Plant Physiol. (1984) 74, 984–988
0032-0889/84/74/0984/05/$01.00/0

Reduction of Ferric Leghemoglobin in Soybean Root Nodules[1]

Received for publication October 20, 1983 and in revised form December 9, 1983

KEUK-KI LEE AND ROBERT V. KLUCAS*
Department of Agricultural Biochemistry, University of Nebraska-Lincoln, Lincoln, Nebraska 68583-0718

ABSTRACT

Reduction of ferric leghemoglobin to ferrous leghemoglobin in soybean nodules (*Glycine max* [L.] Merr. cv Woodworth) was studied using a spectrophotometer equipped with an in-cell space diffuse reflectance accessory. Nodule slices prepared and scanned under nitrogen gas showed a ferrous leghemoglobin absorption spectrum. Nodule slices equilibrated with 100% O_2 or air exhibited two absorption bands characteristic of oxygenated leghemoglobin. The addition of CO shifted those bands to CO leghemoglobin absorption bands. Potassium ferricyanide was not effective in oxidizing ferrous to ferric leghemoglobin in nodule slices. However, ferric leghemoglobin was formed by treating the nodule slices with hydroxylamine, and this was confirmed by complexing the ferric leghemoglobin to acetate, fluoride, or nicotinic acid. The diminution of ferric leghemoglobin was monitored as a function of time, and in the presence of nicotinic acid, the conversion of ferric to ferrous leghemoglobin was monitored by the appearance of ferrous leghemoglobin nicotinate complex as a function of time. Ferric leghemoglobin reduction was also confirmed by direct transmission spectrophotometry. The evidence presented here suggests that ferrileghemoglobin reduction occurs in nodule slices.

Leghemoglobin, a myoglobin-like pigment, is an essential component for nitrogen fixation by leguminous nodules but its role is indirect since nitrogen fixation is accomplished by isolated bacteroids devoid of Lb[2] (7). An apparent function of Lb is to facilitate the diffusion of O_2 to O_2-requiring bacteroids (24). This requires reversible binding of O_2 with Lb^{+2} and is dependent on the concentration of Lb^{+2}. LbO_2 and Lb^{+2} are the predominant forms of Lb in young soybean nodules as determined spectrophotometrically on minimally disturbed nodules (2) and nodule segments (5). However, other forms of Lb do exist *in vitro* and possibly *in vivo*.

Direct evidence for the presence of Lb^{+3} in legume nodules is not available, but several indirect lines of evidence suggest that Lb^{+3} may exist in nodules. Lb^{+3}, or one of its complexed forms, is always observed in nodule extracts prepared aerobically. Lb^{+3} is thought to be an artifact of the extraction procedure, but it does suggest that Lb^{+3} is readily produced from forms that occur in the nodule by a typical electron oxidation of Lb^{+2}. The midpoint potential for the components of soybean Lb (12) and for lupin Lb (4) is close to +0.22 v at pH 7. The change in redox potential *versus* change in pH is invariant with a pH range of 5 to 6, but becomes −0.05 between pH 6 to 7 (12). Lb^{+3} can also be formed by autoxidation of LbO_2 (14, 19, 22) similar to autoxidations of oxygenated Hb and Mb. The rate of *in vitro* autoxidation varies with changes in pH, temperature, and experimental conditions. With purified Lb, the half-time for autoxidation is reported to be 50 min at pH 5.5 and 37°C in the presence of EDTA (19) and 102 to 134 min at pH 6.3 and 40°C (14). Conditions in nodules such as acidic pH values, estimated to be 6.4 in young nodules (2), and abundant nonheme ion (6, 23) are conducive for autoxidation. Unless LbO_2 is stabilized in nodules, it is expected to autoxidize.

