Biochimica et Biophysica Acta, 292 (1973) 271–282

BBA 46466

STUDIES OF THE PHYSIOLOGICAL ROLE OF LEGHAEMOGLOBIN IN SOYBEAN ROOT NODULES

F. J. BERGERSEN, G. L. TURNER and C. A. APPLEBY

Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, Canberra (Australia)

(Received August 28th, 1972)

SUMMARY

Evolution of H_2 by nitrogenase in intact soybean nodules was consistently inhibited by CO when the nodules were equilibrated with argon–CO mixtures for 1 h prior to adding O_2 to initiate the reaction. Evolution of H_2 by nitrogenase in bacteroid suspensions prepared from nodules, was not inhibited by CO.

Dense, slowly shaken suspensions of bacteroids, with 12% O_2 in the gas phase maintained slow rates of H_2 evolution and acetylene reduction for up to 12 h. Addition of leghaemoglobin to these assays greatly enhanced the nitrogenase-mediated reactions. CO prevented stimulation by leghaemoglobin of H_2 evolution by bacteroids. Stimulation of acetylene reduction by bacteroid suspensions was dependent upon leghaemoglobin concentration up to about 1 mM. Increased shaking rates gave greater rates of acetylene reduction and O_2 uptake. Stimulation of nitrogenase activity in bacteroid suspensions by leghaemoglobin was much greater than stimulation of O_2 uptake. Increasing acetylene reduction in response to increasing agitation was accompanied by increasing oxygenation of the leghaemoglobin.

The significance of these results in relation to the physiological role of leghaemoglobin in symbiotic N_2 fixation by legume root nodules is discussed.

INTRODUCTION

The presence of the myoglobin-like haemoprotein leghaemoglobin in the N_2-fixing tissues of legume root nodules is well known, and a number of physiological roles have been proposed to explain the observed correlations between leghaemoglobin concentration and nitrogen fixation (reviewed by Bergersen[1]). Leghaemoglobin does not have a direct role in the N_2-fixing system[2]. The role which seems most likely at present was proposed by Yocum[3], who suggested that leghaemoglobin may act by facilitating the diffusion of O_2 into nodule tissue under the low pO_2 existing in this dense tissue. The production of ATP for N_2 fixation by root nodules appears to be limiting under atmospheric O_2 concentrations (*e.g.* ref. 4). This proposed role for leghaemoglobin would be similar to the facilitation, by myoglobin, of O_2 diffusion into mammalian muscle fibres (reviewed by Wittenberg[5]). The physicochemical properties of leghaemoglobin and its state in the nodule are compatible with such a role[6–10] but there is little experimental evidence to support it. Several

authors[11–14] have shown that haemoglobins enhanced O_2 uptake by cultured *Rhizobium* spp., by suspensions of nodule bacteroids and by other species of bacteria at low pO_2. Burris and Wilson[15], although confirming these results considered that they may have been due to a non-specific effect similar to that obtained with amino acids and other nitrogen compounds. More recently Tjepkema and Yocum[16] and Tjepkema[17] have produced supporting evidence for facilitated diffusion from optical and respirometrical studies of soybean nodule slices.

The N_2-fixing enzyme complex (nitrogenase) of nodules is located wholly within the bacteroids[2,18]. It catalyzes the reduction of N_2 to NH_3, acetylene to ethylene and other ATP- and reductant-dependent reactions[1]: in the absence of reducible substrates, H^+ is reduced to H_2 by nitrogenase [19,20]. In this paper, we present results of experiments with intact soybean nodules, in which advantage was taken of the insensitivity to CO of the ATP- and reductant-dependent evolution of H_2 by nitrogenase when reducible substrates (N_2, acetylene, *etc.*) are absent. Leghaemoglobin has a high affinity for CO which blocks any O_2 binding function[9,10,21]. In other experiments, leghaemoglobin is shown to stimulate nitrogenase activities of soybean nodule bacteroid suspensions, as well as stimulating their O_2 uptake. In these experiments, the densities of bacteroid suspensions and the concentrations of leghaemoglobin which were used were selected to be similar to those within the nodule cells.

MATERIALS AND METHODS

Nodules

Nodules were produced on roots of soybeans (*Glycine max* Merr. cv. Lincoln) by inoculation with *Rhizobium japonicum* strain CC705 (syn. Wisconsin 505) or CB1809, as previously described[22]. They were harvested 28–35 days after their appearance on the roots.

