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Effect of pO_2 on the Formation and Status of Leghemoglobin in Nodules of Cowpea and Soybean[1]

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ABSTRACT

Nodulated cowpea (*Vigna unguiculata* [L.] Walp. cv Vita 3: *Bradyrhizobium* strain CB756) and soybean (*Glycine max* [L.] Merr. cv White Eye: *Bradyrhizobium* strain CB1809) were grown with their root systems maintained in a flowing gas stream containing a range of pO_2 (1–80%, v/v) in N_2 for up to 28 days after planting. At the extremes of sub- and supra-ambient pO_2, the levels of leghemoglobin (Lb) in nodules were reduced. However, neither the proportional composition of Lb component proteins (eight in soybean, three in cowpea) nor their oxidation state was affected by pO_2. Short-term changes in pO_2 (transferring plants grown with sub- or supra-ambient pO_2 in the rhizosphere to air or vice versa) caused a significant decline in Lb content and, in cowpea but not soybean, where pO_2 was increased, a higher percentage of oxidation of Lb. Combining data on changes in Lb level of cowpea nodules grown in sub-ambient pO_2 with those for their structural adaptation to an under supply of O_2 indicated that, despite the nodules having a lower level of Lb, the amount per infected cell was increased by up to twofold and per bacteroid up to fivefold (in those from 1% O_2) compared to those grown in air. Progressive decline in pO_2 resulted in a progressive increase on this basis, indicating a close relationship between Lb content and the adaptation of nodule functioning to external O_2 level.

Lb[3] constitutes a buffering mechanism in legume root nodules serving to minimize the O_2 gradient through the infected tissue and to provide sufficient O_2 for bacteroid respiration, albeit at an extremely low free O_2 concentration (2). Although other factors, notably variable mechanical barriers against oxygen penetration (reviewed in ref. 7), are also concerned with the regulation of nodule O_2 supply, both the degree of oxygenation and perhaps the level of Lb might be expected to respond to long-term differences in pO_2. Each could alter the efficiency with which nodules function and contribute to the overall adaptation that results in N_2 fixation being maximized over a wide range of rhizosphere pO_2 (9).

There is considerable evidence that synthesis of the protoheme moiety of Lb occurs principally in the *Rhizobium* symbiont and is stimulated greatly by microaerobic conditions (16). Although this may not be the case for the Lb polypeptides, which are synthesized in the plant host cytosol (23), the rapid assembly of functional Lb in the cytosol of the infected cells appears to require conditions of increasing O_2 demand and consequent lowered O_2 levels (2).

The proportional composition of Lb component proteins has been found to vary with nodule development (14, 27) and in relation to other environmental factors (2, 4, 5). Although adaptation of nitrogenase activity to altered pO_2 (7, 9) is now believed to be due partly to operation of a variable diffusion barrier in nodules, Fuchsman *et al.* (13) suggested that changes in the proportions of Lb components might be involved.

Detopping, which has been shown to reduce nitrogenase activity (18) in legumes through increased diffusion resistance in nodules (7), was accompanied by decreased oxygenation of ferrous Lb (18). Although King *et al.* (17) found that the degree of oxygenation of Lb *in vivo* may change in response to pO_2 without alteration in nodule respiration, it seems possible that O_2 supply could also affect the redox status of Lb and, in this way, modify its function as a nodule O_2 carrier. Ferric Lb does not combine reversibly with O_2 (2). There is experimental evidence for the occurrence of Lb only in its reduced (ferrous) form in unstressed, functioning nodules (1, 22). However, the demonstration of the presence of a nodule cytosolic reductase, able to reduce ferric Lb to ferrous Lb (19, 24), coupled with a recent report of *in vitro* nonenzymatic reduction of Lb in soybeans (26), together suggest the operation of reactions which maintain Lb in its functional, ferrous state.

This investigation reports on two major questions relating to the effect of O_2 on the nodule's O_2 buffering system. First, do the proportions of Lb components change in relation to external gas phase pO_2; and second, does the total amount of Lb and its oxidation state in nodules reflect O_2 supply?

