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Electron-Paramagnetic-Resonance Studies of Leghaemoglobins from Soya-Bean and Cowpea Root Nodules

IDENTIFICATION OF NITROSYL-LEGHAEMOGLOBIN IN CRUDE LEGHAEMOGLOBIN PREPARATIONS

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1. Leghaemoglobins from soya-bean (*Glycine max*) and cowpea (*Vigna unguiculata*) root nodules were purified by chromatography on DEAE-cellulose phosphate columns at pH 8.0 and pH 5.8, to avoid the relatively low pH (5.2) commonly used to purify these proteins. 2. E.p.r. (electron-paramagnetic-resonance) spectra of the fluoride, azide, hydroxide and cyanide complexes of these ferric leghaemoglobins were very similar to the spectra of the corresponding myoglobin derivatives, indicating that the immediate environment of the iron in leghaemoglobin and myoglobin is similar, an imidazole moiety of histidine being the proximal ligand to the haem iron [cf. Appleby, Blumberg, Peisach, Wittenberg & Wittenberg (1976) *J. Biol. Chem.* **251**, 6090–6096]. 3. E.p.r. spectra of the acid-metleghaemoglobins showed prominent high-spin features very near $g = 6$ and $g = 2$ and, unlike myoglobin, small low-spin absorptions near $g = 2.26$, 2.72 and 3.14. The width of the $g = 6$ absorption derivative at 10–20K was about 4–4.5mT, similar to the value for acid-methaemoglobin. In contrast, a recently published (Appleby *et al.*, 1976) spectrum of acid-metleghaemoglobin *a* had less high-spin character and a much broader absorption derivative around $g = 6$. 4. E.p.r. spectra of ferric leghaemoglobin nicotinate and imidazole complexes suggest that the low-spin absorption near $g = 3.14$ can be attributed to a trace of ferric leghaemoglobin nicotinate, and those near $g = 2.26$ and 2.72 are from an endogenous dihistidyl haemichrome. 5. A large e.p.r. signal at $g = 2$ in all samples of crude leghaemoglobin was shown to be from nitrosyl-leghaemoglobin. A soya-bean sample contained $27 \pm 3\%$ of the latter. A previously unidentified form of soya-bean ferrous leghaemoglobin *a* was shown to be its nitrosyl derivative. If this is not an artifact, and occurs in the root nodule, the nitrosyl radical may interfere with the function of leghaemoglobin.

Leghaemoglobin, the haemoglobin of legume root nodules, is an essential requirement for nitrogen fixation in these structures. It does not, however, participate directly in nitrogen fixation, but occurs in the nodule cells that contain the bacteroids, the actual site of nitrogen fixation. Recent evidence suggests that leghaemoglobin functions by facilitating the diffusion of O_2 to the nodule bacteroids, free O_2 being delivered to the bacteroids' surface at a stable low concentration (Bergersen *et al.*, 1973; Wittenberg *et al.*, 1974).

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Crude leghaemoglobin from soya-bean root nodules can be fractionated into two major ferric components, leghaemoglobin *a* and leghaemoglobin *c*, by using a DEAE-cellulose acetate column at pH 5.2 (Ellfolk, 1960). This method, and minor variations of it, is most commonly used to purify leghaemoglobins. Appleby *et al.* (1975) have developed an improved separation of crude soya-bean (*Glycine max*) leghaemoglobin on DEAE-cellulose acetate columns at pH 5.2. Their method resolves leghaemoglobin *c* into two similar but distinct proteins, leghaemoglobins c_1 and c_2. The soya-bean leghaemoglobins have mol.wts. of approx. 15400 (*a*) and 16700 (unfractionated *c*) (Ellfolk, 1961). They contain one protohaem IX group per molecule (Ellfolk & Sievers, 1965) and appear to be monomeric in solution (Behlke *et al.*, 1971). Cowpea leghaemoglobin also has protohaem IX as its prosthetic group (Jack-

son & Evans, 1966) and mol.wt. of 16000–17000 (C. S. Maskall, unpublished work). The leghaemoglobins thus resemble myoglobin. Leghaemoglobins *a*, c_1 and c_2 have two histidine residues per molecule and no sulphur-containing amino acids (Ellfolk, 1961; Appleby *et al.*, 1975). The sequence of leghaemoglobin *a* is known (Ellfolk, 1972), and comparisons with the sequence of the γ-chain from human haemoglobin and with other types of haemoglobin and myoglobin indicate a considerable similarity among leghaemoglobin, myoglobin and haemoglobin (Appleby, 1974). Circular-dichroism spectra of leghaemoglobin *a* show that it has an α-helix content of 55% (cf. 72.5% for myoglobin) and that the association between the haem group and globin is weaker in this and other leghaemoglobins than it is in myoglobin (Nicola *et al.*, 1975). These findings are consistent with those of Ellfolk *et al.* (1974), who demonstrated by urea-denaturation studies that leghaemoglobin *a* is a less stable molecule than myoglobin.

The similarity between the optical spectra of leghaemoglobin derivatives and those of the corresponding haemoglobin and myoglobin derivatives suggests that the immediate environment of the iron atom in the three proteins is similar (Appleby, 1974). X-ray-diffraction studies by Vainshtein *et al.* (1975) on lupin leghaemoglobin indicate that it has a tertiary structure closely resembling that of myoglobin and haemoglobin. The e.p.r. (electron-paramagnetic-resonance) investigation by Appleby *et al.* (1976) has shown that the fluoride and low-spin complexes of leghaemoglobin have very similar e.p.r. spectra to those of the corresponding myoglobin derivatives. However, these authors found that the e.p.r. spectrum of acid-metleghaemoglobin *a* (the ferric form of leghaemoglobin *a* with H_2O attached) differed significantly from that of acid-metmyoglobin.

The aim of the present work was to purify leghaemoglobins by using a procedure not involving prolonged exposure of the proteins to the relatively low pH (5.2) most commonly used, and then to compare by e.p.r. spectroscopy the immediate environment of the iron atom in the soya-bean and cowpea leghaemoglobins with that in myoglobin and haemoglobin.