Bacteroid suspensions

These were prepared anaerobically in 25 mM phosphate buffer (pH 7.4) containing 2 mM $MgSO_4$ and 0.3 M sucrose[2] using a homogenizer in an anaerobic glove box[22] or a sealed homogenizer and filter system[23]. Bacteroids were washed twice by centrifuging and resuspending the pellet in fresh medium and they were finally suspended in the same medium in the ratio of 1:1 packed cells/liquid. Dry weights of bacteroids per ml of suspension were determined.

Leghaemoglobin

This was prepared from centrifuged soybean nodule homogenates by $(NH_4)_2SO_4$ precipitation at pH 6.8 followed by dialysis and concentration in a Diaflo pressure filtration cell over a UM10 membrane (Amicon Corp. Lexington, Mass.) as previously described[24]. Aliquots of these largely ferric leghaemoglobin preparations were stored at -10 °C and immediately before use, a 6.0-ml quantity was passed through a column (2.8 cm × 35 cm) of Sephadex G-10 (Pharmacia, Uppsala), the top of which had been banded with a 10-fold excess of $Na_2S_2O_4$ in 6.0 ml of the column buffer (25 mM Tris–HCl, pH 8.0). The ferrous leghaemoglobin formed during passage through the $Na_2S_2O_4$ band became oxygenated as it passed down the column and the eluted oxyleghaemoglobin was concentrated to the desired volume

in the Diaflo cell. These preparations were at least 95% leghaemoglobin in terms of haematin and protein[25] content.

Gas mixtures

Gas mixtures were prepared from commercial gases containing less than 0.01% impurities and the composition was checked by mass spectrometer analysis. Mixtures containing O_2 and argon were stored over water but those containing acetylene were stored in a specially constructed mechanical reservoir because of the differential solubility of the component gases. CO, when used was added to assays by injection from a hypodermic syringe.

Reactions

The reaction temperature was 25 °C. Most assays were carried out in calibrated Warburg vessels (capacity approx. 15 ml) with rubber capped sidearms, attached to manifolds with facilities for collecting samples of gas from each vessel. Some assays were made in small vials (16 ml capacity) closed with serum stoppers. Intact nodule experiments utilized nodules harvested directly without washing. After the assays, they were washed, blotted and weighed. Aliquots of nodules (usually about 1 g fresh wt) were placed in the reaction vessels without added liquid and the gas volumes in the vessels were calculated from previously determined nodule density measurements and the vessel volume. Intact nodules were not shaken. Bacteroid suspensions were shaken at 50 cycles/min (just sufficient to prevent settling of the suspension) or 150 cycles/min, using reaction volumes of 2.1 ml. The reaction medium contained sodium succinate[2] in 25 mM potassium phosphate (pH 7.4), containing 2 mM Mg^{2+} and 0.3 M sucrose. Assays with leghaemoglobin contained mixed buffer because of the use of 0.5 ml per assay of the haemoprotein in 25 mM Tris–HCl (pH 8.0). The use of these mixed buffers was without effect in the assays. Nitrogenase activity was measured by O_2-dependent H_2 evolution[19,26,27], and by the acetylene-reduction method[28,29].

Analytical

H_2 evolution and O_2 uptake were measured by mass spectrometrical analysis of samples removed from assays at intervals, using an Atlas M86 mass spectrometer, calibrated for the various gases. Acetylene and ethylene were analyzed by gas chromatography as previously described[30]. Spectrophotometric measurements utilized an Optica CF4R for routine recording of the quality of leghaemoglobin preparations, a Shimadzu Model Double-40 for haematin estimations and spectra of reaction mixtures were recorded using a Cary Model 14 spectrophotometer equipped with scattered transmission accessory and high intensity light source. Leghaemoglobin concentrations were determined from pyridine haemochromogen assays, using alkaline pyridine reagent (4.2 M pyridine in 0.2 M NaOH), to which an equal volume of diluted leghaemoglobin was added. The mixture was divided between two cuvettes, one being reduced with a few crystals of $Na_2S_2O_4$ and the other oxidized with potassium ferricyanide. $\Delta A_{556\ nm}$ *minus* $\Delta A_{539\ nm}$ were recorded: ε_{mM} was 23.4.

RESULTS

H_2 evolution

Net H_2 evolution by intact nodules was greater with strain CC705 than with CB1809 although acetylene-reduction rates were similar for both strains. This difference was even more marked with bacteroid suspensions. Therefore CC705 was used for H_2–evolution studies.

