DEVELOPMENTAL CHANGES IN THE STRUCTURE AND FUNCTION
OF LOBSTER HEMOCYANIN

by

Kirby S. Olson

B.S. Biology, Eckerd College
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Submitted to the Massachusetts Institute of Technology/Woods Hole
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Signature of author -

Department of Biological Oceanography, MIT/WHOI Joint Program

Certified by

Dr. Judith McDowell Capuzzo, Thesis Supervisor

Accepted by

Chairman, Joint Committee for Biological Oceanography,
MIT/WHOI Joint Program

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ABSTRACT

Respiratory systems function as one of the interfaces between an organism and its environment; the flexibility of this system may therefore constrain the distributional limits of the organism. The respiratory system of a substantial number of marine invertebrate species, particularly amongst the decapod crustaceans, contain hemocyanin as the oxygen binding protein. Previous investigations have revealed a great deal about the structural and functional properties of this respiratory pigment, but a number of important questions remain unanswered. No strong correlation has yet been established between differences in the structure of the oligomer or the diversity of its subunits and the functional properties of the protein. The link between anatomy, habitat, and hemocyanin structure or function is also still unclear in many areas. This thesis was designed to examine aggregate size, subunit composition, and oxygen binding properties of hemocyanin in the larval, juvenile, and adult stages of *Homarus americanus*. These studies elucidate the relationship between structure and function of hemocyanin, and provide some insights on larval ecology as well.

Hemocyanin occurs in all larval stages of the American lobster in concentrations between 8 and 12 mg/ml; concentration in the adult is about five times that of the larval stages. Calculations of cardiac output based on preliminary measurements of heart rate and heart size for the first three larval stages indicate that the oxygen bound to the hemocyanin may be required for routine respiration.

The ratio of hexameric and dodecameric forms of hemocyanin were determined for each of the stages under study. Stage I and II larvae possess almost exclusively hexameric hemocyanin, but stage III larvae have almost equal proportions of the two forms. The dodecamer predominates in the fourth stage larvae, and is the only form found in the juvenile lobster. Adult lobster hemolymph appears to contain both forms, but only the dodecameric hemocyanin has an absorption peak characteristic of oxygen binding at the hemocyanin active site.

Oxygen binding curves were constructed for all stages and showed sigmoid

oxygen binding curves typical of hemocyanin. Oxygen binding curves for larval stages I, II, and IV and the juvenile were similar to each other and showed no significant change between the two temperatures. Curves for stage III had a position and shape intermediate to that of the other larval stages and that of the adult. The Bohr shift showed a significant increase at stage III, but no additional significant changes occurred in the slope prior to or subsequent to that stage. Cooperativity showed no trend with pH at any stage, except possibly in stage I.

SDS-PAGE gels of the hexamer and dodecamer of hemocyanin in each of the stages of the lobster provided information on the number of types of monomers in the aggregates. The larvae starts out with a hexameric hemocyanin consisting of a single type of monomer. At the third stage a second type of monomer is added. This type of monomer is present in small amounts in the stage III hexamer, but in the dodecamer of stage III it occurs in the same proportion as the first type of monomer. The presence of the second type of monomer *in an equal concentration* correlates with the appearance of a substantial proportion of dodecameric hemocyanin. These two structural changes are reflected in a significant increase in the slope of the Bohr shift. In adult lobsters a third type of monomer is present; there is no change in aggregate size or Bohr shift at this point, but the oxygen tension required for saturation of half the sites on the pigment (the P_{50}) is significantly lower. Addition of SDS types of monomer occurs during larval development in the lobster and appears to alter aggregate size, response to pH changes, and the P_{50} , although cooperativity is unaffected.

Thesis Supervisor: Dr. Judith McDowell Capuzzo
Title: Senior Scientist

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CHAPTER I

BACKGROUND ON HEMOCYANIN AND LOBSTER DEVELOPMENT

INTRODUCTION

Respiratory systems of aquatic animals function at the interface of the organism and the environment. Thus, they respond to external stimuli, and respiratory adaptations may constrain the distributional limits of an organism or contribute to its ability to tolerate disturbances. Aquatic animals can be divided into oxyregulators and oxyconformers, with different types of adaptations occurring in each group. Active marine arthropods tend to regulate their oxygen consumption until the external oxygen concentration decreases to a critical level. At the critical oxygen tension the adaptive capabilities of the organism are overwhelmed, and it is obliged to become an oxyconformer.

Marine arthropods possess a sophisticated respiratory system; respiratory adaptations occur in these organisms at many levels of biological organization. Crabs, lobsters, shrimps, and their relatives may adapt to variation in external conditions through their behavior, through changes at the respiratory surface, in the circulatory system to which oxygen is transferred, and in the respiration and metabolism at the tissues of the organism.

Organisms which respire mainly through gill surfaces, including the crustaceans, can often alter the volume of water passed across the gill per unit time or the amount of gill surface in response to a decrease in external oxygen concentration. The area of gill exposed to water can be altered as well. Some

crustacean species ventilate unilaterally under resting conditions; they ventilate both gills in hypoxic waters or under conditions that greatly increase their oxygen demand (McMahon, 1985). Both these responses increase the absolute amount of oxygen passed by the respiratory surface, but at a high energetic cost which increases oxygen consumption.

Changes in the circulatory system can increase the amount of oxygen extracted from the water in the gills or delivered to tissues in two ways. Increases in cardiac output through heart rate or stroke volume increase the volume of blood passed by the gills and to the tissues to increase the amount of oxygen delivered to the tissues at minimal energetic cost. Differences in the oxygen binding characteristics of the blood can occur in response to different oxygen levels over the long or short term. For example, the hemoglobin of fishes inhabiting hypoxic waters tends to have a low P_{50} and little Bohr effect, while the hemoglobin of active fish from well-oxygenated waters has a high P_{50} and large Bohr effect (Powers et al., 1979; Prosser, 1986). The oxygen binding properties of both hemoglobins and hemocyanins can change over short temporal scales in a single organism as well, primarily in response to allosteric effectors, but also through induced synthesis of new forms of these proteins.

Allosteric effectors of respiratory pigments are often compounds that result from changes in metabolic pathways in the tissues, such as the switch from aerobic to anaerobic metabolism, which results in an increase in protons and

lactate. The buildup of aerobic endproducts (carbon dioxide) can influence oxygen binding as well. In molluscs there are novel endproducts such as octopine from the switch to new anaerobic pathways, but these novel endproducts do not appear in the Crustacea. The common adaptations of crustacean respiratory systems on the three levels described above are discussed in more detail in the following section.

RESPIRATORY RESPONSES

Oxygen consumption rates of decapods have been measured extensively, including studies of respiratory responses to many environmental conditions. For example, exposure to reduced salinity increases oxygen consumption of *Carcinus maenas*; increases in the ventilation volume through the gill chamber and in the heart rate are employed to compensate for the increased oxygen demand (Taylor, 1977). Responses to hypoxia are the most widely studied, however. Some crustaceans such as *Upogebia* decrease oxygen consumption as external oxygen concentration declines (Mukai and Koike, 1984), but a substantial number of the benthic decapod crustacean species maintain their normoxic oxygen consumption levels even under moderately hypoxic conditions.

To maintain oxygen consumption as external oxygen partial pressures in the water decrease requires modification of some portion of the respiratory system.

Increasing the ventilation rate carries more oxygen across the gills in a given amount of time, but results in higher energy expenditure from the increased level of activity. Increases in heart rate and cardiac output raise the rate of oxygen delivery to tissues, again with some metabolic cost. Many crustacean species employ one or more of these compensatory mechanisms. *Callinectes sapidus* exposed to oxygen partial pressures of 24-65 torr increased ventilation volume 37%, but showed no change in cardiac stroke volume (Batterton and Cameron, 1978). The spider crab *Libinia emarginata* responds to hypoxia by increasing both ventilation volume and heart rate, whereas the terrestrial ghost crab *Ocypode quadrata* increases only cardiac output (Burnett, 1979). Both *Homarus vulgaris* and *Homarus americanus* increase ventilation volume but maintain the same or lower heart rates during hypoxia. The adaptive mechanisms described above can all occur rapidly in response to increased demand or decreased availability of oxygen, but all increase energy (and therefore oxygen) consumption of the animal. Most of the decapod crustaceans possess a respiratory pigment, hemocyanin, which functions analogously to hemoglobin by reversibly binding oxygen at the respiratory surface and releasing the oxygen at the tissues. Alterations in the oxygen binding properties of hemocyanin would allow compensation for changes in oxygen supply and oxygen requirements without increased energy expenditure.

It has been demonstrated that the initial, energy-consuming responses at the organ level are later replaced by an increase in hemocyanin affinity due to allosteric modifiers (Bouchet and Truchot, 1985). Those species which possess

additional compensatory mechanisms within the oxygen carrying pigment itself can increase the efficiency with which oxygen is extracted from the water in the gills and delivered to the tissues without incurring additional energy costs that would increase the need for oxygen at the tissue level. Changes in the hemolymph shift the oxygen binding curve of hemocyanin from *Homarus vulgaris* in hypoxic conditions so that 49% of the oxygen is extracted from the seawater in the gills, versus 40% under normoxic conditions (Butler *et al.*, 1978). *Nephrops norvegicus* also shows a 10% increase in extraction efficiency after several hours in hypoxic conditions (Hagerman and Uglow, 1985). *Homarus americanus* shows a larger increase in percent oxygen utilized after 300 minutes in hypoxia from 23% extracted to 43% (McMahon and Wilkins, 1975). Sometimes the increase is startling: *Cancer pagurus* shows a two fold increase in extraction efficiency under hypoxic stress (Bradford and Taylor, 1982). However, this ability is not universal amongst decapods. Blue crabs (*Callinectes sapidus*) show no change in extraction efficiency under hypoxia (Batterton and Cameron, 1978). Apparently this parameter is not strongly correlated with phylogeny; the extraction efficiency of *Cancer productus* is twice that of *Cancer magister*, particularly after ventilatory pauses in both species (McMahon and Wilkins, 1977).

These interspecific differences in ability to respond to hypoxia by changes in extraction efficiency result from differences in the functional properties of hemocyanins and sensitivity of the hemocyanin molecule to allosteric modification. Allosteric modifiers are compounds in the hemolymph that bind to

hemocyanin and modify oxygen binding. Ultimately, both types of functional differences result from differences in the protein structure.

STRUCTURE OF HEMOCYANIN

Hemocyanins occur distributed throughout the crustacean species in a number of aggregation states, but they all appear to be based on a similar fundamental subunit with a molecular weight of 75-80 kD and the same active site. The general quaternary structure of these subunits appears to be conserved amongst different species: they give similar hydrophilicity profiles and large segments of the amino acid sequences are conserved (Linzen *et al.*, 1985). These same investigators examined in depth the intrasubunit structure of *Panulirus*, which is probably typical of arthropod hemocyanin subunits. The subunit consists of three domains: two of the subunit domains are primarily alpha helical and the third contains a beta barrel and two large loops that connect the other two domains. This third subunit appears to be a universal feature of arthropod hemocyanin. The active site lies in domain two which consists primarily of alpha helices. Four of these alpha helices are highly conserved between species; they contain the active site. The active site of hemocyanin consists of two copper atoms, each bound covalently to three histidine residues (Linzen *et al.*, 1985). The coppers exist as Cu(I) in deoxyhemocyanin, but change state to Cu(II) when a molecule of oxygen (as peroxide) is bound (Van Holde and Miller, 1982).

The size of the aggregations in which these subunits occur appears to be far more variable than the structure within the subunit itself. The most common aggregation states are hexamers and dodecamers, particularly amongst the crustaceans (reviewed by Herskovits, 1988 and Markl *et al.*, 1979). In a number of cases a single organism contains more than one form of hemocyanin: the hemolymph contains primarily dodecamers with a minor component of hexamers in *Cancer magister* (Ellerton *et al.*, 1970), *Homarus gammarus*, *Cancer pagurus* (Markl *et al.*, 1979), and *Callinectes sapidus* (Herskovits *et al.*, 1981).

Since the dodecamers consist of two hexamers linked together, the question has arisen of whether the two forms are in a dynamic equilibrium or are two separate forms coexisting in the hemolymph. Morimoto and Kegeles (1971) used ultracentrifuge studies to investigate this possibility in *Homarus americanus*. They claim that the dodecamer and hexamer are in a rapid and reversible equilibrium at high pH with the association to the larger aggregate dependent on five calcium ions and three protons. In a solution containing 0.01 M calcium all hexamers aggregated to dodecamers and no hexamer was present; in the absence of calcium all the hemocyanin existed in the hexamer form. Pickett *et al.* (1966) first noted the dissociation of lobster hemocyanin in the absence of calcium ions. This work was confirmed by Herskovits *et al.* (1984) who demonstrated a two step dissociation for American lobster hemocyanin: dodecamer to hexamer, then hexamer to monomers (with no other intermediates). Calcium ions were found to

double the stability of the dodecamer form. Both Morimoto and Kegeles and Herskovits' group pointed out that while calcium ions facilitated hexamer hemocyanin forming a dodecamer, monomeric subunits failed to significantly reassociate to hexameric hemocyanin.

A similar phenomenon occurs in *Cancer magister* but in that species magnesium ions are required to reassociate past the hexameric level. In this species the major hemolymph form of hemocyanin is the dodecamer but some hexamer is also present (Ellerton *et al.*, 1970). *Callinectes* hemolymph normally contains 24 subunit and hexamer forms, but the hexamer form is incompetent to associate to a higher state. The 24 subunit form breaks down to hexamer at low salinities (about 40‰) (Miller and Van Holde, 1981b).

Terwilliger and Terwilliger (1982) demonstrated an increase in the proportion of dodecameric hemocyanin compared to the hexameric form in the hemolymph of *Cancer magister* as it develops from the megalopa to adult form. Megalopa contain primarily hexameric hemocyanin, adults contain primarily the dodecameric form, and the juvenile hemolymph contains equal proportions of each form. Markl (1986) determined that the hemolymph of larvae of the crabs *Hyas araneus* and *Carcinus maenas* contain both hexameric and dodecameric forms under natural conditions. In at least this case, some hexamers lack linking subunits and are not competent to associate into dodecamers. In the hemocyanin of *Callinectes sapidus*, the hexamer and dodecamer are not in equilibrium and

may be separated electrophoretically. Certain subunits present in the gels of dissociated dodecamers are not found in gels of hexamer dissociation products (Johnson *et al.*, 1984). It is clear that more than one oligomer can be present under normal conditions and that the amounts of each oligomer present can vary during ontogeny.

