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Leghaemoglobin and the Supply of O_2 to Nitrogen-fixing Root Nodule Bacteroids: Presence of Two Oxidase Systems and ATP Production at Low Free O_2 Concentration

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SUMMARY

Studies of rates of consumption of dissolved O_2 by suspensions of bacteroids (*Rhizobium japonicum*, strain CB1809) from soybean root nodules showed the presence of two different terminal oxidase systems. A high-affinity system, sensitive to inhibition by *N*-phenylimidazole and by carbon monoxide, was most active when the dissolved O_2 was between 0·01 and 0·1 μM. At 1 μM-O_2 or higher, this oxidase system had little activity and O_2 was consumed largely by a low-affinity system insensitive to these inhibitors. At low concentrations of dissolved O_2, bacteroid respiration rates appeared to be diffusion-limited. When purified oxyleghaemoglobin was added to such systems, this restriction was relieved and respiration was maintained to much lower concentrations of free dissolved O_2, where nitrogenase activity was greatest.

Analysis of reactions which were terminated at various stages during the depletion of O_2 from oxyleghaemoglobin showed that at low free O_2 concentration, the high-affinity pathway produced up to five times greater bacteroid ATP concentrations than the low-affinity oxidase pathway operating about 1 μM free O_2 in the absence of leghaemoglobin. At intermediate free O_2 concentrations, occurring during the later stages of deoxygenation of oxymyoglobin, intermediate concentrations of ATP were found in the bacteroids.

INTRODUCTION

Several authors have described the stimulation of O_2 consumption and the greatly increased nitrogenase activity which occurs when purified oxyleghaemoglobin (LbO_2) is added to suspensions of bacteroids prepared from soybean root nodules and shaken in closed vessels with gas mixtures containing low concentrations of O_2 (Bergersen, Turner & Appleby, 1973; Wittenberg *et al.* 1974; Appleby *et al.* 1975*a*; Appleby, Turner & Macnicol, 1975*b*). These experiments were considered to provide models for the effects *in vivo* of LbO_2 in legume root nodules, where a solution of leghaemoglobin is believed to be in contact with the bacteroids, within membrane-enclosed vesicles (Truchet, 1972; Bergersen & Goodchild, 1973; Gourret & Fernandez-Arias, 1974). There was greater nitrogenase activity per mole of O_2 consumed when LbO_2 was present. This appeared to be due to a more efficient ATP supply in the bacteroids, through an oxidative phosphorylation pathway which functioned during LbO_2-facilitated respiration (Appleby *et al.* 1975*a*, *b*). After using the specific inhibitor *N*-phenylimidazole, these authors concluded that bacteroid cytochrome P-450 (Appleby, 1969) was a component of the efficient pathway.

Wittenberg *et al.* (1974) concluded that their results from shaken assays were consistent with facilitation by leghaemoglobin of the flux of O_2 across thin, unstirred layers around each bacteroid. Stokes (1975; and personal communication) deduced mathematically that

other effects in the stirred suspension might predominate during the shaken assays; these might have limited relevance *in vivo* in nodule tissue. To minimize such effects and to eliminate gas–liquid interface anomalies, Bergersen & Turner (1975) devised an experimental system with no gas phase and found that bacteroid nitrogenase activity accompanying release of O_2 from LbO_2 or from oxymyoglobin (MbO_2) was enhanced compared with activity in the absence of these O_2 carriers. We now report that the low free dissolved O_2 concentrations occurring during delivery of O_2 from LbO_2 to respiring bacteroids are optimal for the high affinity terminal oxidase system which provides high cellular concentrations of ATP for the support of nitrogenase activity.

METHODS

The preparation of bacteroid suspensions with active nitrogenase from root nodules of soybean (*Glycine max* Merr. cultivar Lincoln, inoculated with strain CB1809 of *Rhizobium japonicum*) and of purified leghaemoglobin and myoglobin were described by Bergersen, Turner & Appleby (1973), Bergersen & Turner (1973), and Wittenberg *et al.* (1974). The experimental systems in which the consumption of dissolved free O_2 and of O_2 released from LbO_2 or MbO_2 could be monitored and nitrogenase activity measured in the absence of a gas phase were exactly as previously described (Bergersen & Turner, 1975). The reaction medium was 25 mM-tris–HCl, pH 7·4, containing 15 mM-sodium succinate; other factors were added to this as required. All measurements were made at 25 °C.

