

Anisakis simplex and *Anisakis physeteris*: Physicochemical Properties of Larval and Adult Hemoglobins

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SUZUKI, T., AND ISHIDA, K. 1979. *Anisakis simplex* and *Anisakis physeteris*: Physicochemical properties of larval and adult hemoglobins. *Experimental Parasitology* 48, 225–234. To resolve the taxonomic relationship between two types of parasitic nematode larvae (Type I and II) and two species of parasitic nematode adults (*Anisakis simplex* and *A. physeteris*) of the aquatic ascarid genus *Anisakis*, collected in Japanese coastal water, a comparison was made of their hemoglobins' physicochemical properties. The larval hemoglobins were more similar to each other in electrophoretic pattern than to either adult, indeed there were few similarities whatsoever in these patterns of larval and adult hemoglobins. However, isoelectric points were 6.2 for the Type I larva and for *A. simplex* and 5.4 for the Type II larva and for *A. physeteris*. All samples showed identical patterns in spectrophotometric scanning. The circular dichroic spectra of the samples were also virtually identical, although slight differences were noted in the oxygenated hemoglobins; the Type II larva and *A. physeteris* exhibited a small positive peak at 575 nm but the Type I larva and *A. simplex* exhibited a much smaller peak (negative position). The sedimentation coefficients of the samples possessed essentially identical values (11.2–12.4). The molecular weights of the samples were estimated, roughly, to be in the range 33 to 43×10^4 by thin-layer chromatography on Sephadex G-200. The evidence suggests that a relationship may exist between the Type I larva and *A. simplex*, and between the Type II larva and *A. physeteris*.

INDEX DESCRIPTORS: *Anisakis simplex*; *Anisakis physeteris*; *Anisakis* larva Type I and II; *Ascaris suum*; Nematodes, parasitic; Dolphin, blue white; Pig; Hemoglobin, nematode; Electrophoresis; Spectra, absorption, id. circular dichroic; Sedimentation velocity; Molecular weight.

INTRODUCTION

Two species of adult nematodes of the aquatic ascarid genus *Anisakis* have been recovered from marine mammals of the coastal areas of Japan and its vicinity, and three types of *Anisakis* larvae have been found in fish caught in these waters. However, knowledge about the development of *Anisakis* spp. larvae into adults is far from complete.

Kagei (1969) has compared in detail the morphology of two types of these larvae (Types I and II) and two species of adults

(*Anisakis simplex* and *A. physeteris*), and suggested that the Type I might be the larval form of *A. simplex* and that the Type II might be that of *A. physeteris*. Further evidence was presented by Pippy *et al.* (1975) who succeeded in rearing stage-3 larvae of Type I into the pre-adult stage of *A. simplex*. Grabda (1976) suggested that the Type I larva is *A. simplex* on the basis of cultivation studies, and Beverley-Burton *et al.* (1977) reached the same conclusion on grounds of morphology and acid phosphatase polymorphism.

Further evidence is provided in this paper. In an attempt to resolve the taxonomic relationship among the four kinds of specimens, a constituent of these nematodes—hemoglobin—was analyzed physicochemically. Partially purified parasite hemoglobins were used in the present study because a preliminary experiment using whole worm extracts gave conflicting results, due to the complex nature of samples.

MATERIALS AND METHODS

Collection of nematodes. *Anisakis* larvae Types I and II (Berland 1961) were obtained from the viscera of fish, Pacific pollocks (*Theragra chalcogramma*) and bonitos (*Katsuwonus palamis*), respectively. These fish were supplied by a commercial fish processor. Adults of *Anisakis simplex* Baylis, 1920 were obtained from the stomachs of blue-white dolphins (*Stella caeruleo-albe*) at a local fishing port. Adults of *Anisakis physeteris* Baylis, 1923 were obtained from a whaling company researcher; according to his information, the specimens had originated in the stomachs of sperm whales (*Physeter catodon*). Terrestrial ascarids, *Ascaris suum* adults, were obtained from swine slaughtered locally.

