Studies on the *Casuarina* - *Frankia* Symbiosis With Special Reference to Hemoglobin

by

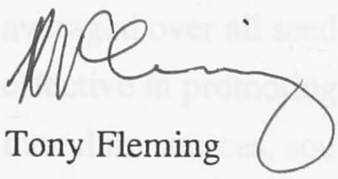
Anthony Ian Fleming

A thesis submitted for the degree of Doctor of Philosophy at the Australian National University.

November 1989

Declaration of originality

This thesis reports original experiments carried out for the degree of Doctor of Philosophy at the Australian National University. The work presented is my own except where due acknowledgement to others is made in the text.



Tony Fleming

SUMMARY

Section I: Host specificity.

Intra-specific variation in the effectiveness of the *Casuarina cunninghamiana* Miq.- *Frankia* symbiosis has been observed. Seed and root nodules (for use as a *Frankia* inoculum) collected from 18 provenances of *Casuarina cunninghamiana* Miq. were used in a complete cross-inoculation trial conducted in a glasshouse. The provenances covered the geographical range of the species and represented major river systems. They were arranged *a priori* into five groups according to geographical location. Seventeen inocula were effective on seed from all provenances; one inoculum failed to nodulate seedlings from any provenance. Inoculum source, seed source and their interaction all affected plant growth. Greatest shoot weight was obtained with seed and inoculum combinations from similar geographical regions. When averaged over all seed sources northern inocula were the most generally effective in promoting plant growth. In contrast, when averaged over all inoculum sources, southern seed sources grew best.

In a second trial, single nodule inoculum sources from a range of southern provenances showed no significant variation in the promotion of plant growth of a single southern seed source. This was in agreement with the results of the first trial.

These results demonstrate the potential for improving the effectiveness of the *C. cunninghamiana* - *Frankia* association in forestry by selection of the symbiotic partners.

Section II: Root nodule hemoglobin purification and characterisation.

The presence of a membrane-bound hemoglobin in aqueous extracts of nitrogen-fixing *Casuarina cunninghamiana* and *C. glauca* nodules (Davenport, H. E. (1960) Nature 186, 653-654) has been confirmed.

By strictly anaerobic grinding and extraction under carbon monoxide, with inclusion of soluble polyvinylpyrrolidone and zwitterionic detergent in the extraction buffer, soluble carboxyhemoglobin was obtained. This was purified by anaerobic 'adsorption' chromatography on Sephacryl S-200 (Pharmacia) followed by aerobic molecular exclusion chromatography on Sephadex G-75 (Pharmacia) to yield very stable oxyhemoglobin . By preparative-scale isoelectric focusing *Casuarina* oxyhemoglobin is separable into three major components comprising approx. 20% of applied protein, and very many minor components. Monomeric *Casuarina glauca* hemoglobin was further characterised and was similar to other plant hemoglobins in respect of molecular weight (17856 Da, inc. heme), optical spectra, extremely rapid kinetics of binding to oxygen and carbon monoxide and high oxygen affinity (P_{50}=0.074 torr). Hence, it is possible that this protein functions in the *Casuarina* symbiosis as does leghemoglobin in leguminous nitrogen-fixing symbioses.

Western blot analysis showed close immunological relationships between the non-leguminous *C. glauca* and *Parasponia* hemoglobins and a weaker relationship between these two proteins and soybean leghemoglobin. The amino acid sequence of the *C. glauca* protein was also determined. The protein is composed of 151 amino acids including a single cysteine, a residue not found in the leghemoglobins. *C. glauca* hemoglobin I shows extensive sequence homology (43-52%) with other plant hemoglobins.

It is proposed that all known hemoglobins, which are from widely separated plant orders, have a common evolutionary origin.

TABLE OF CONTENTS

TABLES

FIGURES

PREFACE

This thesis reports original experiments carried out between November 1982 and February 1986. My supervisor for this work was Professor David Griffin, Department of Forestry. It is submitted for the degree of Doctor of Philosophy at the Australian National University. The work presented is my own, except where due acknowledgement to others is made in the text.

The initial topic for this thesis, suggested by Dr Alan Gibson (CSIRO Division of Plant Industry) was host-endophyte specificity in the *Casuarina-Frankia* symbiosis. I completed two experiments on this subject, including one major and self-contained study of provenance variation in the *Casuarina cunninghamiana-Frankia* symbiosis. This was conducted in collaboration with Dr John Turnbull (CSIRO Division of Forest Research) and Mr Emlyn Williams (CSIRO Division of Mathematics and Statistics). The results are, I believe, of significant interest and may have direct application in the plantation growth of casuarinas.

It was originally intended that the thesis would be devoted entirely to specificity studies. However, in the early stages of my research I also allowed some time to pursue a separate topic, the investigation of *Casuarina* nodules for the presence of hemoglobin, an interest which stemmed from my Honours project.

Research by Dr Cyril Appleby of CSIRO at that time had just confirmed the presence of hemoglobin in nodules of *Parasponia* (a *Rhizobium* - nonlegume symbiosis), muddying the intellectual waters on the origin and distribution of hemoglobin in plants. Probing *Casuarina* nodules for hemoglobin would be a considerable step into the unknown, both because *Casuarina* is phylogenetically distant from either the legumes or *Parasponia*, and also because its symbiotic partner, *Frankia*, is unrelated

to *Rhizobium* . If hemoglobin were found (contrary to popular opinion at the time) and then characterised, it could help resolve many questions concerning nodule function, and improve our understanding of the origin and distribution of hemoglobin in plants.

I sought out Dr Appleby, who readily agreed to my request to spend a "few weeks in his lab", and with his supervision I undertook a preliminary investigation of *Casuarina cunninghamiana* nodules. The results suggested that hemoglobin may indeed be present in the nodules. As the excitement grew, I was encouraged by Dr Appleby to continue this line of research to the extent of purifying and characterising the protein. The "few weeks" turned in to an international collaborative effort over three years, the results of which occupy the main body of this thesis. The other principal participants in this work were Dr Alex Kortt (CSIRO Division of Protein Chemistry), Drs Beatrice and Jonathan Wittenberg (Albert Einstein College of Medicine, New York), Dr Bill Dudman (CSIRO Division of Plant Industry), and the late Dr David Goodchild (CSIRO Division of Plant Industry).

The significance of purifying hemoglobin from *Casuarina* nodules in resolving the origin of hemoglobin in plants is well summed up by Stephen Jay Gould (Natural History 8/1988, pp16-22):

" We welcome .... new and instructive examples [of lateral gene transfer] but must now turn to the oldest and most publicized potential case of lateral transfer - the hemoglobins of leguminous plants...

Globins have now been found in the root nodules of several plants only distantly related to the legumes - *Parasponia* of the Ulmaceae, and in *Casuarina* , a group including the Australian pine, or 'she-oak'. Lateral transfer was attractive when globins seemed confined to legumes - for introduction from animals might place an odd gene into ancestors of one coherent group of plants. But why propose anything but common presence by ordinary descent for a molecule that appears widely, however sporadically, throughout the angiosperms or flowering plants?

Lateral transfer of animal globins is not dead, but the hypothesis has been greatly weakened by these two discoveries."

The hemoglobin research became very time consuming, in part because of the extensive effort required to grow nodule material, and also because I had to develop novel extraction and purification procedures. As the hemoglobin work took over, I discontinued my research on host-endophyte specificity. The thesis is thus separated into two discrete sections. Section I contains the experiments on host-specificity. Section II, by far the major part of the thesis, reports the work on *Casuarina* hemoglobin.

Tony Fleming

The following papers have been published using results presented in this thesis:

Fleming, A.I., Wittenberg, J. B., Wittenberg, B. A., Dudman, W. F. and Appleby, C. A. (1987) The purification, characterisation and ligand-binding kinetics of hemoglobins from root nodules of the non-leguminous *Casuarina glauca - Frankia* symbiosis. *Biochimica et Biophysica Acta* **911** 209-220.

Kortt, A. A., Inglis, A. S., Fleming, A. I. and Appleby, C. A. (1988) Amino acid sequence of hemoglobin I from root nodules of the *Casuarina glauca - Frankia* symbiosis. *FEBS Letters* **231** 341-346.

Fleming, A.I., Williams, E. R. and Turnbull, J. W. (1988) Growth and nodulation of provenances of *Casuarina cunninghamiana* inoculated with a range of *Frankia* sources. *Australian Journal of Botany* **36** 171-181.

The following posters and lectures have been presented using results from this thesis:

Appleby, C. A., Bogusz, D., Dennis, E. S., Fleming, A. I., Landsmann, J. and Peacock, W. J. (1989) The vertical evolution of plant hemoglobin genes. In: *Nitrogen Fixation: Hundred Years After* (Bothe *et al* , Eds.), Gustav Fischer, Stuttgart, pp 623-628.

Fleming, A. I., Wittenberg, B. A., Wittenberg, J. B. and Appleby, C. A. (1985) Hemoglobin in the root nodules of the actinorhizal genus *Casuarina* . *Proceedings of the Australian Biochemical Society* **17**, 24.

Kortt, A. A., Burns, J. E., Inglis, A. S., Appleby, C. A. and Fleming, A. I. (1985) Hemoglobins from the nitrogen-fixing root nodules of non-legumes and their relationship to the legume hemoglobins. *Proceedings of the Australian Biochemical Society* **17**, 43.

Fleming, A. I. (1984) Recent actinorhizal research, with special reference to *Casuarina* hemoglobin. *Australian Institute of Agricultural Science Occasional Publication* **12**, 85-86.

Appleby, C. A., Bogusz, D., Dennis, E. S., Dudman, W. F., Fleming, A. I., Higgins, T. J., Kortt, A. A., Landsmann, J., Peacock, W. J., Tjepkema, T. J., Trinick, M. J., Wittenberg, B. A. and Wittenberg, J. B. (1984) The origin and survival of plant hemoglobin genes. *Australian Institute of Agricultural Science Occasional Publication* **12**, 77-78.

Appleby, C. A., Trinick, M. J. and Fleming, A. I. (1985) Plant hemoglobins and oxygen supply. In: *Limitations and Potentials of Biological Nitrogen Fixation: A Boden Research Conference* (Gibson, A. H., Ed.) p. 9.

Fleming, A. I., Wittenberg, B. A., Wittenberg, J. B., Kortt, A. A. and Appleby, C. A. (1985) Hemoglobin in the Actinorhizal genus *Casuarina* . In: *Limitations and Potentials of Biological Nitrogen Fixation: A Boden Research Conference* (Gibson, A., Ed.) p. P-3.

Wittenberg, J. B., Wittenberg, B. A., Trinick, M., Gibson, Q. H., Fleming, A. I., Bogusz, D. and Appleby, C. A. (1986) Hemoglobins which supply oxygen to intracellular prokaryotic symbionts. In: *Nitrogen Fixation Research Progress* (Evans, H. J., Bottomley, P. J. and Newton, W. E., Eds.) p354. Martinus Nijhoff Publishers, Dordrecht.

ACKNOWLEDGEMENTS

I cannot, in such a short space, properly acknowledge the help which so many people have given me throughout the period of this work. However, there are a few to whom I would like to express special thanks.

I am indebted to Professor David Griffin for supervising such a wayward student. In particular I thank him for giving me the freedom to roam around the general topic of *Casuarina* nodulation, allowing me to find what turned out to be a very interesting area of research.

It is impossible to adequately express my thanks to Cyril Appleby. He taught me an enormous amount about plant hemoglobins, and through his own enthusiasm he opened my eyes to the excitement of science. He is one of those rare inspirational people. He is also an outrageous storyteller!

I owe special thanks to Judy Appleby for her friendship and generosity, for so graciously accepting the endless interruptions to her family life which my research caused, and for accepting me into the Appleby home when I desperately needed a quiet place to write.

There are many people with whom I collaborated in this work and to all of them I say thankyou. I learnt a lot during my time as a PhD student, but perhaps the most important lesson was in communication and collaboration. It is a pleasure to acknowledge the assistance of Drs Jonathan and Beatrice Wittenberg, Dr Alex Kortt, Dr Bill Dudman, Dr John Turnbull and Peter Martensz. I could not have done this work without the support of CSIRO and, in particular the Chief of the Plant Industry Division, Dr Jim Peacock. My thanks also go to the technical staff of the Dept. of Forestry (ANU), Plant Industry Division and Forest Research Division (CSIRO).

I also thank Dr Alan Gibson who first introduced me to the general area of nitrogen-fixation research, and who was always available to provide advice when I needed it.

I would like to express my gratitude to the late Dr David Goodchild. He gave me a great deal of support throughout the PhD, particularily in the difficult early stages, and it was he who first suggested I speak with Cyril about my interest in hemoglobin.

This thesis also bears the mark of my father who kept me going when all I could think of was giving up. Thanks. It was worth the struggle.

There is no doubt that my PhD caused massive intrusion to family life. Helene put aside her career as an artist so that I could complete my studies, and Alex and Adrian hardly saw me for weeks at a time. To Helene all I can say is that I love you and I thank you for your un-ending support. Now it's time to get your career back on the rails!

ABBREVIATIONS AND DEFINITIONS

• Actinorhizal plants	Plants capable of forming a symbiotic relationship with the nitrogen-fixing actinomycete *Frankia*. The microorganism is contained within root nodules, which are sometimes referred to as actinorhizae.
• Amino acids	
Ala	Alanine
Arg	Arginine
Asn (Asx)	Asparagine
Asp (Asx)	Aspartic acid
Cys	Cysteine
Gln (Glx)	Glutamine
Glu (Glx)	Glutamic acid
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Trp	Tryptophane
Tyr	Tyrosine
Val	Valine
• cDNA	Complementary deoxyribonucleic acid. Single-stranded DNA synthesised from a ribonucleic acid (RNA) template by the enzyme reverse transcriptase.
• *Casuarina* hemoglobin	The protein from *Casuarina glauca* root nodules (unless otherwise specified).
• Exon & Intron	An exon is a coding sequence of DNA, i.e. a section of chromosome that codes for the section of the final mRNA that is translated into a polypeptide sequence. Exons are normally separated by introns, which are non-coding regions, in hemoglobin genes.
• Ferric hemoglobin	Unligated ferric hemoglobin.
• Ferrous hemoglobin	Unligated ferrous hemoglobin.

• HbO_2	Oxyferrous hemoglobin.
• HbCO	Carboxyferrous hemoglobin.
• Hemoglobin I, II and III	The principal components of *Casuarina* hemoglobin separable by isoelectric focusing in order of increasing acidity.
• IEF	Isoelectric focusing.
• Kinetics terms K' & L'	Equilibrium dissociation constants for oxygen (K') and carbon monoxide (L') representing the molarity of dissolved ligand at which the protein is half combined.
P_{50}	The partial pressure of oxygen or carbon monoxide at which the protein is half saturated. This is used as a measure of the oxygen affinity of hemoglobin; i.e. the strength with which hemoglobin binds oxygen. P refers to the partial pressure of oxygen, 50 to the 50% saturation point on the O_2 dissociation curve.
k' & l'	The "on" rate; i.e. the association rate for oxygen (k') and carbon monoxide (l') with hemoglobin.
k & l	The "off' rate; i.e. the dissociation rate for oxygen (k) and carbon monoxide (l) with hemoglobin.
• Leghemoglobin (Lb)	Monomeric hemoglobins from legume root nodules.
• Phylogeny	The evolutionary development of a genetically related group of organisms, e.g. plants.

SECTION I

Casuarina cunninghamiana - Frankia

Host Specificity

Chapter One

INTRODUCTION - SECTION I

In Section One of this thesis I report on aspects of host-specificity in the *Casuarina cunninghamiana - Frankia* symbiosis. The term 'host-specificity' is used to describe the relationship between the host plant and the *Frankia* endophyte as measured by infectivity and symbiotic N_2-fixing effectiveness. I am thus assuming that it is possible to observe varying degrees of host-specificity, which would be reflected in plant growth. The term 'host-specificity' is used in the same sense as the term 'cross-inoculation group' is normally applied.

Fundamental to any investigation of host-specificity is an understanding of the taxonomic status of the organisms involved. While this is relatively straightforward for *Casuarina* , the situation is far less clear for *Frankia* , where there is no agreement over what characteristics, if any, adequately delineate species.

Taxonomy of the Casuarinaceae

The taxonomy of the Casuarinaceae used throughout this thesis follows the recent revision by Wilson and Johnson (1989). Thus the casuarinas are split into four genera: *Casuarina* (seventeen species), *Gymnostoma* (eighteen species), *Allocasuarina* (59 species) and *Ceuthostoma* (species number not known). The first three genera have representatives in Australia, the Pacific islands and South-East Asia, with *Allocasuarina* being endemic to Australia. *Ceuthostoma* is restricted to the Pacific islands. The species of concern in this section of the thesis, *Casuarina cunninghamiana,* is divided into two subspecies, subsp. *cunninghamiana* and subsp. *miodon* . Only provenances of *C. cunninghamiana* subsp. *cunninghamiana* were used in these studies.

The concept of species in the Frankiaceae

The endophytes of actinorhizal plants are grouped together in the monotypic actinomycete family Frankiaceae, within the genus *Frankia* (Becking 1970, 1974). This classification is based on gross morphological features of the endophyte in the nodule, and supported by studies of *Frankia* in pure culture. While classification to the genus level is widely accepted, there is considerable confusion and disagreement over the merits of dividing *Frankia* into one or more species. For the purposes of this thesis I have not attempted to classify *Frankia* down to the level of species.

It is not suprising that progress on the specific classification of *Frankia* has been limited. Pure cultures of *Frankia* have been available for study only since Callaham *et al.* (1978) isolated the endophyte from *Comptonia peregrina* nodules, though it is now clear that the first pure culture of *Frankia* was obtained by Pommer (1959) from nodules of *Alnus glutinosa* . At the time of writing this thesis, cultures had been obtained only from a minority of the known actinorhizal species and only about half the known actinorhizal genera, thus making it impossible to develop a comprehensive classification system. To add to the confusion, there is continuing uncertainty over what constitutes a species in the order Actinomycetales (Arai 1976), within which *Frankia* is placed.

The initial taxonomy of* Frankia *rejected.

Becking (1970, 1974) proposed splitting *Frankia* into 9 species, based largely on their ability to infect different hosts. This proposal has been rejected, partly on the grounds that cross-inoculation (host-specificity) groups are not an appropriate descriptor of species (detailed discussion of cross-inoculation below), but more importantly because his classification was based on trials using crushed nodules as an inoculum, which do not always match results found using pure culture *Frankia* strains (e.g. Lalonde and Calvert 1979, Normand and Lalonde 1986). In any event,

such groups cannot be precisely defined because infectivity is affected by a range of environmental factors which bear no relationship to the taxonomic identity of the endophyte.

Becking's classification also relied on *in vivo* morphological characteristics which, again, are problematic as taxonomic characters because the host can express some control over endophyte morphology. A very clear example is the lack of vesicle expression in *Casuarina* nodules, whereas *Frankia* strains isolated from those nodules do produce vesicles under certain environmental conditions.

In 1983, a decision was taken at an International Workshop on *Frankia* held in Wageningen (the Netherlands) to recognise only the genus *Frankia* until and unless reliable characters, which clearly distinguished species, could be identified. This would require the analysis of a large number of strains from a wide range of actinorhizal associations if the classification scheme was to be made comprehensive.

Frankia strains are currently identified by a series of letters and numbers, which ensures consistency of nomenclature without making any presumptions as to specific relationships.

***Two proposed* Frankia *species:* F. alni *and* F. elaeagni.**

A reliable classification system for *Frankia* must be based primarily on pure culture characteristics, rather than those of the organism *in vivo*, and it is only now becoming clear which characters may be effectively used to differentiate strains. Lechevalier (1984) suggested that cell chemistry, physiology, morphology, infectivity, ecology, phage grouping, DNA homology, serology and 16S RNA were all useful for the differentiation of species.

Lalonde *et al.* (1988) reviewed the data on characters they considered suitable as taxonomic indicators (including those identified by Lechevalier and also novel work on whole-cell fatty acid composition) and boldly

suggested the separation of two *Frankia* species, one of which they further subdivided into two sub-species. The proposed species names were *F. alni* (Woronin) von Tubeuf 1895 subsp. *pommerii* (subsp. nov.), *F. alni* subsp. *vandijkii* (subsp. nov.) and *F. elaeagni* (Schroter) Becking 1970. Their preliminary separation was based on host-specificity groups broadly labelled *Alnus* and *Elaeagnus* . They also identified a third host-specificity group, *Casuarina*, but did not attempt to define this as a species. The *Alnus* group included *Frankia* isolated from the nodules of *Alnus, Myrica* and *Comptonia*; the *Elaeagnus* group included *Frankia* isolated from nodules of *Elaeagnus, Shepherdia, Hippophae* and *Colletia* (further discussion on these groups below). Lechevalier (1984) described equivalent groups as A (*Elaeagnus*) and B (*Alnus*), separating them on the basis of physiological characteristics. Group B included (among others) the serotype I strains of Baker *et al.* (1981 - strains identified by immunodiffusion assays) and group A included (among others) Baker *et al.*'s serotype II strains.

The separation of the two host-specificity groups was then matched by Lalonde *et al.* (1988) against a series of data which were found to be generally consistent with the *Alnus* and *Elaeagnus* grouping.

The two subspecies of *F. alni*, as proposed by Lalonde *et al.* (1988) were consistent with the type-N and type-P *Frankia* strains previously described (Normand and Lalonde 1982). This N- P separation was primarily based on the spore-positive (Type-P) and spore-minus (Type-N) characteristic which has been well described for *Alnus* (Van Dijk 1978, Holman and Schwintzer 1987), *Myrica* (Schwintzer *et al.* 1982, Kashanski and Schwintzer 1987) and *Comptonia peregrina* (VandenBosch and Torrey 1985) nodules, all of which are members of the *Alnus* host-specificity group.

It is important to note that the proposed classification scheme of Lalonde *et al.* (1988) does not attempt to describe the phylogenetic relationships between *Frankia* strains. It is a scheme of convenience based on what

they consider to be reliable indicators of taxonomic identity. It must also be realised that their scheme does not consider *Frankia* from a wide range of the actinorhizal symbioses, including *Casuarina* . It relates only to those *Frankia* strains which have been identified as belonging to either the *Alnus* or *Elaeagnus* host-specificity groups. Unfortunately, it is not spelt out in the paper by Lalonde *et al.* (1988) what weight is applied to different characters when deciding whether a given strain fits within one or other of their species. This would seem to be particularly important for the proposed species, *F. elaeagni*, where so many of the characters are highly heterogeneous.

The significance of host-specificity groups

The Lalonde *et al.* (1988) proposal relied heavily on host specificity groups, just as Becking (1970, 1974) had previously done, particularly the *Alnus* and *Elaeagnus* groups. These groups have been recognised for some time (e.g. Baker and Torrey 1980), though as with legume-*Rhizobium* cross-inoculation groups their boundaries are not precise. They are, however, generally consistent with the results of a number of cross-inoculation studies (Normand and Lalonde 1986, Jiabin *et al.* 1985).

Even so, there are numerous exceptions to these groups (including some identified by Normand and Lalonde 1988) such as three strains isolated from *Myrica gale* which did not nodulate *Myrica* but did nodulate *Elaeagnus* (Baker 1987). There are reports of a number of strains isolated from *Casuarina* which were only able to nodulate members of the Elaeagnaceae (Gauthier *et al.* 1981, Diem *et al* 1982, Baker 1987). Baker (1987) also found that a single strain isolated from *Colletia cruciata*, which is supposed to belong in the *Elaeagnus* host-specificity group (Normand and Lalonde 1986) was able to nodulate both members of the Elaeagnaceae and the Myricaceae.

In general, the results obtained by Baker (1987) did not fit comfortably with the *Alnus* and *Elaeagnus* host-specificity groups. Instead, he proposed four host-infectivity groups; viz:

1. strains which nodulate *Alnus* and *Myrica* ,
2. " " " *Casuarina* and *Myrica* ,
3. " " " Elaeagnaceae and *Myrica* , and
4. " " " Elaeagnaceae only.

Thus, for example, the *Myrica* strains described above belong to group four.

Host specificity at the intra-and inter-generic level.

It is clear from the discussion above that the question of host-specificity in actinorhizal plants is extremely complex. It can at best be used only as a rough guide to relationships among *Frankia* isolates. This is strongly reinforced by the confused nature of relationships at the intra- and inter-generic level.

The best studied genus is *Alnus*, where there are many examples of; (a) absolute specificity for one or a few species of *Alnus*, and (b) variation in the degree of effectiveness of different host-endophyte combinations which do produce nodules. For example, Baker (1987) found that of thirteen strains of *Frankia* isolated from six species of *Alnus* , only seven induced nodules on either *A. glutinosa* or *A. rubra* . One strain isolated from *A. glutinosa* failed to nodulate that species. Jiabin *et al.* (1985), however, found that *Frankia* strains isolated from four species of *Alnus* were all able to nodulate *A. cremastogyne* . Normand and Lalonde (1986) isolated a *Frankia* strain from *A. sinuata* that failed to nodulate *A. rugosa* and formed only ineffective nodules on *A. crispa* and *A. rubra* . These and numerous other results (e.g. Dawson and Sun 1981, Nesme *et al.* 1985, Wheeler *et al.* 1986, Hooker and Wheeler 1987, Dillon and Baker 1982, Hahn *et al.* 1988, Sheppard *et al.* 1988, van Dijk *et al.* 1988, Lechevalier *et al.* 1983, Normand and Lalonde 1982, Weber *et al.* 1987) show it is not reasonable to assume that complete cross-inoculation is possible within a family, or even within a genus, and that even when

nodulation occurs both the host and endophyte can influence the effectiveness of the association. It is thus possible that there exist many strains (species?) of *Frankia* within the *Alnus* host specificity group.

Host-endophyte specificity in the Casuarinaceae

Within the Casuarinaceae, specificity between host and endophyte has been observed at both the inter- and intra-generic levels (Table 1.1), despite early reports to the contrary (Mowry 1933, Bond 1957).

Coyne (1973, 1983) was able to divide *Casuarina cristata*, F. Muell. ex Miq., *C. cunninghamiana* Miq., *C. glauca* Sieb. ex Preng., *Allocasuarina verticillata* (Lam.) L. Johnson, *A. torulosa* (Ait.) L. Johnson and *A. littoralis* (Salisb.) L. Johnson into the two genera according to their growth response to inoculation with crushed field nodules from each of the species. He also found that nitrogen fixation was greatest when a species was inoculated with its own inoculum. Coyne interpreted the degree of effectiveness of different host-endophyte combinations as indicating specificity, but apart from two inocula (*A. littoralis* and *A. verticillata)* which failed to nodulate any species, each inoculum was able to induce nodulation on each host species (i.e. there was no absolute incompatability), although the very great time taken for nodulation in some cases (40-80 days) leaves open the possibility of contamination.

It is of historical interest that Coyne was able to distinguish between members of *Casuarina* and *Allocasuarina* on the basis of nodulation; when he did his experiments the Casuarinaceae was considered to be a monotypic family. In fact, most studies which have been done on nodulation in the Casuarinaceae are consistant with the recent taxonomic revision of that family by Wilson and Johnson (1989).

More recent work has reinforced the view that the genera *Allocasuarina* and *Casuarina* represent two host-specificity groups. Gauthier *et al.*

TABLE 1.1: A SUMMARY OF HOST SPECIFICITY TRIALS INVOLVING ISOLATES OBTAINED FROM *Casuarina* NODULES EITHER IN (A) PURE CULTURE, OR AS (B) CRUSHED NODULES.
+ INFECTIVE - NON INFECTIVE

The results are compiled from the following sources:
Zhang *et al.* (1984), Zhang and Torrey (1985 a, b), Rosbrook and Bowen (1987), Gauthier *et al.* (1984), Baker (1987), Coyne (1974), Jiabin *et al.* (1985), Reddell and Bowen (1985a)

	Frankia SOURCE			
HOST				
	(A) Pure culture			
	C. cunn.	C. equis.	C. jung.	A. lehm.
Hippophae	+ + - +	+ + + + +	-	
Elaeagnus	+ - + + -	+ + - + + + + + + +	- -	
Myrica		+ + + + - + +	+	
Alnus		- - - - - - - -	-	
Colletia		+	-	
Casuarina spp				
cunninghamiana	+ + - - - - - - +	+ + +	+	+
glauca	+	+ + +	+	
equisetifolia	+ + - - +	+ + + - - + + - - - - - - + -	+ + +	+
obesa		+ + +	+	
junghuniana		-	- +	
cristata			+	
Allocasuarina spp				
lehmanniana	-			+
decaisneana			-	
distyla			-	
fraseroana			-	
huegliana			-	
littoralis			-	
verticillata			-	
torulosa			-	
deplanchneana			-	

	(B) Crushed nodule inoculum							
	C.cris.	C. cunn.	C. glau.	C. equis.	C. tim.	A. lit.	A. ver.	A.tor.
Casuarina spp								
cristata	+	+	+			-	-	+
cunninghamiana	+	+ +	+	+ - + -	-	-	-	+
glauca	+	+	+			-	-	+
equisetifolia		+		+ + +	+			
Allocasuarina spp								
littoralis	-	-	-			-	-	-
verticillata	+	+	+			-	-	+
torulosa	-	+	+			-	-	+

(1984) found that a pure *Frankia* strain isolated from *C. junghuhniana* Miq. formed nodules on all six *Casuarina* species tested, but failed to nodulate any of the seven species of *Allocasuarina* or one species of *Gynnostoma* L. Johnson used. Zhang and Torrey (1985a) could nodulate only 2/16 *Allocasuarina lehmanniana* (Miq.) L. Johnson seedlings using an inoculum from *C. equisetifolia*, and their attempts to nodulate 9 other *Allocasuarina* species with the same inoculum were completely unsuccessful. In another study, Zhang and Torrey (1985b) found that a pure culture of *Frankia* isolated from *C. cunninghamiana* nodules was infective and effective on both *C. cunninghamiana* and *C. equisetifolia* but was non-infective on *Allocasuarina lehmanniana* and *A. decaisneana* .

Specificity has also been observed within the genus *Casuarina* (Table1.1). Reddell and Bowen (1985a) found that three out of four *C. equisetifolia* crushed nodule inoculum sources were non-infective on *C. cunninghamiana* , and also found that the one *C. equisetifolia* inoculum source that did cause nodulation was less effective than the *C. cunninghamiana* inoculum source also tested. *C. equisetifolia* plants formed ineffective nodules with a *C. cunninghamiana* inoculum, but effective nodules with inoculum from their own species. These results, however, conflict with later work by Rosbrook and Bowen (1987) using three pure cultures of *Frankia* isolated from *C. equisetifolia* , which failed to detect significant differences in effectiveness of these isolates on *C. cunninghamiana, C. equisetifolia* var *equisetifolia, C. equisetifolia* var *incana, C. obesa* and *C. glauca* .

Frankia isolates have also been obtained from nodules of various *Casuarina* species which failed to nodulate *Casuarina* but did nodulate members of the Elaeagnaceae (Gauthier *et al.* 1984, Baker 1987). Baker (1987) has found that the CcI3 isolate originally obtained by Zhang and Torrey (1985b) from *C. cunninghamiana* , could nodulate both *Myrica* and *Casuarina*. Yet another isolate could nodulate *Elaeagnus* , *Hippophae* and *Myrica* , but failed to nodulate *Casuarina* . The CcI3 report is the

first record of a *Frankia* strain which is infective on *Casuarina* and members of any other actinorhizal family.

An understanding of host-specificity is not only important in determining taxonomic relationships, it can also be of great significance in the practical application of the symbiosis. This is true not only at the inter and intra-generic levels, but also at the intra-specific level, and is particularly relevant for the genus *Casuarina* which is widely planted. This matter is further discussed in the next chapter.

Chapter Two

GROWTH AND NODULATION OF PROVENANCES OF *CASUARINA CUNNINGHAMIANA* INOCULATED WITH A RANGE OF *FRANKIA*

Natural populations of *Casuarina cunninghamiana* , the species of concern in the studies reported here, are distributed over a wide latitudinal range in eastern Australia (Figure 2.1). There are two distinct sub-species, *C. cunninghamiana* Miq. subsp. *cunninghamiana* and *C. cunninghamiana* subsp. *miodon* L. Johnson. The distribution of *C. cunninghamiana* subsp. *cunninghamiana* is from southern New South Wales to the Cape York peninsula, and *C. cunninghamiana* subsp. *miodon* from Daly River in the Northern Territory east to the Gulf of Carpentaria (Wilson and Johnson 1989). Typical habitats in which this species is found are shown in Figure 3.3. In deep-sided gorges which occur at the edge of the tablelands in New South Wales trees may occur as either scattered individuals or in small groups on locally favourable sites such as sand or gravel banks. Occasionally, individuals will develop in rock cracks. In broader valleys of the coastal plains, western slopes (down to 300 m) and lower parts of the tablelands (up to 900 m - Beadle 1981) closed forests of young, even-aged casuarinas will develop on terraces. These terraces, composed of small stones and sand, are formed by the deposition and scouring of river bed material. These young forests probably represent a pulse-stable system, being maintained in that state by periodic flooding. Much older trees will line the permanent water courses in many situations.

The distribution of *C. cunninghamiana* is not continuous throughout its range, because the species grows only in riparian habitats associated with freshwater streams. Thus populations in discrete river systems are

On one occasion, field plantings of *Casuarina* sp., inoculated with artificially cultured *Frankia* were made in Senegal (Dommergues, 1984 in Advances in Nitrogen Fixation Research, Eds. C.Veeger & W. E. Newton, pp7-1 3, Nijhoff/Junk/Pudoc, The Hague.)

insert →

geographically isolated from each other and, given the extensive distribution, it is proposed that specificity has evolved between isolated populations of *C. cunninghamiana* and *Frankia.*

The identification of specificity has important implications for the practical use of *C. cunninghamiana* . This species is planted widely for poles, shelterbelts and river bank conservation, often on nutrient-deficient soils where the efficiency of the nitrogen-fixing symbiotic system may be critical to the health and productivity of the plant (Gauthier *et al.*. 1981, Torrey 1983). Many examples of the use of *C. cunninghamiana* are given in a publication by the National Academy of Sciences (1983), and in Midgley *et al.* (1983), including plantings in Israel, Egypt, Kenya, Zimbabwe, Argentina, California, Florida and Hawaii.

Little attention has been paid to the selection of the best combination of host and endophyte. Nodulation of planted trees has been dependent on either the presence of native *Frankia* in the soil, or inoculation with whatever inoculum has been conveniently available, neither of these techniques allowing control over the effectiveness of the association.

The potential for large scale inoculation of actinorhizal plants with *Frankia* has been demonstrated in various genera (Lalonde and Simon 1985, Perinet *et al.* 1985, Berry and Torrey 1985). Similar techniques for *Casuarina* need to be developed if the symbiosis is to be exploited effectively. However, for such large operations to be worthwhile, it is necessary to select an effective combination of host and endophyte to match prevailing conditions.

This study was conducted in two parts. The first experiment examined the growth and nodulation, under glasshouse conditions, of *C.cunninghamiana* from a single tree seed source inoculated with *Frankia* from nine provenances in New South Wales. The second and major experiment examined differences in growth and nodulation, under glasshouse conditions, of eighteen geographically isolated populations (provenances)

of *C. cunninghamiana* each inoculated with *Frankia* from each of the eighteen sources.

Both experiments used crushed, surface-sterilised nodules as inoculum sources. While it is often desirable in experiments manipulating symbiotic partners to use pure culture inocula, at the time when this work was done it was not practicable to do this for *Casuarina,* particularly given the large number of isolates which would have been required. There was thus no certainty that the inoculum from a single location was made up of a single 'strain' of *Frankia*, but this was inconsequential as the experiment was only concerned with the expression of specificity down to the level of provenance.

Material and methods

Experiment one

Seed and inoculum collection.

Seed cones of *Casuarina cunninghamiana* were collected from a single tree at Paddy's River in the A.C.T. (35^{o} 23' E, 148^{o} 58' S). Cones were air-dried to release seed which was then stored at 4^{o}C until use.

Nodules were collected from five sites in New South Wales (Table 2.1). At each site individual nodules from a number of trees were collected, washed clean of soil, lightly dried and stored in separate vials over silica gel. Once back in the laboratory they were stored over silica gel at 4^{o} C until use. One of the nodule-source trees from site 005 was a possible hybrid with *Casuarina cristata* and the tree from site 031 was a hybrid with *C. glauca*. Hybridisation of these species of *Casuarina* is known to occur (Wilson and Johnson 1989).

Experimental design.

The experiment was conducted, using a water-culture system, as five

TABLE 2.1: INOCULUM PROVENANCE VARIATION ON A SINGLE SEED SOURCE (EXPT 1, SECTION I). THE LOCATION OF SITES OF *C. cunninghamiana* FROM WHICH NODULE MATERIAL WAS COLLECTED FOR USE AS AN INOCULUM.

All sites are in New South Wales.

No.	Locality	Latitude (°E)	Longitude (°S)
1.	Dandhara Ck, near Grafton	29° 31'	152° 27'
2.	Dandhara Ck, near Grafton	29° 31'	152° 27'
3.!	Bundella Tamilies Ck, Quirindi Rd	31° 33'	150° 0'
4.!	Bundella Tamilies Ck, Quirindi Rd	31° 33'	150° 0'
5.	Dingo Ck, near Wingham	31° 55'	152° 27'
6.	Dingo Ck, near Wingham	31° 55'	152° 27'
7.*	Broke picnic ground	32° 45'	151° 08'
8.	Kangaroo R.	34° 43'	150° 35'
9.	Kangaroo R.	34° 43'	150° 35'

! Possible x *cristata* hybrid * Possible x *glauca* hybrid

complete replicates of a nine-level, one factor experiment, the factor being inoculum source. An error during inoculation meant that there were only four replicates for inoculum 034/004. There were five replicate control pots which were uninoculated. The pot layout was completely randomised, and re-randomised twice during the experimental period.

Experimental methods.

The experiment was conducted in a glasshouse at the Department of Forestry, Australian National University.

Seed was sown into flat trays of sand and watered regularly with de-ionised water. Seedlings were transferred 43 days after sowing into 2-litre aerated water culture pots containing half-strength complete Hoaglands solution (Table 2.2). Each pot contained three seedlings equally spaced. The complete nutrient solution was replaced every 14 days until inoculation, 84 days after transfer. The inoculation procedure, repeated for each inoculum source, is outlined below:

Inoculation : Individual nodules were used from the sources indicated in Table 2.1. Each nodule was thoroughly cleaned in distilled water, rinsed in 70% ethanol for 30 s - 1 min, rinsed in sterilised water, dried, weighed and ground in a mortar and pestle with sterilised water. The mortar and pestle were thoroughly rinsed in ethanol and sterilised water between grindings. The ground nodule suspension was next filtered through two layers of cheesecloth and the filtrate diluted with sterilised water to give a final inoculum concentration of 1.35 g nodule material per 100 ml of sterilised water. Seedlings were inoculated firstly by placing their roots in a saucer containing 100 ml of the appropriate inoculum suspension for one minute, and secondly by adding 20 ml of the inoculum suspension to the N-free nutrient solution of each of the five replicate pots.

The height of each seedling was measured on inoculation day and thereafter every seven days. The measurement was taken from the level

TABLE 2.2: HOAGLANDS NUTRIENT SOLUTION (COMPLETE AND NITROGEN-FREE) USED IN EXPT 1, SECTION I, AND IN THE PRODUCTION OF NODULE MATERIAL FOR HEMOGLOBIN EXTRACTION (SECTION II)

Stock solutions	Complete ($ml . l^{-1}$) half strength	N-Free ($ml . l^{-1}$) half strength
1M KNO_3	2.5	-
1M $Ca(NO_3)_2$	2.5	-
1M $MgSO_4$	1.0	1.0
1M KH_2PO_4	0.5	0.5
1M KCl	-	2.5
1M $CaCl_2$	-	2.5
Micronutrients	0.5	0.5
FeEDTA	0.5	0.5
Demineralised water	to 1.0 litre	to 1.0 litre

Hoagland, D.R. and Arnon, D.I. (1950) The water culture method for growing plants without soil. Calif. Agric. Expt. Sta. Cir. 347.

of the cotyledons to the apex of the swept-up branchlets.

After inoculation the plants were transferred to half-strength nitrogen-free Hoaglands solution (Table 2.2). This nutrient solution was replaced at fourteen day intervals for the first six weeks, and then every seven days. Plants were harvested 84 days after inoculation (i.e. 30 weeks after sowing). Immediately prior to harvest a final height measurement was taken. The tops were then carefully separated from the roots at the level of the cotyledons, both roots and shoots were then dried at 80^{o} C for 48 h and weighed.

Experiment two

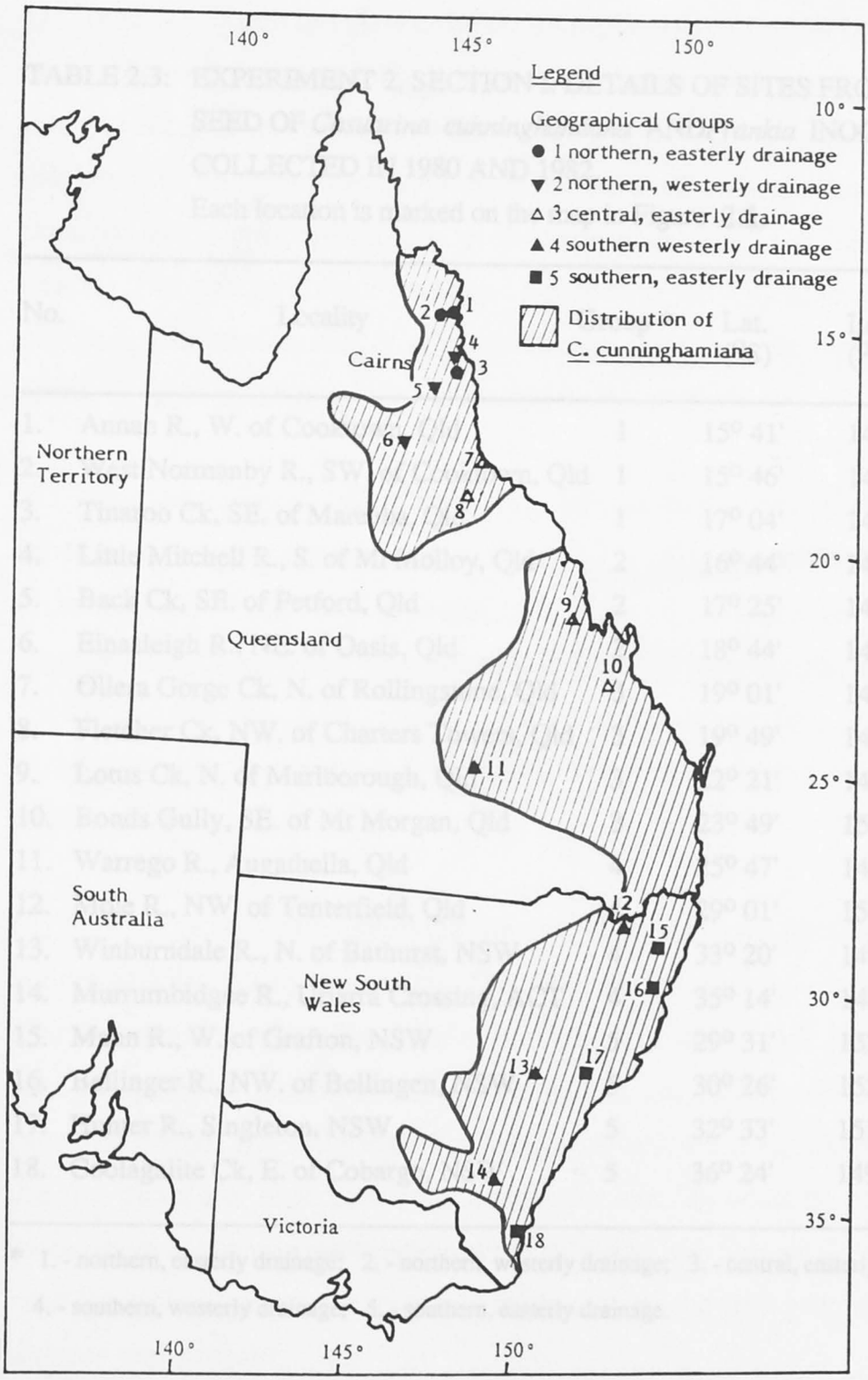
Seed and inoculum collection.

Seed was collected from three trees in each of eighteen natural populations of *C. cunninghamina* subsp. *cuninghamiana* (Table 2.3). *C. cunninghamiana* subsp. *miodon* , from the Northern Territory and northwestern Queensland, was not included. One population was sampled from each of the different river systems throughout the natural range of the species, and more than one population was included from each of the very large Murray-Darling and Fitzroy River systems (Figure 2.1). On the basis of geographic location, the collection sites were separated into five groups: (1) Northern, easterly drainage, (2) Northern, westerly drainage, (3) Central, easterly drainage, (4) Southern, westerly drainage and (5) Southern, easterly drainage (Figure 2.1). Root nodules were taken from one or more trees at each of the eighteen seed collection sites. The nodules to be used as the *Frankia* inoculum source were surface-sterilised to remove external *Frankia.*, thoroughly rinsed in distilled water (*Frankia*-free) and air-dried for 24 h. Once dried they were stored over silica gel in a desiccator at room temperature.

Experimental design.

The experiment was conducted as three complete replicates of a two-factor experiment, the factors being seed source and inoculum

Figure 2.1: Map showing the locations of seed and inoculum collection sites of *Casuarina cunninghamiana*. The natural distribution of *C. cunninghamiana* is indicated by the solid black line. Note that within this area *C. cunninghamiana* is restricted to watercourses.



Group 1

1. Annan R.
2. Normanby R.
3. Tinaroo Ck.

Group 2

4. Little Mitchell R.
5. Back Ck.
6. Einasleigh R.

Group 3

7. Ollera Gorge Ck.
8. Fletcher Ck.
9. Lotus Ck.
10. Bonds Gully

Group 4

11. Warrego R.
12. Mole R.
13. Winburndale Riv.
14. Murrumbidgee R.

Group 5

15. Mann R.
16. Bellinger R.
17. Hunter R.
18. Coolagalite Ck.

TABLE 2.3: EXPERIMENT 2, SECTION I. DETAILS OF SITES FROM WHICH SEED OF *Casuarina cunninghamiana* AND *Frankia* INOCULUM WERE COLLECTED IN 1980 AND 1982.
Each location is marked on the map in Figure 2.1.

No.	Locality	Group *	Lat. (°S)	Long. (°E)	Alt (m)
1.	Annan R., W. of Cooktown, Qld	1	15° 41'	145° 12'	100
2.	West Normanby R., SW. of Cooktown, Qld	1	15° 46'	144° 59'	110
3.	Tinaroo Ck, SE. of Mareeba, Qld	1	17° 04'	145° 28'	400
4.	Little Mitchell R., S. of Mt Molloy, Qld	2	16° 44'	145° 21'	380
5.	Back Ck, SE. of Petford, Qld	2	17° 25'	144° 59'	560
6.	Einasleigh R., NE. of Oasis, Qld	2	18° 44'	144° 19'	460
7.	Ollera Gorge Ck, N. of Rollingstone, Qld	3	19° 01'	146° 20'	20
8.	Fletcher Ck, NW. of Charters Towers, Qld	3	19° 49'	146° 03'	280
9.	Lotus Ck, N. of Marlborough, Qld	3	22° 21'	149° 06'	140
10.	Bonds Gully, SE. of Mt Morgan, Qld	3	23° 49'	150° 18'	120
11.	Warrego R., Augathella, Qld	4	25° 47'	146° 36'	370
12.	Mole R., NW. of Tenterfield, Qld	4	29° 01'	151° 32	320
13.	Winburndale R., N. of Bathurst, NSW	4	33° 20'	149° 37'	700
14.	Murrumbidgee R., Uriarra Crossing, ACT	4	35° 14'	148° 57'	520
15.	Mann R., W. of Grafton, NSW	5	29° 31'	152° 27'	80
16.	Bellinger R., NW. of Bellingen, NSW	5	30° 26'	152° 39'	80
17.	Hunter R., Singleton, NSW	5	32° 33'	151° 10'	110
18.	Coolagalite Ck, E. of Cobargo, NSW	5	36° 24'	149° 56'	100

* 1. - northern, easterly drainage; 2. - northern, westerly drainage; 3. - central, easterly drainage; 4. - southern, westerly drainage; 5. - southern, easterly drainage.

source. The experimental design was based on designs developed by Patterson and Williams (1976). The main features of the design used in the experiment are:

(1) Groups of three benches constituted replicates of the design. Within each replicate the seed-inoculum source combinations were organised into blocking units consisting of benches within replicates and columns (eighteen of them) within benches. This required only six out of the possible seven pots available in each incomplete block (or column) on a bench. The remaining pot was used either as a non-inoculated control , to check for contamination, or as controls with inorganic nitrogen added. None of these extra pots were included in the analysis.

(2) The experimental design was a type of split-plot arrangement where all pots in any incomplete block of six pots (excluding the extra one) received seed of the same provenance. Within the incomplete blocks, the arrangement of inoculum sources maximised the information available for the comparison of inoculum sources and the interaction. The eighteen incomplete blocks on any one bench all received seed from a different provenance.

(3) As well as being designed for the two eighteen-level factors (seed source and inoculum source) and their interaction, the experiment was also organised so that efficient comparisons could be made between the five groups of collection sites. Hence, within any incomplete block of six pots, the design guaranteed that at least one representative from each of the five groups of inoculum sources was present. In addition, the incomplete blocks on each bench were grouped into lots of six so that in each lot, at least one representative from each of the five groups of seed sources was present.

The experimental design was complex, but such detailed blocking maximised the possibility of detecting differences between the seed-inoculum source combinations. The design was generally balanced

and therefore could be analysed using the statistical package GENSTAT (Alvey *et al.* 1977).

Experimental methods.

Washed and stream sterilized (70 C, 2 h) river sand was added to 9cm undrained plastic pots (781 g dry sand per pot). The sand contained 0.012% nitrogen (4.3 mg/g NH_4^+ , 2.2 mg/g NO_3^-), 0.006% phosphorus and 6.0% potassium. Phosphorus, as superphosphate, was added to each pot to give a concentration of 20 ppm.

Seed from each provenance was sown directly into pots immediately after each pot had been watered to field capacity with demineralised water. Demineralised water was used throughout the experiment. Seedlings were progressively thinned to three per pot at four weeks after sowing and one per pot at the time of inoculation (seven weeks). Care was taken to prevent cross-contamination of seedlings by using pots with no drainage holes and by sterilization of all inoculum equipment. To reduce the risk of aerosol disperal of *Frankia* between pots, a misting spray was used to water each pot individually.

Seedlings were inoculated at seven weeks. The inoculum was prepared by grinding the surface-sterilized nodule material in distilled water and the resulting suspension made up to a final concentration of 0.45 g of nodule material per 100 ml distilled water. Insufficient nodule material meant that lower concentrations had to be used for the following five provenances: Back Creek 0.33 g/100 ml; Normanby River 0.29 g/100 ml; Lotus Creek 0.13 g/100 ml; Warrego River 0.08 g/100 ml and Hunter River 0.07 g/100 ml. Analyisis of the results showed inoculum concentration had no effect on plant growth or nodulation. Seedlings were inoculated by injecting 5 ml of the nodule suspension 1 cm below the soil surface on either side of the seedling (i.e. 10 ml/plant) immediately following watering to field capacity.

A complete nutrient solution (NH_4NO_3 472 mg l^{-1}, KNO_3 81 mg l^{-1}, KH_2PO_4 27 mg l^{-1}, K_2SO_4 122 mg l^{-1}, K_2HPO_4 87 mg l^{-1}, $Ca(NO_3)_2.4H_20$ 118 mg l^{-1}, $Mg(NO_3)_2.6H_20$ 179 mg l^{-1}) was injected into each pot at 10ml per seedling at four and eight weeks. A nitrogen-free solution (Mg $SO_4.7H_2O$ 740 mg l^{-1}, KH_2PO_4 200 mg l^{-1}, KCl 560 mg l^{-1}, $CaCl_2.2H_2O$ 1100 mg l^{-1}, Fe EDTA 1.5 ml l^{-1}, 1.5ml l^{-1} micronutrient solution) was applied at 10 ml per seedling at 23 (1/2 strength), 25, 26 and 27 weeks.

Day temperatures throughout were mainly in the range of 25-30° C (with extremes of 20° C to 39° C). Night temperatures were in the range 14-21° C. No artificial lighting was used.

At 25 weeks the height of each seedling was measured from the level of the cotyledons to the apex of the swept-up branchlets. Plants were harvested at 32 weeks. Seedlings were carefully removed from the pots and the shoots cut from the roots immediately below the level of the cotyledons. All plant parts were dried at 80° C for 48 h and then sealed in plastic pouches with silica gel until they could be weighed.

It was impracticable to measure nodulation strictly quantitavely, so a semi-quantitative assessment was made of number and average size of nodules per plant. Nodule number was assessed on a 0-3 scale: 0=no nodules; 1= 1-10; 2 = 11-100; 3 = 100+ per plant. Average nodule size was determined by comparison with selected representative root systems which showed small, medium and large nodule size ratings of 1,2,3 respectively.

After all plants had been dried and weighed, a sub-sample of 132 tops were removed for total nitrogen determination by the sulphuric peroxide digest method (Heffernan 1985). Seed x inoculum combinations were chosen which represented all five geographic groups and spanned the

range of low to high shoot dry weight.

Results

Experiment one

Time to nodulation.

The time taken for each inoculum source to effect nodulation is shown in Table 2.4.

All inoculated plants formed nodules. However, there were significant differences ($p \leq 0.001$) in the time taken for the first nodules to appear (Table 2.5). The greatest difference occured between the two inocula from Dandhara Creek, one taking on average the shortest time for nodulation and the other the longest.

Influence of inoculum source on plant growth.

The shoot weight, root weight, root-shoot ratio and pre-harvest height for each inoculum source is shown in Table 2.4.

All inocula significantly increased plant growth relative to the uninoculated controls, indicating that all symbioses were actively fixing nitrogen. The most striking impact of nodulation was the reduction in root-shoot ratio compared with the uninoculated controls (Figure 2.2).

There was no significant variation between inoculum sources for any of the parameters used to measure plant growth; viz, shoot weight, root weight, pre-harvest height (Table 2.5). There was no relationship between the time taken for nodulation to occur and plant growth (e.g., Figure 2.2).

There was a strong positive correlation between pre-harvest height and shoot dry weight (Figure 2.2).

TABLE 2.4: INOCULUM PROVENANCE VARIATION ON A SINGLE SEED SOURCE (EXPT 1, SECTION I). RESULTS FOR ALL PARAMETERS MEASURED.

The inoculum number relates to Table 2.1. There were five replicates for each inoculum source, except inoculum 9 (four reps). Shoots and roots were separated at the cotyledons. The final height measurement was taken immediately prior to harvesting.

Inoculum number	Shoot dry weight (g)	Root dry weight (g)	Root/Shoot ratio	Time to nodulation (days)	Final height (cm)
1	14.69	5.53	0.38	23	50.5
1	8.46	3.33	0.39	27	46.7
1	8.78	3.30	0.38	26	41.4
1	10.60	4.52	0.42	25	40.5
1	11.15	3.90	0.38	26	48.6
2	9.00	4.15	0.46	17	49.3
2	11.00	3.56	0.31	17	46.1
2	11.00	3.91	0.36	14	46.4
2	16.00	5.19	0.33	15	57.9
2	12.00	4.10	0.35	17	54.3
3	8.53	3.14	0.37	20	52.6
3	13.60	5.03	0.37	18	61.5
3	17.44	5.29	0.30	17	68.0
3	12.70	4.83	0.38	24	68.2
3	9.15	3.42	0.37	20	39.2
4	8.07	2.90	0.36	19	44.6
4	7.92	3.16	0.40	16	40.9
4	10.56	3.53	0.33	17	49.2
4	12.24	4.39	0.36	18	47.8
4	7.91	3.01	0.38	21	38.2
5	12.42	4.35	0.35	21	59.0
5	4.39	1.93	0.44	22	30.0
5	9.95	4.08	0.41	20	51.2
5	7.38	2.44	0.33	25	44.3
5	10.94	4.05	0.37	20	49.8
6	11.24	3.37	0.30	19	46.5
6	11.29	4.40	0.39	23	44.2
6	17.77	6.4	0.36	17	56.1
6	12.80	4.86	0.38	17	50.3
6	17.23	6.72	0.39	17	62.7

Cont'd

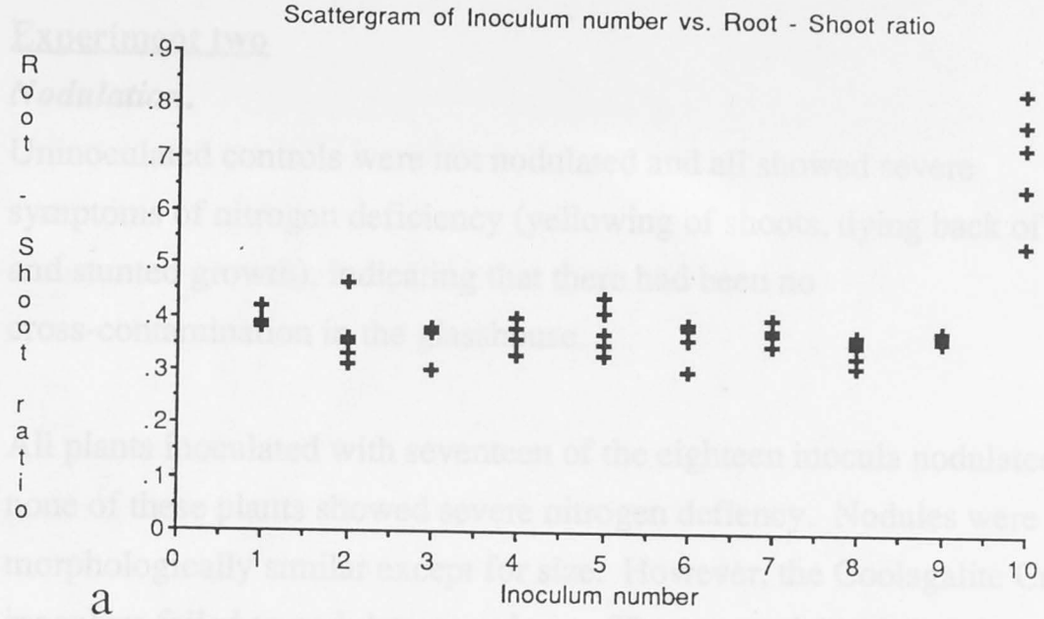
TABLE 2.4: CONT'D

Inoculum number	Shoot weight (g)	Root weight (g)	Root/Shoot ratio	Time to nodulation (days)	Final height (cm)
7	11.07	3.87	0.35	23	52.2
7	8.57	3.19	0.37	22	42.1
7	13.26	5.04	0.38	20	57.7
7	15.21	5.78	0.38	19	53.4
7	6.71	2.68	0.40	23	41.5
8	11.00	3.96	0.36	16	52.9
8	12.96	4.02	0.31	17	56.8
8	11.30	3.96	0.35	17	54.7
8	11.41	4.22	0.37	17	52.2
8	8.38	2.77	0.33	23	40.6
9	8.71	3.22	0.37	19	42.5
9	14.27	5.42	0.38	20	47.8
9	11.14	4.23	0.38	20	46.1
9	9.84	3.54	0.36	21	53.9
10	4.77	3.97	0.83	-	29.8
10	8.79	4.75	0.54	-	43.3
10	4.94	3.82	0.77	-	34.6
10	6.82	4.42	0.65	-	28.2
10	7.63	5.55	0.73	-	38.6

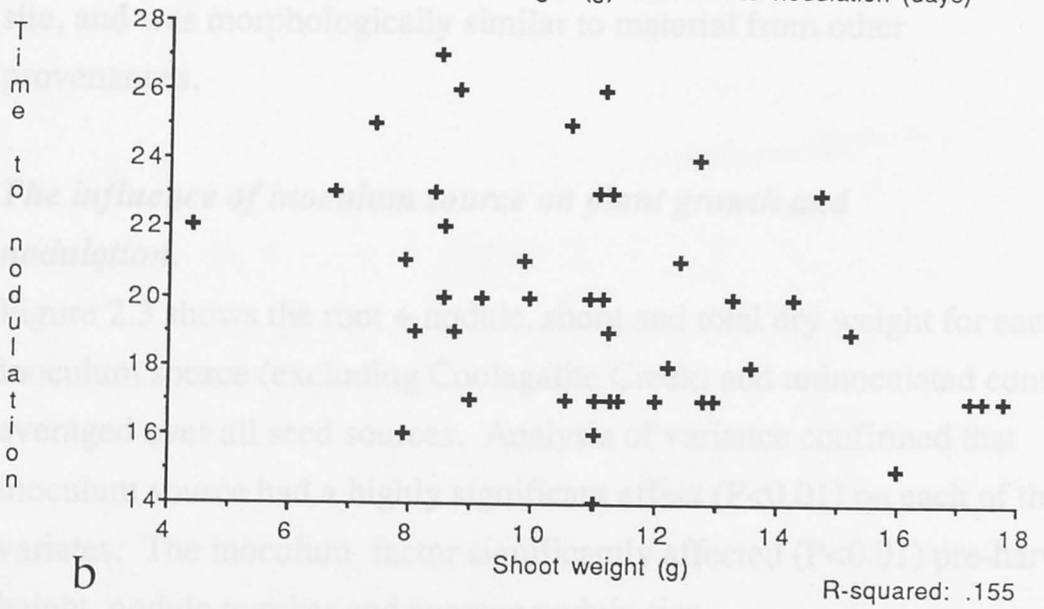
TABLE 2.5: INOCULUM PROVENANCE VARIATION ON A SINGLE SEED SOURCE (EXPT 1, SECTION I). ANALYSIS OF VARIANCE (ANOVA) BETWEEN INOCULUM SOURCES FOR ALL PARAMETERS USED TO MEASURE PLANT GROWTH.
Each parameter is analysed without and with the uninoculated controls. Data are reproduced in Table 2.4.

SOURCE	DF	SS	MS	F-TEST
(a) Shoot weight				
Between groups	8	112.63	14.08	**1.39**
Within groups	35	354.15	10.12	**0.10<p≤0.25**
Total	43	466.78		
	inc. uninoculated controls		F= 2.32	0.03<p≤0.05
(b) Root weight				
	8	12.14	1.52	**1.46**
	35	36.32	1.04	**0.10<p≤0.25**
	43	48.46		
	inc. uninoculated controls		F= 1.50	0.10<p≤0.25
(c) Root-Shoot ratio				
	8	.007	.001	**.801**
	35	.039	.001	**p>0.25**
	43	.046		
	inc. uninoculated controls		F= 24.75	p≤0.0001
(d) Time to nodulation				
	8	294.03	36.75	**8.441**
	35	152.40	4.35	**p<0.0001**
	43	446.43		
(e) Final height				
	8	681.99	85.25	**1.50**
	35	1994.98	57.00	**0.10<p≤0.25**
	43	2676.97		
	inc. uninoculated controls		F= 3.32	0.0001<p≤0.005

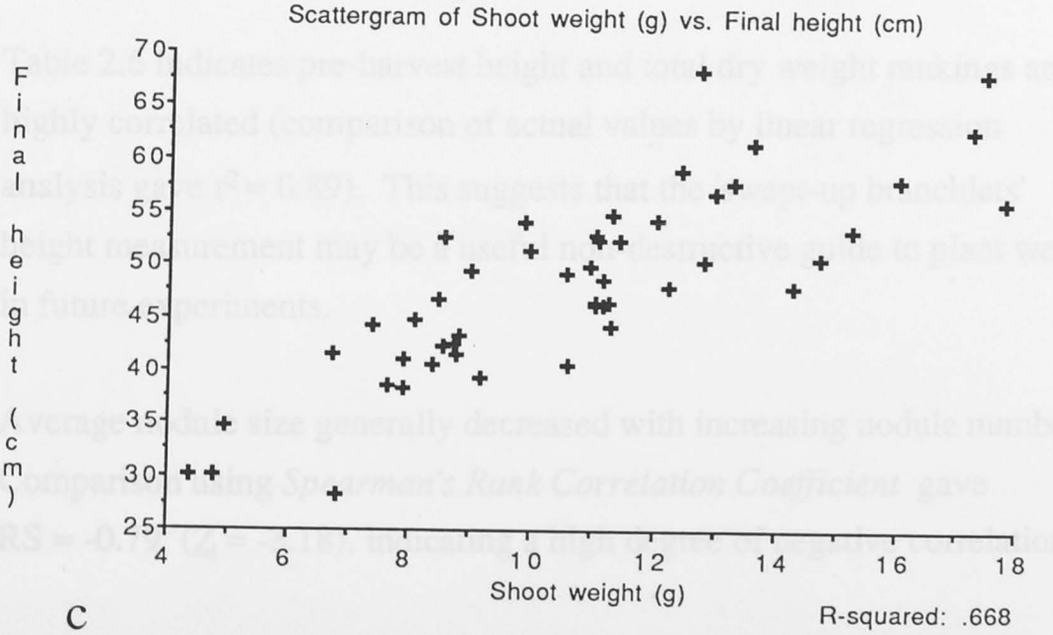
Figure 2.2: Experiment One, Section I. Scattergrams illustrating the interaction between:
(a) root-shoot ratio and source of inoculum. Note the high R:S of the uninoculated controls (inoculum 10).
(b) time to nodulation and shoot dry weight. There was no significant interaction between these two parameters.
(c) final height and shoot weight. A strong correlation was found between these two parameters.


