NATURALLY CRYSTALLINE HEMOGLOBIN OF THE NEMATODE *MERMIS NIGRESCENS*

An In Situ Microspectrophotometric Study of Chemical Properties and Dichroism

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ABSTRACT A dichroic microspectrophotometer was used to measure isotropic and dichroic absorbance spectra of this unique cytoplasmic hemoglobin and its derivatives. A perfusion slide enabled changing the media bathing the *Mermis* head. The native spectrum, which has an exceptionally low α-band extinction, was shown to be entirely due to oxyhemoglobin. The CO-hemoglobin spectrum is more typical, however, the α- and β-bands are unusually closely spaced. A ferric hemochrome was formed on oxidation with ferricyanide or hydroxylamine and was readily converted to ferric hemoglobin cyanide on adding cyanide. Aquoferric hemoglobin and ferric hemoglobin fluoride were not easily formed. Deoxyhemoglobin, identified by its typical absorption spectrum, was formed only under the extremely low O_2 pressures attainable in the presence of dithionite. A glucose oxidase, catalase solution deoxygenated hemoglobin in human erythrocytes but not in adjacent *Mermis* preparations. The affinity for O_2 is much greater than for CO. Also, spectral evidence points to an oxyheme environment that is different than in vertebrate hemoglobin and myoglobin. The polarization ratio (PR) magnitude and the PR spectrum were unaffected by perfusion with high refractive index solvents; therefore, form dichroism due to the rodlike crystals is negligible. Maximum extinction is approximately perpendicular to the long axis of the microscopic crystals, which are oriented parallel to the body axis within the hypodermal cells. The PR spectra of the hemoglobin derivatives strongly resemble the corresponding spectra previously reported of single crystals made of horse hemoglobin, whale myoglobin, or *Aplysia* myoglobin and change appropriately when the ligand is changed. This confirms that the intracellular crystals of *Mermis* are of oxyhemoglobin.

INTRODUCTION

A bright red region develops in the head of the adult female *Mermis* during a dormant period in the soil, several months before the nematode emerges to lay eggs on vegetation. Because it seemed to be associated with photobehavior, Cobb (1929) called it a "chromatrope." It has been suggested that the pigment may be the photoreceptor pigment (Cobb, 1929; Croll, 1966) or an ocellar shading pigment (Burr et al., 1975). Ellenby (1964) identified the pigment as a hemoglobin, showed that it was located in the hypodermis (Ellenby and Smith, 1966), and proposed that it could have a respiratory function. Working with a limited amount of extracted hemoglobin, Burr et al. (1975) found that the hemoglobin binds oxygen and carbon monoxide reversibly and determined the intracellular concentration to be very high, on the order of 10 mM in heme. A large fraction of the cytoplasm of the hypodermal cells is observed to contain microscopic crystals, 0.3–0.9 μm in diameter and up to 20 μm long, whose long axis is oriented roughly parallel to the body axis (Burr, A. H., manuscript in preparation). The cytoplasm has a high electron density similar to that of the crystals.

This paper addresses several questions arising from these studies. Is the unusual absorption spectrum due to contamination with ferric hemoglobin? Is the high oxygen affinity of extracted *Mermis* hemoglobin present also in vivo, where the concentration is much higher and allosteric modifiers could be present? Are the crystals present in vivo and not an artifact of fixation and embedding? Are the crystals composed of hemoglobin as is suggested by the high hemoglobin concentration and high electron density?

We approached these questions using a microspectrophotometer built for measuring dichroic ratio (polarization ratio, PR) spectra of photoreceptor outer segments (Harosi, 1982) and a device for changing the media bathing the *Mermis* head while it was held between coverslips. We show in fresh preparations that the native hemoglobin spectrum is the same as in extracts and entirely due to HbO_2. We show the oxygen affinity in fresh preparations to be higher than that of human erythrocytes in the same chamber. We confirm that the crystals are of HbO_2 by

showing that the PR spectra of native HbO_2 and its derivatives are very similar to the corresponding spectra of all other hemoglobin and myoglobin single crystals investigated (Eaton and Hochstrasser, 1968; Makinen and Eaton, 1973; Eaton et al., 1978; Churg and Makinen, 1978; Makinen and Churg, 1983).

METHODS

Specimens

Mermis nigrescens adult females were collected during a 2-wk period in May, the time when most emerge from the soil to lay their eggs. They were stored 2–3 mo in moist soil at ~4°C in the dark, conditions under which they normally remain dormant. With the worm in a puddle of tap water, an anterior portion was cut off and transferred to a drop of Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.5 mM $MgSO_4$, 5.0 mM HEPES acid, 2.0 mM NaOH, pH 7.4) on a sheet of dental baseplate wax. Under the dissecting microscope, a piece ~0.7 mm long and containing the chromatrope and nerve ring was cut off just anterior to the trophosome with a razor blade. This was washed in another drop of Ringer's solution and mounted in the perfusion chamber described below. Under these conditions, the crystallinity and absorbance spectra remained stable for at least a day.

Human erythrocytes were prepared by diluting a drop of blood in a mammalian Ringer's solution (130 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, 30 mM $NaHCO_3$, 6 mM *d*-glucose, 0.1 mM each of absorbic acid, *d*-α-tocopherol acetate, and EGTA, pH 7.8). The suspension was drawn between the coverslips of the perfusion chamber by absorbing solution from the opposite side with filter paper. To prevent the erythrocytes from being dislodged during perfusion, the lower coverslip was coated beforehand with polylysine (Mazia et al., 1975) to which the erythrocytes adhered tightly. A more uniform coating was obtained after cleaning the coverslip with the flame of an oxygen torch (E. F. MacNichol, Jr., personal communication). Draining-off the solution before drying the slide gave better adherence.

Perfusion Slide

It was desirable to be able to change the solution bathing the preparation without contaminating it with oxygen from the air. This was done using a perfusion slide (Fig. 1) made of two No. 1½ coverslips and a 25 gauge hypodermic needle. The *Mermis* head was placed on the lower, 35 × 50 mm, coverslip in a drop of Ringer's solution and the upper, 24 × 40 mm, coverslip was carefully placed on top and tacked down along the short edges with molten paraffin mixture (65% paraffin, 35% Vaseline). First one end then the other was placed on a hotplate until the paraffin mixture had run under one-third to two-fifths of the area of the coverslip. Each time, the coverslips were removed from the hotplate and pressed together until the paraffin solidified. The channel so formed was filled with Ringer's solution by capillarity. Any trapped bubbles were removed at this stage.

