operation of $O_2$-carrier-mediated effects which may not be relevant to the in vivo system (cf. Stokes, 1975). We now discuss the present results in relation to some of the mechanisms which have been proposed.

Facilitated diffusion in unstirred layers surrounding suspended bacteroids. Appleby (1974) has concisely reviewed current evidence for the proposition made by Yocum (1964) that the mechanism of leghaemoglobin action in nodules is that of a carrier of $O_2$, in a form of the general phenomenon of macromolecular facilitated diffusion (reviewed by Wittenberg, 1970). With their high $O_2$ consumption rates, bacteroids could generate steep gradients of $O_2$ concentration close to their surfaces, where bacteroid oxidases are presumably located. These gradients would not be able to be dispersed by rapid stirring. Wittenberg et al. (1974) interpreted their data to show that leghaemoglobin could be facilitating diffusion of $O_2$ across such unstirred layers, thus bringing about a higher flux of $O_2$ than would occur without the carrier. An added consequence would be a moderate rise in $O_2$ concentration at the outer surface of the bacteroids. This rise was considered to make possible a more efficient oxidase function, giving rise to higher ATP levels within the bacteroids for support of nitrogenase.

The findings presented in Fig. 1, showing increasing $LbO_2$-augmented increments of $O_2$ uptake with increasing $pO_2$ suggest that facilitated diffusion across the unstirred layer surrounding the bacteroids in shaken assays may not be the principal mechanism involved. Contrary to previous conclusions (Wittenberg et al. 1974) Appleby, Turner & Macnicol (1975b) observed that the $O_2$ pressures used in the model experiments were insufficient to saturate the macromolecular carrier with $O_2$ in the bulk liquid of the shaken system, and concentration gradients of $O_2$, particularly near the gas–liquid interface, may have been the dominant cause of the effects. The main effect of leghaemoglobin in the experiments of Wittenberg et al. (1974) is seen by A. N. Stokes (private communication) to have been the establishment within the liquid of a zone of stabilized low $O_2$ concentration which was optimal for nitrogenase activity. The effects of facilitated diffusion through the layer of solution around each bacteroid were considered to be negligible. Our finding, in experiments with no gas phase, that $O_2$ consumption and nitrogenase rates were relatively insensitive to leghaemoglobin concentration tends to support this conclusion.

Stabilization of $O_2$ supply by leghaemoglobin. Stokes (1975) introduced the concept of stabilization of $O_2$ supply by partially oxygenated macromolecular carriers such as leghaemoglobin. Numerically, this effect can be much greater than facilitated diffusion. However, in experiments with no gas phase, stabilization would not be expected to have much effect because there is little resistance to whatever $O_2$ flux is needed to sustain bacteroid activities.

Optimum $O_2$ concentration for nitrogenase. In the absence of a gas phase and with no $O_2$ carrying protein present, as the $O_2$ concentration declines towards a low value (about 1 mM, Fig. 3), limiting inhomogeneity of $O_2$ distribution may cause a premature decline in $O_2$ consumption by bacteroids. With leghaemoglobin present at this low free $O_2$ concentration the total $O_2$ in the system is greater (free $O_2$ plus $LbO_2$), the rate of decline of free $O_2$ concentration is much slower and inhomogeneities are less likely to occur. Consequently, maximum rates of $O_2$ consumption could be extended to lower free $O_2$ concentrations by the ‘$O_2$-buffering’ action of leghaemoglobin (cf. Fig. 11b, d). Since $O_2$ is required for nitrogenase
activity but is also an inhibitor of nitrogenase (Wong & Burris, 1972), it is possible that this extended range of O₂ concentration for bacteroid respiration also coincides with a range optimal for nitrogenase activity (A. N. Stokes, private communication). In the absence of a carrier, such conditions may be so transient that no enhancement of nitrogenase activity could be observed. The results of our experiments, consistent to some extent with this interpretation (Fig. 11), present some difficulties. Experiments such as those of Figs. 9(b) and 10 show transitions from a low steady rate to another higher rate when the O₂-carrying proteins begin to unload O₂ at a constant rate. If the low rates were due to inhibition by O₂, one would expect that the nitrogenase rates would increase progressively as the O₂ concentration declined. (The impression from Fig. 11c that this was so, results from the inclusion of a number of experiments in which the rates of nitrogenase activity between 5 and 10 μM were different.) Further, the ranges of O₂ concentration in which the enhanced nitrogenase rates operated were different for the two carrier proteins of different affinity (Fig. II c, e) and correspond to their respective ‘O₂-buffering’ ranges. Critical establishment of this point must await the development of techniques which will permit the study of steady rates of nitrogenase activity at various O₂ concentrations maintained within these ranges.

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