Experimental

Materials

Reagents were of analytical grade unless otherwise stated and were usually obtained from BDH Chemicals, Poole, Dorset, U.K. Nicotinic acid was from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Polyclar AT was a gift from GAF (G.B.) Limited, Manchester, U.K. DEAE-cellulose (DE52) was obtained from Whatman Products, H. Reeve Angel, London E.C.4, U.K., and Sephadex G-25 from Pharmacia (G.B.), London W.5, U.K. NO gas (minimum purity 99%) was from Matheson Gas Products, Cambrian Chemicals, Croydon CR9 3QL, Surrey, U.K.

All solutions were made up in single-distilled water from an all-glass still. Phosphate buffers were made by mixing stock solutions (0.1 M) of Na_2HPO_4 and KH_2PO_4 to the stated pH (Gomori, 1955), and diluting as required.

Growth of plants

Seeds of soya bean (*Glycine max*), var. Merit, Altona and Chippewa, and cowpea (*Vigna unguiculata*), var. Poona, were surface-sterilized by rinsing quickly with 95% (v/v) ethanol followed by immersion for 3 min in a 0.28% solution of $HgCl_2$. The seeds were then rinsed six times with tap water and inoculated with the required *Rhizobium* strain by immersion in a broth culture of it for 2–3 min. (Cultures of *Rhizobium* were maintained on slants of agar containing mineral salts, yeast extract and mannitol. Flasks of broth containing these nutrients were inoculated either from a slant culture or with the contents of a freeze-dried ampoule and incubated with shaking at 25°C. *Rhizobium* strains used were CC705 and CB1809 for soya beans and CB756 and SU318 for cowpeas.) The seeds were then planted in boxes of Perlite (British Gypsum, Robertsbridge, Sussex, U.K.) which had been previously soaked in a nitrogen-free culture solution (Dart & Pate, 1959). Plants were grown in a glasshouse at about 27°C (day) and 24°C (night). They were watered with the above culture solution, diluted 1:4 (v/v) with tap water which contained 7 p.p.m. of N as NO_3^-. Cowpeas were also grown in 15 cm (diam.) pots containing sand/grit (2:1, v/v) in controlled-environment cabinets maintained at the same temperature as the glasshouse; day length 16 h, illumination 25000 lx from warm-white fluorescent tubes. For more recent work, soya beans (var. Merit and Altona) were grown in the field in a light sandy soil, low in available N. Surface-sterilized seeds were inoculated just before planting with a broth culture of the required *Rhizobium* strain diluted with an equal volume of 20% (w/v) sucrose solution.

Plants were harvested at flowering to early pod-fill stage, and the roots and nodules washed with tap water. Nodules were picked within a few hours of harvesting, immediately stored on ice and crushed on the same day.

Isolation and purification of leghaemoglobin

Nodules were crushed in a Pirie (1961) press or a press based on the modified Pirie press of McArthur & Miltimore (1964) into 20 mM-phosphate buffer, pH 7.2, containing 10% (w/v) of an insoluble cross-linked polyvinylpyrrolidone, Polyclar AT. (In early soya-bean-nodule extractions and all the cow-

pea-nodule extractions, 0.2M-sodium ascorbate and 1mM-$MgCl_2$ were added to the medium; omitting them did not appear to cause any deterioration of the extracted soya-bean leghaemoglobin.) Nodules and ice-cold medium were loaded into the press, which had been cooled to 4°C (about 1g fresh wt. of nodules/ml of medium). The annular gap was approximately 9μm for the Pirie press and 10μm for the modified press. Oxygen-free nitrogen was flushed through the press for at least 5min and the nodules were then crushed into the medium. Subsequent procedures were performed on ice or at 4°C. The homogenate was collected in a N_2-flushed flask, filtered through two layers of bolting cloth to remove solid material and centrifuged for 10min at 12000*g* in gas-tight tubes previously flushed with N_2. The supernatant was then fractionated with solid $(NH_4)_2SO_4$ and the fraction precipitated between 55% and 80% saturation (designated crude leghaemoglobin) was collected. The precipitate was dispersed in 60%-satd. $(NH_4)_2SO_4$ solution and stored in liquid N_2.

Determination of leghaemoglobin

Leghaemoglobin was measured either by the pyridine haemochromogen method by using ε_{mM} at 556nm as 34.6 (Paul *et al.*, 1953) or by the cyanomethaemoglobin method (Wilson & Reisenauer, 1963).

Column chromatography on DEAE-cellulose

Procedures were performed at 4°C unless otherwise stated. Crude leghaemoglobin was dissolved in 20mM-sodium/potassium phosphate buffer, pH7.0, left on ice for 1h and then centrifuged at 20000*g* for 10min to remove the small amount of insoluble protein present. The supernatant was desalted on a column (2cm×28cm) of Sephadex G-25 (fine or medium grade) and the leghaemoglobin fraction concentrated to a volume of less than 3.5ml by ultrafiltration over an Amicon UM10 membrane (Amicon, High Wycombe, Bucks., U.K.). It was then passed through another column (1.2cm×18cm) of Sephadex G-25 (fine grade) to remove any remaining traces of salt, and then oxidized by incubation at 4°C for 2h with $K_3Fe(CN)_6$ (Geyer & Lemberg, 1971). Purified ferric leghaemoglobins were obtained by chromatography on DEAE-cellulose. The oxidized crude leghaemoglobin was equilibrated on a column (2cm×28cm) of Sephadex G-25 (fine or medium grade) with 2mM-phosphate buffer, nominally pH8.0. (At this dilution the actual pH of the buffer was about 7.7.) The solution was left overnight on ice and then centrifuged at 29000*g* for 20min to remove the small amount of insoluble protein present. About 60mg of this leghaemoglobin was loaded on a column (2.2cm×10cm) of DEAE-cellulose, equilibrated with 2mM-phosphate buffer, nominally pH8.0. Starting buffer and stepwise elution were used at a rate of 80ml/h, and 1.5–3.0ml fractions were collected. Tubes containing ferric leghaemoglobin were pooled and concentrated by ultrafiltration over a UM10 membrane.