The air in the needle was replaced by Ringer's solution, the coverslips were fastened to the brass plate with molten paraffin, and the joint between coverslips and needle and between coverslips and plate was sealed with the molten paraffin. A paraffin dike was made to contain the expelled perfusion fluid. The assembly was mounted in the microscope using glycerol as immersion fluid.

The pressure of the coverslip usually held the *Mermis* preparation in place during perfusion although slight movements made it uncertain that the identical spot was being observed each time. The slight flattening of the specimen also improved the preparation optically, by eliminating the cylindrical form of the body and decreasing the thickness.

Perfusion fluids were conveniently prepared in a 5-ml plastic hypodermic syringe, which was then plugged into the Luer fitting of the needle, being careful that no air bubbles were introduced. Dithionite ($Na_2S_2O_4$) solutions were prepared in deoxygenated water or buffer and handled in hypodermic syringes so as to minimize contact with air. There was no spectral evidence of damage to human or *Mermis* deoxyHb so long as precautions were observed. A solution used for enzymatic deoxygenation contained 200 units/ml glucose oxidase (Sigma G-2133; Sigma Chemical Corp., St. Louis, MO) and 19,000 units/ml catalase (Sigma C-10; Sigma Chemical Corp.) dissolved in 200 mM dextrose, 50 mM NaCl, 2 mM NaOH, and 5 mM HEPES (pH = 7.2).

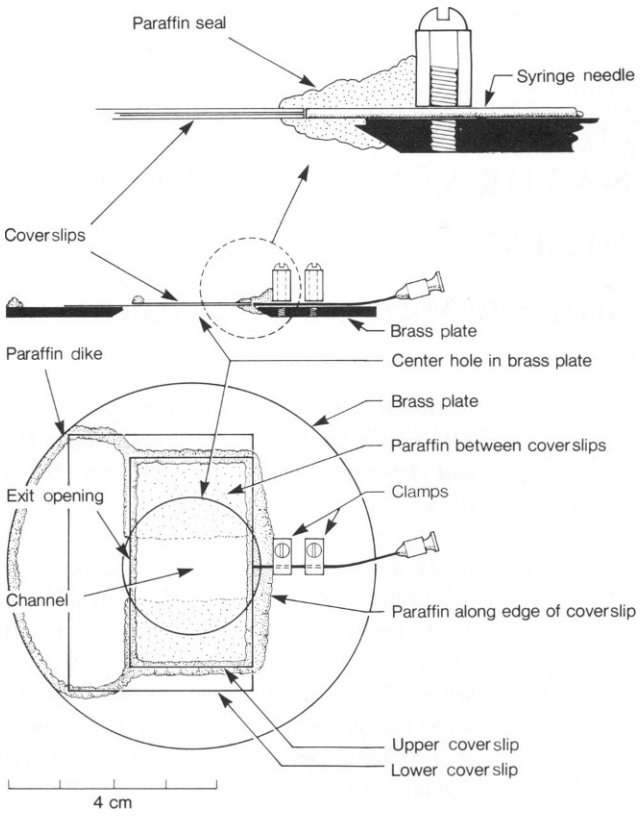

FIGURE 1 Perfusion slide. The edges of two coverslips butt against a hypodermic needle with its tip filed square. Molten paraffin is used to seal this joint, to form a channel in the space between the coverslips, to reinforce the joint between the coverslips, and to form a dike around the exit opening to contain expelled fluids.

Spectrophotometer

A dichroic microspectrophotometer (Harosi and MacNichol, 1974; Harosi, 1982) was used for all spectroscopic measurements. It is a single-beam, wavelength-scanning instrument that simultaneously records average- and polarized-transmittance spectra of microscopic samples. Light from a quartz-iodine (tungsten) lamp is focused by a mirror onto the entrance slit of a motor-driven monochromator having 1.5-mm entrance and exit slits. The exit slit is focused by a quartz lens onto the back focal plane of the microscope condenser. Between the lens and condenser are a rectangular diaphragm serving as the field stop, a quartz beam splitter, a Glan-Foucault type polarizer, and the CaF_2 crystal of a photoelastic device that modulates the plane of polarization. The objective collects the light on the far side of the specimen and projects it toward the cathode of a photomultiplier. A circular diaphragm placed at the image plane serves as an image field stop, corresponding to a 10 μm diameter circle in the plane of the specimen.

After the photomultiplier current is transduced by a wide-band current-to-voltage converter, the alternating current (AC) and average direct current (DC) signals are separately amplified and filtered and the

AC signal is synchronously rectified. The two signals are multiplexed and digitized by the computer at 75 wavelengths between 325 and 695 nm. The data from either 16 or 32 scans were averaged by summing the signals at each wavelength.

Pigment absorbance was assumed to be equal to the optical density of the pigmented area minus that of an equivalent nonpigmented reference area. The average pigment transmittance, T, was computed as the ratio of the two stored DC signals corresponding to average light fluxes transmitted through sample and reference. The average absorbance, A, was computed as log $(1/T)$. Linear dichroism, LD, was obtained by dividing the AC signal by the DC signal (Jasperson and Schnatterly, 1969). With appropriate adjustment of the photoelastic modulator (Goode and Buchanan, 1980), LD is proportional to the polarization of the transmitted light, defined as $p = (T_\parallel - T_\perp)/(T_\parallel + T_\perp)$. Following Land and West (1946), the dichroic ratio ($DR = A_\perp/A_\parallel = \epsilon_\perp/\epsilon_\parallel$) was calculated as

$$PR = DR = \frac{2A + \log k}{2A - \log k}, \text{ where } k = \frac{1 + p}{1 - p} = \frac{T_\parallel}{T_\perp}.$$

Details of these relationships will be given elsewhere (Harosi, F. I., manuscript in preparation). In the present paper, the DR will be called polarization ratio (PR) to conform with the hemoglobin literature.

Automatic baseline correction was used routinely. This is achieved by sampling the dark values of the AC and DC channels before each scan and subtracting their averages from the subsequent sample values of that scan. For reference measurements, the LD was zero and the PR was one at all wavelengths.