Concentrations of free dissolved O_2 were measured by O_2 electrode or calculated from the oxygenation of leghaemoglobin or myoglobin with reference to the equilibrium constants for oxygenation of these haemoproteins. Oxygenation was measured by spectrophotometry. Dissolved C_2H_2 and C_2H_4 were recovered from reaction mixtures following vacuum decompression and then assayed by gas chromatography (Bergersen & Turner, 1975). The production of C_2H_4 was used as an index of nitrogenase activity (Hardy *et al.* 1968).

Concentrations of ATP in extracts of bacteroids were measured by the luciferin–luciferase (firefly lantern extract, Calbiochem) method, using a Packard Tricarb scintillation counter (Stanley & Williams, 1969). Reactions with bacteroid suspensions were terminated by the injection of 1 ml of 2·2 M-perchloric acid per 4·5 ml of assay mixture, followed by rapid agitation and chilling to 0 °C. The vessels were agitated periodically during 30 min extraction and the extracts were then clarified by centrifuging (4000 ***g***, 0 °C) and stored in glass-stoppered tubes at −10 °C until analysed. Portions were analysed for ATP, and for ATP plus ADP, following treatment for 30 min at 25 °C with an excess of an ATP-generating system (creatine phosphate, 10 mg/assay; creatine kinase, 14 units/assay; Calbiochem).

RESULTS

Bacteroid terminal oxidase systems

Oxygen electrode traces of consumption of dissolved O_2 by dilute bacteroid suspensions, with no O_2-carrying haemoproteins present, show declining rates as the O_2 concentration falls below 4 to 5 μM. Near 1 μM the rates become steady before declining to very low rates of O_2 consumption near 0·1 μM (Fig. 1). When O_2 consumption rates were replotted against O_2 concentration on a semi-logarithmic scale (Fig. 2), curves with an inflection near 1 μM-O_2 were obtained, suggesting that two terminal oxidase systems of different O_2 affinity may operate sequentially with increasing O_2 concentration. Appleby *et al.* (1975*b*) chose the cytochrome P-450 inhibitor *N*-phenylimidazole as being least likely to have non-specific

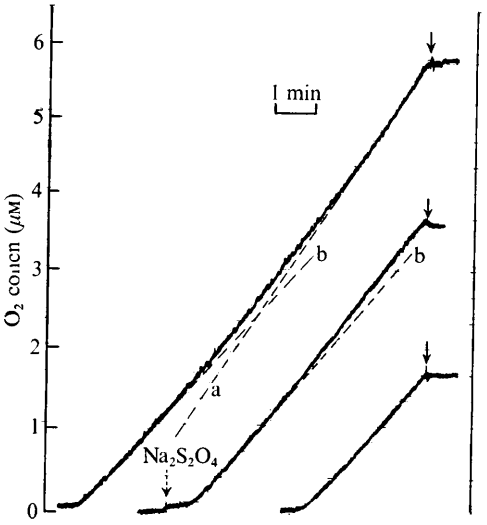

Fig. 1

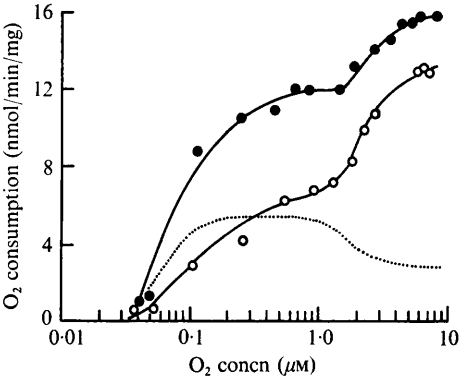

Fig. 2

Fig. 1. Oxygen electrode traces of O_2 consumption by 0·5 mg dry wt bacteroids/4·5 ml, with no O_2-carrying protein present. Zero O_2 was checked by injection of dithionite solution (broken arrow). Broken line 'a' indicates the maximum rate of consumption and line 'b' indicates the second steady rate applying at low free O_2 concentrations. Bacteroids were injected at the times indicated by the arrows.