MATERIALS AND METHODS

Plant Material

Effectively nodulated cowpea (*Vigna unguiculata* L. Walp. cv Vita 3: *Bradyrhizobium* strain CB 756) and soybean (*Glycine max*. L. Merr. cv White Eye: *Bradyrhizobium* strain CB 1809) plants were grown in liquid culture free of combined N with their root systems maintained in a flowing gas stream containing a range of pO_2 (1–80%, v/v) in N_2 (9). Cowpea

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[3] Abbreviations: Lb, leghemoglobin, hence ferric Lb, the species with oxidized heme Fe that is unable to combine reversibly with O_2 or CO; (deoxy) ferrous Lb, the species with reduced heme Fe, able to combine reversibly with O_2 or CO to form, respectively, oxyferrous Lb (Lb O_2) or carboxyferrous Lb (Lb CO); DAP, days after planting.

plants were harvested 18, 24, and 28 DAP and soybean 20 and 28 DAP. The nodules were removed rapidly and stored immediately in liquid N_2. In some cases, the atmospheres surrounding roots were changed, at 25 DAP, from sub-ambient pO_2 (1–10% O_2, v/v, in N_2) to air, for 7 d before harvesting nodules. Similarly, plants were transferred after 20 or 21 d culture with their nodulated roots in air to rooting atmospheres containing a range of pO_2 for 3 to 6 d. In each case where the pO_2 of the rooting atmosphere was changed, controls were maintained under the original conditions of culture, and nodules were harvested at the same times as those subjected to altered pO_2.

Extraction of Leghemoglobin from Nodules

Frozen nodules (0.8–20 g) were ground to a fine powder in liquid N_2 and transferred under Ar to 2 to 10 volumes (per g fresh weight) of 50 mM KPO_4 (pH 7.4) buffer which contained 1 mM EDTA and had been equilibrated with CO. The tube containing this suspension was placed in a 50 L open-topped bucket filled and continually flushed with Ar by upward flow. The mixture was stirred until it thawed into an homogenate (final temperature 2°C), transferred under Ar to centrifuge tubes, and capped under CO. Following centrifugation at 4°C and 100,000*g* for 60 min, the Lb-containing supernatant was collected and stored under Ar. This procedure yielded a clear purple-red solution from cowpea nodules and a clear red solution from soybean nodules.

Estimation of Nodule Leghemoglobin and its Oxidation State

A portion of each nodule extract made under CO (see above) was transferred under Ar to a CO-flushed spectrophotometer cuvette, which was then stoppered under CO. Under these extraction and transfer conditions, it may be assumed that all reduced Lb (*i.e.* Lb O_2 plus deoxyferrous Lb) in the nodule was converted to and maintained as Lb CO in the extract. Any oxidized Lb (*i.e.* ferric Lb) in the nodule would not have been converted to Lb CO. An estimate of Lb CO content (and hence of total Lb O_2 plus deoxyferrous Lb originally present in the nodule) was made by recording the optical spectrum of each extract between 350 and 480 nm, measuring the absorbance difference between the Soret peak (near 417 nm), drawing a line between the inflections on either side of this peak (*cf.* Fig. 1, ref. 12), and assuming a micromolar extinction coefficient of 0.177 for cowpea Lb CO (CA Appleby, unpublished observations) and of 0.180 for soybean Lb CO (2). Ferric Lb in the extract was then converted to Lb CO by addition of a small amount of sodium dithionite to each cuvette followed by reequilibration with CO. The increased absorbance near 417 nm, determined as above, was used as a measure of ferric Lb now converted to Lb CO. Percentage of oxidation of Lb in the extract (and presumably in the nodule) was calculated as ferric Lb/total (ferrous + ferric Lb) × 100.

Gel Filtration Chromatography

Extracts, maintained under CO in anaerobic conditions, were chromatographed on 30 cm columns (final gel-bed dimensions, 20.5 × 1.6 cm) of Sephacryl S-200 HR (Pharmacia, Uppsala Sweden) equilibrated at 1 to 2°C with CO-saturated 50 mM KPO_4 (pH 7.4) buffer containing 1 mM EDTA. The column was eluted with this buffer at 1.2 $mL \cdot min^{-1}$ and the eluate monitored for absorbance at 276 and 408 nm. Such chromatography resolved Lb in both cowpea and soybean extracts as a single band separated from higher and lower mol wt, colored impurities, with a recovery better than 90% in each case.

Ion Exchange Chromatography

Lb-containing fractions from gel filtration were concentrated in Amicon 50 cells over Amicon YM10 membranes, with air instead of N_2 as pressurizing gas to promote conversion of LbCO to LbO_2. The membrane was washed twice (2 × 5 mL) with air-saturated 0.1 M sodium acetate (pH 5.8) solution, and the highly concentrated final Lb solutions were diluted to 1.3 to 1.6 mL with the same buffer and assayed spectrophotometrically to confirm complete conversion from Lb CO to LbO_2 before oxidation to ferric Lb (with approximately 10 μL 0.1 M potassium ferricyanide to 1 mL LbO_2 solution). Complete oxidation of ferrous Lb to ferric Lb was confirmed by comparison with standard spectra for pure Lb. Excess ferricyanide was removed by passage through small Sephadex G-25 columns equilibrated with 10 mM sodium acetate (pH 5.8), and 2-mL samples were concentrated to around 0.05 mL with Amicon Centricon-10 microconcentrator tubes. Spectral analysis of the ferric Lb acetate in these samples showed a Soret:UV ratio of 5.05 (a value close to that for pure soybean Lb; 2), and a recovery from LbO_2 of about 92%.