Preparation of parasite and host hemoglobins. Both types of larvae were washed several times with 0.15 M phosphate buffered saline, pH 7.2 (PBS) and homogenized at 4 C in a small quantity of the same buffer solution. Homogenates were then centrifuged at 47,400g for 30 min and clear pinkish supernatant fluids were obtained. Each species of adult worm was washed several times with PBS and slit transversely, after which the red perienteric fluids were obtained by centrifugation at 850g for 10 min. The fluids were then clarified by further centrifugation at 47,400g for 30 min. From the body fluids thus obtained, the hemoglobins were purified by a technique described previously (Suzuki *et al.* 1971); a saturated ammonium sulfate solution (pH 7.2) was added dropwise to the body

fluid with constant stirring to produce 60% saturation. The mixture was kept at 4 C for 30 min and was then centrifuged at 6700g for 30 min. The resulting supernatant fluid was saturated to 80% with saturated ammonium solution in the same way. The sediment obtained by further centrifugation was dissolved in a small quantity of PBS and dialyzed against several changes of PBS at 4 C for 72 hr. After removal of the sediment by centrifugation at 850g for 10 min, the sample was applied to a 2.5 × 25-cm column of DEAE-cellulose equilibrated with 0.05 M phosphate buffer, pH 7.2 containing 0.1 M sodium chloride, and components not binding to the cellulose were thoroughly washed out with the same buffer. The fraction eluted by the above buffer with 0.3 M sodium chloride was collected and concentrated by ultrafiltration. The concentrated sample was dialyzed against 0.15 M phosphate buffer, pH 7.0, containing 0.05% sodium azide at 4 C for 12 hr and a slight insoluble product was removed by centrifugation at 47,400g for 20 min. The sample was stored at 4 C until used. Hemolysates from Pacific pollock, blue-white dolphin, and pig were used for comparison with parasite hemoglobins.

Electrophoresis. Polyacrylamide gel electrophoresis was carried out by a modification of the procedure of Raymond (1964). A thin-layer, 1.7 mm thick, was prepared using 5% gel in 75 mM sodium barbital, pH 8.6. Ten microliters of sample (about 15 µg protein) was in the 7 × 2-mm slit and electrophoresis proceeded at 25 mA (2.5 mA/cm) for 4 hr. On completion of electrophoresis, the gel plate was scanned in a Shimadzu dual-wavelength TLC scanner CS-900. A long narrow vertical slit was made on the detector side of the gel. The gel was driven with a synchronous motor and scanned at dual wavelengths (410 nm for hemoglobin components in the sample and 750 nm for its absorbance reference) along the direction of electrophoresis. This

