Control of Leghaemoglobin Synthesis in Snake Beans

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1. The finding that the plant is the genetic determinant of leghaemoglobin production in legume nodules was further tested by inoculating snake beans with two strains of *Rhizobium* selected to give large genetic differences. Carbohydrate requirement patterns, immunological techniques and DNA base ratio determinations were used to demonstrate genetic differences between the two rhizobial strains. 2. Partially purified preparations of the haemoglobins from the nodules produced by the two strains showed no differences when examined by electrophoresis, isoelectric focusing or ion-exchange chromatography. 3. Two different leghaemoglobins from each type of nodule were separated by chromatography on DEAE-cellulose. One of these was isolated in the Fe^{3+} form and accounted for two-thirds of the total leghaemoglobin. When it was examined in the analytical ultracentrifuge and by amino acid analysis, this major component did not vary with the inoculant rhizobial strain. The molecule had an $s_{20,w}$ of 1.88S, a diffusion coefficient of $10.7 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ and a mol. wt. of 16700. 4. These results strongly support the hypothesis that the mRNA for leghaemoglobin is transcribed from plant DNA.

Recent evidence has suggested that genetic control of synthesis of the protein moiety of leghaemoglobin resides in the plant rather than the rhizobial symbiont of the legume root nodule.

Cutting & Schulman (1968) used immunological techniques to show that material reacting with anti-leghaemoglobin sera was found only in the supernatant from crushed nodules, and not in extracts from leaves, stems, roots or bacteroids, and that a cell-free system derived from plant tissue would incorporate labelled amino acids into a cross-reacting material. Peive, Yagodin, Zhiznevskaya & Borodenko (1970) presented crystallographic and electrophoretic evidence that two different species of *Lupinus*, when inoculated with the same rhizobial strain, produced different leghaemoglobins. However, they studied crystals of leghaemoglobin from *Lupinus angustifolius* that contained at least two components.

Dilworth (1969) examined the effect on leghaemoglobin of varying the plant species with a constant bacterial component, and of varying the rhizobial strain with the same legume. The leghaemoglobins were examined by ion-exchange chromatography, and by electrophoresis in polyacrylamide gels. Different plants contained differ-

ent haemoglobins, yet different rhizobia, when used to inoculate the same plant, produced similar haemoglobins. It was therefore concluded that the plant was the genetic determinant of leghaemoglobin production. A similar conclusion was reached by Cutting & Schulman (1971) by using different rhizobia to inoculate soya-beans, and by using a single rhizobial strain to inoculate a number of other legumes.

However, whereas Dilworth (1969) and Cutting & Schulman (1971) took care to select plants with considerable genetic differences between them, neither investigation used bacterial strains selected for their genetic disparity. In other words, both series of experiments with lupin, serradella or soya-bean used bacteria from the slow-growing groups of rhizobia. It is therefore possible that the bacteria used were genetically so alike that differences in leghaemoglobins attributable to the bacteria were so slight as to be missed with the techniques employed.

Accordingly, the following experiments were undertaken with the snake bean [*Vigna sinensis* (L.) Savi ex. Hassk ssp. *sesquipedalis* (L.) van Eseltine], which nodulates with representatives of both fast- and slow-growing groups of rhizobia (Trinick, 1968). In this way, the maximum genetic difference between rhizobial strains was possible

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(see Graham, 1964) and analysis of the leghaemoglobins for relatedness made most meaningful.

EXPERIMENTAL

Growth of plants. Snake-bean seeds were surface sterilized, inoculated with appropriate rhizobia, and sown in sterilized sand in pots in the glasshouse. After 7 days, each pot was watered with 200 ml of a rhizobial suspension in water containing approx. 10^9 cells of the appropriate strain/ml.

The plants were watered every second day with a solution of the composition: CaCl_2 , 1 mM; KH_2PO_4 , 0.5 mM; ferrous citrate, 10 μM ; MgSO_4 , 0.25 mM; K_2SO_4 , 0.25 mM; MnSO_4 , 1 μM ; H_3BO_3 , 2 μM ; ZnSO_4 , 0.5 μM ; CuSO_4 , 0.2 μM ; CoSO_4 , 0.1 μM ; Na_2MoO_4 , 0.1 μM . Every other day the pots were soaked with tap water. The plants were grown for up to 8–10 weeks with a mean day temperature of 30°C, and the nodules were removed by hand for storage at –20°C until use.

