HEMOGLOBIN FUNCTION IN A BURROWING SEA CUCUMBER, PARACAUDINA CHILENSIS

by

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The oxygen equilibrium characteristics of the water vascular and perivisceral hemoglobins from the burrowing holothurian, Paracaudina chilensis, were determined tonometrically. The oxygen affinity of P. chilensis perivisceral hemoglobin is higher than that of other holothurians studied. The oxygen affinity is concentration and temperature dependent; affinity decreases with increased hemoglobin concentration or temperature. A normal Bohr shift occurs. This is in contrast to other holothurians whose hemoglobins are insensitive to pH. Cooperativity is comparable to that of other holothurian hemoglobins. The oxygen affinities and cooperativities of water vascular and perivisceral hemoglobins of P. chilensis are indistinguishable. The high affinity hemoglobin could be adaptive for an animal that burrows in an oxygen depleted environment.

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INTRODUCTION

Hemoglobin, an iron-containing respiratory protein, generally transports oxygen from relatively high oxygen tensions in the environment to regions of lower oxygen tensions in the metabolizing tissues of an organism. In some instances hemoglobin also participates in oxygen storage. Hemoglobin is the sole respiratory pigment of the vertebrates but only one of several oxygen transport proteins found among the invertebrates. It is more common among invertebrate species that are regularly exposed to low oxygen tension, but its occurrence otherwise reveals no clear phylogenetic trends. Such varied invertebrates as aschelminthes, platyhelminthes, nemerteans, molluscs, annelids, echiurans, arthropods, phoronids and echinoderms have been found to contain hemoglobin. (For review see: Lankester, 1872; Manwell, 1960a; Edsall, 1980; Terwilliger, 1980; Weber, 1980; Terwilliger et al., 1985; Toulmond, 1985; Bunn & Forget, 1986).

Vertebrate and many invertebrate hemoglobins have similar polypeptide chain structures, suggesting a common evolutionary history (Huber et al.,1971; Dayhoff,1972; Padlan & Love, 1974; Royer et al., 1975). Echinoderms, being deuterostomes, are thought to be closely related to the primitive chordates, and probably they or close relatives were ancestors to the vertebrate lineage (Nichols, 1969; Barnes, 1987). Since most schemes of hemoglobin evolution have been based primarily on vertebrate hemoglobin (Ingram, 1961, 1963;

Zuckerkandl & Pauling, 1965; Goodman et al., 1975), studies of invertebrate hemoglobins, especially those of the echinoderms, should clarify the evolution of the hemoglobin molecule.

Although the basic structures of most hemoglobins are similar, the size of the molecule varies remarkably. While most intracellular vertebrate hemoglobins are heterogeneous tetramers of 64,500 daltons in equilibrium with dimers and monomers (Guidotti, 1964; Perutz, 1983), the molecular weights and aggregation states of invertebrate hemoglobins are diverse. Furthermore, they can be intracellular and/or extracellular (Terwilliger, 1980: Toulmond, 1985). Invertebrate intracellular hemoglobin molecular weights vary from 18,000 daltons for myoglobin of the opisthobranch Aplysia depilans (Rossi-Fanelli et al., 1958) to 430,000 daltons in red cells of the bivalve Barbatia reeveana (Grinich & Terwilliger, 1980). Most echinoderm hemoglobins, found mainly in the holothurian orders Dendrochirotida and Molpadiida (Manwell, 1959; Hetzel, 1960), however, are around 36,000 daltons and are dimeric (Table 1), consisting of two polypeptide chains, each with a heme prosthetic group (Terwilliger, 1975; Roberts et al., 1984). Some holothurian hemoglobins are heterogeneous, producing two protein bands when electrophoresed. It is not known whether the subunits within a dimer differ or whether there are two types of dimers, each with two identical subunits. Some holothurian hemoglobins electrophorese as single protein bands but this is not concrete evidence for a single subunit type. Ligand-linked dissociation, which occurs in the hemoglobin of one of the most primitive vertebrates, the lamprey (Briehl, 1963; Andersen, 1971; Li et al., 1972; Dohi et al.,

Table 1. Characteristics of holothurian hemoglobins.

Species	oxy M.W.	deoxy M.W.	subunit M.W.	heterogeneous /homogeneous
Order Dendrochirotida				
(1) Cucumaria curata	34,500	51,000	17,400 18,200	heterogeneous
(2) Cucumaria miniata				
	37,000			homogeneous
	36,000	55,000	18,000	
			17,400 18,400	heterogeneous
(3) Cucumaria piperata	40,000		20,000	homogeneous
(4) Eupentacta quinquesemita	33,000		17,400 17,000	heterogeneous
(5) Thyone briareus	34,000		17,000	homogeneous
(6) Thyonella gemmata	35,000		17,500	
	33,000	32,000	17,500	heterogeneous
Order Molpadiida				
(7) Caudina sp.				
(8) Molpadia arenicola				
				heterogeneous
(9) Molpadia intermedia	37,000			heterogeneous
(10) Molpadia oölitica	40,000			homogeneous
(11) Paracaudina				

	P50 (mm Hg) (°C)	"n"	ΔH	reference
(1)	7.1 (20°)	1.6		Roberts et al., 1984
(2)	3.8 (10°) 8.2 (25°)	1.3	-8.4	Manwell, 1959
				Terwilliger & Read, 1970
	10.0 (20°)	1.8		Terwilliger, 1975
				Roberts et al., 1984
(3)				Terwilliger & Read, 1970
(4)				Roberts et al., 1984
(5)				Roberts et al., 1984
(6)				Parkhurst & Mobley, 1971
		1.4		Steinmeier & Parkhurst, 1979
(7)		2.0		Manwell, 1959
(8)		1.5		Bonaventura & Kitto, 1973
	3.5 (20°)	1.5		Bonaventura et al., 1976
(9)				Terwilliger & Read, 1970
(10)		1.6	-8.8	Terwilliger & Read, 1972
(11)	8.0 (20°)	0.8		Kawamoto, 1928

1973), is a general characteristic of holothurian hemoglobins. Holothurian hemoglobins reversibly polymerize to tetramers and even oligomers upon deoxygenation (Bonaventura & Kitto, 1973; Terwilliger, 1975; Bonaventura et al., 1976; Roberts et al., 1984). At physiological hemoglobin concentration and pH, holothurian hemoglobins are generally dimeric. However, under hypoxic conditions, further aggregation may take place.

Hemoglobin reversibly binds with oxygen. At high oxygen tensions (PO_2) the hemoglobin (Hb) binds oxygen forming oxyhemoglobin (HbO_2 or oxyHb). At low oxygen tensions, oxygen is released. A plot of percent oxygen saturation at increasing oxygen tensions results in an oxygen-hemoglobin dissociation curve. The oxygen tension at which half the protein is oxygenated is referred to as the half-saturation pressure (P50) and is a measure of the affinity of the hemoglobin for oxygen. A low P50 indicates a high oxygen affinity and vice-versa.

Oxygen affinity of vertebrate hemoglobins can be affected by protein concentration, organic allosteric modulators, pH and temperature. Vertebrate hemoglobins show a decrease in oxygen affinity as hemoglobin concentration is increased and, conversely, an increase in affinity when concentration is decreased. It is proposed that the affinity is decreased at high hemoglobin concentrations because the aggregated state, which has raised structural constraints, is favored (Briehl, 1963; Rossi-Fanelli et al.,1964; Andersen, 1971; Dohi et al., 1973). Among holothurians, the aggregation states and therefore molecular weights of Cucumaria miniata and Molpadia arenicola hemoglobins vary with concentration. The hemoglobin aggregates at high

concentrations and dissociates at low concentrations (Bonaventura & Kitto, 1973; Terwilliger, 1975). The molecular weights of M. intermedia and M. oölitica, however, are independent of concentration over the concentration range examined (Terwilliger & Read, 1970, 1972). At this time, the oxygen affinity of only one of these holothurian hemoglobins, that of Cucumaria miniata, is known to vary with concentration. As in vertebrates, the oxygen affinity of this sea cucumber hemoglobin increases upon dilution (Terwilliger, 1975).