Fig. 2. Effects of the concentration of free dissolved O_2 on rates of O_2 consumption by bacteroids with no O_2-carrying proteins present, and the effect of 1 mM-*N*-phenylimidazole. Reactions contained 0·2 mg dry wt bacteroids/4·5 ml. ●, Unhibited respiration; ○, 1 mM-*N*-phenylimidazole. The dotted line shows inhibitor-sensitive respiration (curve ● minus curve ○).

Table 1. *Effect of N-phenylimidazole on consumption of* O_2

Reactions contained 0·2 mg dry wt bacteroids/4·5 ml. Free O_2 concentrations given are the average values for the periods of each rate measurement. No O_2-carrying proteins were present.

N-phenylimidazole concn (mM)	Rate of O_2 consumption (nmol/min/mg)		
	Free O_2...6 μM	0·5 μM	0·1 μM
0	17·1	15·2	3·9
0·5	14·5	10·7	3·3
1·0	14·3	8·9	2·5
1·5	13·6	1·0	<0·5
2·0	13·5	0·8	<0·5

effects on bacteroid metabolism or on leghaemoglobin, when they studied bacteroid phosphorylation pathways related to nitrogenase function. We therefore used this inhibitor in an attempt to distinguish between the different oxidase pathways. This inhibitor is a dense oily liquid of limited solubility in water, and care had to be exercised to obtain reproducible results. It tended to precipitate from chilled or frozen and thawed solutions (about 10 mM) as almost invisible oily droplets. Therefore it was used from stock solutions kept at room temperature. In the absence of O_2-carrying haemoproteins, a wide range of inhibitor concentrations was applied but in the presence of LbO_2 or MbO_2, only 1·0 to 1·5 mM-*N*-phenylimidazole was used to minimize the possibility of haemochrome formation (Appleby *et al.* 1975*b*). The *R. japonicum* strain used (CB1809) was less sensitive to this inhibitor than

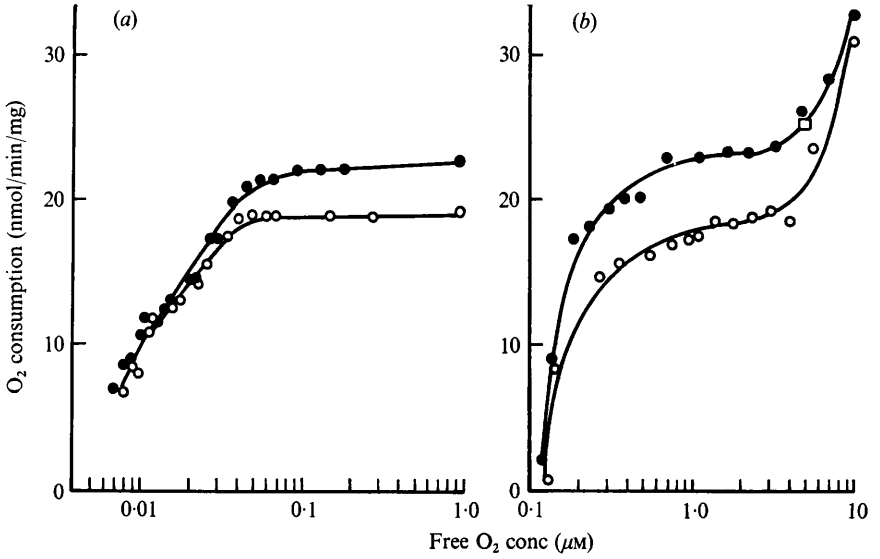

Fig. 3. Relationship between bacteroid respiration and the concentration of free dissolved O_2 with (*a*) 98 μM-leghaemoglobin (●) and (*b*) 94 μM-myoglobin (●) and inhibition by 1 mM-*N*-phenylimidazole (○). In (*b*), □ represents a single value from the leghaemoglobin treatment in this range of O_2 concentration. Reactions contained 1 mg dry wt bacteroids/4·5 ml.