An analogy can be drawn concerning reductions among Lb, Hb, and Mb. Under normal conditions, red blood cells maintain approximately 99% of the Hb in the ferrous state (10) by two systems (13, 26) that reduces Hb^{+3} to Hb^{+2}. Reduction is directly observed if red blood cells are exposed to chemicals that induce the formation of Hb^{+3} such as nitrite. Also, from experiments designed to show that myoglobin acts to increase the flow of O_2 in red skeletal muscle, Wittenberg *et al.* (25) induced the formation of Mb^{+3} by treating pigeon muscle fiber bundles with nitrite or hydroxylamine and followed formation of Mb^{+3} using reflectance difference. The time course for the reduction of Mb^{+3} back to Mb^{+2} was about 20 min at room temperature. Tamura *et al.* (20) also provided direct evidence for the reduction of Mb^{+3} by infusing nitrite into hemoglobin-free perfused rat heart. The rate of Mb^{+3} reduction was about 30 nmol per min per g wet weight of heart. An enzyme, ferric myoglobin reductase, is proposed to be responsible for the reduction (11, 21).

Although Lb^{+3} is not known to exist in nodules, a comparable reductase is proposed for reducing Lb^{+3} to Lb^{+2} in leguminous nodules (1, 15–18). The objective of this paper is to determine if Lb^{+3} is reduced in nodules to Lb^{+2}. Washed segments of soybean nodules were exposed to isotonic solutions containing hydroxylamine to oxidize Lb^{+2} to Lb^{+3} and the subsequent reduction of Lb^{+3} to Lb^{+2} was monitored spectrophotometrically.

MATERIALS AND METHODS

Plants and Nodule Slices. Soybeans (*Glycine max* [L.] Merr. cv Woodworth) seeds were surface sterilized, inoculated with *Rhizobium japonicum* SR and planted into pots containing Turface (IMC Chemical Group, Boston, MA). Plants were grown in plant growth chambers and supplied with a nitrogen-free mineral nutrient solution (9). Nodules were harvested from plants at flowering stage and used immediately.

Nodules approximately 3 to 5 mm in diameter were washed in distilled H_2O, blotted free of water, and cut into slices approximately 1 mm in thickness. A central slice was washed as described below and used for spectrophotometric examination.

In those experiments requiring anaerobiosis, whole plants were placed in a glove bag (Instrument for Research and Industry, Cheltenham, PA), previously purged with N_2, and subsequent manipulations of the nodules were performed while maintaining

[1] This material is based upon work supported by the United States Department of Agriculture under Agreement No. 59-2311-1-1-673-0. Published as Paper No. 7322, Journal Series, Nebraska Agricultural Experiment Station.

[2] Abbreviations: Lb, leghemoglobin; Lb^{+2}, ferroleghemoglobin; LbO_2, oxygenated ferroleghemoglobin; Lb^{+3}, ferrileghemoglobin; Hb, hemoglobin; Hb^{+2}, ferrohemoglobin; Hb^{+3}, ferrihemoglobin; Mb, myoglobin; Mb^{+2}, ferromyoglobin; Mb^{+3}, ferrimyoglobin.

anaerobic conditions.

Washing and Treatment of Nodule Slices. Slices were placed in an isotonic solution (0.7 M sorbitol, 10 mM K_2HPO_4, and 2 mM $CaCl_2$, pH 5.8) and slowly stirred for 10 min. This procedure removed extracellular Lb released from damaged cells. After washing, the slice was placed in the isotonic solution supplemented with 1 mM hydroxylamine to convert Lb^{+2} to Lb^{+3}, 10 mM nicotinic acid for complexing to Lb^{+2} and Lb^{+3}, 0.1 M NaF for complexing to Lb^{+3}, or 10 mM Na-acetate for complexing to Lb^{+3}. When 0.1 M NaF was used, the sorbitol concentration was reduced to 0.5 M. When a slice was subjected to multiple treatments, it was rinsed in the isotonic solution for 10 s between treatments. Prior to spectrophotometric examination, the slice was rinsed in the isotonic solution for 10 s.