Allosteric modulators such as organic phosphates, H^+ ion, CO_2, and inorganic salts may increase or reduce the oxygen affinity of vertebrate hemoglobins. The modulators change the tertiary or quaternary structure and thereby favor the oxy or deoxy state (Weber, 1980; Toulmond, 1985). The organic phosphates, inositol hexaphosphoric acid (IHP), 2,3-diphosphoglyceric acid (DPG) and adenosine 5'-triphosphate (ATP) reduce the affinity of vertebrate hemoglobins by stabilizing the deoxy hemoglobin structure (Perutz, 1970a; Duhm, 1971; Benesch & Benesch, 1974a). Invertebrate hemoglobins, however, are generally insensitive to organic phosphates (Terwilliger & Read, 1971; Weber, 1980).

pH also influences hemoglobin oxygen affinity. Deoxy hemoglobin molecules bind protons, reducing the molecule's oxygen affinity (Bunn & Forget, 1986; Riggs, 1988). When P50 varies inversely with pH a normal Bohr effect is said to occur. Decreased pH shifts the oxygen-hemoglobin dissociation curve to the right and vice versa. The slope of a line on a graph of log P50 vs. pH is called ø, a measure of the Bohr effect. The more negative the ø value, the stronger the Bohr

effect. Hemoglobins with large Bohr effects are generally only present in animals with vascular systems and internal pH gradients (Weber,1980). However, a hemoglobin with a Bohr effect could be advantageous to non-vascular invertebrates if the animal is active or intertidal and therefore encounters temporary situations of decreased oxygen or increased carbon dioxide (Manwell, 1959). For example, a marked Bohr effect occurs in hemoglobins of several intertidal polychaete species (Weber, 1980). No Bohr effect has been found in most holothurian hemoglobins (Manwell, 1959; Manwell, 1964; Terwilliger & Read, 1972; Terwilliger, 1975), although Bonaventura et al. (1976) report some pH dependence of ligand binding for Molpadia arenicola hemoglobin.

Oxygenation of hemoglobin is an exothermic reaction and therefore oxygen affinity varies with temperature; an increase in temperature decreases oxygen affinity (Rossi-Fanelli et al., 1964). A measure of temperature sensitivity is the heat of oxygenation, ΔH. Most vertebrate hemoglobins have a ΔH of about -13 kcal/mol (Rossi-Fanelli et al., 1964). Low values of ΔH reflect molecular adaptations that minimize the effects of temperature on oxygen affinity. These adaptations enable an animal to maintain a constant rate of oxygen transport despite the wide temperature range it may encounter. For example, the intertidal clam, Noetia ponderosa, has a very low ΔH of -2.1 kcal/mole (Freadman & Mangum, 1976).

The oxygenation of hemoglobin initiates structural changes in the molecule, reducing salt-bridge constraints between subunits. Therefore, partially oxygenated molecules are more likely to become

more oxygenated than are deoxy molecules. This enhancement of oxygenation is called cooperativity. Cooperativity aids oxygen transport by facilitating oxygen loading and unloading. A graph of log Y/1-Y (Y = % HbO_2) and log PO_2 is called a Hill Plot. The slope of a straight line drawn tangent to the curve log Y/1-Y is called "n", the Hill coefficient. The Hill coefficient is indicative of the degree of cooperativity. When "n" equals one, there is no cooperativity, and the oxygen-hemoglobin dissociation curve is hyperbolic. When the Hill coefficient is greater than one, the hemoglobin is cooperative, and the oxygen-hemoglobin dissociation curve is sigmoidal. An "n" value less than one suggests the occurrence of negative heme-heme interactions but may be observed when multiple subunits with differing oxygen affinities are present.

Heterogeneous aggregations of subunits are generally required for cooperativity in vertebrate hemoglobins (Perutz, 1970b; Benesch & Benesch, 1974b). Vertebrate hemoglobins are usually composed of two types of subunits, α and β. A human hemoglobin variant, Hb H, is composed of four identical β subunits and does not show cooperativity (Bunn & Forget, 1986). The hemoglobin of the echiuran Urechis caupo is a tetramer made up of homogeneous subunits and also is not cooperative (Mangum et al., 1983; Kolatkar et al., 1988). It is not known if the cooperativity observed in holothurian hemoglobins is due to subunit heterogeneity or ligand-linked dissociation. All but one holothurian species studied to date possess cooperative hemoglobins. The single exception is Kawamoto's report in 1928 of an "n" value of 0.82 for Paracaudina.

Holothurian Morphology and Respiration

Two of the five orders of Holothuroidea possess hemoglobin in nucleated hemocytes. The Dendrochirotida have dendroid tentacles and numerous podia. The Molpadiida possess digitate tentacles, lack podia and have a tapering caudal body region. Both orders have respiratory trees, and their internal anatomy is otherwise similar. Holothurians have three body cavity systems which may contain hemoglobin and be involved in respiration: the perivisceral coelom, the water vascular system and the hemal system. The extensive perivisceral coelom is present between the body wall and the digestive tract, extending from the calcareous ring of the pharynx to the cloacal attachment. It is into this coelom that the arborescent respiratory trees ascend from the cloaca, terminating in thin-walled vesicles (Hyman, 1955; Hetzel, 1960). The cloaca pulsates rhythmically, forcing seawater in and out of the respiratory trees (Brown & Shick, 1979; Hopcroft et al., 1985), as well as serving to circulate the perivisceral fluid in the coelom. The second body cavity, the water vascular system, consists of the ring canal encircling the pharynx, the tentacular canals and ampullae of the buccal podia, the polian vesicle and stone canal, and when podia are present, the radial canals and podial ampullae. The polian vesicle, an elongate sac hanging from the ring canal into the coelom, appears to function as an expansion chamber for the water vascular system. The stone canal is also continuous with the ring canal and terminates in the coelom as a perforated madreporic plate. The third cavity, the hemal system, includes the hemal ring around the pharynx, contractile

sinuses along the digestive tract and a complicated mesh of sinuses in the connective tissue of the gut and mesentaries with which the respiratory trees are in close contact (Kawamoto, 1927; Hyman, 1955; Hetzel, 1960).

Cuénot (1891), Kawamoto (1927) and Hyman (1955) considered these three body cavities in holothurians morphologically continuous, allowing exchange of coelomocytes and fluid, especially between the water vascular system and the perivisceral coelom via the madreporite. However, the existence of holothurian species with hemoglobin in one of these two systems and not the other suggests that the compartments are not continuous, at least with respect to the passage of hemocytes. For example, in Thyone briareus, T. mexicana, Psolus chitonoides and Eupentacta quinquesemita the hemocytes are confined to the water vascular system (Manwell & Baker, 1963; Roberts et al., 1984). In Penamora pulcherrima the hemocytes are present only in the perivisceral coelom (Manwell & Baker, 1963). In those sea cucumbers with hemocytes in more than one system, the hemoglobins may or may not be the same. The water vascular and perivisceral hemoglobins of Molpadia intermedia are electrophoretically indistinguishable (Terwilliger & Terwilliger, in press) while those of Thyonella gemmata differ in electrophoretic pattern in the proportions of two minor hemoglobin components (Manwell & Baker, 1963). The presence of dissimilar hemoglobins in distinct areas of an animal provides the possibility of complementary oxygen affinities and thus an oxygen transfer system (Weber, 1980). Such transfer systems occur in polychaetes (Terwilliger, 1974; Mangum et al., 1975a; Manwell & Baker, 1988a, 1988b) and sipunculans (Manwell,

1960b; Mangum & Burnett, 1987). Oxygen affinities of the hemoglobins from the three compartments in holothurians have not been compared.