Calibration

Wavelength calibration was carried out periodically using a Hg-Cd spectral lamp as the light source. The initial mechanical adjustments provided wavelength accuracy within approximately ±2 nm. For the accurate determination of wavelengths of absorption maxima, correction factors were computed from responses of the microspectrophotometer to the spectral lines and the absorbance maxima of hemoglobin in human erythrocytes.

The LD was calibrated by two independent methods. First, a good quality Rochon prism was placed in the system beyond the photoelastic modulator as a crossed analyzer. This was assumed to produce 100% modulation, and the equivalent gain of the AC channel (amplifiers and rectifiers) was established. Second, the LD spectra obtained using the photoelastic modulator were compared with those obtained under conditions of static polarization. Several *Mermis* heads were measured in two orientations, 90° apart, with the modulator excitation turned on and off. By comparing the polarization modulation spectra with those of static polarization, the linear dichroism could be calibrated. The aforementioned two methods yielded results accurate within the gain calibration of the amplifiers (a few percent).

Spectral Measurements

The light beam, usually measuring ~3 × 4 μm in the specimen plane, was directed through a region of the chromatrope, preferably one of the several streaks due to the location of the pigment in the long, narrow cells of the hypodermal chord. The planes of polarization were oriented perpendicular and parallel to the streaks, the orientation giving maximum polarization ratios.

Conditions were selected to minimize the errors that are always present in absorbance measurements of tissue. Experiments with blocking filters indicated that there was no error due to the presence of light at other than the nominal wavelength for absorbances up to 2. The error due to stray light that is scattered around the pigmented zone could not be determined. It probably becomes significant at absorbances >2. On the other hand, errors due to inhomogeneity in the measured area is more likely in regions of low average pigment density. Therefore, regions were chosen where the maximum observed absorbance was between 1.0 and 1.5 units for recording the Soret band, and the densest streaks were chosen for recording the visible peaks (absorbances between 0.3 and 0.5).

For reference measurements, areas were easily found posterior to the chromatrope that had no detectable pigment and for which 680-nm light was scattered the same amount as in the sample areas. The latter was indicated by a computed absorbance of zero at 680 nm where hemoglobin absorption is negligible. Though the reference and sample areas were matched at this wavelength, a possibly different wavelength dependence of scatter in the two areas could cause a mismatch at the shortest wavelengths. The relative uncertainty due to mismatch in the Soret region is minimized by measuring at a peak absorbance >1.

Another error, a slight flattening of sharp peaks (such as the Soret) due to the finite spectral bandwidth of the instrument (≈5 nm) was unavoidable. For microspectrophotometry of red cells the combination of errors was small as indicated by the ratio of the Soret to β-band absorbance for the human red cell HbO_2 and HbCO. This ratio was within ±5% of that calculated from published spectra of the human hemoglobin derivatives measured in the conventional way (Antonini and Brunori, 1971). The same ratio for *Mermis* HbO_2 and HbCO in situ (low-density spectra) was 6–11% lower. Although the lower ratio may reflect the greater error expected for the thicker, more scattering tissue, it could be due to a real difference between human and *Mermis* hemoglobin. A possible error in relative magnitude of peak heights causes no problem in the characterization of native *Mermis* hemoglobin and identification of the derivatives.

RESULTS

Native Form is Oxyhemoglobin

An absorbance spectrum through the pigmented region of a freshly prepared *Mermis* head is shown in Fig. 2. The spectra and absorbance maxima (Table I) were identical in all healthy individuals examined and are comparable to those obtained of extracts (Burr et al., 1975). A few moribund individuals evidently contained methemoglobin.

The spectrum differs from that of most oxyhemoglobins (e.g., Fig. 3 *A*) in that the maximum extinction of the α-band (581-nm maximum, Q_0 band) is 30% lower than

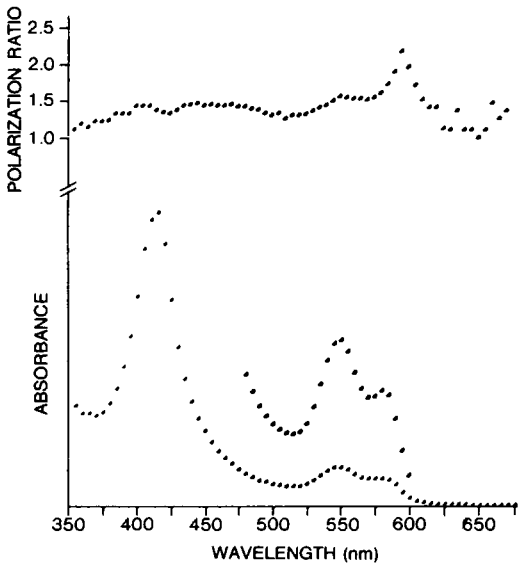

FIGURE 2 Absorbance and polarization ratio spectra of native *Mermis* HbO_2. The partial absorbance spectrum was obtained in a region of higher pigment density.

TABLE I

ABSORBANCE MAXIMA OF VARIOUS DERIVATIVES OF REPRESENTATIVE HEMOGLOBINS: THOSE OF *MERMIS*, *ASCARIS*,* SOYBEAN ROOT NODULES,‡ AND MAN§

	Soret	β	α
Oxyhemoglobin			
Mermis chromatrope	413	548	581
Ascaris body wall	412	543	578
Ascaris perienteric fluid	412	542	577
soybean leghemoglobin	411	541	574
human erythrocyte	415	541	577
Deoxyhemoglobin			
Mermis chromatrope	432	556	—
Ascaris body wall	429	556	—
Ascaris perienteric fluid	430	554	—
soybean leghemoglobin	427	556	—
human erythrocyte	430	555	—
CO-Hemoglobin			
Mermis chromatrope	418	545	563
Ascaris body wall	419	538	566
Ascaris perienteric fluid	418	538	568
soybean leghemoglobin	—	538	563
human erythrocyte	419	540	569
CN-Methemoglobin			
Mermis chromatrope	418	549	—
Ascaris body wall	417	542	—
Ascaris perienteric fluid	417	540	—
human erythrocyte	419	540	—

*From Wittenberg et al. (1965) and Okazaki et al. (1967).
‡From Appleby (1969).
§From Antonini and Brunori (1971).