Two brown–red ferric leghaemoglobins (*a* and *c*) and, unexpectedly, two bright-red ferrous leghaemoglobins were eluted from the soya-bean column. For crude cowpea leghaemoglobin, one ferric and one ferrous leghaemoglobin were eluted. Approx. 80mg of ferric leghaemoglobin, pooled from two pH8.0 columns, was then equilibrated on a Sephadex G-25 column with 2mM-phosphate buffer, nominally pH5.8, and loaded on a column (2.2cm×20cm) of DEAE-cellulose that had been equilibrated with the same buffer. This column was developed by starting-buffer elution, followed by a linear gradient of 2mM- to 20mM-phosphate buffer, pH5.8 (500g of each buffer). Further elution with 20mM-phosphate buffer, pH5.8, was required to elute the second major soya-bean leghaemoglobin (*c*). The contents of tubes containing an individual ferric leghaemoglobin were pooled and concentrated by ultrafiltration to a volume of approx. 1.5ml and then stored in liquid N_2.

Polyacrylamide-gel electrophoresis

Purity of the isolated ferric leghaemoglobins was checked by polyacrylamide-gel electrophoresis: 7.5% (w/v) acrylamide gels were run at pH8.9, the system being similar to that of Davis (1964). Electrophoresis was performed at 4°C for 60min at a voltage of 200V and a current of 5mA/gel. Gels were stained either for protein with Amido Black [1% (w/v) in 7% (v/v) acetic acid] or for peroxidase activity by the method of Seevers *et al.* (1971).

Preparation of ferric leghaemoglobin derivatives for e.p.r. spectroscopy

All procedures were carried out at 0–4°C. Approx. 0.3–0.5ml of purified ferric leghaemogolobin solution (12–18mg of leghaemoglobin) was passed down a column (1.2cm×18cm) of Sephadex G-25 (fine grade) equilibrated with 20mM-phosphate buffer, pH7.0, to prepare the acid-metleghaemoglobin derivatives, and the same buffer containing the appropriate dissolved compound to prepare the F^-, N_3^-, CN^- and imidazole derivatives. Molar ratios of low-molecular-weight ligands to leghaemoglobin were: F^- (NaF), 10:1; N_3^- (NaN_3, Laboratory Reagent grade), 5:1; CN^- (KCN), 3:1 (pH of this solution was 7.4); imidazole (Laboratory Reagent grade), 10:1 (pH of solution adjusted to 7.0 with 0.3M-H_3PO_4); and nicotinate, 3:1 (nicotinic acid dissolved in 20mM-phosphate buffer, pH6.8, and the pH adjusted to 6.9 with 1M-NaOH). For leghaemoglobin hydroxides the column was equilibrated with glycine/NaOH buffer, pH9.6 [0.05M in glycine

(chromatographically homogeneous)]. The solutions of leghaemoglobin derivatives were then concentrated to a volume of about 0.3ml (1–2mM-leghaemoglobin) by using dry Sephadex G-25 (coarse grade), introduced into an e.p.r. tube, frozen and stored in liquid N_2.

Preparation of ferrous nitrosyl-leghaemoglobin

Soya-bean leghaemoglobin *c*, purified by DEAE-cellulose phosphate chromatography at pH8.0, was converted into oxyleghaemoglobin *c* by the method of Dixon & McIntosh (1967). This solution of oxyleghaemoglobin *c* was converted into nitrosyl-leghaemoglobin *c** by the method of Henry & Banerjee (1973).

E.p.r. measurements

E.p.r. spectra were recorded on a Varian E-12 e.p.r. spectrometer by using 100kHz magnetic-field modulation and operating at about 9.2GHz. Samples run above 77K were cooled by using a Varian variable-temperature accessory. Samples run below 77K were cooled by He gas boiled directly from the liquid.

Results and Discussion

Chromatographic separation of soya-bean leghaemoglobin

The order of elution of the leghaemoglobin fractions from the pH8.0 columns was: (i) leghaemoglobin *a* followed by 'oxyleghaemoglobin *a*' (2mM-phosphate, nominal pH8.0) (all 'oxyleghaemoglobin' fractions contained nitrosyl-leghaemoglobin; see under 'Presence of nitrosyl-leghaemoglobin in samples of crude leghaemoglobin'); (ii) leghaemoglobin *c* followed by 'oxyleghaemoglobin *c*' (10mM-phosphate, pH8.0). Two minor leghaemoglobin fractions were eluted with 20mM-phosphate buffer, pH8.0, and then 100mM-phosphate buffer, pH8.0, was used to elute a protein fraction that, when examined by polyacrylamide-gel electrophoresis, showed six bands having peroxidase activity and no red leghaemoglobin bands. It was therefore designated the 'peroxidase fraction'. The two leghaemoglobin *c* components, c_1 and c_2, were not resolved by DEAE-cellulose phosphate chromatography at pH8.0 or pH5.8, and polyacrylamide-gel electrophoresis of crude leghaemoglobin at pH8.9 and pH6.6 gave only one leghaemoglobin *c* band. These results are in agreement with those of Appleby *et al.* (1975).

The mobilities of the leghaemoglobins relative to Bromophenol Blue (polyacrylamide-gel electrophoresis at pH 8.9) were: leghaemoglobin *a*, 0.54; leghaemoglobin *c*, 0.74. The fractions 'oxyleghaemoglobins' *a* and *c* had the same relative mobilities as their respective ferric forms. The elution profile of crude soya-bean leghaemoglobin (Appleby, *et al.*, 1975) shows that the amounts of leghaemoglobin *b* and d_1+d_2 (incompletely separated) are small relative to the amounts of leghaemoglobin *a* and c_1+c_2. Thus these relatively minor components would not have been detectable in the column separation at pH8.0. They may have been present in the 'oxyleghaemoglobin' fractions.

Chromatographic separation of cowpea leghaemoglobin

For crude cowpea leghaemoglobin the order of elution from the pH8.0 columns was: (i) ferric leghaemoglobin (2mM-phosphate, nominal pH8.0); (ii) 'oxyleghaemoglobin' (10mM-phosphate, pH8.0). One minor leghaemoglobin fraction remained on the column.