The molpadiid sea cucumber, Paracaudina, has abundant hemoglobin in all three body cavities (Kawamoto, 1928). Paracaudina lives buried in sand or mud with the tip of its elongated posterior at the surface for respiratory purposes (Yamanouchi, 1926); at low tide the animal may be deprived of respiratory medium (personal observation). Some studies of the hemoglobin of a species of Paracaudina, referred to as P. chilensis in Japan, have been carried out. Kawamoto (1927) reported that the hemocyte count and cell types of the perivisceral and hemal fluid are different. A P50 of 8 mmHg and an "n" of 0.82 for perivisceral hemoglobin in a lysed cell supernatant were measured but the hemoglobin concentration, pH and buffer conditions were not reported (Kawamoto, 1928). Kobayashi (1932) measured the absorption spectra of the perivisceral and water vascular hemoglobins. For both hemoglobins, the α band is at 580nm and the β band at 544nm. Unlike most hemoglobins, the β band has a higher absorbance than the α band, by 8%. Later, Manwell (1959) found the absorbance of the β band to be only 3% higher than that of the α band. Koizumi (1932) discovered that the ion concentrations in the perivisceral coelomic fluid are the same as those in sea water.

Little more has been reported on Paracaudina hemoglobin structure or function since these early studies. The presence of large quantities of hemoglobin and the attention invertebrate hemoglobins have recently been attracting, together with this animal's unusual habitat make a more detailed study of the respiratory physiology of

Parcaudina compelling. In this paper the following questions will be addressed:

1) How is the oxygen affinity of the perivisceral hemoglobin affected by the concentration of the pigment, the presence of organic phosphates, pH and temperature?
2) What is the cooperativity of the perivisceral hemoglobin? Do negative heme-heme interactions ("n" < 1) occur as reported by Kawamoto (1928)?
3) Do the oxygen affinities of the perivisceral and water vascular hemoglobins suggest that an oxygen transfer system occurs?

MATERIALS AND METHODS

Animal Collection and Blood Preparation

Specimens of Paracaudina (10-40g) were collected from the protected muddy-sand beach of Sunset Bay (lat. 43° 4'; long. 124° 3') near Coos Bay, Oregon. This species is referred to as P. chilensis (Müller) in Pacific coast faunal treatments (Kozloff, 1987), and may or may not be the same species given this name in Japan. Animals were kept until use at the Oregon Institute of Marine Biology in Charleston, in a 40 l tank of sand under running sea water at 11°C (± 3°C) and 31ppt (± 1ppt).

Perivisceral Blood Sample

A longitudinal slit was cut between two muscle bands of the bodywall and the perivisceral fluid collected in an ice-cold dish. Hematocrits (packed cell volume in percent) were performed on the perivisceral blood according to Davidsohn (1962). Plain micro-hematocrit capillary tubes were filled with perivisceral fluid and spun 3 minutes in an International Micro-Capillary Centrifuge (Model MB). Colorimetric (methemoglobin method) determination of total hemoglobin was performed according to Sigma Procedure No. 525. Hematocrit and total hemoglobin results were used to calculate the mean corpuscular hemoglobin concentration (M.C.H.C.) according to Davidsohn (1962).

After removal of aliquots for hematocrit and total hemoglobin determinations, the perivisceral fluid was centrifuged at 121xg for 10 minutes in a Sorvall RC2-B centrifuge (4°C) to pellet the hemocytes. The supernatant was removed and the hemocytes resuspended in a Tris-HCl buffer, pH 7.6, formulated to reflect the concentrations of salts found in sea water. The "sea water" buffer concentrations were as follows: 50mM Mg^{+2}; 10mM Ca^{+2}; 10mM K^{+}; 540mM Cl^{-}; 418mM Na^{+} and 29mM SO_4^{+2}. The cells were washed three times by resuspension and centrifugation and then lysed with a tissue homogenizer in a 1:100 dilution of the "sea-water" buffer. The homogenate was centrifuged at 13,300xg for 10 minutes to remove cell debris. The red supernatant was chromatographed on a Sephadex G-25 column (24.0 X 1.8cm) in equilibrium with "sea water" buffer and dialyzed at 4°C against "sea-water" buffers of the desired pH (pH 7.0 to pH 8.0). The dialysis buffers were changed 3 times, with at least 2 hours between changes. The concentration of the pigment was determined using a millimolar heme extinction coefficient at 578nm of 14.2 (Terwilliger & Read, 1970).

Water Vascular Blood Sample

The single polian vesicle was rinsed of perivisceral blood and carefully removed from the animal onto a piece of Parafilm. The water vascular fluid from the vesicle was centrifuged in an Eppendorf 5415 centifuge at 282xg for 3 minutes to pellet the hemocytes. The cells were washed and lysed in a procedure similar to that followed for the perivisceral cells except that no tissue homogenizer was used to avoid the large percent loss involved in this procedure. The lysed cells

were spun for 3 minutes at 11,898xg in the Eppendorf centrifuge and the supernatant dialyzed as described for the perivisceral fluid. The water vascular hemoglobin was not chromatographed in order to avoid diluting the sample.

Perivisceral Hemoglobin Oxygen Equilibrium Studies

Except when modified as discussed below, the following oxygen binding procedure was followed. Oxygen binding was performed tonometrically as described by Benesch et al. (1965), using two-armed tonometers equipped with 1 cm light path cuvettes (hereafter referred to as 1 cm tonometers). Three ml of hemoglobin sample, diluted to the desired concentration (usually 0.062 mM heme $\pm$ 0.009 SE) with "sea water" buffer of the appropriate pH, were introduced into the tonometer and the vessel sealed. The tonometer was alternately evacuated (using a vacuum pump) and allowed to equilibrate on ice. When gas bubbles ceased to develop during evacuation, equilibration and evacuation proceeded at room temperature. Conversion to deoxyhemoglobin (deoxyHb) was determined to be complete when the spectrum resembled that in Figure 1.

Upon complete deoxygenation the tonometer was placed in a gently shaking water bath of the desired temperature (15, 20 or 25°C $\pm$ 1°C) and allowed to equilibrate. Absorbances (A) were recorded at 545, 565 and 579nm using a Beckman DU70 spectrophotometer. After obtaining the deoxyHb absorbance (Ad), known amounts of air were added to the tonometer with a 2.5 ml glass syringe. Following each addition of air the tonometer was allowed to equilibrate in the water bath for 10

minutes before reading the resulting absorbance. After several air additions, the tonometer was opened, equilibrated and the oxyhemoglobin (oxyHb) absorbance (Ao) obtained. Following oxygen binding, the pH of the sample was determined.

The following equations were used to calculate the partial pressure of oxygen (PO_2) after each air addition and the percent hemoglobin saturation (HbO_2) at each PO_2.

