THE EFFECTS OF PARASITISM ON THE HEMOCYANIN OF AN INTERTIDAL HERMIT CRAB, *PAGURUS SAMUELIS*

by

MARK ERIK TORCHIN

A THESIS

Presented to the Department of Biology
and the Graduate School of the University of Oregon
in partial fulfillment of the requirements
for the degree of
Master of Science

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Nora Terwilliger

Chair of the Examining Committee

January 29, 1994

Date

Committee in charge: Dr. Nora Terwilliger
Dr. Richard Castenholz
Dr. David Cox

Accepted by:

Vice Provost and Dean of the Graduate School

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Title: THE EFFECTS OF PARASITISM ON THE HEMOCYANIN OF AN INTERTIDAL HERMIT CRAB, *PAGURUS SAMUELIS*

Approved: ______________________________
Dr. Nora Terwilliger

The hemocyanin from *Pagurus samuelis* parasitized by two rhizocephalans, *Peltogaster paguri* and *Peltogasterella gracilis* was examined for parasite-induced changes. Structural properties including quaternary structure, apparent molecular mass, association-dissociation behavior and subunit composition were compared. *P. samuelis* hemocyanin appears to be composed of both 25S and 16S molecules with apparent molecular weights of 940,000 and 450,000 respectively. The stability of the 25S, two-hexamer hemocyanin is dependent on calcium ion activity. The hemocyanin subunit composition of parasitized individuals differs markedly from non-parasitized individuals, when analyzed electrophoretically. Oxygen equilibrium characteristics of hemocyanin from parasitized and non-parasitized individuals were determined tonometrically. Hemocyanin from both parasitized and non-parasitized groups

exhibits similar oxygen binding characteristics. Both parasitized and non-parasitized *P. samuelis* hemocyanin exhibits high cooperativity and low oxygen affinity.

CURRICULUM VITA

NAME OF AUTHOR: Mark Erik Torchin

PLACE OF BIRTH: Santa Monica, California

DATE OF BIRTH: February 21, 1968

GRADUATE AND UNDERGRADUATE SCHOOLS ATTENDED:

University of Oregon
University of California, Santa Barbara
California State University, Northridge

DEGREES AWARDED:

Master of Science in Marine Biology, 1994, University of Oregon
Bachelor of Arts in Aquatic Biology, 1991, University of California, Santa Barbara

AREAS OF SPECIAL INTEREST:

Parasitology
Biological Invasions
Comparative Physiology and Biochemistry

PROFESSIONAL EXPERIENCE:

Graduate Teaching Fellow, Department of Biology, University of Oregon, Oregon Institute of Marine Biology, Charleston, Spring 1992, Fall 1993

AWARDS AND HONORS:

Summer Science Research Fellowship, American Heart Association, Oregon Affiliate, 1992

Lerner-Gray Award, American Museum of Natural History, 1992

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CHAPTER I

INTRODUCTION

Pagurus samuelis (Stimpson) is an anomuran hermit crab which usually inhabits *Tegula funebralis* shells. It is found from Vancouver Island, British Columbia to Punta Eugenia, Baja California and occurs in tide pools in mid to high rocky intertidal areas, but can extend into subtidal regions (Morris et. al, 1980). *P. samuelis* may be parasitized by a barnacle in the order Rhizocephala. On rocky shores in southern Oregon, near Coos Bay, the degree of parasitism varies seasonally and may be as high as 20 percent (personal observation). The two genera of rhizocephalans infesting *P. samuelis* in this area are *Peltogaster* and *Peltogasterella*. *Peltogasterella gracilis* has been documented as far south as Chile (Morris et. al, 1980). Until this report, *Peltogaster paguri* had only been documented as far south as Alaska on the west coast of the United States (Høeg, J., 1993, Personal communication).

Rhizocephalans are endo- and ectoparasites of marine and freshwater decapods and cirripeds. They may have several free-living planktonic larval stages, consisting of nauplii and cyprids. The female cyprid differentiates into a larval stage called the kentrogon. The kentrogon infects

the host by injecting an undifferentiated cell mass into the host hemolymph, in which the young parasite begins to grow. The cell mass develops into a rootlet network called the interna, which, in most species, ramifies throughout most parts of the host's body (Høeg and Lüzen, 1985). Rhizocephalans are parasitic castrators which many times cause physiological and morphometric changes in their host. These include degeneration of reproductive organs, feminization of male crabs and a cessation of molt cycle in some host species. Upon sexual maturation of the rhizocephalan, a reproductive sac-like structure, called the externa, breaks through the host's abdomen (Figure 1). The externa remains attached to the interna through a narrow stalk. The externa contains the ovary and oocytes. A male cyprid invades special receptacles in the recently emerged female externa and implant cells which develop into spermatozoa. These eventually fertilize the oocytes (Yanagimachi, 1961). Embryonic development takes place within the externa. The roots of the interna are believed to be responsible for the nourishment of the externa. Substances are presumably taken up through the cuticle lining the roots of the rhizocephalan and are transported to the externa and developing embryos. In *Peltogaster* and some other species of rhizocephalan the rootlets are sometimes green in color due to biliverdin, a degradation product of hemoglobin. Hemoglobin exists in the parasite's externa

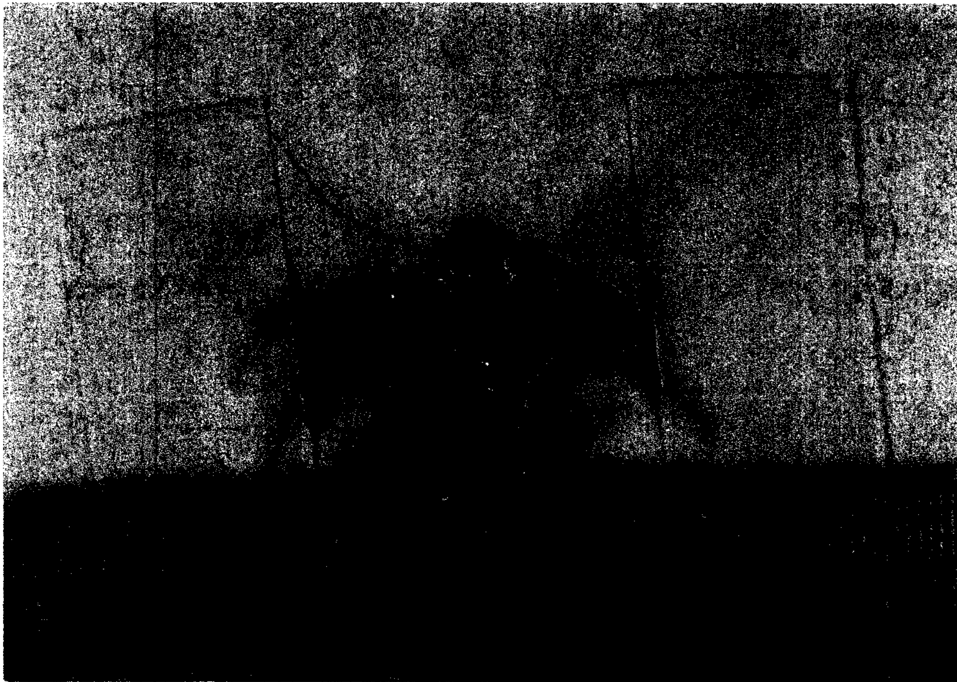

Figure 1. *Pagurus samuelis* infected by *Peltogaster paguri*. Note the presence of the externa on the host's abdomen.

and is responsible for its red color (Fox, 1953; Shirley et al 1986; Terwilliger et al 1986). A red color is also sometimes apparent in the maturing externae of *Peltogasterella,* suggesting that hemoglobin may also be present in *Peltogasterella*.

The hermit crab host, *P. samuelis*, on the other hand, contains hemocyanin, a copper containing respiratory pigment, common among decapod crustaceans.

Hemocyanins are oxygen transport molecules found in the hemolymph of many arthropods and molluscs. In the Arthropoda, the monomeric subunits forming these proteins self assemble into one-hexamer, two-hexamer and even larger aggregates. In the Decapoda, two-hexamer molecules with sedimentation coefficients ($S°_{20},w$) of about 25S and one-hexamer molecules ($S°_{20},w$) of about 16S are present in some combination in the hemolymph (van Holde and van Bruggen, 1971, van Holde and Miller, 1982). Studies have shown that the formation of the dodecameric, two-hexamer hemocyanin molecule can result from several different mechanisms. Specific subunits may be responsible for linking hexamers into two-hexamer or even larger aggregates (Jeffery et al, 1978). Some studies also suggest that in certain species, the subunits linking the higher aggregates may be connected by disulfide bonds (Markl et al, 1981; Pilz et al, 1980; Jeffery et al, 1978). In a few cases, association to dodecamer may involve non-covalent interactions with calcium

ions (Morimoto and Keagles, 1971). Examples in the literature of crustacean dodecameric hemocyanins showing a calcium dependence include; *Homarus americanus* (Morimoto and Kegeles, 1971), *Ligia pallasii* (Terwilliger, 1981) and *Pagurus pollicaris* (Rhodes, 1983). Thus, it is conceivable that the concentration of divalent cations, especially calcium, could have a significant influence on the ratio of the two hemocyanin components, hexamers and dodecamers, in the hemolymph.

One of the most important properties of hemocyanin is that it binds reversibly with oxygen. Oxygen binding occurs at an active site containing two copper atoms which bind to one oxygen molecule. One subunit contains one active site. Hemocyanins, like hemoglobins, often show a sensitivity to hydrogen ions, i.e. a change in oxygen affinity as a function of pH. This is called a Bohr shift. Among the Crustacea, a "normal" Bohr shift usually occurs. Thus, oxygen affinity decreases with a decrease in pH. Cooperativity is another significant feature of respiratory proteins whereby oxygen binding to one active site facilitates the binding of oxygen at other active sites in the molecule. This allosteric effect is usually large in crustacean hemocyanins, with n_{50} ranging from 2 to 6 (Mangum, 1980).

The presence of hemocyanin in the host and hemoglobin in the parasite poses a particularly interesting question in

terms of potential oxygen transport, since hemoglobins tend to have higher oxygen affinities than hemocyanins (Mangum, 1992 and Markl, 1986). If there were oxygen exchange between the hemolymph of the hermit crab and the rootlet network of the barnacle, one could predict that the direction of oxygen transport would be from crab to barnacle. If this were the case, it might then be advantageous to the parasitized hermit crab to have a hemocyanin with a higher oxygen affinity compared to non-parasitized individuals.

The purpose of this thesis is to approach this question by describing some structural properties of *Pagurus samuelis* hemocyanin using column chromatography, gel electrophoresis and ion analysis. This study will also ask whether the one-hexamer / two-hexamer association properties of *P. samuelis* hemocyanin are influenced by divalent cation concentrations in the hemolymph.

Furthermore, this study will examine the oxygen binding properties of *P. samuelis* hemocyanin, including oxygen affinity, Bohr effect and cooperativity. Finally, it will ask whether parasitism by *Peltogaster paguri* and *Peltogasterella gracilis* affects either the structural or functional properties of *P. samuelis* hemocyanin, by comparing hemocyanin from parasitized and non-parasitized hermit crabs.

CHAPTER II

MATERIALS AND METHODS

Animal Collection and Maintenance

Pagurus samuelis were surveyed for presence of *Peltogaster paguri* and *Peltogasterella gracilis* infestation at two mid to high rocky intertidal locations; Sunset Bay and Cape Arago, near Coos Bay, Oregon. Parasitism was determined by the presence of one or more externae on the ventral surface of the host's abdomen. Nauplii and cyprid larvae of *P. paguri* and *P. gracilis* were cultured at the Oregon Institute of Marine Biology and their identity confirmed by J. Høeg (1993).

Both parasitized and non-parasitized *P. samuelis* were collected for further studies and maintained without a shell at the Oregon Institute of Marine Biology. The crabs were kept in individual 0.5L flow-through plastic containers in running seawater at 10-13° C. The crabs were fed chopped mussels twice weekly. The hermit crabs were divided into three treatment groups; non-parasitized, parasitized by *P. paguri* and parasitized by *P. gracilis*. Hereafter they will be referred to as non-parasitized, parasitized (Pp) and parasitized (Pg) respectively. Hemolymph was collected between two and three weeks after animal capture.

Hemolymph Collection and Preparation

Pagurus samuelis was bled by inserting a micropipet into the arthrodial sinus at the base of a walking leg. All samples were collected on ice and diluted with a Tris-HCl buffer (pH 7.5, see below) to reduce clotting. Samples were centrifuged in a refrigerated Eppendorf 5415 centrifuge (10°C) for 2-3 min. at 13,800 x g. The resulting pellet was discarded and the supernatant was used in further studies. Blood samples from 10 to 15 individuals were pooled for all column chromatography and oxygen equilibrium experiments. Both individual and pooled samples were analyzed by gel electrophoresis.

Hemolymph from *Peltogaster paguri* was obtained by puncturing the blood sinus at the base of the stalk on the externa with a micropipet. The externae of *Peltogasterella gracilis* were homogenized, centrifuged and the supernatant was used in all experiments.

Column Chromatography

Pooled samples were chromatographed on a Bio Gel A-5m column (112.0 X 1.88 cm) in equilibrium with a 0.05 I Tris-HCl buffer, pH 7.5 (0.1 M in $NaCl_2$; 0.01 M in $CaCl_2$; 0.01 M in $MgCl_2$) at 10°C. This buffer was used in all experiments and will be referred to as the standard buffer. The column was calibrated with 25S and 16S *Cancer magister* hemocyanin

(Ellerton et al., 1970). Adjustments to pH were made by titrating the standard buffer with a 1.0 M Tris solution at the appropriate temperature. Effluent fractions were collected and the volumes were measured and analyzed for absorption maxima at 280 and 340 nm on a Zeiss PMQ II spectrophotometer.

Non-denaturing Gel Electrophoresis, pH 7.4

Polyacrylamide gel electrophoresis (PAGE) modified from Davis (1969) was used to analyze the native hemocyanin molecules of both whole hemolymph and purified hemocyanin of *P. samuelis*. Non-denaturing, 5% acrylamide gels, pH 7.4, containing either 10 mM $CaCl_2$, 10 mM $MgCl_2$ or 10 mM ethylene-diaminetetraacetic acid (EDTA) were used to determine the structure of the intact hemocyanin molecule. Electrophoresis was carried out in a 0.05 M Tris-HCl/Tris-maleate buffer system. Both upper and lower electrode buffers were modified to contain 10 mM $CaCl_2$, 10 mM $MgCl_2$ or 10 mM EDTA, depending on which gel was run. *Cancer magister* whole hemolymph and purified hemocyanin were used as calibrants. All hemolymph and hemocyanin samples included approximately 20% glycerin to prevent convection anomalies in the wells. Gels were run at 35mA for 3-4 hours and stained with Coomassie Blue (Fairbanks et al., 1971).