Polyacrylamide-gel electrophoresis at pH8.9, of these leghaemoglobins showed that both had the same mobility relative to Bromophenol Blue, 0.64, indicating that cowpea has only one major leghaemoglobin. However, as with soya-bean leghaemoglobin, the amount of crude leghaemoglobin applied to the column was too small to reveal any minor fractions that might have been eluted with the two major fractions.

Purity of the isolated ferric leghaemoglobins

Polyacrylamide-gel electrophoresis of the ferric leghaemoglobins eluted from the pH5.8 columns showed that they were only slightly contaminated with peroxidases. One faint peroxidase band was visible in gels of leghaemoglobins *a* and *c* and cowpea leghaemoglobin, but no corresponding bands were visible in duplicate gels stained for protein. Comparison of gels of ferric leghaemoglobins from the pH8.0 and pH5.8 columns showed that the second column had effected a further purification. Leghaemoglobin *a* and cowpea leghaemoglobin contained a major contaminant that had no peroxidase activity and probably accounted for less than 10% of the total protein. Leghaemoglobin *c* contained much less of this contaminant, probably a product of leghaemoglobin catabolism. The e.p.r. spectra of acid-met-leghaemoglobins *a* and *c* were basically very similar (Figs. 1*a* and 1*b*), indicating that although the contaminant may contain iron, it gives no e.p.r. signal under these conditions.

High-spin ferric derivatives

The three fluoride derivatives of leghaemoglobin had almost identical e.p.r. spectra, closely resembling the e.p.r. spectrum of the fluoride derivative of myoglobin (Peisach *et al.*, 1971), indicating that the fluoride derivative of leghaemoglobin is predominantly,

* The prefix 'ferrous' is omitted from nitrosyl-leghaemoglobin and nitrosylhaemoglobin because only the ferrous haemoprotein NO complexes are considered in the present paper. Also, in 'oxyleghaemoglobin' the prefix 'ferrous' or 'ferric' is omitted because only the ferrous protein binds O_2.

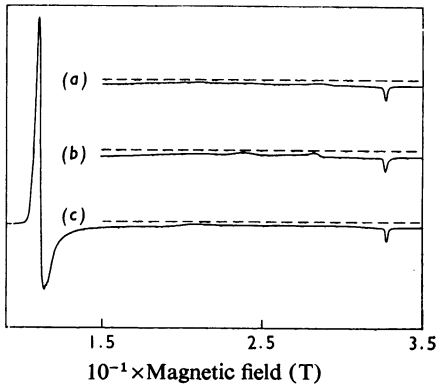

Fig. 1. *E.p.r. spectra of acid-metleghaemoglobin at pH7.0*
Leghaemoglobin samples (1–2mM) were prepared and their e.p.r. spectra recorded as described in the Experimental section. Soya-bean leghaemoglobins were from Merit CC705 nodules; (see under 'Growth of plants'). (*a*) Soya-bean leghaemoglobin *a* at 13K; (*b*) soya-bean leghaemoglobin *c* at 11K; (*c*) cowpea leghaemoglobin at 20K. For all three samples the power was 2mW. The broken line over each trace was drawn parallel to the grid line to accentuate the slight undulations in the trace. The spectra in the region of 0.1T in (*a*) and (*b*) were similar to that shown in (*c*).

if not entirely, high-spin. The doublet at $g=2$ proves that the F^- is ligated to the haem iron. The superhyperfine splitting measured from this doublet was 4.3±0.2mT for all three derivatives; cf. 4.4mT for the fluoride derivative of myoglobin (Peisach *et al.*, 1971).

In agreement with the results of Appleby *et al.* (1976) for leghaemoglobin *a*, this demonstrates that the immediate environment of the iron atom in the fluoride derivatives of leghaemoglobin and myoglobin is similar, with a nitrogen atom of the imidazole ring of a histidine residue occupying the fifth coordination position of the iron and F^- occupying the sixth position. For soya-bean leghaemoglobin *a* this histidine is almost certainly histidine-92 of the protein's sequence, as suggested by Ellfolk (1972).

The *g* values of the prominent high-spin features of the acid-metleghaemoglobins were very close to 6.0 (g_x and g_y) and 2.0 (g_z). (Fig. 1 and Table 1.) The width between the derivative extrema of the absorption derivative at $g=6$ was approx. 4.5mT for leghaemoglobins *a* and *c* and approx. 4.0mT for cowpea leghaemoglobin (spectra recorded at 10–20K). This is compatible with the value of 4.1mT measured for haemoglobin at 1.4K (Peisach *et al.*, 1971), but contrasts with the acid-metleghaemoglobin *a* value of

Table 1. *Effective g values of high-spin ferric leghaemoglobin derivatives*
Derivatives were prepared and e.p.r. spectra recorded at 10–40K as described in the Experimental section. The soya-bean leghaemoglobins were from Merit CC705 nodules. The *g* values of the low-spin features in the acid-metleghaemoglobin spectra were also measured.

Acid-metleghaemoglobin derivative	*g* values			Superhyperfine splitting (mT)
	g_x	g_y	g_z	
Soya-bean leghaemoglobin *a* at pH7.0	5.99	5.99	2.00	
Soya-bean leghaemoglobin *c* at pH7.0	6.01	6.01	2.00	
Cowpea leghaemoglobin, Poona CB756:				
at pH7.0	5.99	5.99	2.00	
at pH5.8	5.98	5.98	2.00	
Cowpea leghaemoglobin, Poona SU318 at pH7.0*	5.97	5.97	1.99	
Fluoride at pH7.0				
Soya-bean leghaemoglobin *a*	6.04	6.04	2.00	4.3±0.2
Soya-bean leghaemoglobin *c*	6.04	6.04	2.00	4.3±0.2
Cowpea leghaemoglobin, Poona CB756	6.05	6.05	2.00	4.3±0.2

g values of low-spin features in the acid-metleghaemoglobin spectra

	g_x	g_y	g_z	g'_x‡	g'_y	g'_z
Soya-bean leghaemoglobin *a*	†	2.26	2.72	†	†	3.14
Soya-bean leghaemoglobin *c*	†	2.28	2.73	†	†	3.14
Cowpea leghaemoglobin, Poona CB756 at pH7.0	†	2.24	†	†	†	3.10

* Sample purified by one chromatographic separation at pH8.0.
† Unobservable.
‡ The primed symbols refer to a second low-spin species.