Cowpea ferric Lb acetate was loaded onto a DEAE-Sepharose (Pharmacia, Sweden) column (9.1 × 1.6 cm) equilibrated with 10 mM sodium acetate (pH 5.8). The column was eluted first with this buffer at 1.5 $mL \cdot min^{-1}$ and then with 0.1 M sodium acetate (pH 5.8) and the eluate monitored at 276 nm (protein) and 408 nm (ferric Lb acetate, Soret absorbance).

Analytical Isoelectric Focusing

After desalting and addition of nicotinic acid, samples of ferric Lb acetate, prepared as above from cowpea or soybean nodules or of Lb components recovered from ion exchange chromatography (8–10 nmol Lb per track for cowpea or 10 nmol per track for soybean), were separated as their ferric Lb nicotinate complexes in polyacrylamide gels containing 2% ampholytes (pH 4.5–5.4; Pharmalyte, Pharmacia, Uppsala Sweden) as described by Fuchsman and Appleby (14). A sample of the mixed components of ferric Lb nicotinate from soybean cv Lincoln (14) was applied to gels for comparison. Gels were fixed in 35% (w/v) methanol containing 10% (w/v) TCA and 3.5% (w/v) sulfosalicylic acid. The separated bands of Lb were quantified by scanning the fixed gels at 400 to 415 nm and integrating the absorbance peaks.

RESULTS

Isolation and Separation of Lb Components

The Lb in crude nodule extracts (stabilized as LbCO) was initially separated from impurities, likely to cause Lb degra-

dation, by gel filtration under anaerobic conditions. The peak fractions showed a relatively constant Soret/UV absorbance ratio between 3.52 and 4.46, with recovery of more than 90% of the Lb in the initial extract from the conversion to ferric Lb acetate. Ion exchange chromatography of this partially purified Lb on DEAE sepharose resolved three distinct components of ferric Lb acetate (Fig. 1), designated LbI, LbII, and LbIII according to their order of elution from the column. The first component, LbI, was followed by the major component, LbII; the minor component, LbIII, was then eluted with 100 mM sodium acetate (pH 5.8). Absorption spectra of the colored fractions were recorded from 240 to 500 nm, and the Soret elution profile and Soret to UV ratio were plotted (Fig. 1). On the basis of Lb concentration and relative purity (as measured by Soret/UV ratio), fractions 12 to 20 (Fig. 1) were pooled as LbI, fractions 31 to 50 as LbII, and fractions 70 to 85 as LbIII. These were concentrated by pressure filtration (Amicon YM10 membrane) to yield 1.0 mL LbI, 2.4 mL LbII, and 0.4 mL LbIII. Spectral analysis of the pooled fractions, representing each of the three Lb components, indicated (for this preparation from nodules grown in air) 177, 602, and 33 nmol for LbI, II, and III or 21.8, 74.2, and 4.0%, respectively. Separation of Lb components by isoelectric focusing of the same extract (Fig. 2) indicated that the three components were in the proportions of 20.9, 77.1, and 2.0% for LbI, II, and III respectively. Consequently, analytical isoelectric focusing was used routinely to separate and assay components of Lb in extracts from 1 g or less of nodules.

Extracts from soybean cv White Eye nodules prepared in similar fashion to those from cowpea yielded eight ferric Lb components following isoelectric focusing; the four major bands (Lb*a*, c_1, c_2, and c_3) and four minor components (Lb*b*, d_1, d_2, and d_3) have been named as for Lb from soybean cv Lincoln by Fuchsman and Appleby (14) (see Fig. 2).

Effect of Long-Term Growth of Nodules in Different pO_2 on Lb Content, Oxidation State, and Proportions of Components

Levels of O_2 from 5 to 60% around nodulated roots during development had relatively little effect on the Lb concentration in nodules of both cowpea and soybean (Table I). However, extremes below 5% and above 60% did have an effect. Cowpea was more sensitive than soybean at low pO_2, with Lb concentration at 1% O_2 being 32% of that at 20% O_2; the corresponding value for soybean was 76%. In both species, the Lb concentration at 80% O_2 was 50% of the controls. Although there was some variation in the proportions of Lb extracted as ferric Lb, generally there was no clear trend with respect to rhizosphere pO_2; the average proportion of Lb in the ferric form being 6.2 ± 0.7% for cowpea at 24 to 28 d and 5.4 ± 0.4% for soybean from 20 to 28 d, respectively (Table I). We consider these amounts to be little above the accidental oxidation level that might occur during extraction.