Scattergram of Inoculum number vs. Root - Shoot ratio
Root - Shoot ratio
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
0 1 2 3 4 5 6 7 8 9 10
Inoculum number

a


Scattergram of Shoot weight (g) vs. Time to nodulation (days)
Time to nodulation
28
26
24
22
20
18
16
14
4 6 8 10 12 14 16 18
Shoot weight (g)
R-squared: .155

b


Scattergram of Shoot weight (g) vs. Final height (cm)
Final height (cm)
70
65
60
55
50
45
40
35
30
25
4 6 8 10 12 14 16 18
Shoot weight (g)
R-squared: .668

c

Experiment two

Nodulation.

Uninoculated controls were not nodulated and all showed severe symptoms of nitrogen deficiency (yellowing of shoots, dying back of tips and stunted growth), indicating that there had been no cross-contamination in the glasshouse.

All plants inoculated with seventeen of the eighteen inocula nodulated and none of these plants showed severe nitrogen defiency. Nodules were morphologically similar except for size. However, the Coolagalite Creek inoculum failed to nodulate any plants. The cause of this failure is not known. The original nodule material was from a number of trees at the site, and was morphologically similar to material from other provenances.

The influence of inoculum source on plant growth and nodulation.

Figure 2.3 shows the root + nodule, shoot and total dry weight for each inoculum source (excluding Coolagalite Creek) and uninoculated controls, averaged over all seed sources. Analysis of variance confirmed that inoculum source had a highly significant effect ($P<0.01$) on each of these variates. The inoculum factor significantly affected ($P<0.01$) pre-harvest height, nodule number and average nodule size.

Table 2.6 indicates pre-harvest height and total dry weight rankings are highly correlated (comparison of actual values by linear regression analysis gave $r^2 = 0.89$). This suggests that the 'swept-up branchlets' height measurement may be a useful non-destructive guide to plant weight in future experiments.

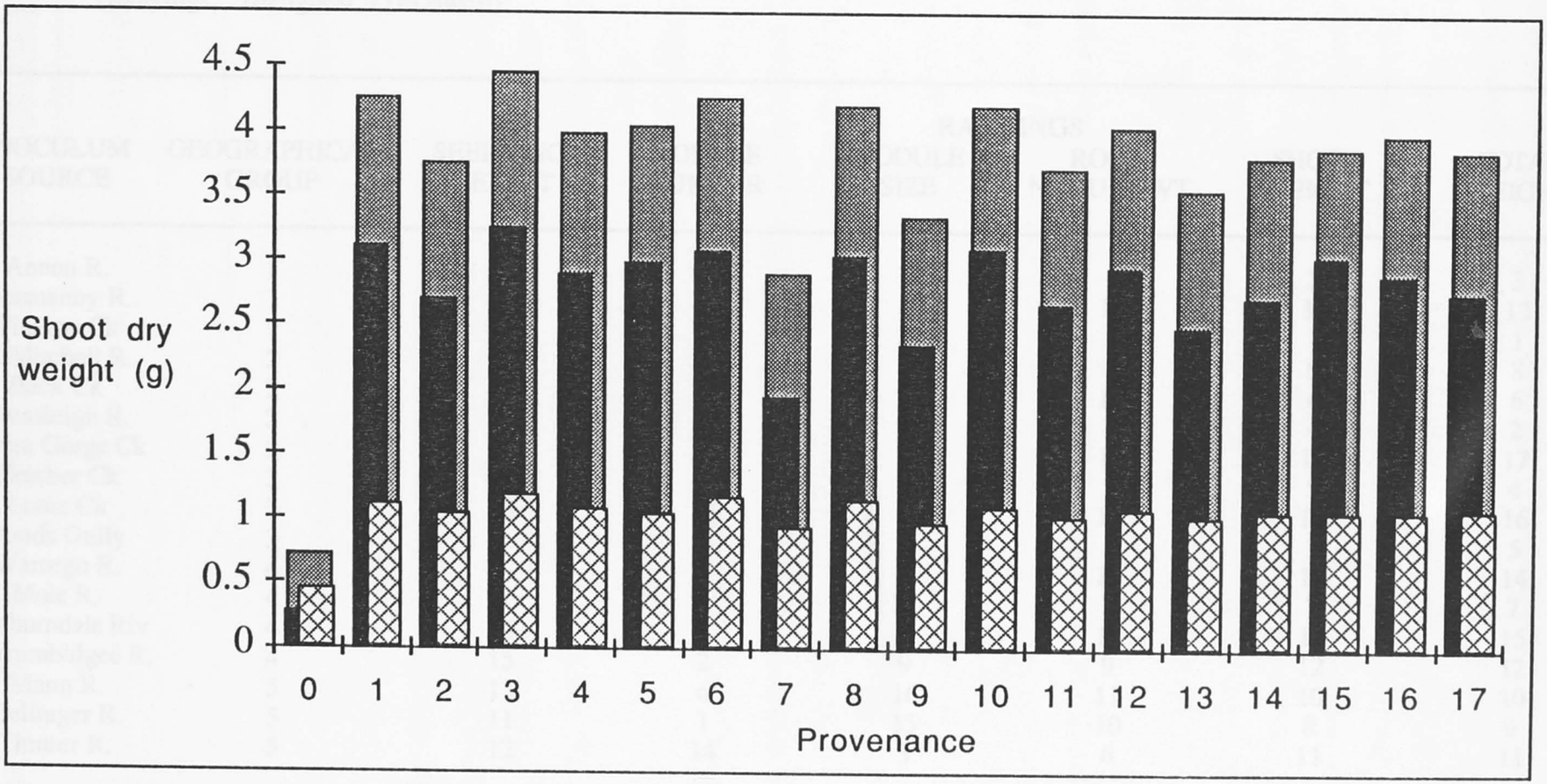
Average nodule size generally decreased with increasing nodule number. Comparison using *Spearman's Rank Correlation Coefficient* gave $RS = -0.79$ ($Z = -3.18$), indicating a high degree of negative correlation.

Figure 2.3: Experiment Two, Section I. Root, shoot and total dry weight associated with the 17* inoculum sources. Plant weight is the average of all seed sources. Provenance '0' is the average of the uninoculated controls.

Provenances are as follows:

1 - Annan River (group 1)
2 - West Normanby River (group 1)
3 - Tinaroo Creek (group 1)
4 - Little Mitchell River (group 2)
5 - Back Creek (group 2)
6 - Einasleigh River (group 2)
7 - Ollera Gorge Creek (group 3)
8 - Fletcher Creek (group 3)
9 - Lotus Creek (group 3)
10 - Bonds Gully (group 3)
11 - Warrego River (group 4)
12 - Mole River (group 4)
13 - Winburndale River (group 4)
14 - Murrumbidgee River (group 4)
15 - Mann River (group 5)
16 - Bellinger River (group 5)
17 - Hunter River (group 5)

*Coolagalite Creek excluded (see text)



The histogram shading should be identified as follows: Cross-hatching is root weight, black is shoot weight, stippled is total weight.

TABLE 2.6: EXPERIMENT TWO, SECTION I. PERFORMANCE RANKING OF INOCULA FROM THE 17 PROVENANCES (EXCLUDING COOLAGALITE CK), AVERAGED OVER ALL SEED SOURCES
(Rankings: 1=Highest, 17=Lowest)

INOCULUM SOURCE	GEOGRAPHICAL GROUP	RANKINGS					
		SEEDLING HEIGHT	NODULE NUMBER	NODULE SIZE	ROOT+ NODULE WT.	SHOOT WEIGHT	TOTAL WEIGHT
Annan R.	1	1	7	12	4	2	3
Normanby R.	1	10	15	3	13	13	13
Tinaroo Ck	1	2	5	14	1	1	1
L. Mitchell R.	2	9	11	10	8	9	8
Back Ck	2	6	10	13	12	6	6
Einasleigh R.	2	3	12	6	2	4	2
Ollera Gorge Ck	3	17	17	1	17	17	17
Fletcher Ck	3	4	13	4	3	5	4
Lotus Ck	3	16	16	2	16	16	16
Bonds Gully	3	8	9	11	5	3	5
Warrego R.	4	7	8	8	15	14	14
Mole R.	4	5	3	17	7	7	7
Winburndale Riv	4	14	4	7	14	15	15
Murrumbidgee R.	4	15	2	9	9	12	12
Mann R.	5	13	6	16	11	10	10
Bellinger R.	5	11	1	15	10	8	9
Hunter R.	5	12	14	5	6	11	11

Thus Bellinger River inoculum had the highest ranking for number, but a low ranking (16) for size. Conversely, Ollera Gorge Creek ranked lowest for number, but highest for size. These results suggest that the host had some control over growth of nodules. This inverse relationship, however, did not hold for all inoculum sources. Winburndale River inoculum produced a relatively high number of nodules (ranked 4) which had a relatively large average size (ranked 7). A similar result was found for Murrumbidgee River. In contrast, Back Creek produced many fewer nodules (ranked 10) which had a small average size (ranked 13).

Winburndale River inoculum was also notable because despite abundant nodulation it was associated with plants which averaged the third lowest total dry weight. Thus it is an inefficient symbiont in terms of plant dry matter produced per unit nodule mass. Back Creek, however,was associated with plants which averaged a total dry weight ranking of seven which is relatively high whencomparedwith its sparse nodulation indicating a much higher degree of efficiency. Because the nodulation assessment was only semi-quantitative it was not possible to make more refined comparisons of symbiotic efficiency.

The rankings for top weight and total weight agreed very closely (Table 2.6) (RS = 0.99, $\underline{Z}$ = 3.94). Root + nodule weight ranking also generally agreed with top and total weight (RS = 0.91,$\underline{Z}$ = 3.63) except for Back Creek and Hunter River. The most generally effective inoculum source, in terms of plant dry weight produced, was Tinaroo Creek. The least effective, averaged over all seed sources, was Ollera Gorge Creek. For top weight, the factor seed and the seed x inoculum interaction were also significant (see below).

Analysis of variance on grouped inoculum sources for each of the variates confirmed that group differences were highly significant ($P<0.01$) for all variates except root + nodule weight, which was significant only at the 5 per cent probability level.

The performance of the factor inoculum at the level of geographical groups for all significant variates is shown in Table 2.7. Northern inocula, groups 1 and 2, were associated with the highest plant dry weight. The southern groups 4 and 5 were intermediate. Nodule number ranking was significantly different for all groups, being highest in group 4 and lowest in group 3. Nodule size was the inverse of nodule number.

The influence of seed source on plant growth and nodulation.

Different seed sources showed highly significant ($P<0.01$) variation in top weight. Top dry weight for each seed source, averaged over all inoculum sources (excluding Coolagalite Creek) is shown in Figure 2.4. Overall, the Hunter River provenance showed greatest growth and Annan River the least. When the seed sources were ranked according to top dry weight (Table 2.8), the rankings suggested performance was once again correlated to geographical groups. This was confirmed by analysis of variance of grouped seed sources, which showed highly significant ($P<0.01$) differences between groups. Plants from geographical groups 5, 4 and 3 grew significantly better than those from groups 1 and 2.

The influence of the seed x inoculum interaction on plant growth.

Top weight showed highly significant differences ($P = 0.001$) due to the two factor interaction. It is not readily possible in an 18 x 18 interaction table to determine the cause of the variation. However, given the prior success of the five geographical groupings the data were condensed into a 5 x 5 table for geographical groups (Table 2.9).

The interaction is complex, with the degree of symbiotic effectiveness, as measured by top weight, varying between inocula and seed sources. In general, inocula from groups 1 and 2 caused significantly greater increases in weight with plants from groups 1, 2 and 3. Inocula from groups 4 and 5 caused significantly greater increases in weight with plants from groups 4 and 5. The same result was evident for seed sources. Thus, top weight was greatest with seed x inoculum combinations from

Figure 2.4: Shoot dry weight for each seed source. Each figure is the average of the 17* inoculum sources.

Provenances are as follows:

1 - Annan River (group 1)
2 - West Normanby River (group 1)
3 - Tinaroo Creek (group 1)
4 - Little Mitchell River (group 2)
5 - Back Creek (group 2)
6 - Einasleigh River (group 2)
7 - Ollera Gorge Creek (group 3)
8 - Fletcher Creek (group 3)
9 - Lotus Creek (group 3)
10 - Bonds Gully (group 3)
11 - Warrego River (group 4)
12 - Mole River (group 4)
13 - Winburndale River (group 4)
14 - Murrumbidgee River (group 4)
15 - Mann River (group 5)
16 - Bellinger River (group 5)
17 - Hunter River (group 5)

*Coolagalite Creek excluded (see text)

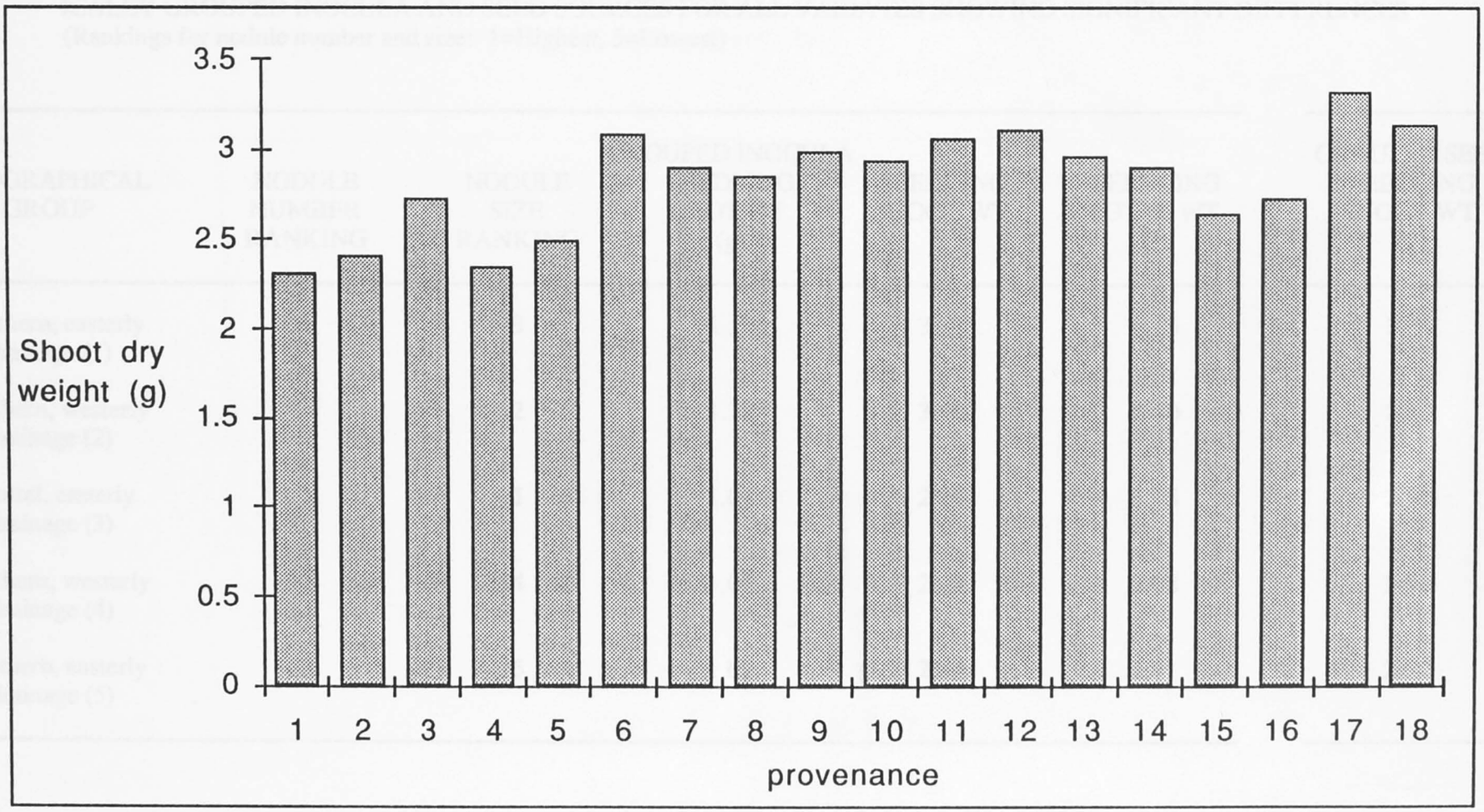

Shoot dry weight (g)
3.5
3
2.5
2
1.5
1
0.5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
provenance

TABLE 2.7: EXPERIMENT TWO, SECTION I. NODULATION RANKINGS AND PLANT DRY WEIGHT ASSOCIATED WITH GEOGRAPHICALLY GROUPED INOCULA AND SEED SOURCES FOR ALL VARIATES SHOWING SIGNIFICANT DIFFERENCES
(Rankings for nodule number and size: 1=Highest, 5=Lowest)

GEOGRAPHICAL GROUP	NODULE NUMBER RANKING	NODULE SIZE RANKING	GROUPED INOCULA SEEDLING ROOT WT. (g)	SEEDLING SHOOT WT. (g)	SEEDLING TOTAL WT. (g)	GROUPED SEED SEEDLING SHOOT WT. (g)
Northern, easterly drainage (1)	3	3	1.12	3.03	4.15	2.50
Northern, westerly drainage (2)	4	2	1.10	3.00	4.10	2.69
Central, easterly drainage (3)	5	1	1.04	2.61	3.65	2.91
Southern, westerly drainage (4)	1	4	1.06	2.72	3.78	2.99
Southern, easterly drainage (5)	2	5	1.07	2.84	2.91	2.92

TABLE 2.8: PERFORMANCE RANKING OF SEED FROM ALL 18 PROVENANCES FOR THE VARIATE TOP WEIGHT, AVERAGED OVER 17* INOCULUM SOURCES.
(Rankings: 1=Highest, 18=Lowest) * Coolagalite Ck excluded

SEED SOURCE	GEOGRAPHICAL GROUP	RANKING FOR TOP WT.	SEED SOURCE	GEOGRAPHICAL GROUP	RANKING FOR TOP WT.
Annan R.	1	18	Bonds Gully	3	8
Normanby R.	1	16	Warrego R.	4	5
Tinaroo Ck	1	13	Mole R.	4	3
L.Mitchell R.	2	17	Winburndale Riv.	4	7
Back Ck	2	15	Murrumbidgee R.	4	9
Einasleigh R.	2	4	Mann R.	5	14
Ollera Gorge Ck	3	10	Bellinger R.	5	12
Fletcher Ck	3	11	Hunter R.	5	1
Lotus Ck	3	6	Coolagalite Ck	5	2

similar geographic regions, particularly southern inocula on southern seed sources. As a corollary to this, worst performance (i.e. lowest plant weight) was found with the combination of groups 4 and 5 inocula on groups 1 and 2 seed; i.e. southern inocula on northern seed sources. Inoculum from group 3, the central region, did significantly better with group 3 seed sources. This is another example of specificity within a geographical region.

Discussion

Neither experiment revealed any absolute specificity in nodule production or incompatibility between provenances of *Frankia* and *C. cunninghamiana*. Incompatibility has been demonstrated between *C. cunninghamiana* and *Frankia* derived from nodules of other *Casuarina* species (Reddell and Bowen 1985a). With the exception of Coolagalite Creek inoculum which failed completely for reasons unable to be determined, all combinations produced functional symbioses. The eighteen x eighteen cross-inoculation trial did, however, demonstrate highly significant variation in the degree of effectiveness of different host-endophyte combinations under nitrogen-limiting factors, which was responsible for the observed variation in plant growth. This is similar to findings of Dawson and Sun (1981) for combinations of two *Frankia* strains with three species of *Alnus*. They found that both inoculum source and host plant genotype accounted for variation in growth, though the interaction of the two was not significant.Thus the potential exists for improving the effectiveness of the association by selection of the symbiotic partners.

Particularly interesting was the finding that greatest biomass production was obtained with host-endophyte combinations from similar geographic regions (Table 2.9). Groups east and west of the Great Dividing Range at simliar latitudes were not generally significantly different from each other, but major differences were found between northern and southern

TABLE 2.9: THE INTERACTION INOCULUM * SEED SOURCE GROUPED ACCORDING TO A PRIORI GEOGRAPHICAL GROUPINGS, FOR THE VARIATE TOP WEIGHT.
Geographical groupings are: 1=northern, easterly drainage; 2=northern, westerly drainage; 3=central, easterly drainage; 4=southern, westerly drainage; 5=southern, easterly drainage

INOCULUM GROUP		SEED GROUP North 1	2	3	4	South 5
North	1	3.12	3.24	3.16	2.71	3.01
	2	3.11	3.26	3.18	2.87	2.69
	3	2.59	2.65	2.90	2.62	2.40
	4	1.81	2.14	2.49	3.41	3.32
South	5	2.07	2.33	2.83	3.42	3.24

groups. Figure 2.1 shows that a disjunction in the distribution of *C. cunninghamiana* separates the northern and southern geographical groups. This is an unusually dry area of the Queensland coastal belt which may have provided a long-term natural barrier to gene flow within the species.

The mechanisms underlying this variation in effectiveness were not determined. However, there was no significant interaction between host and endophyte sources for either nodule number or average nodule size. Thus infectivity and nodule growth were not apparently the points at which variation in effectiveness was expressed. This suggests that the amount of nitrogen fixed g^{-1} nodule tissue varied, which is in agreement with the findings of Reddell and Bowen (1985a). Normand and Lalonde (1982) correlated differences they observed in the symbiotic effectiveness of *Frankia*, originally isolated from 27 provenances of two Alnus species, with the spore+ and spore- endophytic character. In Experiment Two I collected and preserved nodules from all plants. These are available for examination of internal morphology at a future date.

The lack of variation in plant growth associated with different endophytes in Experiment One is consistent with the findings described above, because all endophyte provenances came from a similar geographical region.

Lie *et al.* (1984) have also observed geographic specificity in the *Rhizobium leguminosarum* - pea symbiosis. When they inoculated several ecotypes of wild pea from the Middle East with local and European strains of *Rhizobium,* all combinations formed nodules, but only those with local strains were effective. Nodules with European strains showed very low levels of nitrogenase activity. They suggest this result indicates that some from of co-evolution has taken place between the wild pea ecotypes and the local *Rhizobium* strains. More recent studies (Lie *et al.* 1987) have reinforced this result.

The Winburndale River and Murrumbidgee River inocula displayed extreme variation in effectiveness on different seed sources. In Table 2.10 shoot dry weight is shown for seedlings from each geographical area in combination with these inocula. The variation between groups is highly significant, with best performance (measured by plant dry weight) being given by seed sources from groups 4 and 5. This was accompanied by an apparent substantial increase in the efficiency of the symbiosis; a significant decrease in average nodule size was accompanied by a rise in shoot dry weight (data not shown). It was earlier shown (Table 2.6) that the inoculum from Winburndale River was very inefficient when its performance was averaged over all seed sources. However, Table 2.10 shows that this overall inefficiency was largely the result of its poor performance with northern and central seed sources (groups 1, 2 and 3) with which it was far inferior to other group 4 inocula. This is clearly illustrated in Figure 2.5, which shows profuse nodulation on a Little Mitchell River seedling, which grew very poorly, inoculated with Winburndale River inoculum.

In general, southern inocula showed significantly greater symbiotic effectiveness with southern seed sources than northern inocula did with northern seed sources. The group 3 inocula from the central region were interesting in that they showed little variation in effectivenes on different hosts. In contrast to inocula, northern seed sources displayed best growth with northern inocula. This is most clearly shown by the group 1 provenance Annan River which grew significantly better with inocula from groups 1 and 2 than with groups 4 and 5 inocula (Table 2.10).

The existence of varying degrees of effectiveness has practical implications for selection strategies aimed at applicaton of the symbiosis. Tree characteristics will continue to dominate the choice of seed sources for forestry purposes. However, since most casuarinas are planted on nitrogen-deficient soils, optimization of the symbiosis is highly desirable. The results presented here suggest that if selection for *Frankia* is to proceed, then isolates for testing should first be obtained from a similar

TABLE 2.10: SHOOT DRY WEIGHT ASSOCIATED WITH THREE INOCULUM SOURCES AND TWO SEED SOURCES AVERAGED OVER THE FIVE GEOGRAPHICAL GROUPS
The geographical groups are listed in Table 2.3.

SEED SOURCE	VARIATE	GEOGRAPHICALLY GROUPED INOCULUM SOURCES 1	2	3	4	5	LEVEL OF SIGNIFICANCE
Annan R. (Group 1)	Shoot Dry Weight (g)	2.81	3.31	2.52	1.52	1.48	***
	Av. Weight for Gp 1	3.12	3.11	2.59	1.81	2.07	***
Hunter R. (Group 5)	Shoot Dry Weight (g)	3.33	3.79	2.66	3.55	3.33	*
	Av. Weight for Gp 5	3.01	2.69	2.4	3.32	3.24	***

Cont'd

TABLE 2.10: CONT'D

INOC. SOURCE	VARIATE	GEOGRAPHICALLY GROUPED SEED SOURCES 1	2	3	4	5	LEVEL OF SIGNIFICANCE
Winburndale R (Group 4)	Shoot Dry Weight (g)	1.35	2	1.91	3.64	3.25	***
	Av. Weight for Gp 4	1.81	2.14	2.49	3.41	3.32	***
Murrumbidgee R. (Group 4)	Shoot Dry Weight (g)	1.92	2.04	2.25	3.48	3.66	***
	Av. Weight for Gp 4	1.81	2.14	2.49	3.41	3.32	***
Tinaroo Ck (Group 1)	Shoot Dry Weight (g)	3.36	3.34	3.31	3.18	3.19	NS
	Av. Weight for Gp 1	3.12	3.24	3.16	2.71	3.01	NS

Figure 2.5: The root system of *Casuarina cunninghamiana* (Little Mitchell seed source) inoculated with Winburndale River inoculum. Such profuse nodulation was typical for this inoculum source on northern seed sources.
The nodules were of relatively low effectiveness.

geographical region to the seed required. However, if a multiplicity of seedlots are in use it may be impractical to obtain the best host-endophyte combination for each one, in which case a generally effective inoculum will be preferable. Tables 2.6 and 2.9 suggest that such an inoculum is most likely to be found in the northern region. One inoculum in this trial, Tinaroo Creek, demonstrated overall effectiveness (Table 2.10).

Hunter River stood out as the only generally effective seedlot because this was the only provenance to produce above average shoot dry weight with inocula from all geographical groups (Table 2.10). A possible contributing factor to this result is that the Hunter River provenance of *C. cunninghamiana* may be exhibiting above average vigour due to the effect of some introgression with *C. glauca*, which occurs in the same locality (Doran and Hall 1983). In a previous study, seedlings of this provenance were the tallest of six provenances under test at six months old when grown with complete inorganic fertiliser and without *Frankia* (Shepherd and El Lakany 1983). A seed source such as Hunter River which can develop reasonably effective symbioses with a wide range of *Frankia* and exhibit other desirable characteristics may be useful in places where no inoculation is undertaken and nodulation is dependent on native *Frankia* in the soil.

SECTION II

Casuarina Root Nodule Hemoglobin Purification and Characterisation

Chapter Three

INTRODUCTION - SECTION II

Leghemoglobin, which occurs ubiquitously in nitrogen-fixing legume nodules, shares a common evolutionary origin with animal hemoglobin and myoglobin. Until recently, it was considered to represent the sole occurrence of hemoglobin in plants. The hypothesis of horizontal transfer of the hemoglobin gene from an animal to an ancient legume was proposed to explain its presence.

In 1983, however, hemoglobin was found in the non-legume Parasponia-Rhizobium *symbiosis, casting doubt on the horizontal gene transfer hypothesis. Its occurrence in a non-legume raised the exciting possibility that hemoglobin might be widespread in the plant kingdom, giving rise to an alternative hypothesis that the hemoglobin gene had been present in plants throughout their evolution.*

To test this hypothesis, I decided to examine nodules of the Casuarina-Frankia *symbiosis for the presence of hemoglobin.* Casuarina *is one of a number of distantly related plant genera (actinorhizal plants) which form symbioses with the actinomycete* Frankia, *rather than* Rhizobium. *There had been one previous report of hemoglobin in these symbioses, but others had failed to detect it.*

There were functional reasons to suspect the presence of hemoglobin in Casuarina *nodules. Most species of* Casuarina *Adans. s. str. occur in riparian habitats where roots and nodules may be either submerged or in near-saturated soils for days to weeks at a time. Thus the* pO_2 *in the atmosphere surrounding nodules is often low. Access of oxygen to the endophyte is further limited by suberisation of infected cell walls. Hemoglobin could conceivably enable efficient respiration and nitrogen fixation to occur under these conditions by facilitating the flux of oxygen to the endophyte at a desirable rate. In addition, within* Casuarina *nodules* Frankia *apparently lacks any physical mechanism for protecting nitrogenase from exposure to oxygen. In all other actinorhizal nodules nitrogenase is protected inside impermeable vesicles. A high-affinity hemoglobin, which maintains a very low free oxygen concentration by delivering oxygen to the endophyte at a rate commensurate with efficient respiration, may have rendered the vesicle wall unnecessary.*

If hemoglobin were present in the Casuarina *symbiosis as well as in* Parasponia *and the legumes, then it would indeed be widely, if sparsely, spread through the plant kingdom. If it could be purified in sufficient quantity to determine the amino acid sequence and for*

the production of a specific anti-serum, its phylogenetic relationship with other plant hemoglobins could be determined. The amino acid sequence could also be fundamental to determining the sequence of the gene responsible for its production.

Also, if hemoglobin could be purified from Casuarina *nodules in sufficient quantity, its oxygenation kinetics could be determined. This would give valuable insight into its function within the nodule.*

Evolutionary origin of plant hemoglobin

Hemoglobin was first isolated from legume root nodules by Kubo (1939). Since then, investigations of numerous legume species have led to the conclusion that hemoglobin occurs ubiquitously in nitrogen-fixing legume nodules (Bergersen 1982, Appleby 1984). The finding of hemoglobin in legumes was a genetic puzzle; it had previously been considered to be restricted to the animal kingdom. This raised the possibility of convergent evolution; ie, that animal and legume hemoglobins had evolved from independent origins toward a similar structure, presumably due to functional constraints. It is, in fact, now known from studies of various plant and animal hemoglobins that the functional requirement of reversible oxygen exchange has resulted in a close similarity in tertiary and quaternary structure of these proteins (Perutz 1983). However, this does not by itself indicate convergence.

Analysis of the amino acid sequence of soybean, lupin, broad bean and other leghemoglobins (Hunt *et al.* 1978) did not provide strong evidence for or against convergence. Sequence differences (Table 3.1) within the leghemoglobins (20-50%) were much less than between leghemoglobins and animal globins (77-90%), superficially suggesting two separate families of proteins. However, comparison of individual residues showed that all globins, including leghemoglobin, contain the CD1 phenylalanine and the F8 'proximal' histidine at identical placement; both of these are heme contact residues, and as such probably critical for binding heme in a way which permits it to react reversibly with oxygen. The identical placement of these residues is more readily explained by a single

TABLE 3.1: COMPARISON OF LEGHEMOGLOBIN AND ANIMAL HEMOGLOBIN AMINO ACID SEQUENCES, SHOWING PERCENT DIFFERENCES.

Modified from Hunt *et al* (1978)

	% DIFFERENCE BETWEEN SEQUENCES			
	Leghemoglobin (Lb)			
Globin	Broad Bean	Kidney bean	Soybean	Yellow lupin
Hemoglobin Alpha Chain				
1. Human	86	87	86	87
2. Dog	82	85	87	87
3. Gray kangaroo	82	86	86	85
4. Echidna, alpha-1	82	84	84	84
5. Platypus	82	84	85	83
6. Chicken, alpha-2	86	90	86	88
7. Port Jackson shark	86	90	89	87
Hemoglobin beta chain				
8. Human	79	83	85	85
9. Human, delta	79	81	85	84
10. Human, gamma	77	82	83	82
11. Gray kangaroo	81	83	83	82
12. Platypus	79	85	83	82
13. Echidna	81	86	86	85
14. Frog	84	83	86	85
Myoglobins				
15. Human	91	89	90	83
16. Dog	90	89	89	84
17. Red kangaroo	89	90	89	84
18. Platypus	89	88	89	83
19. Chicken	89	89	88	81
Globins - Invertebrates				
20. Gastropod mollusc I	86	85	85	82
21. Bloodworm	85	85	85	82
22. Midge larva, CTT -II	85	84	86	85
Leghemoglobin				
23. Broad bean	**0**	**36**	**36**	**51**
24. Kidney bean	**36**	**0**	**21**	**49**
25. Soybean	**36**	**21**	**0**	**48**
26. Yellow lupin	**51**	**49**	**48**	**0**

evolutionary origin for all globins. Indeed, Dayhoff *et al.* (1972) believed the amino acid sequence evidence to be sufficient indication that all globins had a common ancestral origin.

Further support for the argument of a common ancestral origin for all globins came from crystal structure studies. The x-ray analysis of crystals of lupins and soybean leghemoglobins revealed an overall similarity in folded structure with the animal globins (Ollis *et al.* 1983; Arutyunyan 1981), not expected if they were the products of convergent evolution.

Gene sequencing confirms common origin for animal and plant hemoglobins.

The phylogenetic relationship of leghemoglobin to the vertebrate globins was resolved by sequencing the genes responsible for soybean leghemoglobins a and c (Jensen *et al.* 1981, Hyldig-Nielsen *et al.* 1982). While the coding region of these genes showed only limited homology with any vertebrate hemoglobin genes (approximately 15%), the gene contained three introns, two of which (IVS I & III) aligned perfectly with the two introns present in all known vertebrate hemoglobin genes. In addition, the central intron of the leghemoglobin genes (IVS II) was found in the position predicted by Go (1981), using protein domain analysis, for a now-absent central intron of vertebrate globin genes. Thus, it became apparent that animal and legume globins were of one family. This immediately raised the question of the origin of the globin gene in legumes.

Horizontal gene transfer.

The hypothesis of horizontal gene transfer was proposed to explain the occurrence of hemoglobin in plants (Hyldig-Nielsen *et al.* 1982, Jeffreys 1981, 1982). This hypothesis assumed that the globins are fundamentally animal proteins, and that a globin gene was transferred from the animal kingdom, via a vector, to an ancient legume. Both Hyldig-Nielsen *et al.* (1982) and Jeffreys (1981) favoured a viral vector, possibly an insect-borne plant pathogenic virus (Jeffreys 1981).

There was, at one stage, considerable support for the horizontal gene transfer hypothesis. Lewin (1982 a,b) viewed leghemoglobin as one of four principal candidates as examples of the process. The other three were the genes for superoxide dismutase in *Photobacter leiognathi* (the symbiotic bacterium of ponyfish), a family of histone genes in sea urchin, and a sub-family of repeated sequences in sea urchin. Syvanen (1985) quotes the occurrence of leghemoglobin as one of "a number of hints that functional genes can spread across species boundaries".

Those who proposed the horizontal gene transfer hypothesis had two basic premises. Firstly, it was assumed that hemoglobin is entirely restricted in its occurrence in plants to legume nodules. Secondly, it was assumed that the sole function of hemoglobin in plants is the facilitation of oxygen diffusion to the endophyte of legume root nodules, a function which was demonstrated by Wittenberg *et al.* (1974). The latter of these two premises is based on ignorance of an alternative function rather than established scientific fact. It remains possible that hemoglobin has a more general, and as yet cryptic function in plants (Landsmann *et al.* 1986). Indeed, recent evidence suggests that a more general function is highly likely (Appleby *et al.* 1988 a,b). I will consider this is greater detail later in the thesis.

Hemoglobin in Parasponia.

The purification of hemoglobin from nodules of the non-legume *Parasponia-Rhizobium* symbiosis (Appleby *et al.* 1983 a) cast doubt on the horizontal gene transfer hypothesis. *Parasponia* is a genus of the family Ulmaceae, and is the only non-leguminous plant known to be nodulated by *Rhizobium* . Ulmaceae is a member of the order Urticales, which is widely separated from Fabales (Figure 3.1), the order which contains the legumes. While each scheme of phylogeny shown in Figure 3.1 views relationships from a different perspective, all imply an ancient divergence of the lines which have led to the extant legumes and *Parasponia*.

Figure 3.1: Phylogenetic relationships of dicotyledons, according to a range of classifications, highlighting orders known to contain nodulating and hemoglobin bearing species. *Casuarina* is a member of the order Casuarinales. Current knowledge restricts nodulating species to the fundamentally woody dicotyledons (see also Table 3.2).

(A) Thaktajan (1980)

(B) Dahlgren (1980)

(C) Thorne (1981)

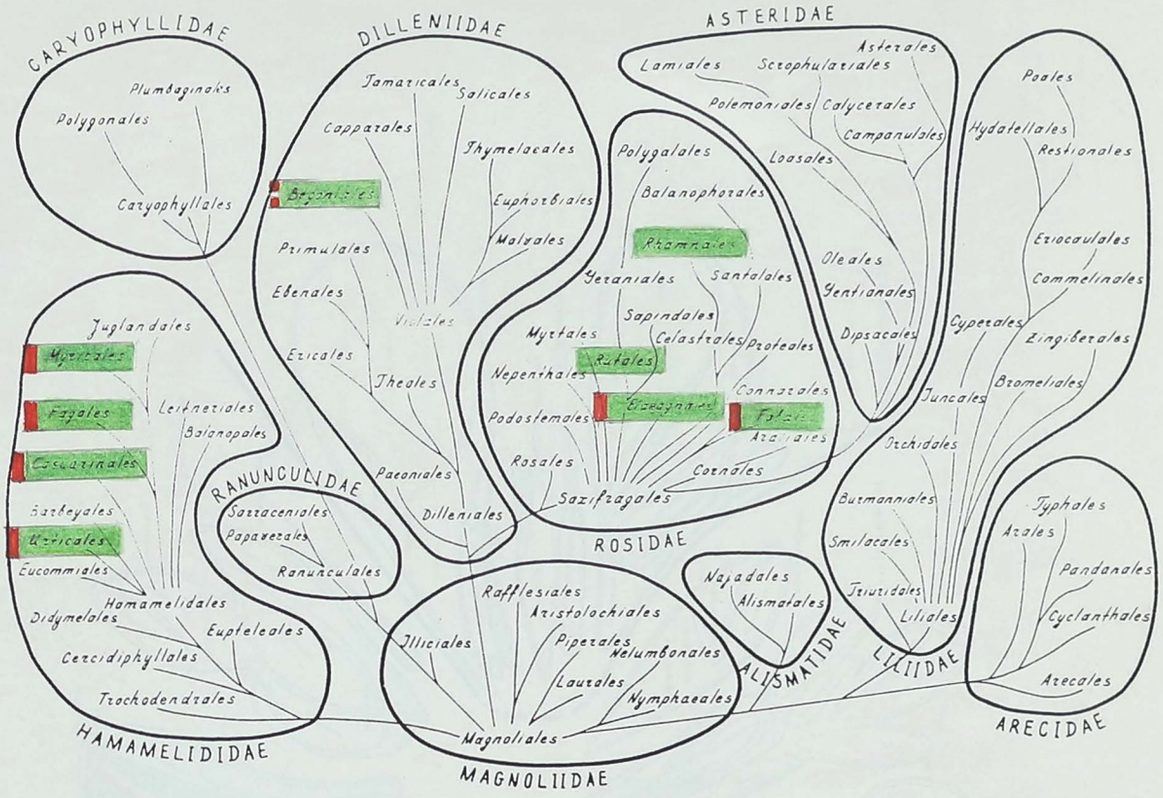
(D) Hutchinson (1973)

(E) Cronquist (1981)

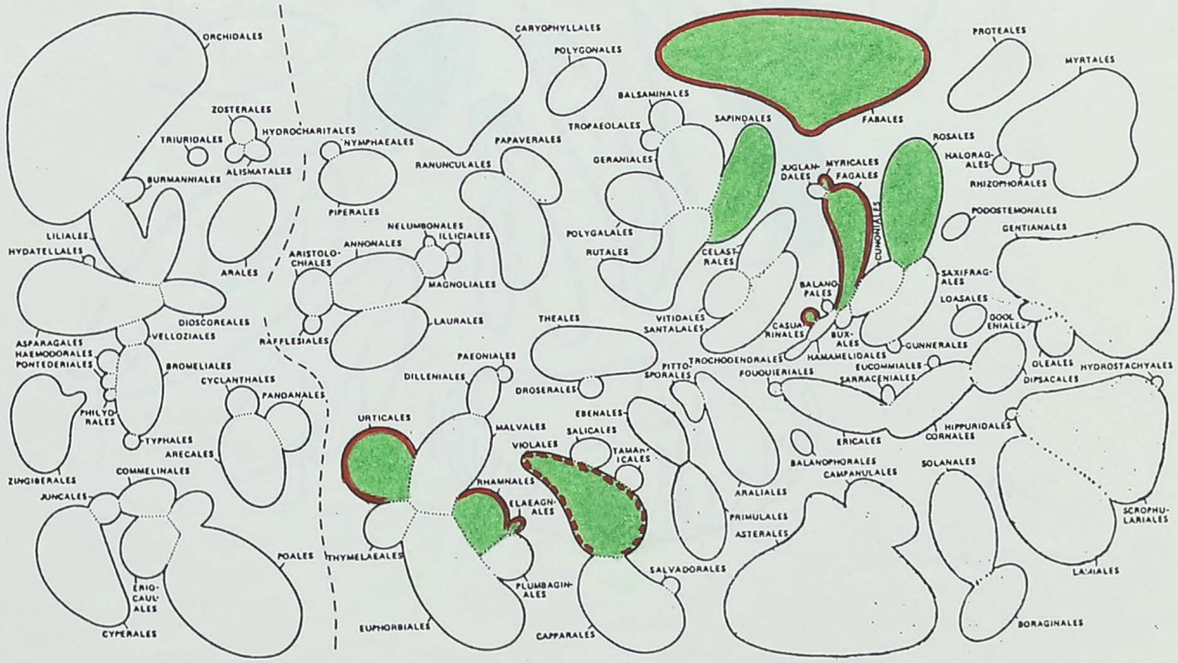
Green: orders known to contain nodulating species.

Red (solid line): presence of hemoglobin confirmed.

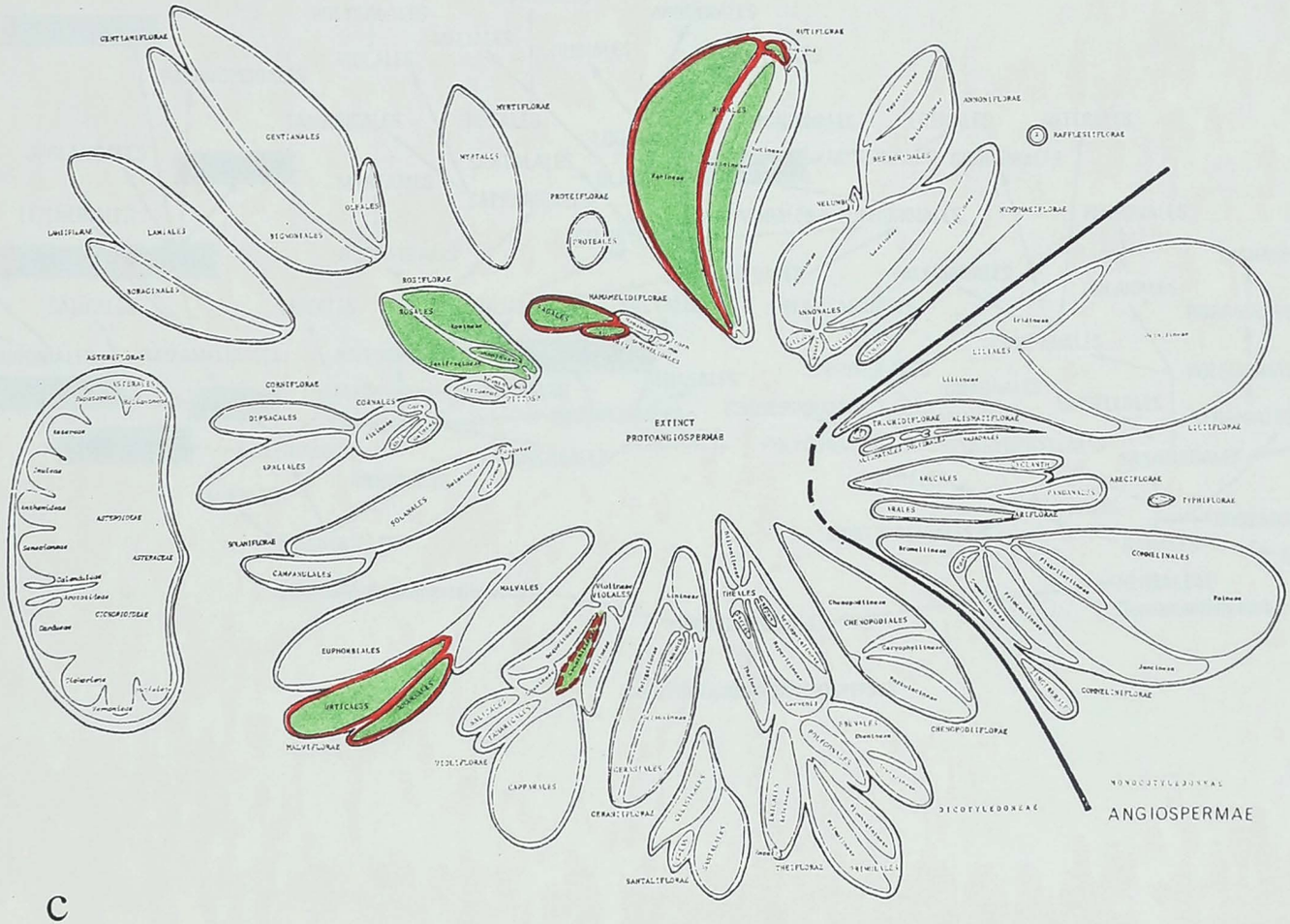
Red (dashed line): possible presence of hemoglobin.


CARYOPHYLLIDAE
Plumbaginales
Polygonales
Caryophyllales
DILLENIIDAE
Tamaricales
Salicales
Capparales
Thymelaeales
Begoniales
Euphorbiales
Primulales
Malvales
Ebenales
Violales
Ericales
Theales
Paeoniales
Dilleniales
ASTERIDAE
Asterales
Lamiales
Scrophulariales
Polemoniales
Calycerales
Campanulales
Loasales
Polygalales
Balanophorales
Rhamnales
Geraniales
Santalales
Oleales
Gentianales
Sapindales
Myrtales
Celastrales
Proteales
Dipsacales
Rutales
Nepenthales
Connarales
Podostemales
Euphorbiales
Fabales
Rosales
Cornales
Saxifragales
ROSIDAE
Poales
Hydatellales
Restionales
Eriocaulales
Commelinales
Cyperales
Zingiberales
Juncales
Bromeliales
Orchidales
Burmanniales
Smilacales
Triuridales
Liliales
LILIIDAE
Typhales
Arales
Pandanales
Cyclanthales
Arecales
ARECIDAE
Juglandales
Myricales
Fagales
Casuarinales
Leitneriales
Balanopales
Garryales
Urticales
Eucommiales
Hamamelidales
Didymelales
Eupteleales
Cercidiphyllales
Trochodendrales
HAMAMELIDIDAE
RANUNCULIDAE
Sarraceniales
Papaverales
Ranunculales
Rafflesiales
Aristolochiales
Illiciales
Piperales
Nelumbonales
Laurales
Nymphaeales
Magnoliales
MAGNOLIIDAE
Najadales
Alismatales
ALISMATIDAE

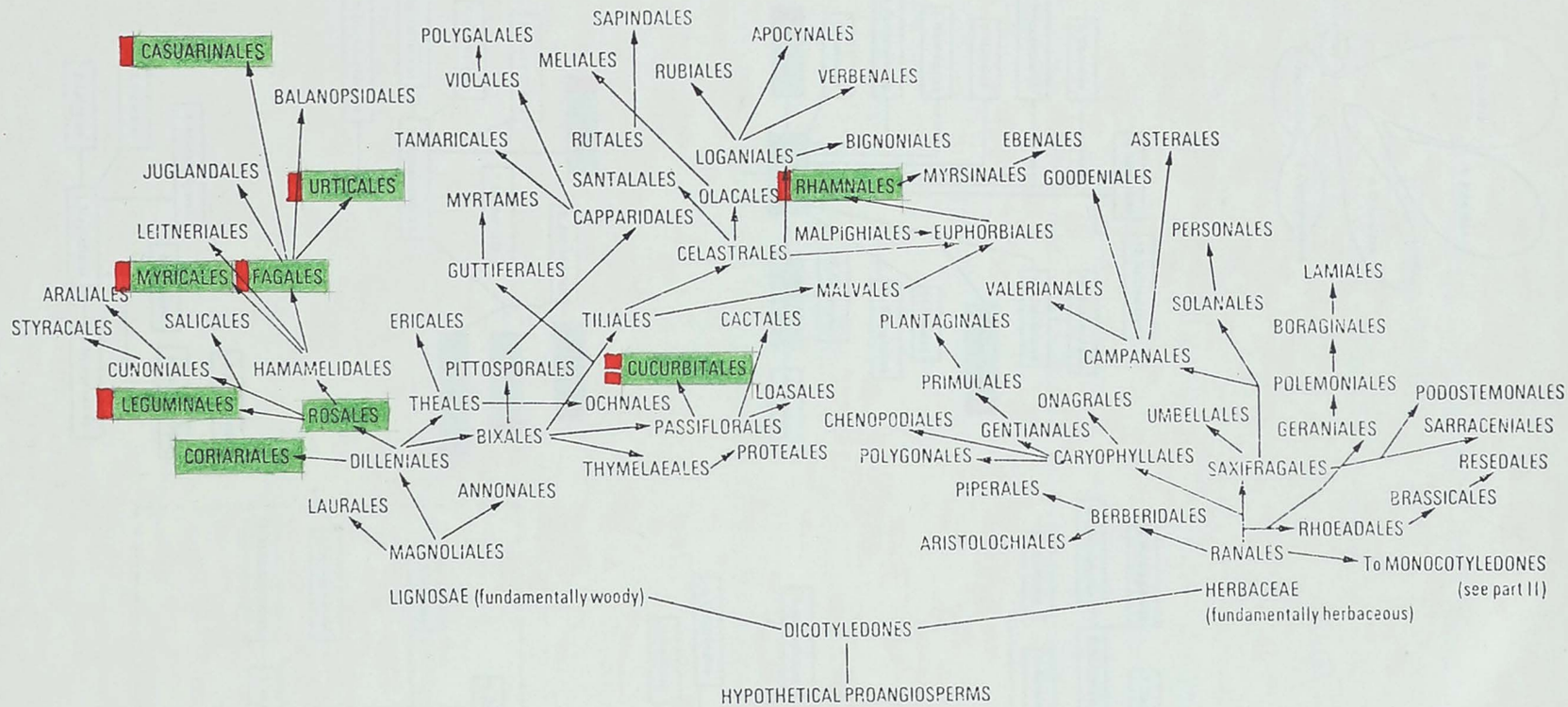
a


ORCHIDALES
ZOSTERALES
HYDROCHARITALES
TRIURIDALES
ALISMATALES
BURMANNIALES
LILIALES
HYDATELLALES
ARALES
DIOSCOREALES
VELLOZIALES
ASPARAGALES
HAEMODORALES
PONTEDERIALES
BROMELIALES
CYCLANTHALES
PANDANALES
PHILYDRALES
TYPHALES
ARECALES
ZINGIBERALES
COMMELINALES
JUNCALES
ERIOCAULALES
CYPERALES
POALES
THYMELAEALES
EUPHORBIALES
URTICALES
CARYOPHYLLALES
POLYGONALES
NYMPHAEALES
PAPAVERALES
RANUNCULALES
PIPERALES
NELUMBONALES
ILLICIALES
ANNONALES
ARISTOLOCHIALES
MAGNOLIALES
LAURALES
RAFFLESIALES
PAEONIALES
DILLENIALES
DROSERALES
MALVALES
VIOLALES
SALICALES
TAMARICALES
EBENALES
RHAMNALES
ELAEAGNALES
PLUMBAGINALES
CAPPARALES
BALSAMINALES
TROPAEOLALES
GERANIALES
POLYGALALES
RUTALES
SAPINDALES
FABALES
JUGLANDALES
MYRICALES
FAGALES
CASUARINALES
CELASTRALES
VITIDALES
SANTALALES
BALANOPALES
BUXALES
TROCHODENDRALES
PITTOSPORALES
PROTEALES
MYRTALES
ROSALES
HALORAGALES
RHIZOPHORALES
PODOSTEMONALES
GENTIANALES
SAXIFRAGALES
LOASALES
HOOKERIALES
GUNNERALES
HAMAMELIDALES
EUCOMMIALES
FOUQUIERIALES
SARRACENIALES
OLEALES
HYDROSTACHYALES
DIPSACALES
ERICALES
HIPPURIDALES
CORNALES
BALANOPHORALES
CAMPANULALES
SOLANALES
ARALIALES
PRIMULALES
ASTERALES
SALVADORALES
SCROPHULARIALES
LAMIALES
BORAGINALES

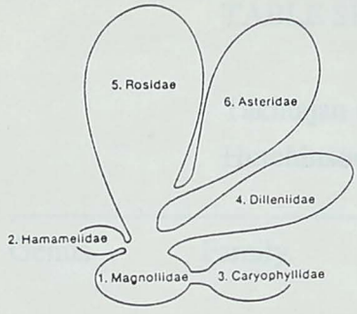
b

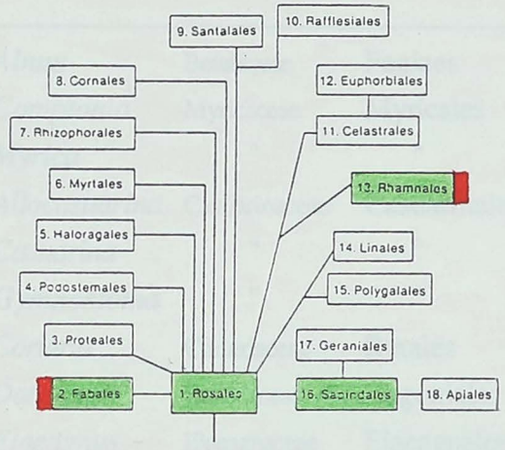

CENTIANIFLORAE
GENTIANALES
OLEALES
BIGNONIALES
LAMIFLORAE
LAMIALES
BORAGINALES
MYRTIFLORAE
MYRTALES
PROTEIFLORAE
PROTEALES
RUTIFLORAE
RUTALES
ANNONIFLORAE
RANALES
MAGNOLIIFLORAE
ROSIFLORAE
ROSALES
HAMAMELIDIFLORAE
EXTINCT
PROTOANGIOSPERMAE
ASTERIFLORAE
ASTERALES
CORNIFLORAE
DIPSACALES
CORNALES
EPACRIDALES
SOLANIFLORAE
SOLANALES
CAMPANULALES
EUPHORBIALES
URTICALES
MALVALES
MALVIFLORAE
PIPERALES
CAPPARALES
VIOLALES
CERASTIFLORAE
SANTALIFLORAE
THEALES
THEIFLORAE
CHENOPODIALES
CHENOPODIIFLORAE
CARYOPHYLLALES
POLYGONALES
PRIMULALES
DILLENIIFLORAE
ARECIFLORAE
TYPHIFLORAE
COMMELINALES
COMMELINIFLORAE
LILIIFLORAE
LILIALES
TRILLIIFLORAE
ALISMATIFLORAE
ARALES
ZINGIBERALES
BROMELIALES
JUNCALES
DICOTYLEDONEAE
MONOCOTYLEDONAE
ANGIOSPERMAE

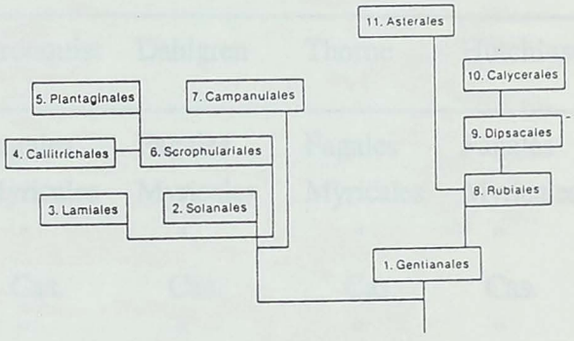
C

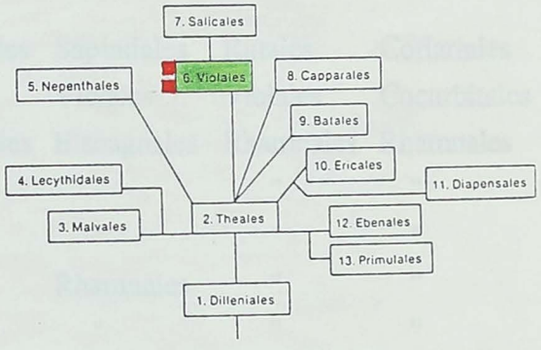

CASUARINALES
BALANOPSIDALES
JUGLANDALES
URTICALES
LEITNERIALES
MYRICALES
FAGALES
ARALIALES
STYRACALES
SALICALES
CUNONIALES
HAMAMELIDALES
LEGUMINALES
ROSALES
CORIARIALES
DILLENIALES
LAURALES
ANNONALES
MAGNOLIALES
POLYGALALES
VIOLALES
SAPINDALES
MELIALES
RUBIALES
APOCYNALES
VERBENALES
TAMARICALES
RUTALES
LOGANIALES
BIGNONIALES
EBENALES
ASTERALES
SANTALALES
OLACALES
RHAMNALES
MYRSINALES
GOODENIALES
MYRTALES
CAPPARIDALES
MALPIGHIALES
EUPHORBIALES
PERSONALES
CELASTRALES
GUTTIFERALES
MALVALES
VALERIANALES
LAMIALES
ERICALES
TILIALES
CACTALES
PLANTAGINALES
SOLANALES
BORAGINALES
PITTOSPORALES
CUCURBITALES
CAMPANALES
PRIMULALES
POLEMONIALES
PODOSTEMONALES
THEALES
OCHNALES
LOASALES
ONAGRALES
CHENOPODIALES
GENTIANALES
UMBELLALES
GERANIALES
SARRACENIALES
BIXALES
PASSIFLORALES
POLYGONALES
CARYOPHYLLALES
SAXIFRAGALES
RESEDALES
THYMELAEALES
PROTEALES
PIPERALES
BRASSICALES
BERBERIDALES
RHOEADALES
ARISTOLOCHIALES
RANALES
To MONOCOTYLEDONES
(see part II)
LIGNOSAE (fundamentally woody)
HERBACEAE
(fundamentally herbaceous)
DICOTYLEDONES
HYPOTHETICAL PROANGIOSPERMS

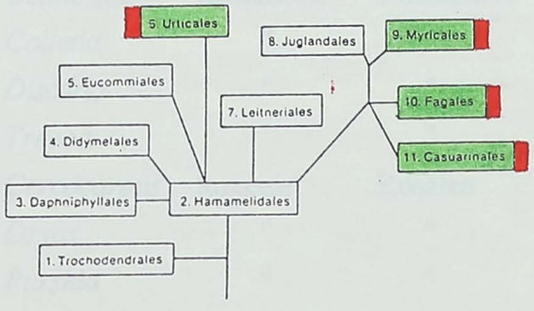
d

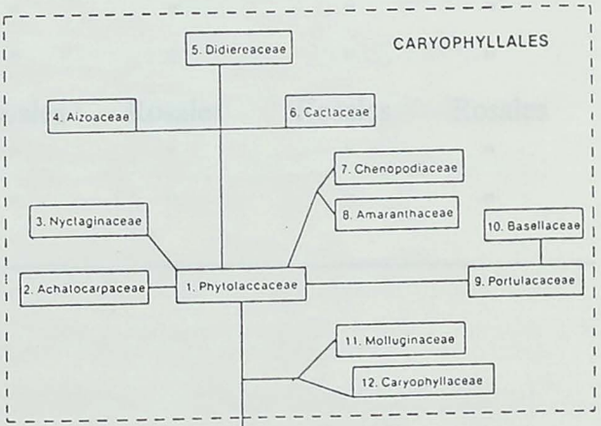

5. Rosidae
6. Asteridae
4. Dillenlidae
2. Hamamelidae
1. Magnoliidae
3. Caryophyllidae

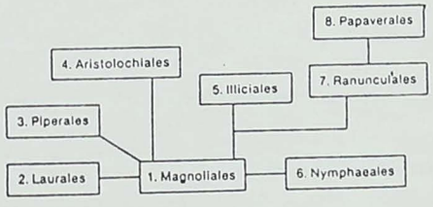

9. Santalales
10. Rafflesiales
8. Cornales
12. Euphorbiales
7. Rhizophorales
11. Celastrales
6. Myrtales
13. Rhamnales
5. Haloragales
14. Linales
4. Podostemales
15. Polygalales
3. Proteales
17. Geraniales
2. Fabales
1. Rosales
16. Sapindales
18. Apiales


11. Asterales
10. Calycerales
5. Plantaginales
7. Campanulales
9. Dipsacales
4. Callitrichales
6. Scrophulariales
8. Rubiales
3. Lamiales
2. Solanales
1. Gentianales


7. Salicales
5. Nepenthales
6. Violales
8. Capparales
9. Batales
4. Lecythidales
10. Ericales
11. Diapensales
3. Malvales
2. Theales
12. Ebenales
13. Primulales
1. Dilleniales


5. Urticales
8. Juglandales
9. Myricales
5. Eucommiales
7. Leitneriales
10. Fagales
4. Didymelales
11. Casuarinales
3. Daphniphyllales
2. Hamamelidales
1. Trochodendrales


CARYOPHYLLALES
5. Didiereaceae
4. Aizoaceae
6. Cactaceae
7. Chenopodiaceae
3. Nyctaginaceae
8. Amaranthaceae
10. Basellaceae
2. Achatocarpaceae
1. Phytolaccaceae
9. Portulacaceae
11. Molluginaceae
12. Caryophyllaceae
Polygonales
Plumbaginales


8. Papaverales
4. Aristolochiales
7. Ranunculales
5. Illiciales
3. Piperales
2. Laurales
1. Magnoliales
6. Nymphaeales

e

TABLE 3.2: THE TAXONOMIC POSITION OF ALL GENERA KNOWN TO CONTAIN ACTINORHIZAL SPECIES, ARRANGED ACCORDING TO FIVE DIFFERENT ANGIOSPERM CLASSIFICATION SYSTEMS.THIS TABLE SHOULD BE READ IN CONJUNCTION WITH FIGURE 3.1.

Takhtajan (1980); Cronquist (1981); Dahlgren (1980); Thorne (1981); Hutchinson (1973).

Genus	Family	Order				
		Takhtajan	Cronquist	Dahlgren	Thorne	Hutchinson
Alnus	Betulaceae	Faglaes	Fagales	Fagales	Fagales	Fagales
Comptonia	Myricaceae	Myricales	Myricales	Myricales	Myricales	Myricales
Myrica	"	"	"	"	"	"
Allocasuarina	Casuarinaceae	Casuarinales	Cas.	Cas.	Cas.	Cas.
Casuarina	"	"	"	"	"	"
Gymnostoma	"	"	"	"	"	"
Coriaria	Coriariaceae	Rutales	Sapindales	Sapindales	Rutales	Coriariales
Datisca	Datiscaceae	Begoniales	Violales	Violales	Violales	Cucurbitales
Elaeagnus	Elaeagnaceae	Elaeagnales	Rhamnales	Elaeagnales	Rhamnales	Rhamnales
Hippophae	"	"	"	"	"	"
Shepherdia	"	"	"	"	"	"
Ceanothus	Rhamnaceae	Rhamnales	"	Rhamnales	"	"
Colletia	"	"	"	"	"	"
Discaria	"	"	"	"	"	"
Trevoa	"	"	"	"	"	"
Cercocarpus	Rosaceae	Rosales	Rosales	Rosales	Rosales	Rosales
Dryas	"	"	"	"	"	"
Purshia	"	"	"	"	"	"

Both the amino acid sequence (Kortt *et al.* 1985) and the gene sequence (Landsmann *et al.* 1986) (Figure 3.2) of *Parasponia* hemoglobin confirm its common ancestry with leghemoglobin. The amino acid sequence shows 40% homology with soybean leghemoglobin a, and the gene sequence is more than 50% homologous in the coding region. More importantly, the gene sequence is also punctuated by three introns which occur in the same positons as they are found in leghemoglobin.