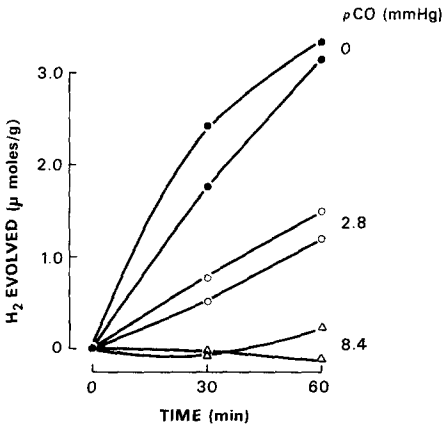

Fig. 1. Inhibition by CO of H_2 evolution by detached soybean nodules. Assays contained approx. 2 g (fresh wt) of nodules aged 30 days. The vessels were of 70 ml capacity and the gas phase was 20% O_2 in argon; 1 atm = 700 mmHg. The nodules were equilibrated with argon–CO mixtures at the indicated CO pressures and O_2–CO mixtures were injected to initiate the reaction. Results for duplicate reactions. The apparently negative values with 8.4 mmHg CO pressure are due to analytical errors at very low H_2 concentration.

H_2 evolution by intact nodules was consistently inhibited by CO (Fig. 1, Table I). Generally, the inhibition was greater at high pO_2 than at 0.2 atm O_2 (*cf.* refs 17 and 26). However, this pO_2 effect was almost eliminated by equilibration of the nodules with argon: CO mixtures for 1 h prior to initiating H_2 evolution by the addition of O_2 to the vessels. When this was done, CO-inhibited rates were low, at all pO_2 values. H_2 evolution by nitrogenase in bacteroid suspensions was not inhibited by CO (Table I), in agreement with previously reported work[19,31]. No change in O_2 uptake was detected when intact nodules were inhibited by CO. The anaerobic period during which CO equilibration took place did not affect subsequent H_2 evolution by control nodules when compared with nodules assayed immediately.

When dense bacteroid suspensions were gently shaken, H_2 evolution proceeded at a slow rate for several hours. The addition of leghaemoglobin (0.2 mM) to these reactions resulted in a 3-fold increase in H_2 evolution. This stimulation was reversed in the presence of CO, a partial pressure of 25 mmHg giving complete inhibition of the leghaemoglobin effect (Table II).

Acetylene reduction by bacteroid suspensions

Acetylene reduction by nitrogenase in dense suspensions of bacteroids (approx. 70–100 mg/ml), shaken gently at 50 cycles/min under atmospheres containing O_2

TABLE I

EFFECTS OF O_2 AND CO UPON H_2 EVOLUTION BY INTACT NODULES AND BY BACTEROID SUSPENSIONS

(a) Intact nodules (1 g fresh wt) were equilibrated with argon or argon – CO mixtures for 1 h before adding O_2 to initiate the reaction. (b) Assays contained 49 mg bacteroids and 100 μmoles succinate in 2.0 ml of 25 mM phosphate (pH 7.4). The gas phase was argon *plus* the indicated O_2 and CO concentrations. The shaking rate was 150 cycles/min and the fluid depth in the vessels was 2.5 mm.

	pO_2 (mmHg)	*pCO (mmHg)*	*H_2 (nmoles/h)*
(a) Intact nodules	70	0	1557
	70	14	433
	140	0	2050
	140	14	194
	210	0	2674
	210	14	137
(b) Bacteroid suspension	27	0	840
	27	14.2	710
	62	0	2100
	62	14.2	1920
	111	0	1190
	111	14.2	1160

TABLE II

STIMULATION OF BACTEROID H_2 EVOLUTION BY LEGHAEMOGLOBIN UNDER NEAR-STAGNANT CONDITIONS AND INHIBITION OF THE EFFECT BY CO

Assays contained 100 μmoles succinate in 2.1 ml of 25 mM phosphate buffer containing 0.3 M sucrose and 2 mM Mg^{2+}. Reaction vessels (15 ml) were shaken at 40–50 cycles/min and the liquid depth was 2.5 mm.

Expt	*Bacteroids (mg/assay)*	*Assay time (h)*	*pO_2 (mmHg)*	*pCO (mmHg)*	*Leghaemoglobin (mM)*	*H_2 (nmoles/assay)*
1	183	5.8	84	0	0	209
				0	0.21	540
				1.0	0.21	359
2	179	6.2	84	0	0	58
				0	0.18	219
				1	0.18	134
				5	0.18	81
				25	0.18	58

(85 mmHg), was maintained for up to 12 h at slow rates (approx. 0.05–0.1 nmole acetylene·h^{-1}·mg^{-1}). Addition of leghaemoglobin to these assays gave large increases in acetylene-reduction rates (Fig. 2). In this experiment (Fig. 2) total soluble

protein was maintained at 0.36 mM in all assays by addition of bovine serum albumin (mol. wt 68000) in order to avoid possible non-specific effects of the presence of quite high concentrations of soluble protein in the leghaemoglobin treatments. Later experiments showed that this precaution was not necessary.