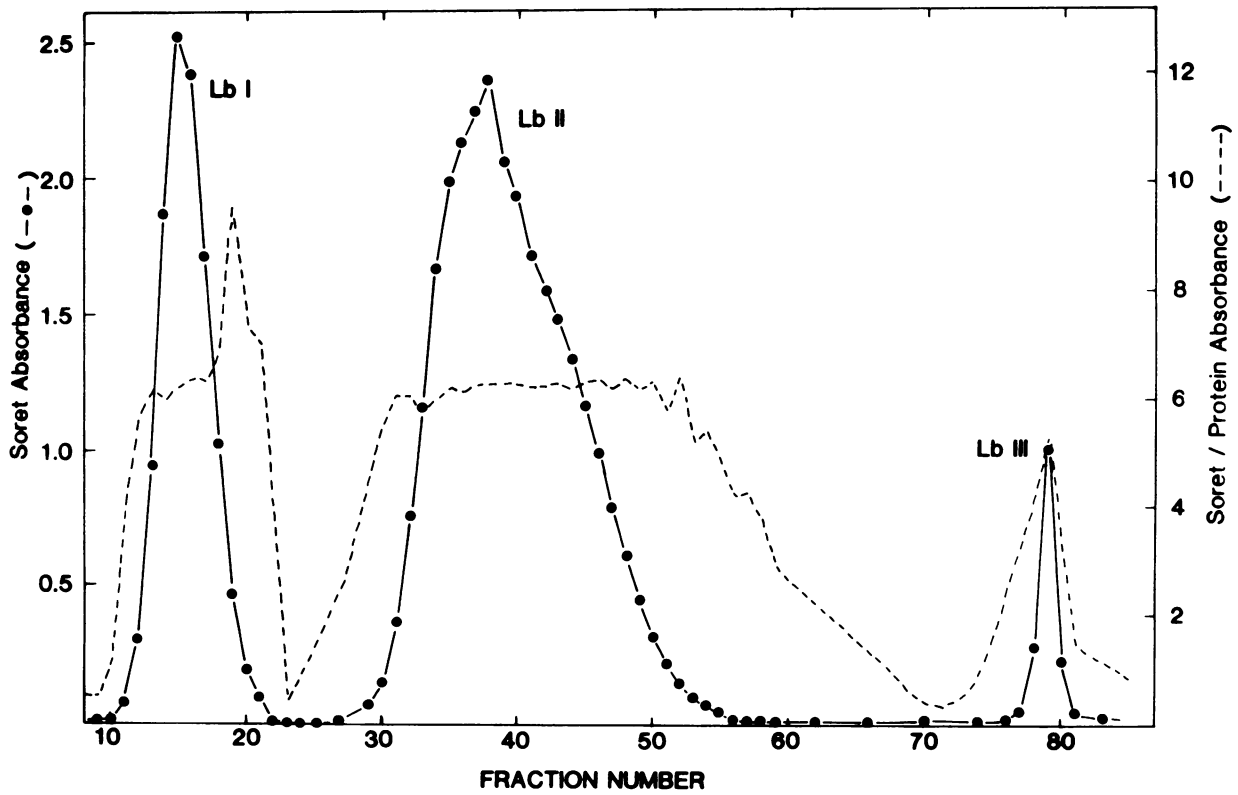

Figure 1. Separation of CO-stabilized components of ferric Lb in extracts from cowpea nodules by chromatography on DEAE-Sepharose. Plants were cultured with their nodulated roots exposed to air for 28 d. Soret peak absorbance, measured at 408 nm, is a measure of Lb CO concentration. Protein was measured by absorbance at 276 nm.

