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Hemoglobin Derivatives of the Zooparasitic Nematode *Anisakis physeteris*[1] and the Sperm Whale Host[2]

D. R. Viglierchio and J. H. Gortz

Department of Nematology, University of California, Davis, California 95616

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VIGLIERCHIO, D. R. AND GORTZ, J. H. 1972. Hemoglobin Derivatives of the Zooparasitic Nematode *Anisakis physeteris* and the Sperm Whale Host. Experimental Parasitology **32**, 211–216 (1972). Hemoglobin is present in *Anisakis physeteris* perienteric fluid and methods are described for extraction and partial purification of the pigment. Absorption spectra for the principal derivatives of hemoglobin of *A. physeteris, Physeter catadon,* sperm whale, and *Baleenoptera physalus,* fin whale, were examined. These spectra were compared with those of myoglobin derivatives of sperm and baleen whales. The hemoglobins of the nematode, sperm and baleen whale and the myoglobins of both whales showed resistance to deoxygenation by vacuum for 3 hr. After this period a slight shift in the absorption peaks was observed except for the sperm and baleen whale oxymyoglobin solutions. Hemoglobins of all zooparasitic nematodes studied show similarities in spectroscopic properties.

In the presence of $Na_2S_2O_4$ at 20°C, pH 7.0, *Anisakis* perienteric fluid hemoglobin deoxygenated instantly, whereas the hemoglobin of the related *Ascaris* more slowly: t_{50} at pH 5.5, 16°C = 440 sec and t_{50} at pH 9.0, 16°C = 220 sec (Davenport, H. E. 1949, *Proceedings of the Royal Society, Series B* **136**, 255–270).

The velocity of oxidative conversion of oxyhemoglobins to methemoglobins in the presence of $K_3Fe(CN)_6$ was measured for the fractions from *Anisakis.*

INDEX DESCRIPTORS: *Anisakis physeteris;* Hemoglobin; Myoglobin; Absorption spectra; Sperm whale; Fin whale; Metabolism; Iron; Copper; *Baleenoptera physalus* (fin whale); *Physeter catadon* (sperm whale); Electrophoresis, paper.

Hemoglobin has been found in a number of nematodes, in tissue as well as in perienteric fluid of *Ascaris* (Davenport 1949a; Smith and Lee 1963; Hamada *et al.* 1962, 1963a,b) in *Haemonchus* (Rogers 1949a) in *Camallanus* (Wharton 1941) in *Nematodirus* (Davey 1938; Rogers 1949a) in *Nippostrongylus* (Davenport 1949b and Rogers 1949a). The hemoglobin from tissue has been differentiated from that in perienteric fluid by spectral properties and reaction velocities (Davenport 1949a; Lee and Smith 1965).

All these investigations involved land inhabiting mammals. It was of interest therefore to explore the hemoglobin parasite–host relations of a deep-sea inhabiting mammal with its unusual respiratory characteristics for comparison with the observations with land mammals.

Materials and Methods

Extraction and purification of the hemoglobin. Whale hemoglobin was collected from the circulatory system; myoglobin was obtained by squeezing fluids from the muscle tissue. Composite samples were

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taken from *Physeter catadon,* the sperm whale, and *Baleenoptera physalus,* the fin whale.

Anisakis physeteris Baylis, 1923 obtained at the Richmond, CA Whaling Station,[3] from the stomachs of pregnant female sperm whales being processed, were kept in 0.75% saline solution. Within 24 hr the live worms were sexed then slit transversely and the perienteric fluid was collected and stored at 4°C until purified.

Purification of the samples was accomplished by the procedure of Davenport (1949a). The perienteric fluid obtained was centrifuged at 17,300 *g* for 20 min. The supernatant liquid was dialyzed overnight against glass distilled water at 5°C and then centrifuged. The precipitate was discarded and the supernatant fraction at pH 6.7 was adjusted to pH 7.0 with 1 *M* K_2HPO_4. Solid ammonium sulfate salt was added to bring the solution to 53% saturation. The precipitate was discarded and the supernatant was adjusted to pH 7.0 with aqueous ammonia. Then $(NH_4)_2SO_4$ was added to bring the solution to final saturation of 73%. The colored precipitate then formed was collected and redissolved in 0.1 *M* phosphate buffer (pH 7.0). The solution was dialyzed overnight at 5°C then adjusted to pH 7.0 and retained for spectral analysis.