the strain CC705 used by Appleby *et al.* (1975*b*). The results showed that there was inhibition of O_2 consumption by *N*-phenylimidazole at less than 1 to 3 μM free O_2. At higher concentrations of free dissolved O_2, inhibition was much less (Figs. 2 and 3, Table 1). These results suggest that cytochrome P-450 was involved in a terminal oxidase system of high affinity, which was replaced by a different system, of lower affinity for O_2, at free O_2 concentrations in excess of about 1 to 3 μM, and which was insensitive to the inhibitor. Cytochrome P-450 also reacts with CO (Appleby, 1969), and experiments were therefore conducted without O_2-carrying haemoproteins (which also react with CO), using solutions equilibrated with gas mixtures containing 99 % CO and 1 % O_2 (about 10 μM-O_2 and 0·9 mM-CO). Again, comparing reactions with or without the inhibitor, O_2 consumption by bacteroids was strongly inhibited by CO, with a peak at 0·2 to 0·3 μM free O_2; there was little inhibition near 10 μM-O_2 (Fig. 4).

Relationship between free O_2 concentration and ATP concentration in bacteroids

Concentrations of ATP and of ATP plus ADP in bacteroids were measured in experiments without O_2-carrying proteins, or using approximately 100 μM-LbO_2 or -MbO_2, to extend the time course and allow more accurate estimation of free O_2 concentrations. Reactions commenced at approximately 10 μM free dissolved O_2 and were terminated at intervals during depletion of O_2 from the systems. With no O_2-carrying haemoproteins present, the rate of change of free O_2 concentration was so fast that only one value for ATP could be obtained before O_2 consumption declined to a low rate (Fig. 5). In the presence of the haemoproteins, the rates of change of free O_2 concentration were slower during their deoxygenation: less than 0·5 μM/min with MbO_2 and about 0·1 μM/min with LbO_2 (Fig. 5). The results are also presented in terms of free O_2 concentration at termination of the reactions (Fig. 6). Bacteroid ATP concentrations were greatest in reactions terminated when the free O_2 concentration was 0·02 to 0·1 μM, i.e. during maximum rates of deoxygenation of

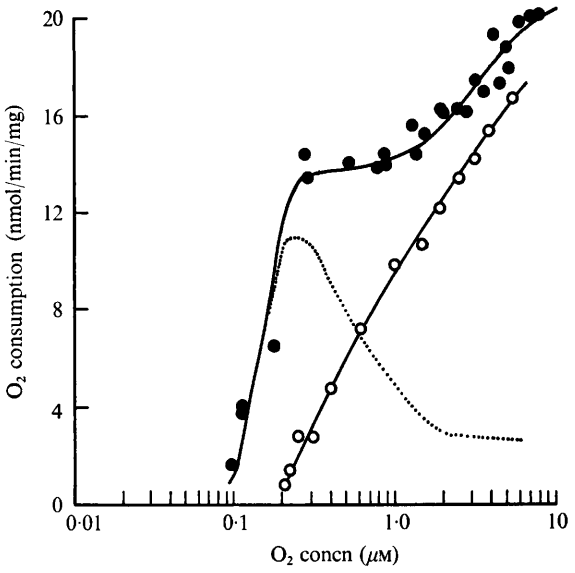

Fig. 4. Inhibition of bacteroid respiration by CO. Reactions contained no O_2-carrying proteins and 0·2 mg dry wt bacteroids was used in 4·5 ml. The liquids were equilibrated with gas mixtures containing 1 % O_2, 69 % N_2 and 30 % argon (●), or 1 % O_2 and 99 % CO (about 0·9 mM) (○) at 700 mmHg and 25° C. The dotted line represents CO-sensitive respiration (curve ● minus curve ○).