Stimulation of acetylene reduction in these experiments was dependent upon leghaemoglobin concentration up to about 1 mM and O_2 uptake was also stimulated,

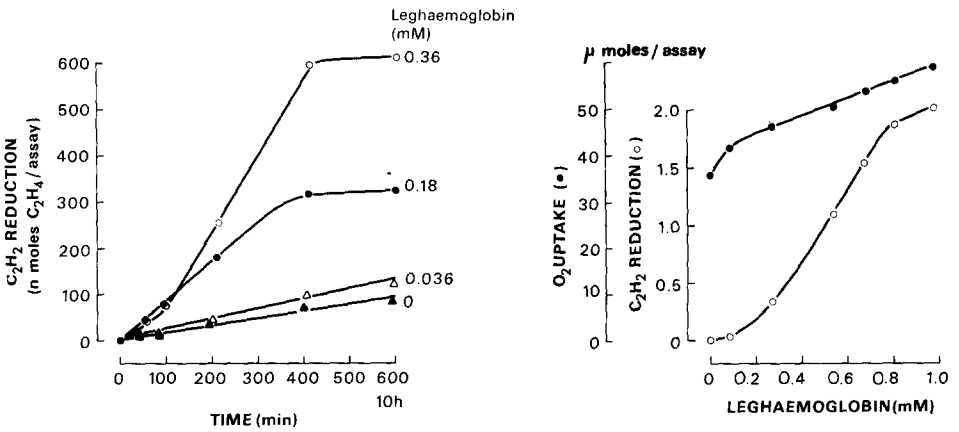

Fig. 2. Stimulation by leghaemoglobin of acetylene reduction by bacteroid suspensions in near-stagnant conditions. Assays contained 216 mg bacteroids, 100 μmoles sodium succinate and the indicated leghaemoglobin concentrations in 2.1 ml 25 mM buffer (pH 7.4). The shaking rate was 50 cycles/min and the vessels were 16-ml vials and the gas phase contained O_2 (84 mmHg) and acetylene (70 mmHg) in argon. The soluble protein content was maintained constant at 0.36 mM with bovine serum albumin. This was omitted as unnecessary in later experiments.

Fig. 3. The effect of leghaemoglobin concentration upon acetylene reduction and O_2 uptake by bacteroid suspensions in near-stagnant conditions. Assays contained 139 mg bacteroids, 100 μmoles sodium succinate and the indicated leghaemoglobin concentrations in 2.1 ml of 25 mM buffer (pH 7.4). The shaking rate was 50 cycles/min and the assays proceeded for 5.5 h. The gas phase contained O_2 (84 mmHg) and acetylene (140 mmHg) in argon.

TABLE III

EFFECT OF LIQUID DEPTH UPON LEGHAEMOGLOBIN-STIMULATED ACETYLENE REDUCTION BY A BACTEROID SUSPENSION, UNDER NEAR-STAGNANT CONDITIONS

Assays (3.5 h) contained 50 μmoles/ml succinate in 25 mM phosphate buffer containing 0.3 M sucrose, 2 mM Mg^{2+} and 0.28 mM leghaemoglobin. The gas phase contained acetylene (70 mmHg), O_2 (84 mmHg) in argon. Rubber-capped glass vials were used as reaction vessels.

Liquid		*Bacteroids* (*mg/assay*)	*Acetylene reduction*	
Volume (*ml*)	*Depth* (*mm*)		*nmoles ethylene/assay*	*nmoles ethylene/mg*
2.1	7.1	109	850	7.8
1.1	3.5	55	919	16.7
0.6	1.7	27	430	15.9

but to a lesser extent (Fig. 3). The liquid depth over which the reaction in these near-stagnant assays was operating was investigated. At depths greater than 3.5 mm, acetylene reduction per mg of bacteroids was diminished (Table III). This result suggested that increasing the shaking rate would bring more of the assay volume into the reaction. Fig. 4 shows the result of one experiment. Shaking at 150 cycles/min increased acetylene reduction 40-fold and O_2 uptake 7-fold compared with shaking at 50 cycles/min (based on values after 1 h). At 50 cycles/min, equilibrium conditions were only slowly established, but 0.6 mM leghaemoglobin increased O_2 uptake rates