SDS Gel Electrophoresis

Sodium dodecyl sulfate (SDS) polyacrylamide gels (7.5 %) were run essentially as described by Laemmli (1970). Hemocyanin samples were denatured for 1.5 min. in boiling incubation buffer containing 2% SDS, 10% glycerin, 1 mM EDTA, 0.01% bromphenol blue, 62.5 mM Tris-HCl, 50 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 1.5 min. and stored at -20°C. The running buffer (pH 8.3) contained 0.025M Tris-OH, 0.192 M glycine, 0.1% SDS, 0.1M EDTA. Gels were run at 100 volts for approximately 4 hours. All gels were stained with Coomassie Blue according to Fairbanks et al. (1971). The calibrants for the SDS gels were the six polypeptide chains of *Cancer magister* hemocyanin (Larson et al, 1981).

Oxygen Equilibrium Studies

BioGel A-5m purified hemocyanin samples from both parasitized and non-parasitized crabs were concentrated in Centricon 30 (Amicon Inc.) microconcentrator tubes in a Sorvall RC2-B refrigerated centrifuge (10°C) at 4000x g. Concentrated samples were resuspended in standard Tris-HCl buffer of the desired pH, centrifuged and resuspended three times, and then diluted to an appropriate concentration (approximately 0.11 - 0.22 µg / µl).

Oxygen binding was conducted tonometrically as

described by Benesch et al. (1965). Two armed tonometers with 1 cm light path cuvettes with moveable stopcocks were used. Between 2.25 to 3 mls of hemocyanin sample diluted to the desired concentration were placed into the tonometer, and air was repeatedly evacuated using a vacuum pump. The tonometers were allowed to equilibrate for 10 minutes on ice between evacuations. The last two evacuations were done at room temperature. After complete deoxygenation, the tonometers were placed in a circulating water bath at 10°C.

Absorbances were measured at 350, 340 and 330 nm using a Beckman DU70 recording spectrophotometer with a thermostatted cuvette holder set at 10°C. After each measurement, a known aliquot of water-saturated air was injected into the tonometer using a 10 ml glass syringe. After each addition of air, samples were allowed to equilibrate in the 10°C water bath for 10 minutes before measuring the resulting absorbance. The final absorbance was measured after the tonometer was opened and flushed with pure oxygen. Immediately following oxygen binding, the pH of each sample was measured at 10°C and recorded.

The partial pressure of oxygen (P_{o2}) and the percent saturation of hemocyanin (Y) were calculated using the following equations:

(1) P_{50} = mls air injected $\cdot$ [(B-P) $\cdot$ (0.21) $\cdot$ Ti $\div$ (V$\cdot$To)]

Where B= barometric pressure (mm Hg); P = vapor pressure at room temperature (mm Hg); 0.21 = percent of oxygen in air; Ti = temperature inside, i.e. sample temperature (K); V = tonometer volume - sample volume (mls) and To = temperature outside, i.e. air temperature (K).

(2) $$Y = (Ax - Ad \div Ao - Ad) \cdot 100$$

Where Ax = absorbance maxima after (x) mls of air injected; Ad = absorbance maxima of deoxygenated hemocyanin and Ao = absorbance maxima of oxygenated hemocyanin.

Ion Analysis

Calcium activity was determined by diluting 10 µl hemolymph with 990 µl 0.3 M KCl, placing the mixture into a 1 ml electrode dish and measuring the voltage potential between a double junction reference electrode (Orion # 900200) and a calcium Selectrode (Radiometer, Copenhagen) using an Ion 83 ion analyzer (Radiometer, Copenhagen). Voltage measurements were taken three times for each sample and averaged. Measured potentials were then plotted on a graph of calcium standard solutions ranging from 1 to 100 mM.

Analysis of Results

The ratio of total protein versus hemocyanin in hermit

crab hemolymph was calculated by the peak absorbance ratio of measured wavelengths (280/340 nm). These ratios were then analyzed by analysis of variance (ANOVA) to compare the treatment groups. The oxygen binding data for hemocyanin from the three treatment groups was analyzed using Quattro Pro 3.0 (Borland Int. Inc.).
Regression lines described the relationships between the Bohr coefficients (the slope of Δlog P50 vs. ΔpH) and cooperativity (n_{50}) versus pH. Bohr coefficients and cooperativities of the three treatment groups were compared by analysis of covariance (ANCOVA). Statistical analyses were done using SYSTAT version 4.1 (SYSTAT Inc.).

CHAPTER III

RESULTS

Parasitism of *Pagurus samuelis* by the rhizocephalan barnacles was determined by the presence of one or more rhizocephalan externae on the ventral surface of a hermit crab's abdomen. A non-parasitized hermit crab was defined as one lacking both externae and a visible internal rootlet network typical of the rhizocephalan's internal phase. Early stages of parasitism, in which the parasite's internal rootlet network was not fully developed, may have been overlooked. These stages might be detected in histological sections or through scanning electron microscopy, techniques not used in this study. *Peltogaster paguri* was present at both collecting sites, while *Peltogasterella gracilis* was only found at Sunset Bay.

Pagurus samuelis hemolymph chromatographs as a single asymmetrical peak on BioGel A-5m (Figure 2). There were no apparent differences among the chromatographic patterns of non-parasitized and parasitized treatment groups [n=8, non-parasitized; n=7, parasitized (Pp); n=9, parasitized (Pg)]. Each chromatography consisted of a pooled sample (n= 10-15 individuals); there were a total of 24 columns run (see Appendix). The main peak (peak I) had an apparent molecular

weight of 940,000 and corresponded to the 25S (two hexamer) fraction of *Cancer magister* hemocyanin. The trailing shoulder (peak II) had an apparent molecular weight of 450,000 and corresponded to the 16S (one hexamer) component of *C. magister* hemocyanin. The ratio of total protein versus oxy-hemocyanin (absorbance at 280/340 nm) averaged 7.2 for peak I and 8.2 for peak II with no significant difference ($p \leq 0.05$) among any of the treatment groups.

<u>One Hexamer - Two Hexamer Association Behavior</u>

Ion analysis of hemolymph from both parasitized and non-parasitized *P. samuelis* individuals revealed an average calcium ion activity of 10 mM (SE= ± 2), n=6 (3 non-parasitized and 3 parasitized), (Figure 3). Aliquots from BioGel A-5m peak I (Figure 2), electrophoresed on a non-denaturing pH 7.4 polyacrylamide gel (pH 7.4 PAGE) in the presence of 10 mM $CaCl_2$ as a single band corresponding to the 25S component of *C. magister* hemocyanin (Figure 4, lanes 1-5,9). Aliquots from peak II (Figure 2), corresponding to the 16S component of *C. magister* hemocyanin, electrophoresed as two bands (Figure 4, lanes 6,7,8,10). The faster migrating band corresponded to the 16S (one-hexamer) molecule, while the slower one corresponded to the 25S (two-hexamer) molecule of *C. magister* hemocyanin (figure 4, lane 12).

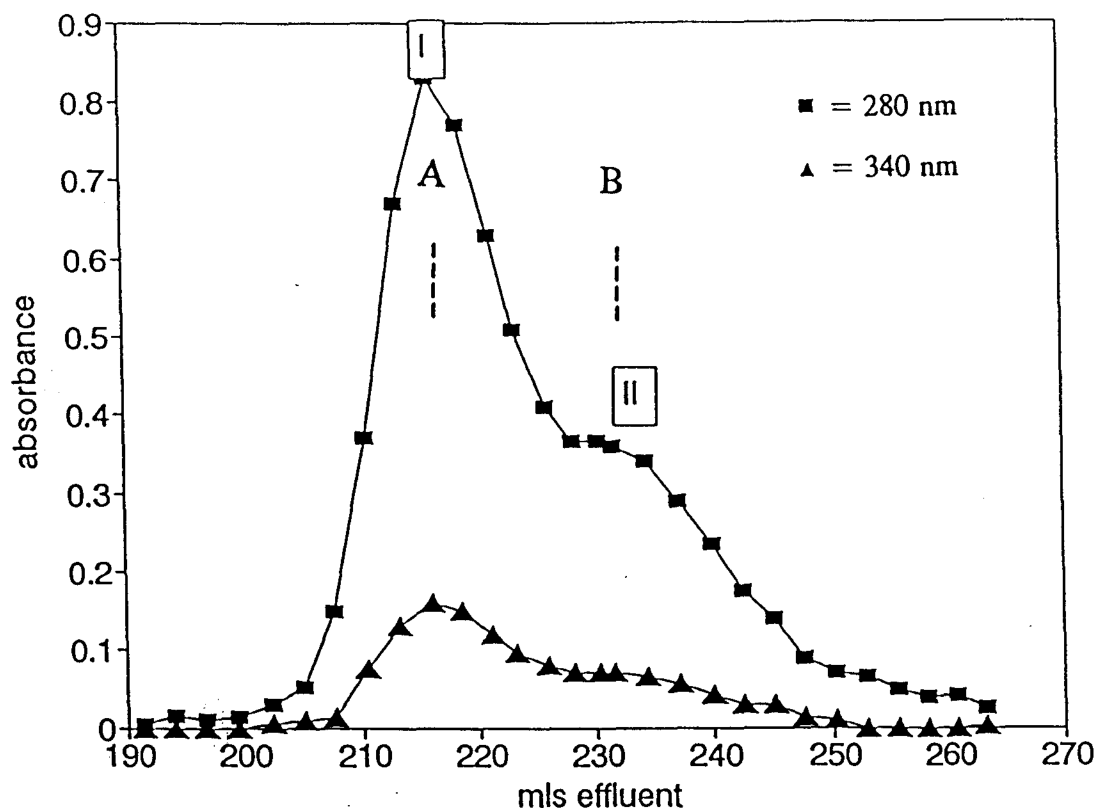

Figure 2. Chromatography of pooled *P. samuelis* hemolymph (parasitized (Pg), n=7) on a column of BioGel A-5m (112.0 x 1.88 cm) Buffer, 0.1 Ionic strength Tris-HCl (pH 7.5), 0.1 M in $NaCl_2$, 0.01 M in $CaCl_2$ and 0.01 M in $MgCl_2$. Absorbance at 280 (■) and 340 nm (▲). Calibrants, A. *Cancer magister* 25S hemocyanin, Mw = 940,000, B. *C. magister* 16S hemocyanin, Mw = 450,000 (Ellerton et al., 1970).

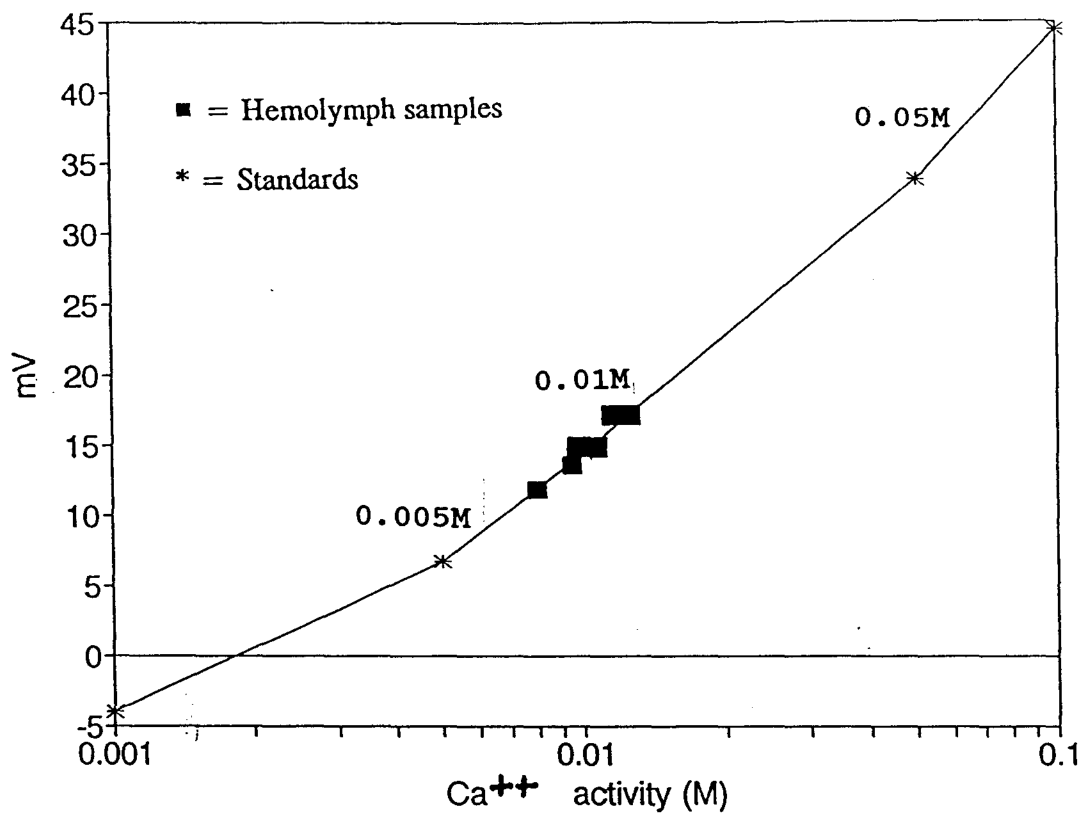

Figure 3. Calcium ion activity in *P. samuelis* hemolymph. Semi log plot of Ca^{2+} activity versus millivolts recorded on an Ion 83 ion analyzer. Standard solutions (✱); *P. samuelis* hemolymph samples (■).