$$\frac{\%HbO_2}{100} = \frac{(Ad - A)}{(Ad - Ao)}$$

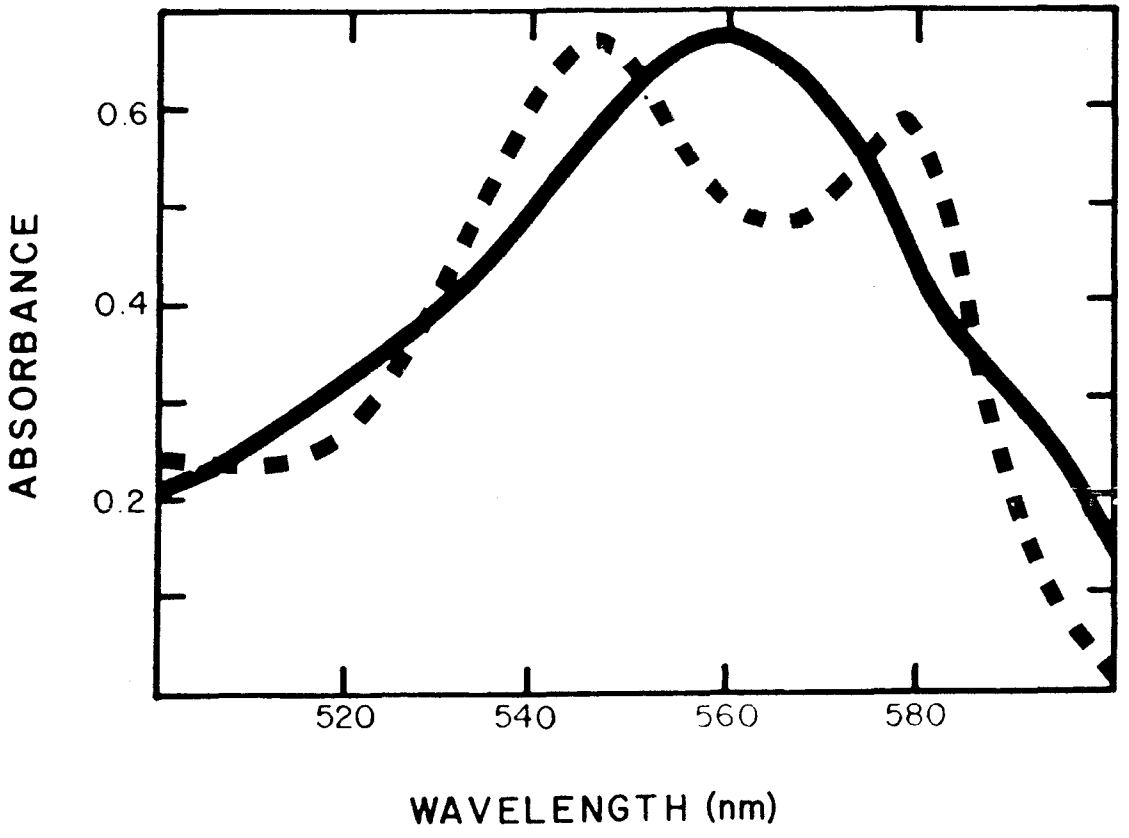
$$PO_2 = \text{ml air injected} \times [0.21 \times (B - H \times P) \times Ti/(V \times To)]$$

Where 0.21 = percent oxygen in air; B = barometric pressure (mm Hg); H = relative humidity; P = vapor pressure at room temperature (mm Hg); Ti = water bath temperature (^{o}K); V = tonometer volume - sample volume (ml) and To = room temperature (^{o}K).

Oxygen Equilibrium at Various Hemoglobin Concentrations

To determine how oxygen affinity of perivisceral hemoglobin is affected by concentration of the pigment, oxygen binding was performed on samples of various mM heme concentrations. When working with dilute samples (0.018mM heme $\pm$ 0.004 SE), conversion to deoxyHb was determined to be complete when the absorbance scan ceased to change shape between evacuations. Absorbances were recorded at 417, 435, 545, 565 and 579nm. For concentrated samples (0.32 mM heme $\pm$ 0.04 SE), one-armed tonometers equipped with 1 mm light path cuvettes requiring only 0.3 ml of sample (hereafter referred to as 1 mm tonometers) were used. When

Figure 1. Example of *Paracaudina chilensis* perivisceral hemoglobin (0.052mM heme) spectrum. Symbols: ▬, deoxyhemoglobin; ■ ■, oxyhemoblobin.


ABSORBANCE
0.6
0.4
0.2
520
540
560
580
WAVELENGTH (nm)

using the 1 mm tonometers, absorbances were recorded as for the dilute samples. Oxygen bindings were performed at 20°C over a pH range of 7.0 to 8.0.

Oxygen Equilibrium and Organic Phosphates

To test for the presence of allosteric modulators within the hemocytes, oxygen binding was performed on the supernatant of cells that were merely lysed and neither chromatographed nor dialyzed. Dilution with "sea water" buffer was required. The characteristics of these bindings were compared to those of bindings using hemoglobin samples that had been chromatographed and dialyzed, essentially stripping them of probable allosteric modulators.

Sensitivity to organic phosphates was tested by performing bindings on samples that included 0.15mM of either adenosine 5'-triphosphate (ATP), disodium salt; 2,3-diphosphoglyceric acid (2,3-DPG), pentasodium salt or inositol hexaphosphoric acid (IHP), dodecasodium salt. Following dialyzation of a hemoglobin sample in pH 7.6 "sea-water" buffer, either ATP or 2,3-DPG in "sea-water" buffer was added to the sample, giving final concentrations of 0.15mM ATP or 2,3-DPG. When IHP was to be tested, the hemoglobin sample was first dialyzed against a pH 7.6 Tris-HCl buffer containing 550mM Cl^- and 500mM Na^+ since addition of IHP to "sea-water" buffer resulted in precipitation. IHP, in Tris-HCl buffer, was then added to the hemoglobin sample to give a final concentration of 0.15mM IHP. The control for the IHP experiment was treated in the same way without IHP added. These oxygen bindings were performed at 20°C in 1 cm tonometers

on samples containing 0.064mM heme (± 0.009 SE) and having initial pH of 7.6.

Water Vascular Hemoglobin Oxygen Equilibrium Studies

Oxygen bindings of water vascular hemoglobin were performed at 20°C in 1 mm light path tonometers. Samples had a hemoglobin concentration of 0.058mM heme (± 0.010 SE) and an initial pH of 7.6.

Analysis of Results

The data for temperature and concentration were described by regression lines (log P50 and "n" versus pH). Student-t test was used to determine if the slopes of these lines were significant at $\alpha = 0.1$. Concentration sensitivity of oxygen affinity and cooperativity was described at three pHs in graphs of log P50 and "n" versus the log of the heme concentration. Similarly, temperature sensitivity was described at three pH in plots of log P50 and "n" versus 1/T. The data points used in these two graphs were obtained from the regression lines of the corresponding plots of log P50 or "n" versus pH. This allowed determination of temperature and concentration effects at constant pH. Log P50 and "n" data appearing in tables of water vascular hemoglobin characteristics and temperature and concentration effects are also for a constant pH.

The data points describing the effects of organic phosphates on oxygen binding characteristics were obtained by plotting "raw" data points on graphs of log P50 and "n" versus pH. Then lines having slopes the same as those found for the stripped control were drawn

through the points. It was then determined what P50 and "n" would be at pH 7.6. This allowed description of the effects of organic phosphates at a constant pH and allowed determination of standard errors.

RESULTS

Spectra

Spectra of perivisceral and water vascular hemoglobins of Paracaudina chilensis are identical. Oxyhemoglobin has absorbance maxima at 417nm (soret), 579nm (α band) and 545nm (β band). The β band absorbance is 9% higher than that of the α band. The minimum absorbance between the α and β bands is at 565nm. Deoxyhemoglobin has absorbance maxima at 435nm (Soret) and 558nm (α-β band).

Perivisceral Hematocrit, Hemoglobin Concentration, and M.C.H.C.

Hematocrits ranged from 0.6% to 3.5% with a mean (n = 14) of 1.5%. The hemoblogin concentration of the perivisceral fluid ranged from 0.04g hemoblogin/dL to 0.74g hemoglobin/dL with a mean (n = 14) of 0.32g hemoglobin/dL. The mean corpuscular hemoglobin concentration (M.C.H.C.) ranged from 4.5g/dL to 34.0g/dL with a mean (n = 14) of 20.1g/dL.

Perivisceral Hemoglobin Oxygen Equilibrium

Concentration Effects

The perivisceral hemoglobin of Paracaudina chilensis reversibly binds oxygen. At 20°C, dilute hemoglobin has a very high oxygen affinity, while samples of high hemoglobin concentration have a lower

oxygen affinity. The oxygen affinity of P. chilensis hemoglobin shows a normal Bohr shift; between pH 6.9 and pH 8.1, oxygen affinity increases as pH is increased. The Bohr shift is most significant for the highest hemoglobin concentration examined (0.32mM heme ± 0.04 SE), especially below pH 7.6 (Table 2; Figures 2a, 3a).