Spectral determinations and preparation of hemoglobin and myoglobin derivatives. Absorption spectra of purified hemoglobin and myoglobin fractions were obtained using a Beckman D. B. spectrophotometer with recorder. The reproducibility of the recorded peaks fell within the limits of experimental error; imprecision resulting from peak shifts of 1–2 nm were therefore ignored. Spectral observations and chemical reactions were carried out in modified Thunberg tubes possessing two diametrically positioned side bulbs facing downwards at different angles. In one side bulb there was sufficient $K_3Fe(CN)_6$ (to give an initial conc, 100–150 millimoles/liter) to oxidize the corresponding hemoglobin solution and the other side tube sufficient $Na_2S_2O_4$ (to give an initial conc, 20–30 millimoles/liter) to reduce the oxyhemoglobin. The quantities of oxidizing and reducing agents were adjusted to given optimum absorption spectra for the hemoglobin derivatives from each source after the methods of Hartridge and Roughton (1923) and Davenport (1949a). Degasing but not deoxygenation was accomplished by repeatedly placing the Thunberg tube, containing the oxyhemoglobin solution, under vacuum. Carboxyhemoglobin was prepared by bubbling gaseous CO through a deoxygenated hemoglobin solution for 30 min at a rate of 125 ml/min. A pH of 9.0 was obtained by adjusting with 0.067 *M* Na_2HPO_4 buffer; pH 7.0 with 0.067 *M* K_2HPO_4 buffer.

Electrophoretic evaluations. Paper electrophoresis was carried out in a RECO electrophoresis apparatus model E-800-2. A potential gradient of 8.8 V/cm was employed through a 0.2 *M* sodium acetate buffer (pH 6.8) as electrolyte at room temperature. The migration matrix was cooled by circulating tap water. Aliquots (equivalent in mg atomic Fe/ml) of purified hemoglobin or myoglobin were spotted on the electrophoretogram.

Determination of the copper and iron of hemoglobin and myoglobin. Copper was determined spectrophotometrically by the dithizone method and iron by the phenanthroline method after Ballentine and Burford (1957). The samples of purified hemoglobin and myoglobin for the determination of iron and copper were digested with 9*N* $HClO_4$ and 16*N* HNO_3 after the method of Noyes and Bray (1927).

Results

Copper and iron content. It is evident from Table I that the iron and copper concentrations in the blood of sperm and fin

[3] This whaling station, the last remaining in continental United States, ceased operations as a result of Congressional Action in 1970.

whales are comparable. The iron content of the muscle extracts of both whales are the same but only about a quarter of that in the blood. No copper was detected in the muscle extracts by our technique.

The iron content of perienteric fluid is low; however, the copper content is relatively high. The proportion of copper to iron in the perienteric fluid of the nematode is over 30-fold greater than that in the circulatory blood of the host whale.

Electrophoretic properties. Of a number of buffers, acetate buffer permitted the best separation and anodic migration for comparison of the 4 hemoglobins and 2 myoglobins. The initial spots developed into long fingers with varying color intensity. The myoglobin patterns were similar except that the sperm whale fraction moved slightly faster. The whale hemoglobins were similar but gave somewhat different patterns than the myoglobins and again the sperm whale fraction moved slightly faster. Protein stains indicated that each of the whale fractions contained at least 3 components. The *Anisakis* hemoglobins gave a very short narrow fringe pattern approximately 40% the length of the sperm whale hemoglobin pattern. When the proteins were stained, the nematode fractions appeared to consist of one component but in a rather diffuse pattern. The electrophoretograms were of poor quality because no precautions were taken to prevent air oxidation of the heme; the fractions were insufficiently purified or perhaps there was a lack of homogeneity of the components, even though the spots were equivalent in terms of added Fe. Nevertheless the chromatograms did show differences among the various fractions, consistent with observations from absorption spectroscopy and reaction kinetics.