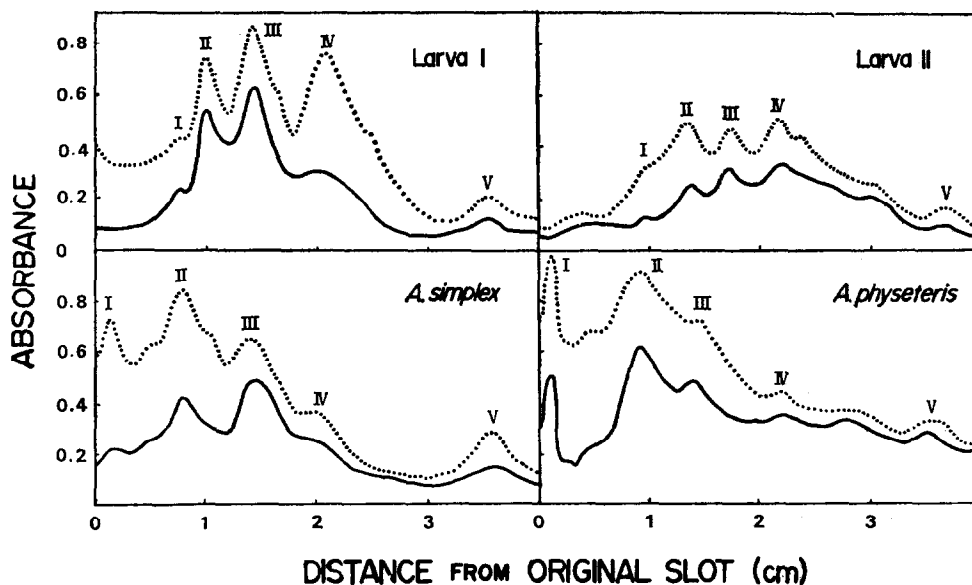


FIG. 1. Densitometric patterns of the electrophoresed *Anisakis* hemoglobins. The gel plate was scanned at two wavelengths, 410 (solid lines) and 280 nm (dotted lines).

procedure was repeated, at 280 nm for the sample and at 350 nm for its reference, to record all protein components. The electrophoresed proteins were then stained with amido black 10 B.

Isoelectric points. Hemoglobins were electrofocused by the method of Vestberg and Svensson (1966) using carrier ampholytes (Ampholine, LKB-Produkter AB). Solutions and density gradients were prepared as described by LKB Instruments. A 110-ml capacity electrofocusing column (No. 8101) was used. The dense solution employed was a 47% (w/v) sucrose solution containing 1.5% Ampholine (pH range 5–8), while the light solution was 0.5% Ampholine not containing sucrose. The hemoglobin sample (10 mg) was introduced into the column with the light solution before gradient formation. The cathode was uppermost and the final steady state was reached after 50 hr with a potential difference of 600 V and 0.4 mA. On completion of the run, the column was drained from the bottom with a peristaltic pump at a constant flow rate of 30 ml/hr. Portions of 1 ml were collected in a frac-

tion collector. The protein concentration in the effluent was measured at 280 nm with a LKB UV cord II and recorded with a LKB 6500 recorder. The pH measurements of each collected fraction were performed at 20 C.

Absorption and circular dichroic spectra. Absorption spectra were measured with a Cary Model 14 recording spectrophotometer, and circular dichroism was measured on a JASCO J-200 recording spectropolarimeter. All measurements were carried out in 0.1 M phosphate buffer, pH 7.0 at 20 C. Deoxygenation was done by adding a few grains of sodium dithionite. Molar concentrations of hemoglobins were determined spectrophotometrically, after conversion into pyridine hemochrome, on the basis of a millimolar extinction coefficient (ϵ mM) of $34 \text{ mM}^{-1} \text{ cm}^{-1}$ at 557 nm. The circular dichroism measurements were expressed in degree-centimeters squared per decimole on a heme basis.

Ultracentrifugation. Measurement of sedimentation velocity was performed with a Hitachi Model UC-L ultracentrifuge equipped with Schlieren optical system at

60,000 rev/min at 20 C. The protein concentration was about 1% in 0.1 M phosphate buffer, pH 7.0. The sedimentation values were converted to S values in water at 20 C ($s_{20,w}$).

Thin-layer Chromatography. Chromatography was performed on a 0.5-mm thin layer of Superfine Sephadex G-200 in Wako plate gel-filtration equipment. The solvent used was 0.15 M phosphate buffer, pH 7.4. The plate was set at 20° angle in a moist chamber. Five microliters of each sample solution was spotted on the plate, which was then developed by descending chromatography. As references, thyroglobulin, human γ -globulin, bovine serum albumin, ovalbumin, pepsin, trypsin, bovine myoglobin, and cytochrome C were used. Development was continued until the thyroglobulin had run 13 cm (about 4 hr). The plate was then removed from the chamber and the pattern was transferred by blotting to a sheet of filter paper. The filter paper was subsequently dried at 60 C and the