Cooperativity is exhibited by perivisceral hemoglobin. Samples of high hemoglobin concentration show more cooperativity in binding than do samples of low hemoglobin concentration. In dilute hemoglobin samples, cooperativity is insensitive to pH. However, cooperativity may be sensitive to pH at some hemoglobin concentrations. For instance, the cooperativity of samples of 0.062mM heme concentration increases slightly with increased pH while cooperativity of 0.32mM heme concentration samples decreases slightly with pH. In all hemoglobin samples studied, "n" values were greater than one (Table 2; Figures 2b, 3b).

Temperature Effects

Temperature affects the oxygen binding characteristics of perivisceral hemoglobin. For hemoglobin samples of 0.058mM heme (± 0.007 SE) concentration, oxygen affinity decreases as temperature is increased. The heat of oxygenation, ΔH, is -11.2 kcal/mole. A significant Bohr shift occurs at 25°C, the highest temperature examined (Table 3; Figures 4a, 5a).

At the three temperatures examined, cooperativity increases slightly with pH. Cooperativity is generally insensitive to temperature between 15° and 25°C but may increase slightly under conditions

Table 2. Oxygen equilibrium characteristics of perivisceral hemoglobin at three heme concentrations. *

hemoglobin concentration (mM heme)	# of trials	P50 (mmHg)	"n"	ø
0.018 (± 0.004 SE)	7	0.97	1.26	-0.19
0.062 (± 0.009 SE)	12	1.99	1.67	-0.07
0.32 (± 0.04 SE)	8	2.96	1.96	-0.48 **

* 20°C, pH 7.6.

** Significant at $\alpha = 0.1$ using Student-t test.

Figure 2. A. The effect of perivisceral hemoglobin concentration on oxygen affinity (log P50) as a function of pH at 20°C.

B. The effect of perivisceral hemoglobin concentration on cooperativity ("n") as a funtion of pH at 20°C.

Symbols: ■ , 0.018mM heme (± 0.004 SE); △ , 0.062mM heme (± 0.009 SE); ● , 0.32mM heme (± 0.038 SE).

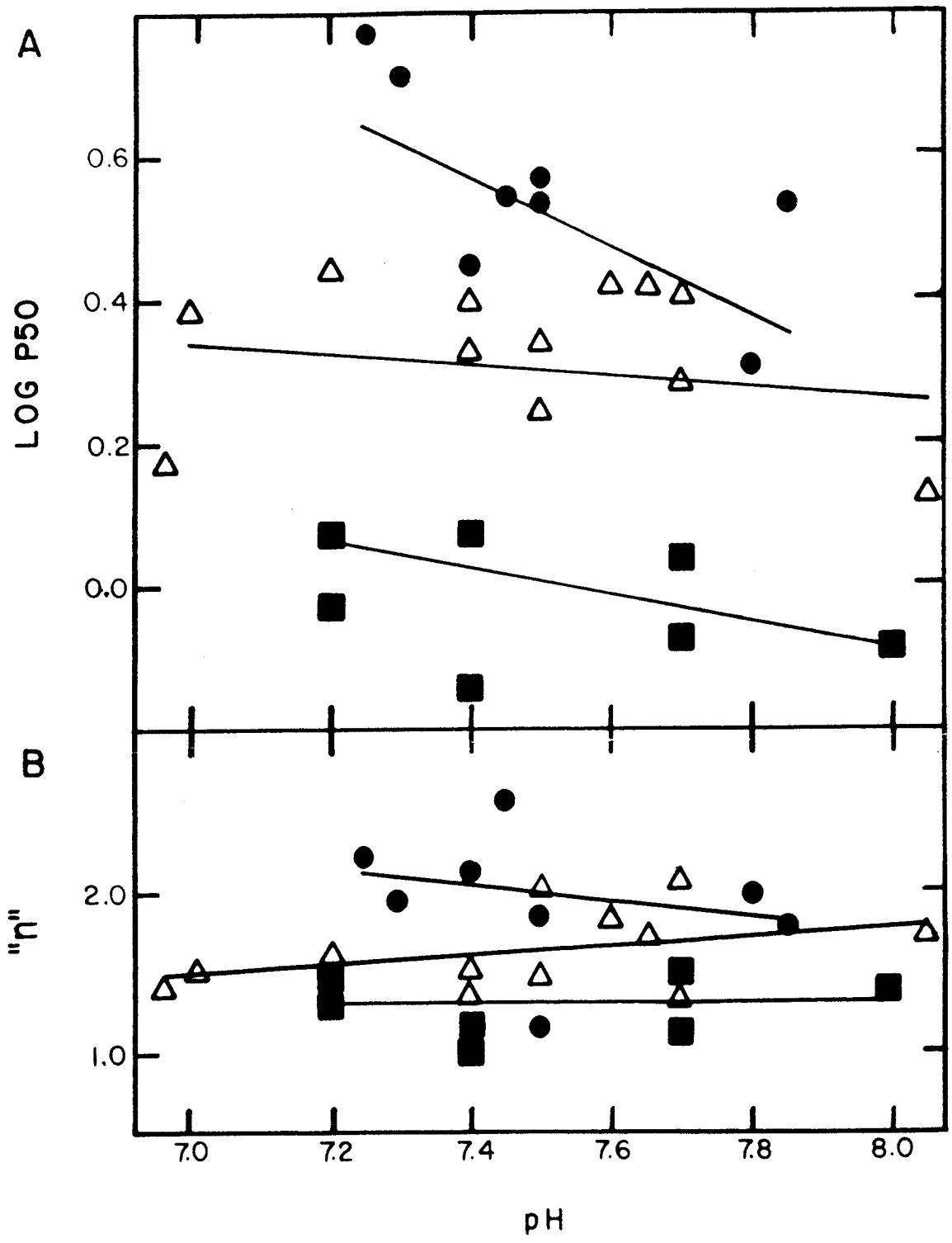

A
B
LOG P50
"n"
0.6
0.4
0.2
0.0
2.0
1.0
7.0
7.2
7.4
7.6
7.8
8.0
pH

Figure 3. A. The effect of perivisceral hemoglobin concentration on oxygen affinity (log P50).

B. The effect of perivisceral hemoglobin concentration on cooperativity ("n"). Oxygen binding was performed at 20°C.

Symbols: ■, pH 7.2; Δ, pH 7.6; ●, pH 8.0.