Spectrophotometric Techniques. Spectra were obtained using Cary Model 219 spectrophotometer in the normal absorbance mode or with an in-cell space diffuse reflectance accessory equipped detector (Varian Co.). For spectra obtained with the diffuse reflectance accessory, small pieces of moistened filter paper and a piece of 25 mesh iron screen, 6 mm width and 50 mm height, were placed in a glass culture tube, 10 mm in outer diameter and 75 mm height. A nodule slice was positioned vertically in the glass tube so that it was supported by the iron screen. The glass tube was stoppered with a serum septum and positioned at the sample port of the in-cell space reflectance accessory by supporting the tube with a rubber stopper having a groove and covered with aluminum foil. The nodule slice received direct incident light and reflected it. Gas composition in the glass tube was changed, if necessary, by flowing the desired gas through hypodermic needles which pierced the serum septum. A base line spectrum was obtained using the glass tube with the iron screen in it. Generally, the spectral bandwidth was set at 3.6 nm with a variable gain for the diffuse reflectance spectra. Reduced slit widths gave somewhat better spectral resolution but increased noise levels. For direct spectra measurements, a washed slice was placed in a rectangular demountable cell of 1.0 mm pathlength (Wilmad Glass Co., Buena, NJ), and masked with black tape possessing a 1-mm diameter slit. The masked slice with the central red tissue totally covering the slit was placed adjacent to the entrance to the photomultiplier tube in the sample compartment and in line with the incident light. A rear beam attenuator was used to compensate for the high absorbance of the nodule slice. Spectral bandwidths were maintained at less than 1 nm.

Pertinent absorption maxima of purified Lb-ligand complexes have been presented by Dilworth and Appleby (8). Band positions for Lb^{+3} complexed to (a) water is 625 nm with weak bands at 560 and 530 nm, (b) acetate are 618 to 622, 569 and 530 nm, (c) fluoride is 605 nm with weak bands at 555 and 525 nm, and (d) nicotinate are 557 and 528 nm. Band positions for Lb^{+2} complexed to (a) nothing is 555 to 556 nm, (b) O_2 are 574 to 575 and 540 to 541 nm, (c) CO are 562 to 563 and 538 nm, and (d) nicotinate are 554 and 525 nm.

RESULTS

Spectra of Nodule Slices Equilibrated with Various Gases. Nodule slices prepared and scanned under N_2 gas exhibited a single absorption peak at 549 nm (Fig. 1A, line 1) near the absorption peak of Lb^{+2}. All diffuse reflectance spectra reported in this study exhibited peaks at 563 and 640 nm which were artifacts from the instrument and not attributable to nodule slices. If the nodule segment was then equilibrated with 100% O_2, absorption peaks appeared at 539 and 574 nm (Fig. 1A, line 2), the peaks for LbO_2. Frequently a slight increase in absorption at 625 nm was also observed, presumably attributable to slight autoxidation of LbO_2 to Lb^{+3}. If CO was added to an anaerobically prepared slice, the spectrum was shifted to a major peak at 534, a minor peak at 560, and a shoulder at 550 nm (data not shown). This is consistent with the formation of LbCO from most of the detectable Lb^{+2}. Spectra of a nodule slice prepared aerobically and maintained under air or 100% O_2 are shown in Figure 1B. The spectrum in Figure 1B, line 1 is from a mixture of Lb^{+2} and LbO_2 when exposed to air and a conversion to more LbO_2 when exposed to 100% O_2 (Fig. 1B, line 2). If CO replaced O_2, spectral change typical for the formation of LbCO was obtained (data not shown).

Spectra of Nodule Slices Exposed to Nicotinic Acid or Hydroxylamine. Nicotinic acid complexes to Lb^{+3} and Lb^{+2} and not to other hemoproteins (3) and therefore is useful to substantiate that Lb is the endogenous hemoprotein being monitored in nodule slices. A nodule slice treated with 10 mM nicotinic acid exhibited absorption maxima at 554 and 525 nm (Fig. 1C, line 2) as expected for the Lb^{+2}-nicotinate complex.

Hydroxylamine oxidizes ferrous hemoproteins such as myoglobin to the ferric forms (25). Lb in soybean nodule slices, which were prepared aerobically and exhibited spectra indicative of a mixture of LbO_2 and Lb^{+2} as well as insignificant levels of Lb^{+3} (Fig. 1D, line 1), was oxidized by exposure to 10 mM hydroxylamine. As shown in Figure 1D, line 2, a distinct increase in absorption at 625 nm (Fig. 1D, line 2) showed that Lb^{+3} had been formed. Subsequent treatment of the nodule slice with Na-acetate or NaF shifted the peak observed at 625 to 622 nm or 608 nm, respectively. Incubation of nicotinic acid with the nodule slice which had been treated with hydroxylamine resulted in the loss of the 625 nm (Fig. 2, line 2). These observations are consistent with the suggestion that Lb^{+3} is formed when nodule slices are exposed to hydroxylamine.