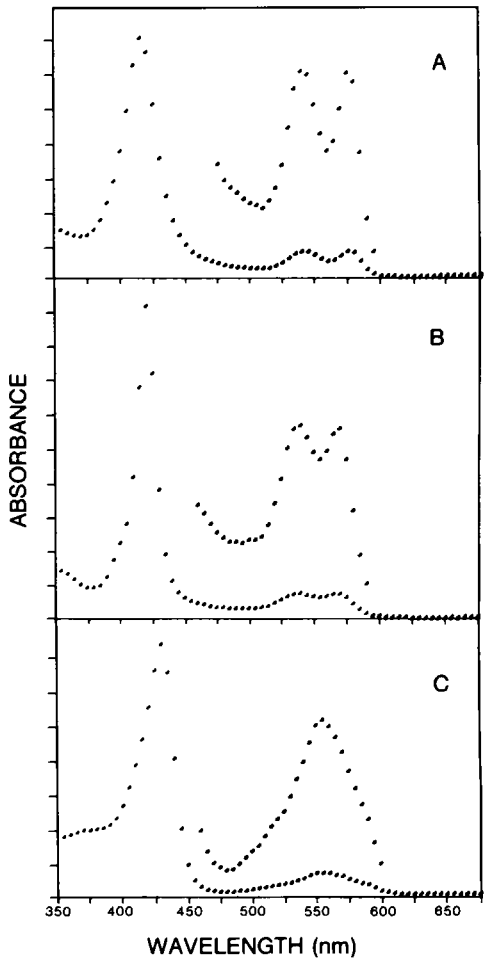

FIGURE 3 Absorbance spectra of human HbO_2 (*A*), HbCO (*B*), and deoxyHb (*C*), in red cells. The partial absorbance spectra were obtained by multiplying the data by 8. The absolute absorbance levels partly reflect cell-to-cell variations in path length or concentration. Spectra identical to *B* and *C* were obtained in the presence of dithionite.

that of the β-band (548-nm maximum, Q_v band) rather than being somewhat greater. Because this feature is reminiscent of low-spin forms of ferric hemoglobin, it was necessary to prove that the native form is purely HbO_2. This was done by showing that native hemoglobin binds ligands characteristic of ferrous hemoglobin and not ferric hemoglobin, and that it is reformed after deoxygenation with dithionite.

Deoxygenation of native hemoglobin was possible only when the oxygen partial pressure (P_{O_2}) was decreased to extremely low levels by reaction of O_2 with dithionite. This method has been used successfully in previous studies of hemoglobin with very high oxygen affinity (Davenport, 1949*a*, *b*; Smith, 1963; Gibson and Smith, 1965). The *Mermis* preparation was first perfused with N_2-equilibrated Ringer's solution, then with a 1:1 mixture of Ringer's solution and 0.15 M Na dithionite. The spectrum of Fig. 4, obtained reproducibly, resembles that of human deoxyhemoglobin (Fig. 3 *C* and Table I). The same dithionite treatment produced spectra in human red cells, adjacent to the *Mermis* head, that were the same as deoxyhemoglobin spectra obtained without dithionite (Fig. 3 *C*).

A spectrum identical to the native spectrum was obtained after the *Mermis* preparation was next perfused with N_2-equilibrated Ringer's solution to wash out the dithionite anaerobically, then with air-saturated Ringer's solution. If the flushing step was omitted, a strikingly different spectrum of ferrous hemochrome was obtained (λ_{max} 535 and 560 nm), evidence that the flushing is necessary to prevent damage to the hemoglobin.

When the *Mermis* head was perfused with Ringer's solution equilibrated with reagent grade CO gas, the native spectrum changed to that of a mixture of native hemoglobin and HbCO. Complete conversion did not occur in CO-saturated Ringer's solution. Perfusion with a 1:1 mixture of the CO-saturated Ringer's solution and 0.15 M sodium dithionite (which lowered the oxygen tension further) produced the spectrum of Fig. 5 reproducibly. The spectrum resembles that of human HbCO (Fig. 3 *B*) except that the α- and β-bands more closely spaced (Table I).

To show that the exchange of CO for O_2 is reversible, the

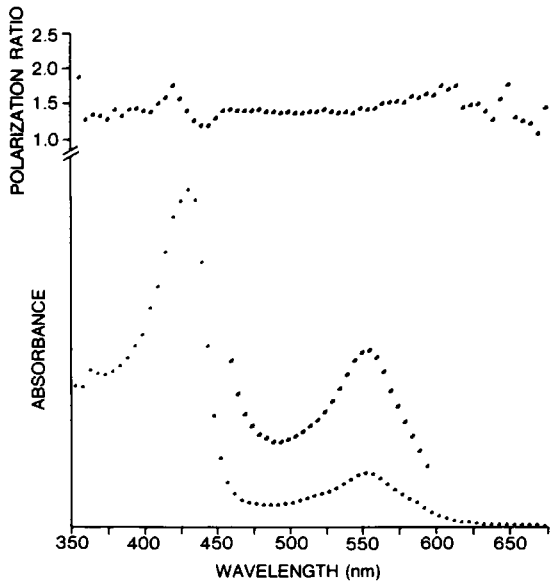

FIGURE 4 Absorbance and polarization ratio spectra of *Mermis* deoxyHb obtained by perfusing with a dithionite-Ringer's solution. Presentation as in Fig. 2.

preparation was next perfused with N_2-equilibrated Ringer's solution (to wash out the dithionite anaerobically) then with air-equilibrated Ringer's solution. The spectrum thus obtained was indistinguishable from the native spectrum.

Further proof that the native hemoglobin is HbO_2 uncontaminated with methemoglobin or ferric hemochrome is provided by the failure of native hemoglobin to react with fluoride and cyanide. After soaking *Mermis* head preparations with isotonic (0.15 M) solutions of NaF or KCN for 50 min, no change from the native spectrum was observed. This is contrary to the results obtained with ferric *Mermis* hemoglobin as described below. Cyanide is a particularly strong ligand for ferric hemoglobin and is capable of displacing the intrinsic ligand of ferric hemochrome (see below and Perutz et al., 1974).