The occurrence of hemoglobin in *Parasponia* does not by itself disprove horizontal gene transfer from animals to plants, but it does require that the transfer event either took place much earlier than had previously been thought, or that transfer has occurred more than once. The latter possibility seems an overly complicated explanation.

Evolution by vertical descent.

An alternative hypothesis to explain the occurrence of hemoglobin in plants is that the gene has been present in plants since their divergence from an ancestor common to plants and animals. Dayhoff *et al.* (1972) compared amino acid sequences to estimate the divergence times of various hemoglobins and myoglobin. They estimated that plant and animal globins diverged approximately 1500 million years ago, which would predate the divergence of plant and animal lines. This implies evolution of hemoglobin in plants by vertical descent, rather than horizontal transfer.

The structure of the legume and *Parasponia* hemoglobin genes apparently supports this view. The presence of an additional intron in plant hemoglobin genes suggests they have a more primitive form than animal globin genes, assuming that in general terms introns have been deleted from proteins rather than inserted during evolution (Doolittle 1978, Gilbert 1981). This is in agreement with the view of Go (1981) that the ancestral gene of animal globins once had a third, central intron. Landsmann *et al.* (1986) considered that the unusually long polypeptide chain of *Parasponia* hemoglobin gene may also indicate a relatively

Figure 3.2: Comparison of the amino acid sequences of Lupin leghemoglobin II, Soybean leghemoglobin a and *Parasponia andersonii* hemoglobin I.

Sources of sequence data:
Parasponia andersonii : Kortt *et al* (1988)
Soybean : Hyldig-Nielsen *et al* (1982)
Lupin : Egorov *et al* (1980)

```
P. andersonii I   S S S E V N K V F T E E Q E A L V V K A W A V M K K N S A E L G L Q F F
Lupin II          + + + + + + G A L T E S Q A A L V K S S W E E F N A N I P K H T H R F F
Soybean a         + + + + + + V A F T E K Q D A L V S S S F E A F K A N I P Q Y S V V F Y

P. andersonii I   L K I F E I A P S A K N L F S Y L K D S P + V P L E Q N P K L K P H A T
Lupin II          I L V I E I A P A A K D L F S F L K G T S E V P + Q N N P D L Q A H A G
Soybean a         T S I I E K A P A A K D L F S F L A N G + + V D P T + N P K L T G H A E

P. andersonii I   T V F V M T C E S A V Q L R K A G K V T V K E S D L K R I G A I H F K T
Lupin II          K V F K L V Y E A A I Q L E V T G V V A S D A T + L K N L G S V H V S K
Soybean a         K L F A L V R D S A G Q L K A S G T V V A D A A + + + + L G S V H A Q K

P. andersonii I   G V V N E + H F E V T R F A L L E T I K E A V K E M W S P E M K N A W G
Lupin II          G V A D A + H F P V V K E A L L K T I K E V V G A K W S E E L N S A W T
Soybean a         A V T D P + Q F V V V K E A L L K T I K A A V G D K W S D E L S R A W E

P. andersonii I   V A Y D Q L V A A I K F E M K P S S T + +
Lupin II          I A Y D E L A I V I K K E M D D A A + + +
Soybean a         V A Y D E L A A A I K K A + + + + + + + +
```

primitive form.

Survival of plant hemoglobin genes during evolution.

The estimate of Dayhoff *et al.* (1972) raises an important question: if hemoglobin has been present throughout the evolution of the plant kingdom, how has it survived? While there is still some debate about the possibility of genes surviving unexpressed for millions of years ('atavisms', e.g. see Hall 1984), a more likely explanation is that hemoglobin has always had a function in plants. Currently, the only known function of hemoglobin in plants is the facilitation of oxygen diffusion to the nitrogen-fixing endophyte of root nodules. While fossil evidence of root nodules is scanty, it is reasonable to suggest that these symbioses have evolved only since the diversification of the angiosperms, given the absence of any such symbioses in gymnosperms. This diversification commenced only 120 million years ago. Presumably, for the previous 1400 million years hemoglobin had some other function in plants, an unidentified function which it may retain today.

How widespread is the occurrence of hemoglobin in plants?

If hemoglobin has been present in plants since their divergence from the animal line, another important question follows: how widespread is it today? Its presence in the phylogenetically disparate legumes and *Parasponia* has been taken to suggest that the genes for plant hemoglobin are indeed widespread and may have survived throughout the plant kingdom (Appleby 1984, Landsmann *et al.* 1986).

Support for this argument can be sought from the taxonomically highly diverse actinorhizal plants, which form nitrogen-fixing root nodule symbioses with the actinomycete *Frankia* . These plants are widely scattered throughout the dicotyledon branch of the plant kingdom, according to any of the major phylogenetic schemes (Figure 3.1).

There have been two reports of hemoglobin occurring in actinorhizal nodules (Davenport 1960, Tjepkema 1983), but in neither case was the protein purified (see Chapter Four for extended discussion). Therefore,

there has remained doubt (eg Hattori and Johnson 1985, Torrey 1985) as to whether these nodules do contain hemoglobin, or rather another molecule with some similar spectral properties.

If hemoglobin were present in these symbioses, as well as in legumes and *Parasponia* , then it must be considered widespread. Further, if this hemoglobin could be shown to share the same origin as legume and *Parasponia* hemoglobin, this would strongly support the hypothesis that the hemoglobin gene has been present in plants since their divergence from the animal line.

To test this hypothesis I decided to examine nodules of the *Casuarina - Frankia* symbiosis for the presence of hemoglobin. When I commenced this study the only report of hemoglobin in actinorhizal plants was by Davenport (1960), who had recorded a positive result for *Casuarina cunninghamiana* nodule slices. However, as I reported above this result had been discounted by other workers who failed to observe hemoglobin.

If hemoglobin could be purified from *Casuarina* nodules in sufficient quantity, it would be possible to determine its amino acid sequence, which could be compared with other plant hemoglobin sequences to derive their phylogenetic relationships. The amino acid sequence could also be fundamental to gene isolation and characterisation, by allowing the synthesis of oligonucleotide probes deduced from knowledge of that sequence (cf Landsmann *et al.* 1986). Sufficient pure hemoglobin would also allow development of an anti-hemoglobin anti-serum which could be used in immunological studies to confirm these relationships.

Oxygen transport within the nodule: the roles of hemoglobin and physical barriers

The oxygen paradox.

The requirement for oxygen in nitrogen-fixing nodules has been

regarded as paradoxical (Appleby 1984). On the one hand it must be available in sufficient amount to meet the respiratory needs of endophyte and host cells, while on the other hand the oxygen-labile nitrogenase (Robson and Postgate 1980) must be maintained in an environment of low free oxygen concentration. The resolution of this paradox is incompletely understood, but on available evidence appears to involve more than one mechanism. As pointed out by Wheeler *et al.* (1979), this is not suprising: " Although the control of oxygen access may be centred on one major property such as the occurrence of leghemoglobin in legume root nodules, it is to be expected that the integration of structural and biochemical properties which must have taken place during the evolution of the symbiotic relationship will have produced many features which participate in this function."

Physical barriers to oxygen movement in legume and* Parasponia *nodules.

In legumes and *Parasponia* the physical structure of the nodule limits oxygen movement. A layer of cortical cells, unbroken by air spaces, surrounds the infected zone and provides a gaseous diffusion barrier (Tjepkema and Yocum 1974, Tjepkema and Cartica 1982). There is empirical evidence to suggest that some legume nodules may be able to regulate the operation of this diffusion barrier so that the influx of oxygen is continuously matched to respiratory oxygen consumption of the endophyte and its host (Layzell *et al.* 1988, Sheehey *et al.* 1983). Layzell *et al.* (1988) found that if they raised the partial pressure of oxygen in the atmosphere surrounding soybean nodules by 5 Kpa, the internal oxygen concentration quickly rose from 22 to 260 nM but dropped back to 22 nM in 8 to 10 minutes. Because this drop was not accompanied by a rise in respiration they concluded that it was due to increasing the resistance of the diffusion barrier. Whether this diffusion barrier is regulated or not, the average free oxygen concentration maintained within the infected zone of the nodule is very low (~7nM - Bergersen 1982, pp 115-118)

TABLE 3.3: KINETIC AND EQUILIBRIUM CONSTANTS FOR THE REACTIONS OF VARIOUS LEGHEMOGLOBINS WITH OXYGEN. HORSE MYOGLOBIN IS INCLUDED FOR COMPARISON.

For sources of data see Table 5.4.

Protein	k' "on"	k' "off"	K' (=k:k')	P_{50}
	$M^{-1}s^{-1}.10^{-6}$	s^{-1}	nM	torr
Lupin I	540	19.5	36.1	0.020
Broad Bean V	200	18.7	93.5	0.051
Kidney Bean a	100	6.22	62.2	0.034
Soybean a	118	5.55	48	0.026
Green Pea I	250	16.3	65.2	0.036
Horse Mb	14	11	770	0.700

However, the presence of a physical barrier alone does not resolve the oxygen paradox. There are two major reasons for this. Firstly, if there were only a physical barrier to oxygen movement in the nodule, then the oxygen concentration within the infected zone would be non-uniform: some bacteroids would be almost anaerobic because the very limited amount of oxygen which would diffuse to them would be rapidly respired. The nodule would therefore function very inefficiently. Secondly, if a physical barrier coupled with endophyte respiration were the only mechanism to maintain low free oxygen concentration, then if respiration were reduced,eg due to a drop in temperature or a reduction in supply of photosynthate, there would be a gradual buildup in oxygen concentration, leading to destruction of the nitrogenase enzyme.

Leghemoglobin and facilitated oxygen diffusion.

Thus the resolution of the paradox within legume nodules seems to rely on the presence of both a physical barrier and an oxygen carrier, leghemoglobin, which can facilitate the diffusion of oxygen to the *Rhizobium* bacteroids at a rate commensurate with efficient respiration (Wittenberg *et al.* 1974, Appleby 1984). Because leghemoglobin is an extremely high affinity oxygen carrier (Table 3.3), with a moderately fast dissociation rate constant, it can function in facilitated oxygen transport at very low free oxygen concentration. This is supported by *in vitro* studies of nitrogen fixation by *Rhizobium* bacteroids bathed in leghemoglobin solutions at various degrees of oxygenation (Bergersen *et al.* 1973, Wittenberg *et al.* 1974), and by the observation that under normal conditions in the nodule, leghemoglobin is maintained in a substantially deoxygenated state (Appleby 1969).

Physical barriers to oxygen movement in actinorhizal nodules.

Less is known about oxygen movement in actinorhizal nodules. In general, these nodules do not appear to have any specialised host morphological structure to limit oxygen diffusion into the infected zone (Tjepkema and Yocum 1974, Winship and Tjepkema 1983, Tjepkema 1979). Tjepkema and Yocum (1974) showed that there were continuous

intercellular air spaces between the external atmosphere and infected cells of *Alnus*, and that some infected cells were exposed to near-atmospheric concentrations of oxygen. A similar result was reported for *Myrica gale* (Tjepkema 1979). However, Wheeler *et al.* (1979) found that in *Alnus rubra* access of air to infected cells was restricted by the reduced number and size of air spaces in the infected region, compared to the uninfected outer cortex. This result is not directly applicable to *Casuarina* nodules, though, because of gross differences in morphological structure which affect access of oxygen into the nodule. *Alnus* nodules have lenticels on their surface, whereas *Casuarina* nodules do not, but do have aerenchymatous, apogeotropic nodule roots (for further discussion, see below).

Winship and Tjepkema (1983) showed that in *Alnus rubra* nodules oxygen protection of nitrogenase may involve a permeability barrier, which they believed might be the vesicle envelope of the endophyte (Winship and Tjepkema 1985). Torrey and Callaham (1982) have also suggested that the vesicle wall structure may provide a permeability barrier to oxygen. This is based on the view (which has a considerable body of supporting evidence) that vesicles are the primary site of nitrogenase within actinorhizal nodules (Tjepkema *et al.* 1980, Meesters *et al.* 1985, Noridge and Benson 1986, Meesters 1987). Winship and Tjepkema (1983) have further suggested that this barrier may be able to undergo rapid reversible oxygen permeability change providing continuous protection for nitrogenase even as respiration becomes oxygen saturated. Recently, it was found (Parsons *et al.* 1987) that when *Frankia* (isolated from *Casuarina* (HFPCcI3) was grown at a range of oxygen levels from 3kPa to 70kPa, it had maximum nitrogenase activity close to the oxygen level at which it was grown. They found that vesicle walls increased in thickness with increasing oxygen concentration, apparently by laying down additional lipid layers. This is unlikely to explain the rapidly-variable diffusion barrier observed by Winship and Tjepkema (1983), however, because of the time delay involved in synthesising lipids.

Frankia vesicles can be compared with the heterocysts of cyanobacteria (Tjepkema 1979, Silvester *et al.* 1988), which also house the nitrogenase enzyme system. Walsby (1985) believes that glycolipid layers in the walls of heterocysts of *Anabaena flos-aquae* reduce gas diffusion into the cell. The resultant permeability coefficient is low enough for the oxygen concentration in the heterocyst to be maintained close to zero by the probable rate of respiration, providing an anaerobic environment for nitrogenase.

A posible role for hemoglobin in* Casuarina *nodules.

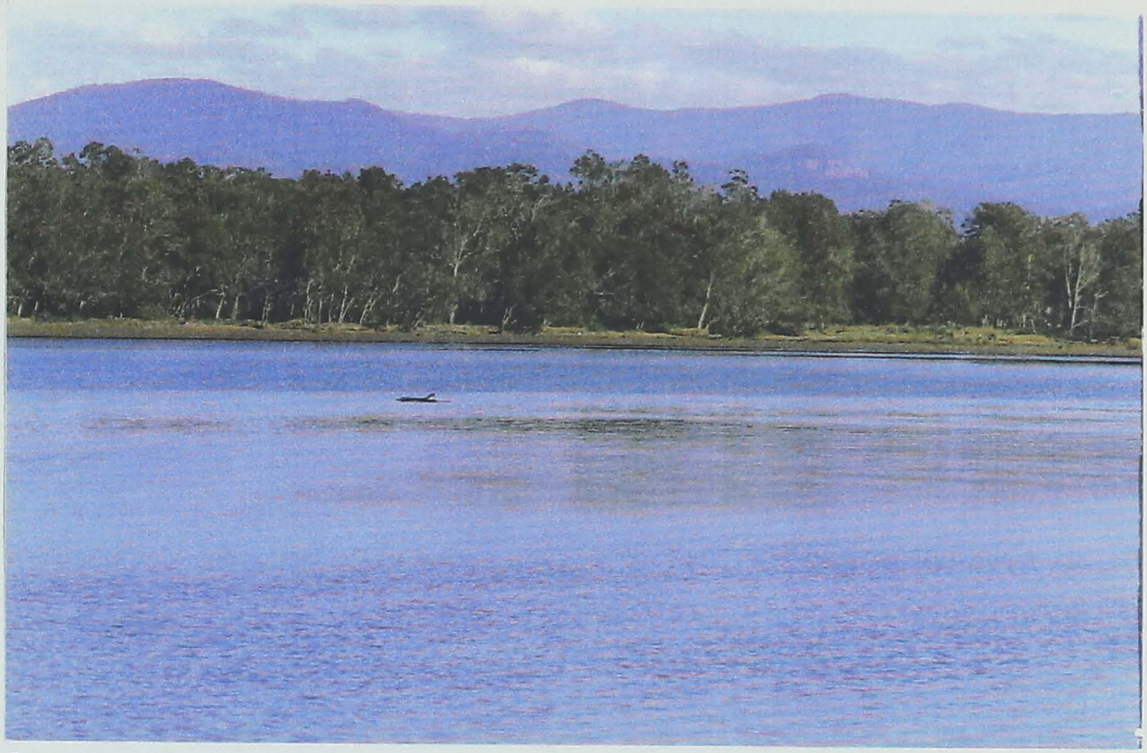
Within legume nodules leghemoglobin facilitates the diffusion of oxygen to the *Rhizobium* endophyte at a rate commensurate with efficient respiration, in an environment of low free-oxygen concentration. There are both environmental and structural reasons to suspect that hemoglobin with a similar function may be important in *Casuarina* nodules.

Both *C. glauca* and *C. cunninghamiana* occur naturally along the edges of watercourses, as can be seen in Figure 3.3. *C. cunningamiana* is restricted to freshwater streams, and *C. glauca* occurs along tidal streams and marshes. In these riparian habitats roots and nodules are often submerged for days to weeks at a time, and thus the pO_2 in the atmosphere surrounding the nodules is often low. One physical mechanism which has apparently evolved to help cope with low soil oxygen levels is the apogeotropic nodule roots. These roots are filled with aerenchyma cells (Figure 3.4) and extend beyond the surface of saturated soils (Figure 3.4), thus providing a pathway for limited oxygen diffusion to the nodule (Tjepkema 1978). They are either withered or absent (Figure 3.4) from nodules which occur in more aerobic environments such as dry soil or the soil surface. Even given the presence of nodule roots, however, the oxygen concentration within the nodule is likely to remain low. Berg (1983) has reported suberization of the walls of infected nodule cells in three *Casuarina* species, including *C. glauca* ,

Figure 3.3: Examples of the natural habitat of *C. cunninghamiana* ('a' and 'b') and *C. glauca* ('c' and 'd'). These photos were taken in southern New South Wales. Note how both species are almost entirely restricted to water courses. *C. cunninghamiana* occurs along fresh water systems in New South Wales and Queensland. *C. glauca* grows in brackish areas along the coast of southern Queensland and New South Wales. The root systems of both species are occasionally inundated during periods of flood (eg, 'b').

a

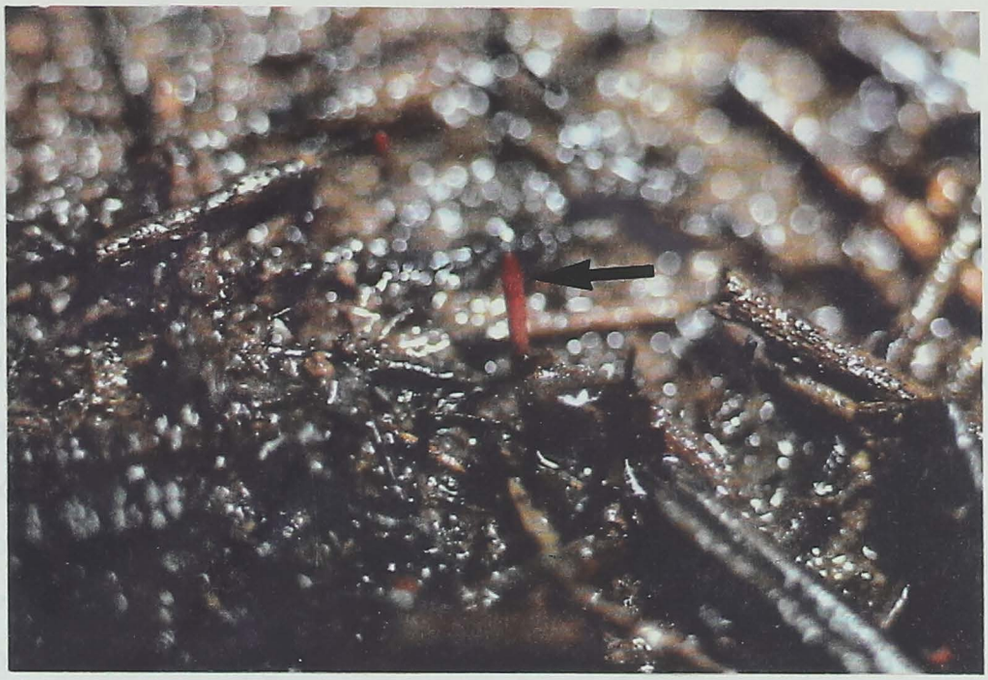
b



c

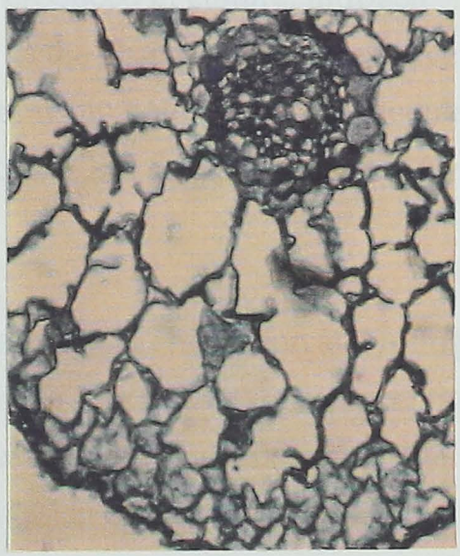
d

Figure 3.4: Presence and absence of apogeotropic nodule roots (*Casuarina glauca*).
(a) Undisturbed site showing a nodule root (arrowed) extending above surface of saturated soil and plant litter.
(b) Litter and surface soil removed to show connection of root with nodule.
(c) Tranverse section of nodule root showing large aerenchymatous cells and absence of endophyte.
(d) Undisturbed site showing absence of nodule roots on nodules growing on the surface of drier soil.



a

b



c

d

which may restrict diffusion of oxygen into these cells. A similar compound has also been reported in the walls of ramifying infection threads in *Parasponia rigida* nodule cells (Smith *et al.* 1986).

The absence of endophyte vesicles in *Casuarina* nodules (N.B. Torrey *et al.* 1988 has observed facultative production of vesicles in the related species *Gymnostoma papuanum* , but there are no reports of vesicles in *Casuarina s. str.*) supports the hypothesis that a low free oxygen concentration is maintained within infected cells. Vesicle production by *Frankia* isolated from *Casuarina* nodules has been shown to be correlated with oxygen concentration (Murry *et al.* 1985). While nitrogen-fixing vesicles are generally produced in culture, nitrogen fixation has also been shown to occur in the absence of vesicles at very low oxygen concentration. If this relationship holds within the nodule, then the absence of vesicles may indicate a low free oxygen concentration within the nodule. *

Hemoglobin could conceivably enable efficient respiration and nitrogen fixation to occur under these conditions of low free oxygen concentration by facilitating the flux of oxygen to the endophyte at a desirable rate. The rates of oxygen uptake and nitrogen fixation by actinorhizal and legume nodules appear to be quite similar (Tjepkema and Winship 1980).

* Tjepkema *et al* (1988) reported that there were limited gas filled intercellular spaces in the cortex of nodules of *Casuarina*, but these were too few to significantly effect the oxygen concentration within the nodule.

Chapter Four

THE IDENTIFICATION, EXTRACTION AND PURIFICATION OF *CASUARINA* ROOT NODULE HEMOGLOBIN

The first significant evidence for the occurrence of hemoglobin in the nodules of actinorhizal plants came from Davenport in 1960. He reported observing absorption bands characteristic of hemoglobin in whole and crushed nodules of *Casuarina cunninghamiana*, *Alnus* and *Myrica* . By hand spectroscopy, he established that the pigment he observed in these nodules could undergo alternate deoxygenation and oxygenation without change in the valency of the heme iron. To quote from his 1960 paper:

' With the untreated [*Casuarina*] nodules a spectrum with two absorption bands, at 580 mμ and 542 mμ, could easily be observed. When a small amount of sodium dithionite was added to the fluid surrounding the nodules the two bands were slowly replaced by a single diffuse band at 562 mμ. What is more significant, however, is that when the dithionite solution was replaced by a fresh *tris* medium the two-banded spectrum reappeared. This sequence of events also occurred in the absence of a reducing agent if the nodules were allowed to respire the dissolved oxygen in the fluid surrounding them and air was then readmitted. Confirmation that the hæm iron of the pigment is in the ferrous state *in vivo* was obtained by treating the nodules with carbon monoxide, when two diffuse absorption bands at 572 mμ and 542 mμ replaced the characteristic spectra of either the oxygenated or deoxygenated derivative. The hæm pigment of *Casuarina* root nodules appears, therefore, to possess the essential properties of hæmoglobin.'

Unfortunately, this putative hemoglobin remained firmly bound to cell

debris under all extraction conditions tried, and hence resistant to purification, in contrast to the readily soluble legume nodule leghemoglobin or animal hemoglobin. Davenport thus lacked critical proof that the pigment he was investigating was a true hemoglobin and as consequence his important observations were generally disregarded. The failure of other workers to detect hemoglobin in a number of actinorhizal species reinforced the opinion that it was absent from these plants (Egle and Munding 1951, Smith 1949, Moore 1964, Becking 1970). In 1983, however, Tjepkema confirmed the observations of Davenport, recording spectra characteristic of a reversibly-oxygenating hemoglobin in nodule segments of *Casuarina cunninghamiana*, *Myrica gale*, *Comptonia peregrina*, *Alnus rubra* and *Elaeagnus angustifolia* . He was also able to extract a small amount of crude *Casuarina* hemoglobin by following the procedure of Appleby *et al.* (1983), which involves the use of soluble polyvinyl pyrolidone in the extraction medium.

The results of both Davenport (1960) and Tjepkema (1983) suggested that hemoglobin occurs in actinorhizal nodules. In the absence of pure protein, however, there has remained doubt as to whether the observed pigment is hemoglobin, or a membrane-bound molecule with some similar spectral properties (Torrey 1985, Hattori and Johnson 1985, Zhang and Torrey 1985). The distinction is critical to a resolution of questions raised in Chapter 3, particularily the evolutionary relationships of plant and animal globins, the breadth of occurrence of the hemoglobin gene(s) in plants and the possible role of this protein in nodule function. In order to answer these questions, purified protein in quantities sufficient for characterisation is required.

In this Chapter I report the solubilisation and purification of hemoglobin from root nodules of the *C. glauca -Frankia* symbiosis. Hemoglobin has also been purified from nodules of *C. cunninghamiana*, but because most subsequent studies were conducted with *C. glauca* hemoglobin, I have concentrated on its' purification. Where possible, information on the properties of *C. cunninghamiana* hemoglobin has been included for

comparison.

Materials and Methods

Growth of nodules.

In an initial trial, nodules of *Casuarina cunninghamiana* were harvested from naturally growing plants at Paddy's River, ACT (35° 23' E, 148° 58' S). Nodules were picked from the roots, rinsed in water to remove soil, then placed directly into liquid nitrogen. Only the young outer lobes of nodules, showing the least visible tannin, were selected. However, these nodules failed to yield any hemoglobin (see Results) and all subsequent extraction experiments used nodules obtained from glasshouse grown plants.

Seeds of *Casuarina glauca* Sieb ex Spreng and *Casuarina cunninghamiana* Miq. were sown, germinated and grown in a 50:50 Perlite:vermiculite substrate in a glasshouse under natural light (temp. 28°-30° C day, 22°-24° C night). They were watered twice weekly with a 1/4 strength complete nutrient solution (Table 2.2) until 4 weeks old, then inoculated with *Frankia* sp. using crushed nodule preparations derived from naturally grown *C.glauca* or *C. cunninghamiana* nodules (inoculum from same species as seed), following the procedure described below. Plants were regularly watered and fertilised twice weekly with 1/2 strength nitrogen-free nutrient solution (Table 2.2). Nodules, which began to appear from 15 days, were harvested 14-16 weeks after inoculation by picking directly into liquid nitrogen, in which they were stored.

Inoculation of plants: Nodule material of *C. cunninghamiana* for use as an inoculum was obtained from Paddy's River, ACT (35° 23' E, 148° 58' S), and *C. glauca* from Nelligen River, NSW. It was stored (for no longer than three weeks) in moist soil at 4° C until required. Nodules were washed free of soil, surface sterilised to remove external *Frankia* by immersion for sixty seconds in 70% (v/v) ethanol, then thoroughly rinsed

three times in distilled water (*Frankia* -free). The inoculum was prepared by grinding the surface sterilised nodule material in distilled water and the resultant suspension made up to a final concentration of 1.0 g nodule material per 100 ml distilled water. Seedlings were inoculated by injecting 10 ml of the nodule suspension just below the soil surface next to the seedling immediately following watering of the substrate to saturation.

Hemoglobin standard extraction procedure.

All manipulations during extraction were done in ice in a fume hood under strict anaerobic conditions with C.P grade CO or argon in the gas phase. The manipulative procedures are modified from those developed earlier for anaerobic extraction of *Parasponia* root nodule hemoglobin (Appleby *et al.* 1983).

The standard extraction procedure described below was generally followed in all experiments, with only minor variation in the amount of nodule material (± 2 g) added to the extraction chamber. In early trials nodules of *C. cunninghamiana* were used. However, *C. glauca* was later chosen for routine use as it grew more rapidly in the glasshouse and produced a greater mass of nodules.

In a typical experiment, 16 g of nodules (nodule aerial roots were removed by abrasion under liquid nitrogen in a sieve) were added to a Sorvall Omnimixer 70ml steel chamber, with gassing ports, containing 60ml of CO saturated extraction buffer (50 mM potassium phosphate, 1 mM EDTA, 4% (w/v) Kollidon 25 [BASF batch S55314], 1.5 mg/ml sodium dithionite, 0.1% (w/v) Zwittergent 3-12 (Calbiochem Lot 903843), pH 7.2). The vessel was sealed under a slight positive pressure of CO and the nodules ground for two minutes at full speed. The suspension was transferred under an argon blanket in an open top 50 L plastic bucket into an argon-equilibrated Beckman Spinco 21 centrifuge tube, bubbled for a few seconds with CO, sealed and centrifuged for 60 min at 40,000 xg at 4^{o} C to yield 56 ml of red-brown supernatant.

A sample of this supernatant was transferred under argon to a 10 mm quartz cuvette, bubbled gently with CO, sealed under CO, and the spectrum recorded from 700-250 nm for calculation of putative hemoglobin concentration and estimation of purity.

Detergent trials: In initial experiments, including the attempted extraction of hemoglobin from field-grown nodules, no detergent was included in the extraction buffer. However, in the absence of a detergent there was a relatively low yield of hemoglobin from *Casuarina* nodules, compared with legumes (see Results), and very poor recovery of hemoglobin during subsequent chromatography on Sephacryl S200 (Table 4.2). In an attempt to increase yield and improve the purification procedure, three trial experiments were conducted, each including one of three detergents at just below their critical micelle concentrations (CMC). The detergents were added to the extraction buffer and then standard extraction and purification procedures were followed. The detergents tested were:

1. 6 mM CHAPS : 3-[(3-cholamidopropyl)dimethylammonio]1-propane sulphonate (SIGMA Cat. No. 3023)
2. 3 mM dodecyl ß-D-maltoside (Calbiochem Cat. No. 324355)
3. 3 mM Zwittergent 3-12 : N-dodecyl-N,N-dimethyl-3-ammonio 1-propanesulphonate (Calbiochem Cat. No. 903843)

CHAPS is a zwitterionic derivative of cholic acid (Hjelmeland 1980). Dodecyl ß-D-maltoside is a non-ionic detergent (Stubbs *et al.* 1976). Zwittergent 3-12 is a member of the series of zwitterionic detergents known as the sulfobetaines (Gonenne and Ernst 1978).

The nodules used in this trial were from *C. cunninghamiana*. Nodules from *C. glauca* , the species of major interest, were not used because then they were available in only very limited amount. Hemoglobin from both species had previously been shown to behave similarly during extraction and Sephacryl S200 chromatography.

Inclusion of soluble polyvinyl pyrrolidone. Casuarina nodules contain considerable amounts of phenolic material, as can be demonstrated by

staining a section of nodule with safranin (Figure 4.1). Phenols, which stain deep red, filled many of the uninfected cells. If these phenols were allowed to come into contact with hemoglobin during extraction, they could cause its inactivation by combining either reversibly by hydrogen bonding, or irreversibly by oxidation (Loomis and Battaile 1966). Polyvinyl pyrrolidone (PVP) has the ability to adsorb these potentially harmful phenolic compounds, thereby removing them from solution (Loomis and Battaile 1966). It was included in the extraction medium primarily for this purpose.

Loomis and Battaile (1966) had used insoluble polyvinyl pyrrolidone for most of their studies. Appleby *et al.* (1983), however, found that soluble polyvinyl pyrrolidone effectively removed phenols, but was also involved in the solubilisation of *Parasponia* hemoglobin, though they could not explain the nature of this involvement. To test the significance of soluble polyvinyl pyrrolidone in the solubilisation of *Casuarina* hemoglobin a trial extraction was conducted as follows. The standard extraction procedure was followed except that soluble PVP was excluded from extraction buffer. After extraction and centrifugation the pellet was resuspended in extraction buffer without soluble PVP but with the concentration of Zwittergent 3-12 raised from 0.1% to 1.0%, and then recentrifuged at 40,000 rpm for 20 minutes. The pellet from this centrifugation was then resuspended in an extraction buffer containing 1.0% Zwittergent 3-12 and 4% soluble PVP and centrifuged at 40,000 rpm for 20 min. After each extraction the spectrum of the supernatant was recorded and the concentration of hemoglobin determined.

Sephacryl S200 SF Column chromatography.

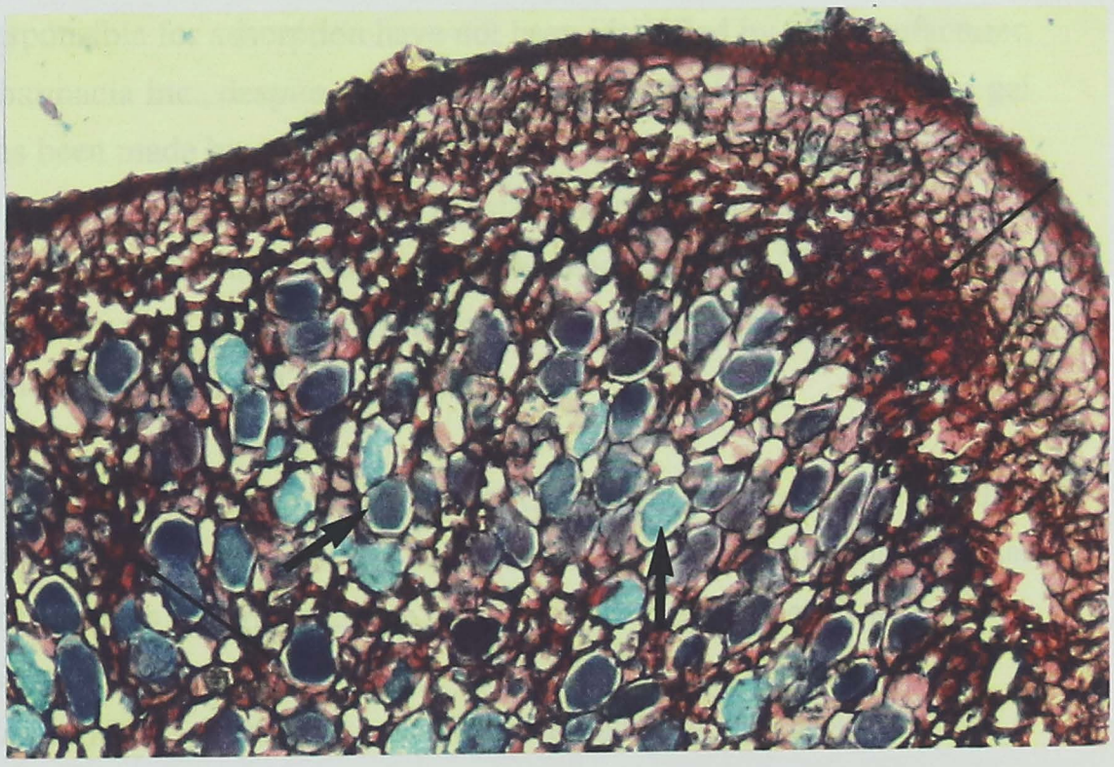
All chromatography was conducted in a coldroom at 4^{o}C.

In the typical experiment 55 ml of CO-saturated supernatant obtained by the hemoglobin standard extraction procedure was transferred to a CO-flushed syringe and pumped anaerobically onto a 45.0 x 5.0 cm column of Sephacryl S200 SF (Pharmacia) which had been equilibrated

Figure 4.1: (a) Equipment used for the collection of nodule material from *Casuarina cunninghamiana* in the field. The nodules were washed clean with stream water and then immediately placed in the liquid nitrogen container.

(b) Transverse section of a field-grown *Casuarina cunninghamiana* nodule. The short arrows indicate cells containing *Frankia* , and the long arrows indicate oil and tannin filled cells. Hemoglobin could not be extracted from field-grown nodules.

a



b

overnight with 3 volumes of CO-saturated column buffer (50 mM potassium phosphate, 1 mM EDTA, pH 7.2). The column flow rate was 3.4 ml/min and 6 min (20.4 ml) fractions were collected. Absorbance was monitored at 405 nm (the shoulder of the HbCO Soret peak) and 280 nm (protein peak) using a UVICORD III flow monitor (LKB). Fractions containing the putative hemoglobin were selected and the spectra of samples recorded from 700-250 nm after re-equilibration with CO. On the basis of hemoglobin concentration and relative purity (measured as the ratio R of 420 nm : 280 nm absorbance) fractions were selected, pooled and concentrated to small volume by pressure filtration over an Amicon YM10 membrane (10,000 mw exclusion). This procedure is illustrated in Figure 4.2. Once the part-purified HbCO had emerged from the S200 column it was not necessary to maintain anaerobic conditions or to use CO-saturated buffers.

Sephacryl S200 'batch trials'. The batch of S200 routinely used for purification was found to weakly adsorb *Casuarina* hemoglobin (Results, Figure 4.5). This adsorption was unexpected because S200 is designed to act as a passive molecular weight seive. The specific groups on S200 responsible for adsorption have not been identified by its manufacturer, Pharmacia Inc., despite repeated requests. From 1980, however, the gel has been made by a slightly modified procedure which apparently eliminates these active sites (Pharmacia Technical Note A 681, and additional information supplied by the manufacturer). The batch of S200 routinely used for the purification of *Casuarina* hemoglobin was a mixture of lots manufactured before 1980.

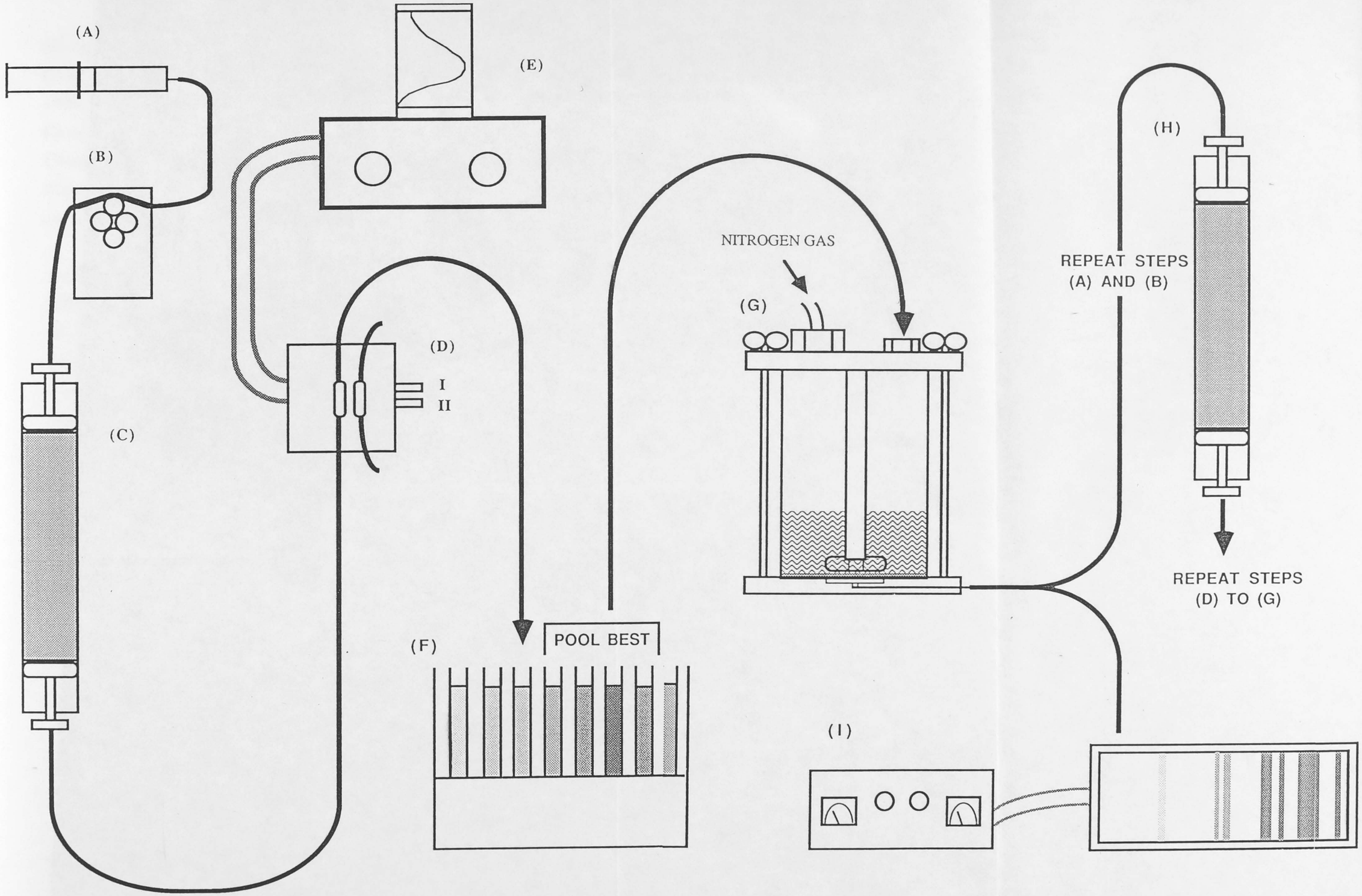
To determine whether adsorption of *Casuarina* hemoglobin to S200 was a general phenomenon, or was restricted to particular lots of the gel, a series of trials was undertaken. Five columns were prepared, each with a different gel lot:

Column I - S200 lot # unknown manufactured after 1980.

Column II - S200 lot # 6720 manufacured before 1980.

Column III - S200 lot # 13560 manufactured before 1980 (provided by

Figure 4.2: Diagramatic representation of the steps involved in the purification of *Casuarina* hemoglobin. After anaerobic centrifugation, the crude nodule extract is taken up in a syringe (A) and pumped (B) onto a column of Sephacryl S200 (C). The steps A-C were performed under strict anaerobiosis, using CO-equilibrated buffers. The effluent from the column passes through a Uvicord UV monitor (D,E) and samples are then pooled in a fraction collector (F). The hemoglobin-containing samples are then pooled, concentrated (G) over an Amicon YM-10 membrane (10,000 MW exclusion) and frozen in liquid nitrogen. The sample can then be subject to further purification on a Sephadex G75 column (H), or by isoelectric focusing (I).


(A)
(B)
(C)
(D)
I
II
(E)
(F)
POOL BEST
NITROGEN GAS
(G)
(H)
REPEAT STEPS
(A) AND (B)
REPEAT STEPS
(D) TO (G)
(I)

the Dept of Physical Biochemistry, Australian National University).
Column IV - S200 mixed lots (numbers unknown) manufactured before 1980. This is the batch which was routinely used for the purification of *Casuarina* hemoglobin.
Column V - S300 lot # 1128820 manufactured before 1980 (provided by Pharmacia Inc.). This gel lot was included because it was reported to contain the same active sites found in pre-1980 S200.

A sample of *Casuarina cunninghamiana* hemoglobin was extracted following the standard procedure. After centrifugation, 4 x 5 ml aliquots of the supernatant were transferred under CO and argon into small tubes, then immediately stored in liquid nitrogen.

Each column (43.5 cm x 1.6 cm) was washed overnight with CO-saturated buffer at 60 ml/hr. The flow rate was then reduced to 21 ml/hr (0.35 ml/min). This was one-tenth the rate of the 50 cm column which had been used in the standard extractions, and which had ten times the cross-sectional area. Column loading and operating procedures were as described in Materials and Methods (see *Sephacryl S200 SF column chromatography*). Ten minute (3.5 ml) fractions were collected.

Sephadex G75 SF column chromatography.
The hemoglobin from S200 column chromatography was sometimes further purified by reconcentration over a YM10 membrane, buffer exchange to 0.1 M KCl, 1 mM EDTA, pH 7.2 in the concentration cell, with a second reconcentration in this cell. The hemoglobin was then run through a 38.7 x 1.6 cm Sephadex G75 SF (Pharmacia batch 456) column equilibrated with the same buffer, following the procedure described above for Sephacryl S200 chromatography, except that the flow rate was 0.15 ml/min and ten minute (1.5 ml) fractions were collected. The hemoglobin-containing fractions were selected, pooled and concentrated also as described above.

Preparative-scale isoelectric focusing.

Preparative-scale isoelectric focusing was conducted on concentrated hemoglobin from Sephacryl S200 chromatography, after buffer exchange to a low salt strength (<10 mM), using an LKB 2217 Ultrophor flat bed isoelectric focusing apparatus and the general procedures of Fuchsman and Appleby (1979). All components of HbO_2 appeared stable at their isoelectric points, so this was the species of choice for isoelectric focusing. In a typical run, 4.4 ml of 2.9 μM HbO_2 in water plus 2% (w/v) Ampholine 4-6, LKB 1809-116, was used. This was added to a flat bed gel (100ml of 4% (w/v) Ultrodex, LKB 2117-510, plus 2% (w/v) of Ampholine 4-6, LKB 1809-116; volume and concentrations before standard pre-evaporation) via an LKB sample applicator temporarily located near the cathode. The anode electrode strip was soaked in 1.0 M H_3PO_4 and the cathode strip in 1.0 M NaOH. The gel was run at 4° C for 12 h with an ISCO 494 electrophoresis power supply set to the following limits: 8 W, 1600 V, 15 mA. At the completion of focusing the gel pattern was either photographed or drawn and Hb-containing bands were excavated, slurried with water, filtered to remove the gel and isoelectric points determined by pH measurement at 4° C, then spectra recorded from 700-250 nm at 20° C. Hemoglobin components were then concentrated by pressure filtration over Amicon YM10 membranes, stabilised by the addition of 50 mM phosphate buffer (pH 7.0) and stored frozen in liquid nitrogen for further use.

Analytical scale isoelectric focusing.

Heterogeneity of *Casuarina* hemoglobin was first demonstrated by preparative scale isoelectric focusing. This method was not appropriate for a more thorough investigation, however, because of the very limited amount of hemoglobin available. Analytical scale isoelectric focusing was therefore adopted because of its more conservative use of resources, and also because it offered much higher resolution. It was first used to further investigate possible artifactual causes of heterogeneity. It was also used to analyse fractions eluted from affinity chromatography columns (see

below), in a study of possible glycosylation of *Casuarina* hemoglobin.

Analytical scale isoelectric focusing was conducted using an LKB 2117 Ultrophor apparatus following the published procedures of Fuchsman and Appleby (1979). In a typical run dilute HbO_2 samples (2 ml) were first concentrated to approximately 45 µl using Amicon Centricon Microconcentrators (containing YM-10 membranes), diluted with Milli-Q deionised and distilled water, with the final concentration of hemoglobin being approximately 0.2 mM in water. The manufacturer's recommended procedures were followed for microconcentration.

In all runs, 20 µl samples, as HbO_2, were applied via filter wicks 4 cm from the cathode onto a 0.4 mm polyacrylamide gel (T=7.5%, C=3.0%) containing 3% (w/v) ampholite. This gel had been prepared and polymerised according to the manufacturer's instructions in an Ultromold apparatus (LKB 2217-100), except that urea was excluded from the gel formulation. The ampholite routinely used was Pharmalyte pH 4-6.5 (Pharmacia 17-0452-01). Such gels were run for 2-4 hours at 4° C with the following limits set on the ISCO power supply; 15 W, 2000 V, 30 mA. The anode strip was soaked in 1 M H_3PO_4 and the cathode strip in 1 M NaOH.

When focusing was complete the gel was photographed using a Nikon FM 2 single lens reflex camera fitted with a 55 mm f 2.8 Micro-NIKKOR lens, using transmitted light and Kodacolor VR 100 *iso* (Kodak) colour print film. The gel was then placed in fixative (35% (v/v) methanol, 10% (v/v) trichloroacetic acid, 3.5% (v/v) sulphosalicylic acid) for more than 15 min. and then stained for heme and protein following the method of Thomas *et al.* (1976). This uses 3-3!,5-5! tetramethyl benzidine for heme recognition and Comassie Blue R-250 for protein. The complete fixation and staining schedules are reproduced in Table 4.1 . After staining, the gel was re-photographed.

TABLE 4.1: PROTEIN AND HEME STAINING SCHEDULE FOR PLANT HEMOGLOBIN SAMPLES ON ANALYTICAL SCALE ISOELECTRIC FOCUSING GELS.

Process	Time
(i) fix gel in 35% methanol, 10% trichloroacetic acid, 3.5% sulphosalicylic acid	15 min
(ii) replace fixative with benzidine stain (place on shaker in the dark): 60ml 6.3mM TMBZ (made fresh in methanol) 140 ml 0.25 M Na Acetate, pH 5.0 (adjusted with glacial acetic acid)	2 hr
(iii) add H_2O_2 to stain to a final concentration of 30 mM (replace on shaker)	10-15 min
(iv) once bands sufficiently visible, place gel in wash 3 : 7 isopropanol : 0.25 M Na Acetate pH 5.0	indefinite
(v) wash in 70 mM sodium sulphite (to remove heme stain)	3 x 10 min
(vi) rinse in 30 % (v/v) isopropanol (to remove $NaSO_3$)	3 x brief
(vii) flood gel with Coomassie Blue 450 ml ethanol, 90 ml acetic acid, 2.5g Coomassie Blue R-250 (dissolved in the ethanol o'night), 450 ml H_2O	10 min
(viii) transfer into destain (90 ml acetic acid, 450 ml ethanol, 450 ml water) until optimum resolution obtained	variable
(ix) photograph	
(x) preserve in 1 % glycerol, 35 % ethanol, 10 % acetic acid	30 min
(xii) dry on glass plate in fume hood	o'night

Variations. The gel composition and sample application pattern were varied in three trials to test possible causes of the microheterogeneity observed during standard runs (Results and Discussion). The variation applied in each trial is outlined below.

1. The routinely used Pharmalyte 4-6.5 was replaced by blended Ampholine 4-6.5 (LKB 1809-116).
2. Pharmalyte 4-6.5 was replaced by Ampholine pH 3.5-10 (LKB).
3. Aliqouts (20 µl) of the same sample were applied to five tracks at 8 cm, 6 cm, 5.5 cm (two tracks) and 3.5 cm from the cathode.

Ammonium sulphate fractionation.

18.6 ml of 5 mM hemoglobin in 50 mM phosphate buffer, pH 7.3, was subjected to ammonium sulphate fractionation following chromatography on Sephacryl S200 and concentration over an Amicon YM10 membrane. Ammonium sulphate was added from 30% to 70% saturation in 10% increments (Green and Hughes 1955). After each addition the solution was centrifuged at 20,000 rpm for 10 min at 4° C and the spectrum of the supernatant recorded. The supernatant was then decanted for the next stepwise addition of ammonium sulphate. The hemoglobin precipitates from each step were redissolved in a minimum volume of buffer, pooled and frozen in liquid nitrogen.

Spectrophotometry and preparation of standard spectra.

Spectra were recorded from 700-250 nm or lesser wavelength interval with a Hitachi PE557 microprocessor-controlled spectrophotometer normally set at 2 nm slit width and 60 nm/min scanning speed, using 0.6-1.0 ml samples in stoppered 10 mm light path semi-micro black-sided quartz cuvettes. The spectrophotometer was connected via a serial interface to a PDP 11-03 computer and Hewlett Packard 7221 B four-pen plotter. In this way selected spectra could be replotted after subtraction, adjustment to standard concentration or other manipulation.

Oxyhemoglobin. In aerobic 50 mM potassium phosphate, 0.1 mM EDTA pH 7.2 or other pH>7.0 buffers (preferably containing EDTA) the part

purified HbCO from Sephacryl S200 or Sephadex G75 chromatography was slowly but quantitatively converted to HbO_2. This occurred during standing in air at 0-4° C, or subsequent aerobic purification procedures (see Results and Discussion). Alternatively, pure HbO_2 could be regenerated from ferric hemoglobin by the procedures used for *Parasponia* HbO_2 (Appleby et al. 1983a) or legume nodule LbO_2 production (Appleby et al. 1983b), involving anaerobic dithionite reduction and passage through aerated buffer in small Sephadex G25 columns.

Ferric hemoglobin. HbO_2 solutions in 50 mM potassium phosphate, pH 7.2 were oxidised by mixing with a five-fold molar excess of powdered potassium ferricyanide. After 5 min at 0-4° C the resulting ferric hemoglobin was separated from ferricyanide and other low molecular weight impurities by passage through Sephadex G25, medium grade, then centrifuged for 10 min at 10,000 xg before storage at 0° C or in liquid nitrogen.

Ferrous hemoglobin: All other *Casuarina* hemoglobin valence states and ligand complexes were stable when stored frozen in liquid nitrogen but ferrous hemoglobin was not, and was prepared only as required for procedures such as spectrophotometry. Typically, 8 µl of 0.1 M sodium dithionite in anaerobic 0.02 M NaOH was added under argon to 0.7 ml of 11 µM HbO_2 or ferric hemoglobin being gently stirred at 0-4° C then transferred to and sealed under argon in spectrophotometer cuvettes.

Carboxy ferrous hemoglobin: Freshly-prepared ferrous hemoglobin (preferably), or HbO_2, was transferred to a CO-equilibrated spectrophotometer cuvette and gently equilibrated with CO by 5 min. gas flow into the head space, with occasional stoppering and inversion of the cuvette, or by gentle CO bubbling into the solution.

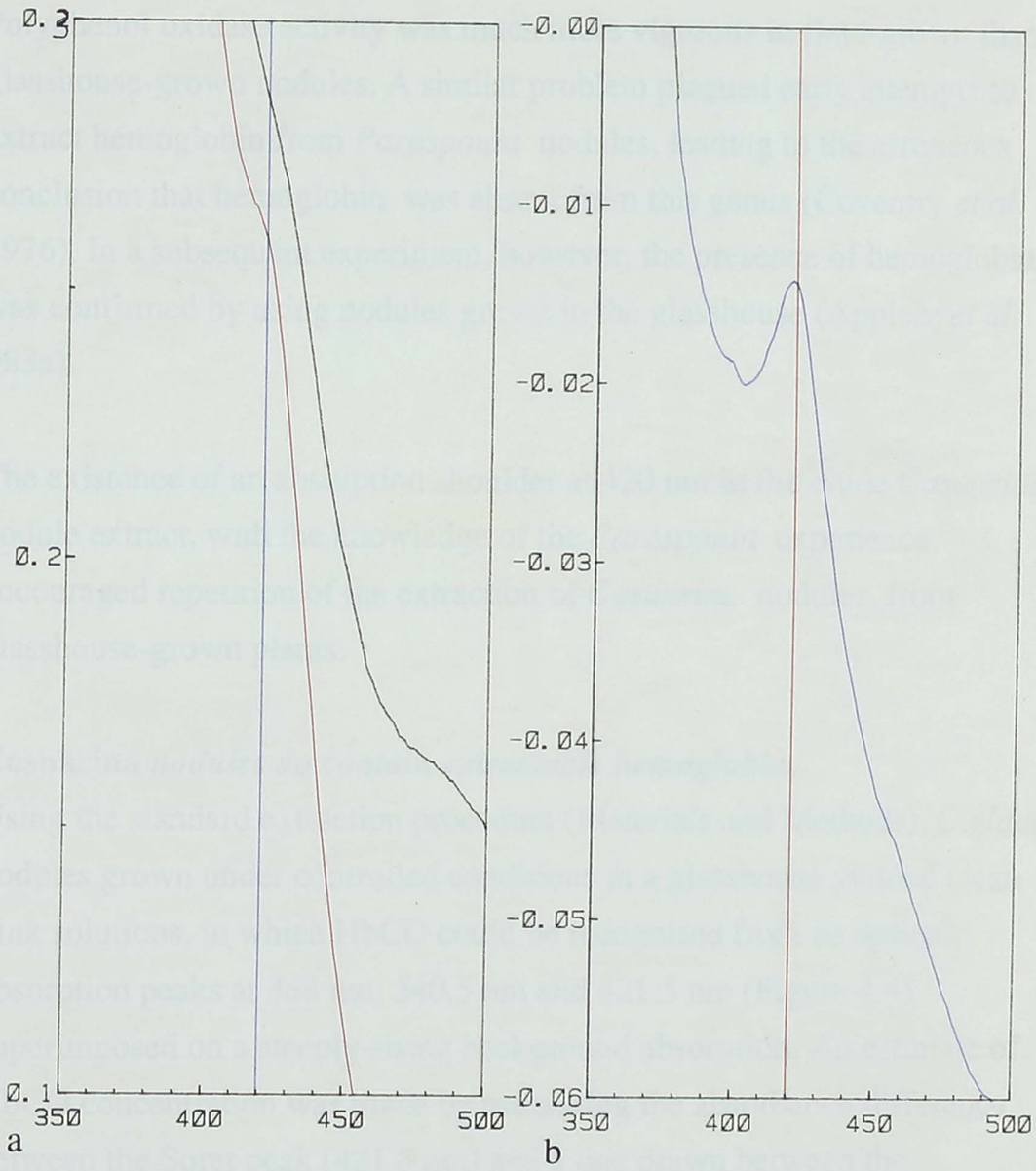
Calculation of hemoglobin concentration. Hemoglobin concentration was determined using a modification of the pyridine hemochrome procedure (Ohlsson and Paul 1976). Equal volumes (300 µl) of a 0.2 M NaOH/4.2 M pyridine mixture and hemoglobin species other than HbCO were mixed in an argon-flushed stoppered cuvette. This mixture was reduced under argon with 8 µl of 0.1 M sodium dithionite (Fluka) dissolved in argon-flushed 0.02 M NaOH. The spectrum was recorded from 700-500 nm against a reagent blank. Assuming ΔE mM=24.5 for the pyridine proto hemochrome alpha peak(556 nm) minus 539 nm trough (Ohlsson and Paul 1976), the concentration of hemoglobin was thereby determined.

Results and Discussion

Field-grown nodules failed to yield hemoglobin, but gave an indication of its presence.

Using the standard extraction procedure (Materials and Methods) with field-grown nodules, a clear brown supernatant was obtained following centrifugation, but no hemoglobin was detected by spectrophotometric examination . When this solution was chromatographed on Sephacryl S200 it spread to form a large, brown zone, with no distinct pink band of HbCO being evident. To check for the possible existence of hemoglobin, the spectrum of the most strongly coloured effluent fraction was recorded from 500 - 250 nm, after re-equilibration with CO. There was an absorption shoulder at approximately 420 nm, superimposed on a steeply rising background (Figure 4.3). Because of the possibility that some oxidation of the sample may have occurred, it was next reduced with sodium dithionite and reequilibrated with CO. The spectra of this sample showed a more pronounced shoulder at 420 nm. When the second spectrum was subtracted from the first, the resultant difference spectrum showed a distinct peak at about 420 nm (Figure 4.3), which is near the characteristic position for the Soret peak of hemoglobin CO complexes. While this peak was very small and could also indicate the presence of the CO complex of ferrous peroxidase, it suggested that hemoglobin might be

Figure 4.3: Ultra-violet absorption spectra of a crude extract of field grown nodule material from *Casuarina cunninghamiana*
(a) the extract equilibrated with CO (black line) and CO plus dithionite (red line)
(b) the difference spectrum of CO plus dithionite - CO, showing a slight peak at 420 nm, the expected position of the soret peak of hemoglobin.
This was the first indication that hemoglobin may be present in *Casuarina* nodules.


0.3
0.2
0.1
350
400
450
500
a
-0.00
-0.01
-0.02
-0.03
-0.04
-0.05
-0.06
350
400
450
500
b
Wavelength (nm)

present in the nodule extract. Similar spectra were obtained from the samples which eluted immediately before and after the peak fraction.

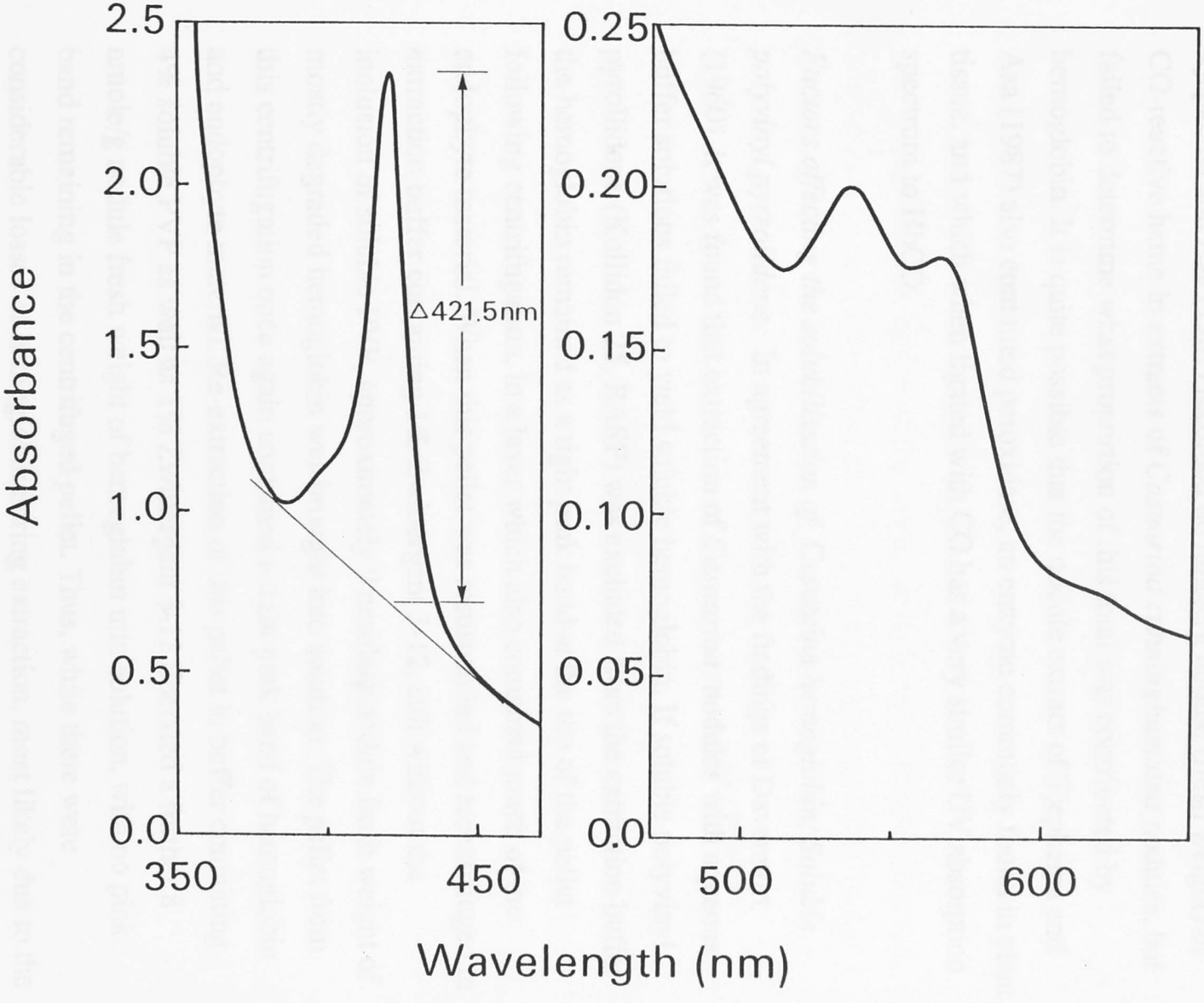
The failure to extract a significant amount of hemoglobin into solution may have been due to the products of polyphenol oxidase activity (Davenport 1960) masking, denaturing or polymerizing the hemoglobin. Polyphenol oxidase activity was much more vigorous in field-grown than glasshouse-grown nodules. A similar problem plagued early attempts to extract hemoglobin from *Parasponia* nodules, leading to the erroneous conclusion that hemoglobin was absent from this genus (Coventry *et al.* 1976). In a subsequent experiment, however, the presence of hemoglobin was confirmed by using nodules grown in the glasshouse (Appleby*et al.* 1983a).

The existence of an absorption shoulder at 420 nm in the crude *Casuarina* nodule extract, with the knowledge of the *Parasponia* experience, encouraged repetition of the extraction of *Casuarina* nodules, from glasshouse-grown plants.