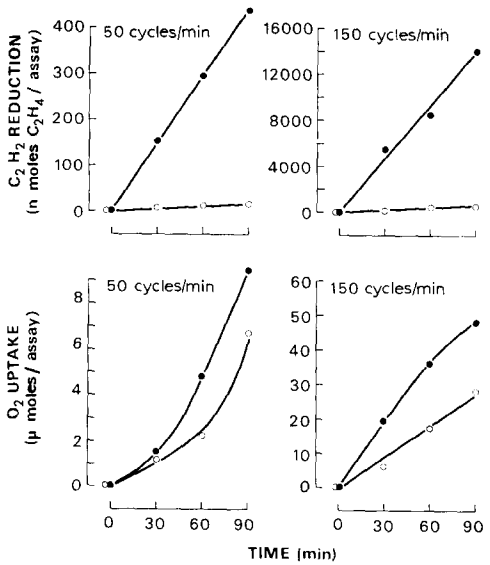

Fig. 4. Effects of shaking rate and leghaemoglobin upon acetylene reduction and O_2 uptake by soybean bacteroids. Assays contained 121 mg bacteroids, 200 μmoles sodium succinate and 0.5 mM leghaemoglobin in 2.1 ml of 25 mM buffer (pH 7.4). The gas phase contained O_2 (84 mmHg) and acetylene (140 mmHg) in argon. ●—●, leghaemoglobin treatments; ○—○, controls

TABLE IV

EFFECTS OF DENSITY OF BACTEROID SUSPENSION AND LEGHAEMOGLOBIN UPON ACETYLENE REDUCTION AND O_2 UPTAKE

Assays as in Fig. 4. The shaking rate was 150 cycles/min and the assay period 1 h.

Bacteroids (mg/assay)	*Leghaemoglobin (mM)*	*O_2 uptake (μmoles/assay)*	*Acetylene reduction*	
			nmoles ethylene/assay	*nmoles ethylene/mg*
24.7	0	19.4	125	5.1
	0.45	27.1	4953	200.4
123.6	0	20.0	336	2.7
	0.45	39.6	7215	58.4

by a factor of about 2 and acetylene-reduction rates by a factor of 27. At 150 cycles/min, 0.6 mM leghaemoglobin increased O_2-uptake and acetylene-reduction rates by factors of 2 and 21, respectively. The O_2-solution rates in these assays, measured by the sulphite oxidation method[32] were 30 and 84 μmoles/h, respectively, at 50 and 150 cycles/min. Even when more dilute bacteroid suspensions were used, with shaking at 150 cycles/min, O_2 uptake was not decreased and the assays were therefore still O_2 limited (19.4 μmoles O_2 uptake with 24.7 mg bacteroids per assay compared with 20.0 μmoles O_2 with 123.6 mg per assay) and leghaemoglobin produced a 40-fold increase in acetylene reduction (Table IV).

The CO sensitivity of the acetylene-reduction reaction precluded CO inhibition studies of leghaemoglobin-stimulated acetylene reduction by bacteroids