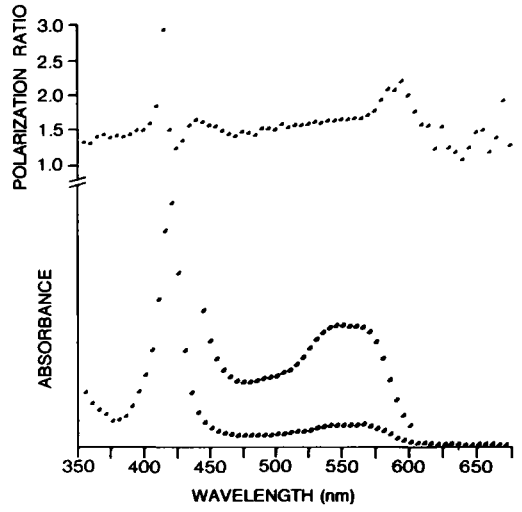

FIGURE 5 Absorbance and polarization ratio spectra of *Mermis* HbCO obtained by perfusing with 1:1 CO-saturated Ringer's solution: 0.15 M dithionite. Presentation as in Fig. 2.

Formation and Reactions of Ferric *Mermis* Hemoglobin

When an oxidant, ferricyanide or hydroxylamine, was perfused in the absence of a ligand, a spectrum resembling that of ferric hemochrome was frequently obtained (λ_{max} = 535 nm). Simultaneous or subsequent perfusion with ~0.07 M cyanide always produced the metHbCN spectrum (Fig. 6 and Table I). Though a variety of oxidants and ligands were tried, a good spectrum of aquoferric hemoglobin was difficult to obtain. After perfusing with a 1:1 mixture of 0.15 M hydroxylamine and 0.15 M NaF, a spectrum was obtained that could be interpreted as being that of a mixture of metMbF and ferric hemochrome. Subsequent perfusion with pure 0.15 M NaF increased the proportion of metMbF as indicated by the difference spectrum. When the preparation was subsequently perfused with 0.07 M cyanide ion, complete conversion to metHbCN occurred.

Affinity for O_2 and CO

Burr et al. (1975) found that the oxygen affinity of extracted *Mermis* hemoglobin was higher than that of horse hemoglobin at the same concentration (~2 μM). The question remained whether this was true also in vivo. We now find that the HbO_2 does not deoxygenate when the *Mermis* head is perfused for 2 h with Ringer's solution equilibrated with high purity nitrogen, yet does within 3 min when the P_{O_2} is reduced to extremely low levels by dithionite in the perfusate.

In another attempt at deoxygenation, fresh *Mermis*

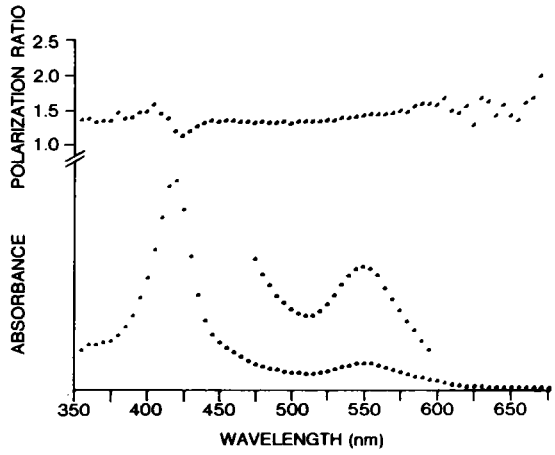

FIGURE 6 Absorbance and polarization ratio spectra of *Mermis* metHbCN obtained after oxidation and in the presence of 0.075 M KCN. Presentation as in Fig. 2.

preparations and human erythrocytes were mounted in the same slide, washed with N_2-equilibrated mammalian Ringer's solution, then perfused with an isotonic solution of glucose oxidase, glucose, and catalase (described in Methods). In the erythrocytes, human HbO_2 was completely deoxygenated within 3 min (Fig. 3 *C*), however, no change occurred in the spectrum of *Mermis* HbO_2. The possibility that this spectrum was of ferryl Hb formed in *Mermis* by reaction with H_2O_2 (Wittenberg et al., 1972) is ruled out by the fact that a different spectrum is obtained on perfusing with H_2O_2. After further perfusion and 30 min in the enzyme solution the *Mermis* hemoglobin remained fully oxygenated though the human hemoglobin remained fully deoxygenated.

The affinity of *Mermis* hemoglobin for oxygen is even greater than for carbon monoxide. A mixture of HbO_2 with some HbCO was formed very slowly after perfusion with mammalian Ringer's solution equilibrated with reagent grade CO gas. An equilibrium was reached in 100 min, which remained unchanged after perfusion with fresh CO-saturated Ringer's solution. Since this equilibrium point was on the side of HbO_2 when the P_{CO} = 1 atm and the P_{O_2} was low, the affinity of *Mermis* hemoglobin for O_2 must be greater than for CO. Human hemoglobin in adjacent erythrocytes, however, was fully CO-liganded within 20 min (Fig 3 *B*).

Subsequent perfusion with nitrogen-equilibrated mammalian Ringer's solution, then with 0.15 M sodium dithionite, yielded a mixture of deoxyHb and some HbCO in the *Mermis* preparations. Then, perfusion with a 1:1 mixture of CO-equilibrated Ringer's solution and 0.15 M sodium dithionite (P_{CO} = 0.5, P_{O_2} = 0) yielded *Mermis* HbCO (Fig. 4) within 3 min. The same spectrum was observed with P_{CO} = 1. Evidently it is fully saturated at P_{CO} = 0.5 when O_2 is absent. After subsequent perfusion with nitrogen-equilibrated Ringer's solution, then air-saturated Ringer's solution (P_{O_2} = 0.21), the CO was slowly replaced by O_2, and the native HbO_2 spectrum was completely restored.

Dichroism

The PR spectra of native *Mermis* hemoglobin and its derivatives consist of a wavelength-dependent fluctuation superimposed on a background level amounting to 1.3–1.6 (Figs. 2, 4–6). Both the background level and nature of the fluctuations depended on the derivative and were fairly consistent within each derivative. In the Discussion, these features will be compared with similar features in published PR spectra of mammalian hemoglobin and myoglobin single crystals. The amplified noise in the PR spectra at the longest wavelengths is caused by division of the AC signal by the near zero DC signal at these wavelengths where the isotropic OD is very low.