Evidence for Detecting Intracellular Lb. It was critical to establish that intracellular and not extracellular Lb was responsible for the observed spectra because diffuse reflectance is mainly reflectance at the cut surface of the nodule. Extracellular Lb resulting from cell damage from slicing the nodule was removed initially by rinsing the slices in isotonic solution for 10 min and placing the slices in fresh isotonic solution. Evidence that intracellular Lb was responsible for spectral shifts was provided by treating washed nodule slices with $K_3Fe(CN)_6$, an oxidant that readily converts Lb^{+2} to Lb^{+3}, but does not penetrate nodule cells (2). The solution from the initial washing of the nodule slice exhibited a typical Lb^{+3} spectrum when $K_3Fe(CN)_6$ was added but washed nodule slices, such as shown in Figure 1A, line 1, exhibited identical spectra in the presence or absence of $K_3Fe(CN)_6$.

Diminution of Lb^{+3} and Appearance of Lb^{+2} as a Function of Time. A washed nodule slice was exposed to hydroxylamine to form Lb^{+3} and then rinsed with isotonic solution to remove excess hydroxylamine. The spectrum of the slice (Fig. 3, line 1) exhibited a 625-nm absorption, indicative of Lb^{+3}. Sequential scanning of the slice at 15, 30, and 60 min showed a decrease of the absorption peak at 625 nm (Fig. 3, lines 2, 3, and 4). If nicotinic acid was added to the slice after 60 min, the prominent bands of the Lb^{+2}-nicotinate complex appeared (Fig. 3, line 5). Although large levels of nicotinic acid resulted in the spectra typical for Lb^{+2}-nicotinic acid complex, the loss of the 625 nm peak with time suggests, but does not prove, that Lb^{+3} was converted to Lb^{+2}. The binding constant at pH 5.3 between nicotinic acid and Lb^{+3} is 24.3-fold greater than between nicotinic acid and Lb^{+2} (3), so the availability of low endogenous levels of nicotinic acid to Lb^{+3} may explain the observation. However, after hydroxylamine treatment, all Lb appears to be Lb^{+3} as indicated by the absence of any prominent peaks attributable to Lb^{+2} or LbO_2 (Fig. 1D, lines 2; Fig. 2, line 1; Fig. 3, line 1).

The conversion of Lb^{+3} to Lb^{+2} was confirmed by the addition of nicotinic acid to a rinsed nodule slice, which converted Lb^{+3}