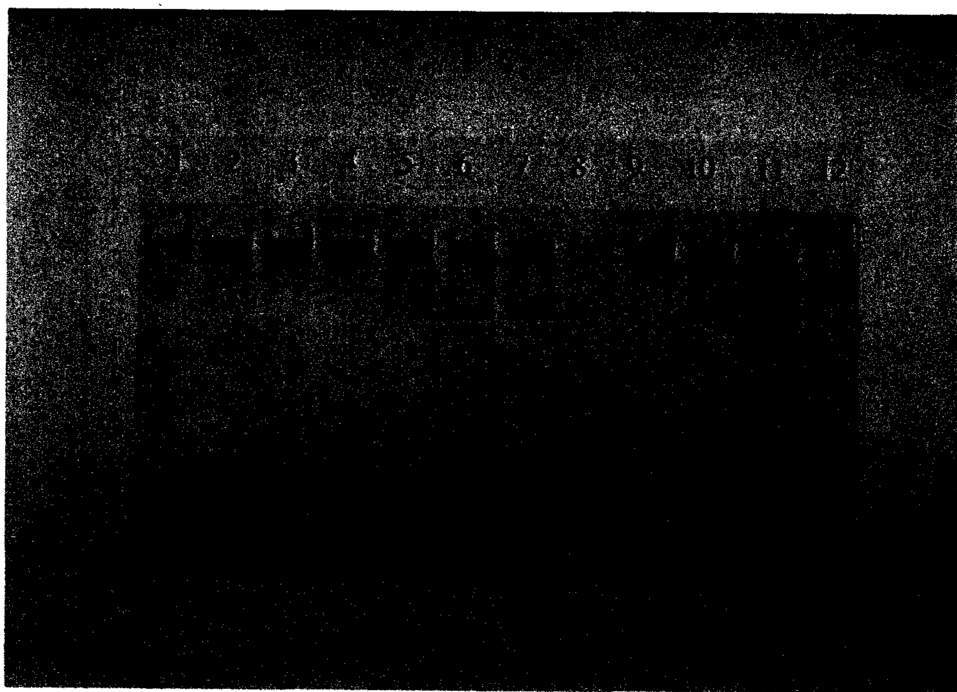

Figure 4. Non-denaturing PAGE (pH 7.4) + 10 mM $CaCl_2$ of purified *P. samuelis* hemocyanin. Samples from chromatographed fractions in Figure 2. Lanes 1-5, 9, peak I; Lanes 6-8, 10, peak II; Lane 11, whole hemolymph from *P. samuelis;* Lane 12, whole hemolymph from *Cancer magister*.

When similar Biogel A-5m aliquots were electrophoresed on pH 7.4 PAGE in the presence of 10 mM $MgCl_2$, a second pattern resulted (Figure 5). The aliquots from peak I showed a very faint band corresponding to a 25S, two-hexamer molecule and a major band corresponding to a 16S, one-hexamer molecule. The aliquots from peak II showed only one band corresponding to a 16S, one-hexamer molecule.

When electrophoresed on pH 7.4 PAGE in the presence of 10 mM EDTA (Figure 6), aliquots from peak I and peak II showed band patterns similar to those electrophoresed in the presence of $MgCl_2$. When *C. magister* 25S and 16S hemocyanin are electrophoresed on pH 7.4 PAGE, each migrates as a single band corresponding to its original molecular weight, regardless of whether $CaCl_2$, $MgCl_2$, or EDTA is present.

Electron micrographs indicate that in *P.samuelis* hemolymph, negatively stained with 1% uranyl acetate, the majority of the hemocyanin occurs as two-hexamer aggregates (Figure 7). Both PAGE, pH 7.4 and Electron microscopy results suggest that experiments conducted in the presence of 10 mM calcium, the physiological calcium ion activity level, most closely mimic the *in vivo* aggregation state.

The formation of a two-hexamer hemocyanin is sometimes due to the presence of a particular "linker" subunit (Jeffrey et al, 1978). In some cases, the two-hexamers are made up of hexamers that differ in subunit composition from

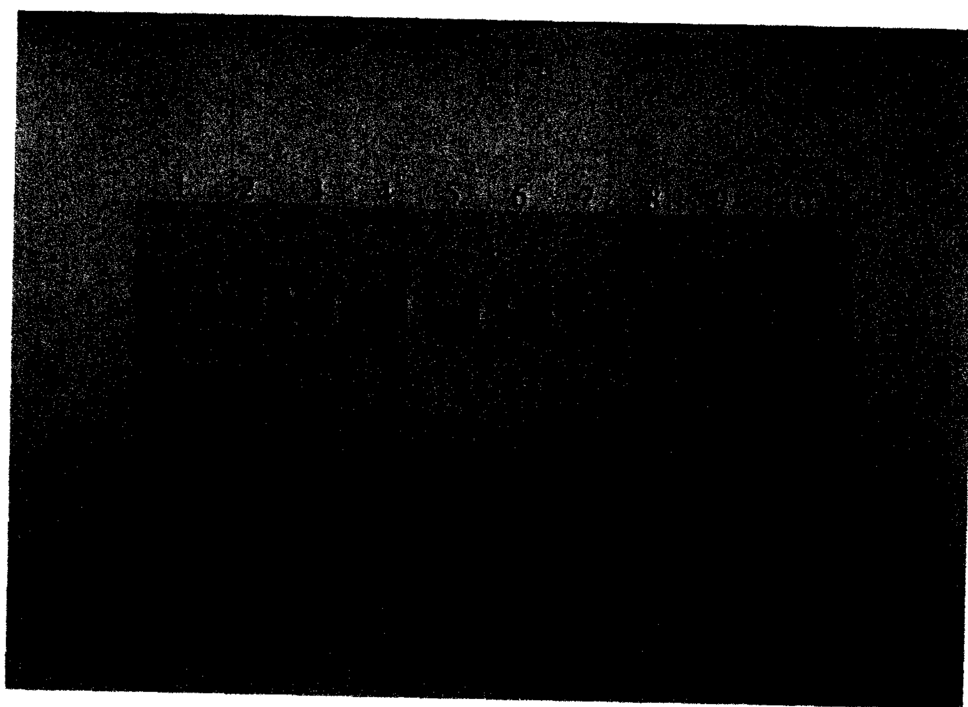

Figure 5. Non-denaturing PAGE (pH 7.4) + 10mM $MgCl_2$ of purified *P. samuelis* (non-parasitized) hemocyanin. Samples from chromatography fractions of pooled hemolymph (n=10) on a BioGel column. Lanes 1-5, peak I (25S fraction); Lanes 6-9, peak II (16S fraction); Lane 10, whole hemolymph from *Cancer magister*.

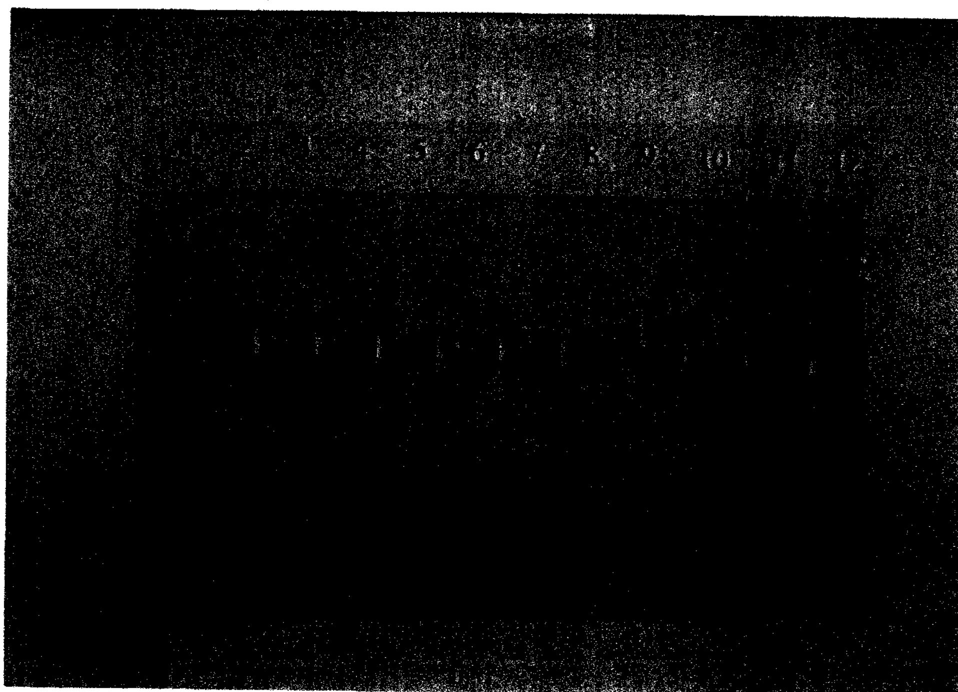

Figure 6. Non-denaturing PAGE (pH 7.4) + 10mM EDTA of purified *P. samuelis* hemocyanin. Samples were taken from chromatographed fractions in Figure 2. Lanes 1-5, 9, peak I; Lanes 6-8, 10, peak II; Lane 11, whole hemolymph from *P. samuelis;* Lane 12, whole hemolymph from *Cancer magister*.

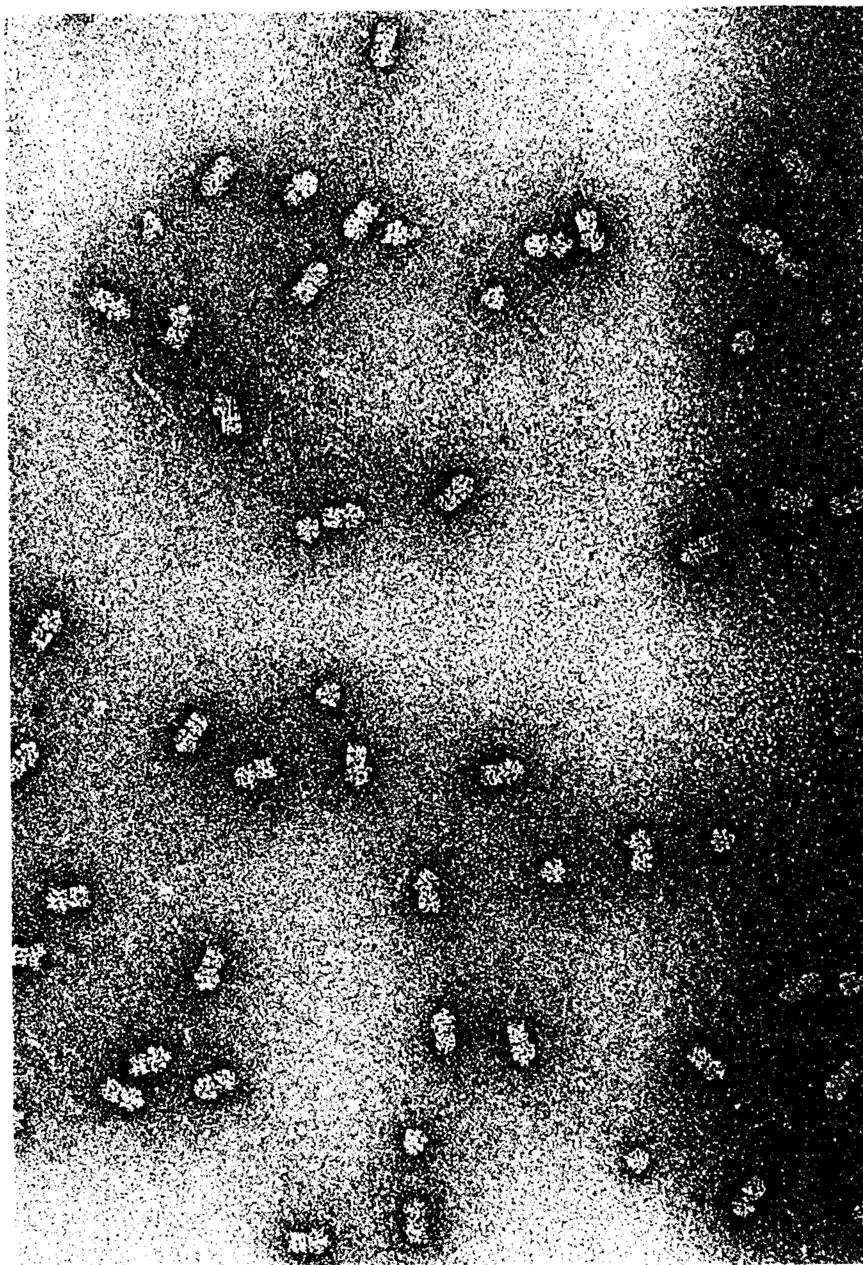

Figure 7. Electron micrograph of crude *Pagurus samuelis* hemolymph at 250,000x magnification. Electron microscopy by Eric Schabtach.

other non-associating hexamers in the hemolymph (Jeffrey et al, 1978, Markl et al, 1981, Terwilliger et al, 1982). In order to determine whether *P. samuelis* hemocyanin was composed of different hexamer populations with different subunit compositions, two-hexamer and one hexamer samples were compared by SDS-PAGE. Purified peak I and peak II hemocyanin from both parasitized (Pp) and parasitized (Pg) hermit crab samples, electrophoresed as 3 distinguishable bands on SDS-PAGE (Figure 8). Hemocyanin from non-parasitized hermit crabs electrophoresed as 6 distinguishable bands (Figure 10, lanes 2-6), as will be discussed later. No difference could be discerned between subunit compositions of peak I or peak II fractions within treatment groups.

Gel Electrophoresis of Whole Hemolymph

Pagurus samuelis crude hemolymph from individual hermit crabs electrophoresed as two components on a pH 7.4 PAGE in the presence of 10mM $CaCl_2$. As shown in Figure 9, the upper band corresponded to Biogel A-5m peak I (two-hexamer). The lower corresponded to Biogel A-5m peak II (one-hexamer). There were no apparent differences in electrophoretic patterns of hemolymph from non-pooled individuals among the three treatment groups.

When crude hemolymph was electrophoresed under dissociating and denaturing conditions (SDS-PAGE), a

Figure 8. SDS PAGE of purified *P. samuelis* [parasitized (Pg)] hemocyanin. Samples were taken from chromatographed fractions in Figure 2. Lanes 2-6, peak I; Lanes 7-9, peak II; Lane 10, whole hemolymph from *P. samuelis;* Lane 11, whole hemolymph from adult *Cancer magister*.

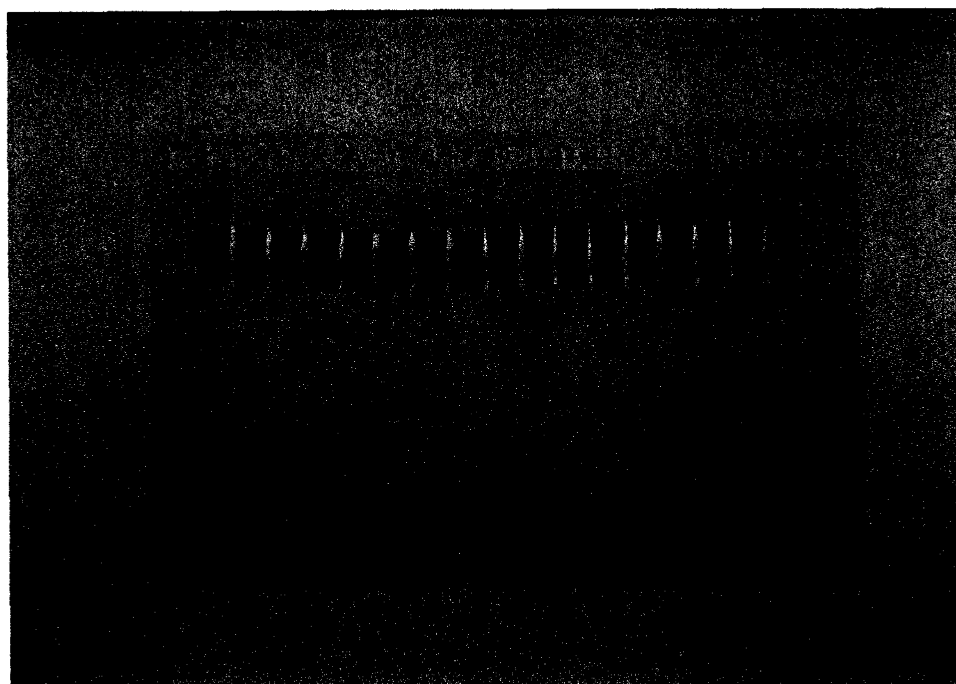

Figure 9. Non-denaturing PAGE (pH 7.4) + 10 mM $CaCl_2$ of whole hemolymph from individual *P. samuelis*. Lanes 2-5, Parasitized (Pp); Lanes 6-9 , Non-parasitized;Lanes 10-13, Parasitized (Pg); Lanes 14 and 15, Parasitized (Pp); Lanes 16 and 17, Parasitized (Pg); Lane 18, *Cancer magister* whole hemolymph.