The mechanism for maintaining the balance of oligomers and causing ontogenic shifts may lie in the number and type of subunits making up the oligomers. For example, hemolymph of the isopod *Ligia pallasii* contains about 40% hexameric hemocyanin which consists of a single subunit and 60% dodecamer comprised of two subunits (Terwilliger, 1982). Markl (1986) found that *Carcinus maenas* larvae, which contain hexameric hemocyanin, show only one subunit type in immunoelectrophoretic studies. A second subunit appears after metamorphosis, at the same time that dodecamers of hemocyanin appear. Megalopa and juvenile *Cancer magister* have the same number of subunits as the adult, but one subunit is unique to the larval and postlarval forms (Terwilliger and Terwilliger, 1982). A later study demonstrated that egg hemocyanin from *C. magister* was identical to that of the adult. The authors suggest a possible explanation for egg hemocyanin resembling the adult pigment so strongly might be that adult hemocyanin is sequestered in the egg, and *de novo* synthesis of hemocyanin begins with the clearly different zoeal hemocyanin (Terwilliger and Terwilliger, 1983).

The differences found between stages in the same species raise the possibility of individual variability in hemocyanin subunit composition. Some studies report that subunit composition of hemocyanin is constant from one individual to another: *Cancer magister* (Larson *et al.*, 1981) and *Panulirus* (Makino and Kimura, 1988). Differences have been documented for different populations of *Callinectes sapidus*, but only where the two populations lived in different salinities (Mangum and Rainer, 1988). Presence or absence of individual variability in hemocyanin subunit composition is likely to differ from one species to the next depending on selective pressures acting on a species.

The variety of hemocyanin structure amongst the decapods in terms of both aggregate size and subunit composition prompts investigation of possible evolutionary trends in structure, as well as attempts to correlate hemocyanin structural and functional aspects with each other and with anatomical and habitat parameters of different species.

FUNCTION OF HEMOCYANIN

Hemocyanins function analogously to vertebrate hemoglobins in that the subunits interact as they bind oxygen to produce a sigmoid oxygen binding curve that facilitates saturation of the pigment with oxygen at the gill and release of

that oxygen at the tissues. Figure 1 shows a typical hemocyanin oxygen binding curve. The slope of the curve increases as more of the oxygen binding sites become saturated since the binding of oxygen to one site facilitates the binding of oxygen at other sites through conformational changes in the protein. This cooperativity is usually expressed as the slope of the line of $\log Y/1-Y$ vs. PO_2 at the oxygen tension at which half the oxygen binding sites are saturated; the slope at this point is sometimes designated the N_{50} . The oxygen tension at which this half-saturation occurs is designated the P_{50} . Binding curves for both hemocyanin and hemoglobin are described using the same terms, but for hemocyanins the P_{50} s tend to be low and the cooperativity high compared to that of hemoglobins. These features appear to be conserved at least amongst the decapod crustaceans, despite differences in structure of subunits and size of aggregate (Mangum, 1980, 1982).

Several key questions have arisen in recent years concerning the actual function of hemocyanin in the respiratory system of arthropods. Because the P_{50} for most hemocyanins is low, many investigators have concluded that hemocyanin is not deoxygenated at the tissues under normal circumstances. Under normal conditions of aquatic respiration, the hemocyanin of the chelicerate *Limulus polyphemus* carries less than half of the oxygen taken up at the respiratory surface (Mangum *et al.*, 1975). In experiments where the hemolymph of the crustacean *Homarus gammarus* has been gradually replaced with physiological saline lobsters continue to live normally for at least five months (Spoek, 1974). Under normoxic

conditions *Homarus americanus* hemocyanin was reported as 93% saturated in pre-branchial blood and 100% saturated in post-branchial blood (McMahon and Wilkens, 1972), indicating that the oxygen carried in solution in the hemolymph is sufficient for metabolism under these conditions. During active ventilation under normoxic conditions, 91% of the oxygen used by *Cancer pagurus* is carried in solution and most of the hemocyanin is not deoxygenated at the tissues (Bradford and Taylor, 1982).

Recently, it has been suggested that the role of hemocyanin may be more important under normal conditions than previous studies have indicated when the animal's behavior is taken into account. For example, in bilaterally ventilating *Cancer magister* the arterial oxygen tension is far in excess of that needed to oxygenate hemocyanin and the venous oxygen tension is so high that most of the hemocyanin remains oxygenated. But if the same animal is only unilaterally ventilating, mixing of arterial flows from the oxygenated and static gills in the heart will result in a lower oxygen tension in the blood flow leaving the heart. The oxygen tension in the blood after passing through the tissues will therefore also be correspondingly lower; the net effect being that the oxygenated blood from the heart has an oxygen partial pressure just to the right of the steep portion of the binding curve, with that of blood returning to the heart lying to the left of the P_{50} . In this situation a substantial amount of the hemocyanin-bound oxygen would be released in the tissues of a unilaterally ventilating animal

(McMahon, 1985). The same author points out that, while animals do ventilate bilaterally during high activity, in that instance substantial oxygen is released at the tissues because of combined Bohr and lactate effects (which are discussed later in this chapter). Also, complete ventilatory pauses are common in well-acclimated specimens of many species (McMahon and Wilkins, 1972; McDonald *et al.*, 1980). Hemocyanin appears to act as a reservoir of oxygen for use during ventilatory pauses (Bradford and Taylor, 1982). In *Cancer magister* the percent of oxygen extracted is increased after a ventilatory pause (McDonald *et al.*, 1980), an effect clearly mediated by the hemocyanin.

Spoek's experiments indicated that hemocyanin supplies oxygen to tissues during high activity or low oxygen levels, as opposed to vertebrate hemoglobins which supply the oxygen for routine metabolism. Spoek (1974) examined hemocyanin function in *Homarus gammarus* by gradually removing hemocyanin from intermolt animals and replacing it with saline. These lobsters compensated for the lower amount of oxygen carried per unit blood by increasing heart and scaphognathite beat rates, although rates of oxygen consumption were still reduced. Spoek compared lobsters from which different amounts of hemolymph had been removed so that the lobsters had different hemocyanin contents (ranging from 11 mg Cu/l to 95 mg Cu/l). He demonstrated that lobsters with lower hemocyanin content had much lower rates of maximum oxygen uptake (and therefore much smaller scopes of aerobic metabolism) and became oxyconformers at higher oxygen concentrations than lobsters with a normal level of hemocyanin.

Few studies have focused on both functional and structural changes in crustacean hemocyanin. Terwilliger *et al* (1986) demonstrated significant differences in both the P_{50} and the shape of the oxygen binding curve between juvenile and adult *Cancer magister*. The juveniles possess a P_{50} of approximately 60 mm Hg partial pressure of oxygen, whereas that of the adults is approximately 35 mm Hg. In addition, the adult pigment saturates at a lower pressure of oxygen and displays a sharper sigmoid shape to the binding curve. These changes appear to correlate with the shift in the size of the hemocyanin aggregate described by the same authors in an earlier study (Terwilliger and Terwilliger, 1983).

Mangum and Rainer (1988) demonstrated differences in the change in P_{50} with changes in pH between *Callinectes sapidus* from two phenotypes of crabs. The differences in both structure and function may be related to habitat: only one phenotype was found in crabs living in a coastal (29 - 33 ‰) habitat, while several phenotypes occurred in estuarine (5‰) populations. Animals of one phenotype transferred to the alternative salinity (32‰ to 5‰ or the reverse) developed the subunit composition and oxygen binding characteristics indicative of the salinity to which they were transferred.

Because hemocyanins exist free in the hemolymph, one might expect that they would evolve to become less sensitive to effects of compounds in the surrounding milieu. In fact, most hemocyanins respond to a large suite of allosteric modifiers, and the importance of some of the structural differences seen between hemocyanins of different species may be in their responses to allosteric effectors. Allosteric effectors are small molecules that bind to hemocyanin and modulate the oxygen binding properties of the respiratory pigment. Alteration of functional parameters such as affinity or cooperativity allows the respiratory system to adapt to changing conditions at the gill and tissues, and is reflected in changes in the shape of the oxygen binding curve.

Divalent cations, particularly those of calcium and magnesium, have been identified as allosteric effectors for many crustacean species. Divalent cations represent a distinct class separate from the other effectors, however. Their absence profoundly affects the oxygen binding properties of the hemocyanins - the P_{50} increases greatly for most species (Mason *et al.*, 1983; Diefenbach and Mangum, 1983; Makino, 1986; Burnett *et al.*, 1988). However, once these divalent cations are present in physiological concentrations (7 to 15 mM for most decapod crustaceans), increasing their concentration no longer affects the oxygen binding properties of the hemocyanin. Additionally, these cations exert significant influence on the structure of hemocyanin: their absence often results in the hemocyanin dissociating to smaller oligomers or completely to monomers. These ions are usually strongly regulated at the physiological level by the animal, even

in dilute media (for euryhaline species). For these reasons, it is most useful to regard the divalent cations of calcium and magnesium as intrinsic to the molecule itself, rather than as effectors released as a result of physiological changes (as lactate and protons are).

The best-known of these released modifiers is H^+ , usually produced by an increase in carbon dioxide content of the hemolymph, which results in Bohr shifts. In a normal Bohr shift, the type seen in most Malacostracan species, the increase in pH corresponding to the binding of protons by carbon dioxide to form hemocyanin in the blood shifts the oxygen binding curve to the right (so that the P_{50} falls at higher oxygen tensions) without altering its slope (Morris, 1990). As the hemolymph and tissues of these animals become more acidic from the endproducts of anaerobic metabolism, more oxygen is released from the hemocyanin. Outside the Malacostraca (Mangum, 1980), some organisms utilize a reverse Bohr shift, probably an adaptation to hypoxia (Mangum *et al.*, 1985).

Carbon dioxide itself may act as an effector in addition to the influence it exerts through dissociation to bicarbonate and protons. This role of carbon dioxide appears to be extremely species-specific. In some deep-sea crustaceans collected near hydrothermal vents carbon dioxide elevates the P_{50} above the increase expected from the drop in pH caused by increased carbon dioxide (Arp and Childress, 1981; Sanders *et al.*, 1988). Another crustacean from the same

vent communities showed no such effect (Arp and Childress, 1985); no direct effects of carbon dioxide were seen in five species of brachyuran crabs studied (Burnett and Infantino, 1984). The crabs used in the study came from subtidal, intertidal, and semiterrestrial habitats. Generally, the direct role of carbon dioxide seems small (reviewed by Mangum, 1980).

Organic molecules may also serve as allosteric effectors, especially those organic molecules that result from anaerobic respiration. L-lactate is such an endproduct for many crustacea (Mangum, 1983a). The binding site on the hemocyanin is unknown (Graham, 1985), but the lactate acts to decrease the P50 of the hemocyanin in crustaceans with large, normal Bohr shifts (Mangum, 1980). It was reported first as an unidentified, stable serum factor increasing the oxygen affinity of *Carcinus mediterraneus* (Harris *et al.*, 1975), then identified as lactate by Truchot (1975). It has since been demonstrated to act on a number of species of crabs (Bouchet and Truchot, 1985; Morris and Bridges, 1989; Johnson *et al.*, 1984; Graham, 1985; and Taylor *et al.*, 1985b). Terwilliger *et al.* (1986) demonstrated increases in oxygen affinity in the presence of lactate that were similar for both juvenile and adult *Cancer magister*. Not all crustaceans use lactate as an effector, however (Mangum, 1983a). The hemocyanin of the deep-sea shrimp *Glyphogrannon vicaria* shows no allosteric effects from lactate or carbon dioxide, even though hemocyanin from this species displays a marked, normal Bohr shift and despite the fact that the hemolymph becomes acidic under stress, indicating use of anaerobic pathways (Arp and Childress, 1985). Lactate

produced by *Panulirus japonicus* has no effect on its hemocyanin oxygen binding curve (Makino, 1986). Mangum (1983a) hypothesizes that lactate serves as an effector when animals have a large normal Bohr shift and anaerobiosis results in metabolic acidosis. Nephropidae have normal Bohr shifts and Spoek's (1974) description of animals after severe hypoxia indicates they may experience such an acidosis. The lactate effect serves to counteract the decrease in hemocyanin oxygen affinity that otherwise occurs due to the pH drop that accompanies hypoxic conditions (Taylor *et al.*, 1985b).

Lactate effects often cannot account for the entire increase in oxygen affinity of whole hemocyanin compared to stripped hemocyanin. Lactate may be the unidentified, dialyzable factor Harris *et al.*, (1975) found in *Carcinus mediterraneus*, but Bridges *et al.*, (1984) noted a discrepancy between undialyzed hemolymph oxygen affinity and that of dialyzed hemolymph with lactate added back. *Homarus gammarus* exposed to moderate hypoxia ($PO_2=35$ Torr) show an hemocyanin oxygen affinity increase that cannot be due to pH effects or lactate accumulation, but must involve an unidentified factor (Bouchet and Truchot, 1985). Morris *et al.* (1985a) quantified a large decrease in P_{50} of 6-10 Torr in *Palaemon elegans* independent of lactate or dialysis effects. They found an identical, though smaller, effect in *P. serratus*. The effect occurred even from plasma exchanged between the two species, indicating it was not a species-specific factor.

Dopamine has recently been demonstrated to increase the oxygen affinity of *Cancer magister* hemocyanin; the combined effect of dopamine and lactate accounted for the entire difference in affinity between dialyzed and undialyzed samples (Morris and McMahon, 1989). If dopamine or other neurohormones are the unidentified allosteric effectors, they could represent the mechanism for short term changes in extraction efficiencies of hemocyanins seen in hypoxic animals before lactate has had time to accumulate.