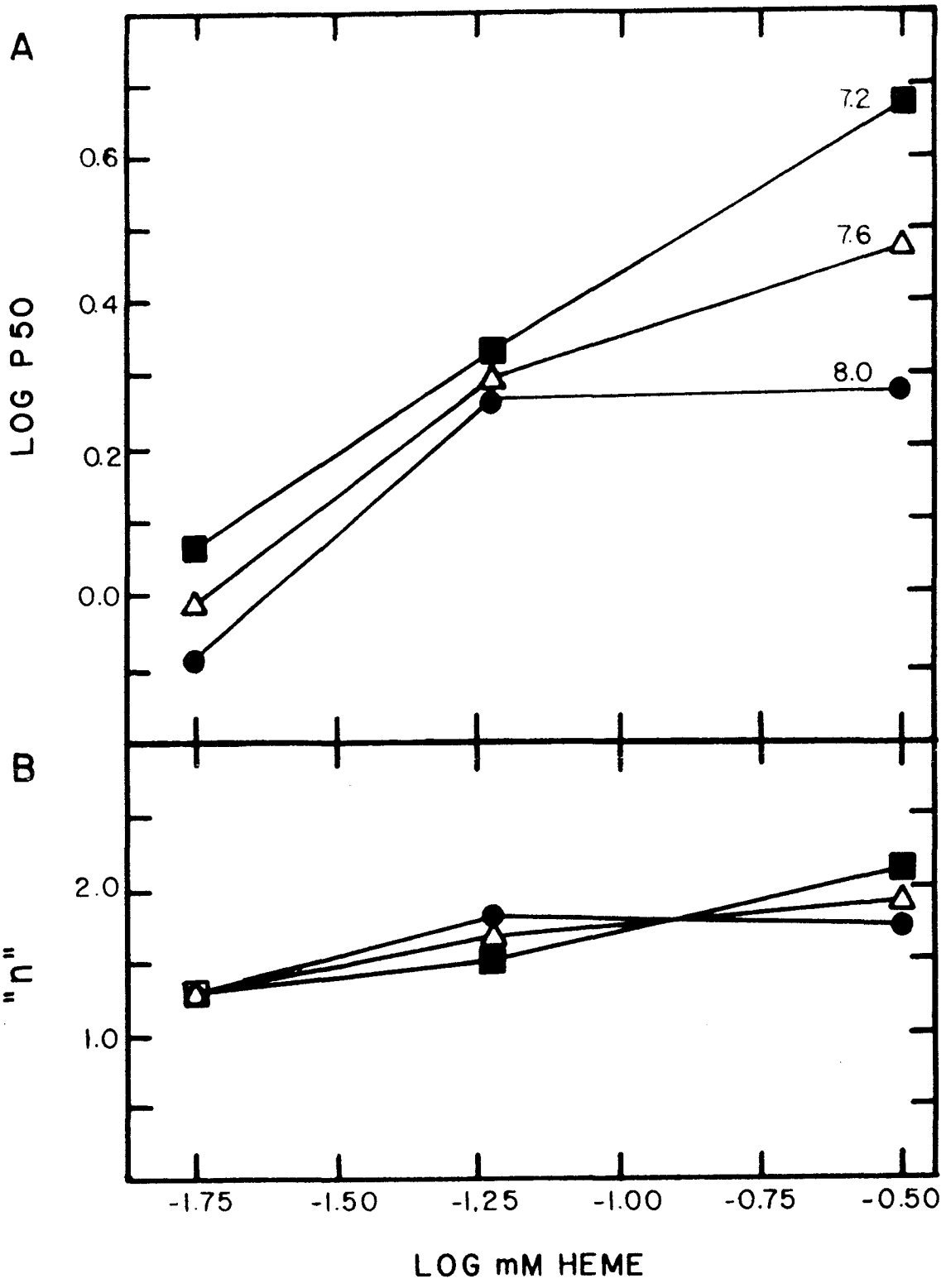

A
B
LOG P50
"n"
0.6
0.4
0.2
0.0
2.0
1.0
7.2
7.6
8.0
-1.75
-1.50
-1.25
-1.00
-0.75
-0.50
LOG mM HEME

Table 3. Oxygen equilibrium characteristics of perivisceral hemoglobin at three temperatures. *

temperature	# of trials	P50 (mmHg)	"n"	ø
15°C	7	1.39	1.74	-0.08
20°C	12	1.99	1.67	-0.07
25°C	8	2.72	1.80	-0.16 **

* 0.058mM heme (± 0.007 SE), pH 7.6.

** Significant at $\alpha = 0.1$ using Student-t test.

Figure 4. A. The effect of temperature on perivisceral hemoglobin oxygen affinity (log P50) as a function of pH.

B. The effect of temperature on cooperativity ("n") of perivisceral hemoglobin as a function of pH. Oxygen binding was performed on samples of 0.0058mM heme (± 0.007 SE) concentration.

Symbols: ■, 15 C; △, 20 C; ●, 25 C.

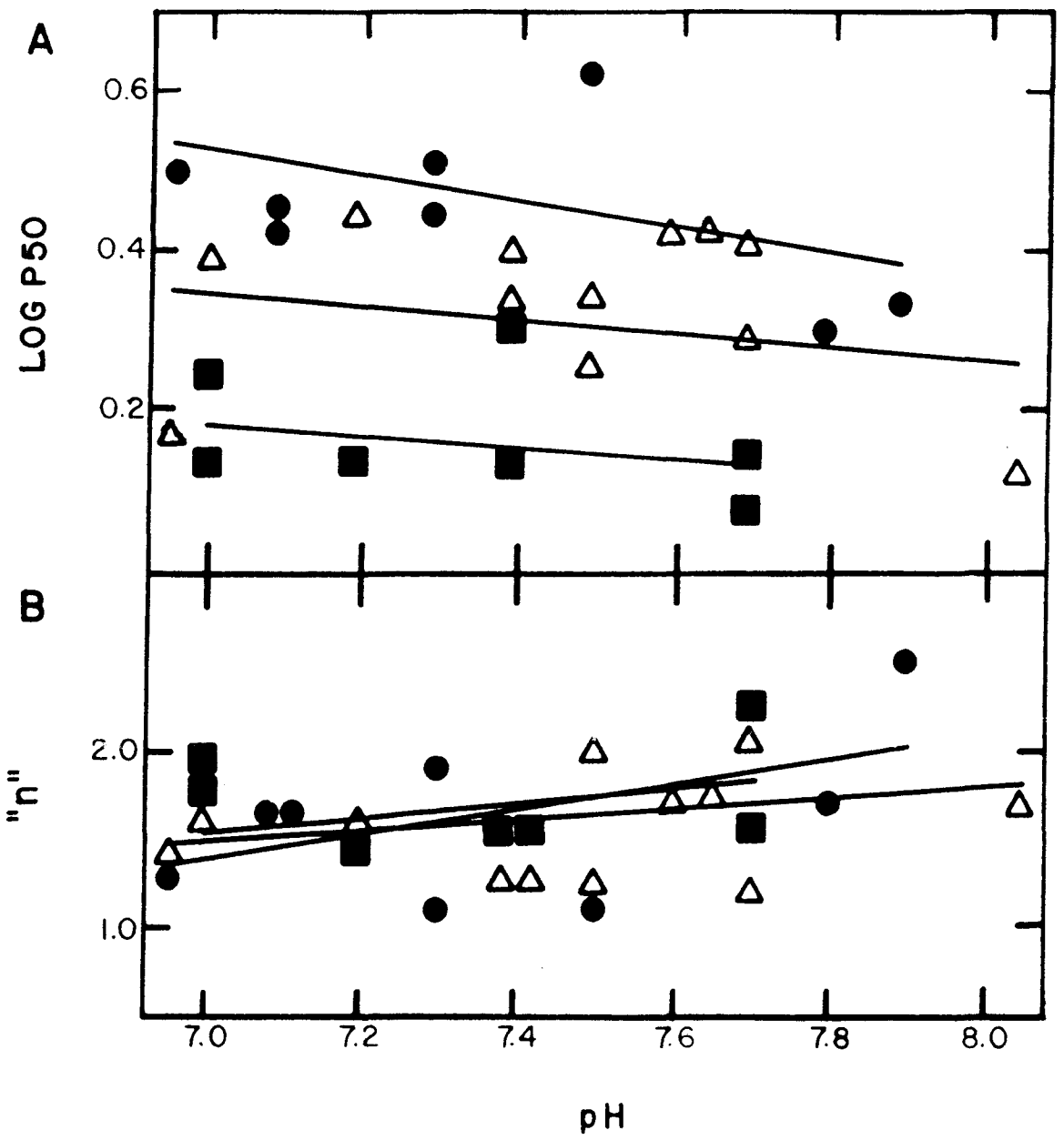
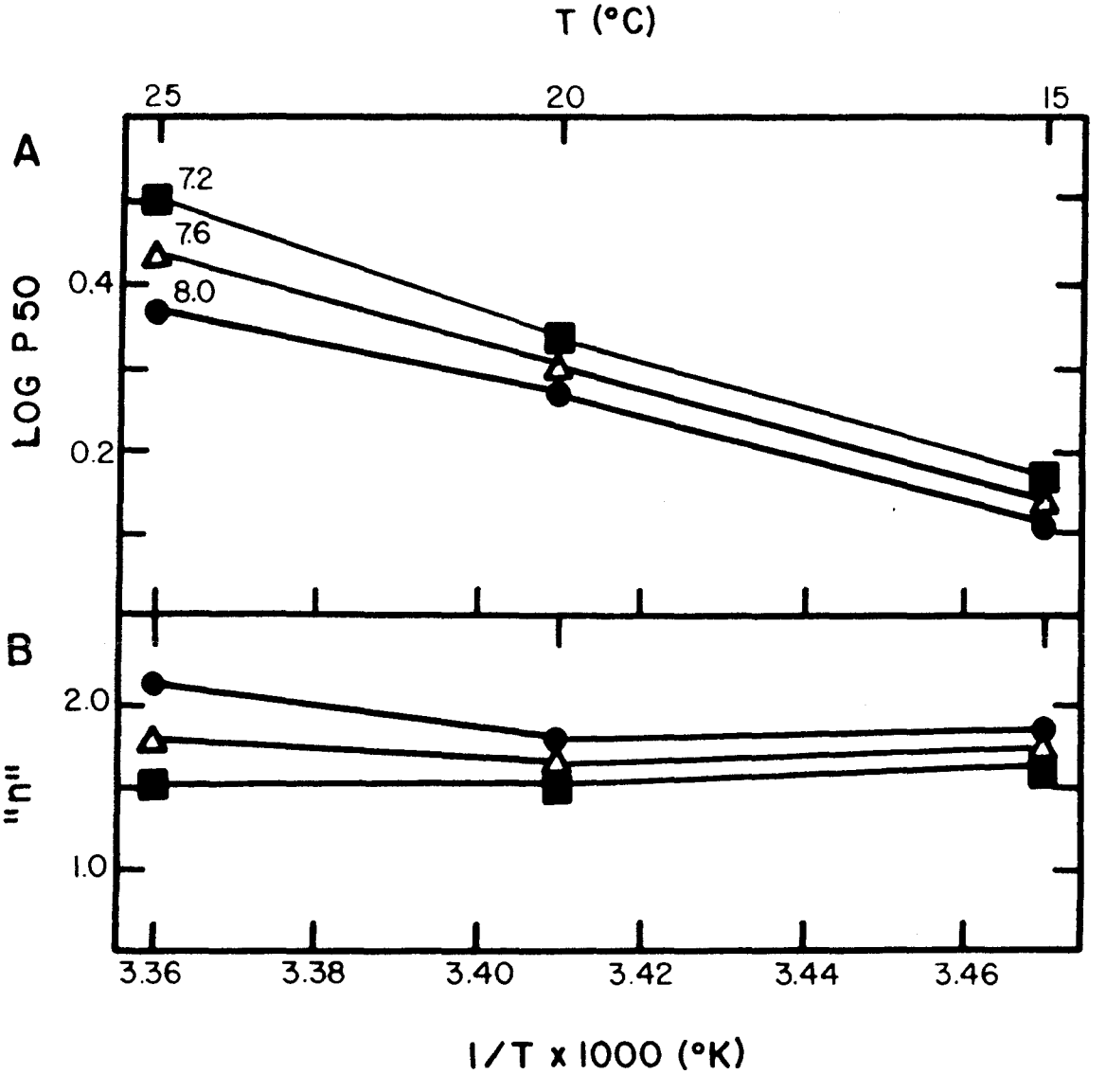