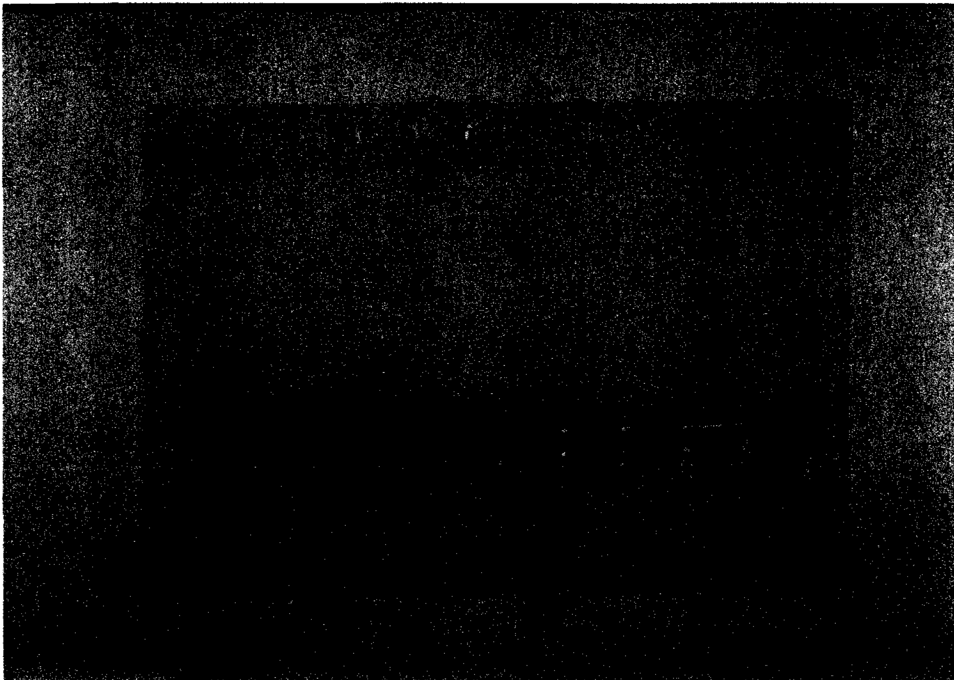

Figure 10. SDS PAGE of whole hemolymph from *P. samuelis*. Lanes 2-6, Non-parasitized; Lanes 7-8, Parasitized (Pp); Lanes 9-10, Parasitized (Pg); Lane 11, adult *Cancer magister* whole hemolymph.

difference in band patterns among parasitized and non-parasitized hemolymph was obvious (Figure 10). Hemolymph of individuals from both parasitized groups consistently showed three bands on SDS gels (Fig. 10) corresponding to molecular weights of approximately 82600 ± 400, 77400 ± 37 and 72200 ± 70 (SE of two determinations). The bands migrated approximately the same distance as *C. magister* hemocyanin controls. Non-parasitized individuals usually showed six bands corresponding to molecular weights of approximately 82200 ± 700, 79800 ± 100, 77100 ± 300, 73700 ± 1300, 72300 ± 1300 and 70100 ± 500 (SE of two determinations), which migrated approximately the same distance as *C. magister* hemocyanin (Fig. 10). The six band pattern was observed in 79 percent of the non-parasitized samples; in 21 percent of the non-parasitized samples, a three band pattern typical of the parasitized individuals was observed (n=62).

Oxygen Equilibrium Studies

The spectra of oxy-hemocyanin samples from all three treatment groups were indistinguishable from one another. A wavelength scan from 300-400 nm reveals one major absorbance maximum at approximately 337 nm (Figure 11).

Oxygen binding spectra revealed that samples at high pH had a higher absorbance at 340 nm than did the same samples at lower pH (Figure 12). The higher absorbance indicates a more fully oxygenated sample. The lower absorbance seen at

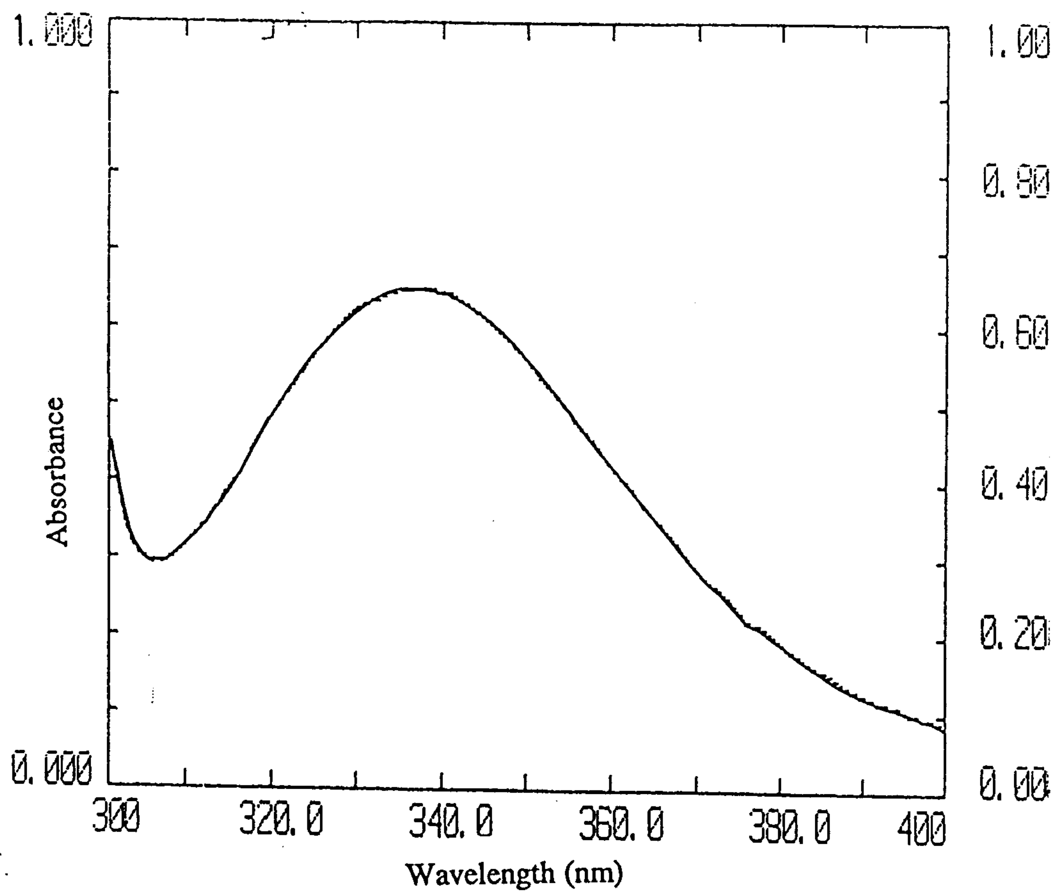

Figure 11. Spectrum of 25S and 16S Para. (Pp) *P. samuelis* hemocyanin purified by BioGel A-5m column chromatography.

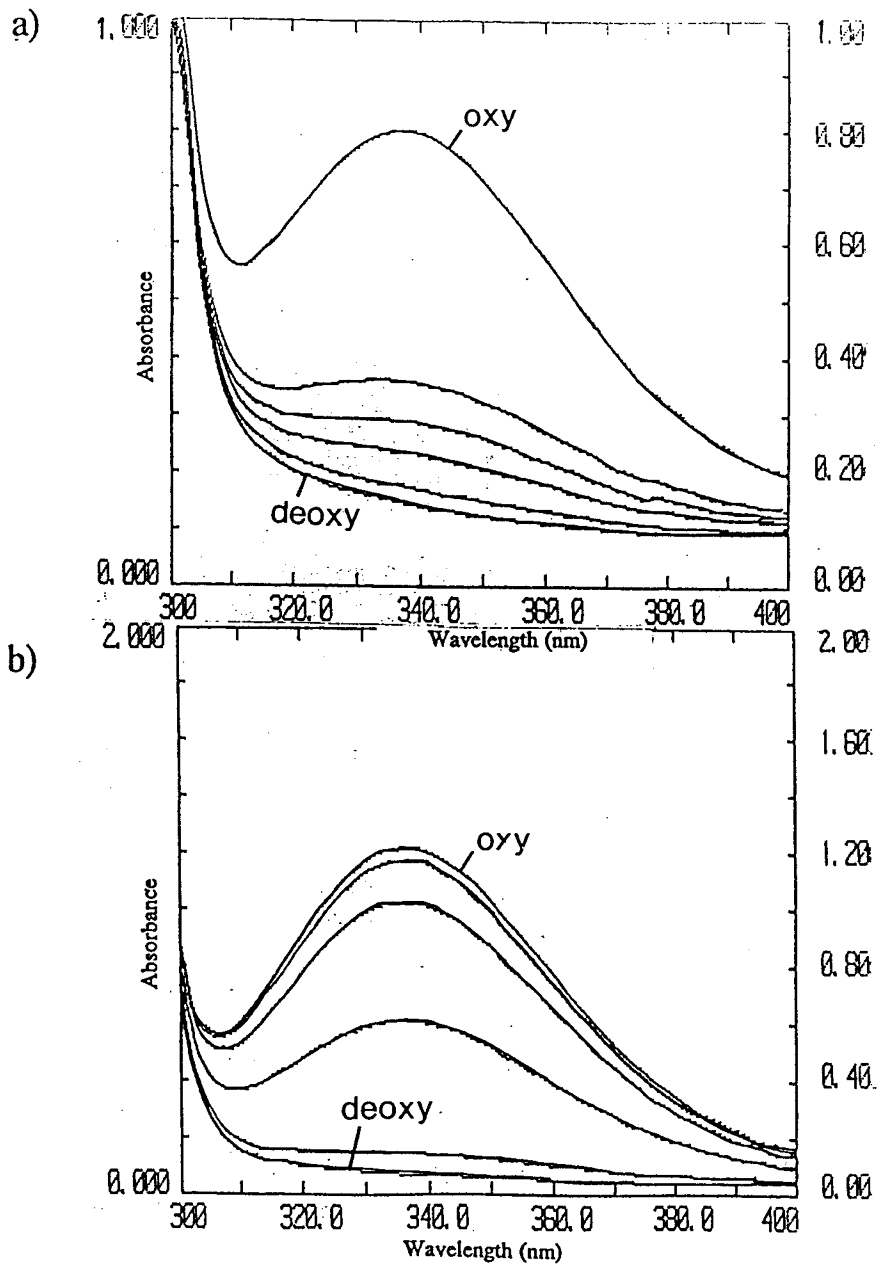

Figure 12. Examples of oxygen binding spectra on hemocyanin from Parasitized (Pg) *P. samuelis*. a) pH 7.25. b) pH 7.94.

low pH suggests that the sample is not fully saturated. Even though the theoretical carrying capacities are equal in both, since the protein concentrations are the same, the sample at lower pH has an apparent lower oxygen carrying capacity in air.

Hemocyanin from *P. samuelis* binds reversibly with oxygen. At 10°C and low hemocyanin concentration (0.11-0.22 µg/µl), the hemocyanin had a very low oxygen affinity and appeared to have a reverse Bohr shift. Samples run at low pH had lower P_{50}'s than samples at higher pH (Figures 13,14 and 15). There are no significant ($p \leq 0.05$) differences among the Bohr coefficients ($\Delta \log P_{50}/\Delta \log pH$) in any of the three treatment groups. All groups appeared to have a reverse Bohr shift.

Pagurus samuelis hemocyanin shows cooperative oxygen binding which is highly sensitive to pH. At low pH, the binding curves appear to have low cooperativity, but as pH increases an increase in cooperativity is apparent (Figures 16, 17 and 18). The binding curve also becomes progressively sigmoidal. As oxygen partial pressure is increased, hemocyanin samples at low pH appear to have a linear binding curve, whereas at high pH the same samples show a sigmoidal binding curve. The pH dependence of cooperativity is manifested in both n_H and the slope of the curve (Figures 19 and 20).