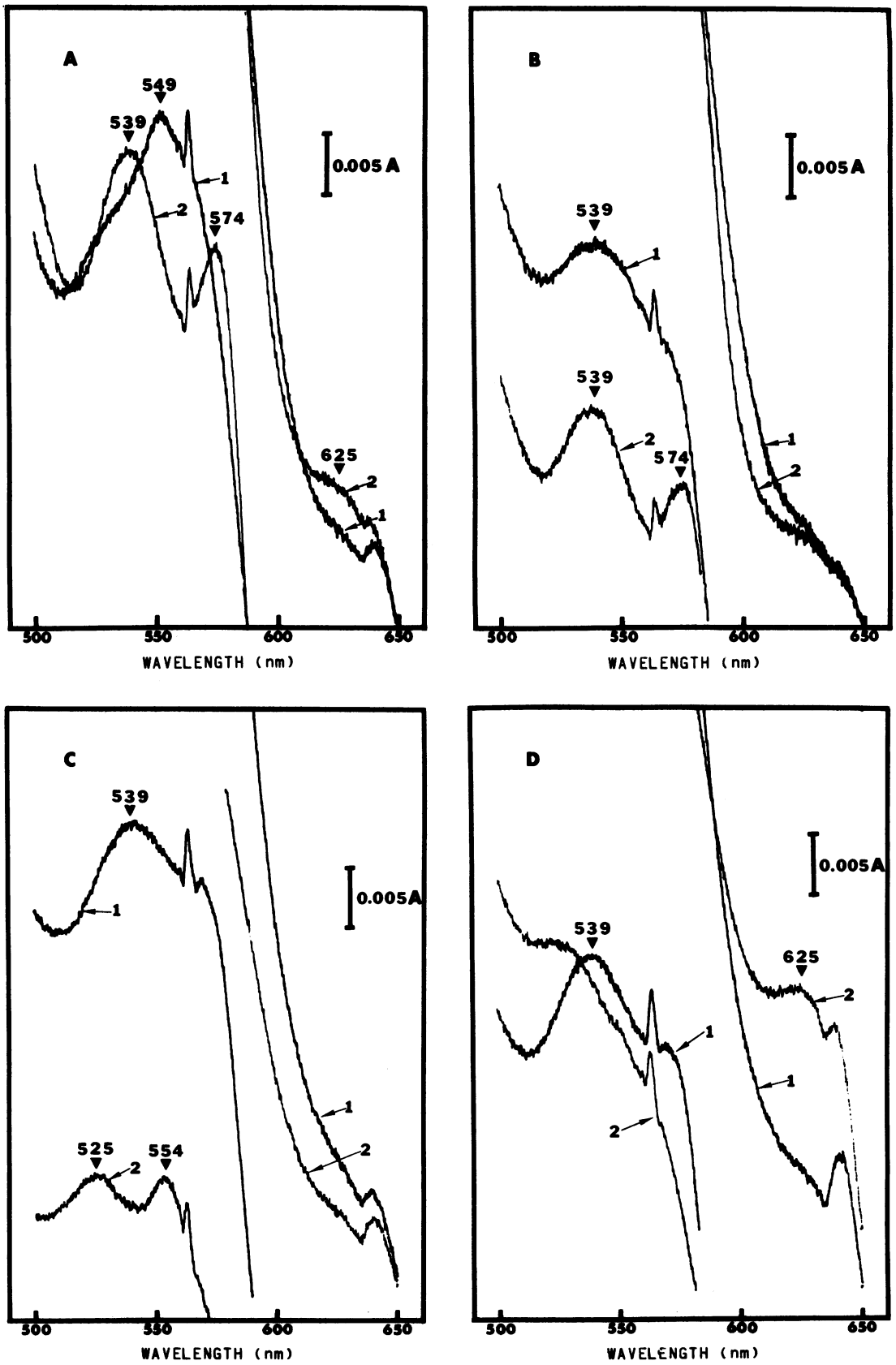

FIG. 1. Diffuse reflectance spectra of Lb in soybean nodule slices incubated in the presence of various gases, nicotinic acid, or hydroxylamine. A, The slice was prepared in N_2 (line 1), and then equilibrated with O_2 (line 2); B, prepared in air and scanned in air (line 1), and equilibrated with O_2 (line 2); C, prepared in air and scanned in air (line 1), and then soaked in nicotinic acid (line 2); D, prepared in air and scanned in air (line 1), and then soaked in hydroxylamine (line 2).

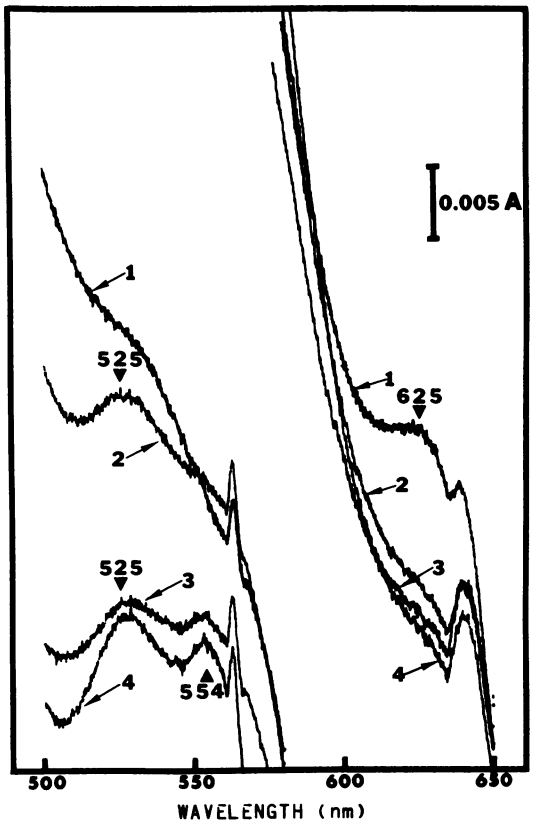

FIG. 2. Appearance of Lb^{+2}-nicotinate complex as a function of time after the addition of nicotinic acid as measured by diffuse reflectance spectrophotometry. The slice was soaked in hydroxylamine and scanned (line 1), and soaked in nicotinic acid for 3 min (line 2), 8 min (line 3), and 15 min (line 4).

