CELLULAR LOCATION AND CONCENTRATION OF LEGHAEMOGLOBIN IN SOYBEAN ROOT NODULES

By F. J. BERGERSEN* and D. J. GOODCHILD*

[Manuscript received 12 December 1972]

Abstract

The reaction of leghaemoglobin (Lb) with oxidized 3,3′-diaminobenzidine has been used to demonstrate the localization of this haemoprotein within the membrane envelopes surrounding the bacteroids in soybean root nodule cells. In fresh tissue sections mounted in a modified Honda medium, the reagent stained the envelope contents a brick-red colour. The cytoplasm between the envelopes and the host nucleus was not stained. Upon fixation with buffered 1–2% glutaraldehyde, purified Lb lost up to 40% of its haem; however, sufficient remained bound to the precipitate to allow the diaminobenzidine to react. The stained precipitate, although not highly coloured by the reagent, reacted strongly with OsO_4 to produce a more electron-dense material than unstained, OsO_4-treated, fixed Lb. This reaction was used to confirm the localization of Lb by electron microscopy of sectioned nodule tissue. It was concluded that precipitated material within the envelope space was Lb.

The growth, C_2H_2-reducing activity, Lb content, and structure of soybean nodules were measured during glasshouse growth of Lincoln soybeans. The volumes of the membrane–envelope space were calculated from measurements of nodule volume and light and electron micrograph profile areas. Lb was detected on the second day after nodules appeared, and C_2H_2-reducing activity was detected on day 4. The number of membrane envelopes per cell was constant from day 4, but multiplication of bacteria within them continued until day 13. As the nodules increased in size, activity and Lb content and concentration within the envelopes increased. At 8 days, when average Lb per nodule was $0{\cdot}17 \pm 0{\cdot}03$ nmole, the concentration within the envelopes was 0·36 mM. At 36 days, when activity reached its maximum, the Lb content per nodule was $19{\cdot}2 \pm 0{\cdot}9$ nmoles and the concentration was 1·53 mM.

I. INTRODUCTION

The red pigment leghaemoglobin (Lb) is a well-known feature of the bacteroid-containing cells of the central tissue of legume root nodules. A number of physiological roles have been proposed to explain the many correlations between Lb concentration and nitrogen fixation which have been observed (reviewed by Bergersen 1971). Recently, reports of a physiological role for Lb in supplying the needs of the bacteroids for oxygen have been published. Tjepkema and Yocum (1970) and Tjepkema (1971) reported that oxygen uptake by nodule slices was inhibited by low concentrations of CO and they attributed this inhibition to inactivation of Lb by CO. Bergersen *et al.* (1973) showed that CO inhibited hydrogen evolution by

* Division of Plant Industry, CSIRO, P.O. Box 1600, Canberra City, A.C.T. 2601.

the nitrogenase of intact nodules but not when washed bacteroid suspensions were used. It was concluded that CO blocked the function of Lb in the tissue. This conclusion was confirmed in experiments in which purified oxyLb was added to bacteroid suspensions at low oxygen pressures; bacteroid oxygen uptake was stimulated and C_2H_2 reduction and hydrogen evolution by the bacteroid nitrogenase were stimulated to a greater extent. In order to confirm the validity of the latter experiments, it is necessary to establish that the Lb is in direct contact with the surface of the bacteroids *in vivo* as it was in the *in vitro* experiments.

The location of Lb in nodule tissue has been a matter of controversy. Smith (1949) showed that the haemoprotein was located within the bacteroid-containing cells of soybean nodule central tissue. Dilworth and Kidby (1968) used electron-microscope autoradiography of *Serradella* nodule tissue in which ^{59}Fe had been used to label the Lb and concluded that the pigment was located between the bacteroid surfaces and the inner surfaces of the membrane envelopes which enclosed them. This location was also favoured by Truchet (1972) who used the pseudoperoxidase activity of denatured haemoproteins in a cytochemical reaction using 3,3′-diamino-benzidine. In contrast, Dart (1968), using the same method with soybean nodules, suggested that Lb occurs in the host cytoplasm and is not enclosed by the membrane envelope.

Lb is easily extracted from nodule tissue, where it appears to be present in solution. We therefore attempted to develop methods which would preserve its location during preparation for electron microscopy. Hirai (1968, 1971) has studied the reactions of various haemoproteins with an autoxidation product of 3,3′-diamino-benzidine formed in light. We have done similar experiments with purified soybean Lb and used the results to develop the techniques which were used with fresh tissue and with fixed tissue prepared for electron microscopy.

The activity, Lb content, and tissue compartment volumes were measured in soybean nodules, grown in a glasshouse experiment, and the physiological concentration of Lb calculated in this nitrogen-fixing system. The same experiment was also used for the investigation of nodule intercellular spaces described in an accompanying paper (Bergersen and Goodchild 1973).

II. Materials and Methods

(*a*) *Plant Growth and Root Nodule Production*

Glycine max Merr. cv. Lincoln seeds were inoculated with strain CB1809 of *Rhizobium japonicum* and grown in a glasshouse as described previously (Bergersen and Turner 1970). The day on which nodules could first be seen was recorded as day 1.

(*b*) *Leghaemoglobin*

Purified Lb was prepared as described previously (Bergersen *et al.* 1973). Amounts of Lb in nodule tissue were measured using 0·5 g (fresh wt.) samples which were crushed and extracted three times with 1 ml of cold (0°C) 25 mM Tris–HCl buffer, pH 7·4. The extracts (total vol. = 4 ml) were filtered under vacuum and then centrifuged in glass tubes at 4000 *g* in a refrigerated centrifuge to sediment the bacteroids. The clear, red-brown supernatant was then assayed for total haematin content by adding 2·5 ml of alkaline pyridine reagent to 2·5 ml of extract and measuring the absorption spectrum of the pyridine haemochromogens as described previously (Bergersen *et al.* 1973). Preliminary tests showed that this method did not extract detectable amounts of other bacteroid or plant haemoproteins.

(c) *Nodule Activity*

All of the results in this paper refer to nodules borne on the top 3·5 cm of soybean tap root. The nitrogenase activity of these nodules was measured by incubating portions of nodulated root for 1 hr at 25°C in a gas mixture containing 10% C_2H_2+20% O_2 in argon. The C_2H_4 produced was analysed by standard methods (Bergersen 1970) and after the assay, the nodules were detached, washed, blotted dry, weighed, and used for Lb estimation.

(d) *Nodule Volume*

This was determined from a calibration curve which related fresh weight of nodules of various ages to volumes obtained by displacement of water.