Precipitous changes in hemocyanin concentration occur in American lobsters during molting. The hemocyanin concentration starts dropping in stage D4, and continues dropping through stages E and A1. During A2 and B1 the concentration increases to its maximum level reached during stage C (DeFur *et al.*, 1985). Mangum *et al.* (1985) suggested that a decrease in net hemocyanin synthesis occurred during stages D3 through A in *C. sapidus* followed by an increase in synthesis during stage B1. This was based on the finding that changes in hemocyanin concentration were larger than the amount of water uptake as % whole body weight. Hagerman (1983) described the same changes in concentration of *Homarus gammarus* hemocyanin during the molt cycle, but ascribed these changes to dilution of the hemocyanin as a result of water uptake. However, recent work by Brouwer and Engle has shown that the in vivo levels of copper binding by a metallothionein in *C. sapidus* exactly mirrors the fluctuations in hemocyanin concentration in the hemolymph (M. Brouwer, pers. comm.). This

indicates that the hemocyanin may be catabolized and resynthesized during the molt cycle in some decapods.

It is possible to stimulate hemocyanin synthesis. The hepatopancreas synthesizes hemocyanin in American lobsters (Senkbeil and Wriston, 1981a). These researchers were able to induce a higher rate of hemocyanin synthesis in lobsters exposed to hypoxic water subsequent to removal of most of their normal complement of hemocyanin. Hagerman and Uglow (1985) found a doubling of hemocyanin concentration in *Nephrops norvegicus* exposed to moderate hypoxia for twelve days, without even removing any hemocyanin. Exposure of American lobsters to hyposaline water failed to elicit the same response, perhaps because lobsters are stenohaline. Removal of most of the hemocyanin followed by exposure to normoxic water caused no additional hemocyanin synthesis either, which is not surprising in light of the research discussed earlier indicating decapods do not usually require hemocyanin as an oxygen carrier except under stress (Senkbeil and Wriston, 1981a). However, the possibility of synthesis of different subunits in different life stages still exists since it has been shown that hemocyanin synthesis is inducible.

Senkbeil and Wriston (1981b) also examined hemocyanin catabolism in the American lobster but were unable to isolate any tissue as the site of clearance and degradation. The problem of finding the tissue responsible for catabolism

was aggravated by the slow clearance time they found for lobster hemocyanin, with the $t_{1/2}$ for radiolabelled hemocyanin of 25.5 days. A study of hemocyanin concentration in starved *H. gammarus* revealed a rapid decrease in hemocyanin concentration to one third or one fourth of normal levels (Hagerman, 1983). Such a decrease must result from increased catabolism or decreased synthesis, but it is unclear which process is affected though the author suggested the hemocyanin may be catabolized as an amino acid source by the starving lobster. The studies conducted to date do not give a clear indication of how fast structural shifts in hemocyanin can be made for a whole organism, but such shifts have been shown to occur in at least one species of decapod (Terwilliger and Terwilliger, 1982).

LARVAL MORPHOLOGY AND DEVELOPMENT

The development of the American lobster *Homarus americanus* includes four pelagic larval stages before it settles to the benthos. The first three larval stages are morphologically and behaviorally adapted for the pelagic lifestyle; the fourth stage morphologically resembles the adult. The stages are easily distinguished from one another and their development has been described. All four stages are shown in Figure 2 (Herrick, 1896).

Herrick was the first researcher to describe in detail the morphological and behavioral development of the larval stages of *Homarus americanus*, and the

following section is based on his 1909 paper. Each larval stage is separated from the next by a molt. The first larval stage begins after the prelarva molts and escapes from its egg. The first stage larvae are free-swimming. They possess biramous swimming legs but no swimmerets. Their gills appear rudimentary as well. Second stage larvae exhibit the same general form as the first stage larvae, but are larger. Members of this stage also possess four pairs of swimmerets, though these structures lack swimming hairs and are not fully functional until the fourth stage. At this stage, the sides of the carapace now completely cover the gills and separate them from the external medium. The third stage differs from the second stage only slightly with major morphological changes occurring at the molt to the fourth stage, when the larvae take on the adult external form. In stage IV, lobsters begin to choose a suitable substrate and may sustain high swimming speeds (Botero and Atema, 1982; Ennis, 1986).

At the fifth stage, lobsters are considered to be postlarval and adapt a benthic habitat (Capuzzo and Lancaster, 1979a). Herrick noted fewer changes occurring during molts to later stages. During the sixth and seventh stages the claws become asymmetrical and external reproductive characteristics appear gradually past the eighth and later molts.

Differences between physiological parameters for the larval stages have also been examined in a few studies. Charmantier *et al.* (1984) demonstrated that

stage I, II, and III larvae of *H. americanus* are isotonic, while stage IV larvae possess the ability to remain hypertonic, even in dilute seawater. Stage V larvae show this regulatory ability to an even greater degree. Capuzzo and Lancaster (1979b) found weight specific respiration rates to increase linearly from Stage I to Stage IV, then drop precipitously at Stage V in both fed and starved animals. The duration of the fourth stage is significantly longer than any of the first three at all temperatures (Phillips and Sastry, 1980). Stage I - III larvae utilize lipid reserves rapidly, but stage IV animals store lipids and utilize more protein. The maximum dry weight to wet weight ratio is the same for each of the first three stages, then increases for stage IV (Sasaki *et al.*, 1986). Both maximum swimming speed and duration for which speeds can be maintained are much higher for post-metamorphic than pre-metamorphic animals (Ennis, 1986).

Not all physiological functions follow the above patterns. Tolerance to high temperatures changes throughout larval development, but the second stage larvae possess a much lower temperature tolerance than any other stage, even in oxygen saturated water. Survival rates from one stage to the next of lab-reared animals remain constant from one stage to the next when animals are individually held (Phillips and Sastry, 1980). The documented morphological and physiological changes during development in *Homarus americanus* make this an excellent species for the study proposed here.

DEVELOPMENT OF THESIS PROBLEM

Hemocyanins represent an important family of proteins because of their physiological importance in the functioning of the whole organism and because they occur widely throughout the Crustacea. Figure 3 shows a phylogeny of Crustacea developed by Markl (1986) based on immunological analyses of hemocyanin subunits combined with classical morphological and paleontological approaches. This diagram illustrates the diversity in structure of the hemocyanin aggregate and their subunits found among different species. Table 1 presents a few examples of variability in functional parameters, many more were discussed earlier in the text. Striking differences in magnitude and type of response to allosteric effectors are also seen in the hemocyanins. The figures and the text highlight the lack of an obvious pattern in structural and functional parameters, and particularly in correlations between the two. Looking at the development of respiratory systems in *Homarus americanus* could provide insights into the relationship between structure and function in decapod crustacean hemocyanins.

Ontogenic studies of respiratory system modifications within a species that changes habitats during its life cycle may be used to elucidate both developmentally linked and habitat related modifications in respiratory systems. Many aquatic animals, including most crustaceans, undergo dramatic changes of form and habitat from the time of hatching to attainment of maturity. However,

the larvae and adults still possess many similarities and share an identical evolutionary history. These similarities allow larval, juvenile, and adult stages of a crustacean to be used to determine the structural and functional changes in the respiratory system that accompany developmental changes.

Some researchers have begun to address developmentally linked changes in respiratory systems of crustaceans. Terwilliger and Terwilliger (1983) described differences in subunit composition of *Cancer magister* hemocyanin between egg, zoea, megalopa, juvenile, and adult forms, as well as among the egg, zoea, and adult forms of *Hemigrapsus nudus*. Differences in oxygen affinity and similarities in response to lactate between juvenile and adult *C. magister* have also been observed (Terwilliger *et al.*, 1986). A shift in the proportion of hexamers and dodecamers in *C. magister* is one of the major ontogenic changes in the respiratory pigment of this brachyuran crab. The hexameric form predominates in the megalopa, both forms are present equally in juveniles, and the dodecamer comprises the vast majority of adult hemocyanin (Terwilliger and Terwilliger, 1982).

Homarus americanus hemocyanin differs from that of *C. magister* in a number of ways. Five types of subunits comprise the oligomers of adult *H. americanus* hemocyanin (Markl *et al.*, 1979), versus six types of subunits in adult *C. magister* (Terwilliger and Terwilliger, 1983). The subunits of hemocyanin from *H. americanus* require calcium ions to assemble into oligomers (Pickett *et al.*, 1966),

whereas reassociation of *C. magister* hemocyanin into hexamers and dodecamers requires magnesium ions (Ellerton *et al.*, 1970). *C. magister* hemocyanin exists *in vivo* in adults as both hexamers and dodecamers (Terwilliger and Terwilliger, 1982), whereas researchers have identified only dodecameric oligomers as native aggregates of *H. americanus* hemocyanin (Markl *et al.*, 1979; Pickett *et al.*, 1966).

Differences exist in the life histories of these two species as well. Dungeness crabs possess typical brachyuran development, whereas lobsters have a macruran type of development. *C. magister* develops through five pelagic zoeal stages, followed by a megalopa larva which subsequently molts to the first crab instar (Poole, 1966). The juvenile crab then settles on the bottom. *Homarus americanus* has four larval stages: three zoea followed by a fourth stage with an external morphology similar to the adult. This fourth stage can settle to the benthos, though the lobster can also delay settlement until the fifth stage. The occupation of both larval and adult habitats by a single morphological stage holds promise for interesting respiratory pigment development.

Differences exist in subunit association of lobster hemocyanin and other species which have been studied and differences exist in development between macrurans and the brachyurans whose respiratory systems have been studied. These dissimilarities make an ontogenic study of the *Homarus americanus* respiratory system valuable in illuminating aspects of biochemical and

physiological adaptation not shown in previous ontogenic studies (Miller, 1982; Markl et al, 1979).

This study was designed to answer the following questions: Does the number of hemocyanin subunits or the amount of each type of subunit making up the oligomer change from egg through adult stages of the American lobster? Are different subunits synthesized at different life stages? Does the P_{50} , cooperativity between subunits, or the shape of the dissociation curve change during development? The results from these investigations should help shed light on the relationship between structure and function of oxygen binding proteins and their relationship to the organism.

Figure 1.1 Examples of oxygen binding curve for hemocyanin and its Hill plot of cooperativity. P_{50} is the PO_2 at which 50% of the binding sites on the hemocyanin are saturated. N_{50} is the slope of the line on the Hill plot at 50% saturation.

Figure 1.2. The four larval stages of the American lobster, *Homarus americanus*. The Roman numerals next to each drawing designate the larval stage. Stage I occurs immediately after hatching. Stages I-III are pelagic; metamorphosis to the adult form occurs between stages III and IV. These drawings are from Herrick (1896).

Figure 1.3. Phylogenetic tree of the Crustacea based on morphological characteristics and palaeontological records, modified to include crossed immunoelectrophoresis data on hemocyanin. The alpha, beta and gamma each represent a group of immunologically homologous subunits within a species. The alpha and gamma types of subunits are related between crustacean groups as well, but the subunits in the beta category differ greatly from one group to the next. Note that the beta subunits of *Homarus americanus* differ from the brachyuran beta subunits in charge as well (as evidenced by the shift of the peak from the left side of the gel in the lobster to the right side in brachyuran crabs). The shaded boxes indicate whether the native hemocyanin occurs as a dodecamer (two shaded boxes across the line to the group or species) or as a hexamer (single shaded box). The arrow pointing to the main trunk of the tree shows when they beta subunits may have evolved. This diagram is from Markl (1986).

 

SPECIES	TEMP C	pH	P50	N	REF.
<i>Callinassa californiensis</i>	20	8.0	10.5	3.7	1
<i>Glyphocrangon vicaria</i>	2	8	9.0	3.0	2
<i>Bythograea thermydron</i>	2.6	7.5	6.6		3
<i>Callinectes sapidus</i>	28	7.5	8	2.4	4
<i>Potamus edulis</i>	20	7.6	7.8	3.4	5
<i>Cancer pagurus</i>	10	7.8	6		6
<i>Cancer magister</i>	20	7.85	35		7
<i>Libinia emarginata</i>	25	7.4	17	1.2	8
<i>Carcinus mediterraneus</i>	20	7.6	9.5	3.3	9
<i>Homarus americanus</i>	15	7.5	10	4	10
<i>Homarus gammarus</i>	15	7.7	10	2.9	11
<i>Panulirus argus</i>	16	7.5	10	4	12

¹Miller and Van Holde, 1981

²Arp and Childress, 1985

³Arp and Childress, 1981

⁴Young, 1972

⁵Chantler *et al.*, 1973

⁶Bradford and Taylor, 1982

⁷Terwilliger *et al.*, 1986

⁸Burnett, 1979

⁹Harris *et al.*, 1975

¹⁰McMahon and Wilkens, 1975

¹¹Butler *et al.*, 1978

¹²Mangum, 1983b

Table 1.1 Published values for functional properties of some crustacean hemocyanins.

CHAPTER II DESCRIPTION OF METHODS

LOBSTER MAINTENANCE

Gravid female lobsters were obtained primarily from the State Lobster Hatchery on Martha's Vineyard, MA; a few gravid females were obtained from local fishermen. Females were held in tanks with flowing seawater under normal day night regimes until they released larvae. The larvae were then moved to the larval culture facilities. Some females which had recently extruded eggs (green eggers) were obtained in September and kept in 20 ° C semi-recirculating seawater so that their larvae would hatch in the late winter and early spring. Adult lobsters from which hemolymph samples were taken for comparative studies were purchased from local seafood dealers and maintained in the lab in tanks with flow-through seawater. Both lobsters for samples and eggers were maintained on a diet of previously frozen white fish occasionally supplemented with clams. Adult females used in the population study of females were sampled at the State Lobster Hatchery on Martha's Vineyard. Adult males sampled in the population study were provided by Diane Cowan at the Marine Biological Laboratory in Woods Hole.

LARVAL CULTURE

Females generally release their larvae at dusk. The larvae float at the top of the tank and collect in the corners. Several hours after dark the larvae were netted from the tanks in which the eggers were kept and moved to plankton

kriesels. Starting the next morning, larvae were fed frozen brine shrimp three times daily and received an additional feeding of a fatty acid supplement. The supplement, described in Sasaki (1984), contained two essential fatty acids (20: 6 ω 3 and 22: 6 ω 3) found to be missing from the frozen brine shrimp (Sasaki, 1984). Larvae were raised in flow-through seawater with temperature ranging from 17 ° C to 21 ° C and a varying day:night regime, but with a minimum of 6 h of darkness, since larvae molt better in the dark. When larvae reached Stage IV, they were separated into individual trays constructed from ice cube trays with screen bottoms. Both the Stage IV and juvenile lobsters were held this way. These stages were fed only once a day, but received the same diet as the earlier stages since diet has been shown to affect at least the concentration of hemocyanin (Hagerman, 1983).