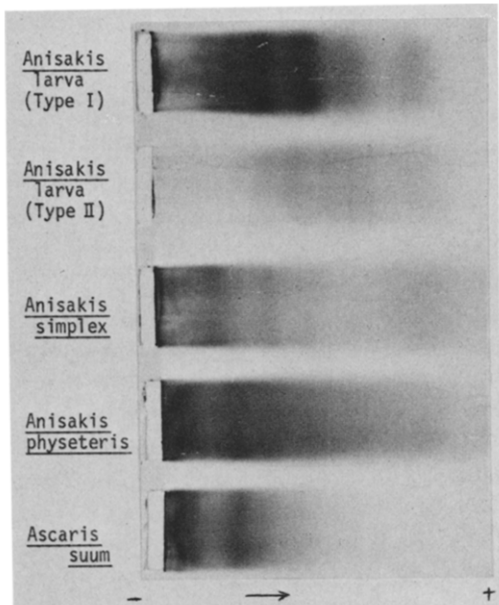


FIG. 2. Polyacrylamide gel electrophoresis of hemoglobin samples from *Anisakis* spp. and *Ascaris suum*. Gel stained with amido black showing proteins.

TABLE I

Isoelectric Points and Sedimentation Coefficients of Parasite and Host Hemoglobins

Species	Isoelectric point	Sedimentation coefficient ($\text{cm} \times 10^{-13}$ / sec/dyne at 20 C)
Parasite		
<i>Anisakis</i> larva I	6.2	12.4
<i>Anisakis</i> larva II	5.4	12.0
<i>Anisakis simplex</i>	6.2	11.6
<i>Anisakis physeteris</i>	5.4	11.2
<i>Ascaris suum</i>	5.1	11.2
Hosts		
Pacific pollock	NT ^a	4.3
Blue-white dolphin	7.2	4.5
Pig	NT	4.5

^a Not tested.

blotted proteins were stained by amido black 10 B.

RESULTS AND DISCUSSION

Identity of Materials

Gordius marinus Linnaeus, 1767 was described as the coiled larva commonly occurring in herring. Van Thiel (1966) contended that the larval material he collected from herring fit the description of Linnaeus and resurrected the species as *Anisakis marina* (Linnaeus 1767). Linnaeus' description could apply to almost any anisakine nematode, and Dollfus (1953) recorded several genera of anisakines from herring. In addition, two species of *Anisakis* were recorded from marine mammals of the North Sea and South Atlantic by Young and Lowe (1969) and Davey (1971). Van Thiel did not provide evidence that his material was conspecific with any adult species. *Gordius marinus* therefore remains a *species inquirenda* awaiting rescription of the type material. In the present study we use the names *Anisakis simplex* and *A. physeteris* as recommended by Baylis (1920, 1923), and