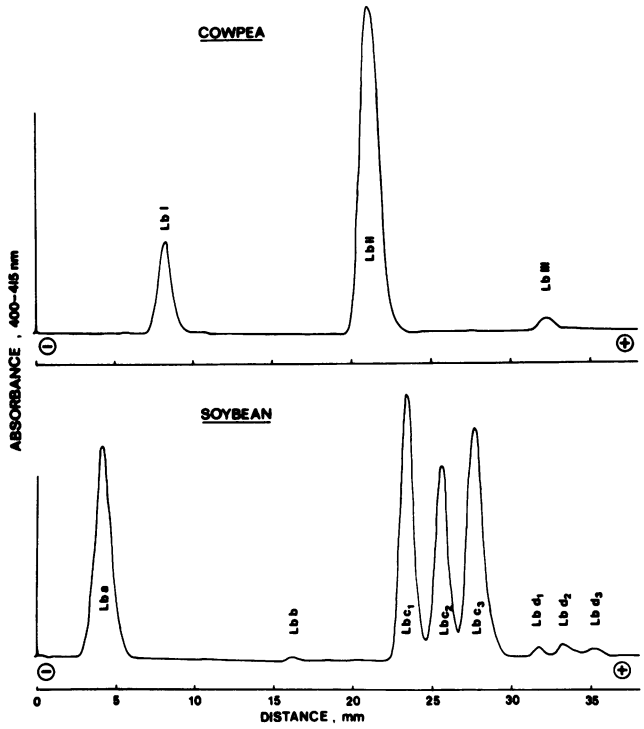

Figure 2. Separation of components of ferric Lb in extracts of cowpea and soybean nodules by analytical isoelectric focusing in polyacrylamide gels (0.5 mm) containing 2% (pH 4.5–5.4) ampholytes. Fixed gels were scanned with a densitometer for absorbance at 400 to 415 nm. In both cases, extracts were prepared from plants grown with their nodulated root systems exposed to air for 28 d.

Table II. *Relative Proportions of Lb Components, Observed by Analytical Isoelectric Focusing of Lb Prepared from Extracts of Cowpea Nodules Grown in Different Rhizosphere pO_2 from 5 to 28 DAP*

Rhizosphere pO_2	Lb component		
	LbI	LbII	LbIII
%		%	
1	17.4	80.1	2.5
5	17.5	80.0	2.5
10	16.5	81.9	1.6
20	16.3	82.9	0.8
60	15.9	82.3	1.8
80	16.6	82.6	0.8

Although in nature plants are not likely to experience significant supra-ambient pO_2, these studies with high O_2 provide information which is important in understanding O_2 regulation of nodule functioning under ambient conditions.

There was no significant effect of rhizosphere pO_2 on the relative proportions of Lb components in extracts from cowpea nodules even at the extremes of sub- and supra-ambient levels used. The data in Table II are for nodules cultured with different pO_2 from 5 to 28 DAP. The same distribution of components was found for nodules cultured similarly and harvested at 18 or 42 DAP (data not shown). In soybean nodules, small changes in the relative composition of Lb components, especially in Lb*a* at 80% O_2, were detected (Table III) as a consequence of pO_2. The differences were, however, relatively small, and from 1 to 60% O_2 the distribution of components was similar. There was a more substantial difference between the previously purified Lb sample from cv Lincoln and those samples prepared from cv White Eye (Table III). In cv Lincoln, Lb*a* comprised a greater proportion of total Lb than it did in cv White Eye with a concomitant decrease in the other components.

Table I. *Concentration of Lb in Cowpea and Soybean Nodules on Plants Grown in Different pO_2 in the Rhizosphere from 5 d to 18, 24, or 28 d for Cowpea and from 8 to 20 or 28 DAP for Soybean*

Rhizosphere pO_2	Cowpea[a]		Soybean[a]	
	Lb Concn.	Oxidation[b]	Lb Concn.	Oxidation[b]
%	*nmol·g⁻¹ nodule fresh wt*	%	*nmol·g⁻¹ nodule fresh wt*	%
1	59.0 ± 4.6	4.5	207.5 ± 1.5	4.9
2.5	132.7 ± 6.2	7.7	236.5 ± 8.5	5.2
5	151.0 ± 16.3	5.5	240.0 ± 10.0	4.2
10	156.3 ± 16.6	8.2	228.0 ± 37.0	5.0
20	183.3 ± 2.6	6.8	271.0 ± 3.0	8.0
40	210.3 ± 9.8	4.4	292.0 ± 0.0	6.3
60	184.3 ± 7.5	3.6	237.0 ± 5.0	4.8
80	94.3 ± 3.2	8.9	113.5 ± 0.5	4.7

[a] Mean ± SE; values were averaged for the three harvests of cowpea and two of soybean. [b] Oxidation % by Lb was calculated as ferric Lb/(ferric + ferrous Lb) × 100 and is the average of values for three harvests of cowpea and two harvests of soybean nodules.