The slopes of cooperativity (n_{50}) versus pH are

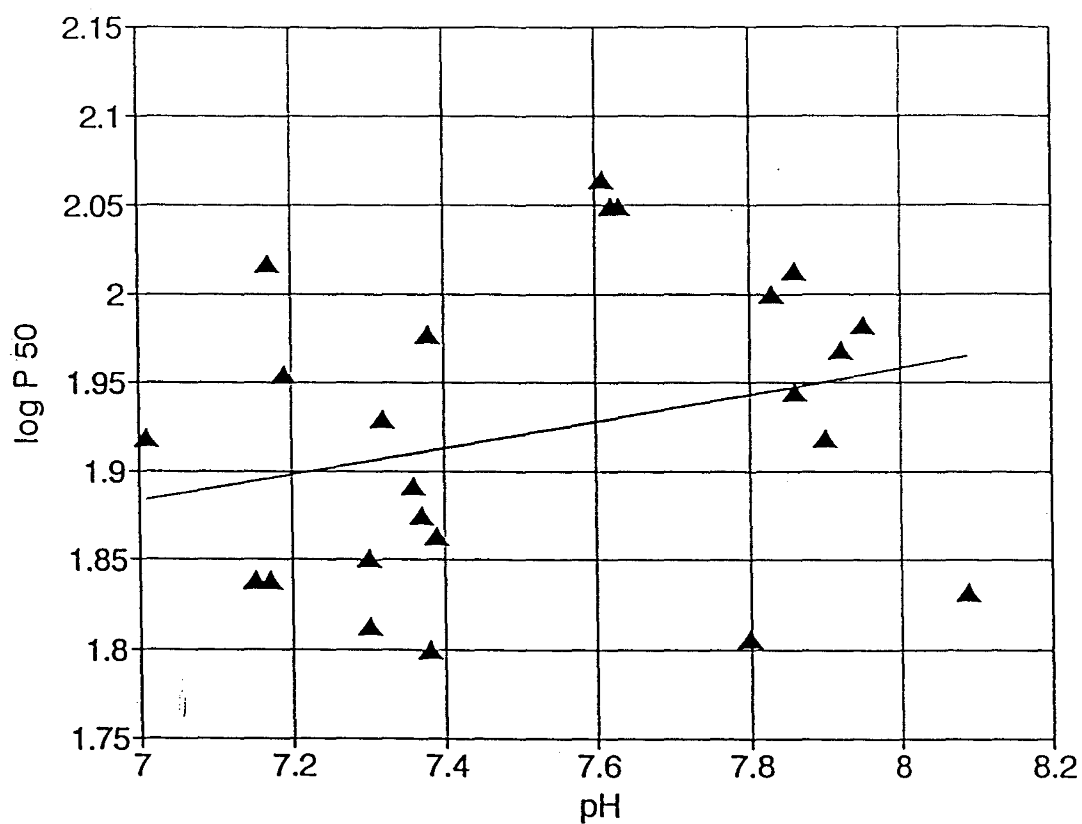

Figure 13. The effect of pH on log P_{50} in purified hemocyanin from Non-parasitized *P. samuelis*. Regression indicates line of best fit (r^2=0.08) through original data points from oxygen equilibrium experiments; Bohr coefficient = 0.074.

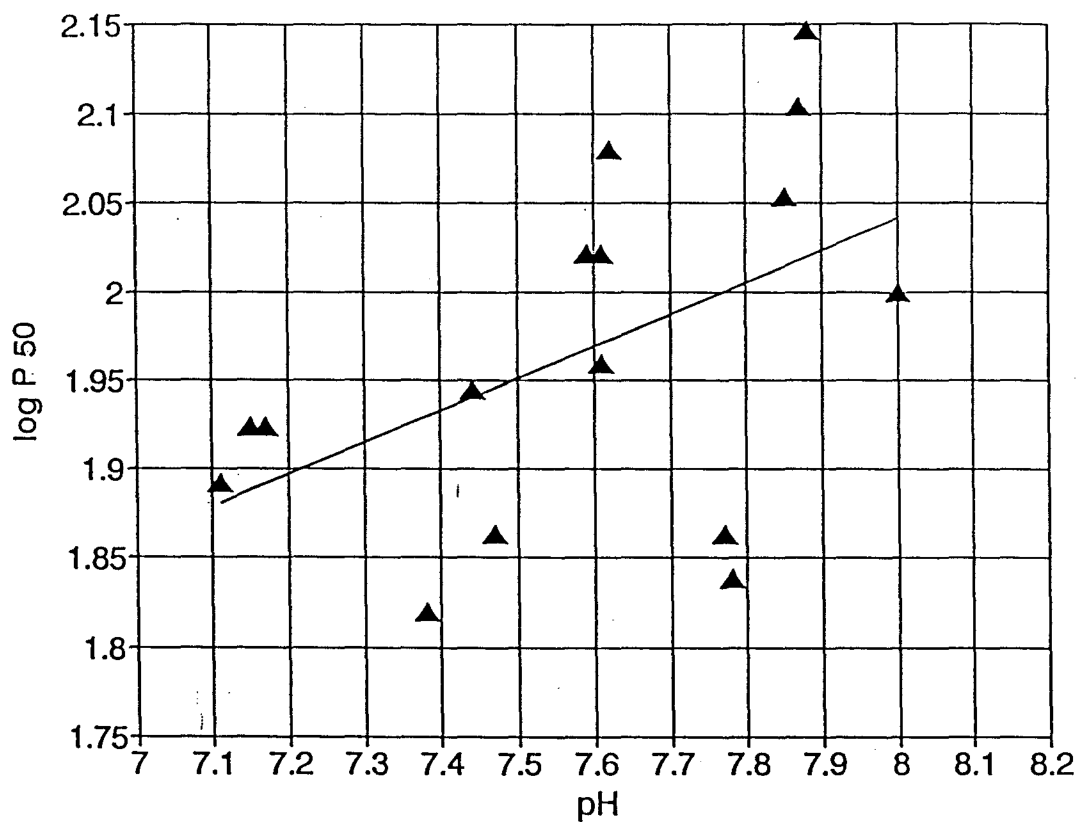

Figure 14. The effect of pH on log P_{50} in purified hemocyanin from Parasitized (Pp) *P. samuelis*. Regression indicates line of best fit (r^2=0.25) through original data points from oxygen equilibrium experiments; Bohr coefficient = 0.18.

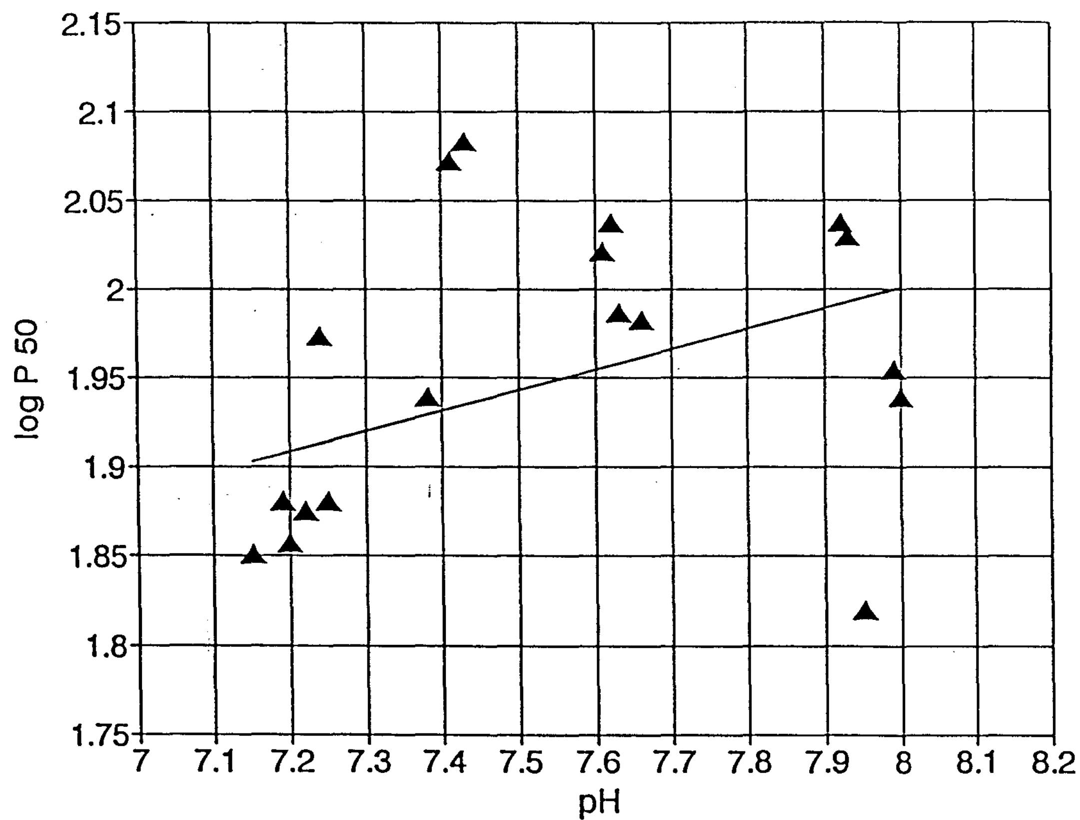

Figure 15. The effect of pH on log P_{50} in purified hemocyanin from Parasitized (Pg) *P. samuelis*. Regression indicates line of best fit (r^2=0.17) through original data points from oxygen equilibrium experiments; Bohr coefficient = 0.11.

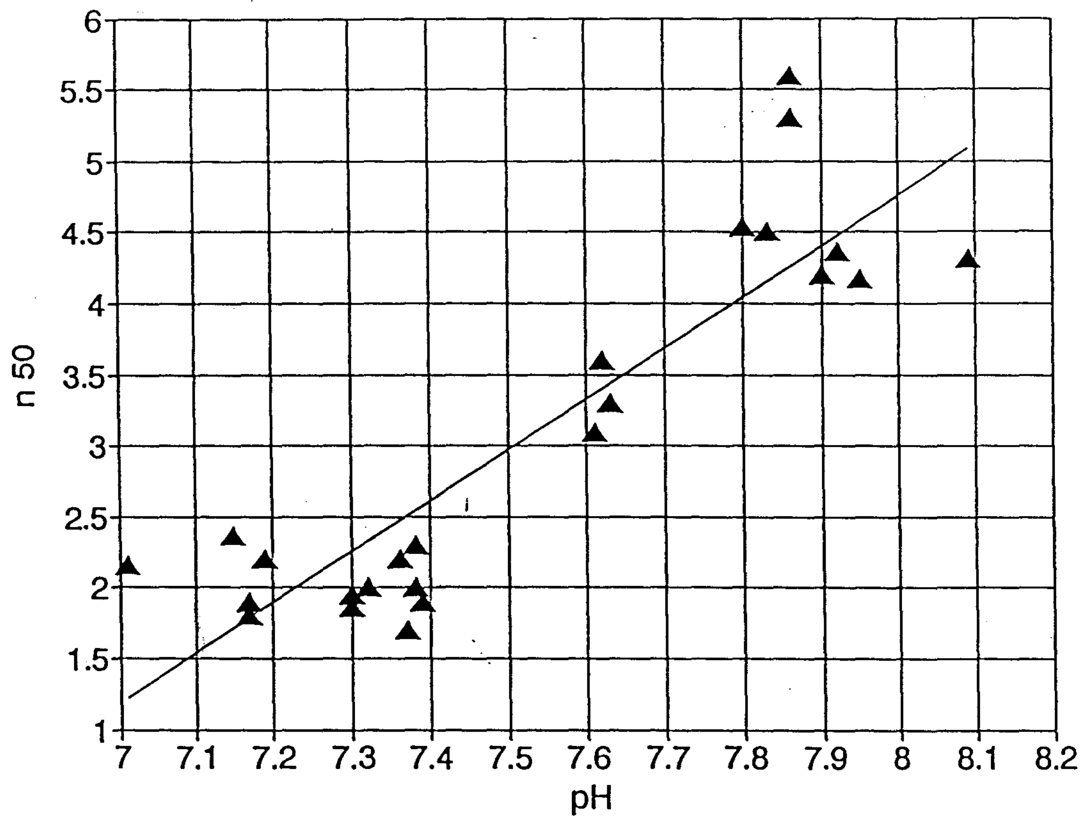

Figure 16. Effect of pH on the cooperativity of purified hemocyanin from Non-parasitized *P. samuelis*. Regression indicates line of best fit (r^2=0.80) through original data points (n_{50}) from oxygen equilibrium experiments.

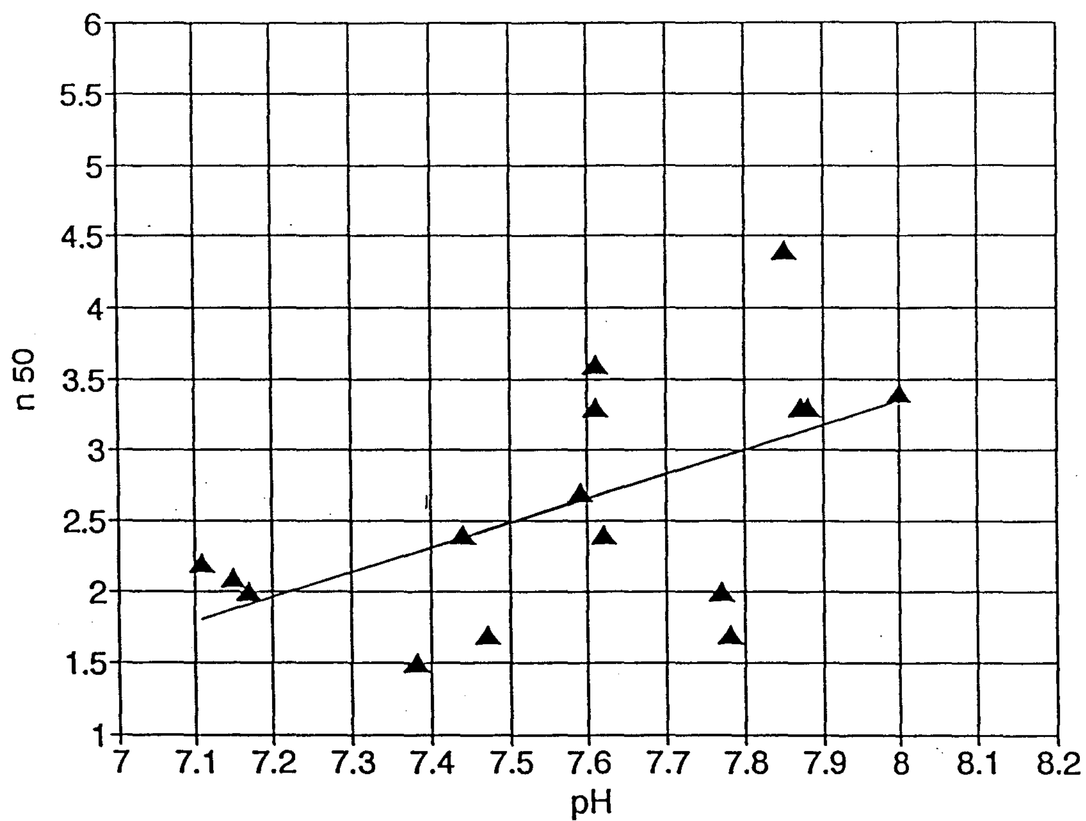

Figure 17. Effect of pH on the cooperativity of purified hemocyanin from Parasitized (Pp) *P. samuelis*. Regression indicates line of best fit (r^2=0.33) through original data points (n_{50}) from oxygen equilibrium experiments.