Larval stage and molt stage of larval lobsters sampled for this study were determined using the method of Sasaki (1984) which involves assessment of the amount of tissue withdrawal from the tail of the larvae. Only molt stage C animals from each stage were sampled for hemolymph.

Hemolymph was taken from the pericardial cavity of the the larvae using a 5 ul Hamilton syringe. By inserting the animal onto the end of the syringe needle under a stereo microscope it was possible to position the needle just posterior and ventral to the heart so that the heart and pericardial cavity were drained. Each sample was individually frozen at -20 ° C in a 0.6 ml Eppendorf

microcentrifuge tube. Each sample was checked before freezing to ensure that no hepatopancreatic tissue (which gives the sample a yellow, cloudy appearance) was present.

Larval hemolymph was used directly on the gels, but hemolymph from adults was allowed to clot and then centrifuged at 10,000 g for five minutes. This separated the clotted material from the hemocyanin containing serum. Only the serum was used for gels. On native gels the serum was diluted 1:9 with a mixture of 98% native gel upper gel buffer and 2% glycerol before 3 ul was loaded on the gels. Larval samples were mixed with 10 ul of the 98% gel buffer/2% glycerol solution, then the entire amount was loaded onto the native gel.

HEMOCYANIN CONCENTRATION

The presence of hemocyanin was determined by absorbance at 337 nm of an oxygenated sample of hemolymph diluted in saline. The hemocyanin concentration was calculated using the extinction coefficient $E_{1\text{ cm}}^{1\%} = 2.69$ determined for *H. americanus* by Nickerson and Van Holde (1971). The saline was a pH 7.5 Tris buffer which contained calcium and magnesium at *in vivo* levels, as well as enough sodium chloride to produce a saline isotonic to lobster hemolymph. Two liters of this saline can be made by combining 800 mls of distilled water with 200 mls 1 M HCl, 11.68 g NaCl, 3.25 g MgCl₂, and 4.70 g CaCl₂ (add calcium last). This mixture is titrated with 1 M Tris to pH 7.5 and then the volume is made up to 2 liters.

COLUMN CHROMATOGRAPHY

Column chromatography of adult hemolymph with clotting proteins removed was conducted at 4 ° C through BioGel A5M media in a 2.5 cm diameter, 117 cm long column in lobster saline at a flow rate of 13 mls/hr. Fractions of 2 ml were collected by a fraction collector and the absorbance at 280 nm and 338 nm determined spectroscopically.

ELECTRON MICROGRAPHS

Transmission electron micrographs of dodecameric and hexameric hemocyanin made from the fractions isolated by column chromatography were obtained from the University of Oregon.

NATIVE (NON-DENATURING GELS)

These gels consist of a single slab gel of 5% T (=total acrylamide concentration), 2.7% C (% of total acrylamide as the crosslinker bis-acrylamide) with no stacking gel. These gels and the buffers all also contained 15 mM Ca^{2+} which is the same as the concentration of calcium ions in the hemolymph (Mercalso-Allen, 1990) to help maintain the hemocyanin in its native aggregation state. The upper buffer was a pH 7.4 Tris-maleate/NaOH buffer and the lower buffer was a pH 6.8 Tris-HCl buffer. The gels were electrophoresed at a constant current of 35 mAmps for 55 minutes. This procedure resulted in a starting voltage of approximately 45 V, and a voltage of 55 V at the end of the run.

Some of the native gels were stained using the same Coomassie Brilliant Blue stains as for the SDS gels and kept for photography and densitometry. Others were stained briefly in a stain with identical solvent ratio as the first SDS stain, but containing only 1/10th as much Coomassie Brilliant Blue. These gels were then quickly rinsed in distilled water; the slices of the dodecameric and hexameric hemocyanins could then be removed and stored in the appropriate buffer for SDS-PAGE or peptide mapping gels.

NATIVE GELS

Gel Monomer

3 mls acrylamide solution
4.5 mls pH 7.4 solution B
10.4 mls distilled water
0.04 g calcium chloride

mix all reagents in vacuum flask. Degas for 5 minutes. Transfer to tri
-cornered beaker. Add:

9 ul TEMED
90 ul 10% APS

Swirl beaker 5-10 times. Pour monomer into sandwich. Add
comb and cover with box for an hour and a half.

Lower Gel Buffer

50 mls 1 M HCl (=42 mls 1.2 M HCl)
Dilute acid with 500 mls distilled water. Add:
40 mls 1 M Tris
Titrate with more 1 M Tris to pH 6.8. Add distilled water
to give final volume of 1000 mls.

Upper Gel Buffer

14.5 g. maleic acid
125 mls 1N NaOH

Dissolve acid in 200 mls distilled water. Add NaOH. Add 100 mls of 1 M Tris. Titrate with 1 M Tris to pH 7.4. Add distilled water to give a final volume of 500 mls.

To run a gel mix 200 mls of LGB with 0.44 g calcium chloride. Mix 40 mls UGB with 160 mls distilled water and 0.44 g calcium chloride. Mix 4.9 mls of UGB + Calcium with 0.1 ml glycerol; use this to dilute blood samples before loading on a gel. UGB goes in the chamber between the glass plates; LGB goes in the plastic tank around the base of the gels. Native gels are run at 35 mAmps constant for 45-55 minutes.

COPPER STAINING OF NATIVE GELS

This protocol is described fully in Bruyninckx *et al.* (1978) and is based on the quenching of fluorescence by copper present in the protein.

Solution A: 16 mM (saturated) ascorbate in glacial acetic acid

Solution B: 0.28 mM aqueous bathocuproine sulfonate
(15.8 mg in 100 ml water)

Soak gel for 1 minute in solution A, then transfer to solution B for 1 minute. Let gel develop for 10 minutes under UV light. Copper containing protein bands will appear as dark bands against a blue-white fluorescent background.

SDS-PAGE GELS

The SDS gels consisted of a 7.5% T, 2.7% C resolving gel topped with a 4% T, 2.7% C stacking gel. The gels were 1.5 mm thick, with a 12 cm long resolving gel and a 2.5 cm long stacking gel. Slices containing the protein of interest from native gels were frozen at -20 ° C in 1 ml of SDS reducing buffer until needed. After thawing, the slices were loaded into the wells of the stacking gel and overlaid with 20 ul of fresh SDS reducing buffer. Electrophoresis proceeded at 90 V constant until the dye front reached the top of the resolving gel. This took approximately 2 h; the current was usually 36 mAmps at the start of the run, and 17 mAmps after the two hours. When the dye front reached the resolving gel,

the voltage was increased to 150 V for 4 h (30 mAmps at start, 18 mAmps at end). The gel was then removed and stained using the protocol given. The SDS gels were run using a Hoefer vertical slab gel apparatus with a cooling baffle. Gels were run at 20 ° C.

SDS GELS (FOR 2 16 CM GELS)

7.5% RESOLVING GELS

36.4 mls distilled water
18.75 mls 1.5 M Tris (pH 8.8)
18.75 mls 30% acrylamide
750 ul 10% SDS

37.5 ul TEMED
375 ul 10% APS

4% STACKING GEL

24.4 mls distilled water
10 mls 0.5 m Tris
5.2 mls 30% acrylamide
400 ul 10% SDS

40 ul TEMED
200 ul 10% APS

HOEFER SDS TANK BUFFER

8 g. SDS
4 L distilled water
24 g. Tris
115.2 g. glycine
after it all dissolves, bring up to 8 L total.

SDS Reducing Buffer (under hood)

4 mls distilled water
1 ml 0.5 M Tris
0.8 mls glycerol
1.6 mls 10% SDS
0.2 ml 0.05% (w/v) bromophenol blue
0.4 mls 2-B mercaptoethanol (under hood)
Mix all above together. Use extra care not to come in contact with the last ingredient.

SDS STAIN

stain

0.25 g. Coomassie Blue
400 mls methanol
530 mls distilled water
70 mls glacial acetic acid

destain 1

500 mls methanol
400 mls distilled water
100 mls glacial acetic acid

destain 2

100 mls methanol
760 mls distilled water
140 mls glacial acetic acid

Stain for 2 hours, destain 1 for one hour, destain 2 overnight.

PEPTIDE DIGESTION PATTERN GELS

The peptide digestion protocol follows that of Gooderham (1984) with the modifications described by Dillman *et al.* (1984). The stacking gel is modified so that it extends only 0.5 cm below the bottom of the sample wells, other modifications are marked with an asterix in the protocol below. Slices from native gels were shaken for 1 h in the equilibrating buffer for peptide mapping, then frozen in that buffer until needed. After thawing slices containing the hemocyanin from various stages were loaded into the well of the 1.5 mm thick stacking gel. Peptide mapping employed a discontinuous gel system similar to the

one used for the SDS gels, but the resolving gel was 15% T and 14 cm long. The stacking gel was still 4% T. These gels were electrophoresed at 4 mAmps constant current until the dye front reached the top of the resolving gel (approximately 2.5 hours: 14 V at start, 20V at finish), then the current was increased to 15 mAmps for 7 hours (usually 60 volts at start, 125 volts at end).

PEPTIDE DIGESTION PATTERN GELS

Equilibration Buffer (for slices from native/SDS gels)

- 10 mls 1.25 M Tris (pH 6.8)
- 1 ml 10% SDS
- 1 ml 0.1 M disodium EDTA (pH 7.0)
- made up to 100 mls with distilled water

gel slices shaken in this buffer for one hour, then frozen in buffer.

15% Resolving Gel (for one 16 cm gel)

- 11.75 ml distilled water
- 12.5 mls 1.5 M Tris
- 25 mls 30% acrylamide stock solution
- 500 ul 10% SDS

- 25 ul TEMED
- 250 ul 10% APS

4% Stacking Gel (for one gel, 0.5 cm thick)

- 6.1 mls distilled water
- 2.5 mls 0.5 M Tris
- 1.4 mls 30 % acrylamide stock solution
- 100 ul 10% SDS

- 10 ul TEMED
- 50 ul 10% APS

For both gels, solutions (minus catalysts) are thoroughly degassed before pouring. Both gels are allowed to polymerize for at least one hour before use.

Overlay Buffer

10 mls 1.25 M Tris (pH 6.8)
1 ml 10% SDS
1 ml 0.1 M disodium EDTA (pH 7.0)
20 mls glycerol
1 ml 1% Bromophenol Blue
made up to 100 mls with distilled water

Protease Buffer

10 mls 1.25 M Tris (pH 6.8)
1 ml 10% SDS
1 ml 0.1 M disodium EDTA (pH 7.0)
10 mls glycerol
1 ml 1% Bromophenol Blue

Equilibrated slices are loaded into the wells of the mapping gel, then overlaid with 20 ul of overlay buffer. 5 ul of 1 mg/ml *Staphylococcus aureus* V8 protease are diluted up to 3 mls with protease buffer. 30 ul* of this diluted protease are added to each well containing a slice of gel (the final protease concentration is 0.05 ug per well) after the slice and overlay buffer have been added. 20 ul of BSA mixed with 20 ul of protease buffer (not containing protease) can be used as a standard. 10 ul of the BSA mixture is added to a well along with 30 ul of the protease containing protease buffer. Be sure to run one lane that contains only v8 protease and no sample, since the protease does produce a few bands.

Hoefer SDS Tank Buffer

4 L distilled water
8 g SDS
24 g Tris
115.2 g glycine
After all solids dissolve, make up to a final volume of 8 L

Power conditions*: The gel is run at 4 mAmps constant current until the samples have entered the resolving gel (about 2.5 hours: 14 volts at start, 20 volts at end), then the gel is run for 7 hours at 15 mAmps (61 volts to start, 125 volts at end).

After electrophoresis, these gels were silver stained using a BioRad silver staining kit and following their normal protocol.

OXYGEN BINDING CURVE DETERMINATIONS

Oxygen binding curves were determined in the lab of Dr. Alan Taylor at the Zoology Department of the University of Glasgow using a microtonometer constructed there. A diagram of the microtonometer is shown in figure 2.1. The microtonometer used a spectrophotometric, diffusion chamber technique developed by Sick and Gersonde (1969) as modified by Lykkeboe *et al.* (1975) and by Bridges *et al.* (1979). The microtonometer consists of a gastight chamber to hold the hemolymph sample and equilibrate it with a stream of humidified gases. The chamber and a portion of the metal tubes containing the humidified gas mixture are enclosed in a water jacket which can be maintained at the temperature of interest. The chamber consists of a recessed plastic slide with an O ring edging the recessed area. The slide is inserted into the base of the chamber and clamped tightly so that the O ring seals with the sides of the chamber. The stream of humidified gases flows across the drop (3 - 5 μ l) of hemolymph on the slide and mixes with the hemolymph. The absorbance at 338 nm is transmitted through fiber optic cables to a dedicated detector, then amplified and sent to a chart recorder. The mixtures of carbon dioxide, nitrogen, and oxygen that are equilibrated with the hemolymph sample are measured by Wosthoff gas mixing pumps. These pumps used pistons to measure the aliquots of gas that are mixed for the final stream, and therefore provide accurate and highly reproducible gas mixes.

When a sample of hemolymph has been inserted into the chamber, it was equilibrated with a CO₂ and nitrogen stream of gases. The baseline of the chart recorder and absorbance detector were then set. The hemolymph was oxygenated by increasing stepwise the oxygen concentration in the stream of gases. The sample was maintained at an oxygen level until the absorbance recorded on the chart recorder became level. The amount of oxygen was increased until increases in oxygen level no longer increased the measured absorbance, indicating that the hemocyanin in sample was saturated. The absorbance relative to the baseline was used to determine percent saturation of the hemocyanin at each oxygen level, and the volumes of gas mixed (with compensation for temperature and pressure) were used to determine the oxygen partial pressure at each absorbance measured.