Appleby *et al.* (1976); see the end of this section. Small low-spin absorptions were also present in the leghaemoglobin *a* and leghaemoglobin *c* spectra near $g = 2.26$, 2.72 and 3.14, those in the leghaemoglobin *c* spectrum being more pronounced. The spectrum of cowpea leghaemoglobin had an absorption near $g = 2.26$ which was smaller than the corresponding absorptions in the soya-bean leghaemoglobin spectra. It had no absorption near $g = 2.7$ but did have an absorption near $g = 3.1$ which was slightly larger than the absorption in the soya-bean leghaemoglobin spectra. [During the initial stages of this work a signal was observed near $g = 2.7$ in the e.p.r. spectrum of cowpea acid-metleghaemoglobin samples isolated by DEAE-cellulose phosphate chromatography at pH7.0. In fact the e.p.r. spectra of all three leghaemoglobins purified at pH7.0 had pronounced absorptions near $g = 1.74$, 2.26, 2.71 and 3.1. This was probably due to the different method of purification and concentration (C. S. Maskall, unpublished work)]. Leghaemoglobin, when pure as indicated by polyacrylamide-gel electrophoresis, thus resembles haemoglobin. The e.p.r. spectrum of acid-methaemoglobin below 77K exhibits low-spin signals at $g = 1.70$, 2.20 and 2.80 (Nakano *et al.*, 1971). Acid-metmyoglobin, however, is entirely high-spin at $\leqslant 77$K (Iizuka & Kotani, 1969).

A comparison of the e.p.r. spectra of acid-metleghaemoglobin and ferric leghaemoglobin acetate (essentially a pure high-spin complex) led to the suggestion that at 77K acid-metleghaemoglobin was approx. 35–50% high-spin (Ehrenberg & Ellfolk, 1963). Our comparison of the e.p.r. spectra of acid-metleghaemoglobin *c* and Lb*c*F$^-$* at 86K likewise indicated that about 20% of the leghaemoglobin *c* was high-spin. Although the e.p.r. spectra and *g* values of the LbF$^-$ derivatives and low-spin leghaemoglobin derivatives (see the next section) obtained in the present study are very similar to those of Appleby *et al.* (1976) obtained with leghaemoglobin *a*, the three acid-metleghaemoglobin spectra (Fig. 1) differ significantly. The spectrum of acid-metleg-

* Abbreviations: Lb, leghaemoglobin; Mb, myoglobin.

Table 2. *Effective g values of low-spin ferric leghaemoglobin derivatives, with g values of some myoglobin derivatives given for comparison*

Details of methods are as for Table 1. Cowpea leghaemoglobin was from Poona CB756 nodules.

Derivative	*g* values			Reference
	g_x	g_y	g_z	
Hydroxides, pH9.6				
Leghaemoglobin *a*	1.86	2.19	2.51	
Leghaemoglobin *c*	1.84	2.19	2.54	
Cowpea leghaemoglobin	1.86	2.18	2.51	
Myoglobin, pH11.3	1.84	2.14	2.57	Gurd *et al.* (1967)
Myoglobin, pH12.8	1.85	2.17	2.54	Gurd *et al.* (1967)
Azides, pH7.0				
Leghaemoglobin *a*	1.72	2.21	2.77	
Leghaemoglobin *c*	1.72	2.21	2.79	
Cowpea leghaemoglobin	1.72	2.20	2.79	
Myoglobin*	1.71	2.19	2.82	Helcké *et al.* (1968)
Myoglobin*	1.72	2.22	2.80	Hori (1971)
Imidazoles, pH7.0				
Leghaemoglobin *c*	1.67	2.27	2.77†	
	1.49	2.27	2.93	
Cowpea leghaemoglobin	1.68	2.27	2.77	
Myoglobin*	1.53	2.26	2.91	Hori (1971)
Haemoglobin*	1.43	2.29	2.93	Rein *et al.* (1975)
Cyanide				
Cowpea leghaemoglobin, pH7.4	‡	1.93	3.33	
Myoglobin*	0.93	1.89	3.45	Hori (1971)
Nicotinates, pH6.9				
Leghaemoglobin *a*	‡	2.17	3.07	
Leghaemoglobin *c*	1.23	2.18	3.14	
Cowpea leghaemoglobin	‡	2.15	3.11	

* pH not specified.
† An absorption at $g = 1.99$ was also present.
‡ Unobservable.

haemoglobin *a* obtained by Appleby *et al.* (1976) had less high-spin and more low-spin character than the spectra of Fig. 1, and a much broader absorption derivative at $g = 6$. These differences probably reflect differences in the conformation of the two leghaemoglobin *a* compounds in the region of the iron atom and presumably arise from the different methods used to extract and purify these leghaemoglobins.

Low-spin ferric derivatives

The three $LbOH^-$ complexes gave similar e.p.r. spectra and had similar sets of g values that most closely resembled the g values for ferric $MbOH^-$ at pH12.8 (Gurd *et al.*, 1967); see Table 2. The typically rhombic e.p.r. spectra for the three azide derivatives of leghaemoglobin were very similar and resembled closely those of myoglobin (Helcké *et al.*, 1968; Hori, 1971). However, the absorption at $g = 2.05$ seen in the spectrum of $Lba N_3^-$ (Appleby *et al.*, 1976) was not present in these spectra (see Fig. 2 and Table 2). Also that of the azide of leghaemoglobin *c* had broader features at $g = 1.72$ and $g = 2.79$ than those of the other two azide derivatives (compare Figs. 2*a*, 2*b* and 2*c*); this is probably because leghaemoglobin *c* consists of two distinct proteins, leghaemoglobins c_1 and c_2.

The e.p.r. spectrum of the cyanide of cowpea leghaemoglobin had g values fairly close to those of the cyanide of myoglobin (Hori, 1971) (Table 2).