Casuarina *nodules do contain extractable hemoglobin.*

Using the standard extraction procedure (Materials and Methods), *C.glauca* nodules grown under controlled conditions in a glasshouse yielded clean pink solutions, in which HbCO could be recognised from its optical absorption peaks at 568 nm, 540.5 nm and 421.5 nm (Figure 4.4) superimposed on a steeply-rising background absorption. An estimate of HbCO concentration was made by measuring the absorbance difference between the Soret peak (421.5 nm) and a line drawn between the inflections either side of the peak (Figure 4.4), assuming ΔE mM=120 as found for similar crude extracts of *Parasponia* HbCO (C. A. Appleby, personal communication). In this way the yield of soluble HbCO was calculated as ~32 nmole/g nodule fresh weight. This is lower than total, 'bound' hemoglobin (83 nmole/g nodule fresh weight) reported by Davenport (1960) and lower than the yield of soluble HbCO (45-70 nmole/g fresh weight) reported from *Parasponia* nodules (Appleby *et al.*

Figure 4.4: Absorption spectrum of a crude extract of HbCO from glasshouse-grown nodule material from *Casuarina cunninghamiana*. Concentration of hemoglobin is ~9 μM in 50 mM potassium phosphate, 1mM EDTA, pH 7.2 at 4° C.


Absorbance
2.5
2.0
1.5
1.0
0.5
0.0
△421.5nm
350
450
0.25
0.20
0.15
0.10
0.05
0.0
500
600
Wavelength (nm)

1983). This lower yield of soluble hemoglobin from *Casuarina* nodules may in part reflect the generally lower percentage of infected cells found in actinorhizal nodules than in *Rhizobium* -induced nodules (Tjepkema 1983), but may indicate also an incomplete solubilisation of the protein. Tjepkema and Asa (1987) recorded 80 nmole/g nodule (fresh weight) of CO-reactive heme in extracts of *Casuarina cunninghamiana* nodules, but failed to determine what proportion of this total was contributed by hemoglobin. It is quite possible that the nodule extract of Tjepkema and Asa (1987) also contained peroxidase, an enzyme commonly found in plant tissue, and which when ligated with CO has a very similar UV absorption spectrum to HbCO.

Factors affecting the solubilisation of Casuarina *hemoglobin: Soluble polyvinyl pyrrolidone:* In agreement with the findings of Davenport (1960), it was found that extraction of *Casuarina* nodules with aqueous buffer solutions failed to yield soluble hemoglobin. If soluble polyvinyl pyrollidone (Kollidon 25, BASF) was excluded from the extraction buffer the hemoglobin remained as a tight pink band at the top of the pellet following centrifugation, in a layer which also contained much of the endophyte material. When this pellet was resuspended and recentrifuged in extraction buffer containing 1% Zwittergent 3-12, still without the inclusion of soluble PVP, approximately 9 nmole/g nodule fresh weight of mostly degraded hemoglobin was brought into solution. The pellet from this centrifugation once again contained a tight pink band of hemoglobin and endophyte material. Re-extraction of this pellet in buffer containing 4% soluble PVP as well as 1% Zwittergent 3-12 liberated a further 8 nmole/g nodule fresh weight of hemoglobin into solution, with no pink band remaining in the centrifuged pellet. Thus, while there were considerable losses of hemoglobin during extraction, most likely due to the extensive manipulations involved, soluble PVP was definitely involved in solubilisation of the protein. The reason for this is unclear, although the possibility exists that in the absence of soluble PVP the hemoglobin became (or remained) adsorbed to the hyphal endophyte. The requirement for soluble PVP in the extraction of *Parasponia* hemoglobin had been taken to

imply that it may be a membrane-bound protein (Appleby *et al.* 1983). PVP has been recorded by its manufacturer (Manufacturer's handbook) as being helpful in converting some substances into a soluble form, but they do not explain why this is so.

Apart from its possible function in displacement or prevention of hemoglobin adsorption to endophyte or host membranes, the use of soluble PVP as a polyphenyl adsorbing agent (C. A. Appleby, pers. comm.; Loomis 1973; Loomis and Battaile 1966), together with strict anaerobiosis with dithionite as an oxygen scavenger, and EDTA and CO as both polyphenol oxidase inhibitors and inhibitors of hemoglobin autoxidation, allowed the production of clear pink solutions containing HbCO. In the absence of one or more of these protective agents (and especially in air) brown, rapidly-darkening extracts, containing oxidised and otherwise-degraded hemoglobin were obtained.

Factors affecting solubilisation of Casuarina *hemoglobin: Detergent:* The role of detergent in the solubilisation of *Casuarina* hemoglobin is not clear. As outlined above, in the absence of soluble PVP, Zwittergent 3-12 was only able to solubilise hemoglobin when included in the extraction buffer at a concentration of 1.0% (w/v). This is in agreement with the work of Gonenne and Ernst (1978) who found that the sulfobetaines ("Zwittergents") with $12 \leq n \leq 18$ are most efficient in their micellar form; that is, when they exceed their critical micelle concentration. They quote as an example the integral membrane protein 5'-nucleotidase which was not released into solution by 0.1% (w/v) Sulfobetaine$_{n=12}$ (equivalent to Zwittergent 3-12, Critical Micelle Concentration 0.12%) but was solubilised and retained full activity when 1.0% (w/v) Sulfobetaine$_{n=12}$ was used. In contrast, *Casuarina* hemoglobin extracted with 1.0% Zwittergent 3-12 was largely degraded, though this may have been due to the experimental procedure employed.

Tjepkema and Asa (1987) have also found recently that CO-reactive

hemoproteins (which they assume to be hemoglobins) could be extracted from the nodules of five actinorhizal genera only when either 5% soluble PVP or detergent, routinely 1% Triton X-100, were included in the extraction buffer. If Tween 80 or CHAPS were substituted for Triton (presumably at the same concentration, though not stated) CO-reactive heme could still be extracted.

The requirement for a high concentration of detergent (in the absence of soluble PVP) to bring any *Casuarina* hemoglobin, albeit degraded, into solution suggests, as does the requirement for soluble PVP, that the hemoglobin may have been strongly attached to a membrane surface, either *in vivo* or as a result of the extraction procedure. There is no previous record of naturally occurring membrane-bound plant hemoglobins, and while it has been suggested that *Parasponia* hemoglobin may be membrane-bound (Appleby *et al.* 1983), immunogold labelling of *P. andersonii* nodule sections showed hemoglobin localised in the cytoplasm of young infected nodule cells (Trinick *et al.* 1989). Similarly, leghemoglobins are soluble proteins which occur in the host cytoplasm of infected legume nodule cells (Appleby 1984). It had been argued that leghemoglobin also occurs in the peribacteroid space (Bergersen and Appleby 1981) but the weight of evidence points against this (Trinick *et al.* 1989; Robertson *et al.* 1978; Verma and Long 1983; Robertson *et al.* 1984).

The type of detergent used made little difference to the extraction of *Casuarina* hemoglobin (Table 4.2), though as reported above the detergent had to be below the critical micelle concentration to avoid degradation. In all cases approximately 20-30 nmole/g nodule fresh weight of hemoglobin were recovered using the standard extraction procedure. The decision for routine use of 0.1% Zwittergent 3-12 in the extraction buffer, in contrast to Tjepkema and Asa (1987) who used other detergents at 1.0% concentration, was based upon its ability to maintain the hemoglobin in a soluble and undegraded form during Sephacryl S200 chromatography (see below).

TABLE 4.2: THE IMPACT OF INCLUDING DIFFERENT DETERGENTS IN THE EXTRACTION BUFFER ON THE RECOVERY OF HEMOGLOBIN FROM SEPHACRYL S200 CHROMATOGRAPHY.

All detergents were included at below their critical micelle concentration. The standard extraction procedure was followed in all cases. Detailed procedures are recorded in the Materials and Methods section.

Detergent	Kd	% recovery off S200
NONE	0.59	4
	1.11	23
CHAPS	0.56	5
	1.01	63
DODECYL MALTOSIDE	0.53	5
	1.10	64
ZWITTERGENT 3-12	0.59	7
	0.99	84

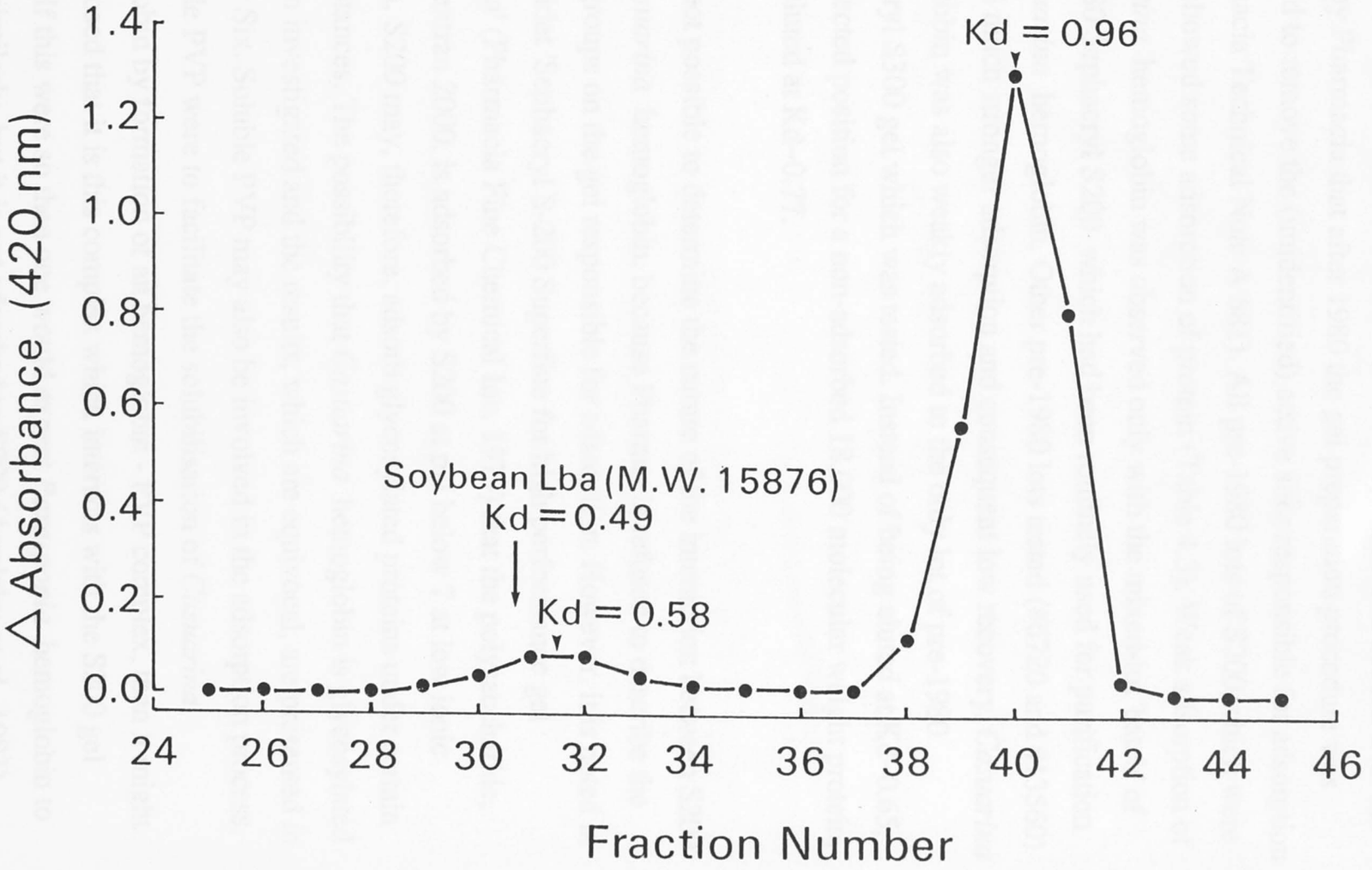
Sephacryl S200 "affinity chromatography".

Adsorption of hemoglobin to Sephacryl S200: Passage of crude extracts of *Casuarina* hemoglobin through a Sephacryl S200 column gave a most unexpected result (Figure 4.5). Although total recovery was usually 95-100%, only about 8% of the applied hemoglobin was eluted at Kd=0.58, the position expected for a monomeric protein of approximately 18,000 mol. wt (from amino acid sequence analysis; we now know that the molecular weight of *Casuarina* hemoglobin, including heme, is 17,856; see Chapter Five). Most *Casuarina* hemoglobin suffered weak adsorption, being eluted with a near symmetrical profile at Kd=0.96. It is important to note that the hemoglobin was not smeared throughout the column, as might be expected for non-specific adsorption. This adsorption phenomenon had not been observed during passage of any legume leghemoglobin (C.A.Appleby, unpublished observations) or of *Parasponia* hemoglobin (Appleby *et al.* 1983) through Sephacryl S200.

Sephacryl S200 is designed for molecular exclusion chromatography and was thus expected to show minimal gel-protein interaction. However, there are other reports of proteins adsorbing to this gel. Ward and Winzor (1982) found that Sephacryl S200 possessed considerable affinity for sites on rabbit muscle lactate dehydrogenase. Adsorption was much greater at pH 5.0 than at pH 7.4. Belew *et al.* (1978) found that at acidic pH (below pH 5.5) the gel acts as a non-specific adsorbent, and at pH 8.0 the gel behaves as a cation-exchanger, adsorbing only basic proteins. They found minimal solute-gel interactions at or near neutral pH. However, Csoma and Polgar (1984) found that mung bean proteinase was adsorbed to S200 at pH 6.5. In the present study also, *Casuarina* hemoglobin was adsorbed at near neutral pH (pH 7.2) Thus, while adsorption has been demonstrated for a number of proteins, a consistent pattern has not emerged.

In a series of trials, it was found that adsorption of *Casuarina* hemoglobin to S200 varied depending on the gel lot being used. This may explain the inconsistency of results found by others, as described above. When a 1985 gel lot was used, following the standard chromatography procedure

Figure 4.5: Chromatography of crude *Casuarina* HbCO on a column of Sephacryl S200 SF as described in Materials and Methods. Fractions were monitored at Δ420 nm absorbance; ie, the difference between the Soret peak (421 nm) and a line drawn between inflections on either side of the peak (Figure 4.4).


Kd = 0.96
Soybean Lba (M.W. 15876)
Kd = 0.49
Kd = 0.58
Δ Absorbance (420 nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
24
26
28
30
32
34
36
38
40
42
44
46
Fraction Number

(Materials and Methods), there was no adsorption of hemoglobin, with approximately 75% of the applied hemoglobin recovered as a single symmetrical peak around Kd=0.62 (Table 4.3). This is in accord with the claim by Pharmacia that after 1980 the gel preparation procedure was changed to remove the (unidentified) active sites responsible for adsorption (Pharmacia Technical Note A 681). All pre-1980 lots of S200 which were tested showed some adsorption of protein (Table 4.3). Weak adsorption of *Casuarina* hemoglobin was observed only with the mixed-lot 'batch' of pre-1980 Sephacryl S200 which had been routinely used for purification of *Casuarina* hemoglobin. Other pre-1980 lots tested (#6720 and #13560) showed much stronger adsorption and consequent low recovery. *Casuarina* hemoglobin was also weakly adsorbed to the only lot of pre-1980 Sephacryl S300 gel which was tested. Instead of being eluted at Kd~0.65, the expected position for a non-adsorbed 18,000 molecular weight protein, it was eluted at Kd~0.77.

It was not possible to determine the nature of the interaction between S200 and *Casuarina* hemoglobin, because Pharmacia refused to describe the active groups on the gel responsible for adsorption. However, it is noted in the booklet 'Sephacryl S-200 Superfine for high performance gel filtration' (Pharmacia Fine Chemical Inc. 1979) that the polysaccharide, Blue Dextran 2000, is adsorbed by S200 at pH below 7 at low ionic strength. S200 may, therefore, adsorb glycosylated proteins under certain circumstances. The possibility that *Casuarina* hemoglobin is glycosylated has been investigated and the results, which are equivocal, are presented in Chapter Six. Soluble PVP may also be involved in the adsorption process. If soluble PVP were to facilitate the solubilisation of *Casuarina* hemoglobin by formation of an hemoglobin - PVP complex, then it might be supposed that it is this complex which interacts with the S200 gel matrix. If this were so then one would expect *Parasponia* hemoglobin to behave similarly, but it is not adsorbed to S200 (Appleby *et al.* 1983). Unfortunately, there was insufficient material available to investigate the possibility of a *Casuarina* Hb-soluble PVP-S200 interaction.

TABLE 4.3: RESULTS OF 'SEPHACRYL' S200 BATCH TRIALS SHOWING THE ELUTION POSITION OF PEAK 412 NM ABSORBING FRACTIONS, AND THE % RECOVERY OF APPLIED HEMOGLOBIN. PRE- AND POST-1980 GEL LOTS WERE MADE TO DIFFERENT FORMULAE.
* The single mixed gel used in standard extraction procedure.

Gel	Date of Manufacture	Kd of peak	% recovery of hemoglobin
S200 lot # ?	post-1980	0.62	75
S200 lot # ?	post-1980	0.6	65
S300 lot # 1128820	pre-1980	0.77	85
S200 lot # 6720	pre-1980	0.6	7.2
		1.25	8.4
S200 lot # 13560	pre-1980	0.6	4.8
S200 mixed lots*		0.53	11.8
	pre-1980	0.91	79
As above		0.58	8
	pre-1980	0.96	90
As above		0.52	7.7
	pre-1980	0.86	92
As above		0.52	5.9
	pre-1980	0.86	73
As above		0.52	10
	pre-1980	0.86	73
As above		0.59	6.7
	pre-1980	0.99	83.6
As above		0.54	6.6
	pre-1980	0.89	73.9

While the nature of the weak interaction between pre-1980 Sephacryl S200 and *Casuarina* hemoglobin was not understood, it was exploited in the purification of this protein. The anomolous elution position of *Casuarina* hemoglobin (Figure 4.5), with consequent near-complete separation from other proteins including polyphenol oxidase, meant that the HbCO, after emerging from Sephacryl S200, was remarkably resistant to autoxidation. When exposed to air such fractions underwent a slow but complete transformation to HbO_2. Also, since the hemoglobin was eluted from S200 with much-smaller molecules as principal remaining impurities, very significant further purification was obtained by pressure filtration through Amicon YM10 (10,000 mol. wt. exclusion) membranes, as shown by the increased ratio of HbCO Soret peak to ultraviolet absorption peak (Figure 4.6). Such a purification procedure was not possible using post - 1980 S200 because it failed to adsorb the protein.

The cleaning of pre-1980 Sephacryl S200 with non-ionic detergent or 0.2 M NaOH did not destroy its adsorptive capacity, nor did long term storage in 0.02% (w/v) sodium azide. Thus, the "best" mixed batch of pre-1980 Sephacryl S200 was repeatedly cleaned and used to purify all crude*Casuarina* hemoglobin extracts made during this study.

Detergent is necessary for maximum recovery from S200. It has been shown above that the presence of either non-ionic or zwitterionic detergent at low concentration (0.1%) in the extraction buffer did not significantly increase the yield of soluble hemoglobin in crude extracts; in fact, detergents at this concentration, without soluble polyvinyl pyrrolidone, completely failed to solubilise hemoglobin. Inclusion of detergent did, however, dramatically improve the subsequent recovery of hemoglobin from Sephacryl S200. Results of the detergent trials can be seen in Table 4.2. With all of these detergents, which were used at less than critical micelle concentration, hemoglobin showed no signs of degradation during chromatography. In the absence of detergent there was only a 23% recovery of applied hemoglobin in the major, adsorbed peak. The best

recovery, 84% of *C. cunninghamiana* hemoglobin from S200, was recorded when 3mM Zwittergent 3-12 was included in the extraction buffer. This detergent was then used routinely in extraction of *C. glauca* hemoglobin, where 95 - 100% recovery of undegraded hemoglobin was obtained during subsequent S200 chromatography.

Perhaps, the role of Zwittergent in chromatography of hemoglobin on S200 concerns maintaining the protein as a soluble unpolymerized molecule, yet without masking the groups responsible for the adsorption phenomenon. This, again, raises questions concerning the nature of *Casuarina* hemoglobin, particularily because neither leghemoglobin nor *Parasponia* hemoglobin requires detergent to ensure good recovery from S200 (C.A.Appleby, pers. comm.). The adsorption of *Casuarina* hemoglobin to S200 is further discussed in Chapter Six.

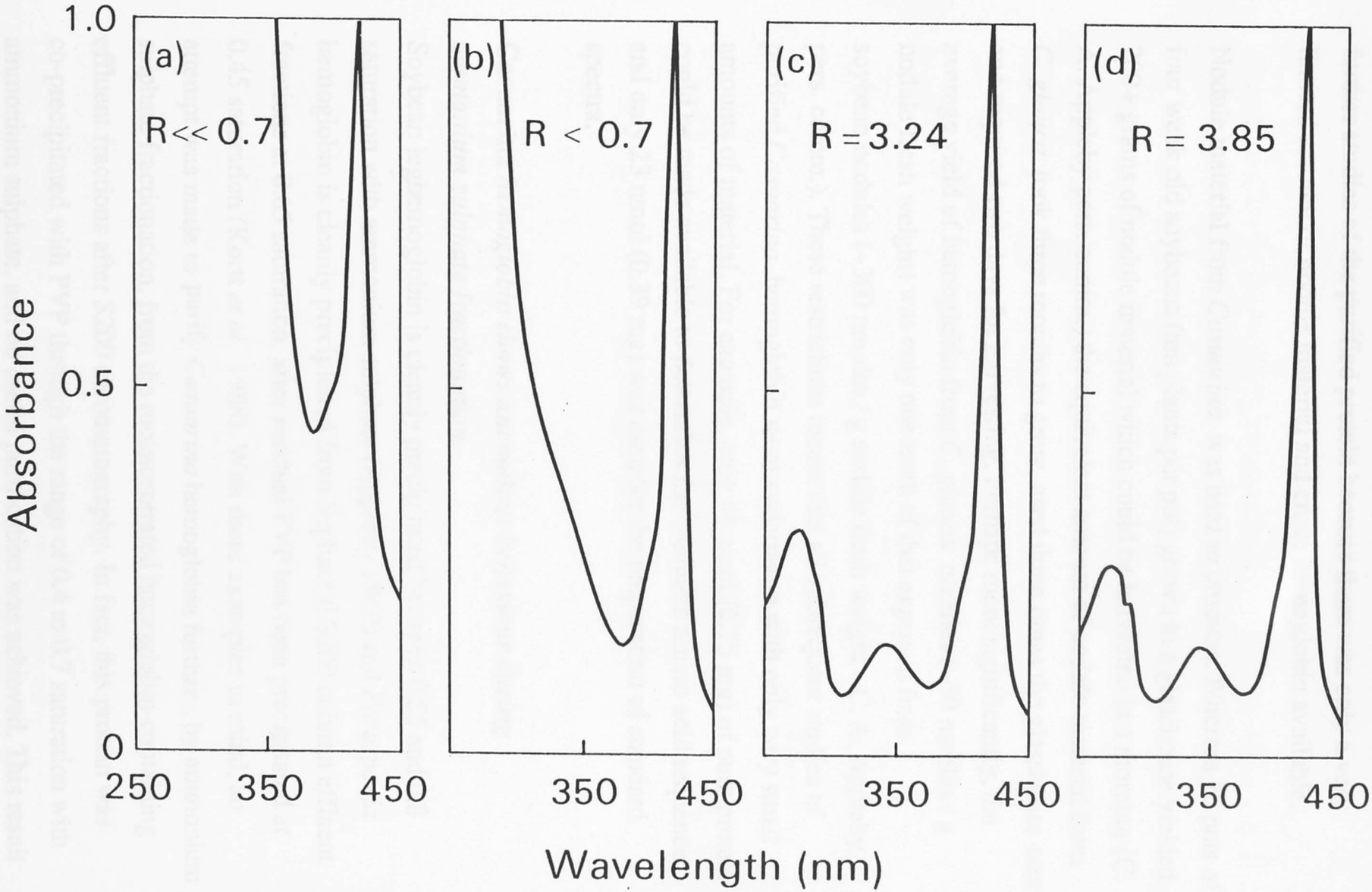
Sephadex G75 molecular exclusion chromatography.

When it was desired to study the properties of the mixed components of *Casuarina* hemoglobin, rather than proceed to final separation by IEF, the product from Sephacryl S200 chromatography and Amicon YM10 pressure filtration was passed through a column of Sephadex G75. In contrast to its behaviour on Sephacryl S200, *Casuarina* hemoglobin was eluted with Kd=0.4 (the expected position for a monomeric hemoglobin with mol. wt. 17000 - 18000) and its optical absorption properties, with the ratio R 420nm : 280nm abs. = 3.85, suggested that it was as free from other proteins or pigments as were the IEF-separated hemoglobin subcomponents (R=3.16 for IEF component I).

Simple 3-step purification.

Pure *Casuarina* hemoglobin was thus obtained by a simple, 3-step purification procedure of Sephacryl S200 "affinity" chromatography, Amicon YM-10 concentration, and Sephadex G75 molecular exclusion chromatography (Figure 4.6; see also Figure 4.2 for outline of the procedure). The significance of this purification process cannot be understated. Reducing the number of steps involved in purification, and

Figure 4.6: Ultra-violet absorption spectra of *Casuarina* HbCO following (a) extraction and centrifugation, (b) Sephacryl S200 chromatography, (c) pressure filtration over an Amicon YM10 (10,000 mol. wt. exclusion) membrane, and (d) Sephadex G75 chromatography. All samples were in 50 mM potassium phosphate, 1mM EDTA, pH 7.2 at 4°C. Techniques of sample preparation are given in Materials and Methods. Spectra were recorded at 20°C and are replotted to give a 420 nm (hemoglobin soret peak) absorbance of 1.0 in each case. The ratio (R) of hemoglobin soret peak (420 nm) to general protein peak (280 nm) is included for all plots.


1.0
0.5
0
Absorbance
(a)
R<< 0.7
(b)
R < 0.7
(c)
R = 3.24
(d)
R = 3.85
250
350
450
350
450
350
450
350
450
Wavelength (nm)

therefore reducing the scope for losses during manipulation, was critical for further studies of the purified protein because there was only a very limited amount of nodule material and crude hemoglobin available.

Nodule material from *Casuarina* was hard to produce. Whereas 20 pots of four week old soybeans (ten plants per pot) grown in a glasshouse yielded 200+ grams of nodule material which could be harvested in a morning (C. A. Appleby pers. comm.) the equivalent amount of nodule material from *C. glauca* took three months to grow, used three times the glasshouse area and required two days for harvesting. Perhaps more significantly, the average yield of hemoglobin from *C. glauca* nodules (~30 nmoles / g nodule fresh weight) was only one tenth of that expected from soybean nodules (~300 nmoles / g nodule fresh weight - C. A. Appleby, pers. comm.). These restrictions meant that all subsequent studies of purified *Casuarina* hemoglobin were undertaken with only very small amounts of material. For example, only 44 nmol (0.75 mg) of pure protein could be made available to determine the complete amino acid sequence, and only 23 nmol (0.39 mg) was used for the preparation of standard spectra.

Casuarina *hemoglobin shows anomalous behaviour during ammonium sulphate fractionation.*

Soybean leghemoglobin is cleanly precipitated between 0.55 and 0.8 saturation with ammonium sulphate (Appleby 1969) and *Parasponia* hemoglobin is cleanly precipitated from Sephacryl S200 column effluent fractions at 0.65 saturation, after residual PVP has been precipitated at 0.45 saturation (Kortt *et al.* 1988). With these examples in mind, an attempt was made to purify *Casuarina* hemoglobin further, by ammonium sulphate fractionation, from the reconcentrated hemoglobin-containing effluent fractions after S200 chromatography. In fact, this protein was co-precipitated with PVP through the range of 0.4 to 0.7 saturation with ammonium sulphate, and no useful purification was achieved. This result gives support to the concept of a *Casuarina* hemoglobin-soluble PVP complex (see above).

Separation of* Casuarina *hemoglobin into multiple components by isoelectric focusing.

The preparative scale isoelectric focusing (IEF) of *Casuarina* HbO_2, already purified by Sephacryl S200 chromatography and Amicon YM10 pressure filtration, resulted in the separation of three major red coloured components with reproducible isoelectric points (Table 4.4, Figure 4.7), a large number of minor components which varied from run to run, and a general background smear of red colour. The three major components, and the many minor components all appeared to be HbO_2 as eluted; they were not oxidised or otherwise degraded species. Notably, even though the oxygenated *Casuarina* hemoglobin appears to be the most stable plant hemoglobin oxygen complex during other preparative or manipulative procedures (C. A. Appleby, personal communication), there was only 20% to 27% recovery of oxyhemoglobin in the three major components (Table 4.4) during IEF. This is in marked contrast with the high yields of major component(s) after preparative scale IEF of soybean ferric leghemoglobin nicotinate (Fuchsman and Appleby 1979) or of *Parasponia* oxyhemoglobin (Appleby *et al.* 1983). Recent experiments with *Parasponia* oxyhemoglobin under identical conditions yielded 80 - 85% recovery (C.A. Appleby, unpublished observations). Included for comparison in Table 4.4 are the results for *C. cunninghamiana* hemoglobin, which are very similar to those obtained for *C. glauca*. There were three major components and very many minor ones, with the total recovery of applied hemoglobin in the three major bands again being very low (27%). The stability of the major components of both *C. cunninghamiana* and *C. glauca* oxyhemoglobins at their isoelectric points argues against the many minor components (also oxy hemoglobins) being artifacts of the experimental procedure.

Despite their low combined yield, the three major components of *Casuarina* hemoglobin were of high purity with reproducible isoelectric points (Table 4.4). All showed spectra typical of HbO_2 as eluted, and could be shown to undergo reversible ligand exchange between oxygen and CO.

TABLE 4.4: PREPARATIVE SCALE ISOELECTRIC FOCUSING OF 2.9 uM *Casuarina* HbO_2 AFTER SEPHACRYL S200 CHROMATOGRAPHY AND PRESSURE FILTRATION OVER AN AMICON YM10 (10,000 MOL. WT EXCLUSION) MEMBRANE

Details of procedures are in Materials and Methods. Only the three major components are recorded.

(A) *Casuarina glauca* hemoglobin

	COMPONENT NUMBER		
	I	II	III
pI	5.92	5.57	5.32
% OF RECOVERED hemoglobin	62	18	20

TOTAL RECOVERY OF APPLIED hemoglobin = 20%

(B) *Casuarina cunninghamiana* hemoglobin

	COMPONENT NUMBER		
	I	II	III
pI	5.92	5.55	5.26
% OF RECOVERED hemoglobin	34	28	20

TOTAL RECOVERY OF APPLIED hemoglobin = 27%

This was also the case for the many minor bands and background smear. Hence, there is no reason to suppose that they represent anything other than undegraded hemoglobin. This result also rules out the possibility that the bands represented various valence states and ligand complexes, a problem which had caused misinterpretation of results on previous occasions (see, eg Fuchsman and Appleby 1979).

Microheterogeneity is not an artifact of isoelectric focusing. Analytical scale isoelectric focusing of *Casuarina* hemoglobin also revealed extensive microheterogeneity. Besides the three major components detectable by preparative scale isoelectric focusing, about thirty minor bands were visible after Coomassie blue protein staining (Figure 4.7), all of which also stained positively for heme (Figure 4.7). Given the purity of the applied sample as measured by spectrophotometry (no other hemoproteins detected), the observation of heme and protein staining suggested that all bands were hemoglobin.

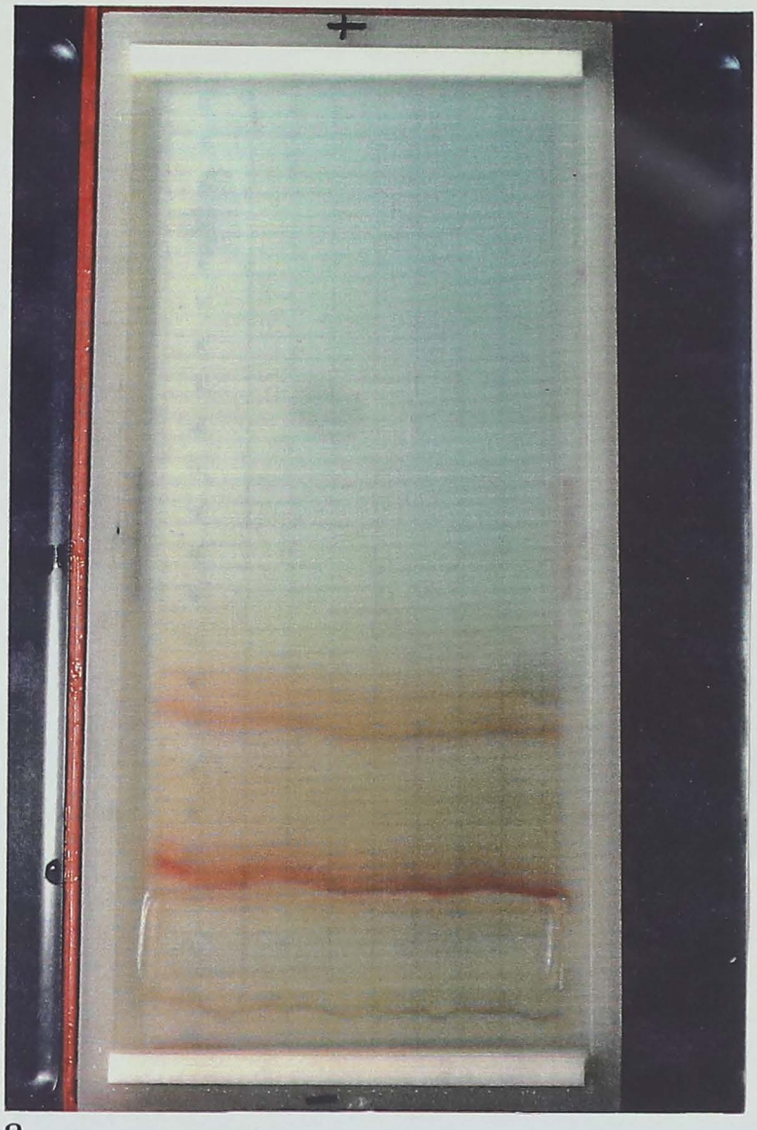
Notably,*Casuarina* hemoglobin was difficult to apply to analytical scale isoelectric focusing gels. Even though highly purified protein was used, it did not migrate cleanly out of the filter wicks used for loading protein onto the gel, unlike the other plant hemoglobins used. A proportion of the hemoglobin was retained on the wick, even if the wick was left on the gel for the entire focusing period. This is yet another indication that *Casuarina* hemoglobin has unique properties. However, this retarded release from the wick could not explain the extensive banding, because the same pattern was obtained no matter where the wick was placed on the gel (data not shown).

It has been suggested that a possible artifactual cause of heterogeneity on isoelectric focusing gels is binding of the protein to carrier ampholytes. However, Rhigetti (1984) and Williamson *et al.* (1973) both consider the evidence for this phenomenon to be scanty, prefering the view that the commonly observed heterogeneity of proteins on isoelectric focusing is an accurate reflection of the natural variation of protein molecules. In my study, interaction with specific ampholyte components as a cause of

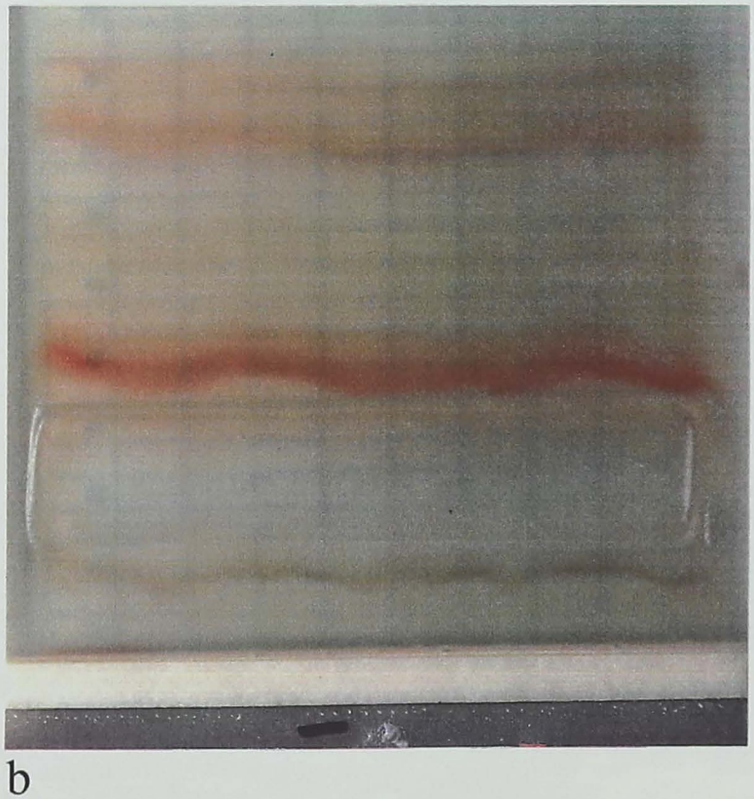
Figure 4.7: (A) Preparative scale isoelectric focusing (pH 4-6.5) of approx. 2.9 μM *Casuarina glauca* hemoglobin purified by Sephacryl S200 chromatography and Amicon YM-10 pressure filtration, showing the three major components visible without staining. Component I was used for most characterisation studies (Chapter 5).

(B) Analytical scale isoelectric focusing (pH 4-6.5) of approx. 12 μM HbO_2 stained for (a) protein with Coomassie Blue R250 and (b) heme with 3-3!, 5-5! tetramethyl benzidine. Track one is the hemoglobin mixture purified by the 3-step purification procedure. The short dark arrows indicate the major hemoglobin components, which stained positively for both protein and heme. Track three is a mixture of *C. glauca* hemoglobin components II and III previously separated by preparative scale isoelectric focusing.Track five is ovalbumin, which stained for protein but not heme (long arrow). Track six is *Parasponia* hemoglobin (open arrow) which stained for both protein and heme.

A

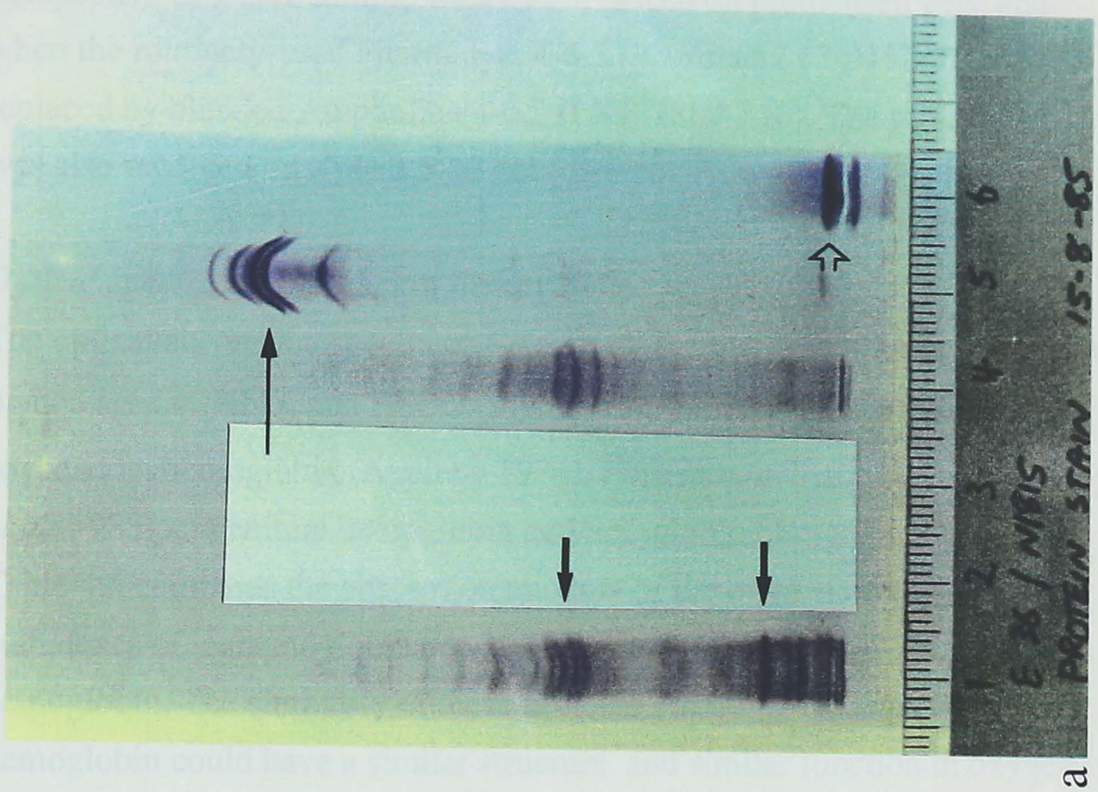


a

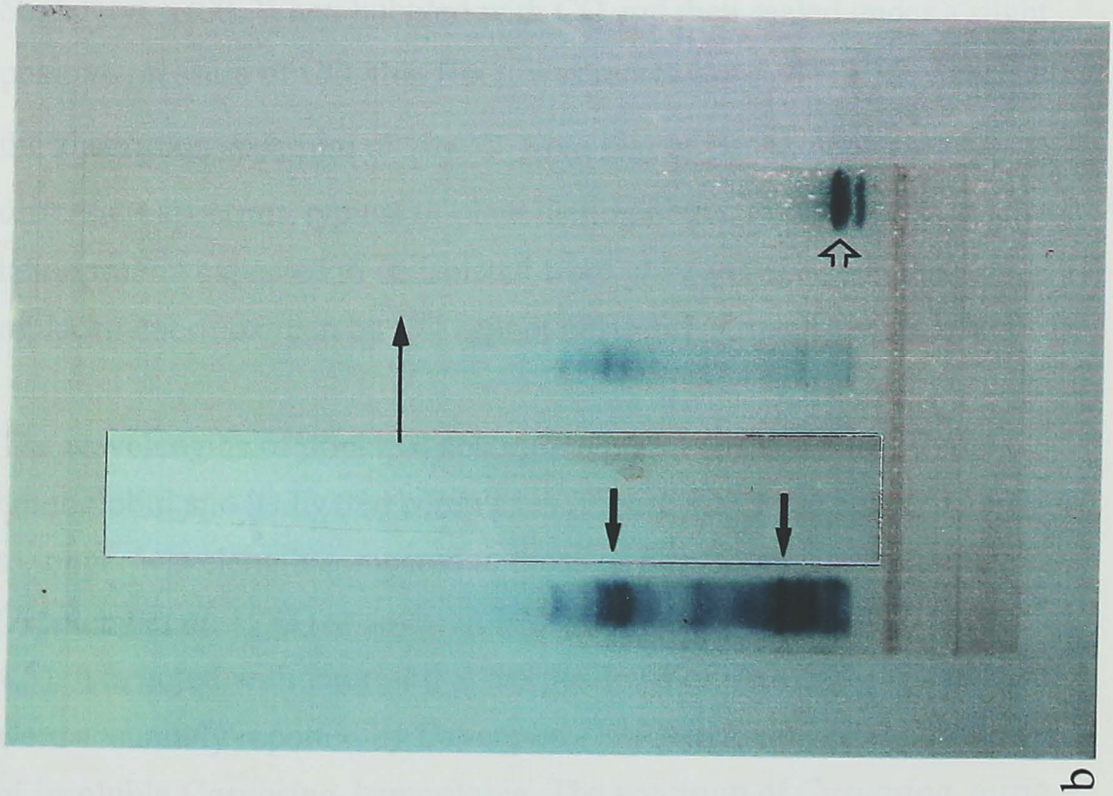


b

B



a



b

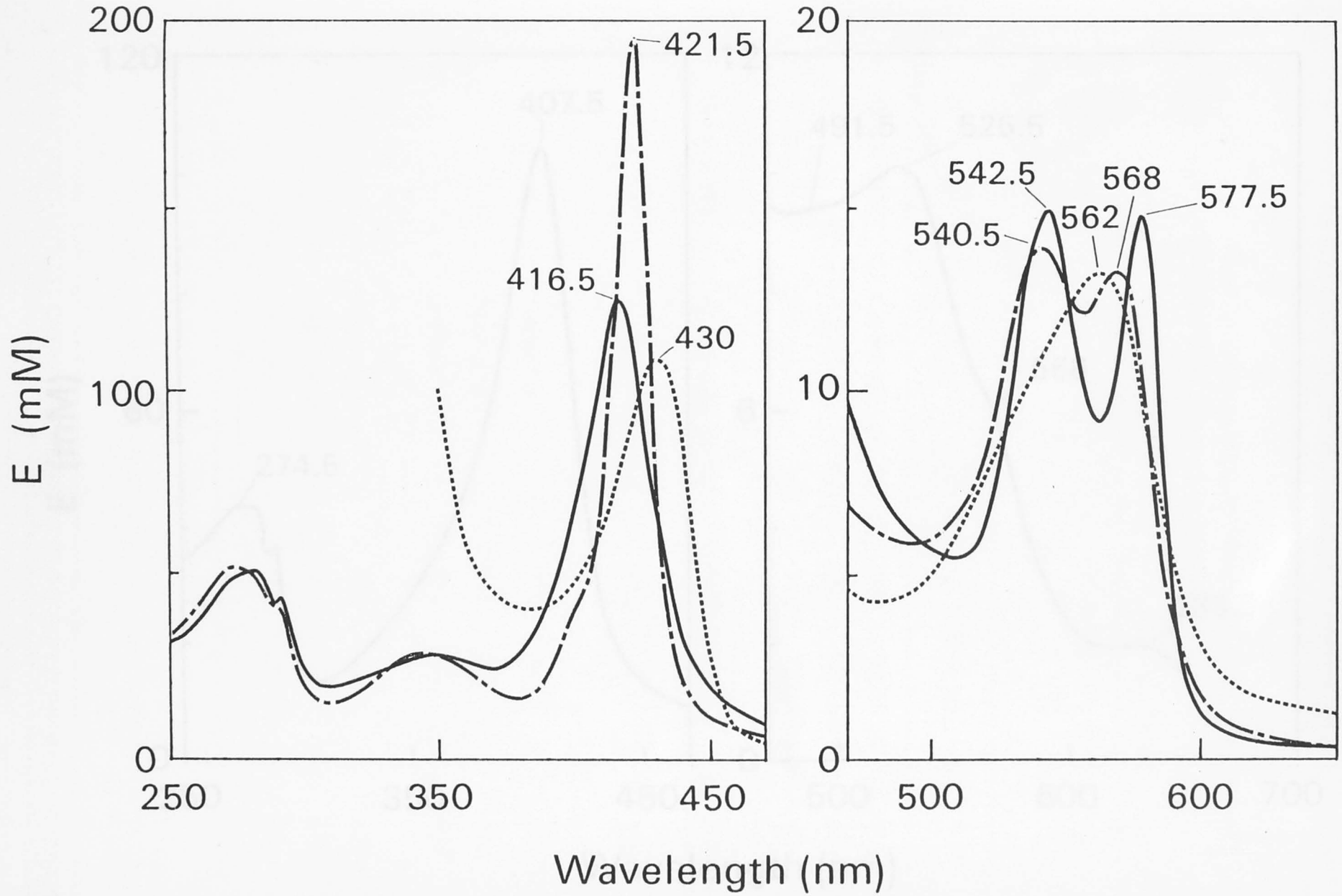
heterogeneity was ruled out by observation of the same banding pattern irrespective of where sample wicks were placed on prefocused gels, and when the routinely-used Pharmalyte 4-6.5 (Pharmacia 17-0452-01) was replaced by blended Ampholine 4-6.5 (LKB 1809-116). The same pattern was also recorded on a pH 3.5-10 gel (data not shown).

Optical spectra of* Casuarina *hemoglobin.

The optical absorption spectra of *Casuarina* ferrous hemoglobin and of the ligated species HbO_2 and HbCO (Figure 4.8), were similar to those of soybean leghemoglobin (Appleby 1974), *Parasponia* hemoglobin (Appleby *et al.* 1983) and animal hemoglobin and myoglobin (Antonini *et al.* 1965). Table 4.5 compares the absorption maxima of the oxygen and CO complexes of leghemoglobin, *Parasponia* hemoglobin and *Casuarina* hemoglobin. The similarity of these proteins allows that *Casuarina* hemoglobin could have a similar structure, and similar function in oxygen transport. This was supported by the observation of reversible ligand exchange between oxygen and CO. Hemoglobin, extracted from nodules as HbCO, was slowly converted to HbO_2 during chromatography on Sephacryl S200. When bubbled with CO and then sealed under a slight positive pressure of CO, this HbO_2 was reconverted to HbCO. Subtracting the absorption spectrum of HbCO from that of HbO_2, generated a difference spectrum typical of plant hemoglobins. In peroxidase, another hemoprotein expected to be isolated from plant extracts, simple replacement of oxygen by CO cannot occur (cf. Appleby *et al.* 1983).

The wavelengths of principal absorption peaks of *Casuarina* ferrous hemoglobin and its ligated complexes (Figure 4.8) were displaced 3-5 nm to longer wavelength compared to those of *Parasponia* hemoglobin (Appleby *et al.* 1983) or soybean leghemoglobin (Appleby 1974) (Table 4.5). It is noted with interest that the positions of these red-shifted peaks were accurately reported by Davenport (1960) in his original description of insoluble *Casuarina* hemoglobin. The spectrum of *Casuarina* ferric hemoglobin (Figure 4.8) is also of interest, insofar as the relative heights

Figure 4.8: (A) Absorption spectra of the pure mixed components of *Casuarina* ferrous hemoglobin (dotted line), HbO_2 (continuous line) and HbCO (dashed line). Sample concentrations were ~11 μM, in 50 mM potassium phosphate, 1mM EDTA, pH 7.2 at 4° C. All spectra were recorded at 20° C and are replotted at millimolar concentration. Positions of absorption maxima are shown on the figure.
(B) Absorption spectrum of the pure mixed components of *Casuarina* ferric hemoglobin. Experimental conditions as for Figure 4.8 a, except that EDTA was excluded from the buffer.


200
100
0
E (mM)
421.5
416.5
430
250
350
450
20
10
0
542.5
568
577.5
562
540.5
500
600
Wavelength (nm)

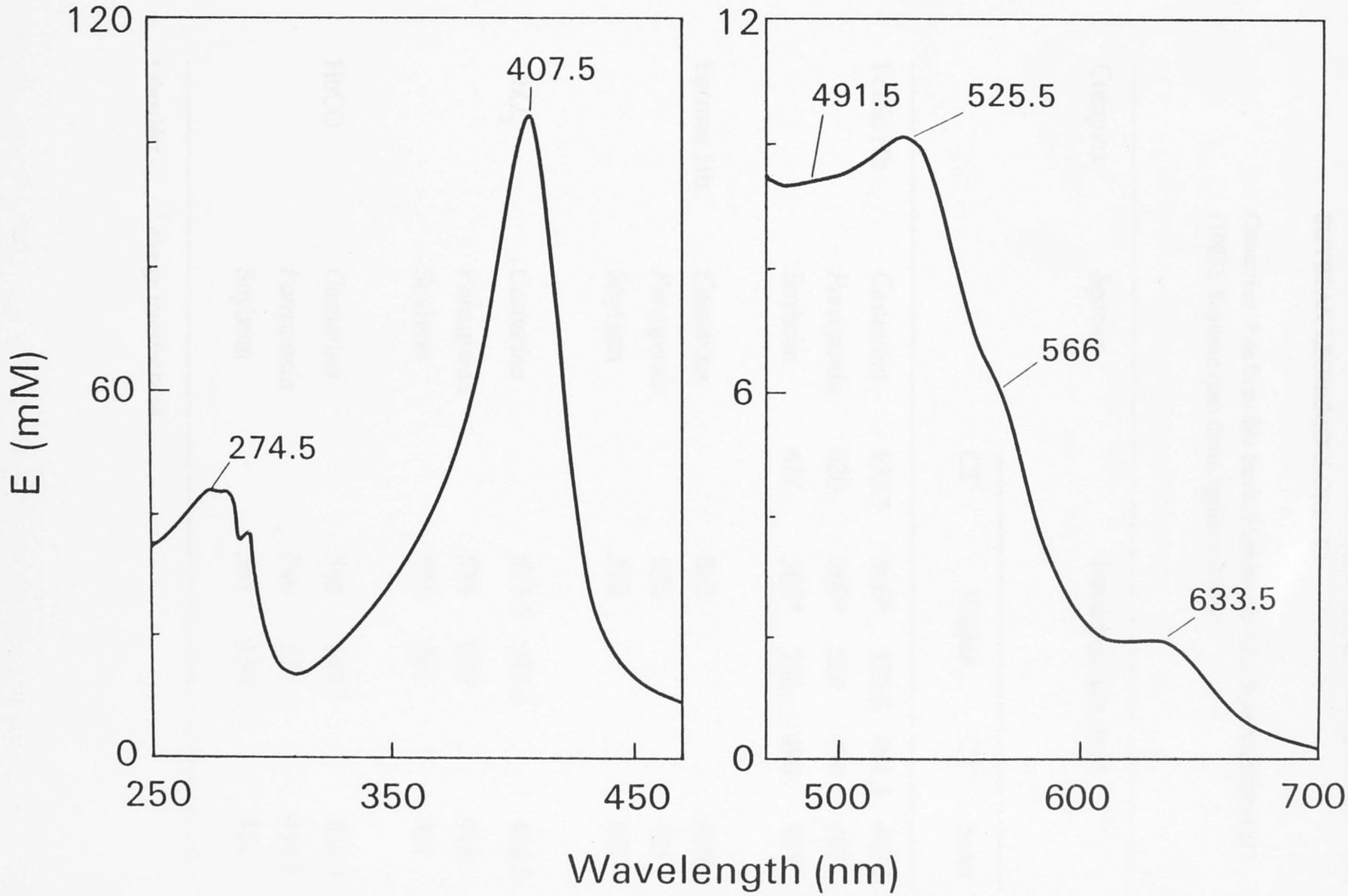

120
60
0
E (mM)
407.5
274.5
250
350
450
12
6
0
491.5
525.5
566
633.5
500
600
700
Wavelength (nm)

TABLE 4.5: OPTICAL SPECTRA OF PLANT HEMOGLOBINS. ABSORPTION MAXIMA OF UNLIGATED, OXYGEN AND CO COMPLEXES OF *CASUARINA* HEMOGLOBIN, *PARASPONIA* HEMOGLOBIN AND SOYBEAN LEGHEMOGLOBIN C.

Casuarina data from this thesis; *Parasponia* data from Appleby *et al* (1983); Soybean data from Appleby (1974).

Complex	Species	Band position (nm)				
		CT!	Visible		CT!	Soret
Ferric Hb	*Casuarina*	633.5	566*	525.5	491.5	407.5
	Parasponia	620	560*	529	484	407
	Soybean	627	565*	532	495	404
Ferrous Hb	*Casuarina*		562			430
	Parasponia		556			425
	Soybean		556			427
HbO_2	*Casuarina*		577.5	542.5		416.5
	Parasponia		574	539		410
	Soybean		574	541		411
HbCO	*Casuarina*		568	540.5		421.5
	Parasponia		564	536		416.5
	Soybean		567	538		421

* shoulder ! charge transfer band

of the ferric hemochrome bands at 525 and 560 nm, and charge transfer bands at 633 and 490 nm (Appleby *et al.* 1983) suggest that this ferric hemoglobin may be a thermal equilibrium mixture of "low spin" and "high spin" species also characteristic of *Parasponia* hemoglobin (Appleby *et al.* 1983) and of soybean leghemoglobin (Appleby *et al.* 1982). In soybean leghemoglobin, at least, this spin equilibrium mixture is associated with an extremely mobile distal histidine, which is thought to be a determinant of the high oxygen affinity of this class of hemoglobins (Appleby *et al.* 1982).

Chapter Five

THE RELATIONSHIP OF *CASUARINA* HEMOGLOBIN TO OTHER PLANT HEMOGLOBINS

In the introductory Chapter to this section of the thesis, a number of questions were raised concerning the possible occurrence and nature of hemoglobin in *Casuarina* nodules. The results presented in Chapter Four have confirmed its occurrence by the purification of a protein which has properties fundamental to any hemoglobin. These properties include its optical spectra, and most importantly its ability to reversibly bind with oxygen. This Chapter presents data which allows a more detailed comparison of *Casuarina* hemoglobin with other plant hemoglobins, from the perspectives of phylogeny and function.

First, I report the amino acid sequence of *Casuarina* hemoglobin I and compare it with the sequences of other plant and animal hemoglobins. Besides showing structural similarity in those parts of the molecule critical to oxygen binding, the overall sequence comparison indicates that the *Casuarina* , *Parasponia* , and legume hemoglobins are closer to each other, as a group, than they are to the animal hemoglobins. This suggests that all plant hemoglobins are inherited from a distant ancestor common to both plant and animals, rather than by repeated acts of horizontal gene transmission. The amino acid sequence of this symbiotic *Casuarina* hemoglobin is significantly different from the deduced amino acid sequence of a presumed non-symbiotic *Casuarina* hemoglobin gene isolated by the K. A. Marcker and W. J. Peacock laboratories (pers. comm.) using gene probes.

Second, I report the immunological relationships that exist between *Casuarina*, *Parasponia* and soybean hemoglobins using the 'western blot'

technique. Considerable cross-reactivity was observed, further supporting the hypothesis that all three proteins share a common origin. In particular, this result is strong evidence against the possibility that the amino acid sequence homology of these proteins is the result of convergent evolution. The results also suggest that *Casuarina* and *Parasponia* hemoglobins may be more closely related to each other than either is to soybean leghemoglobin.

Third, I report the kinetics of the reactions of *Casuarina* hemoglobin with oxygen and carbon monoxide. The reaction rates are very similar to those previously reported for other plant nodule hemoglobins. This suggests that *Casuarina*, *Parasponia* and legume hemoglobins may all perform a similar function in their respective root nodule symbioses;that is, the facilitation of oxygen diffusion to the endophyte. The tertiary structure of all plant hemoglobins is most likely strongly conserved, at least in the critical heme binding region of the protein, to allow this functional similarity.

Materials and Methods

General comments.

Some of the experiments in this section were performed by others at my request because they required specialised equipment not available to me in Canberra. The data obtained from these experiments were necessary for an analysis of the relationship of *Casuarina* hemoglobin to other hemoglobins, and also in a determination of its possible function in the nodule. The decisions to initiate these experiments were my own, and I was involved in frequent consultation during their planning, execution and interpretation. In all cases the experiments used hemoglobin which I had purified .

Determination of the amino acid sequence of* Casuarina *hemoglobin.

This experiment was conducted by Dr Alex Kortt from the Division of Protein Chemistry, C.S.I.R.O., Parkville, Victoria 3052.

* Replace the words 'pure hemoglobin mixture' with purified, unfractionated hemoglobin, throughout.

Casuarina glauca hemoglobin, hereafter referred to as *Casuarina* hemoglobin was isolated and purified as described in Chapter Four. Preliminary amino acid sequence information was determined with ~ 0.8 mg (54 nmole) of the pure *Casuarina* hemoglobin mixture* obtained after sequential Sephacryl S200 and Sephadex G75 chromatography. The complete amino acid sequence of the major component, hemoglobin I, obtained by isoelectric focusing, was determined with ~0.75 mg (44 nmole) of protein.

The hemoglobin I sample (0.75 mg) was dissolved in 0.2 ml of 0.1 M Tris-HCl, 2% SDS, buffer, pH 8.5, reduced with dithiothreitol (0.01 M) at 50° C for 2 h under N_2 and alkylated with iodoacetic acid (0.04 M) for 1 h at 25° C in the dark. The reaction was stopped by the addition of an excess of dithiothreitol and dried under vacuum at 50° C. The reaction mixture was redissolved in 0.1 ml distilled water and the protein precipitated with 0.9 ml cold methanol, washed once with 1.0 ml of cold methanol and dried under N_2. The complete procedure was carried out in a 1.5 ml Eppendorf tube.

The *S*-carboxymethylated hemoglobin I was dissolved in 0.1 M ammonium bicarbonate buffer, pH 8.0, and aliquots (~12 nmol) were digested with trypsin (Worthington), α- chymotrypsin (Worthington) and *Staphylococcus aureus* V8 protease (Pierce) at 37° for 4 h at an enzyme:substrate ratio of 1:50 (w/w). Aliquots (~15 nmol) of native *Casuarina* hemoglobin mixture were also digested with these three enzymes under the same conditions.The digests were dried under vacuum at 50° C, redissolved in 0.25 ml of 0.1% (v/v) trifluoroacetic acid and the soluble peptides isolated by high pressure liquid chromatography in 0.1% (v/v) triflouroacetic acid on a Vydac 218TP54 column using an acetonitrile gradient.

Peptides (using 2-4 nmol) were sequenced either on a gas phase sequencer

or manually by a modified Edman procedure (Peterson *et al.* 1972). Residues were identified as PTH - amino acid derivatives by HPLC as in Woods and Inglis (1984). Amino acid analysis of the protein was performed on a Waters HPLC amino acid analyser after hydrolysis of 1-2 nmol of protein in 6 M HCl for 24 h at 110° C. Tryptophan was determined after hydrolysis of the protein in 4 M methane sulfonic acid/0.2% (w/v) tryptamine for 24 h at 115° C. The carboxyl-terminal residues were determined by amino acid analysis after digestion of the protein with carboxypeptidase Y (1% w/w) in 0.1 M pyridine-acetate buffer, pH 5.6, at 37° C for 1 h and 2 h.

Preparation of anti-sera.

This experiment was conducted by Dr. William F. Dudman of the Division of Plant Industry, CSIRO, Canberra, ACT 2601, with my assistance.

Chapter Four

Casuarina glauca hemoglobin I, purified as described in (0.4 ml of a solution containing 1.67 mg hemoglobin I / 1.0 ml of phosphate buffer), was emulsified with an equal volume of Freund's complete adjuvant (Difco) and 0.4 ml of the mixture injected intra-muscularly into a rabbit. Four weeks later, the immune response of the rabbit was boosted with a sub-cutaneous injection of 0.2 ml of the hemoglobin solution without adjuvant; it was bled from the marginal ear vein on the fourth, sixth and eighth day afterwoods. The resulting antisera were tested individually by immunodiffusion and pooled. The immunoglobulin G fraction was isolated from the whole serum by precipitation with ammonium sulphate following the procedure of Hebert *et al.* (1973). Antisera against *Parasponia* hemoglobin I and soybean leghemoglobin a were prepared earlier by similar schedules (W.F.Dudman, unpublished).

Western blot analysis.

This experiment was undertaken by me in close collaboration with Dr Don Spencer, Division of Plant Industry, C.S.I.R.O, Canberra, ACT 2601.

Highly purified *Casuarina* hemoglobin (mixed components from the G75 Sephadex procedure), Soybean Lba and *Parasponia* hemoglobin I were each applied to three replicate tracks on an SDS-polyacrylamide, 12-22.5% gradient slab gel. *Casuarina* hemoglobin was added at 15 µg per track, and Soybean and *Parasponia* hemoglobin at 12 µg per track. An additional track contained approximately 15 µg of part-purified *Casuarina* hemoglobin (from Sephacryl S200 without concentration over a YM10 membrane). The gel and samples were prepared and run overnight according to the basic procedures of Laemmli (1970).

Following SDS-PAGE the separated proteins were electrophoretically transferred to a nitrocellulose sheet (Bittner *et al.* 1980). This was briefly stained (5 sec.) in Amido black (0.1% (w/v) in 45% (v/v) methanol/10% (v/v) acetic acid), destained with water until optimal resolution was obtained, and then photographed. The stain was largely removed by washing in water, though a visible residue remained throughout. The sheet was then cut into three pieces and each set of replicate tracks was shaken for 30 min in 5% (w/v) skim milk powder (Diploma instant), 0.9% (w/v) NaCl, 10 mM Tris, pH 7.4, and then incubated separately overnight in plastic pouches at room temperature with anti-sera (1/200 dilution in 5% skim milk powder, 0.9% NaCl, 10 mM Tris, pH 7.4) to one of the three proteins (anti-sera preparation described earlier). The strips were then washed (5 x 15 min) in 0.9% NaCl, 10 mM Tris, pH 7.4 and the bound anti-sera recognised by the protein A-peroxidase (Sigma) staining procedure described by Hawkes *et al.* (1982). The sheet was then photographed.

Measurement of ligand reaction rates (oxygen and carbon monoxide).

These experiments were conducted by Dr. Jonathan B. Wittenberg and Dr. Beatrice A. Wittenberg of the Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, New York 10461, USA.

Ligand reaction rates. A Gibson-Milnes (Gibson and Milnes 1964) stopped

flow apparatus with a 2 cm light path in the observation cell, interfaced to an OLIS Data Acquisition/Computation System (On Line Instrument Systems, Route 2, P.O. Box 111, Jeffersen, Georgia 30549, U.S.A.) was used to measure reaction rates.

A standard succinic acid/potassium phosphate buffer system, pH 7.0, was used to enable direct comparison with similar measurements made on soybean leghemoglobin (Appleby *et al.* 1983 b) and *Parasponia* hemoglobin (Wittenberg *et al.* 1986). This buffer mixture contained 100 mM succinic acid (Fluka, purissima grade), 100 mM potassium dihydrogen phosphate (Baker, ultrex grade), and 1 mM EDTA (Fluka, purissima grade) adjusted to pH 7.00 with sodium hydroxide (Baker, ultrex grade).