(e) *Microscopy of Nodule Sections*

Fresh nodule tissue was examined by cutting sections 50 or 100 μm thick with a Reichert hand-microtome. Preliminary experiments showed that Mg^{2+} ions and buffers containing high concentrations of sucrose helped to preserve the membrane envelopes. The use of a modified Honda's medium (Honda *et al.* 1966) was finally adopted, and it permitted the contents of sectioned cells to remain intact, with little loss of Lb for up to 11 hr. The medium contained Ficoll (2·5% w/v), Dextran 40 (5% w/v) (Pharmacia, Uppsala, Sweden), sucrose (0·25M), and $MgCl_2$ (1 mM) in 25 mM Tris–HCl buffer pH 7·8 or in 0·1M potassium phosphate buffer, pH 7·4.

Nodules taken simultaneously with samples for measurement of Lb and C_2H_2-reducing activity were fixed in Fleming's solution (Gurr 1953), embedded in paraffin wax, and serial sections (20 μm thick) were prepared. After de-waxing, the sections were stained in Heidenhain's haematoxylin and safranin (Gurr 1953). Fresh and paraffin sections were examined with a Zeiss model WL microscope and photographed with Reichert Photomatic equipment, using bright field or phase-contrast illumination. Tissue for routine electron microscopy was fixed and dehydrated as described previously (Goodchild and Bergersen 1966) but was embedded in an Epon–Araldite mixture with the aid of vacuum and polymerized at 85°C. Sections were stained with uranyl acetate and lead citrate (Fiske 1966) and examined with a Philips EM200 electron microscope. Tissues stained with 3,3′-diaminobenzidine were treated as described in Section III.

Profile areas of various nodule tissue compartments were measured by weighing excised portions of photographs. Nodules were regarded as being spherical and calculations were based on ratios of areas where possible, in order to avoid problems associated with shrinkage. It was assumed that shrinkage affected all structures equally, in the absence of any observed gross distortions of tissues or of cellular structure.

III. Experimental and Results

(a) *Location of Leghaemoglobin in Nodule Tissue*

(i) *Studies of the Fixation and Staining of Leghaemoglobin*

A purified preparation containing 0·35 mM Lb was studied. Fixation with 1–2% glutaraldehyde in 0·1M phosphate buffer, pH 7·4, produced a gelatinous clot after about 1 hr at 20°C or 2½ hr at 0°C. When this was centrifuged (4,000 *g* for 5 min), a brown supernatant was obtained which contained about 40% of the haematin present in the unfixed Lb, but only about 1% of the protein. Apparently, cross-linking of the protein distorted the molecule sufficiently to release this substantial proportion of the haem. In contrast, the fixation of a sample of bacteroids, which contained substantial quantities of haemoproteins (Appleby 1969*a*), released negligible amounts of haem (Table 1).

A solution of DAB [3,3′-diaminobenzidine hydrochloride (Sigma Chemical Co., St. Louis, Mo.), 15 mM, in 0·1M potassium phosphate, pH 7·0, and brought to pH 7·0 with 40% KOH] was oxidized to a dark-brown liquid when kept in air in daylight

for 1 week. Unfixed oxyLb reacted quickly with DAB oxide to form a brick-red colour but no precipitate. After 45–60 min, the spectrum showed a general darkening between 440 and 680 nm with more intense darkening between 480 and 500 nm. When left overnight at 0°C, further darkening occurred which obliterated the Soret peak [Fig. 1(*a*)]. When a trace of dithionite was added to discharge the oxygen from the Lb before adding oxidized DAB, little reaction occurred in a stoppered tube, until the dithionite had been oxidized [Fig. 1(*b*)]. Lb which had been oxidized with potassium ferricyanide reacted quickly with oxidized DAB to produce a similar colour to that produced by oxyLb. DAB-stained Lb was not precipitated by 2% glutaraldehyde, although some slight turbidity developed overnight.

TABLE 1

LOSS OF HAEM FROM GLUTARALDEHYDE-FIXED LEGHAEMOGLOBIN

The solutions or suspensions were made to 1% with buffered glutaraldehyde. Unfixed bacteroids in ice were disrupted by ultrasonic irradiation at 20 kHz for three 5-min periods and then centrifuged; total haematin was determined on the supernatant which contained mostly cytochrome *c* (cf. Appleby 1969*a*). Soluble haematin was measured as pyridine haemochromogen

Haemoprotein source	Treatment	Soluble haematin (nmoles)
Oxyleghaemoglobin (0·5 ml)	Unfixed	150
Oxyleghaemoglobin (0·5 ml)	Supernatant after fixation	62
Bacteroids (78 mg)	Supernatant after fixation	0·8
Bacteroids (78 mg)	Unfixed, disrupted by ultrasonic irradiation, centrifuged	28

Samples of Lb, which had been fixed with glutaraldehyde (2%) for 2 hr and then washed in three changes of buffer, produced a dark brown colour after staining with oxidized DAB, but did not stain if traces of glutaraldehyde remained. Fixed Lb, stained with DAB oxide, produced a darker precipitate than unstained, fixed Lb when treated with OsO_4 (1% w/v, final concn.). Similar results were obtained when fixed Lb was treated with freshly prepared DAB and then with 0·02% H_2O_2 in buffer.

These results show that Lb reacts with DAB in a similar manner to that described by Hirai (1968, 1971) for other haemoproteins. Reducing conditions inhibited the reaction, perhaps because of reduction of the oxidized DAB.

(ii) *Staining of Fresh Nodule Tissue with Oxidized DAB*

Freshly cut soybean nodule sections were equilibrated for 1 hr in the modified Honda medium and then stained for 1–4 hr in 15 mM oxidized DAB in the same medium. The central tissue stained dark brown, with slight staining of the nodule cortex. Sections were mounted under a coverslip in Honda medium and examined under oil-immersion. Sectioned and intact host cells near the surface of the central tissue of the section were well-stained, the cytoplasmic space being filled with globules

of brick-red-stained material which appeared to correspond to the membrane envelopes and their contents (Figs. 2*A* and 2*B*). This was confirmed by pressing on the coverslip to express some of the cell contents. The bacteroids could then be seen as lighter rods within each envelope. The host-cell cytoplasm between the membrane envelopes was unstained as was the host nucleus. The colour within the envelopes appeared to be evenly distributed, but when tissue stained in this way was fixed and embedded for electron microscopy, the coloured material appeared to condense onto the surface of the bacteroids.