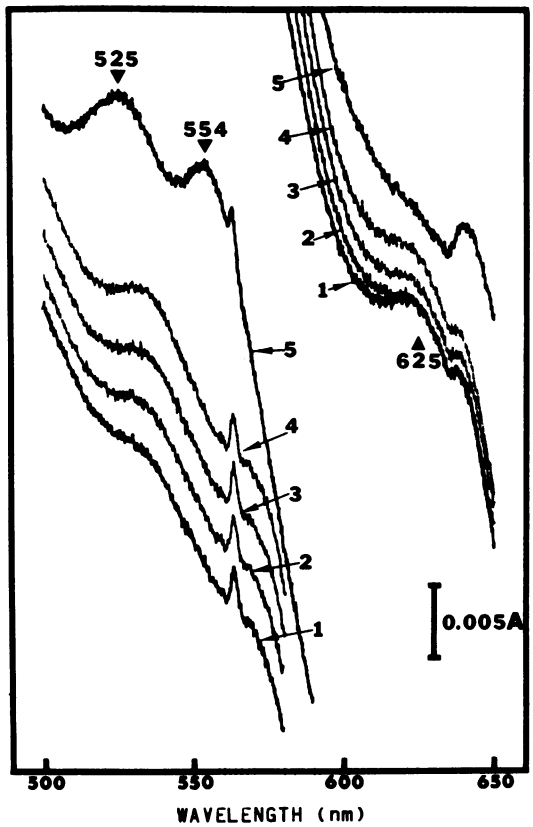

FIG. 3. Diminution of Lb^{+3} in soybean nodule slice as a function of time as measured by diffuse reflectance spectrophotometry. The slice was treated with hydroxylamine and scanned immediately (line 1), at 15 min (line 2), 30 min (line 3), and 60 min (line 4), and then soaked in nicotinic acid and scanned (line 5).

to the Lb^{+3}-nicotinate complex as suggested by the loss of the absorption peak at 625 nm (Fig. 2, line 2). The peaks of Lb^{+3}-nicotinate at 528 and 557 nm were not distinctly observed, presumably because those possess much lower extinction coefficients than the Lb^{+2}-nicotinate complex peaks at 525 and 554 nm. During subsequent incubation, the continued increase in levels of Lb^{+2}-nicotinate was indicated by the obvious enhancement of absorption peaks at 525 and 554 nm (Fig. 2, lines 2, 3, and 4). Because of possible multiple intracellular pools of Lb, diffusion of nicotinate to Lb^{+2} in less accessible pools, presumably inside the membrane envelopes, may explain the gradual increased level of Lb^{+2}-nicotinate complex. However, this explanation is unlikely because most of the observable Lb in the nodule slices are converted to Lb^{+3} when exposed to hydroxylamine.

Direct spectrophotometry was also used to confirm that the conversion of Lb^{+3} to Lb^{+2} (Fig. 4) was not an artifact arising from reflectance spectroscopy. A washed nodule slice exposed to hydroxylamine showed an absorption shoulder around 625 nm (Fig. 4, line 1) and this shoulder decreased with time (Fig. 4, lines 2, 3, and 4), while an absorption increase at 550 nm occurred concurrently.

DISCUSSION

Methods presented in this study were useful for monitoring qualitatively various forms of Lb in nodule slices. Appleby's method (2) in which minimally disturbed nodules are examined by direct spectrophotometric measurements was suited for examining Lb under near-physiological conditions but the procedure restricted manipulations of nodules and worked best with young nodules. Our procedures were designed to facilitate manipulation of nodule slices to oxidize Lb^{+2} by chemical methods. In general, spectra using diffuse reflectance of nodule slices were consistent with direct spectra from minimally disturbed nodules (2) and nodule segments (5). However, the distinct 550-nm peak which Appleby (2) attributed to bacteroidal Cyt *c* was not detected by diffuse reflectance.