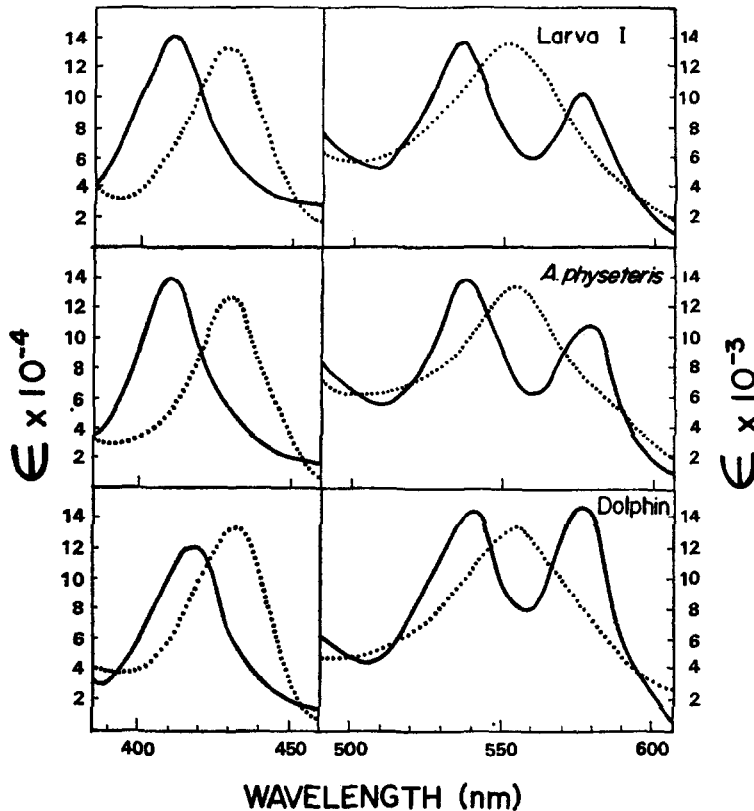


FIG. 3. Absorption spectra of the hemoglobins from *Anisakis* larva Type I, *Anisakis physeteris*, and blue-white dolphin. The cell with 1-cm light path was used for the region between 470 and 610 nm and with 0.1-cm light path for the region between 380 and 470 nm. The extinction (ϵ) is on a molar heme basis. Solid line; oxygenated hemoglobin; dotted line; deoxygenated hemoglobin.

Anisakis Type I larva and Type II larva as recommended by Berland (1961). However, we could not obtain specimens of *A. typica* (Diseing 1860) and *Anisakis* Type III larva (Otsuru 1969).

Occurrence of Hemoglobins

All *Anisakis* spp. used in the present study possessed hemoglobin. The occurrence of hemoglobin in *Anisakis* larva Type I had been already reported by the present authors (1969), and in *A. physeteris* recently by Viglierchio and Gortz (1971). Hemoglobins in the Type II larva and in *A. simplex* are reported for the first time in the present study. There are several reports on *Ascaris* hemoglobin (Daven-

port, 1949; Hamada *et al.* 1962; Okazaki *et al.* 1965; Smith and Morrison 1963; Wittenberg *et al.* 1965), which we use for comparison with *Anisakis* hemoglobins.

Parasite hemoglobins could be separated from the whole worm extracts or perienteric fluids by a previously reported technique (Suzuki *et al.* 1971) used to purify the hemoglobin of the Type I larva.

Electrophoretic Mobilities

The hemoglobin samples from *Anisakis* migrated to the anode on electrophoresis in polyacrylamide at pH 8.6, and each separated into five protein components as demonstrated by densitometric analysis and photography of stained gels (Figs. 1

and 2). We designate these components as Components I to V in order, from origin to anode.

It should be noted that there is a significant gap between the densitometric patterns at 280 and 410 nm, especially in the slow-migrating components. Absorption at 280 nm was always greater than that at 410 nm and the gaps were prominent in the samples from adult worms. It is possible that impurities present in the samples influenced the absorption patterns, however, it is impossible to estimate directly the presence of contaminating components by the 280:410 nm ratio, since polyacrylamide gel absorbs ultraviolet irradiation. In the patterns at 410 nm, a wavelength close to the Soret maxima of oxygenated forms of parasite hemoglobins, all hemoglobin samples from *Anisakis* show similar curves although the samples from larvae tended to move slightly faster than those from adults.

It is interesting to note that somewhat similar patterns were detected between the Type I larva and *A. simplex*. Some differences in the proportions of each component exist for each sample, as judged by the relative heights of each component's densitometric pattern. The most striking differences between the two hemoglobin

samples are that no component corresponding to adult Component I is seen in the larval hemoglobin, and larval Component I is much smaller than the corresponding prominent adult Component II. On the other hand, any appreciable component corresponding to larval Component II is not recognizable in the adult hemoglobin. It is evident that Component III is a prominent component in both hemoglobins, whereas it seems that Component IV and V consist mostly of nonhemoglobin proteins.

In comparing the patterns of the Type II larva and *A. physeteris*, a relation similar to that described above was observed. However, significant differences between both sets of hemoglobins are also observed, especially in the relative contents of the various components. For example, a greater proportion of slow-migrating component is found in the adult compared to the larval hemoglobin.