A
B
LOG P50
"n"
0.6
0.4
0.2
2.0
1.0
7.0
7.2
7.4
7.6
7.8
8.0
pH

Figure 5. A. The effect of temperature (T) on the oxygen affinity (log P50) of perivisceral hemoglobin.

B. The effect of temperature (T) on the cooperativity ("n") of perivisceral hemoglobin. Oxygen binding was performed on samples of 0.058mM heme (± 0.007 SE) concentration.

Symbols: ■, pH 7.2; △, pH 7.6; ●, pH 8.0.


T (°C)
25
20
15
A
7.2
7.6
8.0
LOG P50
0.4
0.2
B
"n"
2.0
1.0
3.36
3.38
3.40
3.42
3.44
3.46
1/T x 1000 (°K)

combining low pH and high temperature (Table 3; Figures 4b, 5b).

Effect of Organic Phosphates

The oxygen affinity and cooperativity of hemoglobin samples stripped of possible allosteric modulators by chromatography and dialysis is indistinquishable from that of hemoglobin samples that have not been stripped.

Paracaudina chilensis appears to be insensitive to organic phosphates. Oxygen affinity and cooperativity of hemoglobin samples with ATP or DPG added are nearly the same as those of controls. Likewise, the cooperativity and oxygen affinity of samples including IHP in Tris-HCl buffer are virtually identical to those of control samples in Tris-HCl buffer. Neither is there a significant difference between the control samples in "sea-water" buffer and those in Tris-HCl buffer (Table 4).

Water Vascular Hemoglobin Oxygen Equilibrium

The water vascular hemoglobin also exhibits reversible and cooperative oxygen binding. Oxygen bindings were performed at 20°C with samples of 0.050mM heme (± 0.007 SE) concentration. Under these conditions, water vascular hemoglobin has a P50 of 1.96mmHg and an "n" of 1.72. Both oxygen affinity and cooperativity of water vascular hemoglobin are indistinguishable from those of perivisceral hemoglobin (Table 5).

Table 4. Oxygen equilibrium characteristics of perivisceral hemoglobin with and without organic phosphates. *

sample conditions	# of trials	P50 (mmHg)	"n"
"sea-water" buffer			
stripped (control)	12	2.20 (± 0.47 SE)	1.66 (± 0.20 SE)
unstripped	6	2.18 (± 0.13 SE)	2.09 (± 0.21 SE)
0.15mM ATP	3	2.84 (± 0.62 SE)	1.80 (± 0.08 SE)
0.15mM DPG	2	2.51 (± 0.08 SE)	1.78 (± 0.25 SE)
Tris-HCl buffer			
stripped (control)	2	2.66 (± 0.13 SE)	2.00 (± 0.21 SE)
0.15mM IHP	2	2.92 (± 0.05 SE)	1.75 (± 0.14 SE)

* 20°C, 0.064mM heme (± 0.009 SE), pH 7.6.

Table 5. Comparison of perivisceral and water vascular hemoglobin oxygen equilibrium characteristics. *

hemoglobin	# of trials	P50 (mmHg)	"n"
perivisceral	12	1.99	1.67
water vascular	5	1.96	1.72

* 20°C, 0.058mM heme (± 0.010 SE), pH 7.6.

DISCUSSION

Spectra

The perivisceral and water vascular hemoglobins of the sea cucumber Paracaudina chilensis have indistinguishable spectral characteristics. If there is any difference between the two proteins, it is not in the heme portions of the molecules. The spectra are similar to those of other invertebrate and vertebrate hemoglobins, again indicating that any differences in the proteins are not in the heme portion.

Perivisceral Hematocrit, Hemoglobin Concentration and M.C.H.C.

The hematocrit and hemoglobin concentration of P. chilensis perivisceral fluid vary a great deal. The variability is comparable to that reported for intracellular annelid hemoglobins but average values for P. chilensis hemoglobin are generally lower (Terwilliger et al., 1985). The mean hematocrit of P. chilensis, 1.5%, is about half that found for Cucumaria curata in which the mean hematocrit is 3.2% (Roberts et al., 1984). The M.C.H.C. (mean corpuscular hemoglobin concentration) variation, as well as the mean value, is comparable to that reported for annelids (Terwilliger et al., 1985). No correlation between season or length of captivity and concentration of hemoglobin or cells was noted.