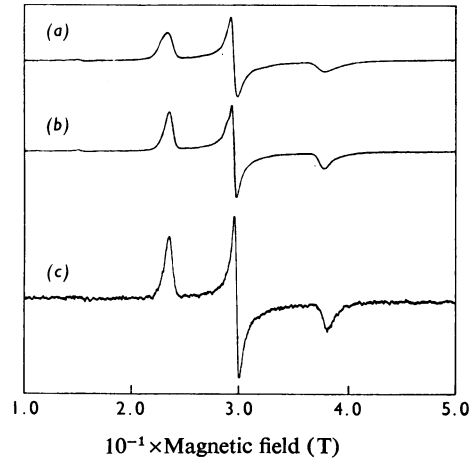

Fig. 2. *E.p.r. spectra of ferric azide derivatives of leghaemoglobin at pH*7.0
Leghaemoglobin samples (1–2mM) were prepared and their e.p.r. spectra recorded as described in the Experimental section. (*a*) Soya-bean azide derivative of leghaemoglobin *c* at 37K and 20mW; (*b*) soya-bean azide derivative of leghaemoglobin *a* at 37K and 20mW; (*c*) cowpea azide derivative of leghaemoglobin at 20K and 0.02mW.

The similarity between the e.p.r. spectra of these low-spin leghaemoglobin derivatives and those of the corresponding myoglobin derivatives again supports the view that the environment of the iron atom in the leghaemoglobins and myoglobin is similar. Like the soya-bean leghaemoglobins, cowpea leghaemoglobin has two histidine residues per molecule and no sulphur-containing amino acids (C. S. Maskall, unpublished work). This finding is also consistent with the great similarity between these proteins.

Origin of the low-spin signals

The probable origin of the low-spin signals in the acid-metleghaemoglobin spectra is suggested by a consideration of the low-spin e.p.r. spectra of imidazole and nicotinate complexes of ferric leghaemoglobin. Appleby *et al.* (1976) detected two low-spin species in the e.p.r. spectrum of soya-bean acid-metleghaemoglobin *a*. They considered that both species were endogenous dihistidyl haemichromes, the imidazole group of the distal histidine (histidine-61) occupying the sixth co-ordination position of the haem iron. The major species had g values of 2.69, 2.24 and 1.72, very similar to the g values of bis-imidazole haem to which base has been added. The minor species had g values of 3.02, 2.24 and 1.34, similar to those of bis-imidazole haem (Peisach *et al.*, 1973).

The low-spin absorptions at $g = 2.26$ and 2.72 of the acid-metleghaemoglobin spectra (Table 1) are similar to the g_y and g_z values of the three azide complexes of leghaemoglobin (Table 2). Cowpea

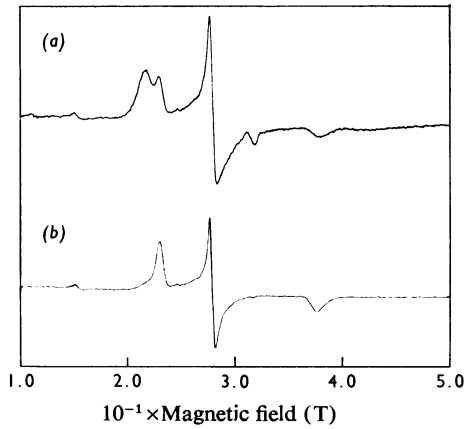

Fig. 3. *E.p.r. spectra of ferric leghaemoglobin imidazole derivatives at pH*7.0
Leghaemoglobin samples (1–2mM) were prepared and their e.p.r. spectra recorded as described in the Experimental section. (*a*) Soya-bean leghaemoglobin *c* imidazole at 32K; (*b*) cowpea leghaemoglobin imidazole at 40K. The power was 0.2mW.

leghaemoglobin imidazole also had an e.p.r. spectrum very similar to that of cowpea azide of leghaemoglobin (cf. Figs. 3*b* and 2*c*), but different from those of myoglobin imidazole and haemoglobin imidazole (see Table 2). Presumably an absorption band near $g = 1.7$ (g_x) is present in the acid-metleghaemoglobin spectra, but it is too small to be detected. Lb*c* imidazole gave a complex e.p.r. spectrum containing absorptions from two low-spin species (Fig. 3*a* and Table 2). One had very similar g values to those of cowpea leghaemoglobin imidazole, the other had g values very close to those of myoglobin imidazole and haemoglobin imidazole. As for the azides noted above, the different species may occur because leghaemoglobin *c* consists of leghaemoglobins c_1 and c_2.

The two forms of azide derivative of myoglobin are attributed to the possibility of electron-pair donation from the distal histidine residue to azide (McCoy & Caughey, 1970). The two forms of Lb*c* imidazole likewise might arise from an interaction involving the distal histidine. In our case, however, the interaction is quite likely to be simple hydrogen-bonding between the lone pair of the nitrogen atom of the distal histidine and the NH group of the iron-bound imidazole; in the second form the hydrogen bond cannot be formed. Fluoride has also been thought to interact with an imidazole NH group in some bis-imidazole haem model complexes (Momenteau *et al.*, 1973).

Of these two imidazole forms, one (and also the cowpea leghaemoglobin imidazole) is a suitable model compound for a low-spin complex present in all three acid-metleghaemoglobin preparations, suggesting that the latter is a dihistidyl haemichrome. In addition (see Table 2), this imidazole complex is clearly unlike the myoglobin and haemoglobin imidazole derivatives. This possibly arises because the haem pocket of leghaemoglobin has greater flexibility than that of myoglobin (Nicola *et al.*, 1975).

The third low-spin absorption near $g = 3.14$ in all the acid-metleghaemoglobin spectra (see Table 2) is probably from a trace of ferric Lb nicotinate, because during purification the nicotinate which might have been present (Appleby *et al.*, 1975) was not removed. The nicotinate complexes of the three leghaemoglobins had very similar g values, with the major absorption near $g = 3.1$ (g_z) (Table 2). Appleby *et al.* (1976) found similar g values for Lb*a* nicotinate and demonstrated that the nitrogen atom of nicotinate is ligated to the haem iron.