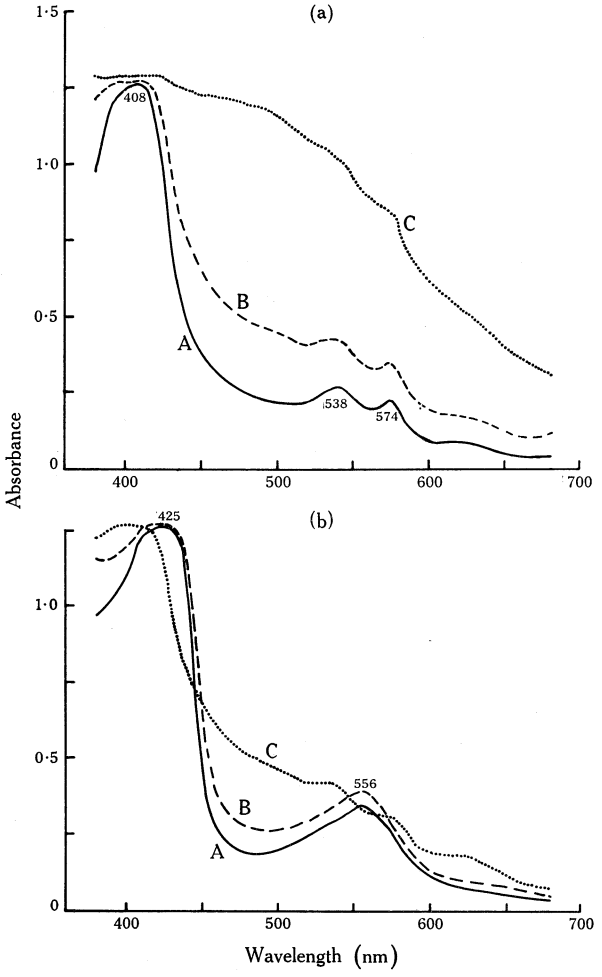


Fig. 1.—Absorption spectra of Lb reacted with DAB oxide. (*a*) Reaction with oxyLb. (*b*) Reaction with Lb reduced with approximately 0·5 mg $Na_2S_2O_4$. *A*, before addition; *B*, 45 min after addition of DAB oxide; *C*, after 9 hr. Reaction contained 0·4 ml of 0·35 mM Lb+0·2 ml of 15 mM DAB oxide: 0·2 ml of this mixture was diluted to 3·0 ml for recording of the absorption spectra (1 cm light path).

When blocks (1 mm cubes) of fresh nodule central tissue were treated with DAB oxide in Honda medium, only the outer edges were stained. It is believed that this effect was due to the reducing conditions, maintained by the endogenous metabolism of the bacteroids, within the tissue. However, at the edge of the blocks, the Lb was apparently oxygenated and able to react with the DAB oxide (cf. Fig. 1).

(iii) *Electron Microscope Observations*

The observations made with DAB-stained fresh sections were confirmed in the electron microscope as follows. It was established that less haem was lost from nodule tissue when the tissue was fixed in Honda medium than when it was fixed in Tris–HCl or phosphate buffers. Blocks of nodule central tissue (age 30 days) 0·2 mm thick and 1 mm square were cut, equilibrated in the Honda medium for 1 hr, and fixed for 2 hr at 20°C in glutaraldehyde (2% v/v) in Honda medium. After fixation, the blocks were washed for 1 hr in frequent changes of decreasing concentrations of Honda medium and finally in 0·1M phosphate buffer pH 7·4 to remove liberated haem and all traces of glutaraldehyde. The blocks were then stained for 4 hr at 20°C or overnight at 4°C with DAB oxide or fresh DAB (both 15 mM) in 0·1M phosphate buffer. The blocks stained with fresh DAB were treated with 0·02% H_2O_2 for 1 hr at 20°C. All blocks were then washed in 25 mM phosphate buffer for 1 hr and then treated with 2% OsO_4 in 25 mM phosphate pH 7·0 for 1 hr. The blocks were then dehydrated, embedded, and sectioned as described above. Unstained control blocks were included.

The results showed that if nodule tissue is fixed in glutaraldehyde in 0·1 or 0·25M phosphate buffer (2% v/v) followed by OsO_4 a small deposit of electron-dense material can be observed in the space between the bacteroids and the membrane envelope (envelope space) (Fig. 3*A*). Deposits of this type have been observed previously (Goodchild and Bergersen 1966) and it has been suggested that they represent the presence of Lb. When Honda medium is substituted for phosphate buffer as the vehicle for glutaraldehyde the amount of deposit within the envelope space increases (Fig. 3*B*) but there is no apparent change in the preservation of other structures. This deposit has a fibrillar appearance. If glutaraldehyde fixation in Honda medium is followed by staining with DAB oxide or DAB–H_2O_2, there is an increase in the staining intensity of the fibrillar deposits within the envelope space (Fig. 3*C*). We consider that the increased intensity is due to a precipitate resulting from the reaction between OsO_4 and DAB-oxide-stained Lb.

In sections not stained with uranyl acetate and lead citrate, an electron-dense deposit was observed in mitochondrial cristae, within the envelope space, and in the bacteroids, where it was particularly dense near the cell wall. The mitochondrial images correspond well with those described by Hirai (1968) who postulated a reaction between the haem of cytochromes and DAB oxide. Cytochromes are associated with bacteroid cell membrane particles (Appleby 1969*a*). Control material did not show any of these precipitates in unstained sections. The density of the precipitates increased with the time of incubation in DAB oxide or DAB–H_2O_2 and reached its greatest density in the H_2O_2 treatment following overnight treatment in DAB. We could not find convincing evidence that the precipitate was absent from the cytoplasm of the host cells in either stained or unstained sections but incubation in DAB oxide or DAB–H_2O_2 changed the appearance of the host cytoplasm as is evident by comparing Figure 3*C* with Figures 3*A* and 3*B*.

The evidence from electron microscopy that the natural location of Lb is in the envelope space agrees with the observations of stained material observed in light microscopy of fresh tissue stained with DAB oxide.