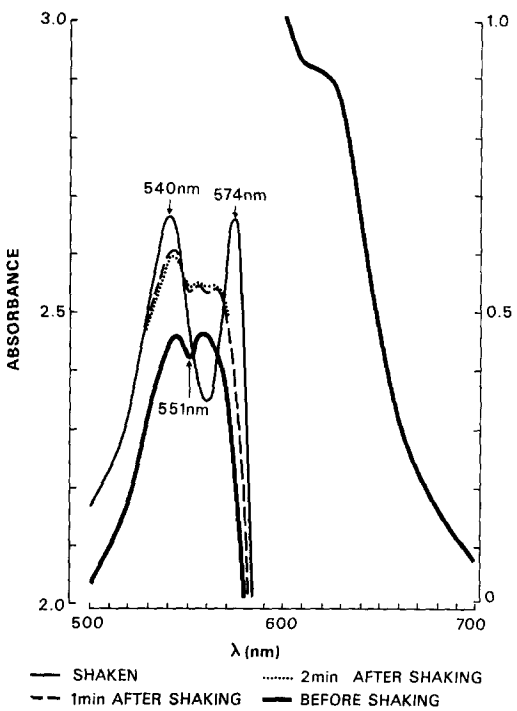

Fig. 5. Spectra of leghaemoglobin in bacteroid suspension assays. Both reference and sample cuvettes (1-cm-light path) contained 2.5 ml of reaction mixture consisting of 33 mg bacteroids, 25 mM phosphate buffer (pH 7.4) containing 2 mM Mg^{2+} and 0.3 M sucrose and sodium succinate (100 μmoles). The gas phase was O_2 (84 mmHg) and acetylene (140 mmHg) in argon. Leghaemoglobin (0.15 mM) was added to the sample cuvette. While spectra were being recorded, the reference assay reduced 15.9 nmoles acetylene and the sample assay reduced 50.7 nmoles acetylene. The thick continuous line indicates the spectrum of leghaemoglobin during stagnant conditions, the thin continuous line indicates the spectrum immediately after shaking. Oxyleghaemoglobin formation is indicated by the appearance of 540- and 574-nm absorption peaks. The dashed and dotted lines were recorded 1 and 2 min later. The top of the light beam passed 1 mm below the liquid surface. The 551-nm absorption trough appearing in stagnant conditions is almost certainly an optical artifact caused by less efficient recording of bacteroid cytochrome *c* absorption in the sample cuvette, where total absorbance is much greater than in the reference cuvette and stray light errors become appreciable.

(14 mmHg CO pressure, as used in Table I, completely inhibited acetylene reduction by bacteroid suspensions in the absence of leghaemoglobin).

Absorption spectra of leghaemoglobin-assisted bacteroid assays

Because of optical difficulties and the strong absorbance of leghaemoglobin only dilute bacteroid suspensions (33 mg per 2.5 ml assay) with 0.15 mM leghaemoglobin could be examined with the Cary spectrophotometer. Under static conditions, using rubber stoppered cuvettes and O_2 and acetylene in the gas phase, leghaemoglobin stimulation of acetylene reduction could be detected in the cuvettes. The leghaemoglobin under these conditions was not detectably oxygenated. However, upon shaking both cuvettes, which as the above results show, would have stimulated the reaction, some oxygenation of the leghaemoglobin was seen, followed by rapid deoxygenation (Fig. 5). It was not possible to record spectra of leghaemoglobin during shaken assays, but examination with the hand spectroscope showed the presence of oxyleghaemoglobin in increasing concentration as acetylene reduction increased in response to increased stirring rates (Table V).

TABLE V

RELATIONSHIP BETWEEN LEGHAEMOGLOBIN OXYGENATION AND ACETYLENE REDUCTION BY BACTEROIDS IN RESPONSE TO STIRRING RATE

Rates of acetylene reduction were determined in a rubber-capped 50-ml round-bottomed flask at different rates of stirring with a teflon-coated magnet. The flask was mounted over a variable speed magnetic stirrer, and the degree of oxygenation of the leghaemoglobin was estimated by spectroscopic examination with a Beck hand spectroscope mounted at the focus of a transmitted light beam. Each stirring rate proceeded for 15 min and gas samples were analysed for ethylene at the beginning and end of each period. The assays contained 108 mg bacteroids and 400 μmoles sodium succinate in 7.5 ml of buffer (25 mM potassium phosphate, pH 7.4, containing 0.3 M sucrose and 2 mM $MgSO_4$). The leghaemoglobin concentration was 0.1 mM. The gas phase contained O_2 (84 mmHg) and acetylene (140 mmHg) in argon.

Stirring rate (arbitrary units)	*Oxyleghaemoglobin (%)*	t_{eq}* *(s)*	*Acetylene reduction (nmoles ethylene · h^{-1} · mg^{-1})*
Nil	Nil	—	0.8
1	Trace	—	1.2
3	Trace	—	6.4
5	Approx. 10	25	19.1
5.5	Approx. 20	20	39.1
7	Approx. 70	20	215.4

* Approximate time taken to reach equilibrium leghaemoglobin oxygenation after each change in stirring rate.

DISCUSSION

N_2 fixation by the symbiotic system of legume root nodules requires O_2. Under atmospheric conditions, nodules of many legumes are O_2 limited and N_2 fixation is stimulated by increased pO_2 (*e.g.* ref. 4). However, the N_2-fixing system of nodule

bacteroids is inactivated by free O_2 (ref. 33) and O_2 is an inhibitor of N_2 fixation[4,34]. Therefore, there would be considerable advantage to the system if it possessed a mechanism enabling satisfaction of its O_2 requirement at a low concentration of free O_2. The data presented in this paper support such a role for nodule leghaemoglobin.