Presence of nitrosyl-leghaemoglobin in samples of crude leghaemoglobin

All samples of crude soya-bean and cowpea leghaemoglobin examined by e.p.r. spectroscopy at temperatures above 77K showed a very large signal near $g = 2$ (Fig. 4*a*). This signal exhibited some structure (see inset in Fig. 4*a* and expanded version of this in Fig. 4*b*) and its shape was almost the same at 10K.

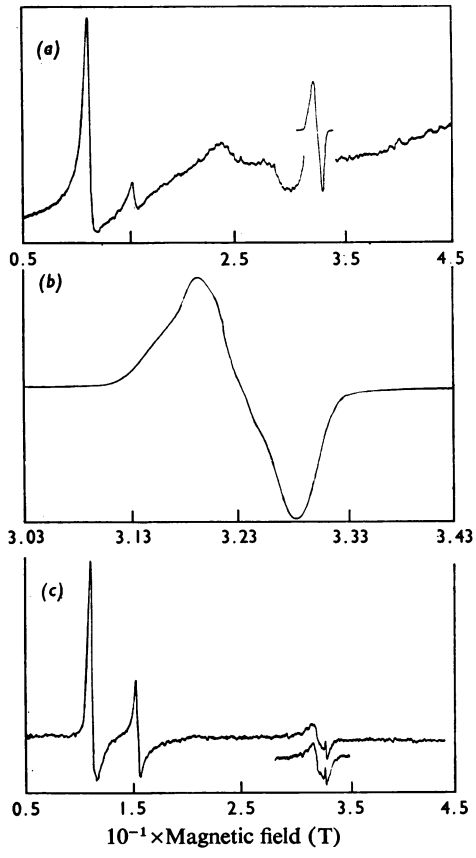

Fig. 4. *E.p.r. spectra of crude soya-bean leghaemoglobin and the peroxidase fraction at pH7.0*

(*a*) Crude Chippewa CC705 soya-bean Lb. Power 200mW, temperature 93K, gain of main spectrum ×1000 and of nitrosyl-leghaemoglobin signal at $g = 2$, ×63. (*b*) Expanded spectrum of nitrosyl-leghaemoglobin signal from a similar crude Chippewa CC705 soya-bean leghaemoglobin preparation. Field sweep 40mT, power 200mW, temperature 86K, gain ×40. (*c*) Peroxidase fraction isolated from crude Merit CC705 soya-bean Lb. Power 2mW, temperature approx. 30K, gain ×5000.

Omitting sodium ascorbate or Polyclar from the nodule extraction medium did not affect the signal. During the development of the DEAE-cellulose phosphate purification procedure, it was found that for soya-bean Altona CB1809 leghaemoglobin [not oxidized with $K_3Fe(CN)_6$] the large signal was not present in the ferric fractions, leghaemoglobins *a* and *c*, but was present in the corresponding bright-red ferrous fractions. The peroxidase fraction isolated from crude soya-bean leghaemoglobin did not show the large signal either (see Fig. 4*c*). The signal at $g = 4.3$ (also present in the spectrum of crude soya-

bean leghaemoglobin) is presumably from non-haem iron. The signals at $g = 6$ and near $g = 2$ are from the high-spin ferric peroxidases. It is noteworthy that the prominent feature at $g = 6$ is not broadened or split. This is in marked contrast with the e.p.r. spectra of the isoenzymes of horseradish peroxidase at 20K, which show a pronounced splitting of the signal in this region (Tamura & Hori, 1972).

Unexpectedly, crude leghaemoglobin treated with $K_3Fe(CN)_6$ still gave bright-red ferrous leghaemoglobin bands on DEAE-cellulose phosphate chromatography. Again, only the ferrous fractions showed the large signal at $g = 2$. The stability towards $K_3Fe(CN)_6$ oxidation and the probable ferrous state of the species responsible for the large e.p.r. signal suggests that this species might be nitrosyl-leghaemoglobin. A comparison of the large signal with the e.p.r. signal of nitrosyl-haemoglobin (Kon, 1968) showed that the two spectra were very similar. Nitrosyl-leghaemoglobin *c* was prepared and its e.p.r. spectrum recorded at 90K and found to be almost identical with the large signal in the leghaemoglobin preparations (Maskall *et al.*, 1974). Thus nitrosyl-leghaemoglobin is present in crude leghaemoglobin and, unless it is an artifact of extraction, in soya-bean and cowpea root nodules too.

Appleby (1974), referring to the work of Melik-Sarkisyan *et al.* (1970), has commented on the possible presence *in vivo* of an as yet uncharacterized ligand of ferrous leghaemoglobin. He had previously noted that soya-bean oxyleghaemoglobin *a* preparations occasionally gave an unusual optical spectrum when deoxygenated with $Na_2S_2O_4$ (Appleby, 1969). This high-spin ferrous leghaemoglobin *a* had a complex spectrum (Soret peak at 418nm with a shoulder at 430nm, plus a peak at 560nm with a shoulder at 545nm). After storage for 5 days at 0°C, addition of $Na_2S_2O_4$ to the same oxyleghaemoglobin *a* preparation gave ferrous leghaemoglobin *a* with the conventional high-spin spectrum (single Soret peak at 427nm plus a single peak at 555nm). These observations, coupled with the fact that our large nitrosyl-leghaemoglobin e.p.r. signal of crude soya-bean leghaemoglobin decayed over a period of 4–5 days storage at 0°C, prompted an examination of the optical spectra of some $Na_2S_2O_4$-treated leghaemoglobin samples possessing the large nitrosyl-leghaemoglobin signal. Crude soya-bean leghaemoglobin and a sample of a cowpea 'oxyleghaemoglobin' fraction from a DEAE-cellulose phosphate column at pH8.0 were deoxygenated with $Na_2S_2O_4$. Both samples gave similar optical spectra. That of crude soya-bean ferrous leghaemoglobin had a peak at 418nm with a shoulder at 425nm, whereas that of cowpea ferrous leghaemoglobin showed a peak at 415nm with a shoulder at 426nm; see Fig. 5. Thus in this region the spectra resemble the unusual ferrous leghaemoglobin *a* spectrum. However, both spectra had only a single peak at 554nm, resembling that of high-spin ferrous leghaemoglobin. This may be because these leghaemoglobin samples contain a lower concentration of the species responsible for the unusual spectrum. The cowpea ferric leghaemoglobin fraction from the same column as the 'oxyleghaemoglobin' fraction gave the conventional high-spin ferrous leghaemoglobin spectrum on addition of $Na_2S_2O_4$. Thus spectra like the unusual ferrous leghaemoglobin *a* spectrum appear to be connected with the presence of nitrosyl-leghaemoglobin. When $Na_2S_2O_4$ and a few crystals