Chemical treatments that apparently dissolve the crystals or denature the HbO_2 resulted in complete disappearance of the dichroism. Dimethylsulfoxide was tried because of its effect on the solubility of gels of sickle-cell hemoglobin (Klotz et al., 1981). Applied to unfixed *Mermis* heads, 50% but not 20% dimethylsulfoxide in Ringer's solution dissolved the crystals. This was indicated by the disappearance of all dichroism. Prior fixation of the tissue with 3% glutaraldehyde prevented this. Treatment with 100% dimethylsulfoxide destroyed the dichroism of even glutaraldehyde-fixed crystals and converted the hemoglobin to a ferric hemoglobin (λ_{max} 407, 535, 575, 615 nm). In the light microscope the pigment streaks and other tissue structures appeared to have remained intact.

To test for form dichroism, water in the tissue was replaced with various solvents having a higher refractive index. To prevent dissolution or alteration of the crystals, the preparation was fixed with 3% glutaraldehye. This converted the hemoglobin to an apparently low-spin ferric hemoglobin (λ_{max} 410 and 540 nm) but did not change the level of dichroism. Soaking in glycerol in a stepwise series from Ringer's solution to 100% glycerol (n = 1.4725 at 25°C) caused no significant change in the dichroic ratio (less than ±0.006). Subsequent treatment with a 1:1 solution of glycerol and aniline, then 100% aniline (n = 1.5863 at 20°C; Lauffer, 1938) also had no effect on the mean level of dichroism. The fluctuations in the PR spectrum also were not changed by these treatments. The refractive indices of these solvents nearly equaled or exceeded the refractive index of the crystals, 1.48. The latter was estimated from the refractive increment for protein, 0.00193 dl^{-1}, and the concentration of protein in crystals of myoglobin (Kendrew and Parrish, 1957). Also, soaking in 1:1 dimethylsulfoxide Ringer's solution (n = 1.4205 at 24°C) had no significant effect. The solvents evidently penetrated the tissue, since the light scattering by the tissue, as seen in the light microscope, was considerably reduced.

DISCUSSION

The technique of the present study had several inherent advantages over that of the previous study done on extracts (Burr et al., 1975). The microspectrophotometer enabled the measurement of the spectra of hemoglobin in a single specimen, containing ~5 pmol heme (Burr et al., 1975), at a higher concentration than in the extracts and under nearly in vivo conditions. Secondly, the perfusion slide facilitated the changing of hemoglobin ligands and oxidation state, the intact tissue acting as a micro-dialysis bag. Thirdly, anaerobic conditions could be maintained. Finally, the dichroic ratio spectra of the native hemoglobin crystals and derivatives were obtainable with this microspectrophotometer.

The presence of both crystalline and dissolved hemoglobin in the optical path could affect the interpretation of the absorption spectra if the hemoglobin in the two states were different. However, there was no evidence of a difference in

spectral properties except for the effect of the orientation of the chromophore in the crystal. Under conditions where the hemoglobin spectra indicated a partially liganded state, the spectra did not vary with location in the chromotrope region even though the fraction that was crystalline would be expected to vary. Further, the spectra were not different in specimens containing only noncrystalline hemoglobin.

Conclusions from the Perfusion Experiments

We were able to prove conclusively that the native form of *Mermis* hemoglobin is HbO_2. It cannot be mixed with ferric hemoglobin because combination with the CN^- could only be achieved after reaction with an oxidant. As would be expected for HbO_2, the oxygen exchanged reversibly with CO on the native hemoglobin. And as would be expected if the native form were entirely in the oxy form and not an admixture with oxidized hemoglobin, an identical spectrum was obtained after treatment with dithionite (a reducing agent for oxidized hemoglobin) and reoxygenation.

The perfusion experiments demonstrated that *Mermis* hemoglobin has a very high oxygen affinity, possibly on the order of that of *Ascaris* body wall hemoglobin. The partial pressure of oxygen necessary to half-saturate body wall and perienteric hemoglobins of *Ascaris* is 0.015 kPa (0.11 mmHg) and 2.0×10^{-4} kPa (Gibson and Smith, 1965; Okazaki and Wittenberg, 1965). Davenport (1949*a*) noted the impossibility of deoxygenating these hemoglobins by conventional methods. Both hemoglobins could be deoxygenated, however, by a glucose oxidase, catalase solution as well as dithionite (Smith, 1963). The fact that *Mermis* hemoglobin was not deoxygenated by a glucose oxidase, catalase solution, though human hemoglobin in adjacent red cells was fully deoxygenated, shows that its oxygen affinity must be high, but a direct comparison with that of *Ascaris* perienteric fluid hemoglobin is not possible because Smith gave no details of his enzyme solution. This high oxygen affinity is not due to the interaction of the HbO_2 molecules with their neighbors in the crystal, since even in dilute solution, *Mermis* hemoglobin exhibits a much higher oxygen affinity than horse hemoglobin at the same concentration (Burr et al., 1975).

Another property of *Mermis* hemoglobin, shared with the *Ascaris* hemoglobins, is a very low relative affinity for CO. The CO-O_2 partition coefficient, M, is 0.82 (at 20°C) and 0.075 (at 27°C) for body wall and perienteric fluid hemoglobin, respectively, as calculated from kinetic data (Gibson and Smith, 1965). For vertebrate hemoglobins, in comparison, M ranges between 125 and 550 (Keilin and Wang, 1946). For *Mermis* hemoglobin it must be much <1.0 since only a small fraction is present as CO-hemoglobin in perfusate saturated with reagent grade CO. Assuming an oxygen impurity of 1%, M is estimated to be <0.01. This unusually low partition coefficient probably reflects the very high affinity for oxygen, since CO affinities do not vary so much between hemoglobins (Gibson and Smith, 1965).

Significant Features of the Isotropic Absorption Spectra

The unusually low extinction of the α-band of *Mermis* HbO_2 cannot be due to the crystallinity of the hemoglobin because it is also seen in extracts (Burr et al., 1975). A reduced α-band is also observed of the *Ascaris* oxyhemoglobins (Wittenberg et al., 1965; Okazaki et al., 1967) and *Aplysia* MbO_2 (Makinen et al., 1978).

Another interesting feature of *Mermis* hemoglobin is the presence of a significant absorption above 600 nm by the oxy- and CO-derivatives (compare Figs. 2, 3, and 5). This is seen also in spectra of *Ascaris* body wall hemoglobin, but not human or *Ascaris* perienteric fluid hemoglobin (Okazaki et al., 1967). For *Mermis* this feature is observed in (*a*) all native spectra, (*b*) after treatment with dithionite followed by reoxygenation, and (*c*) in CO spectra in the presence of dithionite (but not in CO spectra of human erythrocytes in dithionite). Therefore it cannot be due to the presence of oxidized hemoglobin. Okazaki et al. (1967) also dismiss this possibility for *Ascaris* body wall hemoglobin.