Bacteriological techniques. The cultures used were strains WU515 (=NGR 46, a slow-growing isolate from *Crotolaria anagyroides* H.B. & K.) and WU508 (=NGR 234, a fast-growing isolate from *Dolichos lablab* L.) both isolated by Dr M. J. Trinick. The rhizobia were grown on solid and liquid media that contained (per litre): 5 g of sucrose; 5 g of mannitol; 0.3 g of K_2HPO_4 ; 0.1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.1 g of CaCO_3 ; 0.05 g of NH_4Cl ; 0.03 g of urea; 0.1 g of NaCl ; 0.01 g of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$; 2.5 g of yeast extract (Difco); extract from 200 g of fresh potato autoclaved for 15 min at 15 lb/in² and filtered through cheesecloth, with or without 20 g of agar. Slope cultures were used to inoculate snake-bean seed after being dispersed in sterile 5% (w/v) sucrose.

The nodular bacterial component was identified by reaction with the specific antiserum (dilution 1:200) after crushing the nodules in wells on Perspex plates (Parker & Grove, 1970). In no case did the bacteroid component fail to react with the homologous antiserum. Carbohydrate utilization tests were performed on agar plates by the method of Graham (1964).

Leghaemoglobin isolation. About 200 g of nodules was homogenized in 4 vol. of 0.1 M-potassium phosphate buffer, pH 6.8, at 4°C in an MSE Ato-Mix blender. The homogenate was centrifuged at 10 000g for 20 min, the supernatant was fractionated with solid $(\text{NH}_4)_2\text{SO}_4$, and the fraction precipitating between 50 and 80% saturation was dissolved in a small volume of buffer and dialysed with stirring against 0.1 M-EDTA, pH 6.8, for 16 h. The dialysed material was then centrifuged at 25 000g for 15 min and loaded on to a column (10 cm $\times$ 3 cm diam.) of DEAE-cellulose equilibrated with 5 mM-potassium phosphate buffer, pH 6.8. The leghaemoglobins were eluted from the column with 50 mM-phosphate buffer, pH 6.8, and dialysed with stirring for 16 h against 5 l of 0.1 M-EDTA, pH 6.8. This procedure separated the leghaemoglobins from contaminating polysaccharides that would have clogged the analytical columns. All operations were performed at 4°C.

Yields were 0.65–0.80 mg of leghaemoglobin/g fresh weight of nodules.

Column chromatography. The leghaemoglobins were fractionated on a column (40 $\times$ 2 cm diam.) of DEAE-cellulose (acetate form) equilibrated with 13 mM-acetate

buffer, pH 5.2 (Dilworth, 1969). The eluent fractions of interest were pooled and concentrated on a Diaflo ultra-filtration cell equipped with a UM 10 membrane.

Electrophoresis. Polyacrylamide-gel disc electrophoresis was performed as described by Dilworth (1969). Gel strength was 7.5% (w/v) in the resolving gel.

Three buffer systems were used: a discontinuous β -alanine-acetate system, pH 4.8, in which the leghaemoglobins migrate as cations, a continuous histidine-glycylglycine system, pH 7.0, and a discontinuous tris-glycine system, pH 8.9, in which the leghaemoglobins run as anions. Gels were photographed before and after staining with benzidine.

Analytical techniques. Total amino acid composition was determined by hydrolysing 6–8 mg of leghaemoglobin in constant-boiling HCl for 18 h at 108°C in tubes sealed under vacuum. The amino acids were separated on a Beckman model 120C Amino Acid Analyzer, and the values were extrapolated to zero time by using correction factors derived from analyses of similar leghaemoglobins hydrolysed for 18 and 36 h.

DNA base ratios were determined on unpurified DNA by measurement of the adenine and guanine content (Broughton, Dilworth & Passmore, 1971).