The Lb being detected in this study was restricted by a diffusion barrier as shown by the lack of an effect by $K_3Fe(CN)_6$, whereas other compounds including hydroxylamine, acetate, fluoride, and nicotinate were reactive with Lb. The evidence indicates that the pool of Lb being detected was intracellular and that most of the Lb being detected was oxidized by hydroxylamine.

The data demonstrate that Lb^{+3}, induced by chemical oxidation of Lb^{+2}, disappeared with the reappearance of Lb^{+2}. Because of the length of the experiments, biosynthesis of new Lb^{+2} would require too much time, therefore Lb^{+2} appears to be formed by endogenous reduction of Lb^{+3}. Lb^{+3} has not been reported to be a constituent of legume nodules. However, a lack of detection does not rule out the existence of Lb^{+3} in nodules. The 625-nm charge transfer absorption peak which is normally used as the diagnostic test for Lb^{+3} has a low extinction coefficient and this limits the sensitivity for its detection. Also, as suggested by Dilworth and Appleby (8), some complex forms of Lb^{+3}, such as the nicotinate complex do not exhibit the 625-nm peak. Enzyme systems are known to exist for the reduction of Hb^{+3} in erythrocytes (13, 26) and have been suggested to exist in lupine (15) and soybean (17, 18) nodules. The evidence presented here suggests that Lb^{+3} reduction occurs in nodules. Future experiments will be required to establish if the *in vivo* reduction is

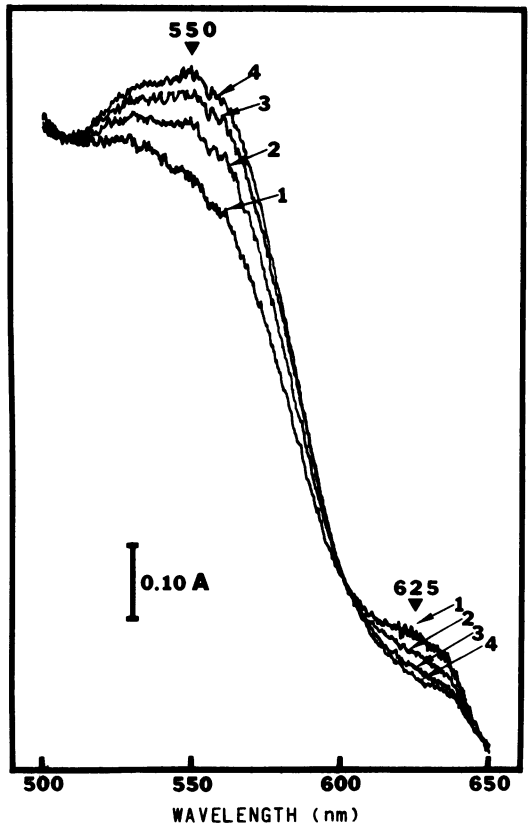

FIG. 4. Diminution of Lb^{+3} and appearance of Lb^{+2} as a function of time by direct spectrophotometry. The slice was treated with hydroxylamine and scanned at 5 min (line 1), 10 min (line 2), 20 min (line 3), and 35 min (line 4).

performed by the proposed enzyme, ferric leghemoglobin reductase.