Oxygen dissociation rate. Solutions of *C.glauca* HbO_2 (0.7 μM HbO_2, 2.8 μM free oxygen in buffer) were mixed rapidly with solutions of CO in buffer and the reaction followed at 421 nm, a maximum in the difference spectrum of *C.glauca* HbCO minus HbO_2. The rate was the same at 0.5 and 1.0 mM CO (before mixing).

Oxygen combination rate. Solutions of *C.glauca* ferrous hemoglobin (0.68 or 0.83 μM hemoglobin, 15 μM dithionite in buffer) were mixed rapidly with solutions of oxygen (2.9 to 11.6 μM in buffer, before mixing) and the reaction followed at 417 nm, a wavelength isosbestic for ferrous and high-spin ferric *C.glauca* hemoglobin, or at 434 nm, a maximum in the difference spectrum ferrous hemoglobin minus HbO_2. Under these conditions, the reaction of oxygen with ferrous hemoglobin is very much more rapid than the reaction of ferric hemoglobin with dithionite, and excess dithionite present in the solution does not interfere. The second order combination rate constant was obtained graphically as the slope of a plot of the observed pseudo-first order rate versus oxygen concentration (after mixing).

Carbon monoxide dissociation rate. A solution of *C.glauca* HbCO (prepared by adding HbO_2 to a solution containing 8μM CO and 34 μM dithionite in buffer to give a mixture containing 3 μM HbCO, 5 μM free CO and 10-20 μM residual dithionite) were mixed rapidly with an oxygen-free solution of nitric oxide (approximately 2 mM) in buffer. The reaction was followed at 420 nm, an absorption maximum of HbCO.

Carbon monoxide combination rate. Solutions of ferrous hemoglobin (1 μM hemoglobin, 17 μM dithionite in buffer) were mixed rapidly with solutions of CO (10-80 μM CO, 17 μM dithionite in buffer, before mixing) and the reaction followed at 417 nm, a wavelength near the 420 nm maximum in the difference spectrum HbCO minus ferrous hemoglobin, and isosbestic for ferrous hemoglobin and high-spin ferric hemoglobin. The observed rate was the same at 434 nm, a maximum in the difference spectrum ferrous hemoglobin minus HbCO. The second order combination rate constant was obtained graphically as the slope of a plot of the observed pseudo-first order rate versus CO concentration (after mixing).

Results and Discussion

Amino acid sequence of* Casuarina *hemoglobin.

The complete amino acid sequence of *Casuarina* hemoglobin I is shown in Figure 5.1. The peptides sequenced to establish unequivocally the primary sequence are illustrated in Figure 5.2. The polypeptide chain contains 151 amino acid residues corresponding to a molecular weight of 17, 856 (including the heme group). This is agreement with the molecular weight of ~16,900 for the heme-free protein determined by SDS-PAGE (see below) and the estimate of 17,000 - 18,000 by Sephadex G75 chromatography (Chapter Four). The sequence is in agreement with the amino acid composition of the protein (Table 5.1). There were several positions of heterogeneity within the sequence (Figure 5.1), indicating a multi-gene family responsible for hemoglobin production. This point is

Figure 5.1: The amino acid sequence of *Casuarina glauca* hemoglobin I. Hemoglobin I is the major component separated by isoelectric focusing.

Also shown are the seven positions of heterogeneity present in the unfocused hemoglobin mixture. There was no heterogeneity in hemoglobin I.The amino terminal sequence (residues 1-38) of intact hemoglobin I was determined using a gas phase sequencer. The complete sequence was established by sequencing overlapping peptides derived from tryptic, chymotryptic and staphylococcal protease digest of the protein (see Figure 5.2). For more details see Materials and Methods.

1									10									20
Ala	Leu	Thr	Glu	Lys	Gln	Glu	Ala	Leu	Leu	Lys	Gln Glu	Ser	Trp	Glu	Val Ile	Leu	Lys	Gln	Asn
									30									40
Ile	Pro	Ala Gly	His	Ser	Leu	Arg	Leu	Phe	Ala	Leu	Ile	Ile Leu	Glu	Ala	Ala	Pro	Glu	Ser	Lys
									50									60
Tyr	Val	Phe	Ser	Phe	Leu	Lys	Asp	Ser	Asn	Glu	Ile	Pro	Glu	Asn	Asn	Pro	Lys	Leu	Lys
									70									80
Ala	His	Ala	Ala	Val	Ile	Phe	Lys	Thr	Ile	Cys	Glu	Ser	Ala	Thr	Glu	Leu	Arg	Gln	Lys
									90									100
Gly	His	Ala	Val	Trp	Asp	Asn	Asn	Thr	Leu	Lys	Arg	Leu	Gly	Ser	Ile	His	Leu	Lys	Asn
									110									120
Lys Gly	Ile	Thr	Asp	Pro	His	Phe	Glu	Val	Met	Lys	Gly	Ala	Leu	Leu	Gly	Thr	Ile	Lys	Glu
									130									140
Ala	Ile	Lys	Glu	Asn	Trp	Ser	Asp	Glu	Met	Gly Glu	Gln	Ala	Trp	Thr	Glu	Ala	Tyr	Asn	Gln
									150									
Leu	Val	Ala	Thr	Ile Gly	Lys	Ala	Glu	Met	Lys	Glu									

Figure 5.2: Peptide mapping of *Casuarina glauca* hemoglobin I. The vertical bars indicate the amino terminus of the peptide and the residues sequenced are indicated by the solid line. Peptides are numbered from the N-terminus of the protein and T, C and S signify tryptic, chymotryptic and staphylococcal protease peptides, respectively.

Casuarina
glauca Hb I

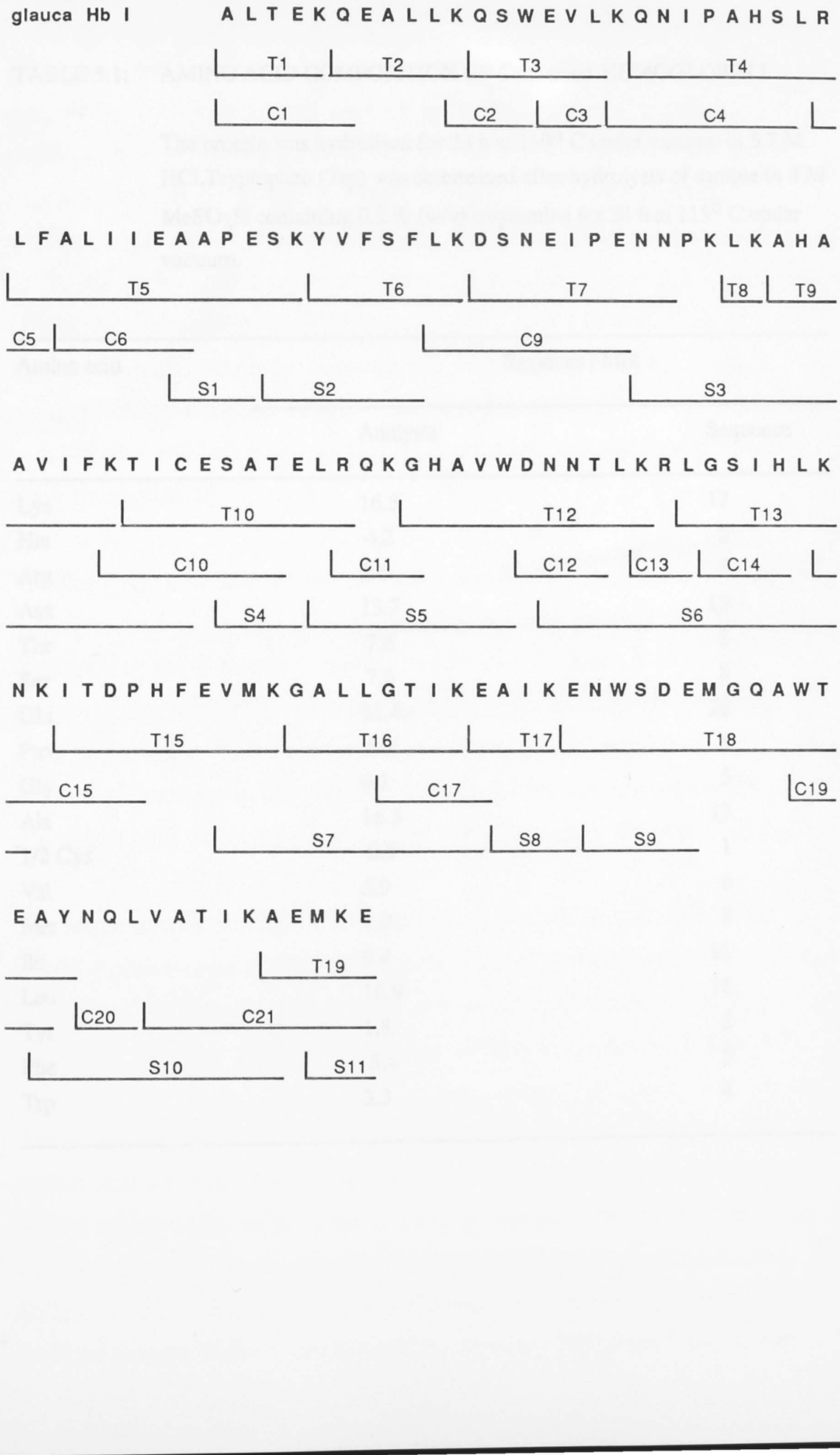


TABLE 5.1: AMINO ACID COMPOSITION OF *Casuarina* HEMOGLOBIN I.

The protein was hydrolised for 24 h at 110° C under vacuum in 5.7 M HCl. Tryptophan (Trp) was determined after hydrolysis of sample in 4 M $MeSO_3H$ containing 0.2 % (w/v) tryptamine for 24 h at 115° C under vacuum.

Amino acid	Residues / Mol	
	Analysis	Sequence
Lys	16.3	17
His	4.2	5
Arg	3.6	3
Asx	13.7	13
Thr	7.6	8
Ser	7.6	8
Glx	21.4	22
Pro	5.2	5
Gly	6.1	5
Ala	16.5	17
1/2 Cys	0.8	1
Val	5.9	6
Met	1.9	3
Ile	9.4	11
Leu	16.9	16
Tyr	1.8	2
Phe	5.4	5
Trp	3.3	4

further discussed below.

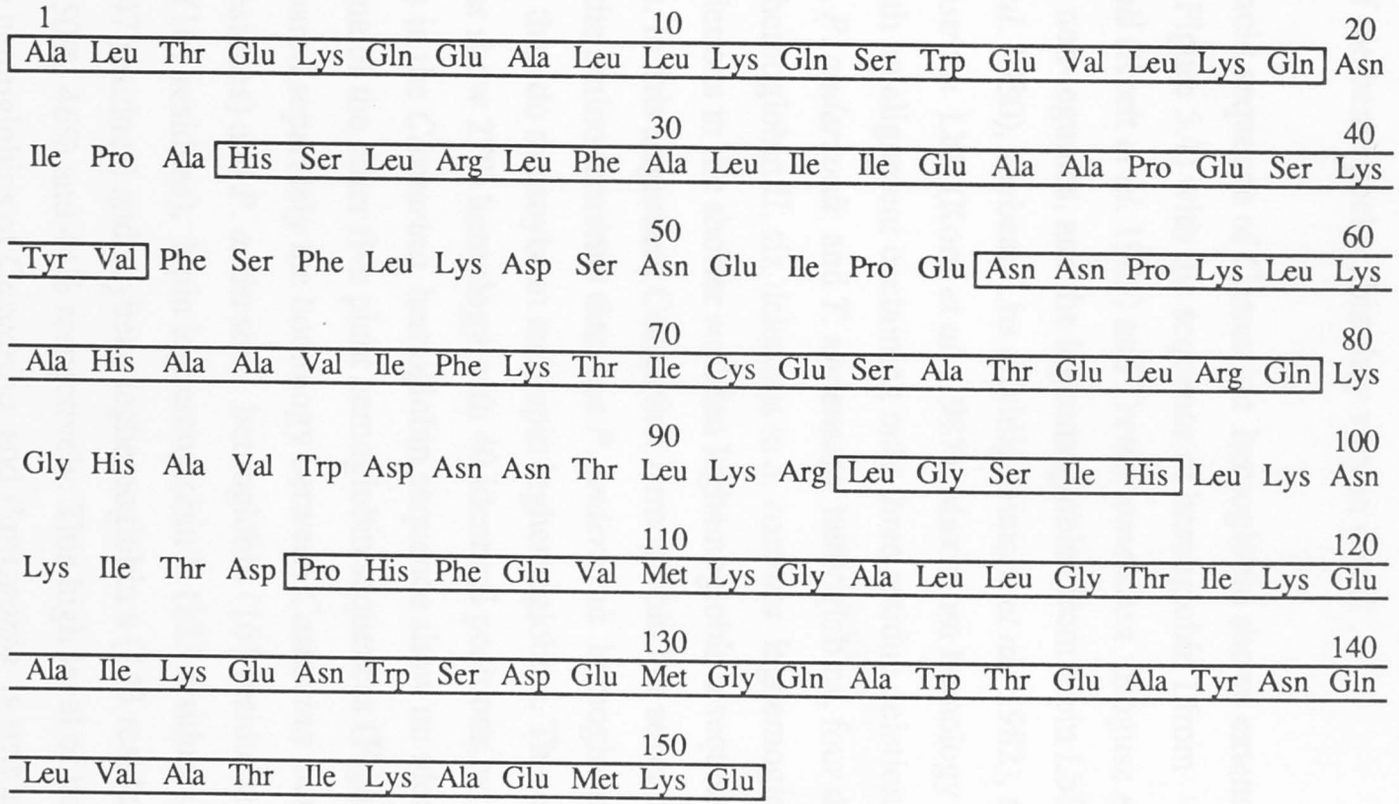
The tryptic peptides from a digest of the pure hemoglobin I accounted for 147 residues of the molecule. Alignment of the tryptic peptides was established with overlapping chymotryptic and *S. aureus* protease peptides, and by homology (Figure 5.2). All tryptic, chymotryptic and *S. aureus* peptides are shown in Figure 5.2. For most of the sequence overlapping peptides were obtained. T7, T8, T9 and T10 were located in the sequence by homology with other plant globins (Figure 5.2). This alignment is consistent with the overlap of T9 and T10 with C10, in agreement with homology. Although S7 and S8 provided only a single residue overlap between T16, T17 and T18 the placement of these peptides is consistent with the overlaps and the homology comparisons with other plant hemoglobins.

The carboxyl terminal amino acid was identified as glutamic acid from the sequence data of peptide C21. Some difficulty was encountered in determining the C-terminal residue by carboxypeptidase Y digestion. No major residue was released upon digestion of intact hemoglobin II with carboxypeptidase Y. A comparison of 1 h and 2 h digests, however, showed an increase in the yields of Glu, Lys, Met and Ala released consistant with the C-terminal sequence deduced from the peptide data.

The sequences of peptides T10 and C10 confirmed the presence of the single cysteine residue (identified as the PTH of carboxymethyl-S-cysteine) at position 71. The cysteine residue in *P. andersonii* hemoglobin I is located in the same position in a highly conserved sequence (Figure 3.2).

The secondary structure of *Casuarina* hemoglobin predicted from the amino acid sequence has a high content of α-helix (75%). The predicted helical regions (Figure 5.3), are in good agreement with the predicted and known helical structures of other plant hemoglobins (Arutyunyan 1981, Ollis *et al.* 1983, Kortt *et al.* 1985). Conservation of hemoglobin structure is also a feature of the animal kingdom. Perutz (1983) found that during

Figure 5.3: The predicted α-helical segments of *Casuarina* hemoglobin I, calculated using the model developed by Garnier *et al* (1978). Boxes enclose the segments predicted as helices. Hemoglobin I is the major component separated by isoelectric focusing.

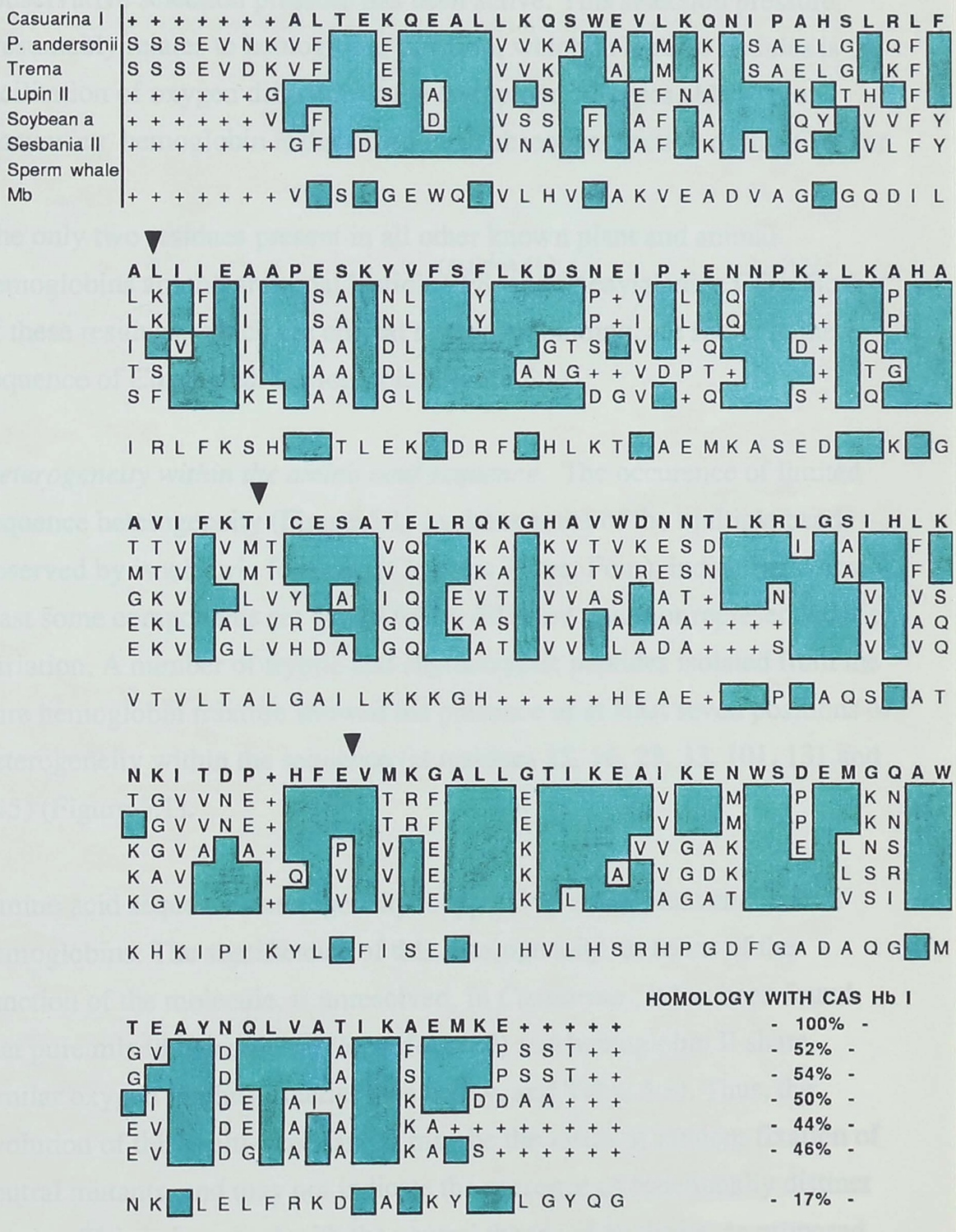

1 10 20
Ala Leu Thr Glu Lys Gln Glu Ala Leu Leu Lys Gln Ser Trp Glu Val Leu Lys Gln Asn
30 40
Ile Pro Ala His Ser Leu Arg Leu Phe Ala Leu Ile Ile Glu Ala Ala Pro Glu Ser Lys
50 60
Tyr Val Phe Ser Phe Leu Lys Asp Ser Asn Glu Ile Pro Glu Asn Asn Pro Lys Leu Lys
70 80
Ala His Ala Ala Val Ile Phe Lys Thr Ile Cys Glu Ser Ala Thr Glu Leu Arg Gln Lys
90 100
Gly His Ala Val Trp Asp Asn Asn Thr Leu Lys Arg Leu Gly Ser Ile His Leu Lys Asn
110 120
Lys Ile Thr Asp Pro His Phe Glu Val Met Lys Gly Ala Leu Leu Gly Thr Ile Lys Glu
130 140
Ala Ile Lys Glu Asn Trp Ser Asp Glu Met Gly Gln Ala Trp Thr Glu Ala Tyr Asn Gln
150
Leu Val Ala Thr Ile Lys Ala Glu Met Lys Glu

vertebrate evolution, the tertiary and quaternary structures of oxy- and deoxyhemoglobin have remained almost invariant. He points out: '... the tertiary structures of vertebrate myoglobins, of the monomeric lamprey hemoglobin, and of invertebrate and leguminous plant hemoglobin all resemble those of the α- and ß-chains of vertebrate hemoglobin to the extent necessary to preserve the vitally important geometry of the heme pocket, but they vary in detail' .

The amino acid sequence of *Casuarina* hemoglobin shows extensive homology (Figure 5.4) with the sequence of hemoglobin I from *P. andersonii* (Kortt *et al.* 1985) and *Trema tomentosa* (Bogusz *et al.* 1988), both non-legumes, and the leghemoglobins from lupin LbII (Egorov *et al.* 1980), soybean Lba (Hyldig-Nielsen *et al.* 1982), and *Sesbania rostrata* LbII (Kortt *et al.* 1987). Maximum homology was obtained with an alignment containing only three residue deletions in the *Casuarina* , *P. andersonii* and *T. tomentosa* hemoglobins, four deletions in lupin leghemoglobin II, six deletions in *S. rostrata* leghemoglobin II and nine deletions in the shorter soybean leghemoglobin a sequence (Figure 5.4). In this alignment *Casuarina* hemoglobin has seven fewer residues at the amino terminus than has *P. andersonii* hemoglobin and one less residue than do the soybean and lupin leghemoglobins. These six hemoglobins show 27% homology with 40 identical positions, while only 46 positions in the *Casuarina* hemoglobin sequence show no identity with a residue in one of the other five plant hemoglobin sequences (Figure 5.4). When compared separately the homology between *Casuarina* hemoglobin (with 151 residues) and *P. andersonii* hemoglobin (161 residues), *T. tomentosa* (161 residues), lupin leghemoglobin II (153 residues), *S. rostrata* (147 residues) and soybean leghemoglobin a (143 residues) is 52%, 54%, 50%, 46% and 44% respectively. This high level of homology between the hemoglobins of *Casuarina* and *Parasponia* is striking and is similar to the level of homology found between the leghemoglobins (Table 3.1). In contrast, there was only 17% homology with sperm whale myoglobin (Figure 5.4).

Figure 5.4: Sequence alignment indicating the homology between *Casuarina* hemoglobin I, *Parasponia andersonii* hemoglobin I, *Trema tomentosa* hemoglobin, lupin leghemoglobin II, soybean leghemoglobin a, *Sesbania* leghemoglobin II and sperm whale myoglobin. Residues identical with those in the *Casuarina* hemoglobin sequence have been boxed and coloured. The symbol (+) indicates gaps introduced to optimise alignment . The symbol ▼ indicates the intron insertion point, which is identical for all the plant hemoglobins shown.

Sources of sequence data:
Parasponia andersonii : Kortt *et al* (1988)
Trema tomentosa : Bogusz *et al* (1988)
Lupin : Egorov *et al* (1980)
Soybean : Hyldig-Nielsen *et al* (1982)
Sesbania : Kortt *et al* (1987)
Sperm whale Mb : Edmonson (1965)


Casuarina I
P. andersonii
Trema
Lupin II
Soybean a
Sesbania II
Sperm whale
Mb
HOMOLOGY WITH CAS Hb I
- 100% -
- 52% -
- 54% -
- 50% -
- 44% -
- 46% -
- 17% -

The high degree of homology amongst the plant hemoglobins shown in Figure 5.4 is particularly surprising considering the phylogenetic distance which separates these plants (see Figure 3.1 and Chapter Three for discussion of phylogeny of these plants), suggesting that a strong conservative selection pressure has been active. This selection pressure presumably relates to hemoglobin function, which in legume nodules is the facilitation of oxygen diffusion to the endophyte. The possible role of *Casuarina* hemoglobin is discussed in a subsequent section of this Chapter.

The only two residues present in all other known plant and animal hemoglobins are the proximal histidine F8 (residue 62) and phenylalanine CD1 (43). Both of these residues, which are critical to oxygen binding, are found in the sequence of *Casuarina* hemoglobin (Figure 5.1).

Heterogeneity within the amino acid sequence. The occurence of limited sequence heterogeneity (Figure 5.1) explains some of the multiple bands observed by isoelectric focusing (Chapters Three, Four), indicating that at least some components are coded for by different genes or represent allelic variation. A number of tryptic and chymotryptic peptides isolated from the pure hemoglobin mixture showed the presence of at least seven positions of heterogeneity within the sequence (at residues 12, 16, 23, 33, 101, 131 and 145) (Figure 5.1).

Amino acid sequence heterogeneity is typical of many plant and animal hemoglobins. The significance of this heterogeneity, in terms of the function of the molecule, is unresolved. In *Casuarina* , it has been found that pure mixed hemoglobin (ie, unfocused) and hemoglobin II share similar oxygen binding kinetics (see below, and Table 5.3). Thus, the evolution of this multi-gene family may be the result of random fixation of neutral mutants, and may not indicate the presence of functionally distinct species. This is in accord with the neutral theory of evolution as proposed by Kimura (1982, 1986), which claims that:
'.... the great majority of evolutionary changes at the molecular level are caused not by Darwinian selection acting on advantageous mutants, but by

random fixation of selectively neutral or near neutral mutants.'

Likewise, it has been shown recently that soybean Lb a, b, c_1, c_2, c_3, d_1, d_2 and d_3 all share similar oxygen kinetics (Martin *et al.* 1989), as do the seven purified components of *Sesbania* hemoglobin (Gibson *et al.* 1989) (Table 5.4). The evolutionary changes leading to the production of these hemoglobins may also have been selectively neutral.

There is evidence, also, that in animal hemoglobins most of the observed structural variation between species is functionally unimportant (neutral mutations), and that adaptation of the molecule to different environments has been brought about by only a few amino acid substitutions in key positions (Perutz 1983).

The immunological relationship of* Casuarina *hemoglobin to other plant hemoglobins.

Hemoglobins were first separated by SDS-PAGE, resulting in the pattern shown in Figure 5.5. *Casuarina* hemoglobin was resolved into one major and two minor bands, with the major band being midway between *Parasponia* HbI (M.W. of monomeric sub-unit 19,319) and Soybean Lba (M.W. 15,876). This indicates a molecular weight of approximately 17,500, which is in good agreement with the calculated molecular weight of the amino acid sequence (17,856). Sephadex G75 molecular exclusion chromatography gave an estimated molecular weight for the undenatured protein of 17,000 to 18,000. This suggests that native *Casuarina* hemoglobin is a monomeric protein (cf. *Parasponia* HbI, a dimer; Appleby *et al.* 1983, Wittenberg *et al.* 1986). The fast-running minor band on SDS-PAGE is most likely slightly degraded hemoglobin. The nature of the slower running hemoglobin is not known. It may be either a separate gene product or a conjugated form of the protein.

Following SDS-PAGE the hemoglobins were transferred by electro-blotting to a nitrocellulose sheet and incubated with antisera developed against each of these three proteins (Figure 5.5). Strong

Figure 5.5: Western blot of plant hemoglobins.

(A) Sodium dodecyl sulphate polyacrylamide gel electrophoresis of *Casuarina* hemoglobin, *Parasponia* hemoglobin and soybean leghemoglobin seen after electro-blotting to a nitro-cellulose sheet, as described in Materials and Methods. The sheet was stained for five seconds in 0.1% Amido black.

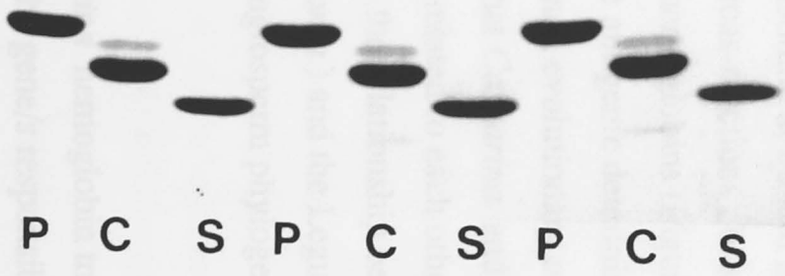
(B) Reaction of proteins with anti-sera developed against each hemoglobin. Bound anti-sera were recognised by the protein-A peroxidase staining procedure (see Materials and Methods). The faint bands in the *Casuarina* and *Parasponia* tracks incubated with anti-soybean leghemoglobin are residual Amido black staining. P= *Parasponia* HbI (mol. wt of native protein monomeric sub-unit 19319)

S= Soybean Lba (mol. wt. of native protein 15876)

C= *Casuarina* hemoglobin

A

PROTEIN



B

ANTI-SERA

anti Casuarina

anti Soybean

anti Parasponia

homologous reactions were observed between each of the proteins and their respective antisera. In addition, reciprocal cross-reactions occurred between *Casuarina* hemoglobin and anti-*Parasponia* hemoglobin antiserum, and also between *Parasponia* hemoglobin and anti-*Casuarina* antiserum. Very weak non-reciprocal cross-reactions occurred between Soy leghemoglobin and both the anti-*Casuarina* and anti-*Parasponia* antisera. The anti-Soybean antiserum was specific for Soybean leghemoglobin, giving no detectable reactions with either of the other hemoglobins.

Evolutionary implications of amino acid sequence homology and immunological cross-reactivity amongst plant hemoglobins.

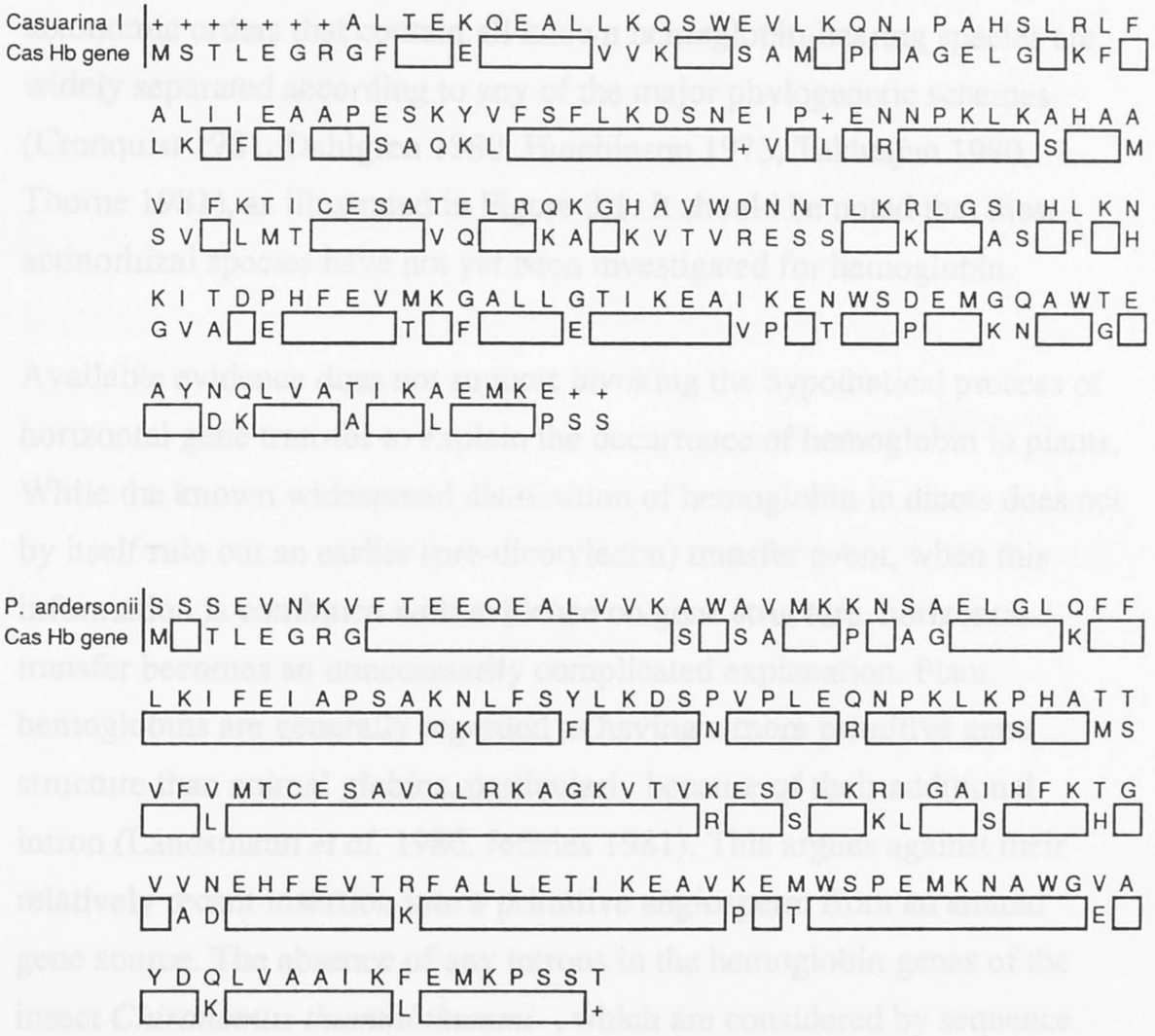
It was argued in Chapter Three that leghemoglobin, *Parasponia* hemoglobin, animal hemoglobin and myoglobin share a common evolutionary origin. The results presented in this Chapter strongly suggest that *Casuarina* hemoglobin is also a member of this single globin family. The similarity in primary structure and predicted tertiary structure between *Casuarina* hemoglobin and other plant hemoglobins (Figures 5.1 and 5.3) is unlikely to be the result of convergent evolution, particularily given the identical placement of the proximal histidine at F8 and the phenylalanine at CD1.Further, the serological cross-reactions observed between legume, *Casuarina* and *Parasponia* hemoglobins (Figure 5.5), indicate that they have at least some of the same antigenic determinants, a highly unlikely event unless they shared a common evolutionary origin. While not conclusive, this result also suggests that *Casuarina* and *Parasponia* hemoglobins may be more closely related to each other than to Soybean leghemoglobin. This is consistent with the relationship between Casuarinales, Urticales (which includes *Parasponia*) and the Leguminales (or Fabales/Fabineae) in most of the proposed angiosperm phylogenies (Figure 3.1).

Final confirmation of the relationship of *Casuarina* hemoglobin to other plant hemoglobins awaits sequence analysis of the gene/s responsible for its production. This work is in progress in the laboratories of Dr W. J.

Peacock (CSIRO Division of Plant Industry, Canberra) and Dr K. A. Marcker (University of Åhrus, Denmark) with plant material initially supplied by me. It has already been shown that *Parasponia* hemoglobin cDNA hybridises to a *Casuarina* hemoglobin gene which has positions of intron insertion identical to *Parasponia* and the legume genes (Landsmann *et al.* 1986). Suprisingly the sequence of this gene, which was established only very recently (K. A. Marcker and W. J. Peacock, pers. comm.) codes for a protein with an amino acid sequence which has greater homology to *Parasponia* hemoglobin I than *Casuarina* hemoglobin I (Figure 5.6). It is clearly not the gene coding for *Casuarina* hemoglobin I, or any other member of the family of *Casuarina* symbiotic hemoglobins which I have observed in my studies; it codes for a larger protein. The protein which this gene theoretically codes for was not seen during amino acid sequencing of the purified *Casuarina* nodule hemoglobin mixture. The sequence heterogeneity observed in that mixture bears no relationship with the derived amino acid sequence of the Marcker-Peacock hemoglobin gene. If this gene is being translated into protein, then that protein was either absent from the nodules or present only in trace amounts. Possibly, the gene codes for a non-symbiotic hemoglobin now known to be present in the tips of *Casuarina* roots and which is larger than the protein I have purified (C. A. Appleby, pers. comm. Discussed further later). This work is continuing.

The use of soybean and *Parasponia* cDNA probes has, so far, not led to the isolation of a *Casuarina* symbiotic hemoglobin gene coding for the protein which I have purified from root nodules. It may well be that the synthesis of specific oligonucleotide probes (with sequences deduced from the known amino acid sequence - Figure 5.1) will lead to eventual isolation and characterisation of the *Casuarina* symbiotic hemoglobin gene(s). A similar strategy led to cloning and characterisation of the *Parasponia* hemoglobin gene, after unsuccessful use of soybean hemoglobin cDNA as a gene probe (Landsmann *et al.* 1986). There will be much to gain from knowing the sequence of both symbiotic and non-symbiotic *Casuarina* hemoglobin genes. By comparing these genes with other plant hemoglobin

Figure 5.6: Amino acid sequence comparison of *Casuarina glauca* hemoglobin I and *Parasponia andersonii* hemoglobin I with the derived amino acid sequence of a hemoglobin gene isolated from *C. glauca* roots (Tove Christiansen, pers. comm.). Note that this hemoglobin gene has a much higher degree of homology with *P. andersonii* hemoglobin than *C. glauca* hemoglobin, even though it is a *Casuarina* gene.


Casuarina I
Cas Hb gene
+ + + + + + + A L T E K Q E A L L K Q S W E V L K Q N I P A H S L R L F
M S T L E G R G F E V V K S A M P A G E L G K F
A L I I E A A P E S K Y V F S F L K D S N E I P + E N N P K L K A H A A
L K F I S A Q K L + V L R S M
V I F K T I C E S A T E L R Q K G H A V W D N N T L K R L G S I H L K N
S V L M T V Q K A K V T V R E S S K A S F H
K I T D P H F E V M K G A L L G T I K E A I K E N W S D E M G Q A W T E
G V A E T F E V P T P K N G
A Y N Q L V A T I K A E M K E + +
D K A L P S S
P. andersonii
Cas Hb gene
S S S E V N K V F T E E Q E A L V V K A W A V M K K N S A E L G L Q F F
M T L E G R G S S A P A G K
L K I F E I A P S A K N L F S Y L K D S P V P L E Q N P K L K P H A T T
Q K F N R S M S
V F V M T C E S A V Q L R K A G K V T V K E S D L K R I G A I H F K T G
L R S K L S H
V V N E H F E V T R F A L L E T I K E A V K E M W S P E M K N A W G V A
A D K P T E
Y D Q L V A A I K F E M K P S S T
K L +

genes, the nature of hemoglobin inheritance in plants, from ancient non-symbiotic to recent symbiotic, might at last be revealed.

One implication of finding a typical plant hemoglobin in *Casuarina* is that the observations of Davenport (1960) and later Tjepkema (1983) were correct, and will lead eventually to the characterisation of similar hemoglobins in other actinorhizal plants. Thus hemoglobin is far more common in the plant kingdom than had previously been assumed. The taxonomic orders that contain all known hemoglobin-bearing species are widely separated according to any of the major phylogenetic schemes (Cronquist 1981, Dahlgren 1980, Hutchinson 1973, Takhtajan 1980, Thorne 1981), as illustrated in Figure 3.1. It should be noted that most actinorhizal species have not yet been investigated for hemoglobin.

Available evidence does not support invoking the hypothetical process of horizontal gene transfer to explain the occurrence of hemoglobin in plants. While the known widespread distribution of hemoglobin in dicots does not by itself rule out an earlier (pre-dicotyledon) transfer event, when this information is combined with evidence on gene structure, horizontal transfer becomes an unnecessarily complicated explanation. Plant hemoglobins are generally regarded as having a more primitive gene structure than animal globins, particularly because of their additional intron (Landsmann *et al.* 1986, Jeffries 1981). This argues against their relatively recent insertion into a primitive angiosperm from an animal gene source. The absence of any introns in the hemoglobin genes of the insect *Chironomus thummi thummi* , which are considered by sequence comparison to be of the same origin as animal and plant globins(Antoine and Niessing 1984), is consistent with this argument. Jeffries (1981) had earlier suggested that it could have been an insect hemoglobin gene which was transferred to a legume via a viral vector.

Assuming the genes for hemoglobin have been inherited in the extant genera of nodulating plants by a process of vertical descent, rather than horizontal transfer, the origin of hemoglobin in plants must be extremely

primitive. Given that plant and animal hemoglobins share the same origin (Hyldig-Nielsen *et al.* 1982, Antoine and Niessing 1984, see Chapter Three), the extensive occurrence of hemoglobin in plants supports the hypothesis that the hemoglobin gene has been present in both plant and animal kingdoms since their divergence from a common ancestor. If so, then it is certain that the occurrence of hemoglobin in plants predates the evolution of nitrogen-fixing root nodule symbioses. While it is unclear when the legume-*Rhizobium*, *Parasponia-Rhizobium* or actinorhizal symbioses evolved, hypotheses which have been advanced to explain the steps involved in the evolution of the legume-*Rhizobium* symbiosis all imply the prior existence of a legume or a direct ancestor of the legumes (Parker 1957, Dilworth and Parker1969, Vance 1983, Verma and Long 1983, Sharifi 1984);i.e.,it was a fairly recent event. In this case the hemoglobin gene would have been present in the host genome well before the symbiosis had begun to evolve.

Plant hemoglobin had, until recently, been assumed to occur only in nodule tissues, where it functions as a high affinity oxygen carrier facilitating the diffusion of oxygen to the respiring endophyte. Assuming it was present in plants prior to the evolution of the symbiosis then it presumably had a quite different function. This putative function, so far unidentified, may persist in some or all plants today.

An exciting recent finding in this regard is the presence of an expressed hemoglobin gene, which has been sequenced, in root tissue of the non-nodulating plant *Trema tomentosa* (Bogusz *et al.* 1988). *Trema* is a member of the family Ulmaceae, and is a close relative of the nodulating genus *Parasponia* . The *Trema* hemoglobin gene was located using a *Parasponia* hemoglobin cDNA probe. This probe has also successfully located a hemoglobin gene in another non-nodulating member of the Ulmaceae, *Celtis australis* (Bogusz *et al.* 1988).

The *Trema* root and *Parasponia* nodule hemoglobin genes show a high level of homology. Each have three introns identically placed, with

approximately 80% nucleotide homology, and there is 93% nucleotide homology in the coding regions. There is also approximately 80% homology in the untranslated leader and 3' sequences. The*Trema* gene sequence is consistent with a functional hemoglobin. Expression of the *Trema* gene was confirmed by western blot analysis which showed the presence of a very small amount of monomeric hemoglobin. The same procedure was used to confirm the presence of a very small amount of dimeric hemoglobin in the roots of unnodulated *Parasponia* , which had an identical molecular weight to root nodule *Parasponia* hemoglobin I (Appleby *et al.* 1988a). As mentioned earlier, hemoglobin has also recently been found in the root tips of water culture grown *Casuarina glauca* seedlings. This protein has approximately the same molecular weight as the *Parasponia* root and nodule hemoglobins and may be the product of a *Casuarina* hemoglobin gene which was recently isolated and sequenced(K. A. Marcker and W. J. Peacock, pers. comm.). The theoretical protein deduced from the gene sequence shows greater homology with *Parasponia* nodule hemoglobin than *Casuarina* nodule hemoglobin (Figure 5.6 for derived amino acid sequence).

The oxygenation kinetics of the *Trema* , *Parasponia* and *Casuarina* root proteins are yet to be determined: it will be extremely interesting to see whether they indicate functional properties quite distinct from nodule hemoglobin, possibly relating to the original non-symbiotic function of hemoglobin in plants alluded to above. Appleby *et al.* (1988b) are assuming that the oxygenation kinetics of *Parasponia* root hemoglobin will be similar to *Parasponia* nodule hemoglobin because they are apparently coded for by the same gene. They also assume *Trema* hemoglobin will have a similarly high oxygen affinity because it and *Parasponia* hemoglobin are coded for by almost identical genes. No assumptions have yet been made about the functional characteristics of *Casuarina* root hemoglobin.

Appleby *et al.* (1988b), in a speculative paper, have hypothesised that root hemoglobin may act as a signal molecule indicating oxygen deficit and the

consequent need to shift plant metabolism from an oxidative to a fermentative pathway of energy production. They present no particular evidence to support this function, other than that the volume-averaged concentration of hemoglobin in the root tips is probably too low to indicate a role in facilitating oxygen diffusion for root mitochondrial respiration, which would be the obvious parallel to its function in nodules. However, they did not localise hemoglobin within root tips, which means that the concentration in restricted locations may be much higher. They admit that hemoglobin may be restricted to a small number of cells in the zone of elongation or near the root cap, in which case it could be present in a sufficient concentration to facilitate oxygen flow.

The occurrence of hemoglobin in a range of symbioses, including the *Casuarina-Frankia* symbiosis; the genetic relatedness of all plant hemoglobins; the expression of hemoglobin in the non-nodulating plant *Trema* ; and the expression of hemoglobin in root tissue of *Casuarina* and *Parasponia* all strongly suggest that the hemoglobin gene has been present in plants at least since the diversification of the dicotyledons. This is consistent with, but not proof of, the hypothesis mentioned above that the gene has been present in both the plant and animal lines since their divergence from a common ancestor.

It is unknown whether the hemoglobin gene exists in monocotyledons, an important question because the monocotyledons contain many agriculturally important crop plants into which it is widely hoped to insert the genetic material necessary for nitrogen fixation. It is relevant, though, to look at the evolutionary relationship between monocotyledons and dicotyledons. There is no universally accepted view on the origin of these two groups of plants. The Ranalian Hypothesis, which is the prevailing view (Dahlgren *et al.* 1985), assumes that the monocotyledons evolved from a primitive dicotyledonous group such as the Magnoliiflorae or the Nymphaeiflorae. The alternative hypothesis assumes that monocotyledons are the ancestral angiosperms (Burger 1981 - this is the hypothesis he favours) from which the dicotyledons evolved. In their comprehensive

treatise on the monocotyledons, Dahlgren *et al.* (1985) support the Ranalian Hypothesis and also accept the hypothesis that the monocotyledons are monophyletic. If their view is correct, then it is possible that the hemoglobin gene was present in the genome of the first monocotyledon and has been maintained throughout the evolution of that order of plants, for some non-symbiotic function in roots.

There have been reports of hybridization by a leghemoglobin cDNA probe to the genomic DNA of monocots (Hattori and Johnson 1985, Roberts *et al.* 1985), but this has not been verified by isolation and characterisation of the hybridized genes. Moreover, this work has been criticised on the grounds of poor experimental technique (Landsmann *et al.* 1986) and cannot be accepted until and unless the genes are isolated and sequenced. It seems unlikely that a hemoglobin gene in the distantly related cereals would have retained sufficient homology to hybridize with a legume hemoglobin probe.Bogusz *et al.* (1988) could only obtain hybridization with the *Trema* hemoglobin gene using a cDNA probe derived from the closely related *Parasponia* . No hybridization was obtained using a soybean-derived probe, because the *Trema* and soybean sequences were too divergent. Recently, Tjepkema and Asa (1987) reported a significant amount of CO-reactive heme in the roots of *Zea mays* , but did not identify it as either hemoglobin or peroxidase.

The role of hemoglobin: kinetics of reactions with oxygen and carbon monoxide.

Until hemoglobin was purified from *Casuarina* nodules (Chapter Four) it was widely assumed to be absent from any actinorhizal symbiosis (see Introduction). This led to a variety of mechanisms being proposed to explain the ability of these nodules to fix nitrogen in atmospheric oxygen. Both physical and biochemical mechanisms were proposed as controlling oxygen access to the endophyte (see Introduction for a review). It was even postulated that nitrogenase in actinorhizal nodules may be unusually oxygen tolerant, but this remains unsubstantiated.

It is clear from the work reviewed in Chapter Three that physical barriers do play a role in restricting oxygen access to infected cells in at least some actinorhizal nodules, including those of *Casuarina*. Further, within infected cells, in all actinorhizal nodules other than *Casuarina*, it seems probable that the vesicle wall forms an additional barrier isolating nitrogenase from free oxygen. These barriers may be sufficient to resolve the oxygen paradox when nodules occur in well aerated soils. Conceivably, oxygen concentration, at least at the periphery of infected cells, may be sufficient to meet endophyte needs, though conditions may be almost anaerobic at the centre of cells. This could explain why the 'business end' of the endophyte, the vesicles, are often found in the outer region of infected cells.

However, in soils which are flooded either intermittently or permanently, and which therefore have reduced oxygen concentration, physical barriers alone could significantly inhibit oxygen access to the endophyte, affecting respiration and greatly reducing the rate of nitrogen fixation. Such environmental conditions are typical for *Casuarina cunninghamiana* and *C. glauca* (Figure 3.3), and also for species of *Myrica* and *Alnus*. Under such conditions it is conceivable that a mechanism has evolved to ensure efficient delivery of oxygen to the endophyte at variable, and sometimes very low, free oxygen concentration within infected cells. Hemoglobin is an obvious candidate for this role. A key factor in determining its eligibility for this role is its kinetics of oxygenation. These have now been determined.

The kinetics of the reactions of *Casuarina* hemoglobin with oxygen and carbon monoxide were investigated for both the purified mixed components (unfocused) and the major component hemoglobin II separable by IEF. The values for these two samples did not significantly differ from each other. The oxygen "off" rate was $5.33 \pm 0.23\ \hat{}\ s^{-1}$ for hemoglobin II and $5.59 \pm 0.07\ \hat{}\ s^{-1}$ for the mixed components. The oxygen combination rates used to derive the "on" rate constant are given in Table 5.2.

The figures shown in Table 5.3 are derived from the combined data of

TABLE 5.2: THE OXYGEN COMBINATION RATE OF *Casuarina* HEMOGLOBIN II AND *Casuarina* HEMOGLOBIN MIXED COMPONENTS MEASURED AT 417 nm AND 434 nm in 1%, 2%, 3% AND 4% AIR (BEFORE MIXING).

These values were plotted together (% air vs rate s^{-1}) and the slope of the regression line (r^2=0.99) was used to determine the oxygen combination rate constant (the "on" rate).
n= the number of replicates

% air	Wavelength (nm)	Oxygen Combination Rate (s^{-1})	
		Hb II	Mixed
1.0	417		30 (n=1)
1.0	434		70.6 ± 2.3 (n=4)
2.0	417	109 ± 8 (n=5)	138 (n=1)
2.0	434	126 ± 22 (n=6)	135 ± 5.9 (n=3)
3.0	434		175 ± 14 (n=4)
4.0	417	232 (n=1)	
4.0	434	217 ± 2.8 (n=4)	219 ± 21 (n=3)

TABLE 5.3: KINETIC AND EQUILIBRIUM CONSTANTS FOR THE REACTIONS OF *Casuarina* HEMOGLOBIN WITH OXYGEN AND CARBON MONOXIDE, COMPARED WITH THOSE OF *Parasponia* HEMOGLOBIN I AND SOYBEAN LEGHEMOGLOBIN a.

The values shown are derived from the combined data of hemoglobin II and hemoglobin mixed components. All values for *Casuarina* hemoglobin were determined at pH 7.0. Values for *Parasponia* hemoglobin I and soybean leghemoglobin a were taken at the alkaline limit of pH-dependent change: pH 7.0 to pH 9.0 for *Parasponia* hemoglobin I and pH 7.0 for soybean leghemoglobin a. All data at 20° C. Equilibrium constants are calculated from the kinetic constants. Data for *Parasponia* from Wittenberg *et al.* (1986); data for soybean leghemoglobin a from Appleby *et al* . (1983).

	Casuarina Hb II, and mixed components	Soybean Lb a	*Parasponia* Hb I
Oxygen			
k' "on" $M^{-1} s^{-1}. 10^{-6}$	**41**	118	165
k "off" s^{-1}	**5.5**	5.55	14.8
K' (=k:k') nM	**135**	48	89
P_{50} torr	**0.074**	0.026	0.049
Carbon Monoxide			
l' "on" $M^{-1} s^{-1}. 10^{-6}$	**2.34**	12.7	13.7
l "off" s^{-1}	**0.0027**	0.0078	0.0187
L' (=l:l') nM	**1.2**	0.62	1.4
Partition Coefficient			
$M = \frac{[HbCO] \cdot P_{O2}}{[HbO_2] \cdot P_{CO}}$	**87**	58	50

Casuarina hemoglobin II and mixed components, except for the carbon monoxide "on" rate which is derived only from hemoglobin mixed components data.

Reaction rates (Table 5.3) are of the same order of magnitude as those previously reported for both Soybean Lba (Appleby *et al.* 1983) and *Parasponia* hemoglobin I (Wittenberg *et al.* 1986). The oxygen "on" rate is 41×10^6 M^{-1} s^{-1}, indicating rapid combination rates, though it is the slowest known amongst plant hemoglobins (see Table 5.4 for an extended list), and the "off" rate is moderate (5.5 s^{-1}). The same trend is evident for CO, though the reactions are much slower. Consequently, the derived equilibrium dissociation rate constants for both O_2 (K') and CO (L') are also essentially similar to other plant hemoglobins (Tables 5.3, 5.4). The oxygen affinity (K' is 135 nM dissolved oxygen and P_{50} is 0.074 torr of oxygen partial pressure in the gas phase) is the lowest of all known plant hemoglobins, but is still exceptionally high compared with animal hemoglobin and myoglobin (Wittenberg *et al.* 1972) (Table 5.4). While the oxygen concentration required to meet the respiratory needs of *Frankia* in *Casuarina* nodules has not been established, the similarity between the oxygenation kinetic data for *Casuarina* hemoglobin and other plant hemoglobins suggests that their roles may be similar (Table 5.4).

In legumes, hemoglobin is involved in the facilitated diffusion of oxygen to *Rhizobium* , enabling efficient respiration to occur at low free oxygen concentration (~ 7nM, Bergersen 1982). There is evidence to suggest that in *Casuarina* nodules also, a low free oxygen concentration is maintained within the infected cells. Berg (1983) has shown that the walls of infected cells of a number of *Casuarina* species, including *C. cunninghamiana* and *C. glauca* , become impregnated with a suberin-like compound. The suberization of the wall may affect the physical environment of the infected cell, limiting diffusion of gases (Berg 1983). The absence of vesicles from *Casuarina* nodules provides indirect support for this hypothesis. Whereas pure cultured *Frankia* isolated from *Casuarina* nodules will produce vesicles and fix nitrogen when grown in N-deficient

TABLE 5.4: OXYGEN KINETICS OF A RANGE OF PLANT HEMOGLOBINS AND HORSE MYOGLOBIN

Sources of data: *Casuarina* (this thesis); *Parasponia* (Wittenberg *et al.* 1986); *Sesbania* , lupin, broad bean, kidney bean, Green pea (Gibson *et al.* 1989); Soybean a^1 (Appleby *et al.* 1983); Soybean a^2, b, c, d, mix (Martin *et al.* 1989); Horse myoglobin (Antonini 1965).

Protein	k' "on"	k "off"	K' (=k:k')	P_{50}
	$M^{-1}s^{-1}.10^{-6}$	s^{-1}	nM	torr
Casuarina	**41**	**5.5**	**135**	**0.074**
Parasponia	165	14.8	89	0.049
Sesbania II	210	7.52	35.8	0.020
Lupin I	540	19.5	36.1	0.020
Broad Bean	200	18.7	93.5	0.051
Kidney Bean a	100	6.22	62.2	0.034
Soybean a^1	118	5.55	48	0.026
Green Pea I	250	16.3	65.2	0.036
Horse Mb	14	11	770	0.700
Sesbania I	190	8.9	47	
Sesbania II	210	7.5	36	
Sesbania III	230	6.3	27	
Sesbania IV	230	10	44	
Sesbania V	190	6.8	36	
Sesbania VI	230	7.2	31	
Sesbania VII	235	6.9	29	

TABLE 5.4: CONT'D

Protein	k' "on"	k "off"	K (= k:k')
Soybean a[2]	116 ± 2	5.72 ±0.15	49.3 ± 2.2
Soybean b	113 ± 2	5.61 ± 0.15	49.6 ± 2.2
Soybean c_1	124 ± 2	4.88 ± 0.13	39.3 ± 1.7
Soybean c_2	99 ± 2	6.13 ± 0.16	61.9 ± 2.9
Soybean c_3	121 ± 2	5.41 ± 0.14	44.7 ± 1.9
Soybean d_1	130 ± 2	6.04 ± 0.16	46.5 ± 1.9
Soybean d_2	100 ± 2	6.04 ± 0.16	60.4 ± 2.8
Soybean d_3	121 ± 2	5.23 ± 0.14	43. 2 ± 1.9
Soybean mix	118 ± 2	5.93 ± 0.16	50 .2 ± 2.2

media at atmospheric oxygen concentration, at low oxygen concentrations (0.1-0.3 kPa pO_2) the same *Frankia* isolate fixes nitrogen in the absence of vesicles (Murry *et al.* 1985). The production of vesicles, in pure culture at least, therefore seems to be correlated with oxygen concentration, and the absence of vesicles in nodules may indicate a low free oxygen concentration.

Assuming *Casuarina* nodules do have a low free oxygen concentration within infected cells, then a role for hemoglobin in facilitated oxygen diffusion is entirely possible. In support of this, Tjepkema and Asa (1987) have correlated cell wall suberization with high total and CO-reactive heme content in actinorhizal nodules from *C. cunninghamiana* and *Myrica gale* , in contrast to nodules of *Alnus rubra* and *Datisca glomerata* which do not have suberized walls and contain little or no CO-reactive heme (Table 5.5).

The absence of vesicles in *Casuarina* nodules may be due only in part to the presence of hemoglobin. Hemoglobin is also found in *Myrica* and *Alnus* , but in their cases vesicles are also present. It seems more likely that in *Casuarina* it is the combination of suberization of infected cells (Berg 1983) and hemoglobin which has rendered the vesicle wall unneccesary, given that the production of vesicle wall (Murry *et al.*1985) and wall thickness (Parsons *et al.* 1987) are a function of oxygen concentration.

TABLE 5.5: EVIDENCE FOR THE SUBERIZATION OF THE WALL OF INFECTED CELLS IN SELECTED ACTINORHIZAL PLANTS.

Reproduced from Tjepkema and Asa (1987)

Species	Test result for lignin-suberin		
	Chromic acid	Phloroglucinol	Autofluorescence
Casuarina cunninghamiana	+ +	+	+ +
Myrica gale	+	+	+
Comptonia peregrina	+	+	+
Alnus rubra	-	-	-
Ceanothus americanus	-	-	-
Datisca glomerata	-	-	-

POSSIBLE GLYCOSYLATION OF *CASUARINA* HEMOGLOBIN

Casuarina hemoglobin was found, during extraction and purification (Chapter 4), to exhibit particular behaviour not seen in other plant hemoglobins. Indeed some of the behaviour, outlined below, played a critical part in developing a successful purification process.

* There was an absolute requirement for the inclusion of soluble polyvinyl pyrollidone (PVP) in the extraction buffer to solubilise the hemoglobin. The presence of soluble PVP is also required for the extraction of *Parasponia* hemoglobin (Appleby *et al.* 1983), but it is not necessary with any of the leghemoglobins (C. A. Appleby, pers. comm.).

* A non-ionic detergent was required in the extraction buffer to ensure a high percentage recovery of the protein (in a functional form) from Sephacryl S200 SF column chromatography. Detergent is not required for the successful S200 chromatography of any other plant hemoglobin (C. A. Appleby, pers. comm.).

* The solubilised hemoglobin was found to loosely adsorb to particular batches of Sephacryl S200, made prior to 1980 (Chapter Four), except for a small portion which was eluted at the position expected for a non-adsorbed protein.

* The hemoglobin showed extensive heterogeneity on both preparative scale and analytical scale isoelectric focusing gels. While it was reported in Chapter Five that a proportion of this microheterogeneity is probably the result of amino acid sequence variation (there were seven positions of heterogeneity in the sequence in the 'mixed' components sample of pure hemoglobin), this is not sufficient to explain the 30+ bands which have

been observed by sequential protein and heme staining on analytical scale IEF gels. This heterogeneity is not considered to be an artifact of isoelectric focusing (Chapter Four).

* Hemoglobin was precipitated through a wide range of percent saturation with ammonium sulphate (0.4 to 0.7), unlike either leghemoglobin or *Parasponia* hemoglobin which are cleanly precipitated (Chapter Four).

None of the properties of the purified *Casuarina* hemoglobin, reported in Chapters Four and Five, could explain the observations summarised above. However, some observations were consistent with the hypothesis that *Casuarina* hemoglobin is glycosylated.

Heterogeneity is typical of many glycoproteins, due to variation in the length and content of their carbohydrate chains (Cunningham 1971, Gottschalk 1966, Horowitz 1977, Sharon 1984). Such heterogeneity effects the surface charge of the molecule and can thus result in multiple banding on isoelectric focusing. There are, of course, other possible causes of protein heterogeneity (summarised in Williamson *et al.* 1973) such as deamidation and allomorphism (stable conformational isomers), but insufficient protein meant that not all possibilities could be studied.

Pharmacia has reported (Pharmacia Fine Chemical Inc Technical Note A681) that the polysaccharide, Blue Dextran, is adsorbed by Sephacryl S200 under certain conditions. If hemoglobin were glycosylated, this could explain its loose adsorption to that gel.

During my earlier experimental studies the requirement for PVP and detergent was taken to imply that hemoglobin may have been membrane-bound (Chapter Four), and was perhaps a membrane glycoprotein. This was largely based on both Davenport's observation (Davenport 1960) that hemoglobin remained bound to cell fragments under all the extraction conditions he tried, and similar findings by me that when PVP was excluded from the extraction buffer hemoglobin remained

intimately associated with cell debris (Chapter Four). The structure of PVP in some ways resembles that of a polypeptide and it has been suggested that it can displace extrinsically bound proteins into solution. The manufacturer claims that PVP helps convert some substances into a soluble form which would otherwise be insoluble or only sparingly soluble (PVP: Polyvinylpyrrolidone. Budische Anilin & Toda Fabrik AG, Ludwigshafen Am Rhein, 1966). Detergent would then be important in maintaining hemoglobin in solution, as has been found with many other membrane glycoproteins (Harrison and Hunt 1980). While the membrane glycoprotein hypothesis was later dismissed (immunolocalisation studies showed that *Casuarina* hemoglobin was restricted to the host cell cytoplasm; D. Goodchild, pers. comm.) it was at the time an important factor in deciding to investigate the possibility of glycosylation.

The results presented in this chapter indicate the presence of glucose in the hemoglobin sample, but are inconclusive on the question of glycosylation.

Materials and Methods

Affinity chromatography.

Concanavalin-A Sepharose. Concanavalin-A Sepharose is widely used in affinity chromatography of glycoproteins. It is specific for α-D-mannopyranosyl,α-D-glucopyranosyl and sterically related residues found in a range of glycoproteins.

A 5.6 ml sample of *C.cunninghamiana* HbCO (20 μM) which had been extracted and centrifuged under the conditions described in the Standard Extraction Procedure was added to a 6.1 x 1.6 cm column (12.26 ml) of Concanavalin-A Sepharose (Pharmacia HK26179) which had been pre-flushed with 130 ml of CO saturated starting buffer (0.02 mM Tris/HCl, 0.5 M NaCl, pH 7.4). The column flow rate was 20 ml cm^{-2} hr^{-1} and 3.3 ml fractions were collected. The effluent was monitored for U.V absorbance with a UVICORD III monitor, as described previously, and the hemoglobin elution profile determined. The column was then flushed with

buffer and the procedure repeated, except that NaCl was excluded from the starting buffer so that conditions were more favourable for adsorption. Finally, a 2.3 ml sample of 0.1% (w/v) Blue Dextran (Pharmacia) in buffer (0.02 mM Tris/HCl, 0.5 M NaCl, pH 7.4) was run. The dextran, which adsorbed tightly, was eluted with 0.1 M 1-O Methyl 1-∝-D-Glucopyranoside (Sigma).

Phenyl Boronate Agarose. Phenyl Boronate Agarose is an affinity chromatography medium specific for *cis* - diol groups.To test for the presence of these groups on the hemoglobin molecule (for example, due to conjugated glucose), a 0.2 ml sample of ~50 µM *C.glauca* HbO_2 in 50 mM glycine buffer (pH 9.0 at 4^{o} C), which had been purified by Sephacryl S200 chromatography and ultrafiltration, was applied to 2 ml columns of Phenyl Boronate Agarose-30 and -60 (Amicon Corp.). PBA-30 contained 30-50 µmol boron/ml and PBA-60 contained 60-100 µmol boron/ml. The columns had been pre-flushed with ~30 ml of the same buffer. After application of the sample it was run in with 0.1 ml of buffer and left for 45 min. The hemoglobin was then eluted under gravity with 0.2 ml aliquots of the glycine buffer, collecting 0.2 ml samples. Hemoglobin which remained tightly adsorbed to the column was eluted with 1.0 mM mannitol. The UV absorption spectra of all samples was recorded from 250-500 nm and the Δ420 nm absorbance used to plot the elution profile. Hemoglobin remaining bound after elution with mannitol was removed with 1.0 M NaOH.