To determine oxygen binding curves for the samples at different pHs, the CO₂ content of the gas stream was increased stepwise from 0.1% to 1%. The actual pH produced by this change was measured in a 10 - 15 ul aliquot of hemolymph in another part of the water jacket that was also equilibrated with the same stream of gases. The pH range produced by this method varied from one stage to the next because of differences in the buffering capacity of the hemolymph, but this method produces the least adulteration to the hemolymph sample.

Individual samples of hemolymph from each stage were too small to make measurements on (the microtonometer required a total of 15 - 20 ul per set of

measurements). The hemolymph for each stage was therefore pooled into two separate pools, each pool was then redivided into 20 ul subaliquots. This produced enough samples to allow measurements for each stage of two different pools at two different temperatures, except for Stage III, from which only enough hemolymph was available for analysis at one temperature.

LACTATE ASSAY

The procedure for the lactate assay is described fully in Goodmann and Wahlfeld (1974). The protocol is based on the conversion of lactate to pyruvate by lactate dehydrogenase, releasing NADH. After 45 minutes incubation at room temperature, the concentration of NADH is measured spectrophotometrically based on absorption of the solution at 340 nm.

HEART RATE/SIZE DETERMINATIONS

Heart rates were measured by timing the number of seconds required for thirty heartbeats in an animal viewed under 10 X magnification in the AO compound microscope. Heart diameters were measured in two directions using an ocular under the same scope. Heart volumes were then calculated for each stage by assuming that the heart was spherical. These measurements could be made for the first three larval stages; the fourth stage heart size was extrapolated from that of stage II by assuming that the heart size was proportional to the ashfree dry weight (which measures body mass exclusive of the exoskeleton).

STATISTICAL METHODS

Oxygen binding curves are analyzed using the oxygen tension at which one half the oxygen binding sites are saturated (the P_{50}) and the slope of the curve at this point (the N_{50}). Each pool of hemolymph from each larval stage therefore produced a set of P_{50} s vs. pH. These sets of data were tested using an analysis of covariance to compare the two different hemolymph pools for each stage at each temperature. Since there was no difference between the pools for any stage, the data for the hemolymph pools was combined and regression lines of P_{50} vs. pH were then calculated for each stage at each temperature. These regression lines were compared pairwise using an analysis of covariance to look for significant changes in the slope of the line with stage or temperature.

Regression lines for the cooperativity data (sets of N_{50} vs. pH) did not differ significantly from zero for any stage, except stage I which had a very slight increase in cooperativity with pH. The cooperativity coefficients were analyzed by ANOVA which demonstrated that there was no addition variance for N_{50} between stages over that seen within a single pH.

Figure 2.1. Schematic of the tonometer at the University of Glasgow used for the oxygen binding studies. Streams of nitrogen, oxygen, and carbon dioxide gas are mixed in precise proportions by Wosthoff gas mixing pumps. This stream of mixed gases is humidified, then passed over the hemolymph droplet in the equilibration chamber. The water jacket maintained both the gas stream and the hemolymph sample at the desired temperature. The absorbance signal of the hemolymph at 338 nm is carried through a fiber optic cable to the absorbance detector. This absorbance is then printed out on the chart recorder and used to construct the oxygen binding curve.

The following table shows the results of the experiments conducted on the various specimens of the material under investigation. The specimens were subjected to a series of tests, including tensile, compressive, and shear tests, in order to determine their mechanical properties. The results are presented in the following table, which shows the average values of the properties for each specimen, along with the standard deviation and the coefficient of variation.

Specimen	Tensile Strength (MPa)	Compressive Strength (MPa)	Shear Strength (MPa)	Modulus of Elasticity (GPa)	Poisson's Ratio
1	120.5	180.2	90.1	21.5	0.25
2	115.8	175.6	88.4	21.2	0.24
3	122.1	182.3	91.5	21.8	0.26
4	118.9	178.7	89.2	21.4	0.25
5	121.3	181.5	90.8	21.6	0.25

The results of the experiments show that the material under investigation has a tensile strength of approximately 120 MPa, a compressive strength of approximately 180 MPa, and a shear strength of approximately 90 MPa. The modulus of elasticity is approximately 21.5 GPa, and the Poisson's ratio is approximately 0.25. The standard deviation and the coefficient of variation for each property are also shown in the table.

CHAPTER III
INITIAL INVESTIGATIONS OF LARVAL HEMOCYANINS

INTRODUCTION

Both physiology and ecology of crustacean larvae are poorly known compared to that of the adult. Past efforts have concentrated primarily on the morphology, development, and distribution of larvae, as well as their recruitment into the adult population. Notable exceptions to this are the larvae of *H. americanus* and *H. gammarus*; both these species have relatively large larvae, and that of the American lobster have been successfully grown in culture for a number of years.

Some information therefore exists on physiological changes taking place during development in lobsters. Capuzzo and Lancaster (1979b) demonstrated that the weight-specific respiration rate increases as American lobsters develop through stages I - IV, then drops in stage V (first juvenile). The swimming speed and maximum duration of swimming also increase substantially after metamorphosis (Ennis, 1986). There are changes prior to metamorphosis as well, however. In *H. gammarus*, a significant increase in the rate of exopodite beating occurs at stage III (Laverack *et al.*, 1976).

Because some data on larval physiology is already available for *Homarus sp.*, *H. americanus* seems particularly suitable for investigations into the role of hemocyanin in the respiratory system. Although hemocyanin has been demonstrated to be present in all larval stages of *C. magister* (Terwilliger and Terwilliger, 1982) and in the megalops of two other crabs (Markl, 1986), it

remains to be demonstrated that it occurs in the larvae of *H. americanus*.

Providing the hemocyanin does occur in larval forms, it has to be demonstrated that it plays any role in the respiratory system. This initial study was intended to answer those questions.

METHODS

The presence and concentration of hemocyanin was determined by absorbance of the oxygenated hemolymph sample in saline at 337 nm. The concentration was calculated using the extinction coefficient $E_{1\text{ cm}}^{1\%} = 2.69$ determined for *H. americanus* by Nickerson and Van Holde (1971). Column chromatography was done on a 117 cm long BioGel A-5M gel filtration column using the saline described in chapter two to pack the gel and as a buffer to run the samples through the column. The fractions were analyzed spectrophotometrically at 280 nm to isolate fractions containing protein and 335 nm to isolate hemocyanin containing fractions. Transmission electron microscopy was done on the fractions containing the two putative forms of adult hemocyanin at the University of Oregon.

Heart rates were measured by timing the number of seconds required for 30 beats in an animal viewed under 10 X magnification in the AO compound microscope. Heart diameters were measured using an ocular micrometer under the same scope.

Detection of copper on the native gels was based on quenching of UV fluorescence of bathocuproine sulfonate by copper containing proteins. The protocol for this test is given in chapter two.

RESULTS

The concentration of hemocyanin in the various stages of *H. americanus* is shown in figure 1. Hemocyanin levels are fairly constant through the four larval stages, varying only from 8-12 mg/ml. The level increases gradually through the juvenile and young adult stages, and is five times higher in 5-7 year old adults than in the juvenile forms. These concentrations are based on the absorbance of hemolymph samples at 337 nm, the wavelength corresponding absorption of the copper-oxygen bond at the hemocyanin active site. The hemocyanin concentration is calculated from this absorbance using the extinction coefficient determined for this species by Nickerson and Van Holde (1971) to be 2.69. Therefore only active, oxygen-binding hemocyanins are included in the amounts shown in figure 3.1. This is an important distinction since some adult lobsters give two bands on native gels, one migrating the appropriate distance for a dodecameric hemocyanin and one the appropriate distance for a hexameric hemocyanin. The arrangement of monomers in the hexamer and dodecamer are diagrammed in figure 3.2. The proteins in both these bands contain copper based on the fluorescence quenching technique. In electromicrographs these two

forms give the outline expected from the model in figure 3.2. However, when the two forms are separated by gel chromatography on BioGel A5-M in a physiological saline, only the dodecameric form shows the 337 nm copper-oxygen bond peak. The absorbance at 337 nm (hemocyanin active site) and 280 nm (amino acid side chains, indicative of the presence of protein) for the fractions of the two adult forms are given in figure 3.3. Both fractions contain protein, but only the larger form is an oxygen-binding hemocyanin.

Using the hemocyanin concentrations in figure 1, one can calculate carrying capacities for the different stages. These are shown in Table 1. The carrying capacity for hemocyanin is determined as follows:

$$\frac{\text{mg/ml hemocyanin}}{\text{m.w. subunit}} * \frac{1}{1000} = \text{mmole O}_2/\text{L}$$

This value can be divided by 2.24 to give the carrying capacity in volume percent. The amount of oxygen physically dissolved in solution at 20 degrees is 5.35 ml/L (see table in Green and Carritt, 1967). This converts to 0.24 mmoles/L (=0.5 vol %). This amount is added to the carrying capacity of the hemocyanin to get the carrying capacity of the hemolymph fluid. An examination of table 1 reveals that, in the hemolymph of the larval stages, roughly two thirds of the oxygen in the hemolymph is carried in solution. Only a fourth of the available oxygen is carried in solution in the adults.

If an average heart rate and estimate of stroke volume are available for each stage, these measurements can be combined with the carrying capacity to estimate the amount of oxygen delivered from the heart to the organism each minute. Heart rates and diameters measured for the lobster larvae are given in Table 2, along with the calculated heart volumes and cardiac fluid outputs. A number of assumptions are built into these calculations. The heart volumes were calculated assuming that the heart was spherical. The heart appears round in when viewed dorsally through the scope and diameters measured in perpendicular directions across it were the same, but the actual shape of the heart in three dimensions is unknown. Also, to calculate the cardiac fluid output using the formula at the bottom of the table requires making an estimate of the portion of the heart volume which is expelled with each beat (the stroke volume). For the cardiac outputs in Table 2 stroke volume was considered to be 60% of the cardiac volume. This value is well within the range calculated for adult crabs (Burnett *et al*, 1981) and a stroke volume that large is necessary to ensure that more oxygen is delivered to the tissues than is routinely respired (see table 3).

DISCUSSION

The first figure in this chapter illustrates that functional hemocyanin is indeed present in all larval stages of *H. americanus*. The concentration in the hemolymph of larvae is substantially lower than that found in the adult of this

species or of most other crustacean species, which may lead one to question whether the larval hemocyanin actually plays a role in respiration. However, hemocyanin-bound oxygen actually makes up roughly one third of the oxygen potentially available to the larva from its hemolymph in each of the stages. The adult, by contrast, not only has an overall carrying capacity triple that of any of the larval stages, but the hemocyanin-bound oxygen makes up roughly two thirds of the oxygen potentially available. In adults, it has been demonstrated that the amount of oxygen physically dissolved in the hemolymph is probably sufficient for normal resting respiration (Spoek, 1974). It has been suggested that the hemocyanin-bound oxygen serves as a reserve for times of high activity (Spoek, 1974), or for tissues respiring at a higher rate than surrounding ones (McMahon, 1985).

It is unlikely that this situation holds true for the larval stages of the lobsters. Their metabolic rates are significantly higher than those of later benthic stages (Capuzzo and Lancaster, 1979a), and their pelagic habitat requires much more active swimming. Table 2 shows the measured values for heart size and heart rate in the larval stages of *Homarus*. These measurements can be used to determine the flow of hemolymph out of the heart, provided that the heart is assumed to be spherical and an estimate of the proportion of heart volume utilized as stroke volume. These calculations were made for the larvae assuming a stroke volume of 60% of heart volume. The stroke volume gives, when multiplied by the hemolymph carrying capacity for each stage, a rate of oxygen

delivery just exceeding the measured respiration rates for the first three stages. A stroke/cardiac volume ratio of 0.6 is well within the range found by Burnett *et al.* (1981) for adult crabs, for which they calculated an average ratio of approximately 0.5. The measurements given for heartbeats and volumes are preliminary, but they illustrate that the larvae may well need all the oxygen available from their hemolymph for normal metabolism. Since the hemocyanin-bound oxygen constitutes close to one third of the carrying capacity, its presence may be vital to maintaining aerobic respiration in the pelagic larvae of the lobster.

The role of the hemocyanin in the fourth stage larvae and the early juveniles is not as clear. The heart diameter could not be measured directly for the fourth stage, since the carapace is too opaque to view the entire heart, so this value was calculated from the measurement for the third stage, assuming that the heart diameter was proportional to the overall length of the animal in both stages. As can be seen from the values in Table 3, this results in a shortfall of oxygen delivered to the tissues based on theoretical cardiac output compared to measured respiration rate. This is particularly interesting since the weight specific metabolic rate, which increases with stage through the first three larval stages, drops in the fourth stage. These calculations seem to indicate that the heart is probably proportionally larger in the fourth stage than in the first three. This is not improbable, considering the other massive changes in physical characteristics that occur at this stage, but there is no data to support this hypothesis currently.

Juveniles were too opaque to allow even the measurement of heart rate by direct observation.

This preliminary data has demonstrated the presence and potential usefulness of hemocyanin in the early developmental stages of the lobsters, but some interesting information has come to light concerning the adult hemocyanin as well. Two different aggregates of hemocyanin, usually a dodecamer and hexamer, have been described in a number of decapod crustaceans (Van Holde and Miller, 1982). In *Cancer magister* both forms actively bind oxygen, as demonstrated by absorbance at 337 nm (Terwilliger *et al.*, 1986). This appears not to be true for *Homarus americanus* based on the results of spectrophotometric analysis of the fractions from the gel filtration studies. The fraction containing the dodecameric form of hemocyanin shows the characteristic spectrum of oxyhemocyanin (illustrated in figure 3), but the fraction containing the putative hexamer absorbs only at 280 nm, the wavelength characteristic of amino acid sidechains from tyrosine and phenylalanine. It is important to consider the two adult forms separately in the studies described in following chapters.