LITERATURE CITED

1. APPLEBY CA 1962 The oxygen equilibrium of leghemoglobin. Biochim Biophys Acta 60: 226–235
2. APPLEBY CA 1969 Properties of leghemoglobin *in vivo*, and its isolation as ferrous oxyleghemoglobin. Biochim Biophys Acta 188: 222–229
3. APPLEBY CA, BA WITTENBERG, JB WITTENBERG 1973 Nicotinic acid as a ligand affecting leghemoglobin structure and oxygen reactivity. Proc Natl Acad Sci USA 70: 564–568
4. ATANOSOV BP, AYV PEIVE 1974 Redox equilibrium of Lupin (*Lupinus luteus* L.) leghemoglobin and certain related heme-containing proteins. Translated from Koklady Akad. Nauk SSSR 216: 1161–1164
5. BERGERSEN FJ 1962 Oxygenation of leghemoglobin in soybean root-nodules in relation to the external oxygen tension. Nature 194: 1059–1061
6. BERGERSEN, FJ 1963 Iron in the developing soybean nodule. Aust J Biol Sci 16: 916–919
7. BERGERSEN FJ, GL TURNER 1967 Nitrogen fixation by the bacteroid fraction of breis of soybean root nodules. Biochim Biophys Acta 141: 507–515
8. DILWORTH MJ, CA APPLEBY 1977 Leghemoglobin and *Rhizobium* hemoproteins. *In* RC Burns, ed, A Treatise on Dinitrogen Fixation. John Wiley and Sons, New York, pp 691–764
9. EVANS HJ, B KOCH, RV KLUCAS 1972 Preparation of nitrogenase from nodules and separation in components. Methods Enzymol 24: 470–476
10. GIBSON QH 1948 The reduction of methaemoglobin in red blood cells and studies on the cause of idiopathic methaemoglobinaemia. Biochem J 42: 13–23
11. HAGLER L, RI COPPES JR, RH HERMAN 1979 Metmyoglobin reductase. Identification and purification of a reduced nicotinamide adenine dinucleotide-dependent enzyme from bovine heart which reduces metmyoglobin. J Biol Chem 254: 6505–6514
12. HENDERSON RW, CA APPLEBY 1972 The redox potential of leghemoglobin. Biochim Biophys Acta 283: 187–191
13. HULTQUIST DE 1978 Methemoglobin reduction system of erythrocytes. Methods Enzymol 52: 463–472
14. IMAMURA T, A RIGGS, QH GIBSON 1972 Equilibria and kinetics of ligand binding by leghemoglobin from soybean root nodules. J Biol Chem 247: 521–526
15. MELIK-SARKISYAN SS, BASHIROVA NF, ZAURALOVA NO, KRETOVICH VL 1976 Enzymatic reduction of leghemoglobin. Biokhimiya 41: 1330–1333 (English Edition, 41: 1085–1087)
16. NASH DT, HM SCHULMAN 1976 The absence of oxidized leghemoglobin in soybean root nodules during nodule development. Biochem Biophys Res Commun 68: 781–785
17. PUPPO A, J RIGAUD, D JOD 1980 Leghemoglobin reduction by a nodule reductase. Plant Sci Lett 22: 1–6
18. SAARI LL, RV KLUCAS 1981 Leghemoglobin reductase from soybean nodules. *In* KW Clark, JHG Stephens, eds, 8th North American *Rhizobium* Conference, The University of Manitoba, Winnipeg, Canada, p 56
19. STETZKOWSKI F, R BANERJEE 1977 Autooxydation de la leghemoglobine. *In* D Labie, C Poyart, J Rosa, eds, Interaction Moleculaires de L'hemoglobine, Vol 70. INSERM, Paris, pp 107–112
20. TAMURA M, R ARAKI, T ISHIKAWA, K SAGISAKA, I YAMAZAKI 1980 Direct observation of reduction of met- and ferrylmyoglobins in the hemoglobin-free perfused rat heart. J Biochem 88: 1211–1213
21. TAYLOR D, P HOCHSTEIN 1978 Inhibition by adriamycin of a metmyoglobin reductase from beef heart. Biochem Pharmcol 27: 2079–2082
22. THOROGOOD E 1957 Oxygenated ferroheme proteins from soybean nodules. Science 126: 1011–1012
23. WITTENBERG JB 1980 Utilization of leghemoglobin-bound oxygen by *Rhizobium* bacteroids. *In* WE Newton, WH Orme-Johnson, eds, Nitrogen Fixation, Vol 2. University Park Press, Baltimore, pp 53–67
24. WITTENBERG JB, FJ BERGERSEN, CA APPLEBY, GL TURNER 1974 Facilitated oxygen diffusion: the role of leghemoglobin in nitrogen fixation by bacteroids isolated from soybean root nodules. J Biol Chem 249: 4057–4066
25. WITTENBERG BA, JB WITTENBERG, PRB CALDWELL 1975 Role of myoglobin in the oxygen supply to red skeletal muscle. J Biol Chem 250: 9038–9043
26. YUBISUI T, M TAKESHITA, Y YONEYAMA 1980 Reduction of methemoglobin through flavin at the physiological concentration by NADPH-flavin reductase of human erythrocytes. J Biochem 87: 1715–1720