nol is substituted for Dibal in step (ii), followed by deprotection. This results in the complete epimerization of the C(2) center (*7*), and provides L-altrose (**17**) (*11*).

The sequences leading to the other hexoses have also been carried out satisfactorily in the manner described above for L-allose and L-altrose (*12*). All steps in Fig. 2 except for the step **9** → **13** proceed with remarkable regio- and stereoselection. Since the mirror image of every compound in Fig. 2 can be prepared by simple exchange of the chiral ligand (tartrate ester) in the AE reaction, the formal synthesis of the D-hexoses has also been achieved. Thus, our two-carbon extension methodology has proved to be generally applicable and efficient in controlling stereochemistry in the construction of acyclic, polyhydroxylated carbon frameworks.

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1. This report is part 5 in a series on the synthesis of saccharides and polyhydroxylated natural products. For part 4, see L. A. Reed, III, Y. Ito, S. Masamune, K. B. Sharpless, *J. Am. Chem. Soc.*, in press.
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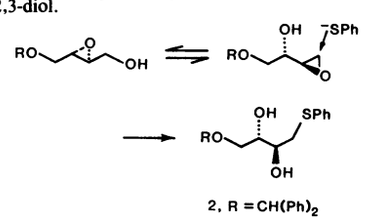
7. See Lee *et al.* (*6*). Although the hydrolysis with epimerization of the 4-carbon Pummerer product proceeds rapidly, completely, and in excellent yield, some complications are observed in the 6-carbon case. In general, this hydrolysis with epimerization proceeds in somewhat lower yield, primarily due to instability and difficulties with isolating the partially hydrated aldehyde product. The epimerization, however, proceeds to give an isomer ratio of > 95:5 in all these 6-carbon cases.
8. Available from Aldrich Chemical Co.
9. See Lee *et al.* (*6*) and references cited therein. Treatment of epoxy alcohol **2** with 0.5*N* NaOH (2.5 equivalents) in *t*-butyl alcohol followed by slow addition of thiophenol (1.2 equivalents) in moist *t*-butyl alcohol and subsequent trapping of the primary epoxide by thiolate anion lead to the 2,3-diol.



2, R = $CH(Ph)_2$

Treatment of the newly formed diol with 2-methoxypropene in the presence of a catalytic amount of camphorsulfonic acid provides **3** in 71 percent overall yield with a 4:1 selectivity.
10. S. Iriuchijima, K. Maniwa, G. Tsuchihashi, *J. Am. Chem. Soc.* **96**, 4280 (1974).
11. Attempts to deprotect the L-altrose derivative with trifluoroacetic acid, followed by catalytic hydrogenation, invariably gave 1,6-anhydro-β-L-altropyranose, which is known to exist in acid solutions in equilibrium with the free sugar [S. Peat, in *Advances in Carbohydrate Chemistry*, W. W. Pigman and M. L. Wolfram, Eds. (Academic Press, New York, 1946), vol. 2, p. 38]. Exposure of commercially obtained D-altrose to acid also produced the 1,6-anhydro-β-D-altropyranose, which upon acetylation proved identical to the synthetic peracetylated β-L-1,6-anhydro derivative.
12. All eight synthetic L-hexoses had identical mobility on thin-layer chromatographic plates and had identical 270-MHz nuclear magnetic resonance (NMR) spectra in D_2O (except for slight differences) as commercially obtained samples of the D- or L-hexoses. In addition, all eight aldehydes (**16** to **23** in Fig. 2) were reduced and successfully transformed into their corresponding peracetylated hexitols, six out of the seven (altrose and talose give the same hexitol) of which were previously known. The synthetic hexitol acetates were judged on the basis of infrared spectra, NMR, melting points, and optical rotation (where applicable) to be identical with the naturally derived materials. This transformation unequivocally confirms the stereochemistry of the critical C(2) through C(5) centers. We were, however, unable to obtain satisfactory optical rotations for several of the synthetic L-hexoses, due primarily to the inaccuracies associated with weighing small quantities of hydrated samples. These results will be reported in full elsewhere.
13. We are grateful to the National Institutes of Health (grant GM 31124) and to the National Science Foundation for financial support. High-resolution mass spectra were provided by a facility supported by the National Institutes of Health (grant RR 00317).