Iron contents of the leghaemoglobins were determined after splitting off the haem with acid-acetone. Haemin standards were similarly prepared as follows: 3–7 mg of leghaemoglobin in 1 ml of water was treated with 0.025 ml of acetic acid, 1.0 ml of acetone and 0.05 ml of pyridine, and the resulting solution adjusted to 5 ml with 50% (v/v) acetone. Standard solutions of haemin were prepared by dissolving approx. 14 mg of haemin in 1 ml of pyridine, then adding 0.5 ml of acetic acid and diluting the solution to 100 ml with 50% (v/v) acetone. Further dilutions were prepared with the same concentrations of pyridine, acetic acid and acetone. Iron contents were measured on a modified Techtron AA3 atomic-absorption spectrophotometer by using the 2484 Å line. With this technique we obtained a value for the protein combining weight of 18 400/atom of iron for horse heart myoglobin, which compares favourably with the value of 18 800 obtained from iron determinations by Theorell & Åkeson (1955) and a value for the molecular weight of 18 400 based on osmotic pressure measurements (Boardman & Adair, 1956).

Isoelectric points of the various leghaemoglobin fractions were determined by isoelectric focusing in 5% (w/v) polyacrylamide gels with pH 4–6 Ampholine (Catsimpoolas, 1968).

Sedimentation and diffusion coefficients were determined on a Beckman Analytical Ultracentrifuge (Spinco model E) by using schlieren optics as described by Schachman (1957). Sedimentation-velocity runs were performed at 59 780 rev./min and diffusion runs at 15 200 rev./min. The samples were dissolved in 0.1 M-potassium phosphate buffer, pH 6.8, and artificially layered with the same buffer in a rubber-valve-type synthetic boundary cell at approx. 8000 rev./min. Photographs were taken at 8 or 16 min intervals on Kodak Tri-X sheet film and with a deep-red filter. Diffusion coefficients were calculated by plotting the square of the second moment against time, and the partial specific volume was determined from the amino acid composition (Schachman, 1957).

Protein was measured by using the E_{280}/E_{260} ratio

(Layne, 1955) and haemoglobin as the pyridine haemochromogen (Paul, Theorell & Åkeson, 1953).

Chemicals. Ampholine (pH 4–6) was from LKB Produkter, Broma 1, Sweden; haemin (97.5% pure by iron analysis) from Gurr's Ltd., London, U.K.; and myoglobin from Miles Laboratories, Kankakee, Ill., U.S.A.

RESULTS

Bacterial strains. Although the two bacterial strains selected behaved as typical fast- and slow-growing rhizobia on yeast-mannitol media, it was necessary to confirm this as well as to measure the degree of homology between them. For these purposes the following criteria were used.

First, the results presented in Table 1 show the ability of the two strains to utilize different carbon sources for growth. Strain NGR 234 was able to grow on all the carbohydrates tested except xylose, whereas strain NGR 46 could utilize only glucose, mannitol, sorbitol and xylose. The pattern of utilization of carbohydrates suggests that the organisms conform to the general pattern of fast- and slow-growing rhizobia (Graham, 1964).

Secondly, antiserum prepared against *Rhizobium trifolii* strain SU 329 reacted with antigen prepared from broken cells of strain NGR 234, but not with antigen from broken cells of NGR 46. Thus strain NGR 234 shows the group-specific antigen of the fast-growing group, whereas strain NGR 46 does not (Vincent & Humphrey, 1970).

Finally, DNA base ratio analysis of strain

NGR 46 gave a value of $64.3 \pm 0.3\%$ guanine + cytosine (G+C) and strain NGR 234 a value of $62.2 \pm 0.3\%$, a difference that is statistically significant. Thus strain NGR 46 is within the range 62.8–65.5% (G+C) observed for subpolarily flagellated slow-growing strains of *Rhizobium*, and NGR 234 is within the range 58.6–63.1% (G+C) found for the peritrichously flagellated fast-growing group (De Ley & Rassel, 1965). We therefore conclude that the two strains of rhizobia are genetically different and that strains NGR 234 and NGR 46 are representative of the fast- and slow-growing rhizobial groups respectively.