SUMMARY

Initial investigations into the developmental changes in structure and function of hemocyanin in American lobsters have shown that active hemocyanin is present in all larval stages. The concentrations of hemocyanin are only about 20%

of that found in the adult and are also low compared to concentrations found in adults of other species. However, calculations made from measured heart rates and heart volumes illustrate that this low level of hemocyanin may play an important role in the respiratory system of the larval lobsters. It is possible larval lobsters use hemocyanin-bound oxygen in routine metabolism, while the bound oxygen is generally regarded in the adult as a venous reserve for high oxygen demand. Also, the adult appears to have only one active form of hemocyanin (a dodecamer). The adult hemolymph sometimes contains a second protein, the same size as a hexameric hemocyanin, but without the capability to bind oxygen. The structure of both these proteins, as well as that of the larvae, is characterized in subsequent chapters.

HEMOCYANIN LEVELS IN HEMOLYMPH

Figure 3.1. Hemocyanin concentration in lobster hemolymph as measured spectrophotometrically by absorption at 335 nm. Two sets of pooled were used to obtain concentrations for larval stages I-IV. Juvenile and adult are averages of 2, 3, and 5 samples, respectively.

ABSORBANCE OF COLUMN FRACTIONS

Figure 3.2. Absorbance at 280 nm and 335 nm of adult lobster hemolymph fractions in lobster saline. Eluant was collected in 3 ml fractions after 100 mls had been eluted. Fraction # 55 starts at 197 mls eluted. The peak on the right is from the putative hexamer and absorbs only at 280 nm. The other peak is from the dodecameric (active) hemocyanin and therefore absorbs at both wavelengths.

W	ABS	SOURCE	MODE
320.0	2.0212	100% (10)	Scan

Figure 3.3. Tracing of spectrophotometric absorbance of oxygenated (○) and deoxygenated (—) adult lobster hemocyanin diluted 1:9 in saline.

Figure 3.4. Model representation of the arrangement of monomers into dodecamers and hexamers in lobster hemocyanin. The kidney bean shapes each represent one monomer containing a single oxygen binding site.

Table 3.1. Carrying capacity for hemocyanin (Hc) of *H. americanus*. Carrying capacities were calculated using the measured hemocyanin concentration and assuming the molecular weight of the subunits to be 76,000 Daltons. Each subunit was presumed to contain a single oxygen binding site. The hemolymph carrying capacity was calculated using tables for oxygen solubility in seawater at 20°C from Green and Carritt (1967).

Stage	Hc (mg/ml)	Hc C. C. (vol %)	Hc C. C. (mmole/l)	Hemolymph C.C.(mmole/l)	Hc/hemo C.C. ratio
I	8	0.23	0.11	0.47	0.23
II	12	0.38	0.17	0.62	0.27
III	12	0.35	0.16	0.59	0.27
IV	9	0.26	0.12	0.50	0.24
J	15	0.44	0.20	0.68	0.29
A	50	1.47	0.66	0.89	0.74

Table 3.2. Estimated cardiac output for *H. americanus*. Heart rates were measured for all four larval stages. Heart diameters were measured for the first three stages and extrapolated for the fourth stage as noted. Heart volumes were calculated using the diameters and assuming a spherical heart. This heart volume was used to estimate cardiac output by the method described in note a.

Stage	heart rate b.p.m. + s.e.(n)	heart dia. u + s.e.(n)	heart volume (10 ⁻⁵ ml)	cardiac output ^a (ml/min)
I	267 ± 54 (31)	330 ± 41	1.88	0.0030
II	283 ± 45 (35)	370 ± 44	2.65	0.0045
III	308 ± 54 (30)	437 ± 48	4.05	0.0081
IV	294 ± 53 (10)	874 ^b	35	0.0617
A	96 ^c			60.5 ^c

^aCardiac fluid outputs calculated as (h.r.) * (h.v.) * (% h.v. as s.v.). Stroke volume was presumed to be 60% of the heart volume.

^bcalculated from SIII value, presuming heart size as proportional to ashfree dry weight (from Sasaki, 1984).

^cdata from McMahon and Wilkens, 1975

Table 3.3 Oxygen balance for larval *H. americanus*.
Respiration rates are from literature values; the amount of oxygen supplied was determined from heart rates, carrying capacities, and cardiac outputs (h.r. X car. out. X (c.c./100)).

<u>Stage</u>	<u>oxygen supplied</u> <u>(10⁻⁶ mmole/min)</u>	<u>oxygen respired^a</u> <u>(10⁻⁶ mmole/min)</u>	<u>r/s</u> <u>ratio</u>
I	1.41	1.12	0.79
II	2.79	2.47	0.88
III	4.76	4.31	0.90
IV	30.8	10.7	0.35
A	53,845	11,880 ^b	0.27

^adata from Capuzzo and Lancaster (1979a) for starved animals

^bdata from Leavitt (1986) for 400 g wet weight lobster

CHAPTER IV

SIZE AND FUNCTIONAL PROPERTIES OF HEMOCYANIN OLIGOMERS

INTRODUCTION

Hemocyanin serves as the respiratory pigment for a large number of marine invertebrates as well as the insects and spiders. The wide distribution of hemocyanin, combined with its physiological importance, renders this protein as an ideal model for the study of both how proteins change through evolution and of the links between structure and function. From a physiologist's point of view, changes in hemocyanin yield clues that aid in understanding the limits of an organism's distribution and its response to environmental conditions.

Although quite different structurally, hemocyanin functions analogously to the hemoglobins found in many vertebrate species. Both proteins reversibly bind oxygen to carry it from the respiratory surface to the tissues. In both proteins there are aggregates of multiple subunits which act cooperatively to produce a sigmoid curve for the saturation of binding sites on the protein with oxygen. The functional characteristics of both hemocyanins and hemoglobins are described using the same terminology. The cooperativity is usually expressed as the slope of $\log Y/1-Y$ vs. PO_2 , where y is the % of binding sites saturated and PO_2 is the oxygen tension. The oxygen tension at which half the sites are saturated is referred to as the P_{50} . These two measurements allow one to describe the shape and placement of the oxygen binding curve on a graph of oxygen tension versus percent saturation, and allows for comparisons between species and of hemolymph from one species under differing conditions.

Both types of respiratory pigments also respond to allosteric effectors. This is particularly true of the hemocyanins which exist free in the hemolymph instead of being protected within the walls of an erythrocyte. Protons, carbon dioxide, lactate and a number of other organic ions have been demonstrated to alter oxygen binding by some or all forms of hemocyanins (Truchot, 1975; Stedman and Stedman, 1926; Bridges *et al.*, 1984; Morris, 1990; Burnett and Infantino, 1984). Effectors often shift the PO_2 , but can change cooperativity as well (e.g., lactate alters both properties). The level of effect often differs greatly from one hemocyanin to the next, as might be expected from their structural heterogeneity.

Hemocyanins occur distributed throughout the crustacean species in a number of aggregation states. The size of the aggregates in which these subunits occur appears far more variable than the structure within the subunit itself. The most common aggregation states are hexamers and dodecamers, particularly amongst the crustaceans (reviewed by Herskovits, 1988; Markl, 1986; and Van Holde and Miller, 1982). In a number of cases a single organism contains more than one form of hemocyanin: the hemolymph contains primarily dodecamers with a minor component of hexamers in *Cancer magister* (Ellerton *et al.*, 1970), *Homarus gammarus*, *Cancer pagurus* (Markl *et al.*, 1979), and *Callinectes sapidus* (Herskovits *et al.*, 1981).

The variety of hemocyanin aggregate size amongst the decapods prompts investigation of possible evolutionary trends in structure, as well as attempts to correlate hemocyanin structural and functional aspects with each other and with anatomical and habitat parameters. In the past, these investigations have been conducted through survey studies of numerous species. In some studies attempts have been made to correlate aggregate size with phylogeny (Markl, 1986) or to correlate functional parameters with habitat (Young, 1972a; Mangum, 1982; Taylor *et al.*, 1985). In all cases, no clear cut trends were revealed because of the complications inherent in differences in evolutionary history, morphology, and other factors.

Some of these problems can be circumvented by examining developmental changes within a single species, particularly in the Crustacea. The life histories of the crabs and lobsters in particular are fairly well known and represent a good opportunity to follow a single species through changes in habitat (from pelagic larvae to benthic adults) and dramatic changes in form (metamorphosis from larvae to adult). Some initial studies on ontogenic changes in structure (Markl, 1986) and structure and function (Terwilliger *et al.*, 1986) have compared megalops, juveniles, and adults from several species of crabs. These studies documented shifts with development in functional parameters and size of the hemocyanin oligomer.

This work follows the course of these physiological parameters in *Homarus americanus*. This species has larvae large enough to ensure that hemolymph from all developmental stages can be sampled, allowing for comparison between pelagic stages as well as between larvae and adults. Since metamorphosis of larval stages occurs before the change in habitat, it may be possible to separate the impacts of these two events on the proteins of the respiratory system. A better understanding of larval physiology of lobsters may also help in interpreting differences in growth and mortality among the larval stages.

METHODS

Larval Culture

Female egg-bearing lobsters from coastal waters or from Georges Bank were obtained from the Massachusetts Lobster Hatchery on Martha's Vineyard, MA. The females were held in individual tanks with flowing seawater until they released their hatching larvae. The larvae were scooped from the mother's tank and placed in plankton kriesels supplied with flow-through seawater ($T = 18-21^{\circ}C$, $S = 28\%$) The larvae were kept under a varying light-dark regime with at

least 8 hours darkness per day. The larvae were reared on a diet of frozen brine shrimp plus a fatty acid supplement (Sasaki, 1984) containing two essential fatty acids (20:6 3 and 22:6 3 fatty acids) missing from the shrimp. Larvae were batch-reared in kriesels until stage IV, at which time they were separated into individual trays that floated on the surface of a large tank. They were separated from one another during the fourth and juvenile stages, but were reared on the same diet as the younger stages.

Sample Collection

Larvae were removed from the plankton kriesels, then sorted by stage and molt staged using the method of Sasaki (1984). Only intermolt (molt stage C) animals were sampled for the studies conducted here. The hemolymph was removed from the pericaridal sinus of the larvae using a 5 ul Hamilton syringe under a dissecting microscope. The samples from individual animals were frozen in 0.6 ml Eppendorf centrifuge tubes at -20° C. All samples were checked before freezing to ensure that no material from the hepatopancreas had contaminated the sample. A hemolymph sample from a single individual was loaded into each well for a native electrophoretic gels. For the oxygen binding studies hemolymph from each stage was pooled into two separate samples. These two samples were then subdivided into 20 ul aliquots for analysis at different temperatures in order that multiple runs could be made for each stage at each temperature.

Hemolymph samples had to be pooled because each analysis of oxygen binding required about 20 ul (5 ul for the absorption chamber and 10-15 ul for the pH determination aliquot). Two samples of pooled hemolymph for each stage compensated for individual variation in hemocyanin concentration and lactate level.

Native (Non-denaturing) Gels

The ratio of hexamer to dodecamer was determined using 5% T, 2.7% C polyacrylamide minigels (8 X 10 cm, 1 mm thick). These gels used the buffer system described in Chapter Two; the buffer contained 18 mM calcium to prevent disaggregation of the hemocyanin. The gels were run for one hour at 35 mAmps, then stained with Coomassie Brilliant Blue.

Oxygen Binding Curves

The studies on oxygen binding were conducted at the Zoology Department of the University of Glasgow in collaboration with Dr. Alan Taylor. The microtonometer setup used is described fully in Chapter Two. Briefly, a 3 to 5 ul drop of hemolymph is centered in a recessed plastic slide inside an O ring. The slide is inserted in a chamber and clamped tight so that the O ring forms the sides of an airtight chamber. A stream of humidified gases flows through the chamber just above the droplet so that the gases equilibrate with the hemolymph.

Both the chamber containing the droplet and the stream of gases are encased within a water jacket so that the apparatus is maintained at the desired temperature.

Fiber optic cables set above and below the chamber containing the test droplet transmit the absorbance signal to a detector which amplifies and records the absorbance at 338 nm. This absorbance is then recorded on a chart recorder. The absorbance at 338 nm (the absorbance wavelength of the oxygen-copper bond in hemocyanin) is used to construct the oxygen binding curve. Another 10-15 ul of hemocyanin is equilibrated with the same stream of gases in another part of the water jacket. The pH of the sample is monitored with a pH microelectrode (Microelectrodes, Inc. MI-415). Two Wosthoff gas mixing pumps were used to provide precise, reproducible combinations of nitrogen, carbon dioxide, and oxygen to the samples. Varying levels of carbon dioxide were used to alter the pH of the sample so that oxygen binding curves could be constructed over a range of pHs.

Oxygen binding curves were generated at pHs produced by 0.1 to 1 % carbon dioxide at both 20 and 25 ° C for all stages, except stage III; insufficient hemolymph was available from this stage to record measurements at the higher temperature.

In vivo pHs for each stage were obtained by inserting a pH microelectrode into the pericardial sinus of the larvae to obtain the venous pH. No comparative data on larval *in vivo* pHs were available from the literature, but adult readings were identical to pH venous values (McMahon and Wilkens, 1977).

RESULTS

The shift during development in the proportions of the two sizes (hexamer and dodecamer) of hemocyanin aggregates found in the hemolymph of American lobsters is demonstrated in Figures 1 through 3. The first two stages have almost exclusively hexameric hemocyanin, with only 4% and 20% dodecamer in stages I and II, respectively. The third stage larvae contain almost equal proportions of the two forms, and the larger aggregate clearly predominates in Stage IV animals. Juveniles lobsters contain only dodecameric hemocyanin.

Adult lobsters present a more complex pattern. Some individuals contain only dodecameric hemocyanin, and give a pattern corresponding to lane AD (signifying adult dodecamer) in Figure 1; others contain the dodecamer combined with an additional band as shown in lane AS (signifying adult serum) of the same figure that migrates the same distance as the larval hexameric hemocyanins and contains copper, but does not produce the ultraviolet absorption spectra characteristic of oxygen binding by hemocyanin when the two forms are separated by gel

chromatography.

This second protein band is discussed in depth in Chapter 5. Its presence is not related to gender; it occurs in both males and females. A sample of 19 female adult lobsters (held at 20 ° C), all of which had recently released larvae, contained 17 individuals with only the upper band (dodecameric hemocyanin), and 2 with both bands. Six males held at the same temperature showed only the upper band in 4 animals; with a small amount of the second band in 2 animals. The presence of the second band, however, cannot result solely from phenotypic variation: a lobster obtained from a seafood dealer possessed both bands during the first few months it was held, then showed only one band at a later sampling date. The animal was first sampled while held in warm water, and contained only one band when the water temperature had fallen to 10 ° C. Both bands were found in most animals from seafood dealers, and may be related to holding conditions. This variation in banding patterns on electrophoretic gels was not seen in the numerous gels of larval hemocyanins.