8 November 1982

Hemoglobin in a Nonleguminous Plant, *Parasponia*: Possible Genetic Origin and Function in Nitrogen Fixation

Abstract. *A dimeric hemoglobin was purified from nitrogen-fixing root nodules formed by association of* Rhizobium *with a nonleguminous plant,* Parasponia. *The oxygen dissociation rate constant is probably sufficiently high to allow* Parasponia *hemoglobin to function in a fashion similar to that of leghemoglobin, by oxygen buffering and transport during symbiotic nitrogen fixation. The identification of hemoglobin in a nonlegume raises important questions about the evolution of plant hemoglobin genes.*

In higher plants, hemoglobin is generally thought to occur only in the nitrogen-fixing root nodules of legumes (*1*). Because the structure of legume hemoglobin (leghemoglobin) genes is very similar to that of animal globin genes, it has been suggested that the gene for leghemoglobin was transferred to legumes from another eukaryote outside the plant kingdom, relatively recently in evolutionary history (*2*). We report that hemoglobin also occurs in the nitrogen-fixing root nodules of *Parasponia*, a member of the Ulmaceae. This plant is nodulated by strains of *Rhizobium* that also nodulate certain members of the Leguminosae (*3*).

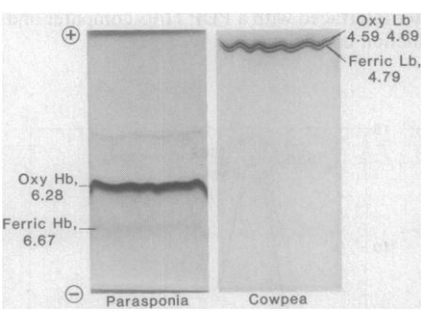


Fig. 1. Preparative-scale isoelectric focusing of oxyhemoglobin (*Oxy Hb*) from *P. andersonii* nodules and leghemoglobin (*Lb*) from cowpea [*Vigna unguiculata* (L.) Walp.] nodules, both inoculated with *Rhizobium* strain CP283 and grown as previously described (*3*). Focusing was performed at 4°C in a 100-ml gel slurry bed of Ultrodex (LKB) containing 0.5 percent Ampholine (LKB) (*p*H 4.0 to 6.0) and 1.5 percent Ampholine (*p*H 5.0 to 7.0) (*14*). The gel plate was loaded with approximately equal amounts of the total hemoglobin purified by Sephacryl S200 chromatography from *Parasponia* and cowpea nodule extracts. Isoelectric points, measured at 4°C, are marked.

The absorption spectra of *Parasponia* nodule segments are consistent with the presence of hemoglobin (*4*), and this *Parasponia* hemoglobin may be purified from nodules extracted under strict anaerobic conditions that prevent tannin formation and hemoglobin degradation. Fresh nodules from *Parasponia andersonii* Planch, infected with *Rhizobium* strain CP283 (*3*), or nodules frozen in liquid N_2 were dropped into the steel chamber of an Omnimixer (Sorvall) containing four volumes of extraction buffer [50 m*M* potassium phosphate (*p*H 7.2), 1m*M* EDTA, 4 percent soluble polyvinylpyrrolidone (Kollidon 25, BASF), and 0.1 percent sodium dithionite (Fluka)] equilibrated with pure CO. The mixture was ground at full speed under CO for 2 minutes at 0°C, then centrifuged under CO at 100,000*g* for 60 minutes at 0°C. The yield of crude carboxyhemoglobin was 45 to 70 nanomoles per gram of *Parasponia* nodule tissue (*5*). Anaerobic chromatography on a column of Sephacryl S200 (Pharmacia) equilibrated with CO-saturated 50 m*M* potassium phosphate and 0.1 m*M* EDTA (*p*H 7.2) at 0°C

