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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4. Nodulated plants of *Parasponia rigida* Merr. and Perry were grown as described by J. D. Tjepkema and R. J. Cartica [*Plant Physiol.* **69**, 728 (1982)], and tangential sections (0.6 mm thick) were cut from the zone of rhizobia-containing tissue. A single slice was placed over an aperture 0.8 mm in diameter close to the surface of a photomultiplier tube (Photovolt photometer model 520-M). The nodule slice was enclosed in a gas-tight chamber under an atmosphere of O_2, N_2, or CO. Monochromatic light from a Beckman model DU spectrophotometer was focused on the surface of the slice. Despite an overall absorption rise at lower wavelengths due to light scatter and the presence of polyphenol oxidation products, the absorption maxima of oxyhemoglobin and ferrous hemoglobin (similar to those of Fig. 3) were recognizable under O_2 and N_2, respectively. Reversible oxygenation was readily achieved by exchange of these gases, with no appearance of ferric hemoglobin (similar to Fig. 2). In the presence of CO the absorption maxima of carboxyhemoglobin (similar to those of Fig. 3) were detected.
5. From cowpeas, *Vigna unguiculata* (L.) Walp, inoculated with the same *Rhizobium* strain used to infect *Parasponia*, the yield of leghemoglobin was ~ 160 nmole per gram of nodules.
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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.

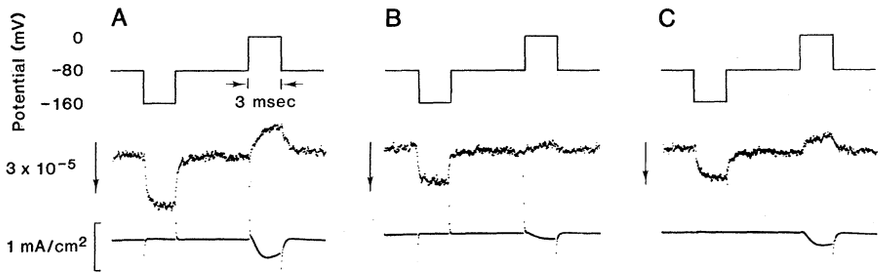

Fig. 1. The effect of colchicine on the birefringence response of an internally perfused squid axon: (A) control frame; (B) after 7 minutes of internal perfusion with 30 m*M* colchicine; (C) 7 minutes after return to the control perfusion fluid. The upper traces represent the potential impressed across the axon membrane. The middle traces are the corresponding birefringence responses, and the lower traces are the membrane currents. The arrows beside the optical records represent a change in light intensity of 3×10^{-5}; an increase in light intensity is plotted downward. Axons were from *Loligo pealii*; diameter, 0.39 mm; temperature, 0°C; 100 Na^+/400 K^+; optical trace filtered at 30 kHz; 1024 sweeps.

Figure 1A shows a control frame in which, during a 3-msec hyperpolarization to −160 mV from a holding potential of −80 mV, the light intensity increased by about 3×10^{-5} (plotted as a downward deflection). The current trace shows the capacitative transients; the "leakage" current cannot be detected at this gain. On return to the holding potential, the light trace returned to its baseline value. When a symmetrical depolarization to 0 mV was applied, the light level decreased by about 1.5×10^{-5} and at a slower rate than the hyperpolarization response. The depolarization is associated with an increase in the Na^+ permeability of the membrane to about 4×10^{-5} cm/sec, seen here as the inward Na^+ current (Fig. 1A).

When 30 m*M* colchicine was added to the internal perfusion fluid, the amplitude of the Na^+ current decreased to about one-third of its pretreatment value in about 3 minutes (Fig. 1B), the time required to change solutions. With longer pulses we observed minor reductions in the K^+ current. The birefringence response to the depolarizing pulse was preferentially and dramatically reduced; the optical response to the hyperpolarizing pulse was also smaller (Fig. 1B). The effect of colchicine on both the optical and the electrical signals was quickly reversible (Fig. 1C). The amplitude of the birefringence responses to the two pulses (hyperpolarizing and depolarizing) did not completely recover. The ratio of the amplitudes was 0.50 before, 0.21 during, and 0.49 after the addition of colchicine. The effect of 10 m*M* colchicine was a similar but smaller diminution of the current and birefringence responses to a depolarization.

The best documented effect of colchicine that we know of is its binding to tubulin. To test for microtubular involvement in the birefringence change, we prepared a saturated solution of β-lumicolchicine (less than 10 m*M*). This solution produced optical and electrical results similar to those obtained with unmodified colchicine; this finding suggests that the colchicine effects that we observed do not reflect its actions on microtubules. The alteration of the optical response is not secondary to the reduction of Na^+ permeability. Adding 300 n*M* tetrodotoxin to the external solution does not obviously change the birefringence response either in the presence or in the absence of internal colchicine.

We repeated this experiment with larger voltage pulses. Normally the initial amplitude of the birefringence change has a parabolic dependence on the voltage. There is a decrease in birefringence for short depolarizing pulses between the holding potential and about +160 mV and an increase for hyperpolarizations (*2*). Internal perfusion with 30 m*M* colchicine changes the birefringence-voltage relationship. The characteristic decrease in birefringence disappears at depolarizations of about +40 mV, and an increase is apparent at +80 mV.

In the presence of colchicine there is a slow decrease in light level during the depolarizing pulse (Fig. 1B). With longer pulses the birefringence continues to decrease for 10 to 15 msec. The effect of colchicine is either to remove a rapid initial component of the birefringence decrease or to slow the early rate of decrease of birefringence.

These experiments demonstrate that at least a portion of the birefringence response is associated with the change in Na^+ permeability, since it seems unlikely that both colchicine and lumicolchicine would have parallel reversible effects on both phenomena. The results also show that the birefringence response is more complex than previously suspected. For example, the simple square law dependence of the amplitude of the birefringence change on membrane potential may well describe the sum of several phenomena.

The pharmacological basis of colchicine's actions on excitability is far from clear. In our experiments it blocked the Na^+ currents preferentially with little effect on the K^+ currents, an effect that is reminiscent of tetrodotoxin. Nonetheless, these two drugs must have quite different modes of action as the alterations of the birefringence response seen with colchicine are not produced by tetrodotoxin.

A similar electrical effect of colchicine has recently been reported (*5*), although with a much lower concentration and much less reversibility. Our results suggest, however, that there is no direct microtubular involvement for one would not expect reversibility after colchicine addition if the tubulin were free to diffuse into the internal perfusion fluid. The similarity of action of lumicolchicine confirms this opinion. There are many actions of colchicine that are not obviously related to its tubulin binding (*6*).

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