The PBA-60 column was then flushed with at least ten column volumes of buffer between application of each of the following 0.2 ml samples in 50 mM glycine buffer; 1mM *Parasponia* $HbIO_2$, 1mM *Sesbania* HbO_2, 1mM Soybean $LbaO_2$.

Wheat germ lectin agarose. Wheat germ lectin is specific for the sugar N-acetyl 1-D-glucosamine found in some glycoproteins, and was chosen for affinity chromatography because inital analysis of *Casuarina* hemoglobin by gas-liquid chromatography indicated the presence of glucosamine.

A 0.2 ml sample of 50 μM HbO_2 (as used on Phenyl Boronate Agarose) in buffer (50 mM phosphate, 1 mM EDTA, 0.2 M NaCl, pH 7.2) was applied to a 3.9 x 0.5 cm (0.8 ml) column of Wheat Germ Lectin Agarose (Pharmacia 423608) which had been pre-flushed with 50 ml of buffer. Then 0.2 ml aliquots of buffer were added and 0.2 ml fractions collected under gravity, and spectra recorded to determine the elution profile. After *Casuarina* hemoglobin had been eluted (as a single symmetrical peak) the column was flushed with 100 ml of buffer and the procedure repeated using a 0.2 ml sample of 1.0 mM sperm whale myoglobin, a protein known not to contain any conjugated groups.

Analysis of sugars by gas-liquid chromatography and mass spectrometry.

This experiment was undertaken with Dr William F. Dudman of the Division of Plant Industry, Canberra, ACT 2601.

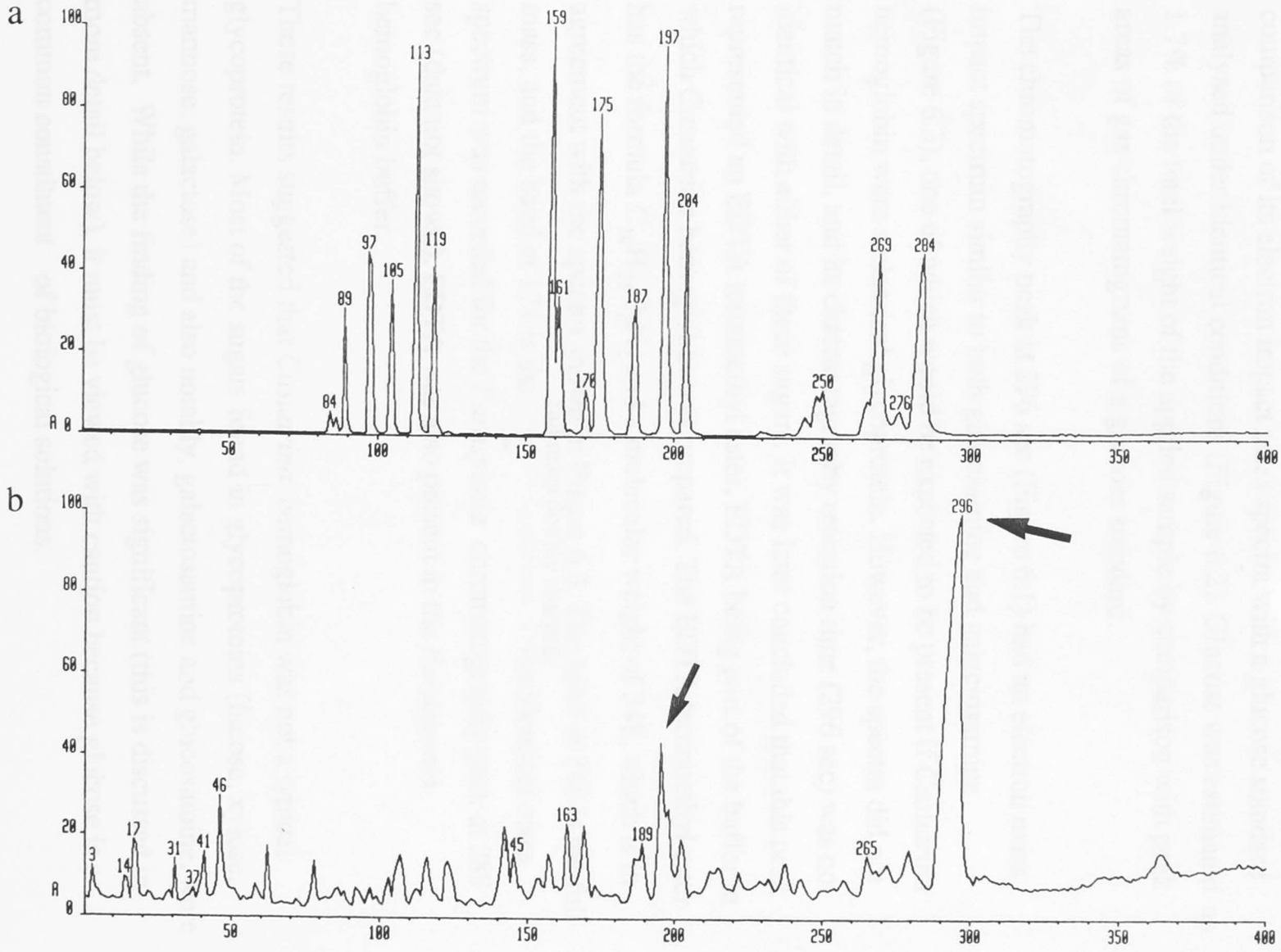
A sample (ca. 0.3 mg) of *Casuarina* hemoglobin was analysed for sugar components by the procedure of Clamp *et al.* (1971). After treatment with 0.9 M hydrogen chloride in methanol at 82° C for 16 h, neutralisation, re-N-acetylation and trimethylsilylation as described, the reaction mixture was analysed by gas-liquid chromatography/mass spectrometry on a BP-5 capillary column in a Varian 1400 gas chromatograph interfaced to a VG Micromass 70-70 mass spectrometer. Reference was made to DeJongh *et al.* (1969) during analysis of the mass spectra.

Results and Discussion

Analysis of hemoglobin for sugars.

Analysis of acid-hydrolysed *Casuarina* hemoglobin by gas-liquid chromatography (by courtesy of Dr W. F. Dudman, CSIRO Division of Plant Industry, Canberra) was initially interpreted as indicating the presence of at least glucose, and possibly other sugars (Figure 6.1), on the

Figure 6.1: Gas chromatography traces of (a) a glycoprotein standard mixture of sugars and (b) *Casuarina glauca* hemoglobin. The *C. glauca* sample contained 0.3 mg of hemoglobin which had been part purified by S200 chromatography and YM10 ultrafiltration (see Materials and Methods, Chapter 4). The sugars in the glycoprotein sample are identified by number (seconds after sample injection) as follows:
89, 97, 105 - fucose; 113, 119 - xylose; 159 - mannose;
175, 187 - galactose; 197, 204 - glucose;
250, 269 - galactosamine; 284 - glucosamine.
The peaks indicated by the two arrows have their mass spectra shown in Figures 6.2 and 6.3 .
The chromatography was undertaken on a Varian 1400 gas chromatograph directly interfaced to a mass spectrometer.


a
100
80
60
40
20
A 0
159
197
113
175
204
97
119
269
284
185
89
161
187
178
250
84
276
50
100
150
200
250
300
350
400
b
100
80
60
40
20
A 0
296
46
17
163
31
41
189
3
14
145
265
37
50
100
150
200
250
300
350
400

basis of retention time of peaks compared with a glycoprotein sugar standard. When the sample was further analysed by on-line mass spectrometry (courtesy of Dr. M. Lacey, CSIRO Division of Entomology, Canberra) however, glucose was the only sugar confirmed as present, by comparison of its electron impact mass spectra with a glucose standard analysed under identical conditions (Figure 6.2). Glucose was estimated as 1.7% of the total weight of the applied sample by comparison with peak areas of gas chromatograms of a glucose standard.

The chromatography peak at 296 sec (Figure 6.1) had an electron mass impact spectrum similar to both glucosamine and galactosamine (Figure 6.3), one of which would be expected to be present if *Casuarina* hemoglobin were a classical glycoprotein. However, the spectra did not match in detail, and its chromatography retention time (296 sec) was not identical with either of these sugars. It was later concluded that this peak represented an EDTA tetramethyl ester, EDTA being part of the buffer in which *Casuarina* hemoglobin was prepared. The EDTA tetramethyl ester has the formula $C_{14}H_{24}O_8N_2$ and a molecular weight of 348, which is in agreement with the spectra shown in Figure 6.3. The band at 348 is the full mass, and the band at 174 is the full mass doubly charged. An identical mass spectrum was recorded for the *Parasponia* chromatography peak at 289 sec (data not shown). EDTA was also present in the *Parasponia* hemoglobin buffer.

These results suggested that *Casuarina* hemoglobin was not a typical glycoprotein. Most of the sugars found in glycoproteins (fucose, xylose, mannose, galactose) and also notably, galactosamine and glucosamine, were absent. While the finding of glucose was significant (this is discussed in more detail below), it must be viewed with caution because glucose is a common constituent of biological solutions.

Con-A Sepharose and Wheat Germ Lectin Agarose Affinity Chromatography.

Affinity chromatography supported the conclusion that *Casuarina*

Figure 6.2: The mass spectra of:
(a) glucose; peak number 196, on the glycoprotein standard chromatogram (Figure 6.1a), and
(b) peak number 195 on the *C. glauca* hemoglobin chromatogram (Figure 6.1b). The mass spectrum of this peak confirms that it is glucose.
The spectra were recorded on a VB Micromass 70-70 mass spectrometer which was interfaced directly to the gas chromatograph.

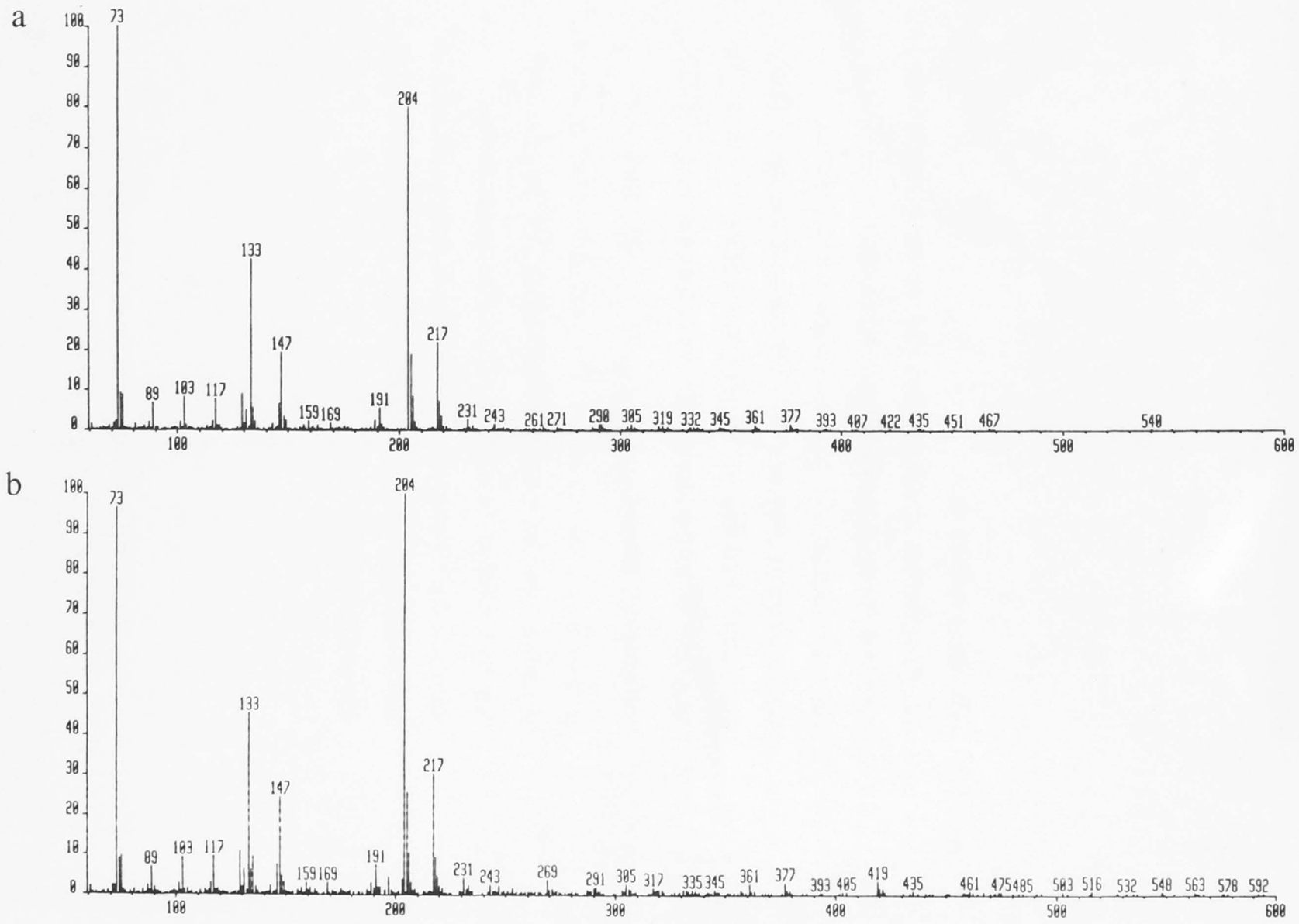
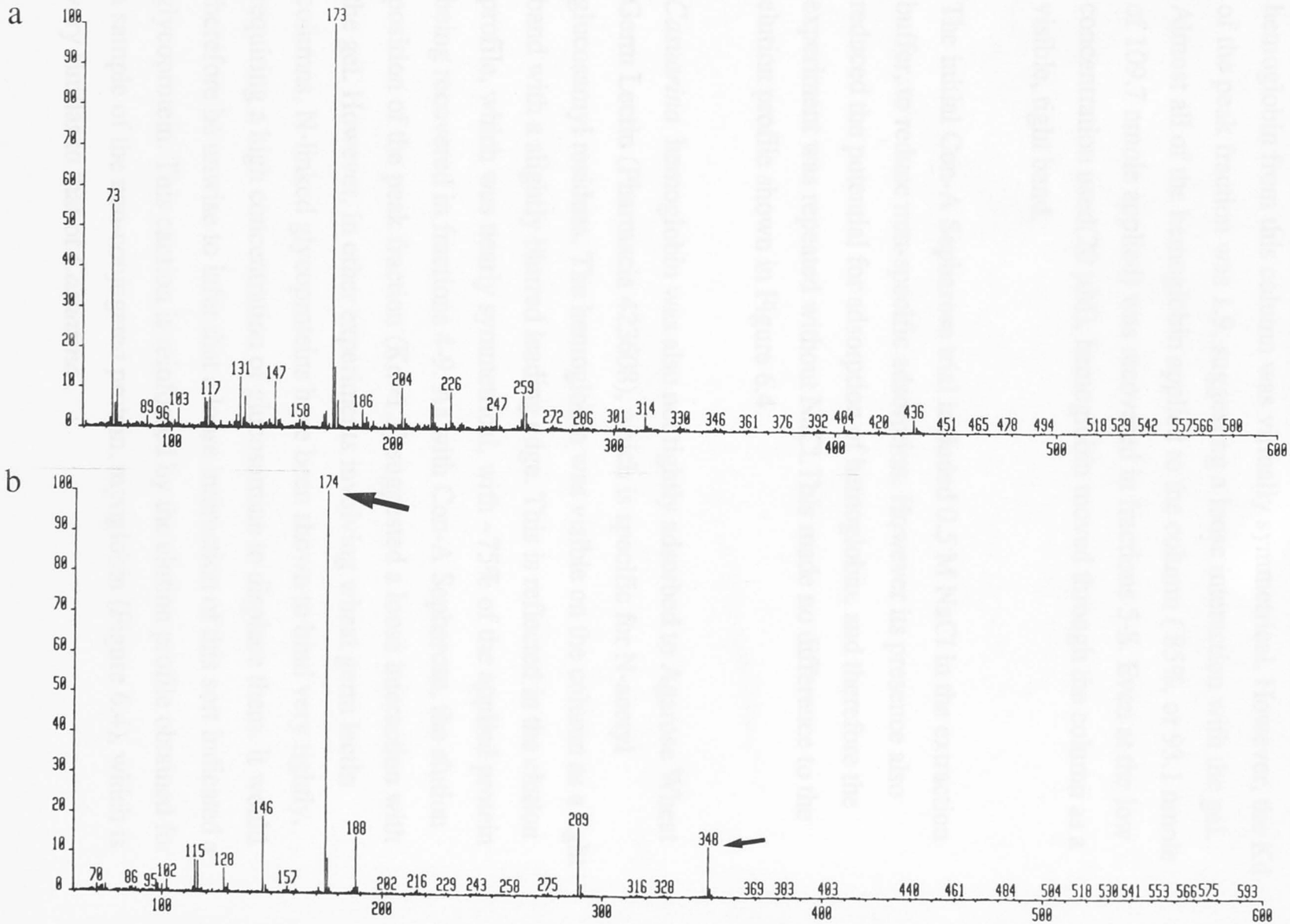

a
b
73
89
103
117
133
147
159
169
191
204
217
231
243
261
271
290
305
319
332
345
361
377
393
407
422
435
451
467
540
269
291
317
335
419
461
475
485
503
516
532
548
563
578
592

Figure 6.3: The mass spectra of:
(a) glucosamine; peak number 284, on the glycoprotein standard chromatogram (Figure 6.1a), and
(b) peak number 296 on the *C. glauca* hemoglobin chromatogram (Figure 6.1b). The mass spectrum of this peak confirmed that it was not glucosamine. However, the spectrum fitted precisely with that expected for the EDTA tetra methyl ester ($C_{14}H_{24}O_8N_2$; MW 348) which was present in the sample buffer. The peak at 348 (small arrow) represents the full mass and the peak at 174 (large arrow) represents the half molecule. The same spectrum was recorded for a *Parasponia* hemoglobin sample which also contained the EDTA tetra methyl ester in the sample buffer (data not shown).


a
b
73
89
96
103
117
131
147
158
173
186
204
226
247
259
272
286
301
314
330
346
361
376
392
404
420
436
451
465
478
494
518
529
542
557
566
580
174
70
86
95
102
115
128
146
157
188
202
216
229
243
258
275
289
316
328
348
369
383
403
440
461
484
504
518
530
541
553
566
575
593

hemoglobin was not a typical glycoprotein. The *Casuarina* protein was not tightly adsorbed to Con-A Sepharose (Pharmacia HK26179), even under favourable conditions. Figure 6.4 shows that the elution profile of hemoglobin from this column was virtually symmetrical. However, the Kd of the peak fraction was 1.9, suggesting a loose interaction with the gel. Almost all of the hemoglobin applied to the column (85%, or 93.1 nmole of 109.7 nmole applied) was recovered in fractions 5-8. Even at the low concentration used(20 μM), hemoglobin moved through the column as a visible, tight band.

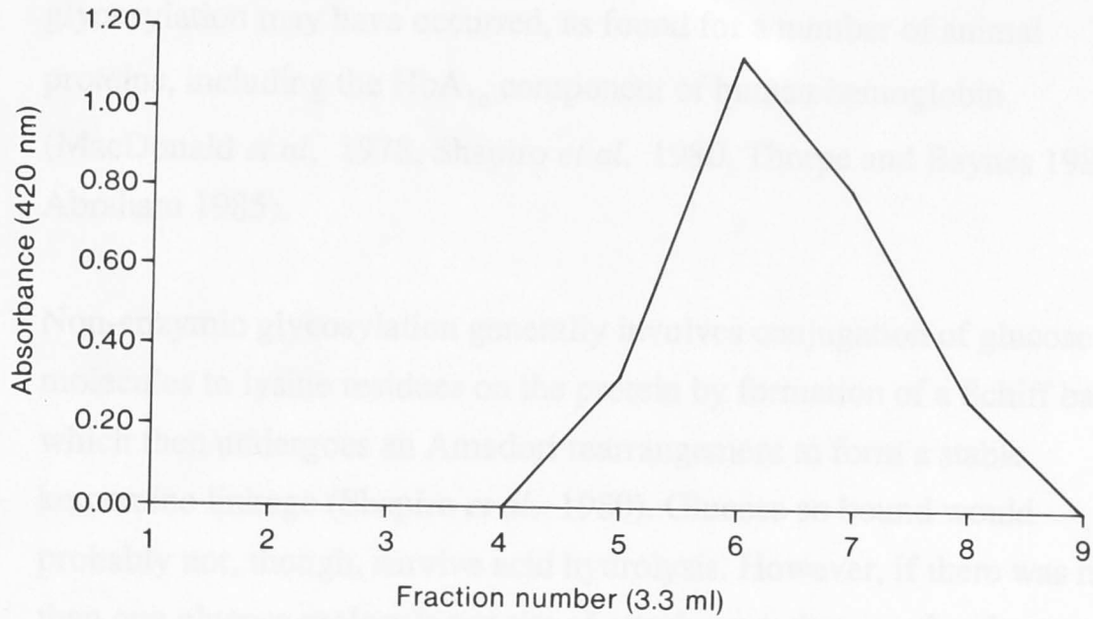
The initial Con-A Sepharose trial included 0.5 M NaCl in the extraction buffer, to reduce non-specific adsorption. However its presence also reduced the potential for adsorption of hemoglobin, and therefore the experiment was repeated without NaCl.This made no difference to the elution profile shown in Figure 6.4.

Casuarina hemoglobin was also not tightly adsorbed to Agarose Wheat Germ Lectin (Pharmacia 423608), which is specific for N-acetyl glucosamyl residues. The hemoglobin was visible on the column as a tight band with a slightly blurred leading edge. This is reflected in the elution profile, which was nearly symmetrical, with ~75% of the applied protein being recovered in fractions 4-9. As with Con-A Sepharose, the elution position of the peak fraction (Kd=1.5) suggested a loose interaction with the gel. However, in other experiments involving wheat germ lectin columns, N-linked glycoproteins have been shown to bind very tightly, requiring a high concentration of glucosamine to displace them. It would therefore be unwise to infer that a loose interaction of this sort indicated a glycoprotein. This caution is reinforced by the elution profile obtained for a sample of the non-conjugated protein, myoglobin (Figure 6.4), which is very similar to that of *Casuarina* .

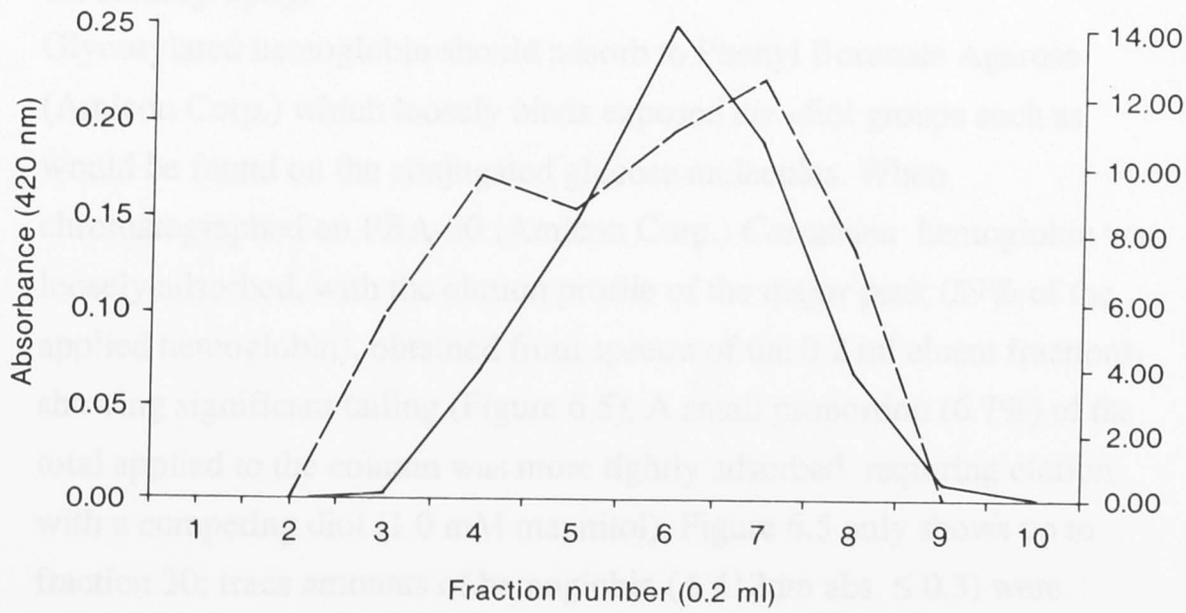
The combined results of sugar analyses, Con-A Sepharose and Wheat Germ Lectin Sepharose 6MB column chromatography effectively ruled out the possibility that *Casuarina* hemoglobin is a classical glycoprotein. This

Figure 6.4: (a) Chromatography of a 5.6 ml sample of 20 μM crude *Casuarina* HbCO on a column of Concanavalin-A Sepharose, as described in Materials and Methods. The hemoglobin sample had been extracted and centrifuged under the conditions described in the Standard Extraction Procedure (Chapter 4). Fractions were monitored at Δ420 nm absorbance; ie, the difference between the Soret peak (421 nm) and a line drawn between inflections on either side of the peak.

(b) Chromatography of 0.2 ml samples of 50 μM *Casuarina* HbO_2 (solid line) and 20 mM sperm whale myoglobin (dashed line) on a wheat germ lectin Sepharose 6MB column, as described in Materials and Methods. The hemoglobin sample had been purified by Sephacryl S200 chromatography and ultrafiltration, under the conditions described in the Standard Extraction Procedure. Fractions were monitored at Δ420 nm absorbance (see above).


1.20
1.00
0.80
0.60
0.40
0.20
0.00
Absorbance (420 nm)
1
2
3
4
5
6
7
8
9
Fraction number (3.3 ml)

a


0.25
0.20
0.15
0.10
0.05
0.00
Absorbance (420 nm)
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
1
2
3
4
5
6
7
8
9
10
Fraction number (0.2 ml)

b

left the observations, summarised in the introduction, unexplained. However, assuming that the glucose identified by sugar analysis was not a contaminant, then a remaining possibility was that non-enzymic glycosylation may have occurred, as found for a number of animal proteins, including the HbA_{1c} component of human hemoglobin (MacDonald *et al.* 1978, Shapiro *et al.* 1980, Thorpe and Baynes 1982, Abraham 1985).

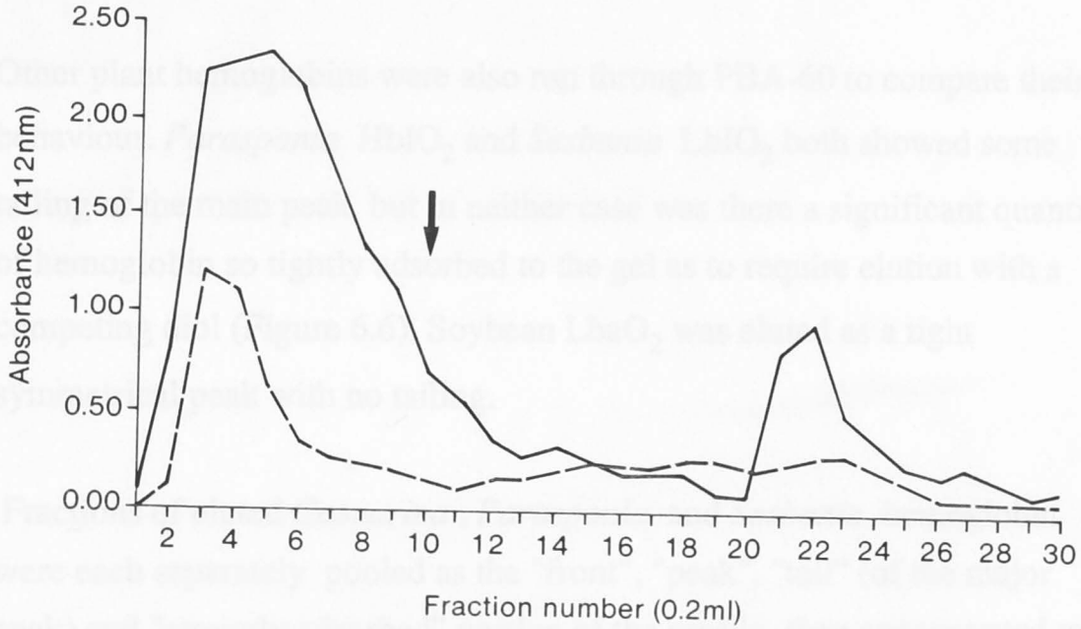
Non-enzymic glycosylation generally involves conjugation of glucose molecules to lysine residues on the protein by formation of a Schiff base which then undergoes an Amadori rearrangement to form a stable ketoamine linkage (Shapiro *et al.* 1980). Glucose so bound would probably not, though, survive acid hydrolysis. However, if there was more than one glucose molecule per site of attachment, those molecules not directly attached to the protein would not be destroyed. Assuming the simple case of two glucose molecules per site, then 1.7% glucose recovered is equivalent to 1.6 molecules of glucose per molecule of hemoglobin.

Possible glycosylation of* Casuarina *hemoglobin: PBA-60 chromatography.

Glycosylated hemoglobin should adsorb to Phenyl Boronate Agarose (Amicon Corp.) which loosely binds exposed *cis* -diol groups such as would be found on the conjugated glucose molecules. When chromatographed on PBA-30 (Amicon Corp.) *Casuarina* hemoglobin was loosely adsorbed, with the elution profile of the major peak (59% of the applied hemoglobin), obtained from spectra of the 0.2 ml eluent fractions, showing significant tailing (Figure 6.5). A small proportion (6.7%) of the total applied to the column was more tightly adsorbed, requiring elution with a competing diol (1.0 mM mannitol). Figure 6.5 only shows up to fraction 30; trace amounts of hemoglobin (Δ 412nm abs. ≤ 0.3) were recorded in each of the next 30+ fractions.

As anticipated, tighter adsorption was obtained on PBA-60, which has a greater number of binding sites than PBA-30. Using this gel, only 16.9%

Figure 6.5: Affinity chromatography of 0.2 ml part-purified *Casuarina* HbO_2 samples on Phenyl Boronate Agarose (PBA) columns (Amicon), which adsorbs *cis* - diol groups. PBA-60 (dashed line) has 60-100 μmol boron/ml and PBA-30 (solid line) has 30-50 μmol boron/ml. Sample concentration was 50 μM in 50 mM glycine buffer (pH 9.0 at 4°C). The major peak of hemoglobin (Fr. 5 on PBA-30, Fr. 3 on PBA-60) was eluted under gravity with 0.2 ml aliquots of the glycine buffer, collecting 0.2 ml samples. The minor hemoglobin peak (Fr. 22 on both PBA-30 and -60), which remained tightly adsorbed to the column, was eluted with 1.0 mM mannitol (a competing diol). Mannitol added from fraction 10 (arrowed).


2.50
2.00
1.50
1.00
0.50
0.00
Absorbance (412nm)
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Fraction number (0.2ml)

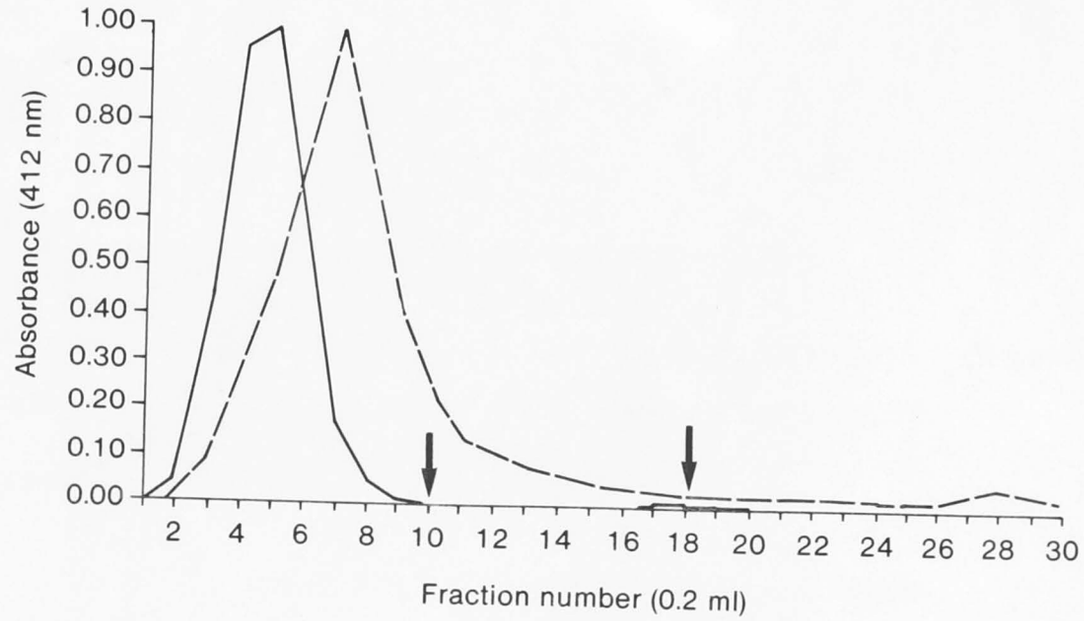
of the applied hemoglobin was eluted in the first peak and, once again, there was pronounced tailing (Figure 6.5).When the first peak had been eluted there remained in the gel a visible, diffuse band of hemoglobin near the bottom of the column. Some of this hemoglobin was eluted with 1.0 mM mannitol, the second peak in the elution profile (Figure 6.5) containing 14.2% of the applied hemoglobin. As with PBA-60, small amounts of hemoglobin were recorded in each of the next 30+ fractions (not shown in the figure).

Other plant hemoglobins were also run through PBA-60 to compare their behaviour. *Parasponia* $HbIO_2$ and *Sesbania* $LbIO_2$ both showed some tailing of the main peak, but in neither case was there a significant quantity of hemoglobin so tightly adsorbed to the gel as to require elution with a competing diol (Figure 6.6). Soybean $LbaO_2$ was eluted as a tight symmetrical peak with no tailing.

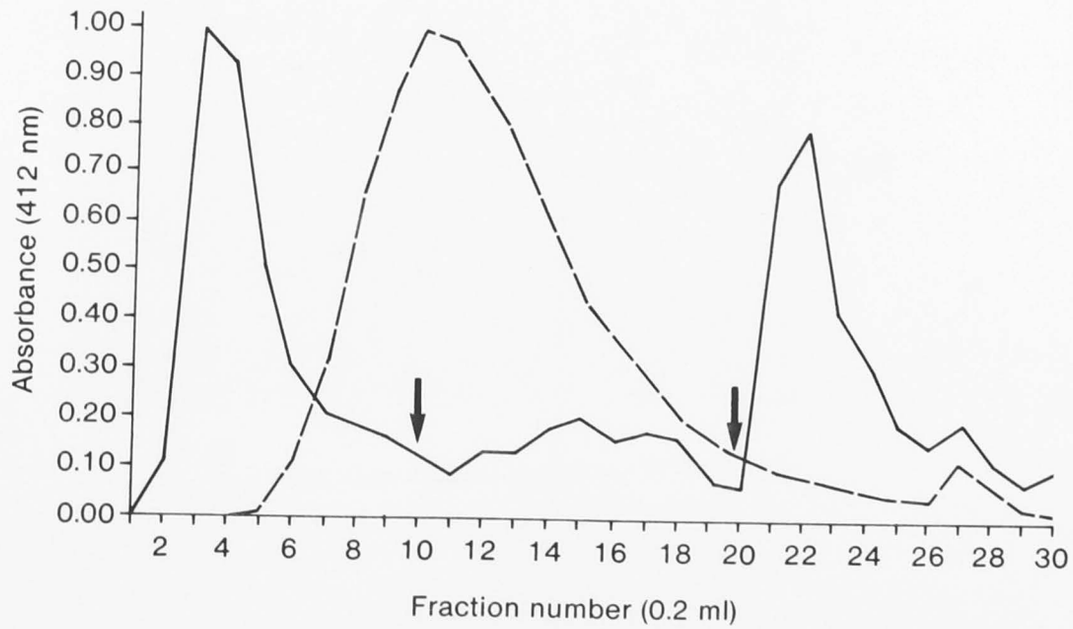
Fractions of eluted *Casuarina* , *Parasponia* and *Sesbania* hemoglobin were each separately pooled as the "front", "peak", "tail" (of the major peak) and "strongly adsorbed" portion of the profile, then concentrated to small volume (approx. 45 µl) in Amicon Centricon Microconcentrators, diluted with water and reconcentrated. These concentrated samples were run on analytical scale isoelectric focusing gels according to the procedures outlined in the Materials and Methods, Chapter Four.

This showed that in the case of *Casuarina* hemoglobin the column had discriminated between the isoelectric focusing components of the sample as applied to the PBA-60 column (Figure 6.7). The more negatively charged components (more acidic pI) were eluted from PBA-60 before the less negatively charged components (more alkaline pI).The same elution pattern was observed when a sample of *Sesbania* Hb was run through the same PBA-60 column (Figure 6.6) and the pooled fractions subjected to isoelectric focusing (pH 4.5-5.4) (Figure 6.8). No such discrimination was observed with *Parasponia* hemoglobin (Figure 6.9)

Figure 6.6: Affinity chromatography of a range of plant hemoglobins (labelled) on Phenyl Boronate Agarose-60 (PBA-60, Amicon Corp.). Graph 'a' includes Soybean Lba (solid line) and *Sesbania* Lb (dashed line). Graph 'b' includes *Casuarina* Hb (solid line) and *Parasponia* Hb (dashed line).
Sample volume in each case was 0.2 ml. *Casuarina* hemoglobin was ~50 μM in 50 mM glycine buffer (pH 9.0 at 4^{o}C). All other samples were ~1.0 mM.
Absorbance of all fractions was normalised to a maximum of 1.0 for easier comparison of elution profiles. *Casuarina* hemoglobin was the only sample to show visible adsorption to the gel.
The dark arrows indicate the point at which the glycine buffer was replaced by a 100 mM mannitol solution to displace loosely adsorbed hemoglobin.


1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Absorbance (412 nm)
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Fraction number (0.2 ml)

a


1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Absorbance (412 nm)
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Fraction number (0.2 ml)

b

Figure 6.7: Analytical scale isoelectric focusing (pH 4-6.5) of *Casuarina* hemoglobin fractions (15 µl samples) eluted from an affinity chromatography column of Phenyl Boronate Agarose - 60 (PBA-60). The elution profile of this column is shown in Figure 6.5. Note the increased relative intensity of bands with a more alkaline pI (arrowed) in the later eluted fractions.

IEF track identification:

Tracks 1	- the mixture as applied to PBA-60
Track 2	- fractions 3, 4, 5 ex PBA-60
Track 3	- fractions 6 to 10
Track 4	- fractions 12 to 19
Track 5	- fractions 21 to 24

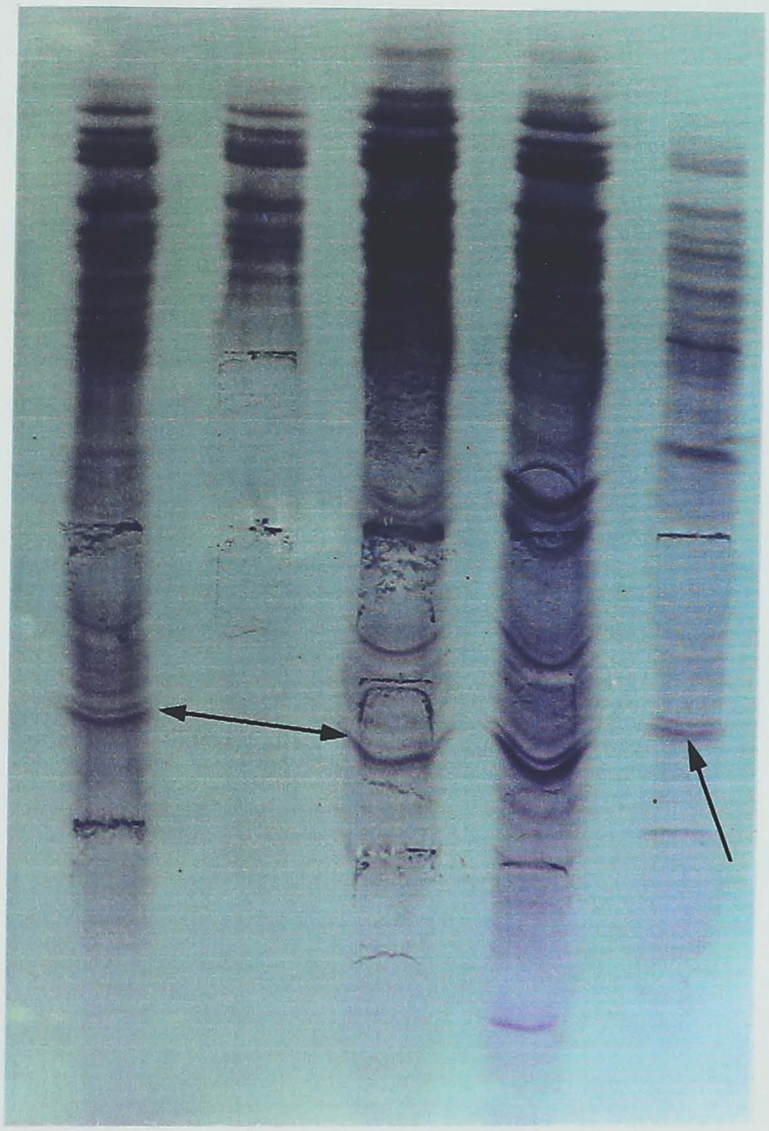
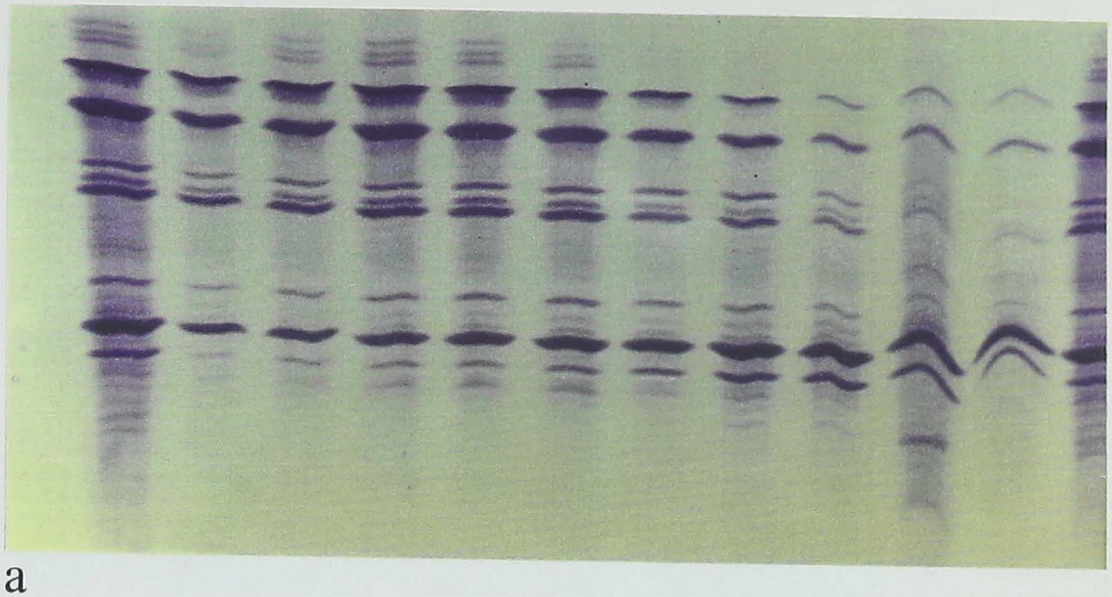


Figure 6.8: Analytical scale isoelectric focusing (pH 4-6.5) of *Sesbania* hemoglobin fractions (15 µl samples) eluted from an affinity chromatography column of Phenyl Boronate Agarose - 60 (PBA-60). The elution profile of this column is shown in Figure 6.6.

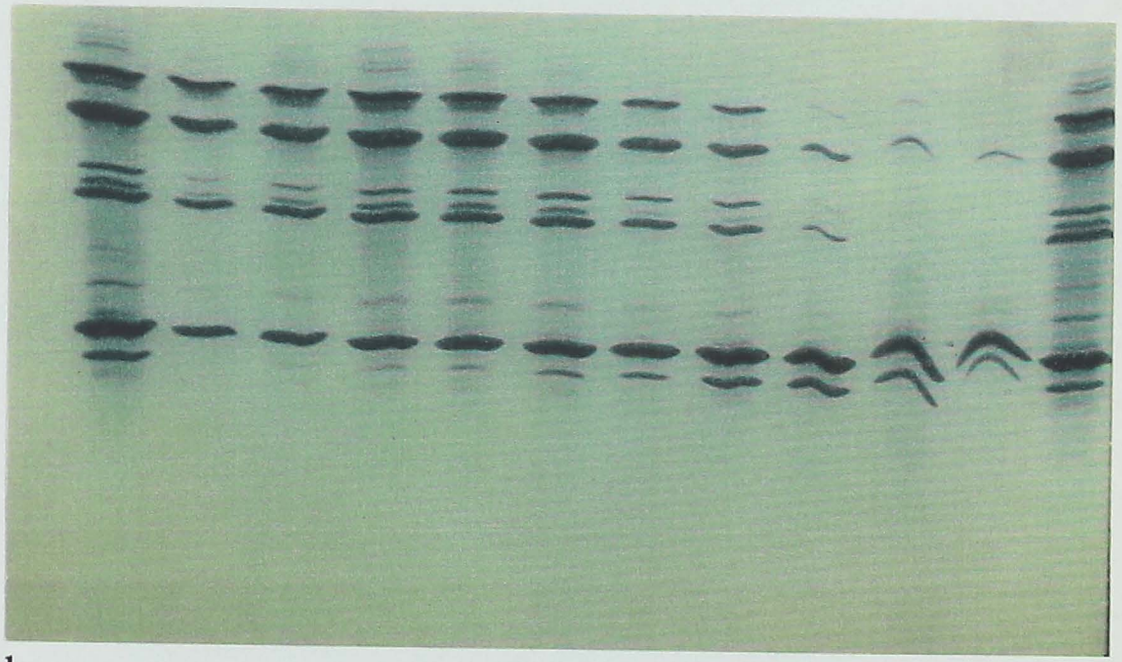
(a) protein stain (Coomassie Blue)
(b) heme stain (3-3$^{!}$, 5-5$^{!}$ tetramethyl benzidine)
(c) unstained hemoglobin

IEF track identification:

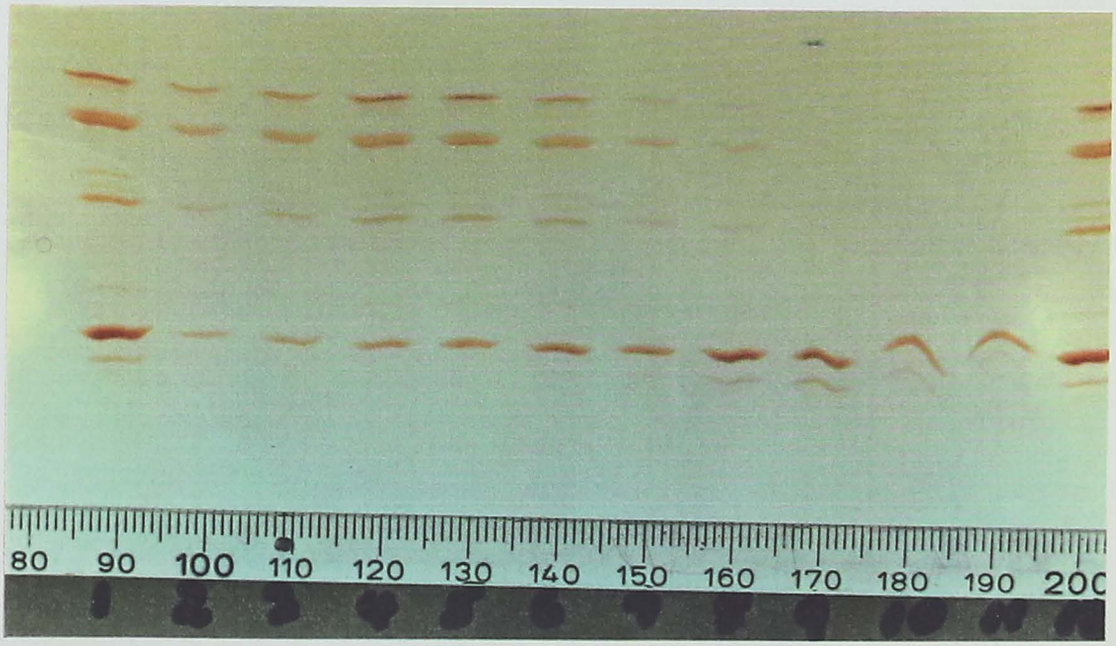
Tracks 1 & 12	- the mixture as applied to PBA-60
Track 2	- fractions 3, 4 ex PBA-60
Track 3	- fraction 5
Track 4	- fraction 6
Track 5	- fraction 7
Track 6	- fraction 8
Track 7	- fraction 9
Track 8	- fractions 10, 11
Track 9	- fractions 12 to 17
Track 10	- fractions 27, 28
Track 11	- fractions 37, 38



a



b



c

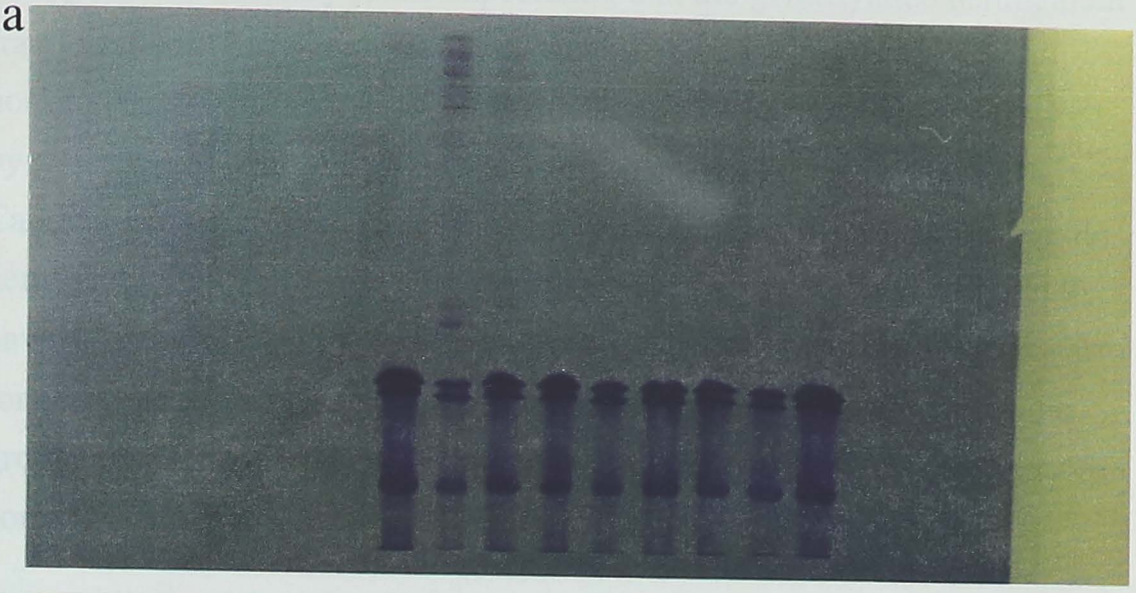
Figure 6.9: Analytical scale isoelectric focusing (pH 4-6.5) of *Parasponia* hemoglobin fractions (15 µl samples) eluted from an affinity chromatography column of Phenyl Boronate Agarose - 60 (PBA-60). The elution profile of this column is shown in Figure 6.6. Note that there are two distinct protein components on IEF, contrary to the findings of Appleby *et al* (1983).

(a) protein stain (Coomassie Blue)
(b) heme stain (3-3$^{!}$, 5-5$^{!}$ tetramethyl benzidine)
(c) unstained hemoglobin

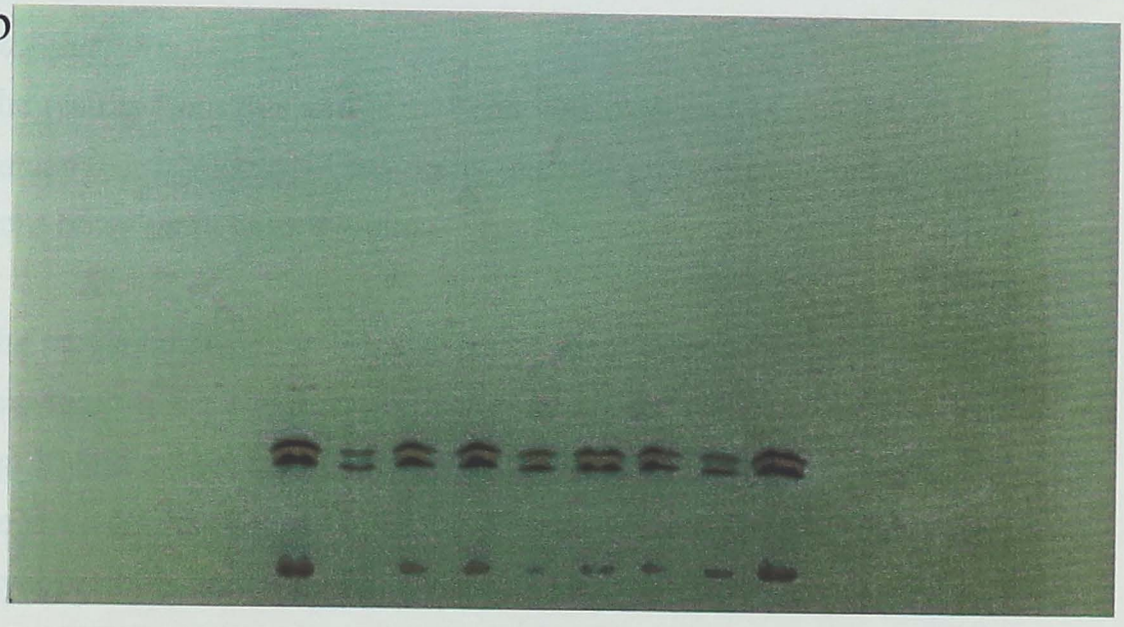
IEF track identification:

Tracks 1 & 9	- the mixture as applied to PBA-60
Track 2	- fractions 5, 6 ex PBA-60
Track 3	- fractions 7, 8
Track 4	- fractions 9,10
Track 5	- fraction 11
Track 6	- fractions 12, 13
Track 7	- fractions 14 to 17
Track 8	- fractions 26, 27

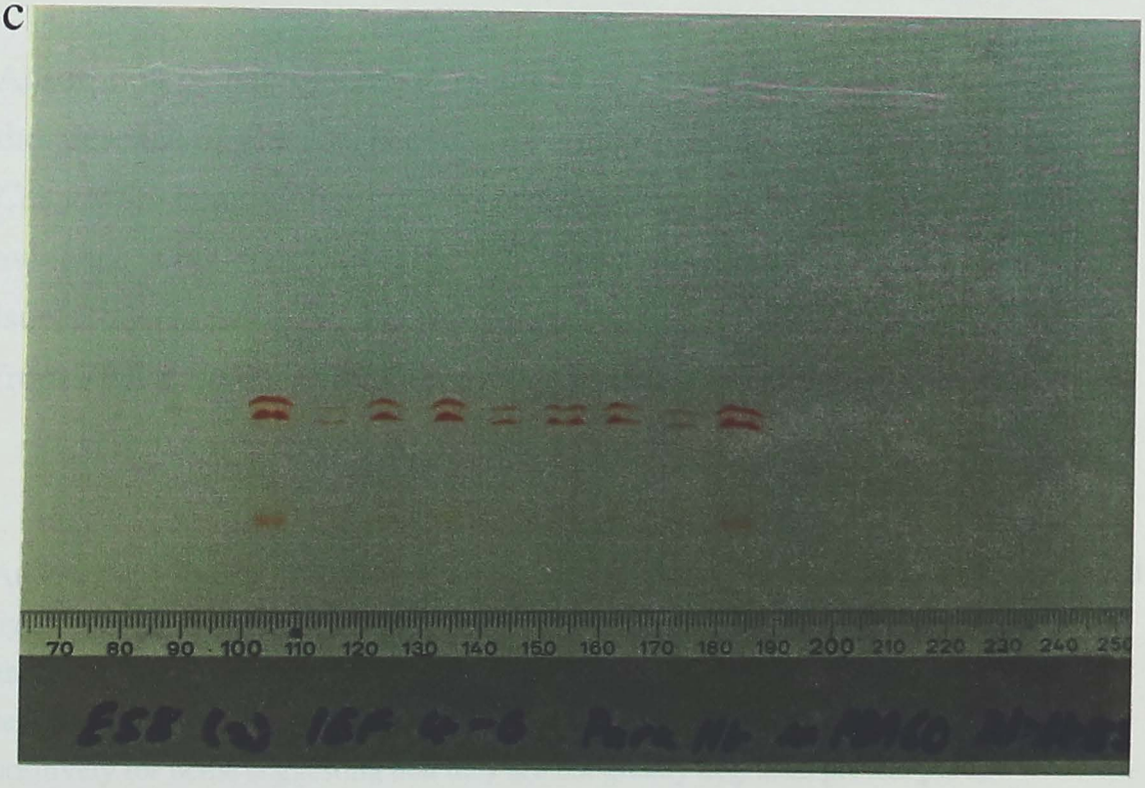
a



b



c


70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 25

This pattern is contrary to the expectation that the glycosylated hemoglobin fraction should have a greater net negative charge than the non-glycosylated fraction, assuming that basic lysine residues are blocked by glucose and their charge partially or completely lost (Abraham 1985). Candiano *et al.* (1984) made an observation similar to mine on *Casuarina* hemoglobin with human serum albumin, finding glycosylated albumin to have a higher pI than unmodified albumin. No explanation can yet be given for this observation. It is possible that either glucose is binding to acidic groups on the protein (carboxyl residues), or the conjugated group is more complex than anticipated and is adding basic charges to the protein.

Conclusions.

The results from this and earlier chapters clearly demonstrate that *Casuarina* hemoglobin has many unusual properties which set it apart from other plant hemoglobins.

The studies reported in this Chapter disprove the hypothesis that *Casuarina* hemoglobin is a classical glycoprotein. It was not adsorbed to any significant extent on affinity columns of either Con-A Sepharose or Wheat Germ Lectin Sepharose 6MB; also, most of the sugars normally found in glycoproteins, including glucosamine and galactosamine, were absent.

Adsorption by hemoglobin to columns of Phenyl Boronate Agarose, plus the presence of glucose, is evidence which supports the hypothesis that *Casuarina* hemoglobin is a non-enzymically glycosylated protein. This evidence is not conclusive, though, and is confused by the anomylous isoelectric focusing behaviour of *Casuarina* hemoglobin fractions eluted from PBA columns. More research is needed to resolve this question.

An incidental finding from this work was that analytical scale isoelectric focusing followed by protein staining revealed hemoglobin bands not previously identified in both *Sesbania* and *Parasponia* hemoglobin (Figures 6.8, 6.9). It is not known whether these bands are separate gene products or the result of post-translational modification, but all stained positively for heme suggesting that they are not greatly degraded protein products.

CONCLUSIONS - SECTION II

The origin and distribution of hemoglobin in plants.

In Chapter Three I summarised the state of knowledge, up to early 1983 when my research began, concerning the origin and distribution of hemoglobin in plants. At that time, considerable uncertainty surrounded this topic, particularily following the purification of hemoglobin from the root nodules of the non-leguminous *Parasponia - Rhizobium* symbiosis. This discovery apparently contradicted the hypotheses of convergent evolution and lateral gene transfer from the animal kingdom, both of which had been previously advanced to explain the occurrence of hemoglobin in plants.

The results presented in this thesis have conclusively proven that hemoglobin does occur in *Casuarina* root nodules. Hemoglobins, purified from both *C. glauca* and *C. cunninghamiana* nodules, were found to have properties typical of other plant and animal hemoglobins, including the form of their optical spectra and the diagnostic feature of reversible oxygen binding which distinguished them from plant peroxidases.

Further characterisation of *C. glauca* hemoglobin revealed many properties which indicated its close phylogenetic relationship with other plant hemoglobins, namely:

* extensive amino acid sequence homology, including residues critical for oxygen binding,
* immunological cross-reactivity, and
* similar kinetics of reactions with oxygen and carbon monoxide.

The detailed characterisation of *Casuarina* hemoglobin described in this thesis gives credence to the observation by Tjepkema *et al.* (1983) of hemoglobin in a range of actinorhizal species. This protein is thus far

more common and widespread in the plant kingdom than had previously been assumed (see Figure 3.1 for phylogenetic schemes).

What does all this imply for the origin of hemoglobin in plants? Firstly, these findings rule out the hypothesis of convergent evolution. There are just too many fundamental similarities between animal and plant hemoglobins and too many occurrences of plant hemoglobins for them to have evolved from separate origins. Secondly, it makes the hypothesis of lateral gene transfer from the animal kingdom very difficult to sustain, particularily when there is the much simpler explanation of evolution by vertical descent from an ancestor common to plants and animals.

The hypothesis of evolution by vertical descent is strongly supported by the results of recent experiments where hemoglobin was identified in root tissue of non-nodulating plants (Bogusz *et al.* 1988, C. A. Appleby, pers. comm.). Not only do these results indicate an even more widespread distribution of hemoglobin in plants than previously recognised, they also, and most importantly, indicate a possible function for hemoglobin prior to the evolution of root nodule symbioses. This is a particularily significant point because the divergence of plants and animals occurred well before the evolution of nitrogen-fixing symbioses, and if hemoglobin is to have survived during the intervening period it must have had an alternative function.

It is clear from the work reported in this thesis and from other recent experimental studies referred to above that hemoglobin is neither fundamentally nor primarily an animal protein.

The purification of hemoglobin from *Casuarina* nodules, reported in this thesis, has thus been important in developing a more comprehensive understanding of the origin and distribution of hemoglobin in plants. This knowledge is also important for the practical application of nitrogen fixation. A major goal of research in this field is to engineer the machinery necessary for nitrogen fixation into non-nodulating plants, e.g.

cereals. Regulation of the oxygen supply in the environment of the oxygen-labile nitrogenase is one of the critical requirements which must be met for this goal is to be achieved. If, as we now suspect, the gene for hemoglobin is already present in all plants, the task of protecting nitrogenase from oxygen is more clearly defined. Molecular biologists can concentrate on learning how to appropriately switch the hemoglobin gene on, rather than unnecessarily importing a gene from another source.

The function of hemoglobin in Casuarina nodules.

Until hemoglobin was purified from *Casuarina* nodules, it was widely assumed to be absent from any actinorhizal symbiosis. This led to a variety of mechanisms being proposed to explain the ability of these nodules to fix nitrogen in atmospheric oxygen, most notably the existence of physical barriers to isolate the oxygen-labile nitrogenase. While physical barriers are undoubtedly important in regulating oxygen movement within the nodule, it seems that in *Casuarina* at least, as in the legumes and *Parasponia* , hemoglobin may also play an important role.

The critical feature of hemoglobin in legumes and *Parasponia* is that it facilitates the diffusion of oxygen to the endophyte at a rate commensurate with efficient respiration, yet with vanishingly small free oxygen concentration maintained in the immediate environment of the bacteroids (Appleby 1974).

The kinetics of the reactions of *Casuarina* hemoglobin with oxygen and carbon monoxide were determined as part of this research project. They were of the same order of magnitude as those previously reported for both soybean leghemoglobin a (Appleby *et al.* 1983) and *Parasponia* hemoglobin I (Wittenberg *et al.* 1986). This striking similarity suggests that their roles may be similar. There is indirect evidence in support of this. The absence of vesicles from *Casuarina* nodules is consistent with a very low free oxygen concentration in *Casuarina* nodules, vesicle production having previously been shown to be oxygen concentration dependent (Murry *et al.* 1985).

There remains, of course, work to be done to conclusively prove the role of hemoglobin in *Casuarina* nodules. The comprehensive studies undertaken by Bergersen and collegues in the study of leghemoglobin function should serve as a model for future reaesrch on the *Casuarina-Frankia* symbiosis. However, in the absence of more detailed work, it is reasonable to say that on the basis of the results presented in this thesis *Casuarina* hemoglobin has the potential to function in the facilitated diffusion of oxygen to the *Frankia* endophyte.

BIBLIOGRAPHY

Abraham, E. C. (1985) Glycosylated Hemoglobins. Methods of Analysis and Clinical Application. Marcel Dekker, Inc., New York, 238 pp.

Alvey, N. G. (1977) GENSTAT. A general statistical program. Rothamsted Exp. Stn, Harpenden, Herts.

Antoine, M. and Niessing, J. (1984) Intron-less globin genes in the insect *Chironomus thummi thummi*. *Nature* **310**, 795-798.