Spectroscopic properties of hemoglobin and myoglobin derivatives. Absorption curves of the oxy-, carboxy-, deoxy- (reduced), met- (oxidized) forms of hemoglobins and myoglobins of the fin and sperm whales and *A. physeteris* were recorded and the absorption peaks are indicated in Table II for comparison to those of some land mammals and their nematode parasites.

TABLE I

Concentrations of Iron and Copper in the Natural Fractions Containing Fin Whale, Sperm Whale Hemoglobin and Myoglobin and Anisakis *Perienteric Hemoglobin*

Source of metallic species	Fe (mg/100 ml nat. fluid)	Cu (mg/100 ml nat. fluid)
Sperm whale blood	62.50	0.102
Sperm whale muscle extract	14.93	—
Fin whale blood	56.25	0.075
Fin whale muscle extract	14.21	—
♂ *Anisakis* perienteric fluid	3.90	0.195
♀ *Anisakis* perienteric fluid	3.00	0.180

The deoxygenation with $Na_2S_2O_4$ of the oxyhemoglobin and oxymyoglobins of both whales and *A. physeteris* occurred immediately, too quickly to be measured by our methods. Oxidation by $K_3Fe(CN)_6$ of hemoglobins and myoglobins from the sperm whale and fin whale also occurred too quickly to be measured by our techniques. However, the oxidation of the hemoglobin from *A. physeteris* occurred at a measurable rate; that of the female oxidized more slowly than that of the male, t_{50} female = 660 sec and t_{50} male = 450 sec (Table III).

Discussion

Hemoglobin and its derivatives from a number of zooparasitic nematodes have been studied in the search for an understanding of the oxygen relations between parasite and host (Davenport 1949a; Rogers 1949a,b; Hamada *et al.* 1962, 1963a,b; Lee and Smith 1965; Okazaki *et al.* 1965; Okazaki and Wittenberg 1965; Wittenberg, Okazaki and Wittenberg 1965; Fernando 1968). Common to these studies was the observation of a higher affinity for oxygen by

TABLE II

Absorption Bands (nm) *of Hemoglobin and Myoglobin Derivatives of Nematode Parasites and Hosts*

Origin	pH	Absorption band	Form				References
			Met-	Oxy-	Deoxy-	Carboxy-	
Hemoglobin							
Ascaris lumbricoides (pig)	7	α	632	576.5	553.5	568	Wittenberg, Okazaki and Wittenberg (1965)
		β	501	542.0	—	538	
Obeliscoides cuniculi (rabbit)	7	α	—	578	554	569	Fernando (1968)
		β	502	542	—	538	
Anisakis physeteris	7	α ♂	630	574	554	572	Present paper
		♀	632	575	555	573	
		β ♂	500	542	—	540	
		♀	—	543	—	541	
Haemonchus contortus (horse)		α	—	576	554	570	Rogers (1949a)
		β	—	541	—	538	
Nematoderus spp.	8	α	—	576	555	570	Rogers (1949a)
		β	—	542	—	537	
Sperm whale	7	α	627	573	560	569	Present paper
		β	—	540	—	538	
Fin whale	7	α	627	576	557	570	Present paper
		β	—	542	—	540	
Sheep	8	α	—	576.6	555	570	Rogers (1949a)
		β	—	542	—	537	
Man		α	635	577	555	572	Lee and Smith (1965)
		β	—	541	—	533	
Myoglobin							
Sperm whale	7	α	630	578	558	576	Present paper
		β	500	542	—	540	
Fin whale	7	α	630	578.5	556	576	Present paper
		β	—	542	—	542	