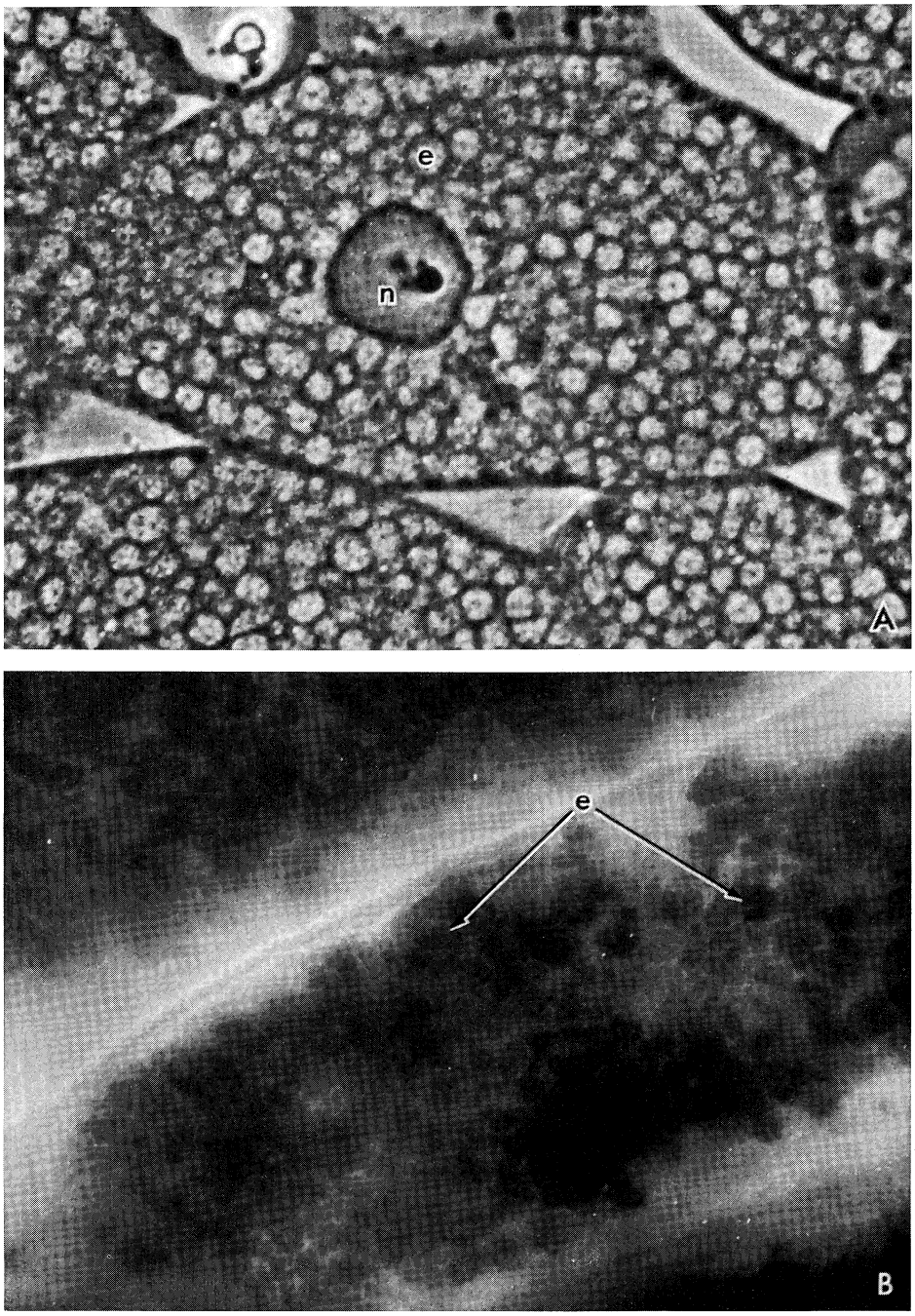


Fig. 2.—Localization of Lb in DAB-oxide-treated nodule tissue. *A*, phase-contrast photomicrograph of unstained, glutaraldehyde-fixed, plastic-embedded central tissue of soybean nodule (age 30 days), showing a single host cell with central nucleus (*n*) and the appearance of the cytoplasm packed with membrane envelopes (*e*). This cell shows the arrangement of the structures seen in stained fresh tissue in Figure 2*B*. ×1370. *B*, Bright-field photomicrograph of a host cell in a fresh unfixed section of nodule central tissue, after staining with DAB oxide in Honda medium. The membrane envelopes (*e*) were stained brick-red. Photograph taken in green light. ×1400.