For *Aplysia* MbO_2 a similar diffuse absorption is observed, and the 925-nm band of vertebrate MbO_2 and HbO_2 is absent. Makinen et al. (1978) interpret these observations, and differences in the PR spectrum, as evidence for a shift of the porphyin(π) $\rightarrow$ $O_2(\pi_g)$ transition to higher energy.

The unusual deoxy spectrum previously obtained in extracts (Burr et al., 1975) is probably that of damaged hemoglobin. In the present study, deoxy-spectra of *Mermis* heads could be obtained with negligible exposure of the dithionite solution to oxygen and appear normal. The spectra of human erythrocytes, adjacent to the *Mermis* preparation and therefore exposed to the identical dithionite treatment, were the same as those following deoxygenation by nitrogen-equilibrated solutions or glucose oxidase/catalase solutions.

In Table I, absorption maxima obtained of *Mermis* hemoglobin derivatives in situ are compared with those of hemoglobins of soybean, *Ascaris*, and man. Other hemoglobins are compared in Rogers (1962) and Lee and Smith (1965). The maxima for human red cells lying adjacent to the *Mermis* heads were within 1 nm of the values given in Table I. Note that (*a*) the Soret maxima of each derivative is essentially the same for all of the hemoglobins listed, but (*b*) the β maxima of the oxy-, CO- and CN-met derivatives of *Mermis* hemoglobin occur at 5–9 nm longer wavelengths, (*c*) the α maximum of *Mermis* HbO_2 occurs at a longer wavelength, and that of *Mermis* HbCO occurs at a

shorter wavelength than for the other hemoglobins (except leghemoglobin). As a result of this, the shift of the α maximum on replacing O_2 with CO (the span), 18 nm, is much larger than for any other hemoglobin reported in the literature.

Origin of the Dichroism

The direction of polarization for maximum absorbance is perpendicular to the direction of the pigmented streaks observable within the pigmented zone. The streaks are parallel to the body axis except at the anterior where they bend outward towards the cuticle. Because the direction of polarization follows the bend, the dichroism must be associated with this structure. The linear dichroism was zero and the dichroic ratio was one when measured outside the pigmented zone.

Electron micrographs of serial sections reveal that each streak is an enlarged region of a hypodermal cell that is packed with elongated crystals, 0.3–0.9 μm in diameter and up to 20 μm long (Burr, A. H., manuscript in preparation). The long axes of most of the crystals are held parallel to the streak by the narrow cylindrical shape of the cell. Organelles such as mitochondria and endoplasmic reticulum are uncommon and are not regularly arrayed. In an analytical study, Burr et al. (1975) estimated that the intracellular hemoglobin concentration is high, on the order of 10 mM (heme).

There could be two different contributions to the observed dichroism: (*a*) a regular orientation of the heme chromophores in the crystal (intrinsic dichroism), and (*b*) a regular array of light-absorbing regions in a medium of different refractive index (form dichroism). However, it is unlikely that there would be any significant form dichroism due to the presence of elongated cells filled with hemoglobin. First, the small (3×4 μm) beam would include only a few cell boundaries as it passes through the material. Second, a few specimens had no dichroism even though they had the normal streaked appearance. Third, perfusion with 50% dimethyl-sulfoxide in Ringer's solution destroys the dichroism without affecting the streaks. Thus a form dichroism due to the parallel cylindrical cells is ruled out. It remains, therefore, that the parallel array of the elongated crystals could contribute a form component to the observed dichroism.

From Wiener's equations for the extinction coefficients of a rodlike array (Fredericq and Houssier, 1973), one can predict for pure form dichroism that $PR = \epsilon_\perp/\epsilon_\parallel = 4/(n_r^2 + 1)^2$, where n_r is the ratio of the refractive indices of the rod and the medium. Thus PR <1 for any $n_r > 1$. In the presence of a PR >1 due to intrinsic dichroism, the PR would be decreased by the form dichroism. In addition to this effect on the average PR level, form dichroism should cause a fluctuation in the region of intense, narrow absorption bands because of the anomalous dispersion in the refractive index. For rodlike HbO_2 crystals in saline, n_r should vary ~1% in the Soret region and the Soret PR should fluctuate ~0.02 units with a local maximum and minimum predicted at approximately the same wavelengths as in the observed spectra (Fig. 2). The fluctuation amplitude should depend on n_r, however.

The traditional method of demonstrating form dichroism is to show that it decreases or even reverses in sign as the refractive index of the solution bathing the structure is increased (n_r decreased). We tested specimens that were glutaraldehyde fixed to prevent dissolution or alteration of the crystals. Soaking in a stepwise series to 100% glycerol, or in 1:1 glycerol/aniline, or 100% aniline, or 50% dimethylsulfoxide had no significant effect on either the mean level of dichroism or the fluctuations. The refractive index of 100% aniline exceeds that of the protein of the crystal, therefore replacing the cytoplasmic water with this solvent should have reversed the phase of the fluctuation in the Soret region if the fluctuation were due to form. Thus we conclude that there is negligible contribution of form dichroism to the observed PR spectra, which must be entirely due to oriented chromophores within the crystals.

Polarization Ratio Spectra

In several publications Eaton, Makinen, and co-workers have analyzed the relationship between the structure of hemoglobin and myoglobin single crystals and the magnitude of the PR (e.g., Makinen and Eaton, 1973). In the Soret region, where only x, y-polarized, $\pi \rightarrow \pi^*$ transitions occur, the PR magnitude (average over wavelength) is determined by the orientations of the hemes of the asymmetric unit relative to the crystal axes. (The electric vector of the measuring beam is aligned with these axes.) For horse hemoglobin the Soret PR varies between 2.1 and 3.0, depending on the ligand-dependent heme orientation in the derivative (Makinen and Eaton, 1974). For orthorhombic crystals of sperm whale and *Aplysia* MbO_2 the x, y-polarized magnitudes are 8.5 and 4.0, respectively (Makinen et al., 1978). The Soret PR for monoclinic crystals of sperm whale MbO_2, on the other hand, is ~1.2 on the *bc* face and ~4 on the *ab* face (Kendrew and Parrish, 1957). By comparison, the corresponding magnitude for measurements through *Mermis* heads ranged from 1.1 to 1.7, depending on location and specimen.