the hemoglobin of the parasite. From these findings it was suggested that the role of hemoglobin in the parasitic animal was to scrub oxygen from the environment and subsequently to make it available for metabolic purposes. Presumably this could be accomplished partly by oxygen storage and partly by oxygen transport. The parasite hemoglobins studied so far have been found to differ from the hemoglobins of their hosts. Direct connection between host and parasite hemoglobin is very unlikely, although indirect connections such as reported by Smith and Lee (1963) for *Ascaris*, where the perienteric hemoglobin of the nematode was found to be dependent on the availability of porphyrins from the host for hemoglobin synthesis may occur. At no time was *A. physeteris* found attached to the nutritive epithelium of the whale stomach (Viglierchio and Gortz 1972), though this cannot be excluded since the parasites may have migrated during the 12–24 hr before the whale was processed. Its habitat would then be similar to that of *Ascaris* rather than *Strongylus* spp. and *Nippo-*

TABLE III

Oxidation and Deoxygenation Times of Invertebrate and Vertebrate Hemoglobins and Myoglobins

Source of hemoglobin (Hb) myoglobin (Mgb)	Deoxygenation t_{50} ($Na_2S_2O_4$) (sec)	Oxidation t_{50} ($K_3Fe(CN)_6$) (sec)	Temperature (°C)	pH	References
Invertebrates					
Anisakis ♂ (Hb)	Immediate[a]	450	20	7.0	Present paper
Anisakis ♀ (Hb)	Immediate	660	20	7.0	Present paper
Ascaris (Hb)	440	440	16	5.5	Davenport (1949a)
	220	220	16	9.0	Davenport (1949a)
Strongylus spp. (Hb)	750	750	11.5	7.0	Davenport (1949b)
Nipostrongylus (Hb)	Deoxygenates in vacuum	—	19.0	9.2	Davenport (1949b)
Vertebrates					
Sperm whale (Hb)	Immediate[a]	Immediate	20	7.0	Present paper
Sperm whale (Mgb)	Immediate	Immediate	20	7.0	Present paper
Baleen whale (Hb)	Immediate	Immediate	20	7.0	Present paper
Baleen whale (Mgb)	Immediate	Immediate	20	7.0	Present paper
Horse (Hb)	0.02	—	20	7.4	Millikan (1936)
Horse (Mgb)	0.01	—	20	7.4	Millikan (1936)
Pig (Hb)	0.047	0.047	22	8.6	Millikan (1933)
Man (Hb)	0.038	0.038	22	8.6	Millikan (1933)
Sheep (Hb)	0.028	0.028	22	8.6	Millikan (1933)

[a] No attempt was made to measure deoxygenation velocity < 1 min.

strongylus spp., which are normally attached to the gastrointestinal mucosa of the host. As with the other nematode parasites and their hosts, the hemoglobin in the perienteric fluid of *A. physeteris* differs in spectral and reactive properties from the hemoglobin and myoglobin of the host, the sperm whale. In addition it appears that the hemoglobin in the perienteric fluid of the male parasite is somewhat different from that of the female.

The relatively large proportion of copper in the perienteric fluid of *A. physeteris* is striking. One might suspect that this nematode may be one of those animals that has 2 oxygen carrying pigments, a hemoglobin and a hemocyanin (Lee and Smith 1965). However, by our methods it was not possible to distinguish two such fractions and the question must be resolved by further investigation.

Though the deoxygenation of the hemoglobin of *A. physeteris* with $Na_2S_2O_4$ takes place rapidly, that of *Ascaris* and *Strongylus* spp. takes place slowly; the oxidation by $K_3Fe(CN)_6$, however, takes place slowly with the hemoglobin of all 3 parasites. It appears therefore that the hemoglobin of *Anisakis* has a much lower affinity for oxygen than the hemoglobin of *Ascaris* and *Strongylus* but more than that of *Nippostrongylus* which deoxygenates in vacuum. It would appear therefore, that there was no survival value in the development of a high oxygen affinity in the perienteric fluid hemoglobin of *Anisakis* as there was in *Ascaris* and *Strongylus* spp.

The function of the hemoglobin of *Anisakis* is less understood than that in some other nematode parasites. In view of the possible dual role suggested by Lee and Smith (1965) the hemoglobin of *Anisakis* may function more in the transport of oxygen than in its storage.

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