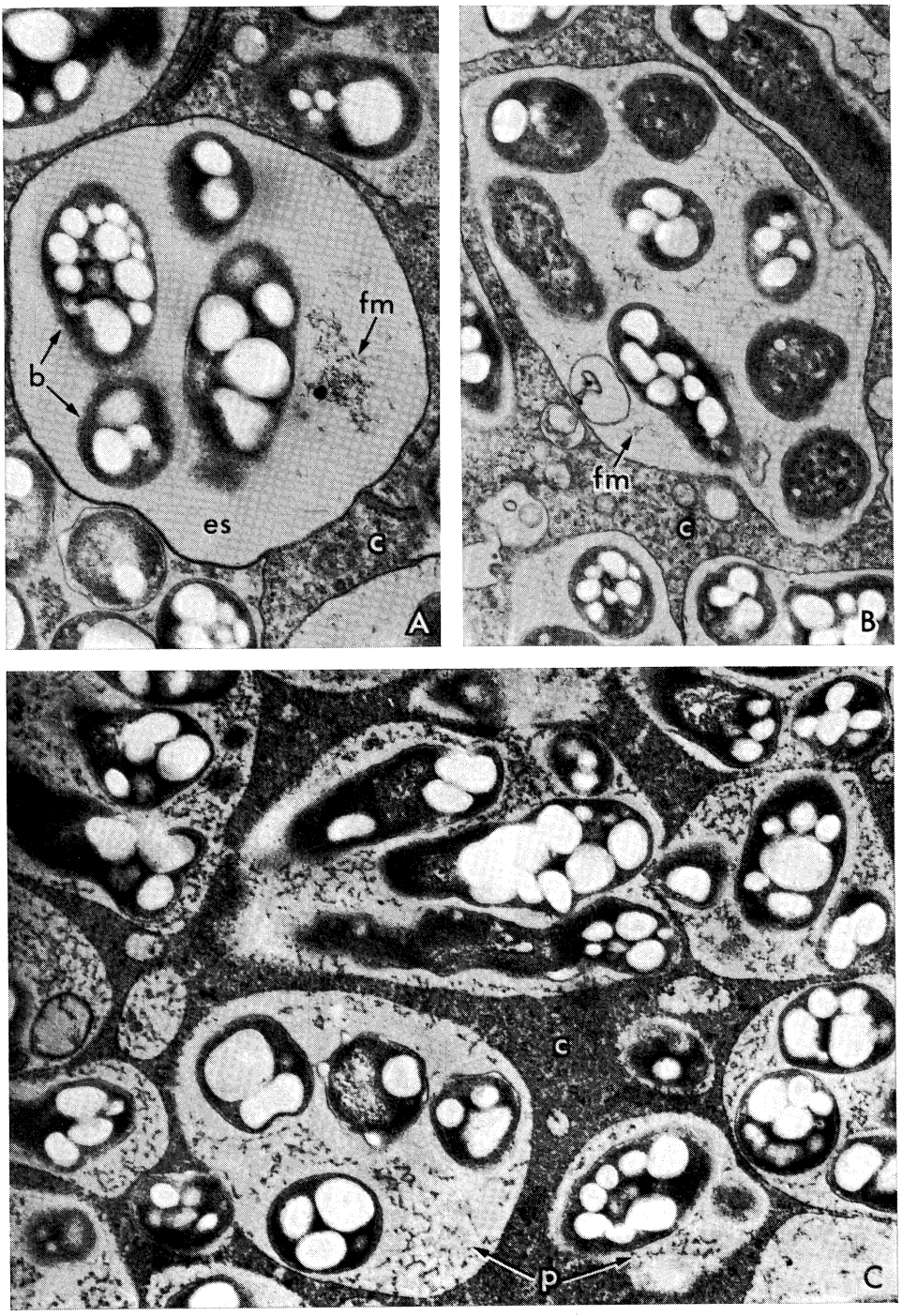


Fig. 3.—Electron micrographs of 30-day nodule tissue showing localization of Lb. ×23,000. *A*, fixation with glutaraldehyde-OsO_4 in 0·025M phosphate buffer. Bacteroids (*b*) within a membrane envelope which encloses the envelope space (*es*) and a small deposition of fibrillar material (*fm*) in the space. Host cytoplast (*c*). *B*, fixation with glutaraldehyde-OsO_4 in Honda

(*b*) *Nodule Activity, Structure, and Leghaemoglobin Content and Concentration during Growth*

In a preliminary experiment, it was found that C_2H_2 reduction was occurring while active multiplication of bacteria was continuing in young nodule cells (Table 2).

TABLE 2

DEVELOPMENT OF BACTEROIDS AND ENCLOSING MEMBRANE ENVELOPES

Counts made on electron micrographs of sections of bacteroid-containing host cells having similar orientation: means and standard deviations for 25 cell profiles of each age

	Nodule age (days)				
	3	4	5	13	22
Envelopes/host cell profile	190±94	323±142	302±95	319±82	333±74
Bacteria/host cell profile	196±96	381±194	354±117	922±270	1008±106
Bacteria/envelope	1·04±0·05	1·15±0·09	1·17±0·06	2·90±0·30	3·03±0·25
Nitrogenase activity per nodule (nmoles C_2H_4/hr)	1·3±0·5	26·7±12·6	27·6±6·2	68·2±21·2	170±30·4

The number of membrane envelopes per cell was constant from day 4 but multiplication of bacteria within them continued until day 13. These results show that increases in numbers of bacteria per envelope were not due to fusion of envelopes as suggested by Gunning (1970). The detailed counts were not repeated in the main experiment but the same trends were evident. The growth of the plants used in the main experiment and the development and activity of nodules on the upper 3·5 cm of tap root are described in Figures 4 and 5. Lb was detected when the nodules were 2 days old (Fig. 6) and increased in amount (nmoles per nodule) throughout the active life of the nodule. Acetylene reduction was detected on day 4, increased to a maximum at day 36, and declined to zero at day 58.

(i) *Relationship between Nodule Radius and the Proportion of Bacteroid-containing Tissue*

Study of median microtome sections of nodules of representative ages showed that there was a good relationship between the proportion of bacteroid-containing zone in a nodule and the radius of the nodule (Fig. 7). In order to calculate the mean volume of bacteroid-containing zone per nodule, the mean volume per nodule was obtained from nodule weight and density data (Table 3) and the radius of the equivalent sphere calculated as $r = (0{\cdot}75v/\pi)^{\frac{1}{3}}$, where v is the nodule volume. The proportion of bacteroid tissue was then read off the curve of Figure 7 and the volume of the tissue calculated. This indirect method was found to give results which were

medium showing an increased deposition of fibrillar material in the envelope space. *C*, tissue fixed with glutaraldehyde in Honda medium then treated with DAB–H_2O_2 followed by OsO_4. A dense precipitate is associated with the fibrillar material in the envelope space and there is a change in the appearance of the host cytoplasm.

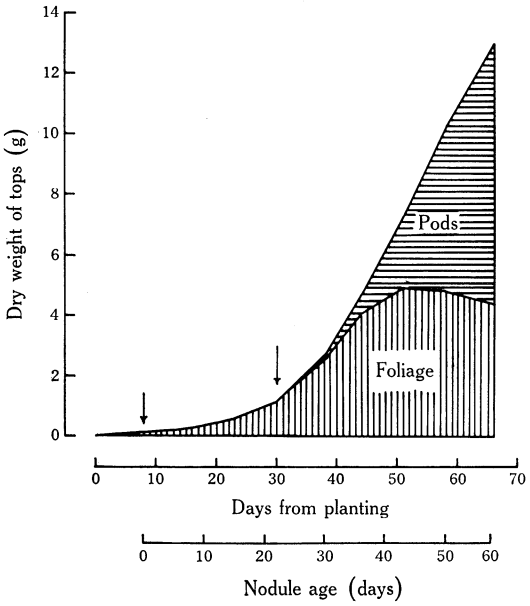


Fig. 4.—Growth of the soybean plants used for the Lb nodule activity and structural measurements in relation to nodule age.