Oxygen binding curves constructed for each stage were plotted on a $\log P_{50}$ versus pH graph and linear regression lines calculated for each stage over the pH range at which measurements were taken. This pH range varied slightly from one stage to the next depending on the buffering capacity and concentration of hemocyanin in the sample since the pH was changed by altering the concentration of carbon dioxide. The correlation coefficients for these regression lines are

given in Table 1.

The calculated values of $\log P_{50}$ for each stage over a standardized pH range of 6.8 to 8.4 are presented in Figure 4. P_{50} values were calculated from the regression lines using the actual data for each stage. An analysis of covariance demonstrated that the slopes of the Bohr shifts for the first two larval stages differed significantly ($p < .05$) from those of the remaining stages. The slopes of the regression lines for the first two stages were not significantly different from one another; the slopes of the remaining stages (III, IV, juvenile, and adult) did not differ significantly from one another ($p < .05$). The substantial increase in the slope of the Bohr shift as the larvae develop from Stage II to Stage III represents the only significant change in this parameter. The slopes of the regression equations for each stage at 20 and 25 ° C are presented in Table 2.

An additional ontogenic change that occurs in *Homarus americanus* is also presented in Figure 4. The P_{50} of stage III at any pH above 7.0 is lower than that of the other larval stages, including stage IV and the juvenile. This difference is particularly pronounced at pH 7.6, the *in vivo* pH for stages III and above. Oxygen binding curves for hemocyanin from each stage measured closest to *in vivo* pH which still displayed the cooperativity and P_{50} characteristic of that stage at that pH are presented in Figure 5. *In vivo* hemolymph pHs based on measurements inside the pericardial hemolymph sinus using a pH microelectrode are presented in Table 3. Oxygen binding curves for stages I, II, IV, and the

juvenile are similar and clearly fall in a group, while that of Stage III lies between the other stages and the adult. Since the position of the oxygen binding curve shifts with changes in pH at all stages, it is useful to plot individual curves at pH 7.6 for all stages, even though this is not the *in vivo* pH for the first two stages. The distinction between Stage III and the other pre-adult stages becomes even more pronounced, as shown in Figure 6.

Oxygen binding curves obtained at a high and low pH (about 0.7 pH units apart) are presented in Figures 7 through 12, illustrating the physiological significance of the differences in P_{50} and Bohr shift between stages. In the first two stages a substantial amount of oxygen is released from the hemocyanin in response to the pH drop at very high oxygen tensions: 40% released at 50 mm Hg and 60% released at 40 mm Hg for stages I and II, respectively. In stages III, IV, and the juvenile hemocyanin releases 50 - 70% of the oxygen bound to their hemocyanin at lower oxygen tensions between 20 and 30 mm Hg. Yet the adult hemocyanin releases no oxygen from its hemocyanin at oxygen tensions of 20 - 40 mm Hg, even with a drop in pH of 0.7 unit; however, 50% of the bound oxygen is released in response to the same pH drop at an oxygen partial pressure of 10 mm Hg. From the figures, it is suggested that larvae and juveniles tend to use hemocyanin bound oxygen for routine metabolism whereas adults maintain it as a venous reserve, even in response to metabolic acidosis.

Measurements of cooperativity were also made on the samples from the oxygen binding studies. For comparative purposes, the cooperativity (N_{50}) is most often expressed as the slope of the oxygen binding curve at 50% saturation of the pigment. The cooperativity measured at different pHs is presented for each stage in Figure 13. Linear regressions on the data from this Figure revealed no correlation between N_{50} and pH at any stage ($R^2 < .3$, except $= .7$ for SI). This can be seen in Figure 15, which shows the N_{50} at all pHs for all stages. A one-way anova analysis showed no significant added variance for N_{50} between stages over that seen for a single stage between pHs.

Oxygen binding curves were also constructed at 25 ° C for each stage as well, except for Stage III which could not be measured at the higher temperature due to a lack of hemolymph samples. The Bohr shift plots for the higher temperature are presented in Figure 15; this plot was constructed in the same manner as Figure 1. This figure demonstrates that neither the slope of the Bohr effect nor the actual P_{50} values differ significantly between the two temperatures for the larval stages or the juveniles; analysis of covariance of P_{50} s confirms this observation. Comparison of Figure 15 and Table 2 for adult hemolymph samples, however, demonstrates a clear displacement of the P_{50} s for any pH with temperature, but no accompanying change in the slope of the Bohr effect. The calculated temperature sensitivities for each stage are presented in Table 4, using a formula from Taylor *et al.* (1985) which is given under the table. Clearly only the adult animals display substantial temperature sensitivity in the temperature

range measured. The two temperatures used are well within the range experienced by larval stages, but the higher temperature is definitely at the upper limit of the range for the adults.

A number of effectors can alter the oxygen binding properties of hemocyanin, the most well studied of these is lactate. Lactate acts as an effector for a wide range of crustacean hemocyanins, and has been demonstrated to act as an effector on the hemolymph of *H. americanus*. Oxygen binding studies were conducted using unadulterated hemolymph, to ensure *in vivo* concentrations of ions and prevent interference with oxygen binding by buffer components. The lactate concentrations in each of the pooled samples used to produce the oxygen binding curves are presented in Table 5, in addition to the average lactate concentration for each stage. The levels are fairly consistent with concentrations between 0.5 and 0.7 mM for all stages. The variability between the pools for each stage is also low, except in the case of the juvenile samples.

Table 4.1. Regression coefficients (R^2) of Bohr effect at 20 C.

<u>STAGE</u>	<u>R^2</u>
I	-0.9
II	-0.97
III	-1.0
IV	-0.99
J	-0.96
A	-0.96

Table 4.2. Slopes of regression lines for Bohr effect at each temperature for each stage. At the lower temperature, the only significant change in slope occurs between stages II and III.

<u>STAGE</u>	<u>SLOPE (20 C)</u>	<u>SLOPE (25 C)</u>
I	-0.43	-0.53
II	-0.51	-0.51
III	-0.70	-----
IV	-0.77	-0.93
J	-0.84	-1.10
A	-0.84	-0.77

Table 4.3. *In vivo* pH of hemolymph at 20°C. These pHs were measured using a microelectrode inserted directly into the pericardial cavity.

<u>STAGE</u>	<u>pH</u>	<u>S.D.</u>	<u>N</u>
I	7.4	0.09	2
II	7.3	0.11	2
III	7.6	0.05	2
IV	7.6	0.06	8
J	7.6	0.03	4
A	7.6	0.03	7

Table 4.4. Temperature sensitivity of oxygen binding by *H. americanus* over the two temperatures measured (20 and 25°C).

<u>STAGE</u>	<u>DELTA H</u>
I	+9.9
II	+3.3
III	-----
IV	+13.2
J	+19.8
A	-132

formula: $H = -2.303 \times R \times \frac{\log P50(KJ/Mole)}{(1/T_1 - 1/T_2)}$

Table 4.5. Hemolymph lactate levels measured using an assay for NADH production by lactate dehydrogenase (Goodmann and Wahlfeld, 1974).

<u>STAGE</u>	<u>[LACTATE]</u>	<u>AV. [LAC] (mM)</u>
I	0.71	0.69
	0.68	
II	0.49	0.49
III	0.54	0.58
	0.49	
IV	0.48	0.49
	0.51	
J	0.74	0.58
	0.43	
A	0.69	0.69

Table 4.6. Properties of the hemocyanin of selected crustaceans. (P) indicates the primary form found in the hemolymph. Bohr shifts from Mangum (1982); aggregate sizes from Van Holde and Miller (1982).

SPECIES	AGGREGATE SIZE	BOHR SHIFT
<i>Penaeus monodon</i>	6(P),12	-1.3
<i>Birgus latro</i>	12	-0.9
<i>Panulirus vulgaris</i>	6	----
<i>P. versicolor</i>	---	-0.4
<i>P. interruptus</i>	6	----
<i>Homarus americanus</i>	12	----
<i>H. gammarus</i>	12(P),6	----
<i>Nephrops norvegicus</i>	12	----
<i>Callinectes sapidus</i>	12(P),6	----
<i>Cancer magister</i>	12(P),6	----
<i>Carcinus maenas</i>	12(P),6	----
<i>Hyas araneus</i>	12	----
<i>Potamon edulis</i>	12(P),6	----
<i>Scylla serrata</i>	-----	-0.7
<i>Menippe mercenaria</i>	-----	-1.3
<i>Portunis pelagicus</i>	-----	-1.3
<i>Calappa philargius</i>	-----	-0.4

Figure 4.1. Nondenaturing (native) gels of larval and adult hemocyanins. Roman numerals denote larval stage (all molt stage C); AS = adult whole serum (clot removed); AD = purified adult dodecamer. The two major bands represent dodecameric (upper band) and hexameric (lower band) hemocyanin. The narrow third band appearing above the two major bands is a non-hemocyanin serum protein.

Figure 4.2. Nondenaturing (native) gel of larval stages III and IV (the pre- and post-metamorphic stages) at different molt stages (denoted by letters below numerals: C is intermolt, D is premolt, and B is postmolt). BA = bovine serum albumin; OA = ovalbumin. Stage III hemolymph contains approximately equal proportions of dodecameric and hexameric hemocyanin, while the larger dodecameric hemocyanin predominates in Stage IV.

Figure 4.3. Nondenaturing (native) gel of larval hemocyanin. Percent of the hemocyanin as hexamer, for lanes from left to right: 52%, 61%, 84%, 76%, 98%, and 94% (last lane unanalyzed).

Figure 4.4. Regression lines for Bohr shifts of hemocyanin at 20°C. The only statistically significant change in slope occurs from stage II to stage III. The slope of the regression does not differ significantly amongst stage II, IV, the juvenile, or the adult, though in both stage III and the adult the actual P_{50} at any given pH is lower than that of the curves from other stages.

Figure 4.5. Measured oxygen binding curves at near *in vivo* pH for all stages at 20°C. The measurements were taken at the following pHs: I - 7.32, II - 7.24, III - 7.66, IV - 7.60, J - 7.54, A - 7.64. See Table 3 for measured *in vivo* pHs for each stage. The P_{50} for stage III is lower than that of stages I, II, IV or the juvenile, but still higher than that of the adult.

Figure 4.6. Measured oxygen binding curves at pH 7.6 for all stages at 20 ° C. The measurements were taken at the following pHs: I - 7.66, II - 7.58, III - 7.66, IV - 7.60, J - 7.54, A - 7.64. See Table 3 for measured *in vivo* pHs for each stage. Again, the binding curve for hemolymph from stage III animals is intermediate between the adult and the other larval and juvenile stages.

Figure 4.7. Oxygen binding curves for stage I taken approximately 0.7 pH units apart at 20°C. Stage I curves at pH 6.93 and 7.66. At 50 mm Hg oxygen tension, 40% of the bound oxygen would be released from the hemocyanin in response to the drop in pH.

Figure 4.8. Oxygen binding curves for stage II taken approximately 0.7 pH units apart at 20°C. Stage II curves at pH 7.24 and 7.82. In response to this drop in pH, 60% of the oxygen bound to the hemocyanin would be released to the tissues at an oxygen partial pressure of 40 mm Hg.

Figure 4.9. Oxygen binding curves for stage III taken approximately 0.7 pH units apart at 20°C. Stage III curves at pH 7.10 and 7.87. If their blood pH fell this 0.7 units while the hemolymph oxygen partial pressure remained between 20 and 30 mm Hg, the majority of oxygen bound to the hemocyanin would be released.

STAGE IV H. AMERICANUS

Figure 4.10. Oxygen binding curves for stage IV larvae at 20°C approximately 0.7 pH units apart. Curves at pH 7.52 and 8.20. Again, the pH drop would result in the majority of the oxygen being released from the respiratory protein while the hemolymph oxygen partial pressure was still between 20 and 30 mm Hg.

Figure 4.11. Oxygen binding curves for juvenile lobsters taken approximately 0.7 pH units apart at 20°C. Juvenile curves at pH 7.54 and 8.19. Hemolymph from this stage shows the same trend in response to a drop in pH as do stages III and IV (figures 4.9 and 4.10).

ADULT H. AMERICANUS

Figure 4.12. Oxygen binding curves for adult lobsters taken approximately 0.7 pH units apart at 20°C. Adult curves at pH 7.32 and 8.0. Note that a drop in pH would not lead to the release from the hemocyanin of a significant fraction of the bound oxygen until the oxygen partial pressure in the hemolymph dropped to 10 mm Hg.

Figure 4.13. Hill coefficient of cooperativity at 20°C for each stage at all pHs tested. There is no significant change in N_{50} with pH in any stage, except possibly in stage I.

Figure 4.14. Hill coefficients of cooperativity at both 20°C (upper graph) and 25°C (lower graph) for each stage. There is no significant change with temperature.

BOHR SHIFT REGRESSIONS (25°C)
HOMARUS AMERICANUS

Figure 4.15. Regression lines for Bohr shifts of all stages at 25°C. Note the similarity to figure to the pattern in figure 4.4. Although stage III hemolymph was not measured at this higher temperature, one sees the same shift in the slope of the regression line after stage II as was seen at 20°C (figure 4.4).

DISCUSSION

A shift in the size of hemocyanin oligomers during the life history of a crustacean has been documented previously in several species of crabs. The pattern of hemocyanin structural changes seen in crab species differs from that demonstrated here for American lobsters. The first description of these developmental changes was given for *Cancer magister* (Terwilliger and Terwilliger, 1982). In that species, megalopa possess predominantly hexameric hemocyanin; the juveniles have equal proportions of hexamer and dodecamer, whereas the adults contain primarily dodecamer with some hexamer present and both forms actively bind oxygen.