Perivisceral Hemoglobin Oxygen Equilibrium

The oxygen affinity of *Paracaudina chilensis* perivisceral hemoglobin, which ranged from a P50 of 0.96 to 2.96 mmHg depending on hemoglobin concentration and temperature, is higher than that of most other holothurian hemoglobins studied. Under all conditions, "n" was greater than one and comparable to the cooperativity of other holothurian hemoglobins. Kawamoto (1928) reported a P50 of 8 mmHg and an "n" of 0.82 for *Paracaudina* hemoglobin (Table 1), values not in agreement with those found in this study. One explanation for the discrepancy is that Kawamoto, working in Japan, may have been studying a different species which had multiple hemoglobin components of differing oxygen affinities, resulting in an apparent low "n" value. However, this seems unlikely to account for the discrepancy in "n" values. No other holothurian hemoglobin studied has an "n" less than one. Furthermore, molpadid species similar to *P. chilensis* such as *Molpadia arenicola* and *Molpadia oölitica* have "n" values of 1.5 and 1.6 (Terwilliger & Read, 1972; Bonaventura & Kitto, 1973). These cooperativity values are similar to that found for *P. chilensis* in this study. Another explanation for the discrepancies between the results of this study and those of Kawamoto is that there are probably differences in experimental procedure. The buffer and experimental conditions of Kawamoto's study are not known.

The oxygen affinity of intracellular invertebrate hemoglobins is generally high, with P50 values ranging from 1.3 to 11.5 mmHg (Mangum, 1976; Weber, 1980; Mangum et al., 1983), but *Paracaudina chilensis*

hemoglobin represents the high end of these values. Animals with hemoglobins of oxygen affinity comparable to that of P. chilensis hemoglobin live buried in muddy, anoxic substrates that may be exposed at low tide. For example, the terebellid polychaetes Enoplobranchus sanguineus and Amphitrite ornata (Mangum et al., 1975), the capitellid polychaetes Capitella "capitata" and Notomastus latericeus (Wells & Warren, 1975) and the phoronids Phoronopsis viridis (Garlick et al., 1979) and Phoronis mulleri (Weber, 1980) have P50 values ranging from 1.32 to 3.0 mmHg. The high affinity hemoglobin (P50 = 2.5 mmHg) of the polychaete annelid Glycera dibranchiata has been shown to serve as an oxygen reservoir during low tide (Hoffmann & Mangum, 1970; Mangum & Carhart, 1972). The fact that the intracellular hemoglobins of many animals living in intertidal and/or muddy, anoxic substrates have a very high oxygen affinity suggests that the high affinity hemoglobin is adaptive to this type of habitat.

The intertidal, somewhat muddy habitat of P. chilensis is similar to that of other animals having high affinity hemoglobins. The animal is often found 50cm deep with the body surrounded by dark, reduced mud and with the tail extended to the surface. The high affinity hemoglobin of P. chilensis may serve as an oxygen reservoir during low tide when the animal is deprived of aerated sea water, as well as to prevent loss of oxygen by diffusion through the thin body wall to the low oxygen tension muds. A similar function has been proposed for the high affinity hemoglobin of the swampworm Alma emini (Mangum et al., 1975b). Further studies of the metabolic rate and hemoglobin oxygen

carrying capacity are required before an oxygen storage function can be confirmed.

Concentration Effect

The oxygen affinity of Paracaudina chilensis perivisceral hemoglobin is dependent upon the protein concentration; oxygen affinity increases upon dilution. The magnitude of the concentration effect is similar to that reported for Cucumaria miniata (Terwilliger, 1975), Glycera dibranchiata (Mizukami & Vinogradov, 1972), G. robusta (Terwilliger et al., 1976) and lamprey hemoglobins (Briehl, 1963). These hemoglobins exhibit ligand-linked dissociation. In hemoglobins that exhibit ligand-linked dissociation, the dependence of oxygen affinity on hemoglobin concentration is due to the aggregation state of the molecule. In high hemoglobin concentrations, the molecules tend to associate to deoxy aggregates and in low concentrations the molecules dissociate to higher affinity dimers or monomers. Hemoglobin aggregates have lowered oxygen affinities because of structural constraints (Briehl, 1963; Rossi-Fanelli et al., 1964; Andersen, 1971; Dohi et al., 1973). The fact that the oxygen affinity of P. chilensis hemoglobin is concentration dependent suggests that the aggregation state of this protein is concentration dependent but does not neccessarily indicate ligand-linked dissociation.

A normal Bohr shift was found for the hemoglobin of P. chilensis. Only one other sea cucumber hemoglobin, that of Molpadia arenicola, is reported to have pH dependent oxygen affinity (Bonaventura et al., 1976). The pH dependence of P. chilensis hemoglobin oxygen affinity

increases with hemoglobin concentration and is probably significant at the hemoglobin concentration found in the hemocytes.

Temperature Effect

The heat of oxygenation of P. chilensis perivisceral hemoglobin is -11.2 kcal/mol, similar to that found for other holothurian hemoglobins (Table 1). Despite this temperature sensitivity, oxygen affinity remains relatively high even at 25°C (P50 = 2.72). The Bohr shift (ϕ = -0.16) is greater at 25°C than at lower temperatures. During exposure at low tide, a decrease in pH due to accumulation of carbon dioxide, combined with an increase in temperature, might lower the oxygen affinity of the hemoglobin enough to allow release of oxygen from the animal's oxygen reservoir. One would need to know the oxygen tension of the tissue to be certain.

Effect of Organic Phosphates

Like hemoglobins of other holothurian, and invertebrate hemoglobins in general, Paracaudina chilensis hemoglobin appears to be insensitive to organic phosphates. Hemoglobins that are phosphate sensitive, such as human hemoglobins, can show a doubling of oxygen affinity when stripped of these allosteric modulators (Dickerson & Geis, 1983). Therefore, although there are very small differences in oxygen affinity between P. chilensis stripped hemoglobin samples and those with organic phosphates added (Table 4), these differences do not indicate hemoglobin oxygen binding regulated by these phosphates.

The small differences in affinities can be attributed to data scatter and/or a possible non-specific salt effect.

Comparison of Water Vascular and Perivisceral Oxygen Equilibria

Under identical experimental conditions, the oxygen affinities and cooperativities of water vascular and perivisceral hemoglobins of Paracaudina chilensis are indistinguishable. This would suggest that an oxygen transfer system does not occur. However, there are differences between the two systems. First, the concentration of the water vascular hemoglobin visually appears to be considerably higher than that of the perivisceral hemoglobin. If the hemoglobin concentration within the water vascular cells is higher than that in perivisceral cells, the hemoglobin may be in higher aggregate states and therefore have a lower affinity. This difference in affinity would not be apparent when performing oxygen binding on samples of similar hemoglobin concentration. Second, preliminary studies indicate that the two systems contain different numbers of polypeptide chain types (Baker, unpublished). The two hemoglobins may react differently to protein concentration, temperature or pH, causing a oxygen transfer under certain conditions. Further studies on the water vascular hemoglobin are necessary to determine whether oxygen transfer occurs.

Conclusions

Paracaudina chilensis perivisceral hemoglobin has a very high oxygen affinity and may serve as an oxygen store during periods of exposure. The oxygen affinity is concentration and temperature dependent; affinity decreases with increased concentration or temperature. A normal Bohr shift occurs and is most pronounced at high temperatures and hemoglobin concentrations, in contrast to the situation in most other holothurians whose hemoglobins are insensitive to pH. *P. chilensis* hemoglobin is not affected by organic phosphates. The cooperativity of the perivisceral hemoglobin is similar to that of other holothurian hemoglobins and does not indicate negative heme-heme interactions as reported by Kawamoto (1928) for *Paracaudina*. The similarity in oxygen affinities of the perivisceral and water vascular hemoglobins do not suggest the occurrence of an oxygen transfer system.

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