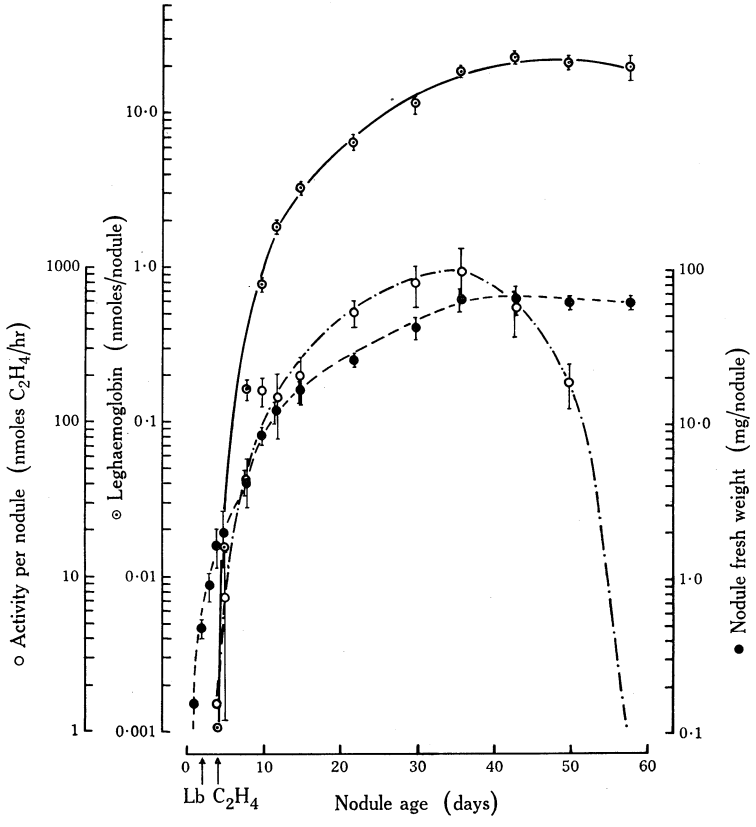


Fig. 5.—Relationship between nodule growth, activity, and Lb content. Vertical bars indicate the magnitudes of ± one standard deviation for each point.

consistent with measurements made on sections of fixed nodules (Table 3) and was much less laborious.

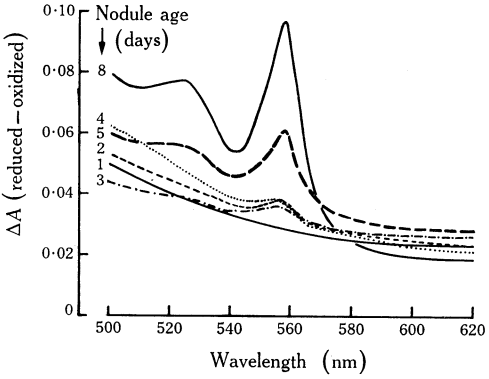
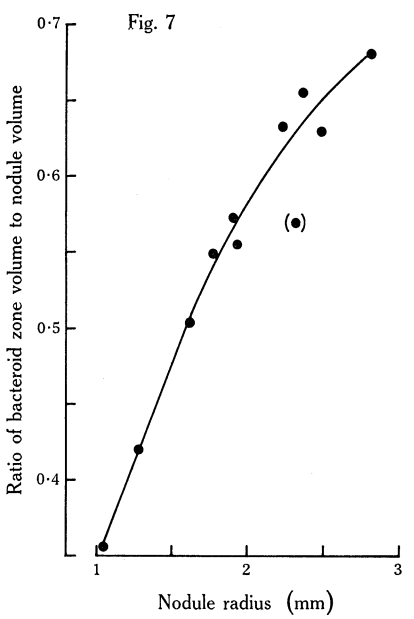


Fig. 6.—Lb in the first 8 days from nodule appearance. Difference spectra (reduced *minus* oxidized) of alkaline pyridine haemochromogens prepared from extracts of 0·5-g samples of nodules, as described in the text.



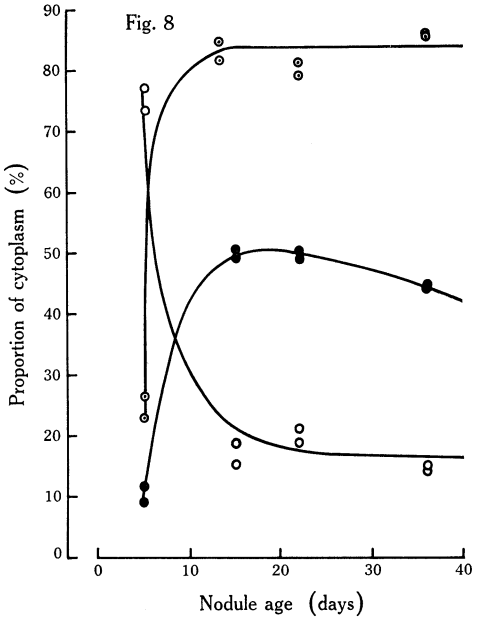


Fig. 7.—Relationship between nodule radius and the bacteroid zone as a proportion of nodule volume. Areas of zones obtained from photographs of medium sections. Radii calculated as in footnote, Table 3, and the spherical volumes calculated from *r*.

Fig. 8.—Distribution of space occupied by cytoplasm, bacteroids, and envelope space in bacteroid-containing nodule cells. ● Membrane envelope space (excluding bacteroids). ⊙ Total membrane envelope space (including bacteroids). ○ Cytoplasm (ground cytoplasm plus amyloplasts, mitochondria, and unidentified cytoplasmic components). Electron micrographs from nodules of various ages were dissected and the profile areas obtained by weighing. The nucleus was not included.

(ii) *Correction for the Interstitial Cell Component of the Bacteroid Zone*

The areas of bacteroid-containing cells and interstitial cells relative to the bacteroid zone were obtained from mosaics of prints (final magnification ×440) of

segments of nodule tissue which had been embedded for electron microscopy. Thick sections (0·15 μm) were mounted in immersion oil and photographed with phase-contrast illumination. The prints were dissected into the various component profiles, weighed, and the areas calculated. The bacteroid-containing cells occupied 75–79%

TABLE 3

ESTABLISHMENT OF NODULE VOLUMES AT VARIOUS AGES

Nodule age (days)	Nodule fresh weight (mg/nodule) ±S.D.	Nodule density (g/c.c.)*	Calculated nodule volume (mm^3/nodule)	
			From density	From sections†
8	4·10±0·79	0·815	5·0	—
10	8·31±1·16	0·865	9·13	—
12	12·48±2·59	0·910	13·72	—
15	16·80±1·79	0·970	17·32	13±3
22	25·31±0·77	1·075	25·54	26±3
30	41·76±5·97	1·135	36·80	43±12
36	65·66±10·25	1·150	57·10	—
43	64·90±6·18	1·155	56·19	57±6

* Obtained from a calibration curve obtained by displacement of water for nodules aged 8, 16, 23, and 35 days.

† Values obtained from areas of median sections, using r obtained from circles of equal area thus: $r = (\text{area}/\pi)^{\frac{1}{2}} \div \text{magnification}$. Values given as ± are the ranges from the mean.