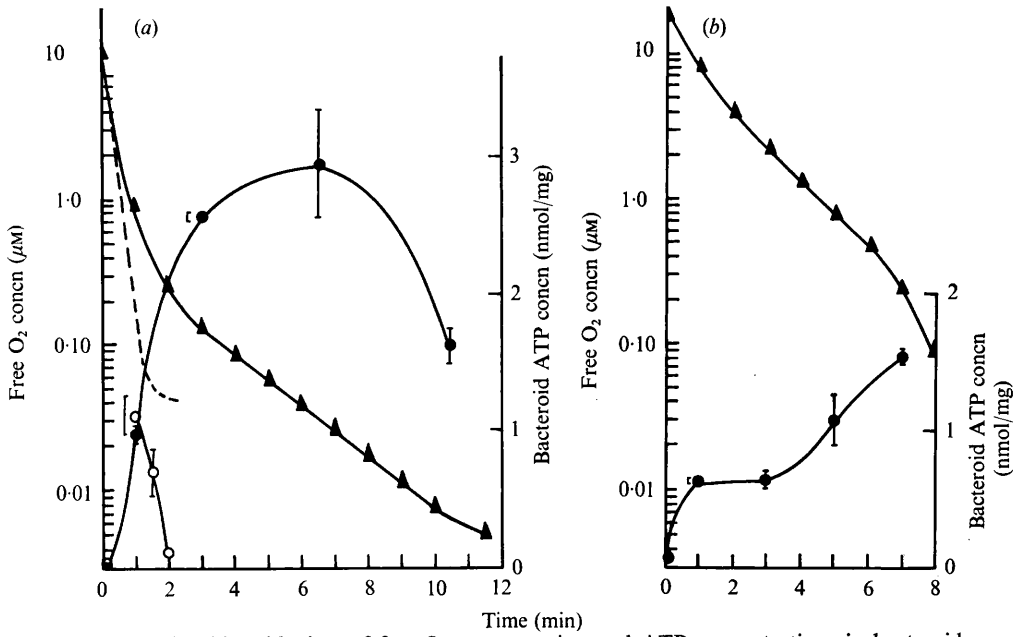

Fig. 5. Relationship with time of free O_2 concentration and ATP concentrations in bacteroids. (*a*) Free O_2 (▲) during deoxygenation of 118 μM-leghaemoglobin or (broken line) in reactions with no carrier; bacteroid ATP concentrations with (●) or without (○) leghaemoglobin. (*b*) Free O_2 (▲) and bacteroid ATP (●) concentrations during deoxygenation of 104 μM-myoglobin. The vertical bars indicate the ranges of duplicate ATP assays.

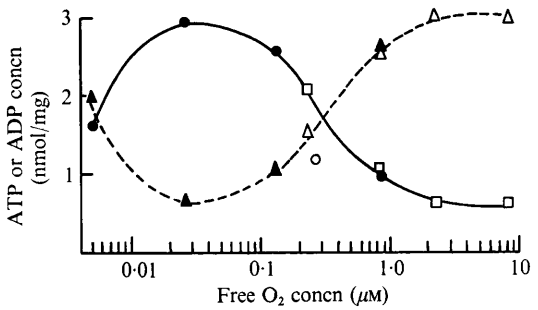

Fig. 6. Relationship between free O_2 concentration and bacteroid ATP (●, □; solid line) and ADP (▲, △; broken line) concentrations. Data for the experiment shown in Fig. 5, using 118 μM-leghaemoglobin (●, ▲) or 104 μM-myoglobin (□, △). The single point (○) is the concentration from a reaction with no O_2-carrying protein. ADP concentrations were the differences between the average ATP concentration at each sampling and the overall average ATP + ADP value (see text).

Table 2. *Inhibition by N-phenylimidazole of ATP formation in bacteroids*

Reactions contained 104 μM-myoglobin and 2 mg dry wt bacteroids/4·5 ml. Concentrations of O_2 are those at termination of the reactions. Duplicate assays agreed within 5 to 8 %.

Free O_2 concn (μM)	*N*-phenylimidazole concn (mM)	ATP concn (nmol/mg)
2·3	0	0·63
1·8	1	0·70
0·8	0	1·08
0·8	1	0·66