Table III. *Relative Proportions of Lb Components Observed by Isoelectric Focusing of Lb Prepared from Extracts of Soybean (cv White Eye) Nodules Grown in Different Rhizosphere pO_2 from 8 to 28 DAP*

Rhizosphere pO_2	Lb component							
	Lba	Lbb	Lbc_1	Lbc_2	Lbc_3	Lbd_1	Lbd_2	Lbd_3
%	%							
1	34.2	1.2	20.8	15.3	25.4	0.6	1.1	1.6
5	36.9	0.5	19.8	13.3	24.8	1.0	1.4	1.6
10	33.6	0.5	20.2	15.0	26.4	1.0	1.3	2.0
20	32.2	0.5	21.9	16.0	26.9	0.8	1.2	1.4
60	32.9	0.5	22.4	15.4	25.8	0.8	1.1	1.1
80	25.9	0.4	25.3	18.3	27.1	0.9	1.2	0.9
20[a]	44.7	2.3	15.5	10.6	17.5	2.3	2.9	3.2

[a] Purified Lb from soybean cv Lincoln as used by Fuchsman and Appleby (14) and run concurrently on the gel with experimental samples from cv White Eye.

Effect of Short-Term Changes in pO_2 on Lb Content and Oxidation State

Cowpea plants, grown for 20 d with their nodulated root systems in air before transfer for 3 to 6 d to rooting atmospheres containing a range of pO_2, showed a marked change in the content of Lb in nodules (Table IV). Those transferred to 1% O_2 showed a 20% lower level compared to those maintained in 20% O_2; otherwise, there was relatively little effect of sub-ambient O_2. However, increase in pO_2 above ambient resulted in progressively lower levels; nodules in 80% O_2 contained only half the concentration of Lb in nodules in 20% O_2. Estimates of percentage of oxidation of Lb, based on measurement of ferric Lb in extracts, indicated that Lb content declined progressively in the extremes of nonambient O_2. This was accompanied by a sharply increasing proportion being oxidized. A similar experiment with 21-d-old soybean plants indicated that Lb concentrations were not different from those in plants maintained in low pO_2 (*cf.* Table I). At high pO_2, a decline occurred which was less severe than for cowpea.

The data in Table V show that, in nodules of cowpea plants cultured at sub-ambient rhizosphere pO_2 for 20 d (5 to 25 DAP) and then transferred to air for 7 d, there was a substantial decline (60–70%) in Lb concentration compared with plants maintained in their original pO_2 atmospheres. The percentage of oxidation of Lb was also greater in nodules transferred from sub-ambient pO_2 to air; the average for nodules maintained throughout at their original pO_2 was 5.69 ± 0.53 (mean ± SE, $n = 5$) and nodules transferred to air for 7 d, 12.76 ± 1.63.

DISCUSSION

Contrary to the previous analyses of Lb components in cowpea (21) it is now clear from DEAE-Sepharose chromatography and analytical isoelectric focusing of partially purified preparations of Lb from cv Vita 3 that nodules of this species have three Lb components, designated according to their elution profile in ion exchange chromatography as LbI, LbII, and LbIII. These results are consistent with those of Gibson *et al.* (15) who found three Lb species in nodules of

Table IV. *Concentration of Lb and Percentage of Oxidation in Cowpea and Soybean Nodules from Plants Grown in Air to 20 (Cowpea) or 21 (Soybean) DAP and Transferred to Different pO_2 in the Rhizosphere for 3 to 6 d*

Rhizosphere pO_2	Cowpea[a]		Soybean[a]	
	Lb Concn.	Oxidation[b]	Lb Conc.	Oxidation[b]
%	*nmol·g⁻¹ nodule fresh wt*	%	*nmol·g⁻¹ nodule fresh wt*	%
1	143.5 ± 9.5	2.9	209.5 ± 7.5	3.5
2.5	185.0 ± 7.0	1.6	264.0 ± 2.0	4.0
5	193.5 ± 14.5	2.7	276.5 ± 3.5	3.8
10	193.0 ± 1.0	3.0	281.0 ± 0.0	5.4
20	176.0 ± 9.0	6.6	267.0 ± 4.0	3.2
40	139.5 ± 20.5	8.5	259.5 ± 5.5	2.7
60	119.5 ± 5.5	15.7	257.0 ± 10.0	2.6
80	76.5 ± 9.5	16.4	201.0 ± 12.0	3.3

[a] Mean ± SD calculated from data of two harvests, one after 3 d, the second after 6 d in altered rhizosphere pO_2. [b] Oxidation % of Lb was calculated as ferric Lb/(ferric + ferrous Lb) × 100 and is the average of values for two harvests, one after 3 d, the second after 6 d exposure to altered rhizosphere pO_2.

Table V. *Effect of Lb Content and Lb Oxidation of Transferring Nodulated Cowpea Plants Grown with Their Root Systems in a Range of Sub-Ambient Rhizosphere pO_2, from 5 to 25 DAP to Air for 7 d*

Control plants were cultured and maintained throughout in similar sub-ambient pO_2 from 5 to 32 DAP.