Identity of leghaemoglobins. This part of the work was concerned with measuring the relatedness of leghaemoglobins produced in plants inoculated either with strain NGR 46 or strain NGR 234. Previously established criteria of polyacrylamide-gel electrophoresis and ion-exchange chromatography (see Dilworth, 1969) as well as such other parameters as amino acid composition, molecular weights and isoelectric points were used for this purpose. Disc-gel electrophoresis of leghaemoglobins isolated from plants inoculated with either strain revealed two major bands that were identical (Plate 1). When leghaemoglobins from plants inoculated with strain NGR 46 and from other plants inoculated with strain NGR 234 were combined and run on the one gel, the same two bands were resolved. Electrophoresis at pH 4.8 and 8.9 produced similar results.

Ion-exchange chromatography of unfractionated haemoglobins (i.e. the concentrated eluate from the DEAE-cellulose phosphate column) displayed two different leghaemoglobins (Fig. 1) each of which was present in two different oxidation states (cf. Appleby, 1969a). The first of the leghaemoglobin peaks was present in the Fe^{3+} form, and comprised about two-thirds of the total leghaemoglobin. This peak consistently eluted around tube 100, a position intermediate between that found for the corresponding serradella and lupin leghaemoglobins (see Dilworth, 1969). Co-chromatography of the pooled proteins extracted from plants inoculated with strain NGR 46 and other plants inoculated with strain NGR 234 did not produce any more bands than were apparent on runs with a single type of plant as the source of leghaemoglobin (Fig. 1c).

Isoelectric focusing of the unfractionated leghaemoglobins produced a multiplicity of bands of which six were quite distinct (Plate 2). Three of these were red in colour and the other three were green with isoelectric points in the range 4.96–5.38 (Table 2). As noted above, only four red bands are observed in ion-exchange chromatography so that the origin of the extra bands generated in isoelectric focusing is obscure. However, leghaemoglobins are known to exist in two different molecular

Table 1. *Ability of strains NGR 46 and NGR 234 of Rhizobium to utilize various carbohydrates*

The medium (after Graham, 1964) contained 20 g of agar, 0.5 g of K_2HPO_4 , 0.2 g of CaCl_2 , 0.2 g of NaCl, 0.1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 25 ml of yeast infusion, and water to 1 litre. Holes (0.5 cm diam.) were cut in seeded agar plates and incubated for 48 h at 28°C. Drops of 10% (w/v) solution were placed in the holes, then the plates were incubated for a further 60 h (strain NGR 234) or 96 h (strain NGR 46) and the zones of growth stimulation recorded.

Strain ... Carbohydrate	Radius (cm)	
	NGR 46	NGR 234
Erythritol	—	2.6
Fructose	—	2.2
Glucose	4.0	2.2
Lactose	—	2.8
Maltose	—	1.9
Mannitol	3.8	2.1
Mannose	—	2.3
Ribose	—	1.6
Rhamnose	—	2.2
Sorbitol	4.0	2.4
Sucrose	—	2.0
Xylose	1.6	—

Fig. 1. Elution patterns of snake-bean leghaemoglobins from columns of DEAE-cellulose equilibrated with 13 mM-acetate buffer, pH 5.2. (a) 100 mg of leghaemoglobin extracted from snake-beans inoculated with NGR 46; (b) 100 mg of leghaemoglobin extracted from snake-beans inoculated with NGR 234; (c) 50 mg of NGR 46 leghaemoglobin plus 50 mg of NGR 234 leghaemoglobin. The eluent buffer concentrations were changed to 25 mM- and 50 mM-acetate as indicated by the arrows. Parts (a) and (b) are LKB-Uvicord traces at 280 nm. —, Leghaemoglobin; ----, protein. Fractions of 10 ml were collected. Abbreviations: LHb^{3+} , ferri-leghaemoglobin; $\text{LHb}^{2+}\text{O}_2$, oxyleghaemoglobin.

Table 2. Isoelectric points (pI) of unfractionated snake bean leghaemoglobins as determined by isoelectric focusing on polyacrylamide gels

The pI values are given $\pm$ s.d. Leghaemoglobins were isolated on DEAE-cellulose phosphate columns.