LbO_2. In reactions terminated at about 1 μM-O_2 or higher, ATP concentrations were only about a quarter to a fifth as great, whether the haemoproteins were present or not. During later stages of deoxygenation of MbO_2 (0·2 to 0·8 μM free O_2), intermediate concentrations of ATP were found. Calculated values for ADP fitted a converse relationship (Fig. 6), since ATP plus ADP was fairly constant (3·6 ± 0·4 nmol/mg dry wt bacteroids) during these experiments. The presence of 1 mM-*N*-phenylimidazole in the reactions lowered steady-state levels of ATP by up to 35 % at free O_2 concentrations below about 1 μM; there was little or no inhibition at higher O_2 concentrations (Table 2). Experiments such as those recorded in Fig. 7 show that nitrogenase activities were increasingly inhibited by *N*-phenylimidazole as the O_2 concentration fell, in similar fashion to the inhibition of ATP production (see Appleby *et al.* 1975*b* and Fig. 6). Rates of acetylene reduction were greatest during deoxygenation of LbO_2 and somewhat less during the later stages of deoxygenation of MbO_2. Inhibition of nitrogenase by *N*-phenylimidazole was associated with diminished rates of O_2 consumption (Fig. 7), especially at low free O_2 concentrations.

Effects of LbO_2 on apparent diffusion limitation of bacteroid respiration

With dilute bacteroid suspensions (about 0·2 mg dry wt/4·5 ml reaction) a linear relationship between free O_2 consumption rates and free O_2 concentration occurred in the range 0·1 to 0·3 μM, when no O_2-carrying proteins were present (Fig. 8, broken line). The linear increase ceased abruptly at about 0·3 μM free O_2. This type of relationship is typical of a diffusion-limited O_2 flux and has been obtained repeatedly. The limitation was relieved by the addition of LbO_2. Fig. 8 shows results from experiments using various concentrations of LbO_2; bacteroid respiration extended well into the range where O_2 consumption was

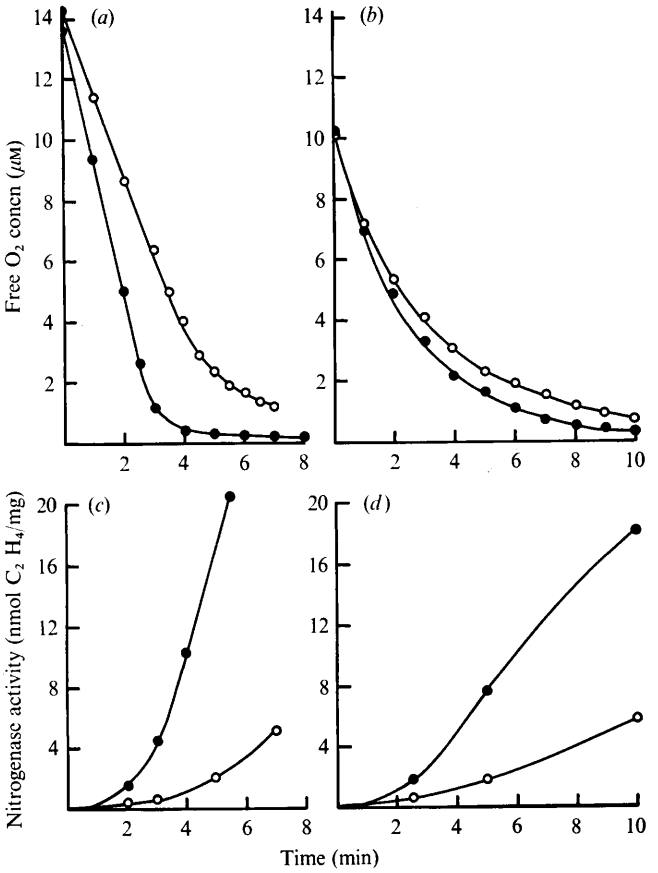

Fig. 7. Effect of *N*-phenylimidazole on nitrogenase activity of bacteroids in the presence of (*c*) 84 μM-leghaemoglobin and (*d*) 97 μM-myoglobin, with (○) or without (●) 1·5 mM-*N*-phenylimidazole. Free O_2 concentration (*a*, *b*) was measured by electrode during measurement of nitrogenase activity.

limited greatly or is undetectable in the absence of the carrier. These results agree well with those in Fig. 11 (*b*, *d*) of Bergersen & Turner (1975). Fig. 9 shows the dependence of bacteroid respiration rate on leghaemoglobin concentration at 0·1 μM free O_2, when the carrier is 73 % oxygenated in the bulk solution, and at 0·04 μM-O_2, when it is 50 % oxygenated.