was used to separate *Parasponia* hemoglobin from remaining impurities that could cause polyphenol oxidation. Deliberate exposure of this chromatographed product to air at 0°C caused the slow conversion of *Parasponia* carboxyhemoglobin to oxyhemoglobin without appreciable production of ferric hemoglobin. Final purification was achieved by preparative-scale isoelectric focusing (Fig. 1). This produced a single major component of oxyhemoglobin having an isoelectric point of 6.28 at 4°C and a much smaller amount of ferric hemoglobin with an isoelectric point of 6.67. Figure 1 also shows that leghemoglobin, prepared in the same way from cowpea nodules induced by the same *Rhizobium* strain, CP283, is focused as a group of several oxygenated and oxidized species with isoelectric points between 4.6 and 4.8. No trace of *Parasponia* hemoglobin is detectable from cowpea nodules, or vice versa. This result indicates that the plant host has *some* control over the types of hemoglobin that are produced in nodules infected with *Rhizobium* strain CP283.

It is possible that, in vivo, *Parasponia* hemoglobin is tightly bound to the *Rhizobium* bacteroid surface, and this may have been the reason for our inability to detect it in a previous investigation (*1*). If insoluble polyvinylpyrrolidone (Polyclar AT, GAF), which appears to be as effective as soluble polyvinylpyrrolidone (Kollidon 25) in suppressing polyphenol oxidation, is substituted for Kollidon 25 in our extraction procedure, then almost all *Parasponia* hemoglobin is found in the bacteroid pellet and not in plant cell debris, plant membrane fragments, or the supernatant. Davenport (*6*) reported the presence of an "insoluble" hemoglobin in nitrogen-fixing root nodules containing actinomycetous endophytes. One of us (J.D.T.) confirmed these observations with segments of nodules from several plant families (*4, 7*), and we have been able to extract "soluble" hemoglobin from *Casuarina cunninghamiana* Miq. nodules by isolation procedures similar to that outlined above (*7*).

Parasponia hemoglobin, purified by isoelectric focusing (Fig. 1) appeared homogeneous when subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis; it contained only one subunit type of ~ 21,000 daltons. Pyridine hemochrome analysis (*8*) of lyophilized, salt-free *Parasponia* hemoglobin showed the presence of 1 mole of protoheme per 22,000 g of protein (equivalent to one heme per subunit). On the other hand, chromatography on calibrated columns of Sephacryl S200 under nondenaturing conditions indicated an apparent molecular weight of 25,000 to 40,000 for *Parasponia* hemoglobin, depending on concentration, valence, and ligand state. This suggests that the functional protein is a readily dissociable dimer. In contrast, SDS–polyacrylamide gel electrophoresis or Sephacryl S200 chromatography showed cowpea leghemoglobin to be a 16,000-dalton monomer.

The absorption spectrum of pure *Parasponia* ferric hemoglobin is shown in Fig. 2. The predominance of hemichrome bands at 560 and 529 nm over charge-transfer bands at 620 and 484 nm (*9*) and the temperature sensitivity of the spectrum identify *Parasponia* hemoglobin as a thermal equilibrium mixture of low-spin and high-spin species. In ferric leghemoglobins similar thermal equilibrium mixtures have been recognized (*1, 9, 10*). As with leghemoglobin (*1, 9*), reduction of *Parasponia* mixed-spin ferric hemoglobin by dithionite produces a high-spin ferrous hemoglobin structure, judged by its optical spectrum (Fig. 3). Equilibration of *Parasponia* ferrous hemoglobin with CO or O_2 (the latter in the strict absence of dithionite) caused rapid formation of carboxyhemoglobin and oxyhemoglobin, respectively (Fig. 3). Exposure of oxyhemoglobin to excess CO caused rapid and complete formation of carboxyhemoglobin with an O_2 dissociation rate constant (k_{OFF}, O_2) of ~ 0.3 sec^{-1}, and exposure of carboxyhemoglobin to excess O_2 caused slower but complete formation of oxyhemoglobin (k_{OFF}, CO, ~ 0.006 sec^{-1}). These observations of reversible oxygenation, and those of nodule slices (*4*), confirm the nature of the new hemoprotein as an O_2 carrier rather than a peroxidase, because in peroxidase simple replacement of O_2 by CO cannot occur (*11*). At *p*H 6.8 and 20°C, the O_2 OFF rate constant (~ 0.3 sec^{-1}) of *Parasponia* oxyhemoglobin is about one-thirteenth that of oxyleghemoglobin, but probably is still sufficient to allow *Parasponia* oxyhemoglobin to function