There are two geometric and optical factors not present in single crystal studies that should affect these measurements: (*a*) the measuring beam passes through many crystals, whose long axes are not exactly parallel and that do not have the same rotation about the long axis (Burr, A. H., manuscript in preparation), and (*b*) the measuring beam also passes through cytoplasm that contains noncrystalline hemoglobin. Both factors can affect the PR by amounts that vary from region to region of the chromatrope and from one specimen to the next. Because we could not be confident that the preparation did not move during perfusion, the similarities or differences in PR magnitude

between the derivatives (Figs. 2, 4–6) may not reliably indicate similarities or differences in heme orientation.

Two observations, however, indicate that the nonuniform orientation of the crystals does not affect the qualitative features of the PR spectra. (*a*) Within each derivative, the main features are very similar regardless of PR magnitude, location, or specimen, and (*b*) the features strongly resemble those determined on single crystals prepared from horse hemoglobin, sperm whale myoglobin, and *Aplysia* myoglobin (Makinen and Eaton, 1973; Churg and Makinen, 1978; Makinen et al., 1978; Makinen and Churg, 1983).

The fluctuation from the x, y-polarized level in the Soret and α-band regions are caused by a splitting of the doubly degenerate excited state, and the α-band region is more sensitive to the splitting (Makinen and Churg, 1983). In *Mermis*, the fluctuations of the Soret PR spectra for HbO_2 are smaller than for the other derivatives. In the α-band region, the PR spectra of the oxy- and CO-derivatives are strikingly similar to the corresponding spectra of horse hemoglobin and sperm whale myoglobin, except that the perturbations occur at a 10–20 nm longer wavelength. The red shift is particularly curious for *Mermis* HbCO because the α-band occurs at 5 nm shorter wavelength than for horse HbCO. The relative heights of the 550 and 595 nm PR peaks of *Mermis* HbO_2 are about the same as for horse HbO_2 and whale MbO_2 in spite of the unusual isotropic absorption spectrum of *Mermis* HbO_2.

The local maximum in PR associated with the β-band is related to the vibronic coupling that produces the β-band absorption (Makinen and Churg, 1983). The low PRs at ~350 and 515 nm for *Mermis* HbO_2 are absent in the HbCO spectrum and correspond to the similar broad depressions in the HbO_2 spectra of the other species. These are ascribed to z-polarized porphyrin → iron charge-transfer bands. The PR spectrum of *Mermis* deoxyHb is qualitatively similar to that of sperm whale deoxyMb (Makinen and Churg, 1983) but differs in some ways from that of human hemoglobin (Hofrichter et al., 1973; Hofrichter, 1979; Eaton et al., 1978).

We can conclude that the main features of *Mermis* PR spectra are similar to those of single crystals of the other hemoglobins and myoglobins that have been investigated. These similarities and the observation that the features of the PR spectra were changed appropriately in the perfusion experiments confirms that the crystals are composed of hemoglobin.

The conversion of *Mermis* HbO_2 to deoxyHb does not cause disintegration of the crystal. Thus, as in myoglobins and some single-chain hemoglobins, conformational changes in the protein molecule are probably not as large as in mammalian hemoglobins.

Three features shared by spectra of *Mermis* hemoglobin and *Aplysia* myoglobin spectra point to an oxyheme environment that is different from that in vertebrate hemoglobins and myoglobins: (*a*) the lower α-band extinction, (*b*) the smaller Soret PR fluctuation, and (*c*) the diffuse absorption above 600 nm (together with the absence of a descrete band at 925 nm observed for *Aplysia*). Makinen et al. (1978) attribute the third phenomenon to a displacement of the infrared band to higher energy. The first two phenomena are consistent with an increase in the axial symmetry of the heme-ligand geometry (Makinen and Churg, 1983, pp. 167, 216).

Biological Implications

Our finding that *Mermis* chromatrope hemoglobin has chemical and spectroscopic characteristics of an oxyhemoglobin, in particular that it reversibly binds oxygen, supports the possibility that it functions somehow in respiration. In a noncirculating cell it can either facilitate the diffusion of oxygen or store oxygen for future conditions of anaerobic stress. Evidence of a temporary oxygen storage has been reviewed for diving mammals (Wittenberg, 1970; Prosser, 1973), and invertebrates (Weber, 1980). It has been estimated that an equivalent amount is stored in the chromatrope of *Mermis* (Burr et al., 1975).

The possibility of facilitated diffusion of oxygen in some nematodes is suggested by the location of hemoglobin in the hypodermal cells, which lie between the porous cuticle and oxygen sinks such as the body wall muscles and nerve cells. In *Mermis*, the portion of the intracellular oxyhemoglobin that is crystalline certainly cannot facilitate the diffusion of oxygen because it is not itself diffusible. The dissolved portion, on the other hand, could function in this capacity.

Our finding that *Mermis* hemoglobin has a very high oxygen affinity, probably on the order of that of *Ascaris* hemoglobins, raises the question of whether the hemoglobin can give up its oxygen at an oxygen tension that is useful in respiration. Atkinson (1980) has reviewed the evidence that this could be so in other nematodes.

Ascaris body wall hemoglobin is nearly as capable as leghemoglobin in augmenting the oxygen uptake by nitrogen-fixing bacteriods of legume root knots (Wittenberg et al., 1974). It is possible that the high oxygen affinity is an adaptation to the low environmental oxygen pressures, which can occur in the water-saturated soil in which *Mermis* is active in the late spring.

The findings of this study confirm that the crystals, seen in electron micrographs to fill the hypodermal chords, are composed of HbO_2. The biological purpose of intracellular crystalline hemoglobin is an interesting question for future studies.

The authors are especially grateful to M. W. Makinen and J. B. Wittenberg who suggested improvements to an earlier version of the manuscript. J. B. Wittenberg gave valuable advice and encouragement throughout the study, and E. F. MacNichol, Jr. provided the laboratory facilities.

The study was supported by grants from the Natural Sciences and Engineering Research Council of Canada and the U.S. National Institutes of Health (grant No. EY 02399).

Received for publication 30 May 1984 and in final form 9 November 1984.

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