DISCUSSION

In the bacteroid experimental system with no gas phase, the presence of LbO_2 or MbO_2 greatly increased bacteroid nitrogenase activity when the free dissolved O_2 concentration in the system fell to levels which permitted maximum rates of deoxygenation of the haemoproteins (Bergersen & Turner, 1975). Initial low rates of activity, early in the time course of depletion of O_2, were shown to be a function of the higher O_2 concentrations prevailing at the time, rather than time-dependent lags. In the absence of O_2-carrying haemoproteins, when changes in dissolved O_2 concentration are rapid, reaction rates may lag behind changes in O_2 concentration. Effects of rates of change of O_2 concentration are minimized by using dilute bacteroid suspensions. However, it was necessary to use at least 2 mg dry wt of bacteroids per assay in order to obtain reliable ATP measurements, and it was therefore not

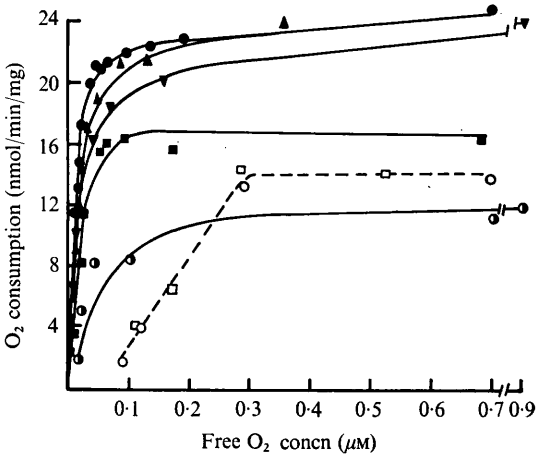

Fig. 8

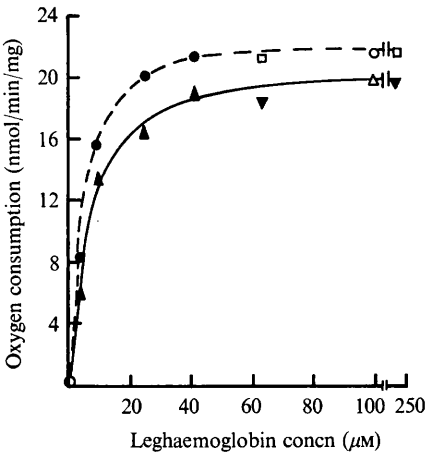

Fig. 9

Fig. 8. Relief of O_2 restriction of bacteroid respiration at low free O_2 concentration, by leghaemoglobin. Results are from two experiments without leghaemoglobin (broken line) and from several experiments with leghaemoglobin at various concentrations (●, 100 μM; ▲, 41 μM; ▼, 25 μM; ■, 9 μM; ◑, 4 μM).

Fig. 9. Relationship between leghaemoglobin concentration and bacteroid respiration at 0·04 μM free O_2 (▲—▲, ▼, △) and 0·1 μM free O_2 (●---●, □, ○). Results are from three separate experiments (▲ and ●; ▼ and □; △ and ○).

practicable to obtain a good set of relationships between O_2 and ATP concentrations in the absence of O_2-carrying proteins. The one value given (Fig. 6) was obtained after 1 min reaction. By 1·5 min, O_2-consumption rates had declined, with subsequent falls in ATP concentration in the bacteroids (Fig. 5).

Previous experiments (Bergersen & Turner, 1975, fig 11 *c*, *e*) suggested that an optimum O_2 concentration range favoured nitrogenase activity by bacteroids.

Bacteroid terminal oxidase systems of different affinity. Appleby *et al.* (1975 *a*, *b*) established that the effect of Lb in shaken assays of bacteroid respiration and nitrogenase activity, with a gas phase present, was related to the efficient production of ATP through an oxidative phosphorylation pathway, for the support of nitrogenase activity. An *N*-phenylimidazole-sensitive component, probably cytochrome P-450, was involved in this pathway, possibly as an intracellular O_2 carrier. Evidence for the involvement of CO-sensitive respiration in soybean bacteroids has previously been inconsistent (reviewed by Bergersen, 1971). Fig. 4 shows that this was the result of uncontrolled O_2 supply.