Antonini, E. (1965) Interrelationship between structure and function in hemoglobin and myoglobin. *Physiological Reviews* **45**, 123-170.

Appleby, C.A. (1969) Properties of leghaemoglobin *in vivo*, and its isolation as ferrous oxyleghaemoglobin. *Biochimica et Biophysica Acta* **188**, 222-229.

Appleby, C.A. (1974) Leghemoglobin. In: *The Biology of Nitrogen Fixation* (Quispel, A., Ed.). North Holland Publishing Company, Amsterdam.

Appleby, C.A. (1984) Leghemoglobin and *Rhizobium* respiration. *Annual Review of Plant Physiology* **35**, 443-478.

Appleby, C.A. (1985) Plant hemoglobin properties, function and genetic origin. In: *Nitrogen Fixation and CO_2 Metabolism.* (Ludden, P.W. and Burris, J.E., Eds.) pp 41-51. Elsevier Science Publishing Co. Inc., Amsterdam.

Appleby, C. A. and Bergersen, F. J. (1980) Preparation and experimental use of leghaemoglobin. In: *Methods for Evaluating Biological Nitrogen Fixation.* (Bergersen, F. J., Ed.) pp 315-335. John Wiley and Sons Ltd, Chichester.

Appleby, C.A., Blumberg, W.E., Bradbury, J.H., Fuchsman, W.H., Peisach, J., Wittenberg, B.A., Wittenberg, J.B. and P.E. Wright (1982) The mobile distal histidine of leghemoglobin. Does it control oxygen binding kinetics? In: *Hemoglobin and Oxygen Binding* (Chien Ho, ed) pp 435-441. Elsevier, Amsterdam.

Appleby, C. A., Bogusz, D., Dennis, E.S. and Peacock, W.J. (1988a) A role for haemoglobin in all plant roots? *Plant, Cell and Environment* **11**, 359-367.

Appleby, C.A., Bogusz, D., Dennis, E.S., Fleming, A.I., Landsmann, J. and Peacock, W.J. (1988b) The vertical evolution of plant hemoglobin genes. In: *Nitrogen Fixation: Hundred Years After* (Bothe *et al*, Eds.) pp 623-628. Gustav Fischer, Stuttgart.

Appleby, C.A., Bradbury, J.H., Morris, R.J., Wittenberg, B.A., Wittenberg, J.B. and Wright, P.E. (1983b) Leghemoglobin: kinetic, nuclear magnetic resonance, and optical studies of pH dependence of oxygen and carbon monoxide binding. *The Journal of Biological Chemistry* **258**, 2254-2259.

Appleby, C.A., Tjepkema, J.D. and Trinick, M.J. (1983a) Hemoglobin in a non-leguminous plant, *Parasponia* : possible genetic origin and function in nitrogen fixation. *Science* **220**, 951-953.

Arai, T. (Ed.) (1976) Actinomycetes. The boundary microorganisms. Toppan Company Ltd, Tokyo.

Arutyunyan, E. G. (1981) The structure of leghemoglobin (translation from Russian) *Molekulyarnaya Biologiya* **15**, 27-44.

Baker, D. and Torrey, J. G. (1980) Characterisation of an effective actinorhizal microsymbiont, *Frankia* sp. AvcI1 (Actinomycetales) *Canadian Journal of Microbiology* **26**, 1066-1071.

Baker, D., Pengelly, W. L. and Torrey, J. G. (1981) Immunochemical analysis of relationships among isolated Frankiae (Actinomycetales). *International journal of Systematic Bacteriology* **31**, 148-151.

Baker, D.D (1987) Relationships among pure cultured strains of *Frankia* based upon host specificity. *Physiologia Plantarum* **70** 245-248.

Beadle, N. C. W. (1981) The Vegetation of Australia. Gustav Fischer Verlag, Stuttgart.

Becking, J. H. (1970) Frankiaceae Fam. Nov. (Actinomycetales) with one new combination and six new species of the genus *Frankia* Brunchorst 1886, 174. *International Journal of Systematic Bacteriology* **20**, 201-220 .

Becking, J. H. (1974) Family III. Frankiaceae Becking, 1970, 201. In: *Bergeys Manual of Determinative Bacteriology* (Buchanan, R. E. and Gibons, N. E., Eds.) pp 701-706 Williams and Wilkins Co., Baltimore.

Belew, M., Porath, J., Fohlman, J. and Janson, J. (1978) Adsorption phenomena on Sephacryl S200 superfine. *Journal of Chromatography* **147**, 205-212.

Berg, R. H. (1983) Preliminary evidence for the involvement of suberization in infection of *Casuarina* . *Canadian Journal of Botany* **61**, 2910-2918.

Berg, R. H. (1987) Endophyte differentiation in *Casuarina* actinorhizae. *Protoplasma* **136**, 104-117.

Bergersen, F. J. (1982) Root nodules of legumes: structure and functions. Research Studies Press, Chichester.

Bergersen, F.J. and Appleby, C.A. (1981) Leghaemoglobin within bacteroid-enclosing membrane envelopes from soybean root nodules. *Planta* **152**, 534-543.

Bergersen, F.J., Turner, G.L. and Appleby, C.A. (1973) Studies on the physiological role of leghaemoglobin in soybean root nodules. *Biochimica et Biophysica Acta* **292**, 271-282.

Berry, A.M. and Torrey, J.G. (1985) Seed germination, seedling inoculation and establishment of *Alnus* spp. in containers in glasshouse trials. *Plant and Soil* **87**, 161-173.

Bittner, M., Kupferer, P. and Morris, C.F. (1980) Electrophoretic transfer of proteins and nucleic acids from slab gels to diazobenzyloxymethyl cellulose or nitrocellusose sheets. *Analytical Biochemistry* **102**, 459-471.

Bogusz, D., Appleby, C.A., Landsmann, J., Dennis, E.S., Trinick, M.J. and Peacock, W.J. (1988) Functioning haemoglobin genes in non-nodulating plants. *Nature* **331**, 178-180.

Bond, G. (1957) The development and significance of the root nodules of *Casuarina* . *Annals of Botany* **21**, 373-380.

Burger, W. C. (1981) Heresy revived: the monocot theory of angiosperm evolution. *Evolutionary Theory* **5**, 189-225.

Callaham, D., Del Tredici, P. and Torrey, J. G. (1978) Isolation and cultivation *in vitro* of the actinomycete causing root nodulation in *Comptonia* . *Science* **199**, 899-902.

Candiano, G., Ghiggeri, G.M., Delfino, G., Queirolo, C., Gianazza, E. and Righetti, P.G. (1984) Glycosylation of human albumin in diabetes mellitus: extensive microheterogeneity of serum and urinary species as revealed by isoelectric focusing. *Electrophoresis* **5**, 217-222.

Clamp, J.R., Bhatti, T. and Chambers, R.E. (1971) The determination of carbohydrate in biological materials by gas-liquid chromatography. *Methods of Biochemical Analysis* **19**, 229-344.

Coventry, D.R., Trinick, M.J. and Appleby, C.A. (1976) A search for a leghaemoglobin-like compound in root nodules of *Trema cannabina* Lour. *Biochimica et Biophysica Acta* **420**, 105-111.

Coyne, P.D. (1973) Some aspects of the autecology of casuarinas, with special reference to nitrogen fixation. *PhD Thesis*, Australian National University, Canberra.

Coyne, P.D. (1983) Specificity between *Casuarina* species and root nodule organisms. In: *Casuarina Ecology, Management and Utilisation* (Midgley, S.J., Turnbull, J.W. and Johnston, R.D., Eds.) pp 205-210. CSIRO, Melbourne.

Cronquist, A. (1981) An integrated system of classification of flowering plants. Columbia University Press, New York.

Csoma, C. and Polgar, L. (1984) Proteinase from germinating bean cotyledons. *Biochemistry Journal* **222**, 769-776.

Cunningham, L. W. (1971) Microheterogeneity and function of glycoproteins. In: *Glycoproteins of Blood Cells and Plasma* (Jameison, G. A. and Greenwalt, T. J., Eds.) pp16-34. Lippincott Company, Philadelphia.

Dahlgren, R.M.T. (1980) A revised system of classification of the angiosperms. *Botanical Journal of the Linnean Society* **80**, 91-124.

Dahlgren, R. M. T., Clifford, H. T. and Yeo, P. F. (1985) The Families of the Monocotyledons: Structure, Evolution and Taxonomy. Springer-Verlag, Berlin.

Davenport, H.E. (1960) Haemoglobin in the root nodules of *Casuarina cunninghamiana* . *Nature* **186**, 653-654.

Dawson, J. O. and Sun, S-H. (1981) The effect of *Frankia* isolates from *Comptonia peregrina* and *Alnus crispa* on the growth of *Alnus glutinosa*, *A. cordata* and *A. incana* clones. *Canadian Journal of Forest Research* **11**, 758-762.

Dayhoff, M.O., Eck, R.V. and Park, C.M. (1972) A model of evolutionary change in proteins. In: *Atlas of Protein Sequence and Structure, Volume 5* (Dayhoff, M.O., Ed.) pp 89-99. National Biomedical Research Foundation, Washington.

DeJongh, D.C., Radford, T., Hribar, J.D., Hanessian, S., Bieber, M., Dawson, G. and Sweeley, C.C. (1969) Analysis of trimethylsilyl derivatives of carbohydrates by gas chromatography and mass spectrometry. *Journal of the American Chemical Society* **91**, 1728-1740.

Dillon, J. T. and Baker, D. (1982) Variations in nitrogenase activity among pure-cultured *Frankia* strains tested on actinorhizal plants as an indication of symbiotic compatibility. *New Phytologist* **92**, 215-219.

Dilworth, M. J. and Parker, C. A. (1969) Development of the nitrogen-fixing system in legumes. *Journal of Theoretical Biology* **25**, 208-218.

Doolittle, W. F. (1978) Genes in pieces: were they ever together? *Nature* **272**, 581-582.

Doran, J.C. and Hall, N. (1983) Notes on fifteen Australian *Casuarina* species. In: *Casuarina Ecology, Management and Utilisation* (Midgley, S.J., Turnbull, J.W. and Johnston, R.D., Eds.) pp 19-52. CSIRO, Melbourne.

Edmonson, A. B. (1965) Amino-acid sequence of sperm whale myoglobin. *Nature* **205**, 883-887.

Egle, K. and Munding, H. (1951) Über den gehalt an häminkörpen in den wurzelknöllchen von nicht-leguminosen. *Naturwissenschaften* **38**, 548-549.

Egorov, T. A., Kazakov, V. K., Shakhparonov, M. I. and Feigina, M. Y. (1980) Amino acid sequence of leghemoglobins I and II from yellow lupine nodules. *Biorg. Chem.* **6**, 666-683.

Fuchsman, W.H. and Appleby, C.A. (1979) Separation and determination of the relative concentrations of the homogeneous components of soybean leghemoglobin by isoelectric focusing. *Biochimica et Biophysica Acta* **579**, 314-324.

Garnier, J., Osguthorpe, D. J. and Robson, B. (1978) Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *Journal of Molecular Biology* **120**, 97-120.

Gauthier, D., Diem, H.G. and Dommergues, Y. R. (1981) *In vitro* nitrogen fixation by two actinomycete strains isolated from *Casuarina* nodules. *Applied Environmental Microbiology* **41**, 306-308.

Gauthier, D., Diem, H. G. and Dommergues, Y. R. (1984) Tropical and subtropical actinorhizal plants. *Pesquisa Agropecuaria Brasieira* **19**, 119-136.

Giannasi, D.E. and Crawford, D.J. (1986) Biochemical systematics. II: A reprise. In: *Evolutionary Biology, Volume 20* (Hecht, M.K., Wallace, B. and Prance, G.T., Eds.) p 25. Plenum Press, New York.

Gibson, Q. H. and Milnes, L. (1964) Apparatus for rapid and sensitive spectrophotometry. *Biochemical Journal* **91**, 161-171.

Gibson, Q.H., Wittenberg, J.B., Wittenberg, B.A., Bogusz, D. and Appleby, C.A. (1989) The kinetics of ligand binding to plant hemoglobin: structural implications. *The Journal of Biological Chemistry* **264**, 100-107.

Gilbert, W. (1981) DNA sequencing and gene structure. *Science* **214**, 1305-1312.

Go, M. (1981) Correlation of DNA exonic regions with protein structural units in haemoglobin. *Nature* **291**, 90-92.

Gonenne, A. and Ernst, R. (1978) Solubilisation of membrane proteins by sulfobetaines, novel zwitterionic surfactants. *Analytical Biochemistry* **87**, 28-38.

Gottschalk, A. (1966) Definition of glycoproteins and their delineation from other carbohydrate-protein complexes. In: *Glycoproteins: Their Composition, Structure and Function.* (Gottschalk, A., Ed.). Elsevier Publishing Company, Amsterdam.

Gould, S.J. (1986) Linnaean Limits. *Natural History* **August**, 16-22.

Green, A. A. and Hughes, W. L. (1955) Protein fractionation on the basis of solubility in aqueous solutions of salts and organic solvents. In: *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., Eds.) Vol 1, pp 67-90. Academic Press, New York.

Hahn, D., Starrenburg, M. J. C. and Akkermans, A. D. L. (1988) Variable compatibility of cloned *Alnus glutinosa* ecotypes against ineffective *Frankia* strains. *Plant and Soil* **107**, 233-243.

Hall, B.K. (1984) Developmental mechanisms underlying the formation of atavisms. *Biol. Rev.* **59**, 89-124.

Harrison, R. and Hunt, G. G. (1980) Biological membranes. Their Structure and Function.

Hattori, J. and Johnson, D.A. (1985) The detection of leghemoglobin-line sequences in legumes and non-legumes. *Plant Molecular Biology* **4**, 285-292.

Hawkes, R., Niday, E. and Gordon, J. (1982) A dot-immunobinding assay for monoclonal and other antibodies. *Analytical Biochemistry* **119**, 142-147.

Hebert, G.A., Pelham, P. L. and Pittman, B. (1973) Determination of the optimal ammonium sulfate concentration for the fractionation of rabbit, sheep, horse and goat antisera. *Applied Microbiology* **25**, 26-36.

Heffernan, B. (1985) A handbook of methods of inorganic chemical analysis for forest soils, foliage and water. *Division of Forest Research, CSIRO* . Canberra, Australia, 281 pp.

Holman, R.M. and Schwintzer, C.R. (1987) Distribution of spore-positive and spore-negative nodules of *Alnus incana* ssp. *rugosa* in Maine, USA. *Plant and Soil* **104**, 103-111.

Hooker, J. E. and Wheeler, C. T. (1987) The effectivity of *Frankia* for nodulation and nitrogen fixation in *Alnus rubra* and *A. glutinosa*. *Physiologia Plantarum* **70**, 333-341.

Horowitz, M. I. (1977) Purification of glycoproteins and criteria of purity. In: *The Glycoconjugates: Vol 1 Mammalian Glycoproteins and Glycolipids* (Horowitz, M. I., Ed.). Academic Press, New York.

Hunt, L.T., Hurst-Calderone, S. and Dayhoff, M.O. (1978) Globins. In: *Atlas of Protein Structure and Function* (Dayhoff, M.O., Ed.) pp 229-249. National Biomedical Research Foundation, Washington.

Hutchinson, J (1973) The families of flowering plants arranged according to a new system based on their probable phylogeny. Clarendon Press, Oxford.

Hyldig-Nielsen, J.J., Jensen, E.O., Paludan K., Wiborg, O., Garrett, R., Jorgensen, P. and Marcker, K. (1982) The primary structures of two leghemoglobin genes from soybean. *Nucleic Acids Research* **10**, 689-701.

Jeffreys, A.J. (1982) Evolution of globin genes. In: *Genome Evolution* (Dover, G.A. and Flavell, R.B., Eds.) pp 157-176. Academic Press, London.

Jeffreys, A.J. (1981) Recent studies of gene evolution using recombinant DNA. In: *Genetic engineering 2* (Williamson, R., Ed.) pp 2-48 Academic Press, London.

Jensen, E.O., Paludan K., Hyldig-Nielsen, J.J., Jorgensen, P. and Marcker, K.A. (1981) The structure of a chromosomal leghaemoglobin gene from soybean. *Nature* **291**, 677-679.

Jiabin, H., Zheying, Z., Guanxiong, C. and Huichang, L. (1985) Host range of *Frankia* endophytes. *Plant and Soil* **87**, 61-65.

Kashanski, C.R. and Schwintzer, C.R. (1987) Distribution of spore-positive and spore-negative nodules of *Myrica gale* in Maine, USA. *Plant and Soil* **104**, 113-120.

Kimura, M. (1982) The neutral theory as a basis for understanding the mechanism of evolution and variation at the molecular level. In: *Molecular Evolution, Protein Polymorphism and the Neutral Theory* (Kimura, M., Ed.) pp 3-56. Japan Scientific Societies Press, Tokyo.

Kimura, M. (1986) DNA and the neutral theory. *Philosophical Transactions of the Royal Society B* **312**, 343-354.

Kortt, A. A., Burns, J. E., Trinick, M. J. and Appleby, C. A. (1985) The amino acid sequence of hemoglobin I from *Parasponia andersonii*, a nonleguminous plant. *FEBS Letters* **180**, 55-60.

Kortt, A. A., Strike, P. M., Bogusz, D. and Appleby, C. A. (1987) The amino acid sequence of leghemoglobin II from *Sesbania rostrata* stem nodules. *Biochemistry International* **15**, 509-516.

Kortt, A.A., Trinick, M.J. and Appleby, C.A. (1988) Amino acid sequences of hemoglobins I and II from root nodules of the non-leguminous *Parasponia rigida - Rhizobium* symbiosis, and a correction of the sequence of hemoglobin I from *Parasponia andersonii*. *European Journal of Biochemistry* **175**, 141-149.

Kubo, H. (1939) The haemoprotein of the root nodules of legumes. *Acta Phytochimica, Tokyo* **11**, 195-200.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lalonde, M. and Calvert, H. E. (1979) Production of *Frankia* hyphae and spores as an infective inoculant for *Alnus* species. In: *Symbiotic Nitrogen Fixation in the Management of Temperate Forests* (Gordon, J. C, Wheeler, C. T. and Perry, D. A., Eds.) pp 95-110. Oregon State University, Corvallis, OR.

Lalonde, M. and Simon, L. (1985) Current reseach strategies for use of actinorhizal symbioses in forestry. In: *Nitrogen Fixation Research Progress* (Evans, H. J. *et al*, Eds.) pp 667-674. Martinus Nijhoff, Dordrecht.

Lalonde, M., Simon, L., Bousquet, J. and Seguin, A. (1988) Advances in the taxonomy of *Frankia* : Recognition of species *alni* and *elaeagni* and novel subspecies *pommerii* and *vandijkii* . In: *Nitrogen Fixation: Hundred Years After* (Bothe, H. *et al*, Eds.) pp 671-680. Gustav Fischer, Stuttgart.

Landsmann, J., Dennis, E.S., Higgins, T.J.V., Appleby, C.A., Kortt, A.A and Peacock, W.J. (1986) Common evolutionary origin of legume and non-legume plant haemoglobins. *Nature* **324**, 166-168.

Layzell, D. B., Gaito, S. T. and Hunt, S. (1988) Model of gas exchange and diffusion in legume nodules. I: Calculation of gas exchange rates and energy costs of N_2 fixation. *Planta* **173**, 117-127.

Lechevalier, M. P. (1984) The taxonomy of the genus *Frankia* . *Plant and Soil* **78**, 1-6.

Lechevalier, M. P., Baker, D. and Horriere, F. (1983) Physiology, chemistry, serology and infectivity of two *Frankia* isolates from *Alnus incana* subsp. *rugosa* . *Canadian Journal of Botany* **61**, 2826-2833

Lewin, R. (1982a) Can genes jump between eukaryotic species? *Science* **217**, 42-43.

Lewin, R. (1982b) On the origin of introns *Science* **217**, 921-922.

Lie, T. A., Goktan, D., Engin, M., Pijnenborg, J. and Anlarsal, E. (1987) Co-evolution of the legume-*Rhizobium* association. *Plant and Soil* **100**, 171-181.

Lie, T. A., Akkermans, A. D. L. and Van Egeraat, A. W. S. M. (1984) Natural variation in symbiotic nitrogen-fixing *Rhizobium* and *Frankia* spp. *Antonie van Leeuwenhoek* **50**, 489-503.

Loomis, W.D. (1973) Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. *Methods in Enzymology* **31**, 528-544.

Loomis, W.D. and Battaile, J. (1966) Plant phenolic compounds and the isolation of plant enzymes. *Phytochemistry* **5**, 423-438.

Malmud, D. and Drysdale, J.W. (1978) Isoelectric points of proteins: a table. *Analytical Biochemistry* **86**, 620-647.

Martin, K.D., Saari, L., Guang-Xin, W, Wang, T., Parkhurst, L.J. and Klucas, R.V. (1989) Kinetics and therodynamics of oxygen, CO and azide binding by the subcomponents of soybean leghemoglobin. *The Journal of Biological Chemistry* , **submitted**

McDonald, M.J., Shapiro, R, Bleichman, M., Solway, J. and Bunn H.F. (1978) Glycosylated minor components of human adult hemoglobin. *The Journal of Biological Chemistry* **253**, 2327-2332.

Meesters, T.M., van Genesen, S.Th. and Akkermans, A.D.L. (1985) Growth, acetylene reduction activity and localisation of nitrogenase in relation to vesicle formation in *Frankia* strains Cc1.17 and Cp1.2. *Archives of Microbiology* **143**, 137-142.

Meesters, T.M. (1987) Localisation of nitrogenase in vesicles of *Frankia* sp. Cc1.17 by immunogoldlabelling on ultrathin sections. *Archives of Microbiology* **146**, 327-331.

Midgley, S. J., Turnbull, J. W. and Johnston, R. D., (Eds.) (1983) Casuarina Ecology, Management and Utilisation. CSIRO, Melbourne.

Moore, A.W. (1964) Note on non-leguminous nitrogen-fixing plants in Alberta. *Canadian Journal of Botany* **42**, 952-955.

Mowry, H. (1933) Symbiotic nitrogen fixation in the genus *Casuarina* . *Soil Science* **36**, 409-426.

Murry, M.A., Fontaine, M.S. and Tjepkema, J.D. (1984) Oxygen protection of nitrogenase in *Frankia* sp. HFPArI3. *Archives of Microbiology* **139**, 162-166.

Murry, M.A., Zhongze, Z. and Torrey, J.G. (1985) Effect of oxygen on vesicle formation, acetylene reduction, and oxygen-uptake kinetics in *Frankia* sp. HFPCcI3 isolated from *Casuarina cunninghamiana* . *Canadian Journal of Microbiology* **31**, 804-809.

National Academy of Sciences (1983) Casuarinas: Tree resources for the future. *National Academy Press* , Washington, D.C..

Nesme, X., Normand, P., Tremblay, F., M. and Lalonde, M. (1985) Nodulation speed of *Frankia* sp. on *Alnus glutinosa* , *Alnus crispa* and *Myrica gale* . *Canadian Journal of Botany* **63**, 1292-1295.

Noridge, N.A. and Benson, D.R. (1986) Isolation and nitrogen-fixing activity of *Frankia* sp strain CpI1 vesicles. *Journal of Bacteriology* **166**, 301-305.

Normand, P. and Lalonde, M. (1982) Evaluation of *Frankia* strains isolated from provenances of two *Alnus* species. *Canadian Journal of Microbiology* **28**, 1133-1142.

Normand, P. and Lalonde, M. (1986) The genetics of actinorhizal *Frankia*: A review. *Plant and Soil* **90**, 429-453.

Ohlsson, P.I. and Paul, K.G. (1976) The molar absorptivity of horseradish peroxidase. *Acta Chem. Scand.* **30**, 373-375.

Ollis, D.L., Appleby, C.A., Colman, P.M., Cutten, A.E., Guss, J.M., Venkatappa, M.P. and Freeman, H.C. (1983) Crystal structure of soybean leghaemoglobin a nicotinate at a resolution of 3.3 Å. *Australian Journal of Chemistry* **36**, 451-468.

Parker, C.A. (1957) Evolution of nitrogen-fixing symbiosis in higher plants. *Nature* **179**, 593-594.

Parsons, R., Silvester, W. B., Harris, S., Gruijters, W. T. M. and Bullivant, S. (1987) *Frankia* vesicles provide inducible and absolute oxygen protection for nitrogenase. *Plant Physiology* **83**, 728-731.

Patterson, H. D. and Williams, E. R. (1976) A new class of resolvable incomplete block designs. *Biometrika* **63**, 83-92.

Perinet, P., Brouillette, J. G., Fortin, J. A. and Lalonde, M. (1985) Large scale inoculation of actinorhizal plants with *Frankia* . *Plant and Soil* **87**, 175-183.

Perutz, M.F. (1983) Species adaptation in a protein molecule. *Mol. Biol. Evol.* **1**, 1-28.

Peterson, J. D., Nehrlich, S., Dyer, P. E. and Steiner, D. F. (1972) *The Journal of Biological Chemistry* **247**, 4866-4871.

Pommer, E. (1959) Uber die Isolierurig des endophyten aus den wurzelknollchen *Alnus glutinosa* Gaertn. und uber erfolgreiche re-infektionsversuche. *Ber. Deutsch Bot.Gesell.* **72**, 138-150.

Reddell, P. and Bowen, G. D. (1985) Frankia source affects growth, nodulation and nitrogen fixation in *Casuarina* species. *New Phytologist* **100**, 115-122.

Rhighetti, P. G. (1983) Laboratory techniques in biochemistry and molecular biology, Vol 11. Isoelectric focusing: theory, methodology and applications. Elsevier, Amsterdam.

Robertson, J. G., Warburton, M. P., Lyttleton, P., Fordyce, A. M. and Bullivant, S. (1978) Membranes in lupin nodules. II. Preparation and properties of peribacteroid membranes and bacterial envelope inner membranes from developing lupin nodules. *Journal of Cell Science* **30**, 151-174.

Robertson, J. G., Wells, B., Bisseling, K., Farnden, J. F. and Johnston, W. B. (1984) Immuno-gold localisation of leghemoglobin in cytoplasm in nitrogen-fixing root nodules of pea. *Nature* **311**, 254-256.

Roberts, M.P., Jafar, S. and Mullin, B.C. (1985) Leghemoglobin-like sequences in the DNA of four actinorhizal plants. *Plant Molecular Biology* **5**, 333-337.

Robson, R. L. and Postgate, J. R. (1980) Oxygen and hydrogen in biological nitrogen fixation. *Annual Review of Microbiology* **34**, 183-207.

Rosbrook, P. A. and Bowen, G. D. (1987) The abilities of three *Frankia* isolates to nodulate and fix nitrogen with four species of *Casuarina* . *Physiologia Plantarum* **70**, 373-377.

Schwintzer, C. R., Berry, A. M. and Disney, L. D. (1982) Seasonal patterns of root nodule growth, endophyte morphology, nitrogenase activity and shoot development in *Myrica gale* . *Canadian Journal of Botany* **60**, 746-757.

Shapiro, R., McManus, M.J., Zalut, C. and Bunn, H.F. (1980) Sites of nonenzymic glycosylation of human hemoglobin A. *The Journal of Biological Chemistry* **255**, 3120-3127.

Sharifi, E. (1984) Parasitic origins of nitrogen-mixing (sic) *Rhizobium* -legume symbioses. A review of the evidence. *BioSystems* **16**, 269-289.

Sharon, N. (1984) Glycoproteins. *TIBS* **April**, 198-202.

Sheehy, J.E., Minchin, F.R. and Witty, J.F. (1983) Biological control of the resistance to oxygen flux in nodules. *Annals of Botany* **52**, 565-571.

Shepherd, K. R. and El-Lakany, M. H. (1983) A provenance trial of *Casuarina glauca* and *C. cunninghamiana* . In: *Casuarina Ecology, Management and Utilisation* (Midgley, S. J. *et al*, Eds.) pp122-125. CSIRO, Melbourne.

Sheppard, L. J., Hooker, J. E., Wheeler, C. T. and Smith, R. I (1988) Glasshouse evaluation of the growth of *Alnus rubra* and on peat and acid brown earth soils when inoculated with four sources of *Frankia* . *Plant and Soil* **110**, 187-198.

Silvester, W. B., Whitbeck, J., Silvester, J. K. and Torrey, J. G. (1988) Growth, nodule morphology and nitrogenase activity of *Myrica gale* with roots grown at various oxygen levels. *Canadian Journal of Botany* **66**, 1762-1771.

Smith, C.A., Skvirsky, R.C. and Hirsch, A.M. (1986) Histochemical evidence for the presence of a suberin-like compound in *Rhizobium*-induced nodules of the nonlegume *Parasponia rigida* . *Canadian Journal of Botany* **64**, 1474-1483.

Smith, J.D. (1949) The concentration and distribution of haemoglobin in the root nodules of leguminous plants *Biochemical Journal* **44**, 585-598.

Stubbs, G.W., Smith, H.G. and Litman, B.J. (1976) Alkyl glucosides as effective solubilising agents for bovine rhodospin: a comparison with several commonly used detergents. *Biochimica et Biophysica Acta* **426**, 46-56.

Syvanen, M. (1985) Cross-species gene transfer; implications for a new theory of evolution. *Journal of Theoretical Biology* **112**, 333-343.

Takhtajan, A.L. (1980) Outline of the classification of flowering plants. *The Botanical Review* **46**, 225-359.

Thomas, P.E., Ryan, D. and Levin, W. (1976) An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulphate polyacrylamide gels. *Analytical Biochemistry* **75**, 168-176.

Thorne, R. F. (1981) Phytochemistry and angiosperm phylogeny: A summary statement. In: *Phytochemistry and Angiosperm Phylogeny* (Young, D.A. and Seigler, D.S., Eds.). Praeger Scientific, New York.

Thorpe, S. R. and Baynes, J. W. (1982) Nonenzymic glycosylation of protens *in vitro* and *in vivo*. In: *The Glycoconjugates, Vol III* (Horowitz, M. I., Ed.) Academic Press, New York.

Tjepkema, J.D. (1978) The role of oxygen diffusion from the shoots and nodule roots in nitrogen fixation by root nodules of *Myrica gale*. *Canadian Journal of Botany* **56**, 1365-1371.

Tjepkema, J.D. (1979) Oxygen relations in leguminous and actinorhizal nodules. In: *Symbiotic Nitrogen Fixation in the Management of Temperate Forests* (Gordon, J.C. *et al*, Eds.) pp175-186. Oregon State University, Corvallis

Tjepkema, J.D. (1983) Hemoglobins in the nitrogen-fixing root nodules of actinorhizal plants. *Canadian Journal of Botany* **61**, 2924-2929.

Tjepkema, J.D. and Asa, D.J. (1987) Total and CO-reactive heme content of actinorhizal nodules and the roots of some non-nodulated plants. *Plant and Soil* **100**, 225-236.

Tjepkema, J.D. and Cartica, R.J. (1982) Diffusion limitation of oxygen uptake and nitrogenase activity in the root nodules of *Parasponia rigida* Merr. and Perry. *Plant Physiology* **69**, 728-733.

Tjepkema, J.D., Ormerod, W. and Torrey, J.G. (1980) Vesicle formation and acetylene reduction activity in *Frankia* sp CpI1 cultured in defined nutrient media. *Nature* **287**, 633-635.

Tjepkema, J.D., Pathirana, M. S. and Zeng, S. (1988) The gas diffusion pathway and hemoglobin content in actinorhizal nodules. In: *Nitrogen Fixation: Hundred Years After* (Bothe, H. *et al*, Eds.) p 701, Gustav Fischer, Stuttgart.

Tjepkema, J.D., Schwintzer, C.R. and Benson, D.R. (1986) Physiology of actinorhizal nodules. *Annual Review of Plant Physiology* **37**, 209-232.

Tjepkema, J.D. and Winship, L.J. (1980) Energy requirements for nitrogen fixation in actinorhizal and legume root nodules. *Science* **209**, 279-281.

Tjepkema, J.D. and Yocum, C.S. (1974) Measurement of oxygen partial pressure within soybean nodules by oxygen microelectrodes. *Planta* **119**, 351-360.

Torrey, J. G. (1983) *Casuarina* : actinorhizal dinitrogen fixing tree of the tropics. In: *Casuarina Ecology, Management and Utilisation* (Midgley, S. J. *et al*, Eds.) pp 193-203. CSIRO, Melbourne

Torrey, J. G. (1985) The site of nitrogenase in *Frankia* in free-living culture and in symbiosis. In: *Nitrogen Fixation Research Progress* (Evans, H.J. *et al*, Eds.) pp 293-299. Martinus Nijhoff, Dordrecht.

Torrey, J. G. and Callaham, D. (1982) Structural features of the vesicle of *Frankia* sp. CpI1 in culture. *Canadian Journal of Microbiology* **28**, 749-757.

Torrey, J.G., Racette, S. and Newcomb, W. (1988) Vesicle formation and dinitrogen fixation by *Frankia* in *Gymnostoma* , a genus in the Casuarinaceae. In: *Nitrogen Fixation: Hundred Years After* (Bothe, H. et al, Eds.) p 702. Gustav Fischer, Stuttgart.

Trinick, M.J., Goodchild, D.J. and Miller, C. (1989) Localisation of bacteria and hemoglobin in root nodules of *Parasponia andersonii* containing both *Bradyrhizobium* and *Rhizobium leguminosarum* biovar *trifolii* .
Manuscript in Preparation

Vance, C.P. (1983) *Rhizobium* infection and nodulation: a beneficial plant disease? *Annual Review of Microbiology* **37**, 399-424.

VandenBosch, K. V. and Torrey, J. G. (1985) Development of endophytic *Frankia* sporangia in field- and laboratory-grown nodules of *Comptonia peregrina* and *Myrica gale* . *American Journal of Botany* **72**, 99-108.

Van Dijk, C. (1978) Spore formation and endophyte diversity in root nodules of *Alnus glutinosa* (L.) Vill. *New Phytologist* **81**, 601-615.

Van Dijk, C., Sluimer, A. and Weber, A. (1988) Host range differentiation of spore-positive and spore-negative strain types of *Frankia* in stands of *Alnus glutinosa* and *Alnus incana* in Finland. *Physiologia Plantarum* **72**, 349-358.

Verma, D.P.S. and Long, S. (1983) The molecular biology of *Rhizobium*-legume symbiosis. *International Review of Cytology* **Supplement 14**, 211-245.

Walsby, A.E. (1985) The permeability of heterocysts to the gases nitrogen and oxygen. *Proceedings of the Royal Society of London B* **226**, 345-366.

Ward, L.D. and Winzor, D.J. (1982) Activation of rabbit muscle lactate dehydrogenase by phosphate: active enzyme gel chromatography and enzyme kinetic studies. *Archives of Biochemistry and Biophysics* **216**, 329-336.

Weber, A., Nurmiaho-Lassila, E-L. and Sundman, V. (1987) Features of the intrageneric *Alnus-Frankia* specificity. *Physilogia Plantarum* **70**, 289-296.

Wheeler, C.T., Gordon, J.C. and Ching, T.M. (1979) Oxygen relations of the root nodules of *Alnus rubra* Bong.. *New Phytologist* **82**, 449-457.

Wheeler, C. T., Hooker, J. E., Crowe, A. and Berrie, A. M. M. (1986) The improvement and utilisation in forestry of nitrogen fixation by actinorhizal plants with special reference to *Alnus* in Scotland. *Plant and Soil* **90**, 393-406.

Williamson, A.R., Salaman, M.R. and Kreth, H.W. (1973) Microheterogeneity and allomorphism of proteins. *Annals of the New York Academy of Sciences* **209**, 210-224.

Wilson, K. L. and Johnson, L. A. S. (1989) Casuarinaceae. In: *Flora of Australia, Volume 3*, pp100-175 Australian Government Publishing Service, Canberra.

Winship, L. J. and Tjepkema, J. D. (1985) Nitrogen fixation and respiration by root nodules of *Alnus rubra* Bong. : Effects of temperature and oxygen concentration. *Plant and Soil* **87**, 91-107.

Winship, L.J. and Tjepkema, J.D. (1983) The role of diffusion in oxygen protection of nitrogenase in nodules of *Alnus rubra* . *Canadian Journal of Botany* **61**, 2930-2936.

Wittenberg, J.B., Bergersen, F.J., Appleby, C.A. and Turner, G.L. (1974) Facilitated oxygen diffusion. The role of leghemoglobin in nitrogen fixation by bacteroids isolated from soybean root nodules. *The Journal of Biological Chemistry* **249**, 4057-4066.

Wittenberg, J. B., Wittenberg, B. A., Trinick, M., Gibson, Q. H., Fleming, A. I., Bogusz, D. and Appleby, C. A. (1985) Hemoglobins which supply oxygen to intracellular prokaryotic symbionts. In: *Nitrogen Fixation Research Progress* (Evans, H. J. *et al*, Eds.) p 354. Martinus Nijhoff, Dordrecht.

Wittenberg, J.B., Appleby, C.A. and Wittenberg, B.A. (1972) The kinetics of the reactions of leghemoglobin with oxygen and carbon monoxide. *The Journal of Biological Chemistry* **247**, 527-531.

Wittenberg, J.B., Wittenberg, B.A., Gibson, Q.H., Trinick, M.J. and Appleby, C.A. (1986) The kinetics of the reactions of *Parasponia andersonii* hemoglobin with oxygen, carbon monoxide and nitric oxide. *The Journal of Biological Chemistry* **261**, 13624-13631.

Woods, E. F. and Inglis, A. S. (1984) *International Journal of Biological Macromolecules* **6**, 277-283.

Zhang, Z., Lopez, M. F. and Torrey, J. G. (1984) A comparison of cultural characteristics and infectivity of *Frankia* isolates from root nodules of *Casuarina* species. *Plant and Soil* **78**, 79-90.

Zhang, Z. and Torrey, J. G. (1985a) Studies of an effective strain of *Frankia* from *Allocasuarina lehmanniana* of the Casuarinaceae. *Plant and Soil* **87**, 1-16.

Zhang, Z. and Torrey, J.G. (1985b) Biological and cultural characteristics of the effective *Frankia* strain HFPCcI3 (Actinomycetales) from *Casuarina cunninghamiana* (Casuarinaceae) *Annals of Botany* **56**, 367-378.

Appendix I

Nitrogen and Phosphorus Content of
Selected Root and Shoot Samples from the
18 x 18 Host Specificity Trial.

(Experiment Two, Section One)

REP/PROV/STRAIN	DRY WT ROOTS (GRAMS)	DRY WT SHOOTS (GRAMS)	TOTAL DRY WT (GRAMS)	SHOOT N (%)	N (mg)	SHOOT P (%)	P (mg)
2/2/3	1.30	2.69	3.99	1.88	50.57	0.17	4.68
6/2/3	1.45	3.24	4.69	1.54	49.90	0.13	4.08
8/2/3	1.27	3.46	4.73	1.77	61.24	0.13	4.57
2/2/8	0.81	1.17	1.98	1.44	16.85	0.38	4.46
5/2/8	0.54	1.13	1.67	0.95	10.74	0.38	4.32
9/2/8	0.65	1.15	1.80	1.15	13.23	0.29	3.37
3/2/9	1.19	1.20	2.39	1.06	12.72	0.25	3.02
6/2/9	0.93	1.15	2.08	0.82	9.43	0.31	3.55
7/2/9	0.61	0.93	1.54	0.80	7.44	0.62	5.77
3/2/10	1.51	4.09	5.60	1.82	74.44	0.11	4.54
4/2/10	1.74	3.89	5.63	1.38	53.68	0.13	4.86
9/2/10	0.88	1.47	2.35	1.44	21.17	0.20	2.98
2/2/13	0.73	0.58	1.31	1.08	6.26	0.84	4.88
4/2/13			0.00				
7/2/13	1.04	3.30	4.34	1.83	60.39	0.19	6.34
3/2/14	1.42	3.99	5.41	1.68	67.03	0.08	3.07
5/2/14	1.22	3.54	4.76	1.53	54.16	0.12	4.25
8/2/14	1.27	2.93	4.20	1.82	53.33	0.19	5.45
1/2/15	1.85	3.66	5.51	2.44	89.30	0.12	4.54
6/2/15	0.81	1.25	2.06	1.99	24.88	0.30	3.74
9/2/15	0.72	0.73	1.45	0.79	5.77	0.38	2.80
1/2/16	0.75	1.92	2.67	1.69	32.45	0.23	4.32
4/2/16	1.27	3.99	5.26	1.87	74.61	0.11	4.39
8/2/16	1.39	3.54	4.93	1.48	52.39	0.12	4.25

REP/PROV/STRAIN	DRY WT ROOTS (GRAMS)	DRY WT SHOOTS (GRAMS)	TOTAL DRY WT (GRAMS)	SHOOT N (%)	N(mg)	SHOOT P (%)	P (mg)
3/2/18	1.12	2.48	3.60	2.14	53.07	0.28	7.04
6/2/18	0.91	3.02	3.93	1.87	56.47	0.22	6.70
7/2/18	1.17	3.94	5.11	1.91	75.25	0.13	5.08
3/8/3	0.24	0.95	1.19	2.59	24.61	0.53	5.07
4/8/3	0.77	2.46	3.23	1.98	48.71	0.19	4.60
9/8/3	0.94	3.52	4.46	1.74	61.25	0.16	5.63
3/8/8	0.34	1.24	1.58	1.75	21.70	0.36	4.50
6/8/8	0.43	1.50	1.93	1.61	24.15	0.31	4.61
7/8/8	0.98	2.82	3.80	1.70	47.94	0.17	4.77
1/8/9	0.97	3.29	4.26	1.91	62.84	0.12	3.92
4/8/9	0.57	2.60	3.17	1.85	48.10	0.21	5.36
8/8/9	0.85	3.26	4.11	2.02	65.85	0.19	6.26
1/8/10	1.05	3.34	4.39	1.96	65.46	0.20	6.71
5/8/10	0.55	1.57	2.12	1.38	21.67	0.36	5.57
7/8/10	0.45	1.27	1.72	1.47	18.67	0.46	5.78
3/8/13	1.04	2.08	3.12				
5/8/13	0.57	0.98	1.55	0.85	8.33	0.52	5.08
8/8/13	0.12	0.10	0.22	0.98	0.98	1.89	1.89
1/8/14	0.74	1.80	2.54	1.48	26.64	0.23	4.05
6/8/14	0.61	2.47	3.08	1.73	42.73	0.28	6.97
9/8/14	0.84	2.08	2.92	1.33	27.66	0.21	4.35
2/8/15	1.21	3.68	4.89	1.89	69.55	0.17	6.22
4/8/15	0.84	2.83	3.67	1.66	46.98	0.16	4.64
7/8/15	1.13	3.84	4.97	1.58	60.67	0.21	8.22

REP/PROV/STRAIN	DRY WT ROOTS (GRAMS)	DRY WT SHOOTS (GRAMS)	TOTAL DRY WT (GRAMS)	SHOOT N (%)	N(mg)	SHOOT P (%)	P (mg)
2/8/16	0.79	2.88	3.67	1.56	44.93	0.20	5.79
5/8/16	1.06	3.24	4.30	1.77	57.35	0.22	7.03
9/8/16	0.97	3.61	4.58	1.59	57.40	0.15	5.56
1/8/18	1.21	3.19	4.40	1.55	49.45	0.14	4.43
4/8/18	1.28	3.97	5.25	1.39	55.18	0.12	4.64
8/8/18	0.93	3.01	3.94	1.53	46.05	0.24	7.22
2/12/3	0.78	2.48	3.26	1.82	45.14	0.17	4.12
6/12/3	1.09	3.22	4.31	1.61	51.84	0.16	5.02
8/12/3	1.46	4.38	5.84	2.20	96.36	0.16	7.18
2/12/8	0.80	2.14	2.94	1.32	28.25	0.16	3.38
5/12/8	0.75	2.08	2.83	1.10	22.88	0.18	3.81
9/12/8	1.48	3.41	4.89	1.65	56.27	0.14	4.74
3/12/9	0.52	0.62	1.14	0.72	4.46	0.33	2.06
6/12/9	0.76	1.05	1.81	1.03	10.82	0.31	3.28
7/12/9	1.04	1.36	2.40	0.97	13.19	0.19	2.54
3/12/10	1.08	3.06	4.14	1.73	52.94	0.14	4.28
4/12/10	1.33	4.21	5.54	1.82	76.62	0.12	4.84
9/12/10	1.32	3.64	4.96	1.99	72.44	0.18	6.62
2/12/13	1.30	3.51	4.81	1.68	58.97	0.13	4.70
4/12/13	1.30	3.70	5.00	1.42	52.54	0.11	3.89
7/12/13	1.22	2.66	3.88	1.64	43.62	0.21	5.51
3/12/14	1.08	1.78	2.86	1.53	27.23	0.27	4.73
5/12/14	1.23	2.76	3.99	1.24	34.22	0.15	4.00
8/12/14	1.11	3.49	4.60	1.76	61.42	0.16	5.41

REP/PROV/STRAIN	DRY WT ROOTS (GRAMS)	DRY WT SHOOTS (GRAMS)	TOTAL DRY WT (GRAMS)	SHOOT N (%)	N(mg)	SHOOT P (%)	P (mg)
1/12/15	0.93	2.60	3.53	1.55	40.30	0.24	6.19
6/12/15	1.20	3.52	4.72	1.85	65.12	0.20	6.93
9/12/15	1.44	3.84	5.28	1.87	71.81	0.13	4.99
1/12/16	1.00	3.64	4.64	1.70	61.88	0.12	4.30
4/12/16	1.08	3.52	4.60	1.65	58.08	0.10	3.59
8/12/16	1.70	4.55	6.25	2.08	94.64	0.11	4.91
3/12/18	1.54	3.93	5.47	1.84	72.31	0.09	3.62
6/12/18	1.01	3.23	4.24	1.83	59.11	0.13	4.13
7/12/18	1.55	4.77	6.32	1.48	70.60	0.09	4.44
1/15/3	1.21	2.39	3.60	1.98	47.32	0.21	4.97
5/15/3	0.96	2.82	3.78	1.73	48.79	0.18	4.94
7/15/3	1.12	2.70	3.82	1.75	47.25	0.24	6.45
1/15/8	0.87	2.30	3.17	1.49	34.27	0.17	3.86
4/15/8	1.13	1.65	2.78	0.94	15.51	0.32	5.30
8/15/8	1.42	2.40	3.82	1.63	39.12	0.22	5.26
2/15/9	1.23	0.82	2.05	1.77	14.51	0.48	3.94
5/15/9	0.89	1.06	1.95	1.05	11.13	0.23	2.41
9/15/9	0.59	0.60	1.19	1.01	6.06	0.63	3.77
2/15/10	1.17	3.47	4.64	1.89	65.58	0.20	6.97
6/15/10	1.13	2.96	4.09	1.68	49.73	0.15	4.50
8/15/10	1.26	3.48	4.74	2.01	69.95	0.17	6.02
1/15/13	0.49	0.63	1.12	1.65	10.40	0.49	3.06
6/15/13	1.31	2.59	3.90	1.48	38.33	0.19	5.02
9/15/13	1.04	1.55	2.59	1.36	21.08	0.28	4.36

REP/PROV/STRAIN	DRY WT ROOTS (GRAMS)	DRY WT SHOOTS (GRAMS)	TOTAL DRY WT (GRAMS)	SHOOT N (%)	N(mg)	SHOOT P (%)	P (mg)
2/15/14	0.68	1.22	1.90	1.62	19.76	0.23	2.75
4/15/14	1.77	3.14	4.91	1.62	50.87	0.18	5.65
7/15/14	0.94	2.87	3.81	1.52	43.62	0.17	4.74
3/15/15	1.38	3.85	5.23	1.65	63.53	0.13	5.12
5/15/15	1.30	3.70	5.00	2.11	78.07	0.14	5.18
8/15/15	0.93	2.73	3.66	2.04	55.69	0.14	3.71
3/15/16	1.05	2.99	4.04	1.47	43.95	0.11	3.20
6/15/16	1.06	3.02	4.08	1.50	45.30	0.20	5.89
7/15/16	1.19	3.03	4.22	1.35	40.91	0.11	3.39
2/15/18	1.25	3.34	4.59	1.84	61.46	0.20	6.71
5/15/18	1.05	3.13	4.18				
9/15/18	1.49	3.22	4.71	1.45	46.69	0.12	3.99
3/16/3	1.20	3.56	4.76	1.64	58.38	0.11	4.06
4/16/3	1.30	3.10	4.40	1.62	50.22	0.12	3.72
9/16/3	1.44	4.35	5.79	2.12	92.22	0.15	6.53
3/16/8	1.30	4.11	5.41	1.64	67.40	0.11	4.32
6/16/8	1.15	3.42	4.57	1.75	59.85	0.14	4.65
7/16/8	1.24	2.99	4.23	1.76	52.62	0.17	5.02
1/16/9	1.11	2.84	3.95	1.64	46.58	0.11	3.12
4/16/9	1.28	2.42	3.70	1.93	46.71	0.13	3.22
8/16/9	1.29	3.86	5.15	1.71	66.01	0.13	5.02
1/16/10	1.04	3.08	4.12	1.71	52.67	0.14	4.19
5/16/10	1.16	2.82	3.98	1.61	45.40	0.15	4.20
7/16/10	1.19	2.72	3.91	1.77	48.14	0.29	7.81

REP/PROV/STRAIN	DRY WT ROOTS (GRAMS)	DRY WT SHOOTS (GRAMS)	TOTAL DRY WT (GRAMS)	SHOOT N (%)	N(mg)	SHOOT P (%)	P (mg)
3/16/13	1.54	3.88	5.42	1.63	63.24	0.10	4.04
5/16/13	1.33	2.89	4.22	1.43	41.33	0.20	5.64
8/16/13	0.58	0.52	1.10	1.11	5.77	0.33	1.74
1/16/14	0.86	1.47	2.33	1.38	20.29	0.27	3.94
6/16/14	1.08	2.81	3.89	1.31	36.81	0.14	3.85
9/16/14	0.55	0.79	1.34	1.33	10.51	0.38	3.02
2/16/15	1.44	3.01	4.45	1.62	48.76	0.17	4.97
4/16/15	1.66	2.68	4.34	1.71	45.83	0.15	4.13
7/16/15	0.95	2.67	3.62	1.69	45.12	0.21	5.53
2/16/16	1.65	2.42	4.07	0.99	23.96	0.09	2.25
5/16/16	1.19	3.47	4.66	1.38	47.89	0.15	5.14
9/16/16	1.40	4.65	6.05	1.20	55.80	0.10	4.51
1/16/18	1.41	2.59	4.00	1.22	31.60	0.22	5.57
4/16/18	1.63	2.89	4.52	1.15	33.24	0.11	3.24
8/16/18	1.22	3.45	4.67	1.57	54.17	0.13	4.52

Biochimica et Biophysica Acta 911 (1987) 209–220
Elsevier

BBA 32693

The purification, characterization and ligand-binding kinetics of hemoglobins from root nodules of the non-leguminous *Casuarina glauca* – *Frankia* symbiosis

Anthony I. Fleming [a], Jonathan B. Wittenberg [b], Beatrice A. Wittenberg [b], William F. Dudman [c] and Cyril A. Appleby [c]

[a] *Department of Forestry, Australian National University, Canberra, ACT 2601 (Australia),* [b] *Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY 10461 (U.S.A.) and* [c] *The Division of Plant Industry, Commonwealth Scientific and Industrial Research Organisation, Canberra, ACT 2601 (Australia)*

(Received 27 August 1986)

Key words: Non-legume hemoglobin; Phylogeny; Ligand binding; Kinetics; (*Casuarina* root nodule)

The presence of a membrane-bound hemoglobin in aqueous extracts of nitrogen-fixing *Casuarina* root nodules (Davenport, H.E. (1960) Nature 186, 653–654) has been confirmed. By strictly anaerobic grinding and extraction under carbon monoxide, with inclusion of soluble polyvinylpyrrolidone and zwitterionic detergent in extraction buffer, soluble carboxyhemoglobin was obtained. This was purified by anaerobic 'adsorption' chromatography on Sephacryl S-200 (Pharmacia) followed by aerobic molecular exclusion chromatography on Sephadex G-75 (Pharmacia) to yield very stable oxyhemoglobin. By preparative-scale isoelectric focusing *Casuarina* oxyhemoglobin is separable into three major components comprising approx. 20% of applied protein, and very many minor components. Monomeric *Casuarina* hemoglobin is similar to other plant hemoglobins in respect of molecular weight (≈ 17500), optical spectra, extremely rapid kinetics of binding to oxygen and carbon monoxide and high oxygen affinity (P_{50} ≈ 0.074 torr). Hence, it is possible that this protein functions in the *Casuarina* symbiosis as does leghemoglobin in leguminous nitrogen-fixing symbioses. Western blot analysis showed close immunological relationships between the non-leguminous *Casuarina* and *Parasponia* hemoglobins and a weaker relationship between these two proteins and soybean leghemoglobin. It is proposed that these hemoglobins from widely separated plant orders have a common evolutionary origin.

Introduction

The ubiquitous occurrence of a monomeric hemoglobin known as leghemoglobin (Lb) in the legume-*Rhizobium* root nodule symbioses was, until recently, considered to represent a rare example of horizontal gene transfer from the animal to the plant kingdom [1,2]. Doubt was cast upon this hypothesis when hemoglobin (Hb) was found in the *Rhizobium*-induced nitrogen-fixing root nodules of the non-leguminous plant *Parasponia andersonii* [3], refuting the common view that Hb was restricted to legumes in the plant kingdom [4].

Abbreviations and definitions: Hb, ferric Hb, ferrous Hb, HbO_2, HbCO represent respectively hemoglobin, unligated ferric Hb, unligated ferrous Hb, oxyferrous Hb, carboxyferrous Hb; the term leghemoglobin (Lb) is used only for the monomeric hemoglobins from legume root nodules; unless otherwise specified *Casuarina* Hb refers to the protein from *C. glauca* root nodules; Hb I, Hb II and Hb III refer to the principal components of *Casuarina* Hb separable by isoelectric focusing in order of increasing acidity; IEF, isoelectric focusing; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Correspondence: A.I. Fleming, Department of Forestry, Australian National University, Canberra, Australia, 2601.

Parasponia Hb was found to be similar in critical respects to Lb, including amino acid sequence homology [5] and kinetics of reaction with oxygen and CO [6]. The *Parasponia* Hb gene sequence also showed considerable homology with Lb genes, including indentical placement of all three introns [7]. All of this supported the concept of a common origin for Lb and *Parasponia* Hb, which in turn suggested that the genes for plant Hb were widespread and might even have survived throughout the plant kingdom since the divergence of plant and animal lines [7,8].

Support for this argument can be sought from the taxonomically highly diverse actinorhizal plants, which form nitrogen-fixing root nodule symbioses with the actinomycete *Frankia.* Davenport [9] reported observing absorption bands characteristic of Hb in whole and crushed nodules of *Casuarina cunninghamiana, Alnus* and *Myrica.* Unfortunately, this putative Hb remained firmly bound to cell debris under all extraction conditions tried, in contrast to the readily soluble legume nodule Lb or animal Hb. Davenport's important observations were generally disregarded, especially so because other workers failed to detect Hb in a number of actinorhizal species (Smith, quoted in Ref. 10) [11,12]. In 1983, however, Tjepkema [13] confirmed the observations of Davenport, recording spectra characteristic of a reversibly oxygenating Hb in nodule segments of *C. cunninghamiana* and at least four other actinorhizal species and also by extracting crude *C. cunninghamiana* Hb in low yield.

In the absence of pure protein there has remained doubt as to whether actinorhizal nodules do contain Hb, or rather a membrane-bound molecule with some similar spectral properties [14–16]. The distinction is critical, both in terms of the evolutionary question and in resolving the possible role of the protein in nodule function. Hemoglobins in legume and *Parasponia* nodules have unique kinetic properties which enable them to facilitate the flux of oxygen to the *Rhizobium* endophyte at extremely low free oxygen concentration, yet at a rate commensurate with efficient endophyte respiration. In this way, the oxygen-labile nitrogenase is apparently protected [8].

There were two principal reasons for our initiation of this study of *Casuarina* Hb purification and properties. First, nitrogen-fixing *Casuarina* root nodules, unlike those from all other actinorhizal plants [17–19], lack vesicles, the apparent physical mechanism for protecting the endophyte nitrogenase from exposure to excess oxygen. If a hemoglobin were present in *Casuarina* nodules, might it have the same rapid kinetics of oxygen binding and high oxygen affinity which characterize legume nodule Lb and so be assumed to have the same function? Second, could Hb purified from this non-leguminous plant actinomycete symbiosis be shown to have amino acid sequence homology and immunological relatedness with the hemoglobins from the legume-*Rhizobium* and *Parasponia-Rhizobium* symbioses? This would be powerful evidence in favour of a common genetic origin for all plant hemoglobins.

We report the solubilization, purification and partial characterization of Hb from the root nodules of the *Casuarina glauca – Frankia* symbiosis. We have also purified Hb from the nodules of *Casuarina cunninghamiana.* A preliminary account of this work has been published [20].

Materials and Methods

Growth of nodules

Seeds of *Casuarina glauca* Sieb ex Spreng were sown, germinated and grown in a 50 : 50 Perlite/vermiculite substrate in a glasshouse under natural light (temp. 28–30°C day, 22–24°C night). They were watered twice weekly with a 1/4 strength Hoagland's complete solution until 4 weeks old, then inoculated with *Frankia* sp. using crushed nodule preparations derived from naturally grown *C. glauca* nodules. Plants were regularly watered and fertilized twice weekly with 1/2 strength Hoagland's nitrogen-free solution. Nodules, which began to appear from 15 days, were harvested 14–16 weeks after inoculation by picking directly into liquid nitrogen, in which they were stored.

Hemoglobin standard extraction procedure

All manipulations during extraction were done in ice in a fume hood under strict anaerobic conditions with C.P-grade CO or argon in the gas phase. The manipulative procedures are based upon those developed earlier for anaerobic extrac-

tion of *Parasponia* root nodule Hb [3].

In a typical experiment, 16 g of nodules (nodule roots removed by abrasion under liquid nitrogen in a sieve) were added to a Sorvall Omnimixer 70 ml steel chamber, with gassing ports, containing 60 ml of CO-saturated extraction buffer (50 mM potassium phosphate, 1 mM EDTA, 4% (w/v) Kollidon 25 (BASF batch S55314), 1.5 mg/ml sodium dithionite, 0.1% (w/v) Zwittergent 3-12 (Calbiochem Lot 903843), pH 7.2). The vessel was sealed under a slight positive pressure of CO and the nodules were ground for 2 min at full speed. The suspension was transferred under an argon blanket in an open top 50-l plastic bucket into an argon-equilibrated Beckman Spinco 21 centrifuge tube, bubble for a few seconds with CO, sealed and centrifuged for 60 min at $40\,000 \times g$ at 4°C to yield 56 ml of red-brown supernatant.

A sample of this supernatant was transferred under argon to a 10 mm quartz cuvette, bubbled with CO, sealed under CO, and the spectrum was recorded from 700 to 250 nm.

Column chromatography

All chromatography was conducted in a cold-room at 4°C.

The remaining 55 ml of CO-saturated supernatant was pumped anaerobically onto a 45.0×5.0 cm column of Sephacryl S-200 SF (Pharmacia; pre-1980 batch) which had been equilibrated overnight with 3 vol. of CO-saturated column running buffer (50 mM potassium phosphate, 1 mM EDTA, pH 7.2). The column flow rate was 3.4 ml/min and 6-min (20.4 ml) fractions were collected. Absorbances were monitored at 405 nm (the shoulder of the HbCO Soret peak) and 280 nm absorbance (protein peak) using a UVICORD III flow monitor (LKB). Fractions containing the putative Hb were selected and their spectra recorded from 700 to 250 nm after re-equilibration with CO. On the basis of Hb concentration and relative purity (measured as the ratio R of 421.5 nm : 280 nm absorbance) fractions were selected, pooled and concentrated to small volume by pressure filtration over an Amicon YM10 membrane (M_r 10000 exclusion). Once the part-purified HbCO was eluted from Sephacryl S-200 neither anaerobic conditions nor CO-saturated buffers were required.

Depending upon experimental requirements, the Hb from S-200 column chromatography was sometimes further purified by reconcentration over a YM10 membrane, buffer exchange to 0.1 M KCl, 1 mM EDTA (pH 7.2) in the concentration cell, with reconcentration over a YM10 membrane. The Hb was then run through a 38.7×1.6 cm Sephadex G-75 SF (Pharmacia batch 456) column equilibrated with the same buffer, and the purest fractions were again concentrated over a YM10 membrane.

Preparative-scale isoelectric focusing

Preparative-scale isoelectric focusing was conducted on concentrated Hb from Sephacryl S-200 chromatography after buffer exchange to a low salt strength (< 10 mM), using an LKB 2217 Ultrophor flat-bed isoelectric focusing apparatus and the general procedures of Fuchsman and Appleby [21]. All components of HbO_2 appeared to be stable at their isoelectric points so this was the species of choice for isoelectric focusing. In a typical run, 4.4 ml of 2.9 mM HbO_2 in water plus 2% (w/v) Ampholine 4-6, LKB 1809-116, were used, added to a flat-bed gel (100 ml of 4% (w/v) Ultrodex, LKB 2117–510, plus 2% (w/v) of Ampholine 4–6, LKB 1809-116; volume and concentrations before standard pre-evaporation) via an LKB sample applicator temporarily located near the cathode. The anode electrode strip was soaked in 1.0 M H_3PO_4 and the cathode strip in 1.0 M NaOH. The gel was run at 4°C for 12 h with an ISCO 494 electrophoresis power supply set to the following limits; 8 W, 1600 V, 15 mA. At the completion of focusing the gel pattern was either photographed or drawn and Hb-containing bands were excavated, slurried with water and filtered to remove the gel, and isoelectric points were determined by pH measurement at 4°C, then spectra were recorded from 700 to 250 nm at 20°C. Hb components were concentrated by pressure filtration over Amicon YM10 membranes, stabilized by the addition of 50 mM phosphate buffer (pH 7.0) and stored frozen in liquid nitrogen for further use.

Analytical-scale isoelectric focusing

Analytical-scale isoelectric focusing was conducted using an LKB 2117 Ultrophor apparatus

following published procedures [21]. In a typical run dilute HbO_2 samples were first concentrated to approximately 45 μl using Amicon Centricon Microconcentrators (containing YM-10 membranes), the final concentration of Hb being approximately 0.2 mM in water. Samples (20 μl) of this HbO_2 were applied via filter wicks onto a 0.4 mm polyacrylamide gel (T = 7.5%, C = 3.0%) containing 3% (w/v) Pharmalyte pH 4–6.5 (Pharmacia 17-0452-01) or Ampholine pH 4–6.5 (LKB 1809-116) and polymerized according to the manufacturer's instructions in an Ultromold apparatus (LKB 2217-100). These gels were run for 2–4 h at 4°C with the following limits set on the ISCO power supply; 15 W, 2000 V, 30 mA. The anode strip was soaked in 1 M H_3PO_4 and the cathode strip in 1 M NaOH.

When focusing was complete the gel was photographed and placed in fixative (35% (v/v) methanol, 10% (v/v) trichloroacetic acid, 3.5% (v/v) sulphosalicylic acid) for more than 15 min. The gel was then stained for heme and protein, using 3-3′,5-5′-tetramethylbenzidine for heme recognition and Coomassie blue R-250 for protein [22].

Spectrophotometry and preparation of standard spectra

Spectra were recorded from 700 to 250 nm with a Hitachi PE557 microprocessor-controlled spectrophotometer, using 0.6–1.0-ml samples in stoppered 10-mm light-path semi-micro black-sided quartz cuvettes. The spectrophotometer was connected via a serial interface to a PDP 11-03 computer and HP 7221 B four-pen plotter.

Oxyhemoglobin. Solutions of part-purified HbCO in aerobic 50 mM potassium phosphate, 0.1 mM EDTA (pH 7.2) or other pH > 7.0 buffers (preferably containing EDTA) were slowly but quantitatively converted to HbO_2, either by standing in air at 0–4°C, or during subsequent aerobic purification procedures (see Results and Discussion). Ferric *Casuarina* Hb could be converted to HbO_2 by the procedures used for *Parasponia* HbO_2 [3] or legume nodule LbO_2 [23], involving anaerobic dithionite reduction and passage through aerated buffer in Sephadex G-25 columns.

Ferric hemoglobin. HbO_2 solutions in 50 mM potassium phosphate (pH 7.2) were oxidized by mixing with a 5-fold molar excess of powdered potassium ferricyanide. After 5 min at 0–4°C the resulting ferric Hb was separated from ferricyanide and other low molecular weight impurities by passage through Sephadex G-25, medium grade, then centrifuged for 10 min at $10\,000 \times g$ before storage at 0°C or in liquid nitrogen.