Isoelectric Points

When hemoglobin samples from *Anisakis*, *Ascaris suum*, and the blue-white dolphin were electrofocused using carrier Ampholine covering the range pH 5–8, the main components of every *Anisakis* hemoglobin showed relatively higher isoelectric point values than those of *A. suum* but lower than those of dolphin (Table I). The presence of at least two additional minor components showing a slightly higher value than that of the main component was seen in the column; however they diffused considerably during the elution and were not clearly separated from each other.

It is of interest to note that essentially identical profiles between the Type I larva and *Anisakis simplex* and between the Type II larva and *A. physeteris* were shown by elution from the column despite the somewhat complicated patterns obtained because of the presence of nonhemoglobin protein contaminants in the samples.

Smith and Morrison (1963) reported that *Ascaris suum* perienteric fluid hemoglobin

TABLE II

Absorption Bands of Oxygenated and Deoxygenated Forms of Parasite and Host Hemoglobins (in nm)

Species	Oxy Hb			Deoxy Hb	
	α	β	Soret	Soret	
Parasites					
<i>Anisakis</i> larva I	575	537	410	553	429
<i>Anisakis</i> larva II	575	541	413	553	429
<i>Anisakis simplex</i>	575	537	410	553	429
<i>Anisakis physeteris</i>	575	540	413	553	429
<i>Ascaris suum</i>	577	541	408	554	428
Hosts					
Pacific pollock	576	540	415	552	430
Blue-white dolphin	576	541	415	555	431
Pig	576	540	415	555	431