The results presented in Figs. 1 to 4 support the concept that there are at least two different terminal oxidase systems in bacteroids from soybean nodules (Appleby, 1969; Appleby *et al.* 1975*b*; Wittenberg *et al.* 1974). These results, plus those in Fig. 6, amplify the conclusions of Appleby *et al.* (1975*b*) and show that a high-affinity oxidase system operates with maximum effect near 0·1 μM free O_2, has little activity above about 1 to 3 μM-O_2, is sensitive to inhibition by *N*-phenylimidazole and CO, and produces high levels of ATP in the bacteroids. The low-affinity system has little activity below about 1 μM free O_2, is insensitive to the inhibitors, and is much less efficient in producing ATP in the bacteroids. Bacteroids *in vivo* or *in vitro*, suspended in a solution of partially oxygenated leghaemoglobin, are thus in an environment in which the free O_2 concentration is poised in the range for optimum ATP production and N_2-fixation. The decline in the activity of the high-affinity

oxidase pathway at free O_2 concentrations above about 1 μM (Figs. 1, 4 and 6) can be used to explain anomalies in previous experiments.

The apparent optimum O_2 concentration for nitrogenase activity near 0·1 μM free O_2 (Bergersen & Turner 1975, Fig. 11 *c*, *e*), is identical with that for ATP production (Fig. 6) and may not be directly related to the sensitivity of nitrogenase to O_2. Inconsistencies, noted in the previous paper, between the effects of LbO_2 and MbO_2 in the range 2 to 4 μM free O_2 are now considered to have been due to a lag in bacteroid response to this range of O_2 concentration, which occurred after a period of rapid change in the presence of LbO_2; with MbO_2, this range occurs during slow controlled decline in free O_2 concentration (see Fig. 5).

Studies of intact soybean nodules have shown inflected curves relating P_{O_2} to respiration rate (Bergersen, 1962; Tjepkema & Yocum, 1973). These were attributed to successive saturation of cortical and bacteroid respiration with O_2. If bacteroid respiration dominates nodule O_2 consumption it seems possible that the inflected curves of nodule respiration may be due to the two bacteroid oxidases. The observed decline in N_2 fixation which accompanied the increased respiration at P_{O_2} above the inflexion may therefore have been due to decreased ATP supply at the higher O_2 concentration rather than to inhibition by excess O_2 (Bergersen, 1962).

Postgate (1974) reviewed respiratory protection of nitrogenase in *Azotobacter chroococcum* and *A. vinelandii*, where ATP production is maximal at low P_{O_2}. Inefficient ATP-producing pathways dominate respiration at high P_{O_2} thus consuming excess O_2 and minimizing O_2 damage to nitrogenase, which appears to be more sensitive to O_2 when ATP levels are high. Our results with soybean bacteroids suggest that a similar mechanism operates in these symbiotic bacteria.

Bacteroid respiration at low O_2 concentration and the effects of LbO_2. Fig. 8 shows evidence of diffusion-limited flux of O_2 in the range 0·1 to 0·3 μM-O_2 when no O_2-carrying haemoproteins are present in the reactions. The sharp change of slope at concentrations close to 0·3 μM-O_2 indicates that the bacteroid high-affinity oxidase is already saturated with respect to O_2 when diffusion becomes non-limiting. When LbO_2 was present, bacteroid respiration was sustained to a much lower range of free O_2 concentrations. This effect of LbO_2 was reported previously (Bergersen & Turner, 1975, Fig. 11 *b*, *d*) but the diffusion-limited flux at low O_2 concentrations was not discerned in the earlier experiments. It is in this low range of O_2 concentration that ATP concentration in the bacteroids and nitrogenase activity are greatest (Fig. 6; see also Bergersen & Turner, 1975, Fig. 11 *c*). The simplest explanation of the increased respiration at low free O_2 concentration is that leghaemoglobin facilitates the diffusive flux of O_2, as previously concluded from the results of shaken assays with a gas phase (Wittenberg *et al.* 1974). The effect of increasing leghaemoglobin concentration (Fig. 9) is less than in model experiments of facilitated diffusion (for review see Wittenberg, 1970). The respiratory capacity of the bacteroids may limit the response to increased leghaemoglobin concentration.

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