Rhizosphere pO_2	Plants Grown Continuously in Different pO_2 from 5 to 32 DAP	Plants Grown in Different pO_2 from 5 to 25 DAP and Transferred to Air for 7 d	
	Lb	Lb	Decline in Lb[a]
%	*nmol·g⁻¹ nodule fresh wt*	*nmol·g⁻¹ nodule fresh wt*	%
1	94 (46)[b]	36 (18)	62
2.5	142 (70)	42 (21)	70
5	162 (80)	53 (22)	67
10	166 (82)	67 (33)	60
20	203 (100)		0

[a] Loss in Lb content compared to plants maintained at their original rhizosphere pO_2 throughout. [b] Values in parentheses are % of plants in 20% O_2 (*i.e.* air control).

cowpea cv Caloona. There was close agreement between anion exchange chromatography and isoelectric focusing so that either technique could be used to estimate the proportions of Lb components in this symbiosis.

Previous studies have demonstrated a recovery in nitrogenase activity after an initial loss following exposure of air-grown, nodulated root systems of soybean and cowpea (8–10) to different levels of O_2. Although there is not a complete understanding of how this adjustment in nodule functioning occurs, the principal mechanism appears to be due to reversible changes in gaseous permeability (10). Fuchsman *et al.* (13) suggested previously that changes in the proportions of Lb components in nodules are involved in adjustment, but the results of the present study do not support this hypothesis. In both symbioses, there is no significant difference in the proportions of their Lb components in nodules of plants cultured in pO_2 ranging from 1 to 80%. In soybean, the proportions of major components had been shown to vary during plant development (14). This is apparently not the case with cowpea, as no significant differences were observed between 18- and 42-d-old nodules (data not shown). The evidence provided by analysis of leghemoglobins in cowpea and soybean nodules, which formed and developed in different pO_2, suggests that transcription of specific Lb genes to make proteins with different functional properties is not influenced by pO_2.

On the other hand, total Lb in nodules is markedly affected by rhizosphere pO_2. The pO_2 at which the highest levels of Lb were recorded in cowpea coincided with those at which the highest nitrogenase activities and rates of N_2 fixation occurred during growth (9, 10). These results are consistent with other experimental evidence which has led to the idea that there is a direct quantitative relationship between Lb content and N_2-fixing capacity of nodules (2, 29). The Lb content of soybean nodules also varied with O_2 tension, but the effect was less marked than in cowpea, consistent with the considerably less severe inhibitory effects of sub- and supra-ambient O_2 on the soybean symbiosis (8). The yields of Lb from nodule extracts were higher from soybean compared to cowpea, and it is possible that these higher levels reduced the impact of changes in the external gas phase pO_2 on nodule functioning.

While rhizosphere pO_2 clearly affected the overall level of expression of Lb genes in both species, it is not possible to ascribe the effect to a direct involvement of O_2 in regulating expression. Lowered N_2 fixation (9) under conditions of extreme O_2 over- or undersupply would also have limited overall levels of protein synthesis.

The consequences of sudden over- or undersupply of O_2 on the status of Lb in nodules were studied in a series of experiments in which the root systems of plants were transferred between rooting atmospheres of different pO_2. A previous study on this cowpea symbiosis (10) has shown that sharp changes in nitrogenase activity and N_2 fixation occur as a consequence of rapidly altered pO_2 whether plants are cultured in air and then transferred to higher or lower pO_2 or vice versa. Lb level as well as percentage of oxidation of Lb were substantially altered in nodules of both species. While this suggests that the nodule controls its content of Lb to cope

Table VI. *Amounts of Lb Expressed on a Plant Basis or on That of g Nodule Fresh Weight, g Weight, or Numbers of Tissue Components in the Central Tissue of Cowpea Nodules Grown in Different pO_2 from 5 to 28 DAP*

Rhizosphere pO_2	Amount of Lb per					
	Plant	g Fresh Wt Nodule	g Infected Tissue[a]	Infected Cell[b]	g Bacteroid[a]	Bacteroid[b]
%	*nmol*	*nmol*	*nmol × 10⁻³*	*nmol × 10⁴*	*nmol × 10⁻³*	*nmol × 10⁹*
1	7.6 (16.6)[c]	50 (27.9)	1.21 (195.2)	2.55 (238.3)	2.22 (211.4)	3.11 (518.3)
2.5	26.0 (56.8)	127 (70.9)	0.95 (153.2)	2.50 (233.6)	2.00 (109.5)	2.09 (348.3)
5	36.3 (79.3)	130 (72.6)	0.61 (98.4)	1.68 (157.0)	1.17 (111.4)	1.18 (196.7)
20	45.8 (100)	179 (100)	0.62 (100)	1.07 (100)	1.05 (100)	0.60 (100)
40	40.2 (87.8)	191 (106.7)	0.74 (119.4)	0.95 (88.8)	1.27[d] (121.0)	ND[e]
60	39.7 (86.7)	185 (103.4)	0.80 (129.0)	0.89 (83.2)	1.37[d] (130.5)	ND
80	17.8 (38.9)	88 (49.2)	0.32 (51.6)	0.33 (30.8)	0.54[d] (51.4)	ND