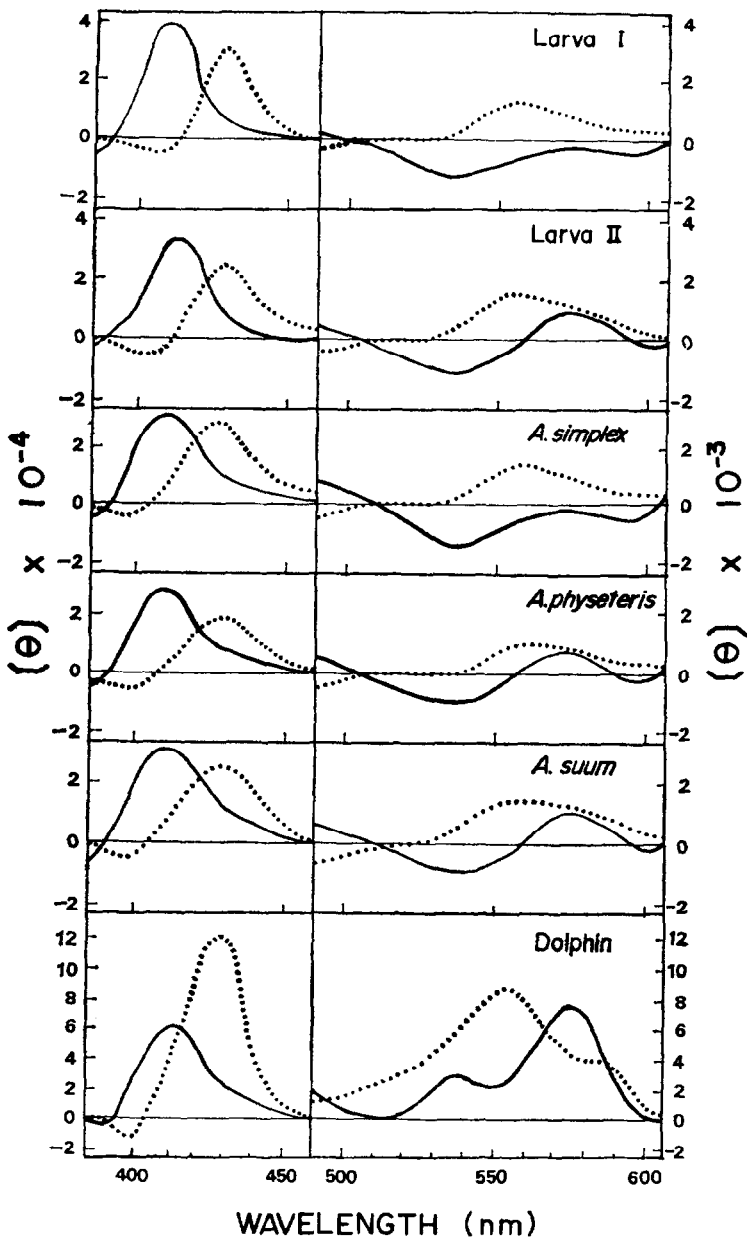


FIG. 4. Circular dichroic spectra of the hemoglobins from *Anisakis* larvae and adults, *Ascaris suum*, and blue-white dolphin. The measurement condition was the same as in Fig. 3. θ is expressed on a molar basis. Solid line; oxygenated hemoglobin; dotted line; deoxygenated hemoglobin.

had an isoelectric point of about 5.0. In close agreement with their report, we obtained a value of 5.1 for *A. suum* hemoglobin in the present study.

Absorption Spectra

The absorption curves of oxygenated and deoxygenated forms in the Type I larva,

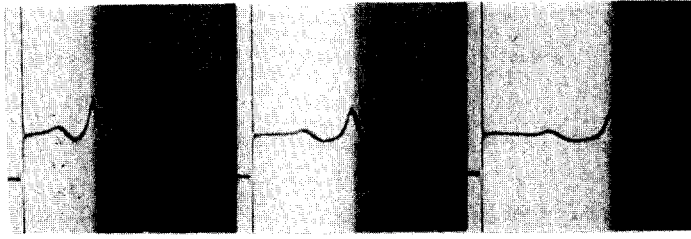


FIG. 5. Sedimentation patterns of hemoglobin from *Anisakis* larva Type II in 0.1 M phosphate buffer, pH 7.0. The plates were exposed at 30, 40, and 50 min after the reaching maximum speed (59,760 rev/min) at 20 C. The phase plate angle was 70°.

Anisakis physeteris, and dolphin over the wavelength range 380 to 610 nm are presented in Fig. 3, and the data for absorption peaks of the remaining samples are shown in Table II. All samples demonstrated an absorption pattern characteristic of hemoglobin, and consequently no clear information was provided to aid in distinguishing between parasite stages. However, there was a slight difference in the height and position of the absorption maxima. A definite difference between parasite and host hemoglobin is that the β peak is higher than the α peak in the parasite hemoglobin, whereas the α peak is higher in the host hemoglobin. This interesting feature has been reported by Davenport (1949) for *Ascaris suum* hemoglobin.

Circular Dichroic Spectra

As far as we know, no information regarding the circular dichroic spectra of parasite hemoglobin has been reported. Patterns of hemoglobin from four *Anisakis* specimen sorts, *Ascaris suum*, and dolphin in the Soret and visible regions are shown in Fig. 4.

All parasite patterns are essentially identical to each other, except for a slight difference of ellipticity values in the peak at about 575 nm. A small positive peak exists in the oxygenated hemoglobins of Type II larva, *A. physeteris*, and *A. suum*, whereas in those of the Type I larva and *A. simplex* the peaks are situated in the negative position.