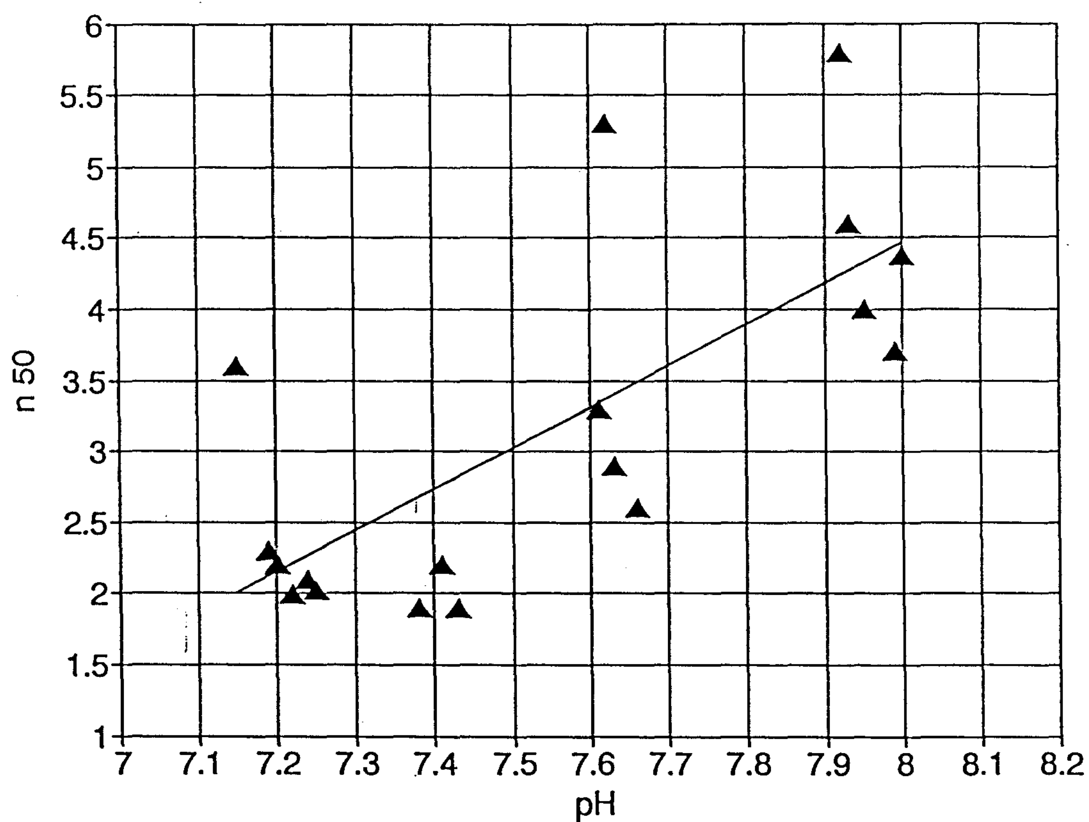

Figure 18. Effect of pH on the cooperativity of purified hemocyanin from Parasitized (Pg) *P. samuelis*. Regression indicates line of best fit (r^2=0.59) through original data points (n_{50}) from oxygen equilibrium experiments.

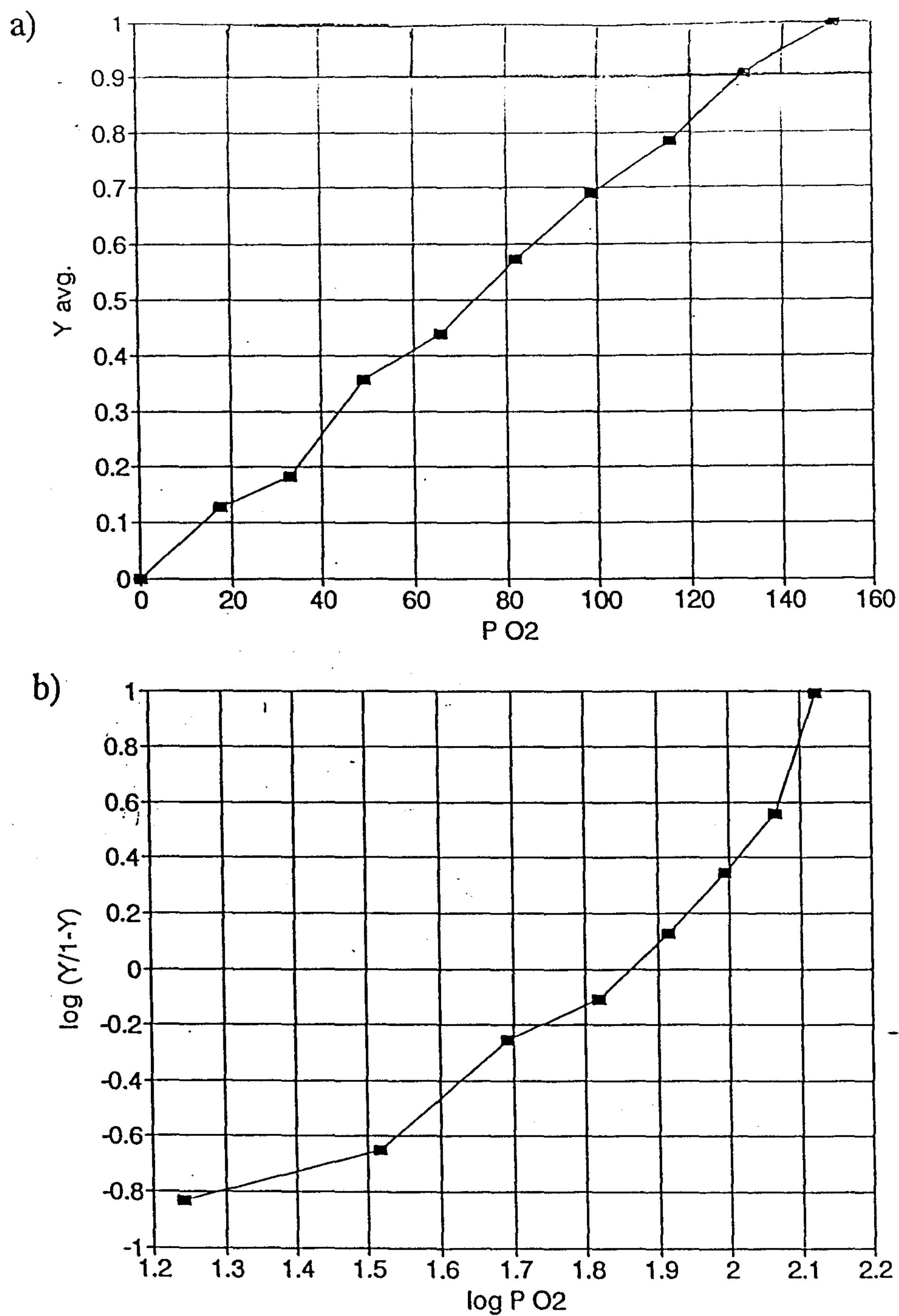

Figure 19. a) Example of an oxygen binding curve of purified hemocyanin from Parasitized (Pg) *P. samuelis* at pH 7.2. Y = percent oxygen saturation of hemocyanin.
b) Hill plot of the above binding curve.

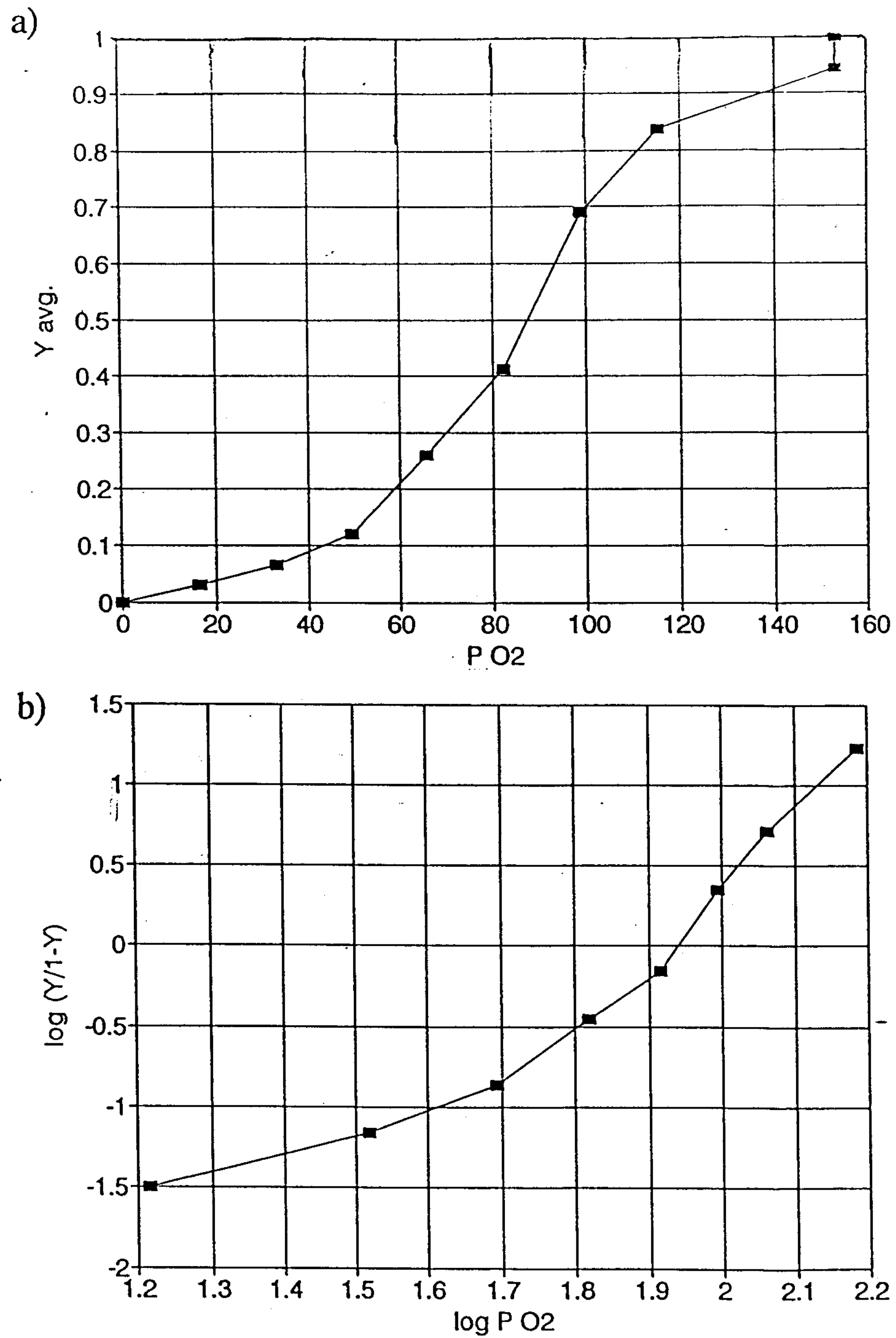

Figure 20. a) Example of an oxygen binding curve of purified hemocyanin from Parasitized (Pg) *P. samuelis*. Same sample as in Figure 16 but at pH 8.0. b) Hill plot of the above binding curve.

statistically homogeneous for all treatment groups, suggesting that the change in n_{50} versus pH is the same. However, the elevation of parasitized (Pp) regression line is significantly ($p \leq 0.05$) less than that of the other two treatment groups. This implies that the cooperativity of parasitized (Pp) for any given pH between 7 and 8 is lower than that of non-parasitized and parasitized (Pg) groups.

The statistical validity of group comparisons for Bohr coefficients and cooperativities may not be adequate since some hemocyanin samples were reused once and data points represented pooled samples. The samples were statistically analyzed as independent samples even though the hemocyanin molecules used were the same. However, their milieu was changed corresponding to the change in pH.

Barnacle Hemoglobin Studies

Spectra of hemolymph from both *Peltogaster paguri* and *Peltogasterella gracilis* reveal peak absorbance maxima at 415, 540 and 580 nm (Figures 21 and 22), the typical absorption maxima of hemoglobin. These results suggest that hemoglobin may be present. Electron micrographs, negatively stained with 1% uranyl acetate, of *P. paguri* hemolymph (Figure 23) exhibit molecules similar to those of *Briarosaccus callosus* hemoglobin (Terwilliger, N., 1994, personal communication). Electron micrographs of *P. gracilis* hemolymph are also shown (Figure 24).

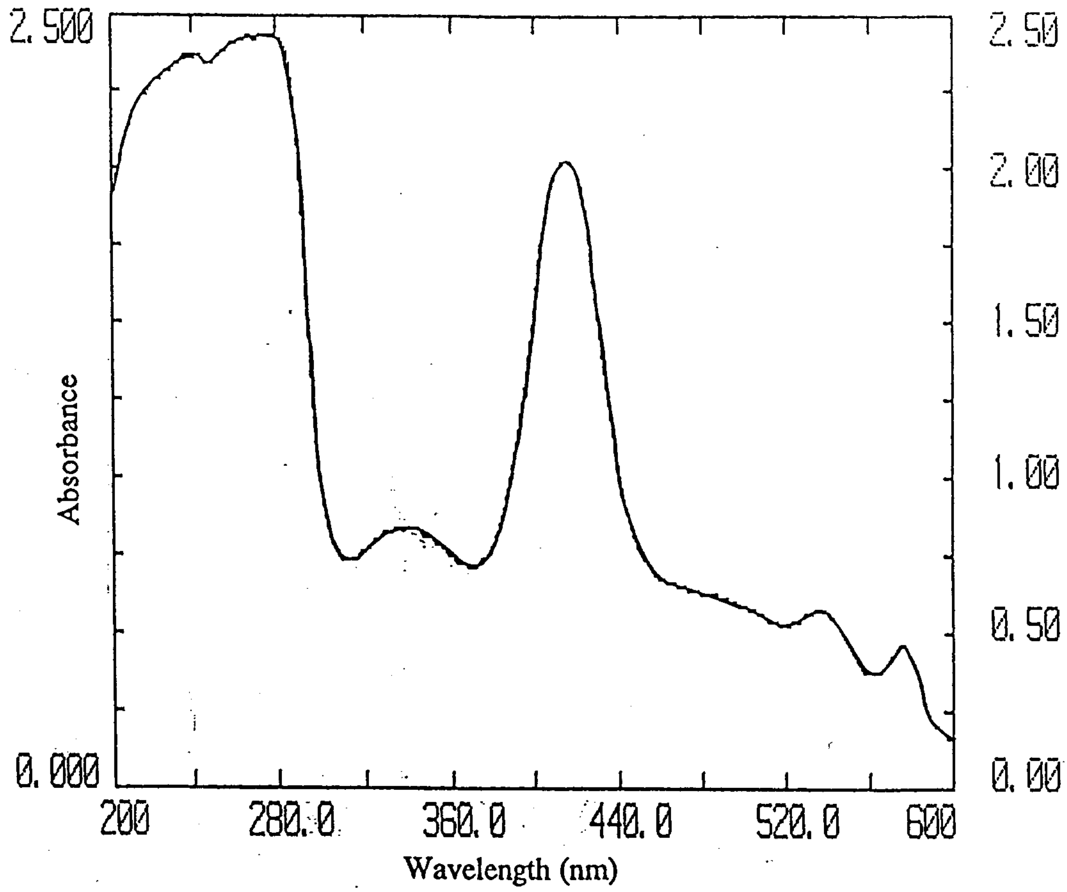

Figure 21. Spectrum of *Peltogaster paguri* crude hemolymph taken from the major blood sinus in the externa.