Slice no.	Colour	pI
10	Green	4.96 ± 0.04
10-11	Red	5.00 ± 0.03
12	Green	5.12 ± 0.03
12-13	Red	5.16 ± 0.03
13	Red	5.19 ± 0.03
15-16	Green	5.38 ± 0.03

orbital forms (Appleby, 1969b) as well as two different oxidation states, and it is possible that electrophoresis in a pH gradient could separate

some of these species. No change in the pattern of protein bands was observed by changing the rhizobial symbiont, nor were extra bands produced by combining and focusing leghaemoglobin from plants infected with strain NGR 46 and from other plants infected with strain NGR 234.

Further evidence of the identity of the leghaemoglobins produced by either type of plant was obtained by measuring the direct chemical and physical properties of the molecules. The major leghaemoglobin in snake beans was isolated by chromatography on DEAE-cellulose acetate and concentrated for further analysis. Hydrolysis of freeze-dried portions of this fraction in 6M-hydrochloric acid under standard conditions for amino acid analysis gave the results presented in Table 3. Tryptophan is destroyed by this process and as only

EXPLANATION OF PLATE I

Polyacrylamide-gel disc electrophoresis of snake-bean leghaemoglobins at pH 7.0. (a) 30 μ g of leghaemoglobin extracted from snake beans inoculated with NGR 46; (b) 15 μ g of NGR 46 leghaemoglobin plus 15 μ g of NGR 234 leghaemoglobin; (c) 30 μ g of leghaemoglobin extracted from snake beans inoculated with NGR 234.

EXPLANATION OF PLATE 2

Isoelectric focusing in polyacrylamide gels of leghaemoglobins purified by passage through DEAE-cellulose/phosphate columns. (a) 185 μ g of leghaemoglobin extracted from snake beans inoculated with NGR 46; (b) 100 μ g of NGR 46 leghaemoglobin plus 100 μ g of NGR 234 leghaemoglobin; (c) 190 μ g of leghaemoglobin extracted from snake beans inoculated with NGR 234; (d) the pH-gradient of the polyacrylamide gel.

Table 3. *Amino acid composition of the major leghaemoglobin fraction isolated from snake beans inoculated with either slow-growing (NGR 46) or fast-growing (NGR 234) rhizobia*

Leghaemoglobins were isolated on DEAE-cellulose-acetate columns. Values are expressed as residues/molecule. The results of single analyses are shown. Tryptophan was not determined.

Amino acid	NGR 46			NGR 234		
	After 18 h hydrolysis	Corrected value	Nearest integer	After 18 h hydrolysis	Corrected value	Nearest integer
Ala	29.45	30.09	30	29.17	29.80	30
Arg	1.85	1.85	2	1.96	1.96	2
Asp + Asn	14.81	14.81	15	15.05	15.05	15
Cys	0	0	0	0	0	0
Glu + Gln	13.31	13.31	13	13.01	13.01	13
Gly	10.67	10.67	11	10.54	10.54	11
His	1.90	1.90	2	2.18	2.18	2
Ile	3.00	3.37	3	3.02	3.39	3
Leu	14.15	14.38	14	14.22	14.45	14
Lys	15.69	16.16	16	15.16	15.61	16
Met	0	0	0	0	0	0
Phe	7.14	7.14	7	7.05	7.05	7
Pro	3.00	3.00	3	2.87	2.87	3
Ser	6.79	7.55	8	7.34	8.16	8
Thr	5.77	6.14	6	5.96	6.34	6
Tyr	4.50	4.50	5	4.83	4.83	5
Val	14.63	15.99	16	14.39	15.72	16

limited amounts of protein were available, the content of this amino acid was not determined.

The amino acid composition of the leghaemoglobins was the same regardless of the rhizobial strain used to inoculate the plants. The protein has a typical leghaemoglobin amino acid composition with high contents of alanine, aspartic acid plus asparagine, glutamic acid plus glutamine, leucine, valine and the basic amino acid lysine. Similarly the protein does not contain any sulphur amino acids and in this respect it resembles the leghaemoglobins from soya-bean (see Ellfolk, 1961).