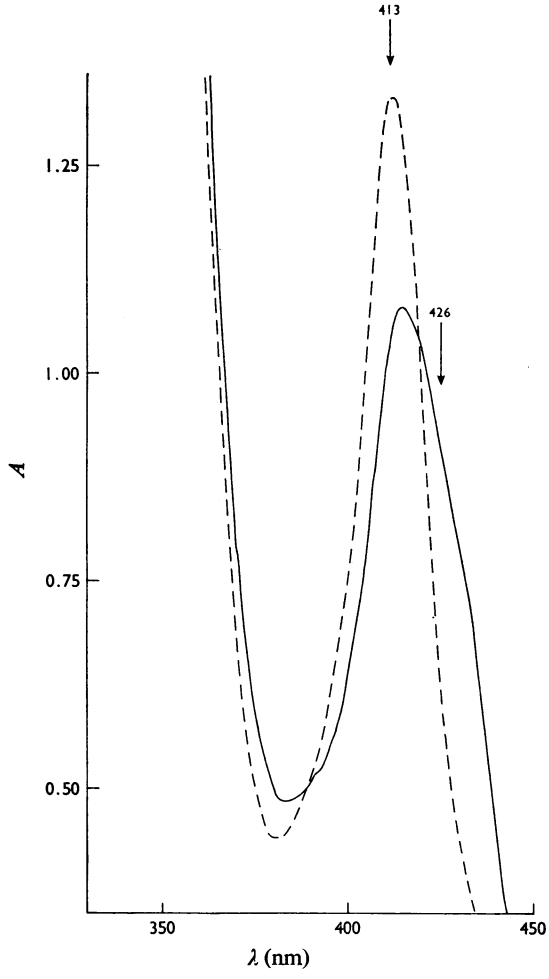

Fig. 5. *Soret region of the optical spectra of purified cowpea ferrous leghaemoglobin and nitrosyl-leghaemoglobin*
——, Spectrum of the 'oxyleghaemoglobin' fraction from a DEAE-cellulose phosphate column at pH8.0 deoxygenated with $Na_2S_2O_4$. – – – –, Spectrum of the same sample after addition of a few crystals of $NaNO_2$ to produce the nitrosyl-leghaemoglobin complex.

of $NaNO_2$ were added to the cowpea ferrous leghaemoglobin sample above (a method used to produce the NO complexes of ferrous haemoproteins; Yonetani *et al.*, 1972), the peak at 415nm shifted to 413nm, increasing in intensity and losing its shoulder at 426nm (see Fig. 5). The peak at 554nm split into two peaks at 542nm and 568nm. These observations indicate that the peak at 415nm in the spectrum of the cowpea ferrous leghaemoglobin sample is due to nitrosyl-leghaemoglobin and the shoulder at 426nm is due to high-spin ferrous leghaemoglobin. They also indicate that the shoulder at 545nm in the unusual ferrous leghaemoglobin *a* spectrum is almost certainly due to nitrosyl-leghaemoglobin *a*. Thus the unstable form of ferrous leghaemoglobin *a* (Appleby, 1969) is almost certainly nitrosyl-leghaemoglobin *a*.

Soya-bean nitrosyl-leghaemoglobin *c*, prepared by the same method as the cowpea nitrosyl-leghaemoglobin above, had absorption bands at 568nm, 545nm and 414nm. Melik-Sarkisyan *et al.* (1970) extracted leghaemoglobin from lupin nodules at pH7.0 in the presence of capron (like Polyclar, an adsorbent of phenolic compounds). Their isolated crude leghaemoglobin gave spectra having absorption bands at 566–572nm and 539–543nm and appeared to contain a form of ferrous leghaemoglobin (about 30%) which was oxygenated only slowly. These observations suggest very strongly that the crude leghaemoglobin preparations of Melik-Sarkisyan *et al.* (1970) also contained nitrosyl-leghaemoglobin.

Nitrosyl-leghaemoglobin was present in crude leghaemoglobin extracted from nodules of plants grown in three different environments, including the field. This indicates that it is not unusual growth conditions which give rise to nitrosyl-leghaemoglobin. Quantitative e.p.r. spectroscopy, with a Cu^{2+}–EDTA standard for comparison, was used to determine the amount of nitrosyl-leghaemoglobin in a sample of crude leghaemoglobin from nodules of 8-week-old Chippewa CC705 soya beans grown in controlled-environment cabinets under similar conditions to those used for cowpeas (see under 'Growth of plants'). It contained $27\pm3\%$ nitrosyl-leghaemoglobin, a considerable fraction of the total leghaemoglobin.

If nitrosyl-leghaemoglobin is an artifact, the mechanism by which it is generated during the extraction of leghaemoglobin is nevertheless worthy of further investigation. If it is actually present in the root nodule, it may originate from NO in the soil. C. S. Yocum (unpublished work), quoted by Tjepkema (1971), has suggested that leghaemoglobin may protect the nodule bacteroids' nitrogenase by combining with the CO and NO that may occur in the soil. [Leghaemoglobin has a higher affinity for CO than for O_2 (Imamura *et al.*, 1972) and presumably it has a higher affinity for NO than for CO (Antonini & Brunori, 1971)]. However, nitrosyl-leghaemoglobin may be produced by NO_3^- or NO_2^- entering the nodule and being reduced to NO. In fact, Virtanen *et al.* (1947) suggested that nitrosyl-leghaemoglobin might be formed in root nodules exposed to NO_3^-, and that this would interfere with the function of leghaemoglobin. This may well be the case, since the presence of the presumed stable nitrosyl-leghaemoglobin complex will decrease the amount of ferrous leghaemoglobin available for facilitating O_2 diffusion to the nodule bacteroids.

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