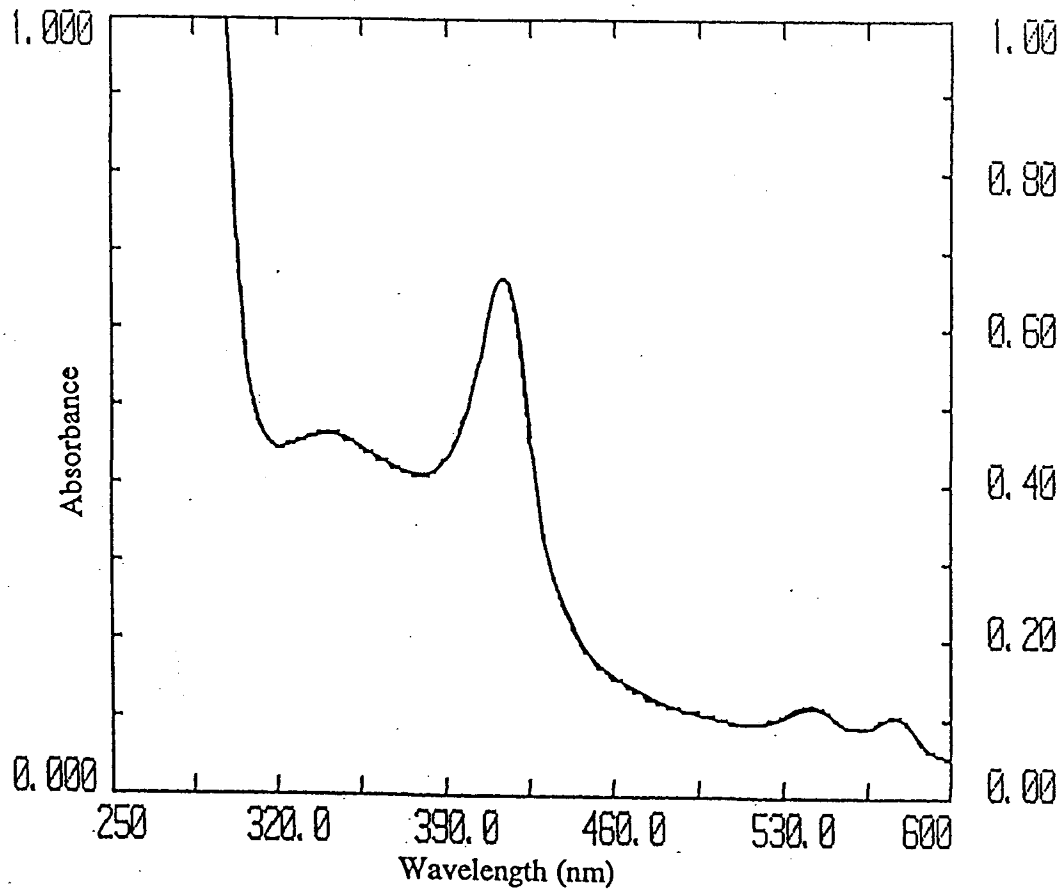

Figure 22. Spectrum of *Peltogasterella gracilis* externa homogenate.

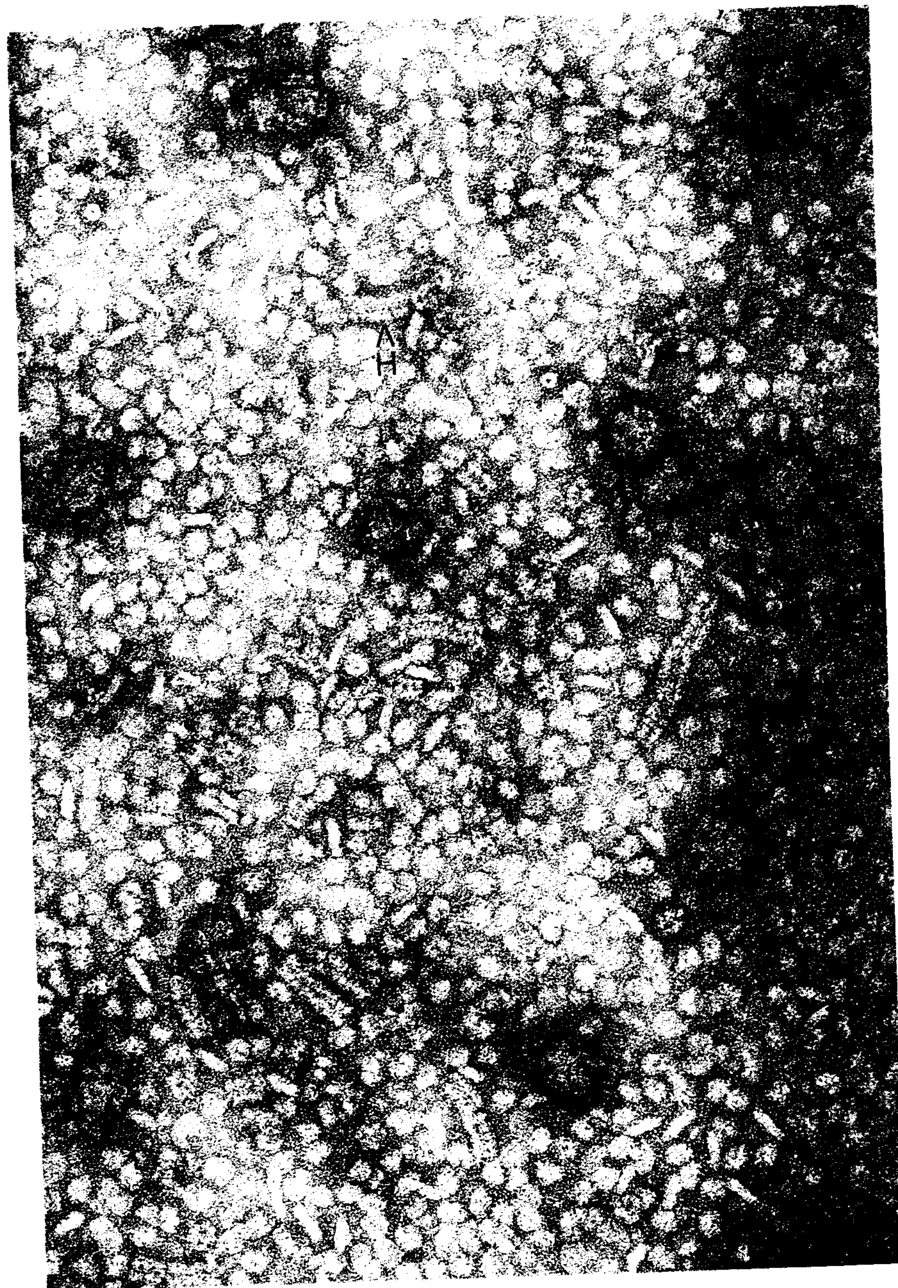

Figure 23. Electron micrograph of *Peltogaster paguri* crude hemolymph at 250,000x mag. H, Hemoglobin. Electron microscopy by Eric Schabtach.

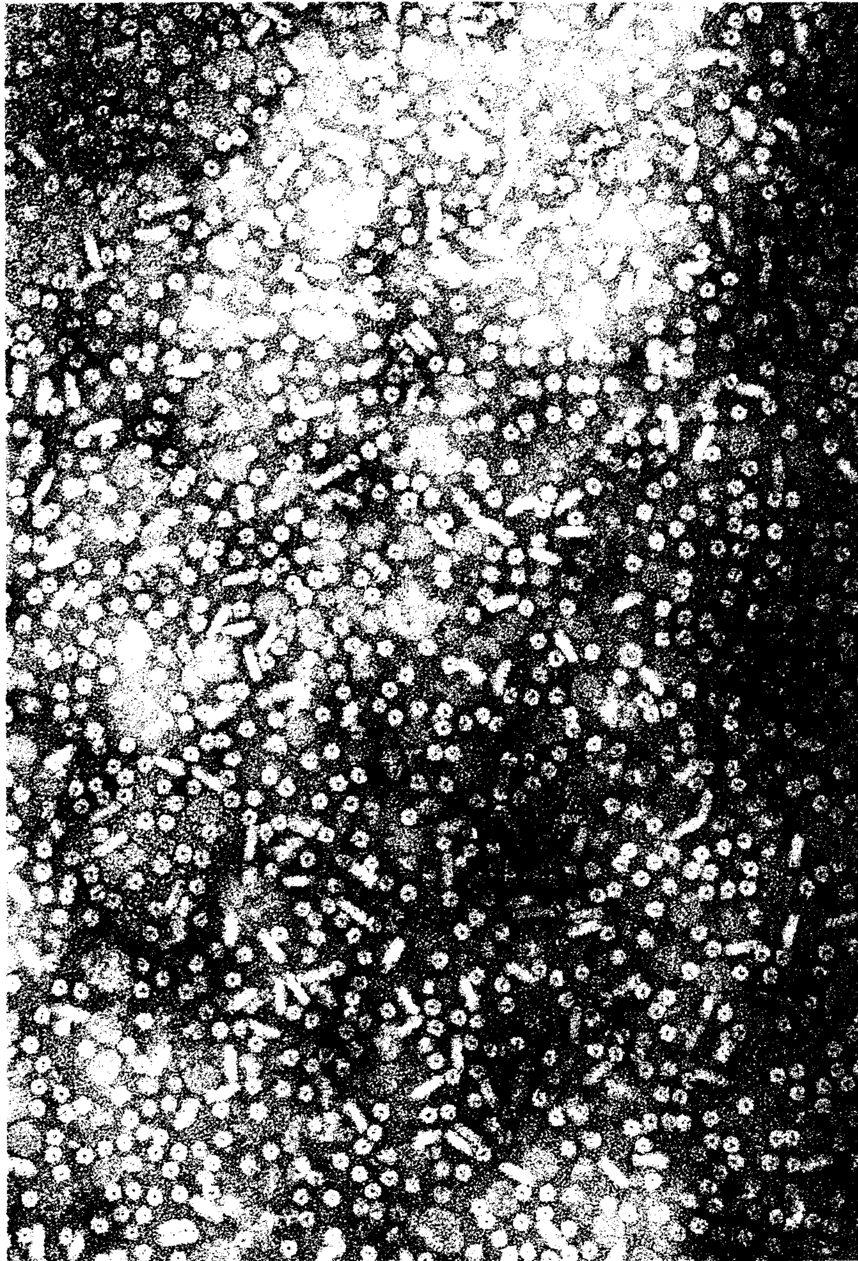

Figure 24. Electron micrograph of *Peltogasterella gracilis* externa homogenate at 250,000x magnification. Electron microscopy by Eric Schabtach.

CHAPTER IV

Discussion

Structural Studies

The two distinct peaks characteristic of chromatographed *P. samuelis* hemolymph indicate that the hemocyanin consists of 25S (940,000 dal) and 16S (450,000 dal) molecules. This is typical of anomuran and brachyuran crustacean hemocyanins. The fact that the hemocyanins of all three treatment groups, ie., non-parasitized, parasitized (Pp) and parasitized (Pg) showed similar patterns when chromatographed and that the ratio of hemocyanin versus total protein in the hemolymph was the same for all treatment groups suggests two points. First, there are no major differences in the proportion of 16S and 25S molecules between parasitized and non-parasitized hemocyanin samples. Second, the proportion of hemocyanin versus total protein is similar among the three groups.

Both parasitized and non-parasitized *P. samuelis* individuals showed a calcium ion activity of 10 mM. This might be expected based on the composition of seawater (Smith, 1974) and when compared to *Pagurus pollicaris* (Rhodes, 1983). However, unexpectedly, *P. samuelis* hemocyanin shows an extreme calcium requirement for

hexameric association into dodecamer when electrophoretically analyzed. Only a few examples of this phenomenon are available in the literature including; Morimoto and Kegeles, 1971; Terwilliger, 1982; Rhodes, 1983. When a mixture of 25S and 16S *P. samuelis* hemocyanin molecules are electrophoresed in the absence of calcium, only one clearly distinguishable band is present. This implies that nearly all 25S dodecamers have disassociated into 16S hexameric molecules. In the presence of calcium, two bands are apparent with a major band corresponding to the 25S (dodecameric) molecule. This suggests that the association of hexameric molecules to dodecamer involves some type of non-covalent interaction involving calcium ions. Electrophoretic evidence (PAGE, pH 7.4) supports chromatographic evidence for the presence of both 16S and 25S hemocyanin molecules with apparent molecular weights of 450,000 and 940,000 respectively. Analyses by pH 7.4 PAGE also show that the band patterns of native hemocyanin molecules among treatment groups are the same. This suggests that the major protein molecules in the hemolymph are 16S and 25S hemocyanin molecules in all three treatment groups.

The question of whether there are separate populations of hexameric molecules, or whether an *in vivo* equilibrium exists between hexamer and dodecamer deserves further investigation. Ultracentrifugation studies, combined with

time-series electron microscopy where calcium and EDTA levels are manipulated would be useful in understanding the extent of this calcium sensitivity.

While both dodecamers and hexamers appear similar in these groups, the hemocyanin subunit compositions of parasitized versus non-parasitized individuals have different patterns when analyzed by SDS-PAGE. In 79 percent of the non-parasitized individuals, a six band pattern indicative of six different monomeric subunits was observed. All parasitized individuals and 21 percent of the non-parasitized individuals showed a three band pattern suggesting that three of the six subunits in non-parasitized hemolymph are present in parasitized hemolymph. If the differences in band patterns between non-parasitized individuals were due solely to chance variation in the population, the different band patterns, ie., six and three band patterns, should be seen in equal proportions. Also, if it were due to variation among *P. samuelis* populations, both phenotypes should be seen in parasitized individuals as well. Several hypotheses could explain the differences among *P. samuelis* populations. 1) The three band pattern is a typical phenotype of individuals that are susceptible to parasitism by these rhizocephalans. The non-parasitized individuals with a six band SDS-PAGE pattern are not susceptible to infection by the parasite. In all assays, individuals with the six band pattern were always free of

parasites. 2) Parasitism somehow affects the monomeric makeup of the hemocyanin molecule such that three electrophoretically distinguishable bands are present instead of six. This could be tested by infecting crabs previously displaying six electrophoretically apparent bands with the rhizocephalan cyprids, waiting until the barnacle infestation is visible, via externa eruption, and then once again analyzing the hemocyanin to determine band patterns. This has not been done, however, due to the difficulty in rearing the barnacle larvae, as well as the fact that it takes several months to two years for sexual maturation, ie., externa eruption of the rhizocephalan. An alternate hypothesis to explain the occasional three band pattern on SDS-PAGE of non-parasitized individuals may be that some of the individuals tested may have been in an early stage of parasitism which was not yet physically detectable (see Chapter III). One way to test this would have been to maintain those 21 percent of non-parasitized individuals whose hemocyanin showed a three band SDS-PAGE pattern and determine whether an externa eventually appeared.