Ferrous hemoglobin. Unlike other *Casuarina* Hb valence states and ligand species, ferrous Hb was not stable when stored frozen in liquid nitrogen, and was therefore prepared only as required for procedures such as spectrophotometry. Typically, 8 μl of 0.1 M sodium dithionite in anaerobic 0.02 M NaOH were added under argon to 0.7 ml of 11 μM HbO_2 or ferric Hb being gently stirred at 0–4°C, then transferred to and sealed under argon in spectrophotometer cuvettes.

Carboxy ferrous hemoglobin. Freshly prepared ferrous Hb (preferably), or HbO_2, was transferred to a CO-filled spectrophotometer cuvette and gently equilibrated with CO.

Calculation of hemoglobin concentration. Hemoglobin concentration was determined using a modification of the pyridine hemochrome procedure of Ohlsson and Paul [24]. Equal volumes (300 μl) of a 0.2 M NaOH/4.2 M pyridine mixture and the sample were mixed in an argon-flushed stoppered cuvette. This mixture was reduced under argon with 8 μl of 0.1 M sodium dithionite (Fluka) dissolved in argon-flushed 0.02 M NaOH. The spectrum was recorded from 700 to 500 nm against a reagent blank. The concentration of Hb was determined assuming $\Delta E_{mM} = 24.5$ for the pyridine protohemochrome alpha peak (556 nm) minus 539 nm trough [24]. Carbon monoxide competes with pyridine for ligation to the heme and must be removed before forming the hemochromogen.

Ligand reaction rates

A Gibson-Milnes [25] stopped-flow apparatus with a 2-cm light-path in the observation cell, interfaced to an OLIS Data Acquisition/Computation System (On Line Instrument Systems, Route 2, P.O. Box 11131, Jeffersen, GA 30549, U.S.A.), was used to measure reaction rates.

A standard succinic acid – potassium phosphate buffer system (pH 7.0) was used to permit direct comparison with similar measurements

made on soybean Lb [23] and *Parasponia* Hb [6]. This buffer mixture contained 100 mM succinic acid (Fluka, purissima grade), 100 mM potassium dihydrogen phosphate (Baker, ultrex grade), and 1 mM EDTA (Fluka, purissima grade) adjusted to pH 7.0 with sodium hydroxide (Baker, ultrex grade).

Oxygen dissociation rate. Solutions of *C. glauca* HbO_2 (0.7 μM HbO_2, 2.8 μM free oxygen in buffer) were mixed rapidly with solutions of CO in buffer and the reaction was followed at 421 nm, a maximum in the difference spectrum *C. glauca* HbCO minus HbO_2. The rate was the same at 0.5 and 1.0 mM CO (before mixing).

Oxygen combination rate. Solutions of *C. glauca* ferrous Hb (0.68 and 0.83 μM Hb, 15 μM dithionite in buffer) were mixed rapidly with solutions of oxygen (2.9 to 11.6 μM in buffer, before mixing) and the reaction was followed at 417 nm, a wavelength isosbestic for ferrous and high-spin ferric *C. glauca* Hb, or at 434 nm, a maximum in the difference spectrum ferrous Hb minus HbO_2. Under these conditions, the reaction of oxygen with ferrous Hb is very much more rapid than the reaction with dithionite, and excess dithionite pre-sent in the solution does not interfere. The second-order combination rate constant was obtained graphically as the slope of a plot of the observed pseudo-first-order rate versus oxygen concentration (after mixing).

Carbon monoxide dissociation rate. A solution of *C. glauca* HbCO (prepared by adding HbO_2 to a solution containing 8 μM CO and 34 μM dithionite in buffer to give a mixture containing 3 μM HbCO, 5 μM free CO and 10–20 μM residual dithionite) were mixed rapidly with an oxygen-free solution of nitric oxide (approximately 2 mM) in buffer. The reaction was followed at 420 nm, an absorption maximum of HbCO.

Carbon monoxide combination rate. Solutions of ferrous Hb (1 μM Hb, 17 μM dithionite in buffer) were mixed rapidly with solutions of CO (10–80 μM CO, 17 μM dithionite in buffer, before mixing) and the reaction was followed at 417 nm, a wavelength near the 420 nm maximum in the difference spectrum HbCO minus ferrous Hb, and isosbestic for ferrous Hb and high-spin ferric Hb. The observed rate was the same at 434 nm, a maximum in the difference spectrum ferrous Hb minus HbCO. The second-order combination rate constant was obtained graphically as the slope of a plot of the observed pseudo-first-order rate versus CO concentration (after mixing).

Preparation of anti-sera

Casuarina glauca Hb (0.4 ml of a solution containing 1.67 mg Hb/ml buffer) was emulsified with an equal volume of Freund's complete adjuvant (Difco) and 0.4 ml of the mixture was injected intra-muscularly into a rabbit. Four weeks later, the rabbit was boosted with a sub-cutaneous injection of 0.2 ml of the Hb solution without adjuvant; it was bled from the marginal ear vein 4, 6 and 8 days later. The resulting antisera were tested individually by immunodiffusion and pooled. The immunoglobulin G fraction was isolated from the whole serum by precipitation with ammonium sulphate following the procedure of Hebert et al. [26]. Antisera against *Parasponia* Hb and soybean Lb were prepared earlier by similar schedules (W.F. Dudman, unpublished data).

Western blot analysis

Highly purified *Casuarina* Hb (mixed components), soybean Lba [21] and *Parasponia* HbI [3] were each applied to three replicate tracks on an SDS-polyacrylamide, 12.5–25% gradient slab gel. *Casuarina* Hb was added at 15 μg per track, and soybean and *Parasponia* Hb at 12 μg per track. An additional track contained approximately 15 μg of part-purified *Casuarina* Hb (from Sephacryl S-200, without concentration). The gel and samples were prepared and run overnight according to the basic procedures of Laemmli [27].

Following SDS-PAGE the separated proteins were electrophoretically transferred to a nitrocellulose sheet [28]. This was briefly stained (5 s) in Amido black (0.1% (w/v) in 45% (v/v) methanol/10% (v/v) acetic acid), destained with water until optimal resolution was obtained, and then photographed. The stain was largely removed by washing in water, though a visible residue remained throughout. Each set of replicate tracks was next shaken for 30 min in 5% (w/v) skim milk powder (Diploma instant), 0.9% (w/v) NaCl, 10 mM Tris (pH 7.4) and then incubated overnight in plastic pouches at room temperature with anti-sera (1/200 dilution in 5% skim milk powder, 0.9% NaCl, 10

mM Tris, pH 7.4) to one of the three proteins (anti-sera preparation described earlier). The strips were then washed (5 × 15 min) in 0.9% NaCl, 10 mM Tris (pH 7.4) and the bound anti-sera were detected by the protein A–peroxidase (Sigma) staining procedure described by Hawkes et al. [29]. The sheet was then photographed.

Analysis of sugars by gas-liquid chromatography and mass spectrometry

A sample (approx. 0.3 mg) of *Casuarina* Hb was analysed for sugar components by the procedure of Clamp et al. [30]. After treatment with 0.9 M hydrogen chloride in methanol at 82°C for 16 h, neutralization, re-*N*-acetylation and trimethylsilylation as described, the reaction mixture was analysed by gas-liquid chromatography/mass spectrometry on a BP-5 capillary column in a Varian 1400 gas chromatograph interfaced to a VG Micromass 70-70 mass spectrometer.

Results and Discussion

Casuarina nodules contain extractable hemoglobin

Using the standard extraction procedure (Materials and Methods) *C. glauca* nodules yielded clean pink solutions in which HbCO could be recognised from its optical absorption peaks at 568 nm, 540.5 nm and 421.5 nm (Fig. 1) superimposed on a steeply rising background absorption. An estimate of HbCO concentration was made by measuring the absorbance difference between the

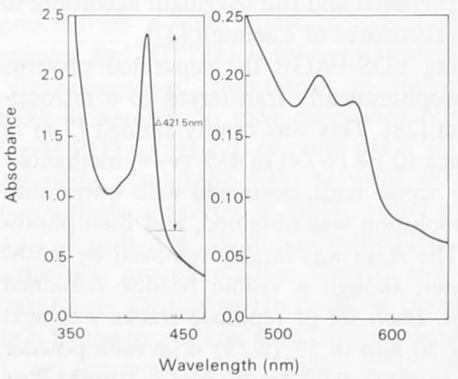


Fig. 1. Absorption spectrum of a crude extract of *Casuarina* HbCO, illustrating the measurement taken for calculation of Hb concentration. Concentration of Hb is approx. 9 μM in 50 mM potassium phosphate, 1 mM EDTA, pH 7.2 at 4°C.

Soret peak (421.5 nm) and a line drawn between the inflections either side of the peak (Fig. 1), assuming $\Delta E_{mM} = 120$ as found for similar crude extracts of *Parasponia* HbCO [3]. In this way the yield of soluble HbCO was calculated as approx. 32 nmol/g nodule fresh weight. This is lower than total bound Hb (83 nmol/g nodule fresh weight) reported by Davenport [9] and lower than the yield of soluble HbCO (45–70 nmol/g fresh weight) reported from *Parasponia* nodules [3]. This lower yield of soluble Hb from *Casuarina* nodules may in part reflect the generally lower percentage of infected cells found in actinorhizal nodules than in *Rhizobium*-induced nodules [12] and also indicate an incomplete solubilization of the protein.

Repeating Davenport's procedure [9], we found that extraction of *Casuarina* nodules with aqueous buffer solutions failed to yield soluble Hb; it remained in a tight pink band at the top of the pellet following centrifugation. This band also contained much of the endophyte material, but it is not clear whether the Hb was actually adsorbed to endophyte. Only when soluble polyvinylpyrrolidone (Kollidon 25) was added to the extraction media, ostensibly as an inhibitor of polyphenol oxidase [31], was Hb obtained in solution. Furthermore, the maintenance of strict anaerobiosis, the inclusion of dithionite, and CO saturation of extraction media were found to be essential for the recovery of undegraded soluble Hb.

Under no extraction condition, however, could soluble undegraded Hb be obtained from field-grown nodules of any age. This was probably due to the products of polyphenol oxidase activity [9] masking, denaturing, or polymerizing the Hb. Polyphenol oxidase activity was much more vigorous in field-grown than in glasshouse-grown nodules.

Sephacryl S-200 'affinity chromatography' and Sephadex G-75 molecular exclusion chromatography of Casuarina Hb

Passage of crude extracts of *Casuarina* Hb through a Sephacryl S-200 column gave an unanticipated result (Fig. 2). Although total recovery was usually 95–100%, only about 8% of the applied Hb was eluted at $K_d = 0.58$, the position expected if *Casuarina* Hb were a monomeric pro-

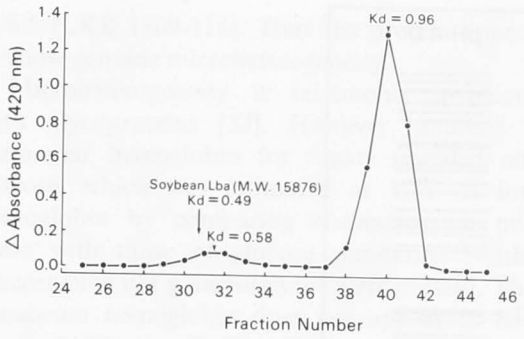


Fig. 2. Chromatography of crude *Casuarina* HbCO on a column of Sephacryl S-200 SF as described in Materials and Methods. Fractions were monitored at 420 nm

tein of the same size as soybean Lb (M_r 15 876 including heme, as calculated from sequence data [1]). The remainder suffered weak adsorption, being eluted as a near-symmetrical profile at K_d = 0.96. This adsorption phenomenon had not been observed during passage of any legume Lb (C.A. Appleby, unpublished data) or of *Parasponia* Hb [3] through Sephacryl S-200. In a subsequent series of trials the weak adsorption of *Casuarina* Hb was observed only with particular batches of Sephacryl S-200 manufactured before 1980. Other batches of pre-1980 Sephacryl S-200 showed much stronger adsorption and consequent low recovery. Post-1980 Sephacryl S-200, made by a slightly modified procedure (information supplied by manufacturer), showed no adsorption. Washing of pre-1980 Sephacryl S-200 with non-ionic detergent or 0.2 M NaOH did not destroy its adsorptive capacity, nor did long-term storage in 0.02% (w/v) sodium azide. Thus, a 'best' mixed batch of pre-1980 Sephacryl S-200 has been repeatedly recycled and used to purify all crude *Casuarina* Hb extracts obtained during this study.

In preliminary trials with *C. cunninghamiana* nodules the use of non-ionic or zwitterionic detergents in extraction buffers did not increase the yield of soluble Hb; in fact such detergents without soluble polyvinylpyrrolidone completely failed to solubilize Hb. Inclusion of zwitterionic detergent in addition to soluble polyvinylpyrrolidone did, however, dramatically improve the subsequent recovery of Hb from Sephacryl S-200. In a trial involving several detergents used at just below critical micelle concentration in extraction media, recoveries from the 'pre-1980' Sephacryl S-200 column were: no detergent, 23%; 6 mM CHAPS (Sigma Cat. No. 3023), 63%; 3 mM dodecyl β-D-maltoside (Calbiochem Cat. No. 324355), 64%; 3 mM Zwittergent 3–12 (Calbiochem Cat. No. 903843) 84%. Consequently, 3 mM Zwittergent 3–12 was then used routinely in extraction media, where 95–100% recovery of *C. glauca* Hb was obtained.

The weak 'affinity chromatography' phenomenon between pre-1980 Sephacryl S-200 and *Casuarina* Hb was exploited in the purification of this protein. The anomalous elution position of *Casuarina* Hb (Fig. 2) resulted in near-complete separation of Hb from other proteins, including polyphenol oxidase. Also, since the Hb was eluted with much smaller molecules as principal remaining impurities, very significant further purification was obtained by pressure filtration through Amicon YM10 (M_r 10 000 exclusion) membranes, as shown by the increased ratio of HbCO Soret peak to ultraviolet absorption peak (Fig. 3). Subsequent passage through Sephadex G-75, from which it was eluted with $K_d = 0.4$, the expected position for a monomeric Hb with M_r 17 000–18 000, further purified the Hb (Fig. 3). Its optical absorption properties, with R 421.5 : 280

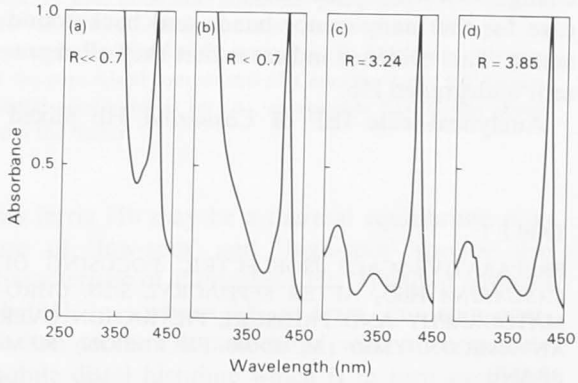


Fig. 3. Ultra-violet absorption spectra of Casuarina HbCO following (a) extraction and centrifugation, (b) Sephacryl S-200 chromatography, (c) pressure filtration over an Amicon YM10 (M_r 10000 exclusion) membrane, and (d) Sephadex G-75 chromatography. All samples were in 50 mM CO-saturated potassium phosphate, 1 mM EDTA, pH 7.2 at 4°C, and were equilibrated with CO. Techniques of sample preparation are given in Materials and Methods. Spectra were recorded at 20°C and are replotted to give a 421.5 nm (Hb soret peak) absorbance of 1.0 in each case. The ratio (R) of Hb Soret peak (421.5 nm) to protein peak (280 nm) is shown on all plots.

nm = 3.85 (Fig. 3), suggested purity at least equivalent to the IEF-separated Hb subcomponents (R 421.5 : 280 nm = 3.16 for IEF component I).

Separation of Casuarina Hb into multiple components by isoelectric focusing

Preparative-scale IEF of *Casuarina* HbO_2 resulted in the separation of three major red-coloured components with reproducible isoelectric points (Table I), a number of minor components which varied from run to run, and a general background smear of red colour. Notably, even though oxygenated *Casuarina* Hb is the most stable root nodule Hb we have handled in preparative procedures, we obtained only 20% recovery (Table I) in the three major IEF fractions, in marked contrast with the high yields of major component(s) after preparative-scale IEF of soybean Lb [21] and *Parasponia* Hb [3]. Recent experiments with *Parasponia* Hb under identical conditions yielded 80–85% recovery in the two major components (C.A. Appleby, unpublished data). Despite their low yield, the three major components of *Casuarina* Hb were of high purity with reproducible isoelectric points (Table I). All showed spectra typical of HbO_2 as eluted, and could be shown to undergo reversible ligand exchange between oxygen and CO. This was also the case for the many minor bands and background smear. This evidence indicates that they all represent undegraded Hb.

Analytical-scale IEF of *Casuarina* Hb mixed components, as purified by Sephadex G-75 chromatography, revealed extensive microheterogeneity. Besides the three major components detectable on unstained gels (analytical or preparative scale), about thirty minor bands were visible after Coomassie blue protein staining (Fig. 4), all of which also stained positively for heme. This suggested that all bands were Hb. Multiple banding also occurred during analytical IEF in the presence of 9 M urea, where the Hb would be unfolded, showing that conformational variation was not a principal cause of heterogeneity. Further, interaction with specific ampholyte components was ruled out by observation of the same banding pattern irrespective of where sample wicks were placed on prefocused gels, and when the routinely-used Pharmalyte 4–6.5 (Pharmacia 17-0452-01) was replaced by blended Ampholine

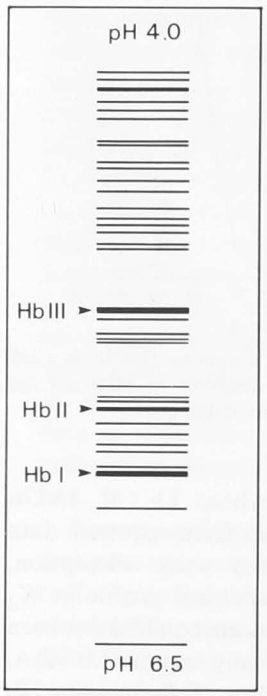


Fig. 4. Analytical-scale isoelectric focusing (pH 4–6.5) of approximately 12 μM *Casuarina* HbO_2 in water, purified by Sephacryl S-200 and Sephadex G-75 chromatography, as described in Materials and Methods. The gel from which the focusing pattern is redrawn was stained in Coomassie blue R250. An identical pattern was obtained when stained for heme with 3-3′,5-5′-tetramethylbenzidine. The positions of the major components (Table I) are indicated.

TABLE I

PREPARATIVE-SCALE ISOELECTRIC FOCUSING OF *CASUARINA* HbO_2 AFTER SEPHACRYL S-200 CHROMATOGRAPHY AND PRESSURE FILTRATION OVER AN AMICON YM10 (M_r 10000 EXCLUSION) MEMBRANE

Details of procedures are in Materials and Methods. Only the three major components are recorded. Total recovery of applied Hb = 20%

	Component number		
	I	II	III
p*I*	5.92	5.57	5.32
% of recovered Hb	62	18	20

4–6.5 (LKB 1809-116). Thus the protein appears to show genuine microheterogeneity.

Microheterogeneity is commonly associated with glycoproteins [33]. However, analysis of *Casuarina* hemoglobin for sugars revealed only glucose, which was estimated as 1.7% of total hemoglobin by comparing chromatogram peak areas with those of glucose standards. Neither glucosamine nor galactosamine were present. Thus *Casuarina* hemoglobin does not appear to be a classical glycoprotein [33]. The possibility that this protein is non-enzymically glucosylated as are certain components of human hemoglobin, or that other conjugated groups are present on the protein and contributing to microheterogeneity, is currently being investigated. Some of the observed microheterogeneity, particularly the three major components separable by IEF, may be attributable to amino acid sequence variation in the protein, reflecting a multi-gene family responsible for hemoglobin production. This is known to be the case with soybean, where there are four Lb genes and two pseudogenes [32].

Optical spectra of Casuarina Hb

The optical absorption spectra of *Casuarina* ferrous Hb and of the ligated species HbO_2 and HbCO (Fig. 5A), by their similarity to those of soybean Lb [4], *Parasponia* Hb [3] and animal Hb and myoglobin, allow that this protein could have a similar structure, and similar function as an oxygen carrier. This was supported by the observation of reversible ligand exchange between oxygen and CO. In peroxidase, another hemoprotein expected to be isolated from plant extracts, simple replacement of oxygen by CO cannot occur (cf. Ref. 3).

The principal absorption peaks of *Casuarina* ferrous Hb and its ligated complexes (Fig. 5A) are displaced 3–5 nm to longer wavelength compared to those of *Parasponia* Hb [3] or soybean Lb [4]. We note with interest that the positions of these red-shifted peaks were accurately reported by Davenport [9] in his original description of membrane-bound *Casuarina* Hb. The spectrum of *Casuarina* ferric Hb (Fig. 5B) is also of interest, insofar as the relative heights of the ferric hemochrome bands at 525 and 560 nm and charge transfer bands at 633 and 490 nm [3] suggest that

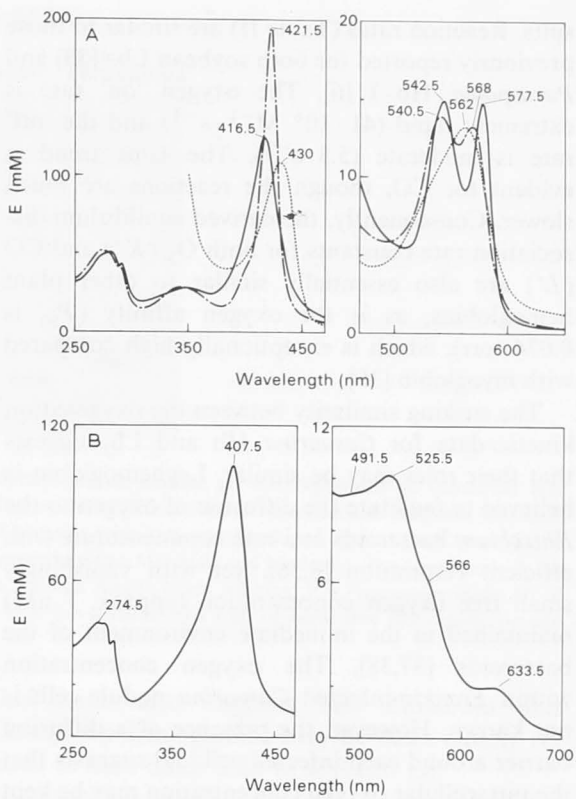


Fig. 5. (A) Absorption spectra of the pure mixed components of *Casuarina* ferrous Hb (dotted line), HbCO (dashed line) and HbO_2 (continuous line). Sample concentrations were approx. 11 μM, in 50 mM potassium phosphate, 1 mM EDTA, pH 7.2 at 4°C. All spectra were recorded at 20°C and are replotted at millimolar concentration. (B) Absorption spectrum of the pure mixed components of *Casuarina* ferric Hb. Experimental conditions as for (A), except that EDTA was absent from the buffer.

this ferric Hb may be a thermal equilibrium mixture of 'low-spin' and 'high-spin' species also characteristic of *Parasponia* Hb [3] and of soybean Lb [34]. In soybean Lb, at least, this spin equilibrium mixture is associated with an extremely mobile distal histidine which is in turn supposed to be associated with rapid oxygen binding [34].

Kinetics of reactions with oxygen and carbon monoxide

The kinetics of the reactions of *Casuarina* Hb with oxygen and carbon monoxide were investigated for both the purified mixed components (unfocused) and the major component Hb II separable by IEF (Table I). Both gave identical re-

sults. Reaction rates (Table II) are similar to those previously reported for both soybean Lba [33] and *Parasponia* Hb I [6]. The oxygen 'on' rate is extremely rapid ($41 \cdot 10^6$ $M^{-1} \cdot s^{-1}$) and the 'off' rate is moderate (5.5 s^{-1}). The same trend is evident for CO, though the reactions are much slower. Consequently, the derived equilibrium dissociation rate constants for both O_2 (K') and CO (L') are also essentially similar to other plant hemoglobins, as is the oxygen affinity (P_{50} is 0.074 torr), which is exceptionally high compared with myoglobin [35].

The striking similarity between the oxygenation kinetic data for *Casuarina* Hb and Lb suggests that their roles may be similar. Leghemoglobin is believed to facilitate the diffusion of oxygen to the *Rhizobium* bacteroids at a rate commensurate with efficient respiration [8,36], yet with vanishingly small free oxygen concentration (approx. 7 nM) maintained in the immediate environment of the bacteroids [37,38]. The oxygen concentration within *Frankia*-infected *Casuarina* nodule cells is not known. However, the presence of a diffusion barrier around each infected cell [39] suggests that the intracellular oxygen concentration may be kept low by rapid respiratory oxygen consumption. This is supported by the observed absence of vesicles on *Frankia* hyphae within the *Casuarina* nodule [19]. In nitrogen-free culture at very low oxygen pressure ($p_{O_2} = 0.001$–0.003 atm) the isolated *Frankia* forms hyphae only, whereas at higher p_{O_2} vesicles are formed [40].

The molecular weight and immunological relationships of Casuarina hemoglobin to other plant hemoglobins: evolutionary implications

A common evolutionary origin has been postulated for all plant hemoglobins on the basis of amino acid sequence homology [5] and gene sequence homology between soybean Lb and *Parasponia* Hb [7]. To further investigate this hypothesis, we have examined the immunological relationships amongst *Casuarina* Hb, *Parasponia* HbI and soybean Lba by the Western blotting procedure [41].

Casuarina Hb is resolved into one major and two minor bands on SDS-PAGE (Fig. 6A), with the major band being midway between *Parasponia* HbI and soybean Lba, indicating a molecular weight of approximately 16 900 for the heme-free protein. This is in broad agreement with its behaviour as an undenatured protein on Sephadex

TABLE II

KINETIC AND EQUILIBRIUM CONSTANTS FOR THE REACTIONS OF *CASUARINA* Hb WITH LIGANDS, COMPARED WITH THOSE OF *PARASPONIA* Hb AND SOYBEAN Lba

All values for *Casuarina* Hb were determined at pH 7.0. Values for *Parasponia* Hb and soybean Lb are taken at the alkaline limit of pH-dependent change: pH 7.0–9.0 for *Parasponia* Hb, and pH 7.0 for soybean Lb. All data at 20 °C. Equilibrium constants are calculated from the kinetic constants. Data for *Parasponia* Hb from Wittenberg et al. [6]; data for soybean Lba from Appleby et al. [23].

	Casuarina Hb II	*Parasponia* Hb I	Soybean Lba
Oxygen			
k' 'on' ($M^{-1} \cdot s^{-1}$)	$41 \cdot 10^6$	$165 \cdot 10^6$	$116 \cdot 10^6$
k 'off' (s^{-1})	5.5	14.8	5.55
K' ($= k : k'$) (nM)	135	89	48
p_{50} (torr)	0.074	0.049	0.026
Carbon monoxide			
l' 'on' ($M^{-1} \cdot s^{-1}$)	$2.34 \cdot 10^6$	$13.7 \cdot 10^6$	$12.7 \cdot 10^6$
l 'off' (s^{-1})	0.0027	0.0187	0.0078
L' ($= l : l'$) (nM)	1.2	1.4	0.62
p_{50} (torr)	$0.84 \cdot 10^{-3}$	$1.0 \cdot 10^{-3}$	$0.45 \cdot 10^{-3}$
Partition coefficient [a]			
$M = [HbCO]\, p_{O_2} / [HbO_2]\, p_{CO}$	87	50	58

[a] These numbers are calculated in terms of the gaseous pressures; alternatively one could define M' in terms of the ratio of the two equilibrium constants L/K, in which case $M' = 1.34$ M at 20 °C.

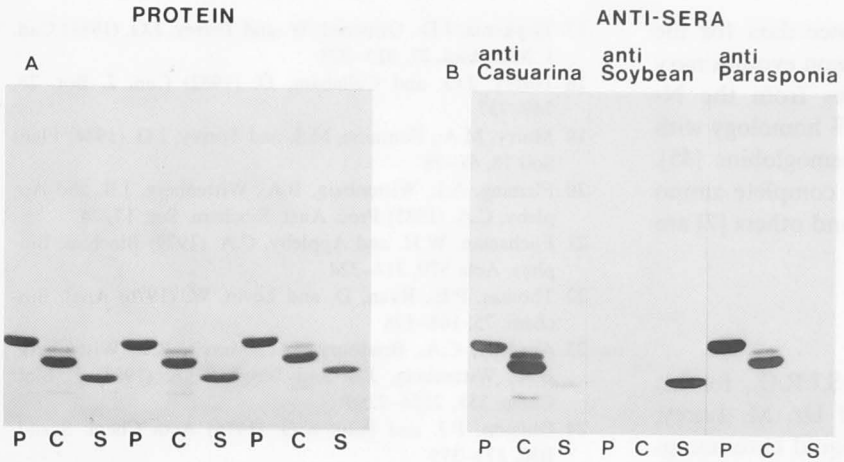


Fig. 6. Western blot of plant hemoglobins. (A) Sodium dodecyl sulphate polyacrylamide gel electrophoresis of *Casuarina* Hb, *Parasponia* Hb and soybean Lb seen after electro-blotting to a nitro-cellulose sheet, as described in Materials and Methods. The sheet was stained for 5 s in 0.1% Amido Black. (B) Pattern of reactions of proteins with anti-sera developed against each hemoglobin. Bound anti-sera were recognised by the protein–A peroxidase staining procedure (see Materials and Methods). The faint bands in the *Casuarina* and *Parasponia* tracks incubated with anti-soybean Lb are residual Amido black staining. P = *Parasponia* HbI (M_r of heme-free monomeric sub-unit 18685, calculated from sequence data [7]), S = soybean Lba (M_r of heme-free monomeric protein 15242, calculated from sequence data [1]), C = *Casuarina* Hb.

G-75, suggesting that it is monomeric (cf. *Parasponia* HbI, a dimer [3,6]). The fast-running minor band (approximate molecular weight 14100) is most likely slightly degraded protein. The nature of the slower running Hb (approximate molecular weight 18000) is not known. It may be either a separate gene product or a conjugated form of the protein.

Strong homologous reactions occurred between each of the proteins and their respective antisera (Fig. 6B). In addition, reciprocal cross-reactions occurred between *Casuarina* Hb and anti-*Parasponia* Hb antiserum, and also between *Parasponia* Hb and anti-*Casuarina* antiserum. Very weak non-reciprocal cross-reactions occurred between soybean Lb and both the anti-*Casuarina* and the anti-*Parasponia* antisera. The anti-soybean antiserum was specific for soybean Lb, giving no detectable reactions with either of the other hemoglobins.

This finding provides further support for the argument of a common evolutionary origin for plant hemoglobins. The serological cross-reactions indicate that the three proteins, particularly *Casuarina* and *Parasponia* hemoglobins, have at least some of the same antigenic determinants, a highly unlikely event unless they shared a common evolutionary origin. While not conclusive, this result also suggests that *Casuarina* and *Parasponia* hemoglobins may be more closely related to each other than to soybean Lb.

Conclusions

The work reported here establishes unequivocally that *Casuarina* root nodules contain a soluble Hb resembling other known plant hemoglobins, with similar oxygen-binding properties. It is thus reasonable to assume that the observations by Davenport [9] and later Tjepkema [13] of Hb absorption bands in the nodules of other actinorhizal plants will lead eventually to the characterization of similar soluble hemoglobins. The taxonomic orders which contain all known Hb-bearing species are widely separated according to any of the major phylogenetic schemes [42–44]. It should be noted that most actinorhizal species have not yet been investigated for Hb and it is thus possible that the distribution of Hb in plants is more widespread than is currently known. Assuming a single origin of the hemoglobin gene in plants, this extensive distribution adds considerable weight to the argument that Hb genes have been present in the plant kingdom since at least early in the evolutionary history of the angiosperms, if not since the divergence of the plant and animal lines [8].

Preliminary amino acid sequence data for the *C. glauca* Hb also support a common evolutionary origin, with the first 42 residues from the N-terminus showing greater than 40% homology with both *Parasponia* and soybean hemoglobins [45]. We are currently determining the complete amino acid sequence of *Casuarina* Hb, and others [7] are determining the gene sequence.

Acknowledgements

We thank Dr. D. Spencer, C.S.I.R.O., for his help with the Western blot, and Dr. M. Lacey, C.S.I.R.O., for undertaking gas-liquid chromatography/mass spectrometry analyses of hemoglobin samples. Dr. A. Kortt, C.S.I.R.O., is thanked for many useful discussions. The technical assistance of Mrs. L. Grinvalds is much appreciated. One of us, A.I.F., was in receipt of a Commonwealth Postgraduate Research Award for most of this work.

References

1 Hyldig-Nielsen, J.J., Jensen, E.Ø., Paludan, K., Wiborg, O., Garrett, R., Jørgensen, P. and Marcker, K.A. (1982) Nucleic Acids Res. 10, 689–701
2 Jeffreys, A.J. (1982) in Genome Evolution (Dover, G.A. and Flavell, R.B., eds.), pp. 157–176, Academic Press, London
3 Appleby, C.A., Tjepkema, J.D. and Trinick, M.J. (1983) Science 220, 951–953
4 Appleby, C.A. (1974) in The Biology Of Nitrogen Fixation (Quispel, A., ed.), pp. 521–554, Elsevier, New York
5 Kortt, A.A., Burns, J.E., Trinick, M.J. and Appleby, C.A. (1985) FEBS Lett. 180, 55–60
6 Wittenberg, J.B., Wittenberg, B.A., Gibson, Q.H., Trinick, M.J. and Appleby, C.A. (1986) J. Biol. Chem. 261, 13624–13631
7 Landsmann, J., Dennis, E.S., Higgins, T.J.V., Appleby, C.A., Kortt, A.A. and Peacock, W.J. (1986) Nature 324, 166–168
8 Appleby, C.A. (1984) Annu. Rev. Plant Physiol. 35, 443–478
9 Davenport, H.E. (1960) Nature 186, 653–654
10 Bond, G. (1951) Ann. Bot. N.S. 60, 447–459
11 Moore, A.W. (1964) Can. J. Bot. 42, 952–955
12 Becking, J.H. (1970) Plant Soil 32, 611–654
13 Tjepkema, J.D. (1983) Can. J. Bot. 61, 2924–2929
14 Zhang, Z. and Torrey, J.G. (1985) Ann. Bot. N.S. 56, 367–378
15 Hattori, J. and Johnson, D.A. (1985) Plant Mol. Biol. 4, 285–292
16 Torrey, J.G. (1985) in Nitrogen Fixation Research Progress (Evans, H.J., Bottomley, P.J. and Newton, W.E., eds.), pp. 293–299, Martinus Nijhoff, Dordrecht
17 Tjepkema, J.D., Ormerod, W. and Torrey, J.G. (1981) Can. J. Microbiol. 27, 815–823
18 Torrey, J.G. and Callaham, D. (1982) Can. J. Bot. 28, 749–757
19 Murry, M.A., Fontaine, M.S. and Torrey, J.G. (1984) Plant Soil 78, 61–78
20 Fleming, A.I., Wittenberg, B.A., Wittenberg, J.B. and Appleby, C.A. (1985) Proc. Aust. Biochem. Soc. 17, 24
21 Fuchsman, W.H. and Appleby, C.A. (1979) Biochim. Biophys. Acta 579, 314–324
22 Thomas, P.E., Ryan, D. and Levin, W. (1976) Anal. Biochem. 75, 168–176
23 Appleby, C.A., Bradbury, J.H., Morris, R.J., Wittenberg, B.A., Wittenberg, J.B. and Wright, P.E. (1983) J. Biol. Chem. 258, 2254–2259
24 Ohlsson, P.J. and Paul, K.G. (1976) Acta Chem. Scand. B30, 373–375
25 Gibson, Q.H. and Milnes, L. (1964) Biochem. J. 91, 161–171
26 Hebert, G.A., Pelham, P.L. and Pittman, B. (1973) Appl. Microbiol. 25, 26–36
27 Laemmli, U.K. (1970) Nature 227, 680–685
28 Bittner, M., Kupferer, P. and Morris, C.F. (1980) Anal. Biochem. 102, 459–471
29 Hawkes, R., Niday, E. and Gordon, J. (1982) Anal. Biochem. 119, 142–147
30 Clamp, J.R., Bhatti, T. and Chambers, R.E. (1971) Methods Biochem. Anal. 19, 299–344
31 Loomis, W.D. (1973) Methods Enzymol. 31, 528–544
32 Bojsen, K., Abildsten, D., Jensen, E.Ø., Paludan, K. and Marcker, K.A. (1983) EMBO J. 2, 1165–1168
33 Gibbons, R.A. (1966) in Glycoproteins; Their Composition, Structure and Function (Gottschalk, A., ed.), pp. 29–95, Elsevier, Amsterdam
34 Appleby, C.A., Blumberg, W.E., Bradbury, J.H., Fuchsman, W.H., Peisach, J., Wittenberg, B.A., Wittenberg, B.J. and Wright, P.E. (1982) in Hemoglobin and Oxygen Binding (Ho, C., ed.), pp. 435–441, Elsevier/North-Holland Inc., New York
35 Wittenberg, J.B., Appleby, C.A. and Wittenberg, B.A. (1972) J. Biol. Chem. 247, 527–531
36 Wittenberg, J.B., Bergersen, F.J., Appleby, C.A. and Turner, G.L. (1974) J. Biol. Chem. 249, 4057–4066
37 Appleby, C.A. (1969) Biochim. Biophys. Acta 188, 222–229
38 Klucas, R.V., Lee, K.K., Saari, L.L. and Erickson, B.K. (1985) in Nitrogen Fixation and Carbon Dioxide Metabolism (Luddon, P.W. and Burris, R.F., eds.), pp. 13–20, Elsevier, Amsterdam
39 Berg, R.H. (1983) Can. J. Bot. 61, 2910–2918
40 Murry, M.A., Zhang, Z. and Torrey, J.G. (1985) Can. J. Microbiol. 31, 804–809
41 Burnette, W.N. (1981) Anal. Biochem. 112, 195–203
42 Hutchinson, J. (1973) The Families of Flowering Plants, 3rd Edn., Clarendon Press, Oxford
43 Dahlgren, R.M.T. (1980) Bot. J. Linn. Soc. 80, 91–124
44 Cronquist, A. (1981) An Integrated System of Classification of Flowering Plants, Columbia University Press, New York
45 Kortt, A.A., Burns, J.E., Inglis, A.S., Appleby, C.A., Trinick, M.J. and Fleming, A.I. (1985) Proc. Aust. Biochem. Soc. 17, 43

Volume 231, number 2, 341–346 FEB 05794 April 1988

Amino acid sequence of hemoglobin I from root nodules of the non-leguminous *Casuarina glauca-Frankia* symbiosis

Alexander A. Kortt, Adam S. Inglis, Anthony I. Fleming* and Cyril A. Appleby+

*CSIRO, Division of Biotechnology, Parkville Laboratory, Melbourne, 3052 Victoria, *Department of Forestry, Australian National University, Canberra, ACT 2601* and *+CSIRO, Division of Plant Industry, Canberra, ACT 2601, Australia*

Received 4 March 1988

The amino acid sequence of hemoglobin I from nitrogen-fixing root nodules of the *Casuarina glauca-Frankia* symbiosis has been determined. The protein is composed of 151 amino acids including a single cysteine, a residue not found in the leghemoglobins. The molecular mass, including heme, is calculated to be 17856 Da. *C. glauca* hemoglobin I shows extensive sequence homology (43–52%) with other plant hemoglobins and this provides further evidence that hemoglobins from distant plant genera and animal hemoglobins share a common evolutionary origin.

Non-legume hemoglobin; Amino acid sequence; Plant hemoglobin sequence homology; Genetic origin; (*Casuarina glauca*)

1. INTRODUCTION

It is well known that hemoglobin occurs in nitrogen-fixing nodules of all legume-*Rhizobium* symbioses [1]. Structural studies [2,3], amino acid and gene sequence analyses [3–5] have confirmed that leghemoglobin is genetically related to the animal globins. On this basis it has been suggested that hemoglobin arose in ancient legumes following an act of horizontal gene transfer from the animal kingdom [4], a suggestion which assumed that the occurrence of hemoglobin in the plant kingdom was unique to legume nodules. This premise was proved false by the isolation of hemoglobin from the *Rhizobium*-induced nodules of the non-leguminous plant *Parasponia* (Ulmaceae) [6]. Both the amino acid sequence [7] and gene sequence [8] of this protein show considerable homology with leghemoglobin, indicating a common ancestral origin.

The finding of a common hemoglobin in two distantly related plant families nodulated by *Rhizobium* raised the question of just how widespread hemoglobin is in the plant kingdom [6]. Recent studies have confirmed Davenport's 1960 claim [9] that hemoglobin is indeed widely distributed in plants. It has been observed in nodules of phylogenetically-disparate species [8] which form nitrogen-fixing symbioses with the actinomycete *Frankia* [10,11].

Hemoglobin purified from the *Casuarina glauca-Frankia* symbiosis has physicochemical properties (molecular mass, optical spectra, kinetics of oxygen binding and oxygen affinity) similar to those of leghemoglobin and *Parasponia* hemoglobin, with which it is immunologically related [11]. These results suggest, but do not prove that *Casuarina* hemoglobin (and, by implication, other actinorhizal hemoglobins) shares a common origin with leghemoglobin and *Parasponia* hemoglobin. The amino acid sequence reported here establishes more firmly the relationship of *Casuarina* hemoglobin to other plant hemoglobins.

Correspondence address: A.A. Kortt, CSIRO, Division of Biotechnology, Parkville Laboratory, 343 Royal Parade, Parkville, 3052 Victoria, Australia

2. EXPERIMENTAL

C. glauca hemoglobin, hereafter referred to as *Casuarina*

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

hemoglobin, was isolated in low yield from fresh nodules of *C. glauca* Sieb ex Spreng inoculated with *Frankia* sp. and purified as in [11]. The *Casuarina* hemoglobin mixture obtained after sequential Sephacryl-200 and Sephadex G-75 chromatography [11] was further fractionated by preparative isoelectric focusing (IEF) as described [11] to yield hemoglobins I, II and III. Because of the low yields obtained for the separated hemoglobins, preliminary sequence information was obtained with the *Casuarina* hemoglobin mixture using ~0.8 mg of protein, and the complete amino acid sequence of the major component, hemoglobin I, was determined with ~0.75 mg (44 nmol) of protein.

The hemoglobin I sample (0.75 mg) was dissolved in 0.2 ml of 0.1 M Tris-HCl, 2% SDS, buffer, pH 8.5, reduced with dithiothreitol (0.01 M) at 50°C for 2 h under N_2 and alkylated with iodoacetic acid (0.04 M) for 1 h at 25°C in the dark. The reaction was stopped by the addition of an excess of dithiothreitol and dried under vacuum at 50°C. The reaction mixture was redissolved in 0.1 ml distilled water and the protein precipitated with 0.9 ml cold methanol, washed once with 1.0 ml of cold methanol and dried under N_2. The complete procedure was carried out in a 1.5 ml Eppendorf tube.

The *S*-carboxymethylated hemoglobin I was dissolved in 0.1 M ammonium bicarbonate buffer, pH 8.0, and aliquots (~12 nmol) were digested with trypsin (Worthington), α-chymotrypsin (Worthington) or *Staphylococcus aureus* V8 protease (Pierce) at 37°C for 4 h at an enzyme/substrate ratio of 1:50 (w/w). Aliquots (~15 nmol) of native *Casuarina* hemoglobin mixture were also digested with these three enzymes under the same conditions and the tryptic digests of native hemoglobin II and III were carboxymethylated immediately following digestion as described [12]. The digests were dried under vacuum at 50°C, redissolved in 0.25 ml of 0.1% (v/v) trifluoroacetic acid and the soluble peptides isolated by HPLC in 0.1% (v/v) trifluoroacetic acid on a Vydac 218TP54 column using an acetonitrile gradient.

Peptides (using 2–4 nmol) were sequenced either on a gas-phase sequencer or manually by a modified Edman procedure [13]. Residues were identified as the PTH-amino acid derivatives by HPLC as in [14]. Amino acid analysis was performed on a Waters HPLC amino acid analyser after hydrolysis of 1–2 nmol protein in 6 M HCl for 24 h at 110°C. Tryptophan was determined after hydrolysis of the protein in 4 M methane sulfonic acid/0.2% (w/v) tryptamine for 24 h at 115°C. The carboxyl-terminal residues were determined by amino acid analysis after digestion of the protein with carboxypeptidase Y (1%, w/w) in 0.1 M pyridine-acetate buffer, pH 5.6, at 37°C for 1 and 2 h.

3. RESULTS

The amino-terminal sequence of intact *Casuarina* hemoglobin I (residues 1–38) was determined using the gas-phase sequencer. The complete amino acid sequence of hemoglobin I was established by sequencing the peptides derived from tryptic, chymotryptic and staphylococcal protease digests of the protein as illustrated in fig.1. The tryptic peptides, isolated by RP-HPLC accounted for 147 residues of the molecule. The alignment of these tryptic peptides and the sequence of the two dipeptides not isolated, T11 and T14, was established with overlapping chymotryptic and staphylococcal protease peptides, and by homology, as shown in fig.1.

Tryptic peptides T1–T5 confirmed the gas-phase sequencer data and peptides T5, T6 and T7 were overlapped by S2 and C9 to establish the sequence to residue 58. Peptides T7, T8, T9 and T10 were located in the sequence by homology with other plant globins (fig.2) and peptide S3 provided the overlap to extend the sequence to residue 78. Peptides T10, T11 and T12 were overlapped by S5 and S6 overlapped T12 and T13 extending the sequence to residue 99. Peptides C15 and S6 connected T13, T14 and T15 and S7 connected T15 and T16 to extend the sequence to residues 119. Peptides T16 and T17 were overlapped by S7 and C17 and T17 and T18 were overlapped by S8. Finally peptides T18 and T19 were overlapped by C19, C21 and S10 to complete the sequence. Although S7 and S8 provided only a single residue overlap between T16, T17 and T18, the placements of these peptides are consistent with homology to other plant globins.

The carboxyl-terminal amino acid was identified as glutamic acid from the sequence data of peptide C21. Some difficulty was encountered in deducing the C-terminal residue by carboxypeptidase Y digestion. No major residue was released upon digestion of intact hemoglobin I with carboxypeptidase Y. A comparison of 1 h and 2 h digests, however, showed an increase in the yields of Glu, Lys, Met and Ala released consistent with the C-terminal sequence deduced from the peptide data.

The sequence determination showed that *Casuarina* hemoglobin I contains 151 amino acid residues corresponding to an M_r of 17856 (including the heme group) which is in agreement with the M_r of 17500 determined by SDS-PAGE [11]. The sequences of peptides T10 and C10 confirmed the presence of the single cysteine residue (identified as the PTH of carboxymethyl-*S*-cysteine) at position 71 which is located in the same region of the molecule, in a conserved sequence (fig.2), as that found in *Parasponia andersonii* hemoglobin I [8]. *Casuarina* hemoglobin I also contains 3 methionine residues in the C-terminal one-third of the molecule and Met-130 and Met-149 are homologous to corresponding

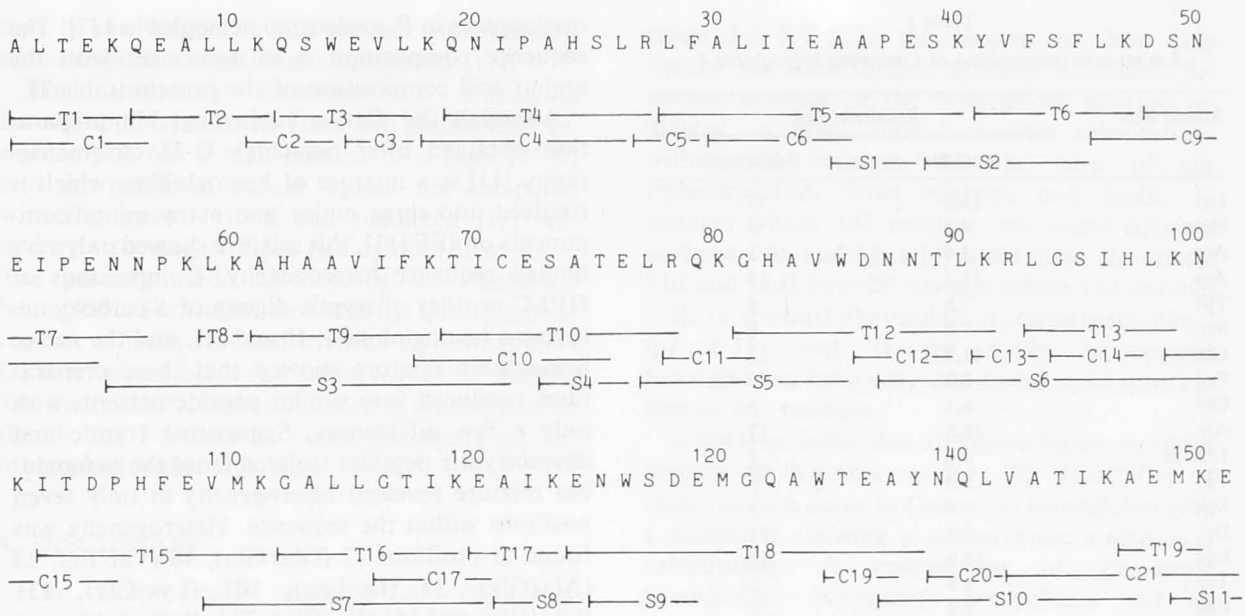


Fig.1. Primary structure of *Casuarina* hemoglobin I. The vertical bar indicates the amino-terminus of the peptide and the residues sequenced are indicated by the solid line. Peptides are numbered from the N-terminus of the protein where T, C and S signify tryptic, chymotryptic and staphylococcal protease peptides, respectively.

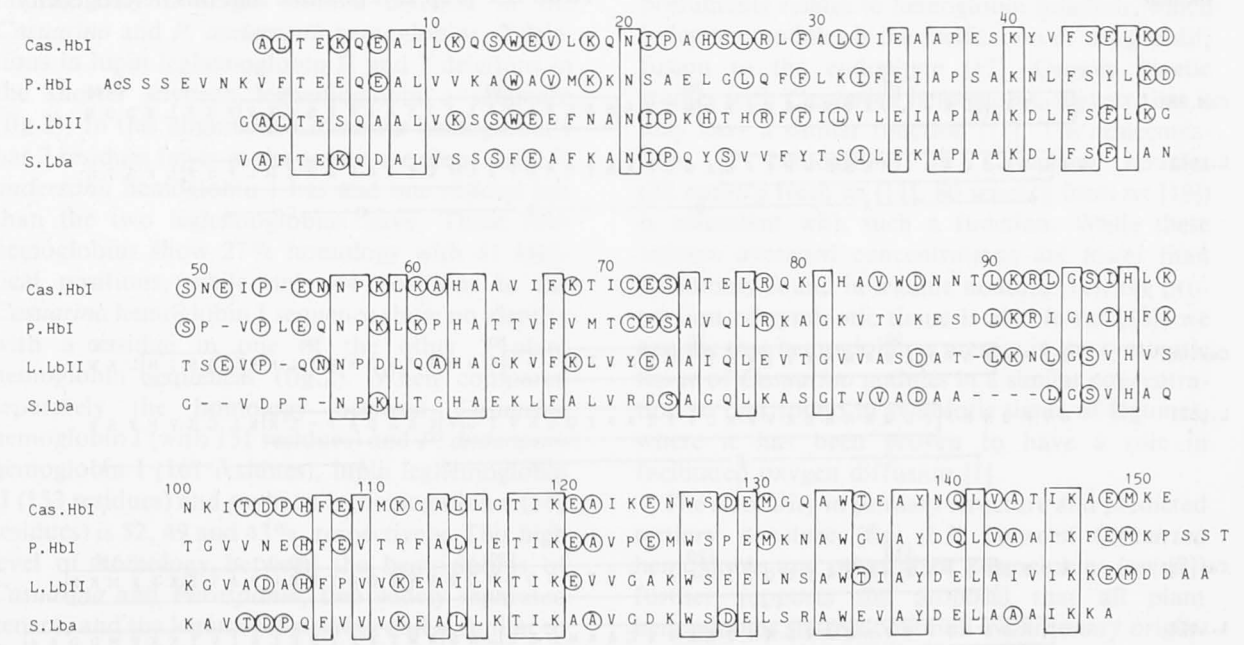


Fig.2. Sequence alignment indicating the homology between *Casuarina* hemoglobin I (Cas HbI), *P. andersonii* hemoglobin I (P.HbI) [7], lupin leghemoglobin II (L.LbII) [16] and soybean leghemoglobin a (S.Lba) [4]. Residues identical in the four plant hemoglobins are boxed. Ringed residues are identical in *Casuarina* hemoglobin I and one of the other hemoglobins. (——) Gaps introduced to optimize alignment.

Table 1

Amino acid composition of *Casuarina* hemoglobin I

Amino acid	Residues/mol	
	Analysis[a]	Sequence
Lys	16.3	17
His	4.2	5
Arg	3.6	3
Asx	13.7	13
Thr	7.6	8
Ser	7.6	8
Glx	21.4	22
Pro	5.2	5
Gly	6.1	5
Ala	16.5	17
1/2Cys	0.8	1
Val	5.9	6
Met	1.9	3
Ile	9.4	11
Leu	16.9	16
Tyr	1.8	2
Phe	5.4	5
Trp	3.3[b]	4

[a] Protein hydrolysed for 24 h at 110°C under vacuum in 5.7 M HCl

[b] Determined after hydrolysis of sample in 4 M $MeSO_3H$ containing 0.2% (w/v) tryptamine for 24 h at 115°C under vacuum

methionines in *P. andersonii* hemoglobin I [7]. The sequence composition is in agreement with the amino acid composition of the protein (table 1).

Although the *Casuarina* hemoglobin preparation obtained after Sephadex G-75 chromatography [11] is a mixture of hemoglobins, which is resolved into three major and many minor components on IEF [11], this mixture showed only very limited sequence heterogeneity. Comparisons of HPLC profiles of tryptic digests of *S*-carboxymethylated hemoglobins I, II and III, and the native hemoglobin mixture showed that these preparations produced very similar peptide patterns with only a few differences. Sequencing tryptic and chymotryptic peptides isolated from the hemoglobin mixture revealed heterogeneity at only seven positions within the sequence. Heterogeneity was found at positions 12 (Gln/Glu), 16 (Val/Ile), 23 (Ala/Gly), 33 (Ile/Leu), 101 (Lys/Gly), 131 (Gly/Glu) and 145 (Ile/Gly). This limited substitution of charged residues may account for some of the apparent heterogeneity of the hemoglobin mixture but it cannot, however, account for the large number of minor hemoglobin components observed on IEF [11]. It is possible that this heterogeneity

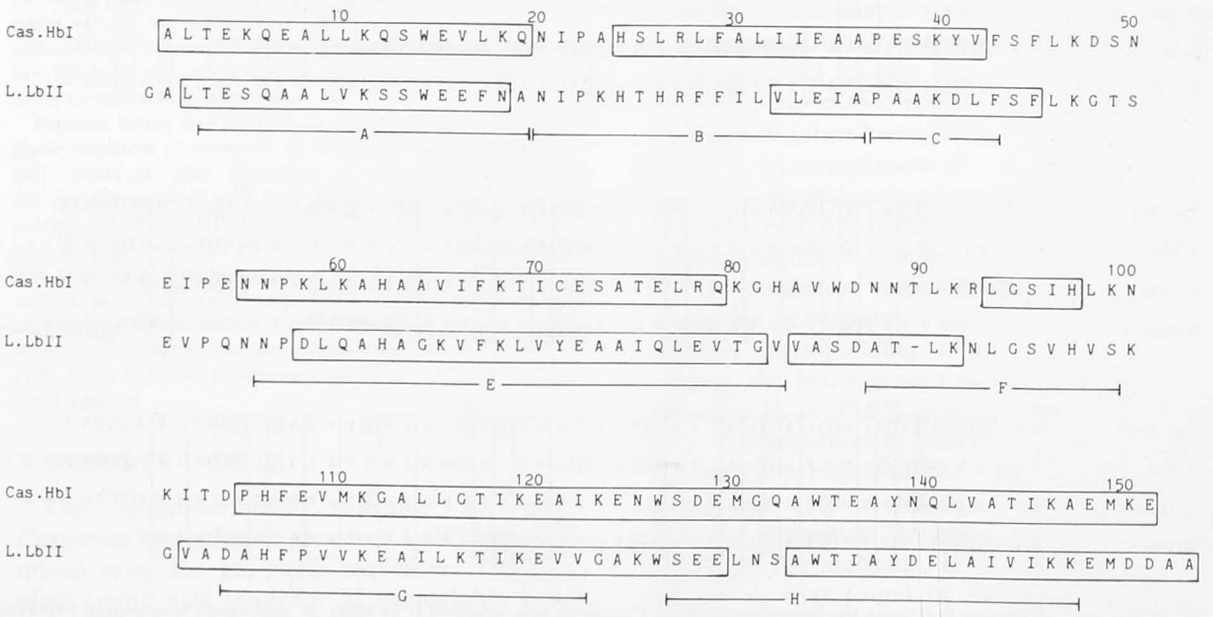


Fig.3. Comparison of the predicted α-helical segments of *Casuarina* hemoglobin I (Cas HbI) and the predicted and known α-helical segments of lupin leghemoglobin II (L.LbII). Boxes enclose the segments predicted as helices [15] and (——) designates the segments identified as helical from X-ray crystallographic studies [3].

may be due in part to non-enzymic glucosylation or other post-translational conjugation [11].

The secondary structure of *Casuarina* hemoglobin I predicted from the amino acid sequence [15] has a high content of α-helix (75%) and the predicted helical regions are compared with the known [3,16] and predicted [15] secondary structure of lupin hemoglobin II in fig.3. The predicted α-helical regions for *Casuarina* hemoglobin I correlate well with the known helical regions of lupin [16] and soybean leghemoglobins [2] determined by X-ray crystallographic studies. The method [15], however, failed to predict part of the B- and F-helices as noted previously [7] for other plant leghemoglobins.

4. DISCUSSION

The amino acid sequence of *Casuarina* hemoglobin I shows extensive homology with the sequence of hemoglobin I from *P. andersonii* [7], another non-legume, and the leghemoglobins from lupin [16] and soybean [4] (fig.2). Maximum homology was obtained with an alignment containing only a single residue deletion in the *Casuarina* and *P. andersonii* hemoglobins, 2 deletions in lupin leghemoglobin II and 7 deletions in the shorter soybean leghemoglobin a sequence (fig.2). In this alignment *Casuarina* hemoglobin I has 7 residues fewer at the amino-terminus than *P. andersonii* hemoglobin I has and one residue less than the two leghemoglobins have. These four hemoglobins show 27% homology with 41 identical positions, while only 44 positions in the *Casuarina* hemoglobin I sequence show no identity with a residue in one of the other 3 plant hemoglobin sequences (fig.2). When compared separately the homology between *Casuarina* hemoglobin I (with 151 residues) and *P. andersonii* hemoglobin I (161 residues), lupin leghemoglobin II (153 residues) and soybean leghemoglobin a (143 residues) is 52, 49 and 43%, respectively. This high level of homology between the hemoglobins of *Casuarina* and *Parasponia*, two widely separated genera, and the legumes is striking and is similar to the level of homology found between the hemoglobins within the legumes.

While IEF analysis of *Casuarina* hemoglobin suggested the presence of at least three major hemoglobin components with p*I* values in the range 5.3–5.9 [11], they appear to show only limited variation in their primary structures. Sequence analysis of the hemoglobin mixture indicated, at most, only 7 possible substitutions within 151 residues. In the case of the leghemoglobins from soybean and lupin, 12 changes within 143 residues are found between soybean Lba and Lbc1 [4] while lupin (*L. luteus*) LbI and LbII have 20 changes within 153 residues [16]. In contrast *Parasponia rigida* hemoglobins I (p*I* 6.15) and II (p*I* 5.64), non-legume hemoglobins, have only one amino acid substitution in 161 residues.

It has been noted that all known leghemoglobins contain 49 invariant residues [2]. Thirty-five of these residues occur in *Casuarina* hemoglobin with a further 6 showing a conservative amino acid substitution. A comparison of *Casuarina* hemoglobin, *Parasponia* hemoglobin and all known leghemoglobins reveals 31 invariant residues. This surprisingly high number, considering the phylogenetic distance which separates these plants, suggests that a strong conservative selection pressure has been active. This selection pressure presumably relates to hemoglobin function, which in legume nodules is the facilitation of oxygen diffusion to the endophyte [17]. Oxygen kinetic studies with *Casuarina* hemoglobin suggest that it may have a similar function [11]. The concentration of hemoglobin in *Casuarina* nodules (32 nmol/g fresh wt [11], 80 nmol/g fresh wt [18]) is consistent with such a function. While these volume averaged concentrations are lower than commonly found in legume nodules [18], the proportion of symbiotic tissue is also lower [10]; we assume that hemoglobin is present in the symbiotic tissue of *Casuarina* nodules in a similar concentration to that found in symbiotic tissue of legumes, where it has been proven to have a role in facilitated oxygen diffusion [1].

The similarity in primary structure and predicted tertiary structure (figs 2,3) between *Casuarina* hemoglobin and other plant hemoglobins (see [7]) further supports the proposal that all plant hemoglobins share a common evolutionary origin. Assuming the genes for hemoglobin have been inherited in these extant genera by a process of vertical descent, rather than horizontal transfer, the origin of hemoglobin in plants must be extremely primitive. Given that plant and animal

hemoglobins share the same origin [4,5], it seems probable that the hemoglobin gene has been present in both the plant and animal kingdoms since their divergence from a common ancestor. The presence of a central intron in the leghemoglobin and *Parasponia* hemoglobin genes [4,8], which has been taken to imply that these genes are more primitive than animal hemoglobin genes [4], supports this argument. It thus seems possible that the hemoglobin gene is widespread in the plant kingdom, a view reinforced by the recent finding of hemoglobin in root tissue of the non-symbiotic genera *Trema* and *Celtis* [19].

Acknowledgements: We thank J.E. Burns for technical assistance and F. Casagranda for gas-phase sequence analyses, P. Somers for HPLC analyses of PTH-amino acids and N. Bartone for amino acid analyses. A.I.F. was in receipt of a Commonwealth Postgraduate Research Award for much of this work.

REFERENCES

[1] Appleby, C.A. (1974) in: The Biology of Nitrogen Fixation (Quispel, A. ed.) pp.521–554, Elsevier, New York.
[2] Ollis, D.L., Appleby, C.A., Colman, P.M., Cutten, A.E., Guss, J.M., Venkatappa, M.P. and Freeman, H.C. (1983) Aust. J. Chem. 36, 451–468.
[3] Arutyunyan, E.G. (1981) Mol. Biol. (USSR) 15, 27–44.
[4] Hyldig-Nielsen, J.J., Jensen, E.Ø., Paludan, K., Wilborg, O., Garret, R., Jørgensen, P. and Marcker, K. (1982) Nucleic Acids Res. 10, 689–701.
[5] Antoine, M. and Niessing, J. (1984) Nature 310, 795–798.
[6] Appleby, C.A., Tjepkema, J.D. and Trinick, M.J. (1983) Science 220, 951–953.
[7] Kortt, A.A., Burns, J.E., Trinick, M.J. and Appleby, C.A. (1985) FEBS Lett. 180, 55–60.
[8] Landsmann, J., Dennis, E.S., Higgins, T.J., Appleby, C.A., Kortt, A.A. and Peacock, W.J. (1986) Nature 324, 166–168.
[9] Davenport, H.E. (1960) Nature 186, 653–654.
[10] Tjepkema, J.D. (1983) Can. J. Bot. 61, 2924–2929.
[11] Fleming, A.I., Wittenberg, J.B., Wittenberg, B.A., Dudman, W.F. and Appleby, C.A. (1987) Biochim. Biophys. Acta 911, 209–220.
[12] Merrill, B.M., Lopresti, M.B., Stone, K.L. and Williams, K.R. (1987) Int. J. Peptide Protein Res. 29, 21–39.
[13] Peterson, J.D., Nehrlich, S., Dyer, P.E. and Steiner, D.F. (1972) J. Biol. Chem. 247, 4866–4871.
[14] Woods, E.F. and Inglis, A.S. (1984) Int. J. Biol. Macromol. 6, 277–283.
[15] Garnier, J., Osguthorpe, D.J. and Robson, B. (1978) J. Mol. Biol. 120, 97–120.
[16] Egorov, T.A., Kazakor, V.K., Shakhparaonov, M.I. and Feigina, M.Y. (1980) Bioorg. Chem. 6, 666–683.
[17] Appleby, C.A. (1984) Annu. Rev. Plant Physiol. 35, 443–478.
[18] Tjepkema, J.D. and Asa, D.J. (1987) Plant Soil 100, 225–236.
[19] Bogusz, D., Appleby, C.A., Landsmann, J., Dennis, E.S., Trinick, M.J. and Peacock, W.J. (1988) Nature 331, 178–180.