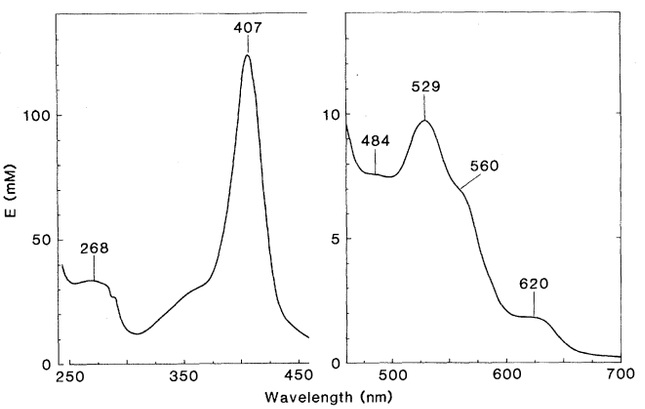


Fig. 2. Absorption spectrum of pure ferric hemoglobin from *P. andersonii* nodules. Oxyhemoglobin in 0.1*M* potassium phosphate (*p*H 6.8) was oxidized with a 20-fold excess of potassium ferricyanide and immediately chromatographed at 0°C on a long, narrow column of Sephacryl S200 equilibrated with the same buffer. This procedure removed residual Ampholine as well as ferri- and ferrocyanides. Hemoglobin concentration was determined by the pyridine hemochrome procedure (*8*), and spectra were recorded at 20°C in a Hitachi–Perkin-Elmer model 557 spectrophotometer interfaced with a PDP 11/03 computer and a HP 7221B plotter. E(m*M*) is the millimolar extinction coefficient.

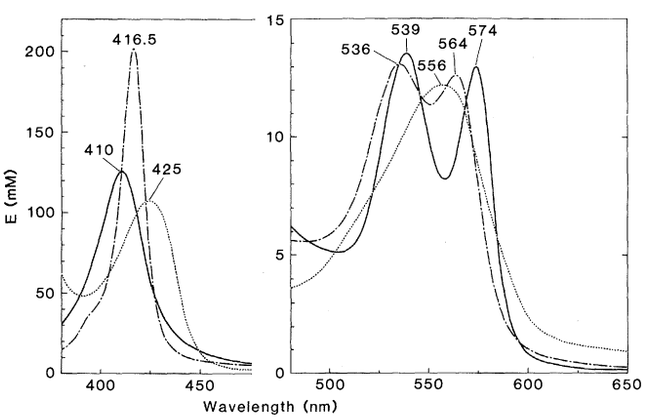


Fig. 3. Absorption spectra of pure ferrous hemoglobin (dotted line), oxyhemoglobin (continuous line), and carboxyhemoglobin (dashed line) from nodules of *P. andersonii*. Ferric hemoglobin in 0.1*M* potassium phosphate (*p*H 6.8) was converted to ferrous hemoglobin by the addition of sodium dithionite under argon. Oxyhemoglobin was prepared by running this ferrous hemoglobin (under argon) into a column of superfine Sephadex G25 (Pharmacia) equilibrated with air-saturated 0.1*M* potassium phosphate and 1 m*M* EDTA (*p*H 6.8) at 0°C. Carboxyhemoglobin was prepared by equilibrating ferrous hemoglobin with CO and passing it through a similar column equilibrated with CO-saturated buffer. Spectra were recorded as for Fig. 2.

in the facilitated diffusion of O_2 to the *Parasponia* bacteroids (*12*).