In comparing the patterns of parasite and dolphin hemoglobins, however, several clear differences are seen, both in the Soret and visible regions: (1) The ellipticities of the parasite hemoglobins are much smaller than that of dolphin hemoglobin; (2) although the circular dichroism extrema of oxygenated and deoxygenated forms nearly correspond in wavelength with the absorption maxima in the Soret and visible regions, the peak at about 540 nm, which corresponds to the β peak on the absorption spectra, is the negative peak and the peak at about 575 nm, seeming to correspond to the α peak, is small, whereas two positive peaks in the visible region are seen in the dolphin hemoglobin; (3) in

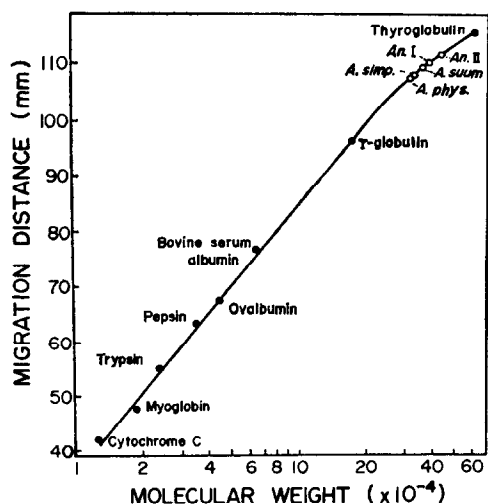


FIG. 6. Estimation of molecular weights of *Anisakis* and *Ascaris* hemoglobins, using a thin-layer of Superfine Sephadex G-200.

the Soret region, the peaks of oxygenated parasite hemoglobin were always larger than those of deoxygenated forms, whereas the reverse is shown for dolphin hemoglobin.

Sedimentation Velocities

A typical pattern of the *Anisakis* Type II larva's hemoglobin is shown in Fig. 5. Two components were always observed in each sample, however the component showing the lower S value was colorless and probably a nonhemoglobin protein contaminant. Therefore, the sedimentation coefficient of hemoglobin was determined by the peak coinciding with the red-colored component.

In Table I, the sedimentation coefficients ($s_{20,w}$) of *Anisakis* spp., *Ascaris suum*, Pacific pollock, dolphin, and pig are shown for comparative purposes. It is evident by this analysis that the S values of *Anisakis* hemoglobin are either nearly equal or somewhat higher than that of *A. suum* hemoglobin, whereas they were much higher than those of their hosts. Furthermore, the values for larvae tend to be slightly higher than those of adults. Sedimentation coefficient values of 11.6 and 11.8 S had been reported respectively by Smith and Morrison (1963) and by Wittenberg *et al.* (1965) for *A. suum* perienteric fluid hemoglobin. In the present study the S value for *A. suum* was calculated to be 11.2 S.

Molecular Weights

The molecular weight of hemoglobin from four different *Anisakis* specimens and *Ascaris suum* was estimated by chromatography on a thin-layer of Superfine Sephadex G-200. A calibration curve between the migration distance of seven reference proteins and the logarithms of their molecular weights was constructed according to the description of Williamson and Allison (1967). Parasite hemoglobins were related to this curve and the molecular

weights of the parasite hemoglobins were roughly estimated at 33 to 34×10^4 , as represented in Fig. 6. Although these values generally correspond to the above sedimentation coefficients, there is no exact correspondence.

In an effort to obtain more reliable values, the Sephadex G-200 gel filtration method (Andrews 1965) and the sodium dodecyl sulfate-polyacrylamide gel electrophoresis method (Shapiro *et al.* 1967) were also employed in the present study (unpublished data). However, more detailed data were not obtained. Further study is needed.

General Considerations

Physicochemical differences in parasite hemoglobins have not been employed for taxonomic distinction. In view of the present findings, it appears that discrimination between genera may not always be fruitful, since considerable similarities with only minor differences were noted between larval stages and adults. However, considerable similarities were observed between larvae and the presumed adults; the isoelectric point and the dichroic spectrum suggest a closer relationship between *Anisakis* Type I larvae and *A. simplex* and between the Type II larva and *A. physeteris* than between each other. This work supports the conspecificity of these larval and adult forms, which were first suggested in Kagei's morphological study. The subtlety of these differences suggests that physicochemical analysis of other substances might prove more generally applicable in the separation of closely related parasite genera, since *Anisakis* hemoglobin was readily distinguished from *Ascaris* spp. and host hemoglobin.

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