TABLE 4

PROPORTION OF BACTEROID-CONTAINING CELLS IN THE CENTRAL TISSUE ZONE OF SOYBEAN NODULES

Areas of profiles of segment shaped samples of median nodule sections were photographed at a magnification of approximately ×440 and dissected into bacteroid-containing and interstitial cells. Areas were obtained from total weights of the profiles and the weight/cm^3 of the printing paper

Nodule age	Sample area (cm^2) (A)	Area: interstitial cells (cm^2)	Area: bacteroid-containing cells (cm^2) (B)	Ratio B/A (%)*
5	420·4	133·6	280·0	66·59
10	485·7	87·6	384·1	79·08
15	583·2	106·3	463·2	79·42
22	1030·9	221·0	772·7	74·95
30	1694·8	296·9	1330·0	78·47
36	2368·6	477·0	1822·4	76·94
43	2317·2	461·8	1789·1	77·21

* Mean and standard deviation, 10–43 days, 77·68±1·63.

of the bacteroid zone after 10 days (Table 4). The mean value of 77·7 is used in Table 5. Although there was no replication at any one age in these measurements, the consistency of the results suggests possible errors of about 2% in any one measurement.

(iii) *Calculation of Proportions of Volumes of Compartments in Bacteroid-containing Cells*

Prints of electron micrographs (final magnification 3700–6750) were examined. Up to 22 days, entire cell profiles were examined but in older nodules, the cells were too large for the largest copper grids and even when slotted grids were used, few entire cell profiles could be photographed because the cells overlapped the edges of the grid. A sampling technique was therefore devised. It was found that samples of about one-quarter of a cell profile enabled the estimation of the ratio of the membrane envelope area to the total cell cytoplasm area, with an absolute error of about $\pm 10\%$ of the value obtained by dissecting the entire cell profile (e.g. value for entire cell 0·799; values for samples 0·78–0·88). Dissection of micrographs of membrane envelope profiles showed that samples of 10 envelopes gave estimates of the ratio of the total bacteroid area to the membrane envelope area which varied by only about $\pm 4\%$ in any one cell (e.g. mean 0·50, range 0·48–0·52). Figure 8 gives the results obtained for the distribution of the various space components within bacteroid-containing cells of a range of ages.

(iv) *Adjustment for Host Cell Nucleus*

The host nucleus did not contain Lb and its volume was excluded from the Lb-containing volume of the bacteroid-containing cells. The volumes of nuclei in nodules of various ages were estimated from electron-micrograph profile areas, as equivalent spheres by applying the relation: volume $= 1 \cdot 382 \times \text{area}^{\frac{3}{2}}$ (Lindberg and Vorwerk 1970). The total nuclear volumes were obtained by multiplying by the number of bacteroid-containing cells per nodule, obtained from counts of median sections. The resulting volumes were not large enough to affect the results very much, but they were included in the calculations.

(v) *Concentration of Leghaemoglobin in the Membrane Envelope Space*

Table 5 gives the derivation of the volume per nodule of the Lb-containing membrane envelope space (V_{Lb}), for nodules aged 8–43 days. It was obtained from the following operational expression:

$$V_{\text{Lb}} = (V_B f - V_N)E,$$

where

V_B = bacteroid zone volume;

f = correction factor relating to the ratio of the volume occupied by bacteroid cells to that of the bacteroid zone;

V_N = nuclear volume per nodule; and

E = ratio of envelope space to host cell volume.

The bacteroid zone volume was read from the curve of Figure 7, using mean radius of equivalent sphere values (Table 5). The Lb concentrations have been presented (Table 6) on a bacteroid zone basis for comparison with other work, on a host cell basis to show the adjustment necessary because of the presence of interstitial cells in the bacteroid zone, and finally on the basis of the Lb being confined within the membrane envelopes. It is seen that the concentration of the haemoprotein increases

throughout the functional life of the nodules, as well as increasing in total amount per nodule (Fig. 5).

TABLE 5

DERIVATION OF THE VOLUME OF LEGHAEMOGLOBIN-CONTAINING SPACE PER NODULE FOR NODULES OF DIFFERENT AGES

See text, Section III(*b*)(v) for definition of symbols and for equation used to calculate V_{Lb}

Nodule age (days)	Nodule radius* (mm)	V_B† (mm^3)	f‡ (%)	E§	V_N‖ (mm^3)	V_{Lb} (mm^3)
8	1·06	1·83	75·0	0·345	0·054	0·455
10	1·30	3·88	77·7	0·435	0·070	1·281
12	1·40	6·20	77·7	0·460	0·086	2·176
15	1·61	8·78	77·7	0·495	0·105	3·324
22	1·78	12·83	77·7	0·495	0·140	4·864
30	2·06	21·90	77·7	0·470	0·157	7·922
36	2·39	36·54	77·7	0·445	0·140	12·569
43	2·38	35·85	77·7	0·400	0·088	11·104

* Calculated as described in Section III(*b*)(i).

† Calculated from nodule radii and the data of Figure 5.

‡ Obtained from dissected phase-contrast micrographs of segments of nodule tissue.

§ Obtained from the curve of Figure 6.

‖ Obtained from electron micrographs of nuclei and counts of cell numbers in median sections.

TABLE 6

PHYSIOLOGICAL CONCENTRATION OF LEGHAEMOGLOBIN

Nodule age (days)	Lb per nodule (nmoles)	Lb concentration (mM)		
		Bacteroid zone basis	Host cell basis	Membrane envelope space basis
8	0·165±0·025	0·09	0·12	0·36
10	0·785±0·024	0·20	0·26	0·61
12	1·87±0·14	0·30	0·39	0·86
15	3·31±0·08	0·38	0·49	1·00
22	6·59±0·68	0·51	0·66	1·35
30	11·77±1·32	0·54	0·70	1·49
36	19·21±0·86	0·53	0·68	1·53
43	23·53±0·78	0·66	0·85	2·12

IV. DISCUSSION

We consider that the evidence presented clearly shows that soybean nodule Lb is located within the space between the bacteroid surfaces and the membrane envelopes which enclose them in groups within the host cytoplasm (Goodchild and Bergersen 1966). We also believe that the haemoprotein is probably confined to this space and is not also present in the host cytoplasm. However, the evidence for this is less conclusive, resting mainly on the observation that the cytoplasm between the

envelopes was unstained when fresh tissue was treated with DAB oxide. It was not possible to say with certainty that the electron-dense precipitate seen in electron micrographs of DAB oxide was present only within the envelope space, because of the electron density of the cytoplasm, which would have obscured it. We attribute previous failure to observe localization of Lb within the envelopes to the following factors: (1) failure to ensure that the haemoprotein did not leak out of the envelopes during fixation; (2) failure to recognize the extent to which haem is lost from the pigment during fixation by glutaraldehyde; (3) failure to appreciate that DAB oxide would not react well with Lb under reducing conditions.