The physical properties of the molecule are presented in Table 4. Snake-bean leghaemoglobin has a sedimentation coefficient of 1.88S, a diffusion coefficient of $10.7 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$, and a molecular weight of 16700 calculated from these values. Molecular weights calculated from the amino acid composition and from the iron determinations were 16400 and 16600 respectively. Thus the molecule is similar in size to the larger soya-bean component (Ellfolk, 1960). Pooled leghaemoglobin purified from plants inoculated with strain NGR 46 and other plants inoculated with strain NGR 234 sedimented as a single homogeneous protein in the analytical ultracentrifuge, further suggesting the identity of the proteins.

Other parameters of the molecule such as the partial specific volume and the Stokes radius are also presented in Table 4. The partial specific volume was calculated from the amino acid com-

position, and would be slightly decreased from the high value reported if the haem portion of the molecule were allowed for in these calculations. A frictional ratio of 1.17 suggests that the protein is globular and slightly ellipsoid.

DISCUSSION

To test fully the hypothesis that the bacterial strain has no influence on the type of leghaemoglobin produced in legume root nodules, the maximum difference in rhizobial strains is desirable. On the other hand, it is necessary that the two strains be effective with the host chosen, and that leghaemoglobin be produced by the symbiosis. The number of cases known where fast- and slow-growing rhizobia produce an effective symbiosis with a single host is relatively small. With strains NGR 46 and NGR 234 nodulating the snake-bean, the requirement for effective nodulation and leghaemoglobin production was met. Our evidence for the genetic difference in the bacterial strains is threefold: (1) the range of carbohydrates that the two strains use is very different and compatible with the slow- and fast-growing groups studied by Graham (1964); (2) the group-specific antigen present in the fast-growing *Rh. trifolii* SU 329 is present in strain NGR 234 but not in strain NGR 46; and (3) the percentage of G+C in the DNA of the two strains places them in the slow- and fast-growing rhizobial groups (De Ley & Rassel, 1965),

Table 4. *Molecular properties of snake-bean leghaemoglobin isolated from DEAE-cellulose acetate columns*

Sedimentation coefficient ($s_{20,w}$)	$1.88 \pm 0.09S$
Diffusion coefficient (D)	$10.7 \times 10^{-7} \pm 0.5 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$
Partial specific volume ($\bar{v}$)	0.747 ml/g
Molecular weight:	
from amino acid composition	16400*
from diffusion and sedimentation values	16700 ± 800
from iron determinations	16600 ± 200
Frictional ratio (f/f_0)	1.17 ± 0.12
Stokes radius	$19.9 \pm 1.0 \text{ \AA}$

* Does not include tryptophan.

and there is a statistically significant difference between the two values.

A number of properties were compared to test as rigorously as possible for potentially small differences in the leghaemoglobins. Two leghaemoglobins were resolved from the mixture recovered from DEAE-cellulose phosphate columns when this was run in disc electrophoresis in polyacrylamide gel, and the leghaemoglobins from strains NGR 46 and NGR 234 nodules were not different. Similarly, chromatography on DEAE-cellulose acetate did not differentiate between the two groups of leghaemoglobins.

Molecular-weight determinations based on sedimentation and diffusion results from the ultracentrifuge experiments, as well as analyses for iron content, indicate that the molecular weights of the major leghaemoglobin component from the two types of nodule were not different within experimental error.

Isoelectric focusing experiments on the total leghaemoglobins from the DEAE-cellulose phosphate columns showed that the bacterial strain used made no difference to the isoelectric points of the separated pigments.

Finally, the identical amino acid composition for the major leghaemoglobin component from both types of nodules is good evidence that the proteins are the same. Although it is possible that the sequences of the two proteins are different, we consider this to be very unlikely.

Accordingly, as we have used rhizobial strains that are as genetically different as is possible, and shown that there is still no influence from the bacterial symbiont on the leghaemoglobin properties, we conclude that complete genetic control of leghaemoglobin composition by the plant is probably general. It remains to be seen whether the actual protein is synthesized on plant or bacterial ribosomes.

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