Functional Studies

The spectra of hemocyanin samples from the three treatment groups of *P. samuelis* are indistinguishable. The peak absorbance maxima (337 nm) are characteristic of decapod crustacean hemocyanins. Thus, it is reasonable to

conclude that the active sites in the hemocyanin molecules do not differ.

The most unique functional feature of *P. samuelis* hemocyanin concerns its pH sensitivity and affinity for oxygen. All treatment groups showed an apparent reverse Bohr shift, in which a decrease in pH facilitates an increase in oxygen affinity. Although relatively small, all Bohr coefficients ($\phi = \Delta \log P_{50} \div \Delta pH$) were positive ($\phi$ non-parasitized = 0.074, ϕ parasitized (Pp)= 0.18 and ϕ parasitized (Pg) = 0.11).

There are few examples of such reverse Bohr shifts in the hemocyanin literature. The only example of a reverse Bohr shift in the Arthropoda occurs in the Chelicerata. In *Limulus polyphemus* hemocyanin, oxygen affinity is high and a large reverse Bohr shift persists throughout the physiological pH range of the organism (Mangum, 1980). In the Mollusca, several examples exist, but the Bohr shifts are usually small and variable in the pH range tested. Some may never be experienced because they are not within the physiological pH range of the organism (Mangum, 1980).

When compared to hemocyanin of another hermit crab, *Pagurus bernhardus* (Jokumsen, A and R.E. Weber, 1982), *P. samuelis* not only exhibits a reverse Bohr shift, which *P. bernhardus* does not, but an extremely low oxygen affinity as well. At physiological pH, pH= 7.8 ± 0.2 (personal observation) and 10°C, the half saturation oxygen tensions

(P_{50}) were approximately 87 mm Hg for non-parasitized, 100 mm Hg for Parasitized (Pp) and 94 mm Hg for parasitized (Pg) hemocyanins. In comparison, the P_{50} for *P. bernhardus* hemocyanin, at physiological pH (7.8 and at 5°, 10°, 15° and 25°C), was 48 mm Hg (Jokumsen, A and R.E. Weber, 1982). The low oxygen affinities were observed in all three *P. samuelis* treatment groups with no significant difference ($p \leq 0.05$) in Bohr coefficients between the groups.

If the apparent reverse Bohr shift in *P. samuelis* hemocyanin holds true, it may be the first incidence recorded among decapod crustaceans. *P. samuelis* is a hermit crab which frequently undergoes air emersion. It sometimes inhabits tide pools in the high intertidal which, in summer months, may become relatively warm and possibly contain little dissolved oxygen. Under these conditions, it is reasonable to assume that the accumulation of carbon dioxide and other acidic metabolites within the hermit crab's hemolymph may lower blood pH. Given these circumstances, a reverse Bohr shift might prove advantageous since as blood pH is reduced, oxygen affinity increases.

A confusing question remains. If there is a true reverse Bohr shift, then why is there an apparent reduction in the oxygen carrying capacity of the hemocyanin at low pH? These two phenomena seem to be contradictory. With a normal Bohr effect, a decrease in pH facilitates a decrease in oxygen affinity. This is consistent with the qualitative

observations made on the oxygen binding curves, which suggest a lower oxygen capacity at low pH. However, in evaluating P_{50}'s a reverse Bohr shift is apparent; the hemocyanin seems to have an increase in affinity at low pH.

One explanation may be that, at low pH, *P. samuelis* hemocyanin may not become fully saturated with oxygen after flushing the tonometer with pure oxygen. If this were the case, a lower apparent P_{50} would result. Comparing the normal 280/340 absorbance, at high pH, to the measured 280/340 value, at low pH, and using that value to extrapolate to a theoretical maximum percent saturation would allow a more accurate calculation of P_{50} (Miller and Mangum, 1988). Correcting partially oxygenated values to 100 percent saturation would result in increased P_{50} values at low pH, perhaps even to the point of demonstrating a normal Bohr effect. Alternatively, injecting measured amounts of pure oxygen instead of air into the tonometer might achieve 100 percent oxygenation levels in the tonometer and probably reach full saturation of the sample. However, the low affinity hemocyanin might require using a pressurized system to reach a P_{O2} greater than one atmosphere. Future investigations should try employing these techniques.

Pagurus samuelis hemocyanin exhibits cooperative oxygen binding which is highly sensitive to pH. At low pH, the hemolymph appears to have low cooperativity. At higher pH

(approximately pH 7.8), within the physiological range of the organism, an increased cooperativity is observed. The slopes of cooperativity versus pH show no significant difference ($p \leq 0.05$) among the three treatment groups, suggesting that a change in pH facilitates a proportionally equivalent change in cooperativity for all three treatment groups. The elevation of the parasitized (Pp) group, however, is significantly lower ($p \leq 0.05$) than the other two treatment groups, suggesting that the hemocyanin of these individuals has a lower overall cooperativity in the pH range tested.

There was a large degree of variation in P_{50} of *P. samuelis* hemocyanin, even within a given treatment group, despite standardization of experimental procedures. The variation may have been a result of pre-experimental history or sex and age differences of the hermit crabs. The variation due to pre-experimental history was presumably reduced by holding the animals in the laboratory for two to three weeks prior to hemolymph collection. Factors such as animal sex or age were not controlled for.

The quaternary structure of *Pagurus samuelis* hemocyanin has a specific requirement for calcium ions for hexameric association into dodecamer. *P. samuelis* appears to have a highly cooperative, low affinity hemocyanin, under the conditions studied. The effect of hydrogen ions on oxygen affinity remains uncertain.

This study suggests that there is an apparent structural difference in subunit composition of the hemocyanin among parasitized and non-parasitized *Pagurus samuelis*. There was no significant difference between the oxygen binding data of parasitized and non-parasitized *P. samuelis* hemocyanin. This suggests that the difference in subunit composition may have little or no effect on the oxygen transport properties studied. Future oxygen binding experiments might reveal differences not seen in this study.

APPENDIX

COLUMN CHROMATOGRAPHY OF PAGURUS SAMUELIS HEMOLYMPH

280/340 ABSORBANCE RATIOS

date	cond.	P I 280	P I 340	PI 280/34	P II 280	P II 340	PII280/34
10/16/92	non	1	0.1975	5.063291	0.69	0.1375	5.018182
12/2/92	non	1.55	0.24	6.458333	0.86	0.15	5.733333
3/2/93	non	1.1	0.15	7.333333	1.6	0.1	16
3/25/93	non	1.8	0.18	10	1.5	0.155	9.677419
4/15/93	non	1.7	0.28	6.071429	1.6	0.24	6.666667
5/6/93	non	1.5	0.18	8.333333	1.5	0.185	8.108108
7/16/93	non	1.1	0.145	7.586207	1.3	0.12	10.83333
7/31/93	non	2	0.37	5.405405	1.4	0.24	5.833333
1/20/93	(Pp) LR	0.74	0.118	6.271186	0.51	0.073	6.986301
1/25/93	(Pp) LR	0.86	0.122	7.04918	0.48	0.056	8.571429
2/4/93	(Pp) LR	1.4	0.135	10.37037	0.75	0.1	7.5
2/15/93	(Pp) LR	1.57	0.195	8.051282	1	0.076	13.15789
4/8/93	(Pp) LR	2	0.25	8	1.05	0.12	8.75
4/22/93	(Pp) LR	2	0.245	8.163265	0.84	0.08	10.5
5/15/93	(Pp) LR	1.8	0.205	8.780488	1.2	0.145	8.275862
11/16/93	(Pg) MY	0.83	0.16	5.1875	0.365	0.07	5.214286
2/22/93	(Pg) MY	2	0.245	8.163265	1.1	0.15	7.333333
4/4/93	(Pg) MY	1.6	0.265	6.037736	1.1	0.16	6.875
4/26/93	(Pg) MY	1.35	0.18	7.5	1.15	0.15	7.666667
5/29/93	(Pg) MY	2	0.33	6.060606	1.15	0.175	6.571429
5/28/93	(Pg) MY	1.6	0.24	6.666667	1.3	0.155	8.387097
6/24/93	(Pg) MY	2	0.33	6.060606	1.1	0.165	6.666667
7/1/93	(Pg) MY	2	0.26	7.692308	1.15	0.115	10
7/31/93	(Pg) MY	2	0.27	7.407407	1.65	0.24	6.875

			P I avg	P II avg
non	P I avg 7.031417	P II avg 8.483797	7.23805	8.216723

	P I avg	P II avg
(Pp) LR	8.097967	9.105927
(Pg) MY	6.752899	7.28772

BIBLIOGRAPHY

Brouwer, M., and B. Serigstad. 1989. Allosteric Control in *Limulus polyphemus* Hemocyanin: Functional Relevance of Interactions between Hexamers. Biochem. 28: 8819-8827.

Benesch R., G. MacDuff, and R.E. Benesch. 1965. Determination of oxygen equilibria with a versitile new tonometer. Analyt. Biochem. 11: 81-87.

Davis, B. 1964. Disc gel electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. 2: 404-408.

Decker H., 1991. Nested allostery of Arthropod hemocyanin. Structure and Function of Invertabrate Oxygen Carriers. Springer-Verlag. New York. pp. 89-98.

Ellerton H.D., D.E. Carpenter, and K.E. van Holde. 1970. Physical studies of hemocyanins. The characterization and subunit structure of the hemocyanin of *Cancer magister*. Biochem. 9: 2225-2232.

Fairbanks, G., T.L. Steck, and D.F.K. Wallach. 1971. Electrophoretic analysis of human erythrocyte membrane. Biochem. 10: 2606-2616.

Fox, H.M. 1953. Hemoglobin and Biliverdin in Parasitic Cirripede Crustacea. Nature 171: 162-163.

van Holde K.E. and Van Bruggen, E.F.J. 1971. Subunits in Biological Systems. Marcel Dekker, New York. pp. 1-53.

van Holde K.E. 1982. Structure and Interactions in Arthropod Hemocyanins. Quart. Rev. Biophys. 15: 9-40.

Jeffrey, P.D., D.C. Shaw, and G.B. Treacy. 1978. Hemocyanin from the Australian freshwater crayfish *Cherax destructor*. Biochem. 17: 3078-3084.

Jokumsen, A., and R.E. Weber. 1982. Hemocyanin-oxygen affinity in hermit crab blood is temperature independent. J. Exp. Zool. 221: 389-394.

Laemmli, H.K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227: 680-685.

Larson, B.A., N.B. Terwilliger, and R.C. Terwilliger. 1981. Subunit Heterogeneity of *Cancer magister* Hemocyanin. Biochem. Biophys. Acta. 667: 249-302.

Mangum, C.P. 1992. Blood and Tissue Oxygen Carriers. Springer-Verlag Berlin Heidleberg.

Mangum, C.P. 1980. Respiratory Function of the Hemocyanins. Amer. Zool. 20: 19-28.

Markl, J. 1986. Evolution and Function of Structurally Diverse Subunits in the Respiratory Protein Hemocyanin from Arthropods. Biol. Bull. 171: 90-115.

Markl, J., H. Decker, W. Stöcker, A. Savel, and B. Linzen. 1981. On the role of dimeric subunits in the quartinary structure of arthropod hemocyanins. Hoppe-Seyler's Z. Physiol. Chem. 362:185-188.

Markl, J., A. Hofer, G. Bauer, A. Markl, B. Kemper, M. Brenzinger, and B. Linzen. 1974. Subunit Heterogeneity in Arthropod Hemocyanins: II. Crustacea. J. Comp. Physiol. 133: 167-175.

Miller, K., and C.P. Mangum. 1988. An Investigation of the Nature of the Bohr, Root, and Haldine effects in *Octopus dolfeni* hemocyanin. J. Comp. Physiology B. 158: 547-522.

Morris R.H., D.P. Abbott, and E.C. Haderlie. 1980. Intertidal Invertebrates of California. Stanford Univ. Press, California, U.S.A. pp. 527.

Morimoto, K., and G. Kegeles. 1971. Subunit Interactions of Lobster Hemocyanin. Arch. Biochem. Biophys. 142: 247-257.

Neilsen, S.O. 1970. The Effects of then Rhizocephalan Parasites *Peltogaster Paguri* (Rathke) and *Gemmosaccus sulcatus* (Lilljeborg) on Five Species of Pagurdian Hosts (Crustacea Decapoda). Sarsia 42: 17-32.

Pilz, I., K.Goral, M. Hoylaerts, R. Witters, and R. Lontie. Studies by small-angle X-ray scattering of the quarternary structure of the 24-S component of the hemocyanin of *Astacus leptodactylus* in solution. Eur. J. Biochem. 105: 539-543.

Rhodes, D.G. 1983. Association Behavior of Pagurus pollicaris Hemocyanin. Ph.D. Thesis, University of Connecticut.

Shirley, S.M., T.C. Shirley, and T.R. Meyers. 1986. Hemolymph Responses of Alaskan King Crabs to Rhizocephalan Parasitism. Can. J. Zool. 64: 1774-1781.

Smith, W.F.G. 1974. Molality of the major constituents in seawater at various salinities. Table 1.2-2. Handbook of Marine Sciences 1: 5 CRC. Press, Cleveland, Ohio.

Terwilliger, N.B. 1982. Effect of subunit composition on quarternary structure of isopod (*Ligia pallasii*) hemocyanin. Biochem. 21: 2579-2586.

Terwilliger, N.B., R.C. Terwilliger, M. Appelstein, C. Bonaventura, and J. Bonaventura. 1979. Subunit structure and oxygen binding by hemocyanin of the isopod *Ligia Exotica*. Biochem. 18: 102-108.

Terwilliger, R.C., N.B. Terwilliger, and E. Schabtach. 1986. Hemoglobin from the parasitic barnacle, *Briarosaccus callosus*. Invertebrate Oxygen Carriers. Ed. Linzen, B. Springer-Verlag. Berlin Heidelberg. pp. 125-127.

Trouchot, J.P. 1992. Respiratory function of Arthropod hemocyanins. Blood and Tissue Oxygen Carriers. Springer- Verlag Berlin Heidleberg. pp. 377-410.

Yanagimachi, R. 1961. Life-cycle of *Peltogasterella* (Cirripedia, Rhizocephala). Crustaceana 2: 183-186.