We are interested in the three-dimensional structure, amino acid sequence, and gene structure of *Parasponia* hemoglobin, and are attempting to purify *Casuarina* hemoglobin. If these proteins and leghemoglobin have overall homology of their folded structures and amino acid sequences and also have the same gene structure, including the "ancient" central intron (*13*) already identified for leghemoglobin (*2*), then ancient hemoglobin genes may have survived in many (or all) higher plant families. Invocation of a recent act of horizontal gene transmission may be unnecessary (*2*). On the other hand, if the three proteins show overall structural homology but differences in gene structure, then more than one event of horizontal gene transmission might have occurred. Another possibility is that differences in protein and gene structure will be sufficient to require the invocation of convergent evolution.

The identification of hemoglobin in *Parasponia* nodules and probably in actinomycetous nodules (*7*) suggests that an O_2 carrier protein might be a necessary part of plant nitrogen fixation symbioses. This finding, and knowledge of plant hemoglobin gene evolution, should influence the strategy of those wishing to achieve nitrogen-fixing *Rhizobium* symbioses with nonleguminous plant families.

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Colchicine Alters the Nerve Birefringence Response

Abstract. *The internal perfusion of squid axons with colchicine reversibly and selectively reduces the transient sodium current and the birefringence response to a brief depolarizing voltage pulse.*

There is a small, brief change in the optical retardation of axons associated with the passage of nerve impulses. Experiments based on the use of the voltage-clamp technique have shown that this birefringence response is correlated with the changes in membrane potential as distinct from the flow of current through the membrane. A disappointing feature of the early optical studies was the absence of any component of the observed structural changes that could easily be linked with the voltage-dependent Na^+ or K^+ conductance of the nerve membrane. The discovery of a physical or pharmacological agent consistently effective in altering both the electrical and the optical signals would have supported the hope that this approach could reveal something useful about the nature of Na^+ channels (*1, 2*). Llano has recently found that colchicine reduces the Na^+ currents of squid axons with little effect on the K^+ currents (*3*). We report here a reversible alteration of the birefringence response associated with a reversible decrease in Na^+ conductance after the addition of colchicine to the internal perfusion fluid.

Squid (*Loligo pealii*) giant axons were internally perfused and voltage-clamped by standard techniques (*4*). The central region of the chamber holding the axon was a cavity (4 by 2 by 3 mm), the walls of which were platinized-silver block electrodes used to measure the voltage-clamp current; the top and bottom of the chamber were made of glass to permit the passage of a light beam. Light from a tungsten-halogen bulb passed through a Glans-Thompson prism polarizer at 45° to the axial direction of the axon and was focused on the axon by a cylindrical lens. The light was collected with a ×10 microscope objective and passed through a second prism at 90° to the polarizer onto a ground-glass screen at the image plane. After field stops were positioned next to the axon, the screen was replaced with a YAG-444 (yttrium-aluminum-garnet) photodiode used in the photoconductive mode. The photocurrent was measured as the potential developed across a load resistor, and the d-c value is thus proportional to the light passing through the system. The light signal was a-c–coupled through a 1-Hz, high-pass resistance-capacitance (RC) filter, amplified, and passed through a 30-kHz, low-pass RC filter. It was then digitized every 40 μsec by means of one input of a Nicolet signal averager; the other input was used to record the voltage-clamp current. One digital count corresponded to a change in light intensity of approximately 10^{-7}. Data were averaged (512 to 4096 sweeps) and then stored on magnetic disks under the control of an Apple][computer.

The temperature was kept near 0°C. Streams of dry nitrogen prevented fogging of the glass surfaces. The external solution was an artificial seawater having less than normal Na^+ to minimize current-dependent artifacts. It contained 100 m*M* Na^+, 400 m*M* tetramethylammonium, 50 m*M* Ca^{2+}, 600 m*M* Cl^-, and 2 m*M* Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), *p*H 7.4. The internal perfusion fluid contained 400 m*M* K^+, 320 m*M* glutamate, 50 m*M* F^-, and 30 m*M* phosphate buffer, *p*H 7.4. Colchicine and β-lumicolchicine were used as obtained from Sigma.