[a] Calculated using values for nodule fresh weight/plant from data in Dakora and Atkins (9) and for % nodule volume as infected tissue from data in Dakora and Atkins (11). [b] Calculated using values for the number of nodules/plant and infected cells/nodule or bacteroids/nodule from data in Dakora and Atkins (11). [c] Values in parentheses are % of that at 20% O_2 (*i.e.* air control). [d] These values were calculated using the volumetric density of bacteroids in air-grown nodules from data in Dakora and Atkins (11). [e] Not determined.

with changes in external pO_2, the changes could reflect reduced protein synthesis under conditions inhibiting nitrogenase activity. The rate of turnover of the two major Lb components of pea nodules has been estimated to be around 48 h (6) and, if similar turnover also occurred in cowpea, the losses of Lb seen on transfer of plants to high or low O_2 could be explained in this way. However, the proportion of Lb as ferric Lb (% oxidation) also increased sharply, indicating that changes in the mechanisms of Lb reduction and/or oxidation (2) could also have contributed to the course of Lb decline. It is interesting that the two symbioses studied differed in their sensitivity to pO_2, and we predict that they might also differ in their ability to alter gaseous diffusion resistance in response to other endogenous factors.

As noted above, under conditions of continuous or sudden oversupply of O_2, the decline in Lb content of cowpea nodules was accompanied by a substantial increase in the proportion which was apparently oxidized *in vivo* to ferric Lb. While percentage of oxidation levels of 2 to 5% might well be accidental, higher levels, like those found here for nodules from high pO_2, are unlikely to be a consequence of extraction. A rise in free O_2 within the nodule would probably be accompanied by increased activity of reactions generating active-O_2 species, such as free radicals of O_2, singlet O_2, or H_2O_2 and, as a consequence, the level of uncontrolled oxidations (20). Oxygen radicals could also be generated by autoxidation of LbO_2 or deoxyferrous Lb (25) so that the capacity for O_2 radical scavenging reactions, such as that catalyzed by superoxide dismutase (EC 1.15.1.1) (25), might be exceeded. The extremely high level of ferric Lb in cowpea nodules after 6 d in 80% probably reflected an increasing level of potentially destructive O_2 radicals as nodule functioning declined. Recent findings have indicated that some of the Lb in soybean may be found in uninfected interstitial cells of the central tissue zone of nodules (28). Extremely high levels of uricase, a flavin-containing oxidase, are also found in these cells (30) and could provide a localized source of active-O_2 species (4). Thus, the increased oxidation of Lb (from 6–16% of total) might conceivably be due to changes in the Lb in these particular cells rather than the nodule as a whole.

Previous studies with the cowpea symbiosis studied here have revealed a complex adaptation of nodules, such that over a wide range of pO_2 (5–60%) normal functioning is maintained and nitrogenase activity maximized (9). Changes included alteration in gaseous permeability and the resistance of diffusion barriers (9, 10), altered structure of both cortical and central infected tissues, lenticel development, and the distribution of extracellular voids (11) as well as adjustments in the activities of enzymes required for ammonia assimilation and formation of translocated solutes of N (3). Combining data on Lb levels in 28-d-old plants (from data used for Table I) with those for the structural adaptation of similar nodules cultured in a range of sub-ambient pO_2 (11) provides a closer insight into the structure/function adaptation of the symbiosis to O_2 (Table VI). When expressed on a plant or nodule weight basis, Lb levels in nodules cultured in sub-ambient O_2 are much lower than those in air. However, expressed on an infected tissue, infected cell, or N_2-fixing unit (bacteroids) basis nodules in low O_2 have more Lb available to the components dependent on O_2 supply. In fact, infected cells in nodules that developed at low rhizosphere pO_2 contain twice as much Lb as those in nodules from plants grown in air, while each bacteroid in nodules grown in 1% O_2 has five times as many Lb molecules potentially available to supply O_2. Thus, subtle adjustments in the relative frequencies of infected and uninfected cells of the central tissue of nodules as well as in the frequency of bacteroids, especially at sub-ambient pO_2, result in substantial changes in relationships between the level of nitrogenase activity and structural (11), diffusional (10), and biochemical (Lb) features which enhance O_2 supply.

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