In intact nodules but not in washed bacteroid suspensions, the presence of a CO-sensitive pathway was demonstrated from measurements of H_2 evolution, and the low partial pressures of CO which were effective indicate the involvement of a system with high affinity for CO. Only leghaemoglobin is known to have this property, other possible host cell systems are much less sensitive to CO. If leghaemoglobin was involved in a mechanism assisting to meet the O_2 requirement of the nodule tissues, it was at first puzzling that CO did not inhibit O_2 uptake by nodules in our experiments (see also refs 13, 14 and 16). However, the later experiments which showed that nitrogenase activity was stimulated much more than O_2 uptake, by the presence of leghaemoglobin in bacteroid suspension assays, may partially explain this. Additionally, in the nodule tissue, some host respiratory systems in the periphery of bacteroid-containing cells or in uninfected interstitial tissue, may also be O_2 limited and these may compete with the bacteroids for a limited O_2 supply when CO blocks leghaemoglobin function. This would tend to keep net O_2 uptake constant in this diffusion limited system.

The apparently slow equilibration of nodule tissue with CO, shown by the need for a period of CO equilibration before commencing our experiments, is in agreement with the observations of Sprent[35] and is probably related to the small carboxyleghaemoglobin dissociation constant (calculated from the equilibrium constant and the CO leghaemoglobin association constant[10]).

The differences in net H_2 evolution observed between nodules produced by the two *R. japonicum* strains did not stem from differential nitrogenase activity, since acetylene-reduction rates were similar. It is possible that strain CB1809 bacteroids have an active hydrogenase, like that described by Dixon[36] in pea nodule bacteroids. Uptake of H_2 by such an enzyme may conserve reducing power which would otherwise be lost because of evolution of H_2 from the nitrogenase.

Leghaemoglobin consistently stimulated acetylene reduction by bacteroid suspensions in our experiments. However, Bergersen and Turner[2] failed to observe any stimulation of N_2 fixation when buffer extracts of nodules containing oxyleghaemoglobin were added to bacteroid suspensions. The presence of other soluble components and/or the relatively dilute leghaemoglobin present (<0.05 mM), may have been responsible for that result.

The concentration of leghaemoglobin in the bacteroid-containing cells of nodules such as those used in the above experiments, has been calculated to be of the order of 1 mM and bacteroids occupy about 30% (v/v) of the cell volume (Bergersen, F. J. and Goodchild, D. J., unpublished). We were not able to use bacteroid suspensions as dense as this but our experiments usually contained 15–20% (v/v) bacteroids/liquid. In these experiments, increments of leghaemoglobin up to about 1 mM final concentration, allowed increased acetylene-reduction rates (Fig. 3). Thus conditions used in the assays were not very different from physiological reality.

The observations that leghaemoglobin stimulated bacteroid H_2 evolution and acetylene reduction much more than O_2 uptake, suggests that there may be a specific

terminal oxidase in the bacteroid membrane which reacts with oxyleghaemoglobin. This pathway may produce ATP in a mechanism which is closely coupled to the nitrogenase. Terminal oxidases reacting directly with O_2 also support bacteroid nitrogenase activity, but the results suggest that the efficiency may be only 1/10 or 1/20 of the efficiency of the oxyleghaemoglobin pathway (Fig. 4, Table IV).

An alternative explanation that ADP/ATP ratios in the bacteroids may regulate the activity of the nitrogenase[37] should be considered also. It is possible that doubling the O_2 consumption for example, may double the ATP production rate and thus decrease the ADP/ATP ratio 4-fold. This could have an effect upon nitrogenase activity. In earlier work, it was found that bacteroid suspensions, unaided with leghaemoglobin, responded to doubling of pO_2 with 4-fold increases in initial N_2-fixation rates[38]. In the present paper, the differences between increases in O_2 consumption and nitrogenase activity produced in the presence of leghaemoglobin were much greater than this, suggesting that a more specific mechanism which involves leghaemoglobin may be operating.

We do not consider that the greater stimulation of acetylene reduction resulted from leghaemoglobin increasing the transfer or activation of acetylene. Firstly, there is no evidence from absorption spectra that leghaemoglobin binds acetylene (Appleby, C. A., unpublished). Secondly, acetylene is about 33 times more soluble than O_2 and the rates of O_2 uptake, even in O_2-limited bacteroid suspensions, were much greater than the acetylene-reduction rates. Thirdly, H_2 evolution was also stimulated by leghaemoglobin. Fourthly, spectroscopic observation suggested a correlation between the degree of oxygenation of the leghaemoglobin and rates of acetylene reduction in bacteroid assays. We therefore conclude that leghaemoglobin functions in intact nodules by means of its capacity for binding and discharging O_2 at low concentrations. The experiments described here do not discriminate between any of the possible mechanisms of leghaemoglobin action, such as facilitated O_2 diffusion to a specific oxidase or direct reaction between oxyleghaemoglobin and an oxidase on the bacteroid surface. Both or either of these functions and others may occur. However, a physiological role for leghaemoglobin has been strongly suggested from studies of CO inhibition of H_2 evolution from intact nodules and confirmed by the experiments with bacteroid suspensions.