The values for Lb concentration on a per bacteroid zone basis (Table 6) are similar to that obtained by Smith (1949). Appleby (1969*b*, Fig. 2) gives an absorption spectrum of pyridine haemochromogen found in 1-mm thick sections of a 2·5-mm diameter nodule, which corresponds to a concentration of 0·14 mM (cf. 0·2 mM in 10-day nodules, Tables 5 and 6). However, when further allowance is made for the cellular compartmentation of the pigment, the functional concentration is seen to be about three times higher (Table 6).

The data presented support the physiological role of Lb in stimulating oxygen-uptake and nitrogenase activity by the bacteroids which was proposed from recent experiments (Bergersen *et al.* 1973), since the haemoglobin is in contact with the bacteroid surface. It is of interest to consider the oxygen-concentrating effect which results from the high affinity of Lb for oxygen. Let us consider the nodule used by Appleby (1969*b*, Fig. 5) which in air contained about 20–25% of its Lb as LbO_2. The concentration of Lb in the membrane envelopes of a nodule of this size was 0·6 mM (Table 6), and the oxyLb concentration would be 0·12–0·15 mM. Under steady-state conditions, the free oxygen concentration giving this degree of oxygenation can be calculated to be 9–12 nM from the equilibrium constant K given by Wittenberg *et al.* (1972) at pH 6·8 as follows:

$$K = [LbO_2]/[Lb][O_2] = 27 \times 10^6 \text{ M}^{-1}.$$

Thus, if a bacteroid oxidase could react with oxyLb as suggested previously (Bergersen *et al.* 1973), respiration could proceed under conditions in which oxygen consumption would otherwise be negligible because of its low concentration. It is noteworthy that the Lb concentration increased as the nodule size increased, thus tending to maintain favourable conditions because free oxygen concentrations within the cells would be expected to fall as a result of the increased length of the diffusion pathway from the nodule exterior.

Nitrogenase activity was detected and increased in the period up to a nodule age of 13 days. During this period the bacteria in the host cells increased fivefold in number (Table 2), and Lb per nodule increased more than 1000-fold (Fig. 5). These observations show that the nitrogen-fixing system is being actively developed during bacterial growth and casts doubt on the long-held view that nitrogen fixation is a property only of the non-growing form of the bacteria, which are known as bacteroids.

V. Acknowledgments

The authors thank Mrs. M. A. Jepperson and Miss Philippa Riddiford for their assistance in this project.

VI. References

APPLEBY, C. A. (1969*a*).—Electron transport systems of *Rhizobium japonicum*. I. Haemoproteins P-450, other CO-reactive pigments, cytochromes and oxidases in bacteroids from N_2-fixing root nodules. *Biochim. biophys. Acta* **172**, 71–87.

APPLEBY, C. A. (1969*b*).—Properties of leghaemoglobin *in vivo*, and its isolation as ferrous oxyleghaemoglobin. *Biochim. biophys. Acta* **188**, 222–9.

BERGERSEN, F. J. (1970).—The quantitative relationship between nitrogen fixation and the acetylene-reduction assay. *Aust. J. biol. Sci.* **23**, 1015–25.

BERGERSEN, F. J. (1971).—Biochemistry of symbiotic nitrogen fixation in legumes. *A. Rev. Pl. Physiol.* **22**, 121–40.

BERGERSEN, F. J., and GOODCHILD, D. J. (1973).—Aeration pathways in soybean root nodules. *Aust. J. biol. Sci.* **26**, 729–40.

BERGERSEN, F. J., and TURNER, G. L. (1970).—Gel filtration of nitrogenase from soybean root nodule bacteroids. *Biochim. biophys. Acta* **214**, 28–36.

BERGERSEN, F. J., TURNER, G. L., and APPLEBY, C. A. (1973).—Studies of the physiological role of leghaemoglobin in soybean root nodules. *Biochim. biophys. Acta* (In press.)

DART, P. J. (1968).—Localization of perioxidase activity in legume root nodules. Proc. 4th Eur. Reg. Conf. Electron Microscopy, Rome. pp. 69–70.

DILWORTH, M. J., and KIDBY, D. K. (1968).—Localization of iron and leghaemoglobin in the legume root nodule by electron microscope autoradiography. *Exptl. Cell Res.* **49**, 148–59.

FISKE, S. (1966).—An adaptation of Reynold's lead citrate stain for high resolution autoradiography. *J. Microscopie* **5**, 355–60.

GOODCHILD, D. J., and BERGERSEN, F. J. (1966).—Electron microscopy of the infection and subsequent development of soybean root nodule cells. *J. Bac.* **92**, 204–13.

GUNNING, B. E. S. (1970).—Lateral fusion of membranes in bacteroid-containing cells of leguminous root nodules. *J. Cell Sci.* **7**, 307–17.

GURR, E. (1953).—"A Practical Manual of Medical and Biological Staining Techniques." (Leonard Hill: London.)

HIRAI, K. (1968).—Specific affinity of oxidized amine dye (radical intermediator) for heme enzymes: study in microscopy and spectrophotometry. *Acta Histochem. Cytochem.* **1**, 43–55.

HIRAI, K. (1971).—Comparison between 3,3′-diaminobenzidine and auto-oxidized 3,3′-diaminobenzidine in the cytochemical demonstration of oxidative enzymes. *J. Histochem. Cytochem.* **19**, 434–42.

HONDA, S. I., HONGLADARUM, T., and LATIES, G. G. (1966).—A new isolation medium for plant organelles. *J. Exp. Bot.* **17**, 460–72.

LINDBERG, L. G., and VORWERK, P. (1970).—On calculating volumes of transected bodies from two dimensional micrographs. *Lab. Invest.* **23**, 315–17.

SMITH, J. D. (1949).—The concentration and distribution of haemoglobin in the root nodules of leguminous plants. *Biochem. J.* **44**, 585–91.

TJEPKEMA, J. D. (1971).—Oxygen transport in the soybean nodule and the function of leghaemoglobin. Ph.D. Thesis, University of Michigan, U.S.A.

TJEPKEMA, J. D., and YOCUM, C. S. (1970).—Leghaemoglobin facilitated oxygen diffusion in soybean nodule slices. *Pl. Physiol.* **46** (Suppl.), 44.

TRUCHET, G. (1972).—Mise en évidence de l'activité peroxydasique dans les différentes zones des nodules radiculaires de pois (*Pisum sativum* L.). Localisation de la leghémoglobine. *C.r. hebd. Séanc. Acad. Sci., Paris* **274**, 1290–3.

WITTENBERG, J. B., APPLEBY, C. A., and WITTENBERG, B. A. (1972).—The kinetics of the reactions of leghaemoglobin with oxygen and carbon monoxide. *J. biol. Chem.* **247**, 527–31.