ACKNOWLEDGEMENTS

The authors thank Mrs L. Grinvalds, Miss P. Riddiford and H. Tantala for technical help in these experiments.

REFERENCES

1 Bergersen, F. J. (1971) *Annu. Rev. Plant Physiol.* 22, 121–140
2 Bergersen, F. J. and Turner, G. L. (1967) *Biochim. Biophys. Acta* 141, 507–515.
3 Yocum, C. S. (1964) *Science* 146, 432
4 Bergersen, F. J. (1962) *J. Gen. Microbiol.* 29, 113–125
5 Wittenberg, J. B. (1970) *Physiol. Rev.* 50, 559–636
6 Appleby, C. A. (1962) *Biochim. Biophys. Acta* 60, 226–239
7 Appleby, C. A. (1969) *Biochim. Biophys. Acta* 188, 222–229
8 Bergersen, F. J. (1962) *Nature* 194, 1059–1061

9 Wittenberg, J. B., Appleby, C. A. and Wittenberg, B. A. (1972) *J. Biol. Chem.* 247, 527–531
10 Imamura, T., Riggs, A. and Gibson, Q. H. (1972) *J. Biol. Chem.* 247, 521–526
11 Kasugai, S., Kubo, H. and Tsujimura, K. (1943) *J. Agric. Chem. Soc. Jap.*, 19, 765–771
12 Little, N. H. and Burris, R. H. (1947) *J. Am. Chem. Soc.* 69, 838–841
13 Smith, J. D. (1949) *Biochem. J.* 44, 585–591
14 Smith, J. D. (1949) *Biochem. J.* 44, 591–598
15 Burris, R. H. and Wilson, P. W. (1952) *Biochem. J.* 51, 90–96
16 Tjepkema, J. D. Yocum, C. S. (1970) *Plant Physiol.* 46 (Suppl.), 44
17 Tjepkema, J. D. (1971), Ph. D. Thesis, University of Michigan, Ann Arbor, Mich.
18 Koch, B., Evans, H. J. and Russell, S. (1967) *Proc. Natl. Acad. Sci. U.S.* 58, 1343–1350
19 Hoch, G., Schneider, K. C. and Burris, R. H. (1960) *Biochim. Biophys. Acta* 37, 273–279
20 Burris, R. H. (1971) In *Chemistry and Biochemistry of Nitrogen Fixation* (Postgate, J. R., ed.) pp. 106–160, Plenum Press, London and New York
21 Keilin, D. and Wang, Y. L. (1945) *Nature* 155, 227–229
22 Bergersen, F. J. and Turner, G. L. (1970) *Biochim. Biophys. Acta* 214, 28–36
23 Bergersen, F. J. and Turner, G. L. (1972) *Biochem. J.* 131, in the press
24 Appleby, C. A. (1969) *Biochim. Biophys. Acta* 189, 267–279
25 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
26 Bergersen, F. J. (1963) *Aust. J. Biol. Sci.* 16, 669–680
27 Turner, G. L. and Bergersen, F. J. (1969) *Biochem. J.* 115, 529–535
28 Dilworth, M. J. (1966) *Biochim. Biophys. Acta* 127, 285–294
29 Hardy, R. W. F., Holsten, R. D., Jackson, E. K. and Burns, R. C. (1968) *Plant Physiol.* 43, 1185–1207
30 Bergersen, F. J. (1970) *Aust. J. Biol. Sci.* 23, 1015–1025
31 Bergersen, F. J. (1969) *Proc. R. Soc. London, Ser. B* 172, 401–416
32 Cooper, C. M., Fernstrom, G. A. and Miller, S. A. (1944) *Ind. Eng. Chem.* 36, 504–509
33 Bergersen, F. J. (1968) *9th Int. Congr. Soil Sci. Trans. Adelaide, 1968*, Vol. II, pp. 49–63, Angus and Robertson, Sydney, London, Melbourne
34 Wang, P. P. and Burris, R. H. (1972) *Proc. Natl. Acad. Sci. U.S.* 69, 672–675
35 Sprent, J. I. (1969) *Abstr. 11th Int. Bot. Congr.* 207
36 Dixon, R. O. D. (1967) *Ann. Bot.* [N.S.] 31, 179–188
37 Kennedy, I. R. (1970) *Biochim. Biophys. Acta* 222, 135–144
38 Bergersen, F. J. and Turner, G. L. (1968) *J. Gen. Microbiol.* 53, 205–220