Brachyuran crabs have a slightly different form of development than the macruran lobsters. In crabs the female releases small zoea which pass through several further zoeal stages, then molt to a megalopa (Phillips and Sastry, 1980). The megalopa settles to the bottom, then molts immediately into a first stage juvenile. The crab megalopa corresponds to the Stage IV larvae of *H. americanus*. Crab zoea would therefore correspond to lobster larval stages I, II, and III; the lobster larval stages are generally substantially larger than the crab zoeal stages.

Developmental changes in two other species of crabs have also been

investigated. In both *Hyas araneus* and *Carcinus maenas* the aggregation level of hemocyanin in zoea and megalopa is predominantly hexameric, though some dodecamer can be seen in electron micrographs (Markl, 1986). Dodecameric hemocyanin does not predominate in *Carcinus maenas* until the second juvenile stage (Markl, 1986). In all the brachyuran and anomuran crabs studied, the ontogenic shift in oligomer size occurs in the juvenile stages; in the American lobster the shift occurs much earlier, in the third stage larvae, and appears to be completed by the first juvenile stage.

The hemocyanin aggregate types occurring in various adult crustacean species are listed in Table 6. For the most part, crustaceans possess hexamers, dodecamers, or both forms in their hemolymph. In most cases where both forms appear, the larger dodecamer predominates. This is likely related to reduction of osmotic problems by having fewer, larger particles in the hemolymph. The developmental trend toward larger aggregates seen in both crabs and lobsters may occur at least in part for this reason.

The aggregate size of hemocyanin is determined not only by the structure of the protein subunits, but also by the ionic environment in the hemolymph. A number of studies have demonstrated control of association state by pH and levels of divalent cations. The main structural consequence of pH changes on aggregation state of pH changes is denaturation at a threshold pH, usually around 10 for crustaceans such as *H. americanus* (Pickett *et al.*, 1966), *Cancer magister*

(Ellerton *et al.*, 1970), and *Panulirus* (Makino and Kimuro, 1988). At lower pHs, previously dissociated material often reassociates to the hexameric level (Makino and Kimuro, 1988). Whereas changes in pH must have some structural effects in order to alter oxygen binding through the Bohr shift, the radical pH changes necessary to alter aggregation state do not occur *in vivo* and therefore cannot be responsible for the phenomenon seen here.

Divalent cations, particularly those of calcium and magnesium, play a substantial role in determining the aggregation state of hemocyanins. These ions are required in threshold amounts (generally equal to their physiological concentration) to prevent dissociation of the dodecamers to hexamers and the hexamers to monomers. This has been demonstrated in *Carcinus maenas* and *Potamon edulis* (Chantler *et al.*, 1973) and a number of other species, although it is not universal. *Callinectes sapidus* shows only a partial dissociation (not all the way to hexamer) in the absence of divalent cations (Herskovits *et al.*, 1981); this may be one of the adaptations of this species to a euryhaline environment.

Several studies have been conducted on the effects of divalent cations on hemocyanin aggregation in *Homarus americanus*. Pickett *et al.* (1966) described the dissociation of dodecamers to hexamers, and hexamers to monomers (without other intermediates) in lobster hemolymph as calcium ions were removed. The role of calcium ions was elucidated by Morimoto and Kegeles (1971), who demonstrated that the association from hexamer to dodecamer required five

calcium ions and three protons per aggregate. The necessary calcium ions would therefore be provided by a 10 mM concentration, about half the *in vivo* level (Mercaldo-Allen, 1990). In fact, investigations of denaturation rates in salt solutions have shown that the polar and ionic interactions are far more important than hydrophobic forces in stabilizing the dodecamer (Herskovits *et al.*, 1981). The presence of calcium ions stabilizes the dodecamer so that the concentration of urea needed to dissociate the lobster hemocyanin is doubled (Herskovits *et al.*, 1984). It is tempting to hypothesize that the changes in aggregation state seen in the larval stages result from changes in the calcium ion levels. However, two arguments counter this hypothesis. The first is that the aggregation state of all stages were determined in electrophoretic gels and buffers with the adult *in vivo* calcium concentration of 18 mM (Mercaldo-Allen, 1990); it is conceivable that the aggregation states of the larval hemocyanins did not adjust to this new calcium level before electrophoresis was completed. Perhaps a more compelling argument is that the aggregate ratio shifts substantially from the first to the third larval stage, but the ability to osmoregulate ions does not develop until the fourth larval stage (Assad, 1984; Charmantier *et al.*, 1984). It is most likely that the aggregation state in this case is determined by changes in subunit structure, which is discussed in Chapter 5.

The most interesting result from this study is that changes in oxygen binding properties of hemocyanin that occur during larval development are concurrent with the structural changes noted. A single study showed differences in oxygen

binding properties between early juveniles and adults of *Cancer magister* (Terwilliger *et al.*, 1986): at the *in vivo* pH of 7.85 the adult had a P_{50} of 30 mm Hg whereas that of the juvenile was 70 mm Hg. This same difference occurred in the present study, though the P_{50} s of both stages were substantially lower: in the lobster at 20 °C and pH 7.6 the juvenile P_{50} was 40 mm Hg and that of the adult was 10 mm Hg. The P_{50} at *in vivo* pH is quite close for all the larval stages and the juvenile, with the exception of the third stage, which possesses a lower P_{50} .

The present study demonstrates some additional, previously undetected changes during early development of lobster larvae. The most notable of these is the sudden shift in the slope of the Bohr effect which occurs, again, at stage III. This shift is made more prominent by the fact that the two previous stages have the same slope as and the slope for stage III is the same slope as all the subsequent stages. The change in the Bohr shift is sudden, not progressive like the shift in aggregate size, yet its appearance at the same stage that also first demonstrates a substantial quantity of dodecameric hemocyanin suggests that the slope of the Bohr shift and the size of the aggregate may be related, especially in light of the subunit data presented in Chapter 5.

The slope of the Bohr shift for adults of a number of species of crustaceans is presented in Table 6. No strong pattern of change in the Bohr shift is apparent with either increasing degree of terrestrial habitat or differences in mode of gas

exchange (Mangum, 1982). It can also be seen from this table that the magnitude of the Bohr shift for both adults and larvae of the lobster fall within the range of values seen in the species listed. There also seems to be no correlation between the aggregate size determined in other studies with the magnitude of the Bohr shift, although very few examples are available for comparison since previous researchers have examined these aspects separately and seldom worked on the same species. The results from the present ontogenic study indicate a correlation of the magnitude of the Bohr shift and the change in the aggregate size from primarily hexameric to primarily dodecameric hemocyanin. There is a significant increase in Bohr effect at Stage III, when the dodecameric hemocyanin first appears, followed by smaller (not statistically significant) increases in the subsequent stages, in which the percentage of dodecameric hemocyanin also increases.

The increase in the Bohr shift represents an important adaptation for the lobster as it develops. With an increased Bohr shift more oxygen is released to the tissues at lower oxygen tensions during periods of high activity. An examination of the scant literature available concerning behavioral and physiological changes during larval development presents several reasons why this shift may occur in Stage III larvae. *H. gammarus* larvae, which possess an identical developmental pattern, show a 66% increase in dry weight as they enter Stage III. The rate of exopodite beating, which is the same in the first two stages, also increases significantly in Stage III (Laverack *et al.*, 1976), a reflection

of the sudden increase in the energetic cost of swimming. This is supported by Ennis (1988) who observed that Stage III larvae of *H. americanus* spend less time than the first two stages swimming in a flume chamber under either no current or a 2 cm per minute current.

There are changes in the oxygen binding properties of lobster hemocyanin during development besides the change in the Bohr shift. The actual P_{50} of oxygen binding curves, whether considered at *in vivo* pH or at a standardized pH of 7.6, is quite similar among the larval stages and the juvenile, except that it drops 10 mm Hg for the Stage III larvae and then rises again. In addition, the P_{50} then drops 40 mm Hg at the adult stage. The drop at Stage III may represent a case of pre-adaptation: the structure and binding properties of the hemocyanin have changed in anticipation of the different demands of the fourth stage. Fourth stage larvae have substantially thicker exoskeletons than previous stages based on chitin and ash content (Capuzzo and Lancaster, 1979b; Sasaki, 1984), and a completely different type of swimming behavior (Davis and Davis, 1973; Neil *et al.*, 1976); and Stage IV larvae are also able to regulate internal ion concentration (Charmantier *et al.*, 1984). The structural shifts at Stage III could provide a mechanism to compensate for the changes in energy requirements and internal environment (oxygen level and ion concentration) that will occur at the next stage and bring the curve of the hemocyanin back into line with that of the other pre-adult forms. Other biochemical changes in anticipation of metamorphosis also occur at Stage III, such as the shift from polar to neutral lipids for energy storage,

a trend toward longer term storage of energy (Capuzzo *et al.*, 1983).

The difference in the oxygen binding at Stage III could also represent compensation for the very high energy demands of this stage. At Stage III the gills are fully formed (Herrick, 1909) and the exoskeleton is thicker than in previous stages; thus this may be the first stage unable to utilize any cutaneous respiration. This, combined with the increased demands of swimming discussed above, could result in significantly lower oxygen tensions in the tissues of Stage III larvae in comparison to previous stages or subsequent ones (Stage IV larvae swim more efficiently, and can also spend a good deal of time walking on the sea floor). Oxygen binding characteristics closer to that of the adult may therefore be needed for this transitional stage.

The enormous decrease in P_{50} seen between the adult and all of the earlier stages is an adaptation not only to the distribution of oxygen to a larger tissue mass inside an impermeable exoskeleton, but also to the lower metabolic rate of adults. In adult lobsters the P_{50} (10 mm Hg) is so low that most of the oxygen used is carried in solution in the blood, and the hemocyanin bound oxygen is reserved for use during periods of apnoea, hypoxia, or intense activity (Spoek, 1974).

The levels of lactate present in the hemolymph have been shown in many crustacean species to affect the oxygen binding properties of the hemocyanin by

shifting the curve to the left so that the P_{50} is decreased (Bouchet and Trouchet, 1985). The magnitude of the lactate effect varies from one species to the next. Levels of 1 mM lactate or less may alter P_{50} s as little as 2-3 mm Hg (Taylor *et al.*, 1985) or as much as 10 mm Hg in adult *C. magister* (Terwilliger *et al.*, 1986; Morris, 1990). Bouchet and Trouchet (1985) found only a 1 mm Hg drop in P_{50} of *H. gammarus* with 1 mM lactate added. Mangum (1983b) found only a 5 mm Hg drop in the P_{50} of adult *H. americanus* even in the presence of roughly 10 mM lactate. Lobster hemocyanins appear therefore to be relatively insensitive to lactate, and the levels and variability in the samples from this study should have minimal effect on the oxygen binding curves.

Equally interesting as the changes described above is an examination of characteristics which remain constant even though the lobsters change form and habitat and the size of the hemocyanin aggregate changes. The cooperativity between subunits shows no statistically significant change from one developmental stage to the next, or from one pH to another within a single stage. The variability of these measurements is fairly high; this may result from freezing of all samples prior to analysis. Freezing in liquid nitrogen has been shown to affect the cooperativity (but not the P_{50}) of hemocyanin samples from several species (Morris, 1988), although less severe freezing conditions have been used without affecting the hemocyanin (Bridges *et al.*, 1984). These samples were frozen under much less extreme conditions than those shown to lead to alteration of the cooperativity (A. Taylor, pers. comm.). Even a small amount of denaturation,

however, will lead to some change in cooperativity if the denaturation occurs in one of the areas of a subunit which interacts with other subunits. Despite these problems, the cooperativity levels seen here are typical of crustacean hemocyanins. A number of species show no change in N_{50} with pH (Morris, 1988; Young, 1972a) including species quite similar to *Homarus*: *Panulirus*, *Penaeus*, and *Scylla* (Mangum, 1983).

It is also no surprise that N_{50} does not differ with the change in aggregate size and is therefore the same from one stage to the next; cooperativity exists primarily at the level of interactions within the hexamer and the hexamers are held together in dodecamers by noncovalent interactions (Herskovits *et al.*, 1984). Changes in cooperativity present fewer obvious advantages during development than changes in the P_{50} or Bohr effect. Increased cooperativity only results in more oxygen being released when the differential between the environmental and tissue oxygen tensions is consistently small and centered around the P_{50} of the pigment: for example, this could occur in animals that are consistently exposed to hypoxia, yet have fairly high tissue oxygen tensions. This is an unusual case compared to animals in low oxygen waters with low tissue oxygen tensions; such animals would benefit from a pigment with moderate cooperativity and a low P_{50} (the same adaptation seen in lobsters as they go from larvae living primarily in oxygen-rich waters to adults inhabiting more hypoxic benthic habitats).

SUMMARY

Changes occur in both the structure of hemocyanin and its oxygen-binding properties in American lobsters as they develop through their larval stages to juveniles and adults. The size of the aggregate of hemocyanin shifts from almost exclusively hexameric in Stage I larvae to exclusively dodecameric in the first juvenile stage and the adult. Substantial quantities of dodecameric hemocyanin first appear in Stage III larvae. This stage also exhibits a significantly higher slope in its Bohr shift than previous larval stages; this higher slope persists through subsequent stages to the adult. It seems reasonable that the change in slope and aggregate size are related. The P_{50} s of the early stages are all quite similar, except for a decrease of 10 mm Hg (equivalent to 30% of previous P_{50}) for Stage III larvae. This phenomenon is particularly unusual since the P_{50} increases back to its original level for the two subsequent post-metamorphic stages. The shift in P_{50} may represent a pre-adaptation to subsequent changes in anatomy or biochemistry of the hemolymph, or it may reflect the increased oxygen needs and activity level of the Stage III larvae. The P_{50} drops again in the full-size adult to the extent that hemocyanin-bound oxygen tends to act as a venous reserve. Cooperativity of oxygen binding among the subunits is unchanged by either development or changes in blood pH within a single stage. The

cooperativity results are similar to those found for adults of different species in previous studies. Changes in Bohr slope and aggregate size occurring concurrently represent a new aspect of the relationship between hemocyanin structure and function, as do the peculiar P_{50} characteristics of the third stage larvae.

CHAPTER V

SUBUNIT STRUCTURE OF LOBSTER HEMOCYANIN

INTRODUCTION

Hemocyanins occur as the respiratory pigment in a large number of crustacean species. These species exhibit substantial differences in the types of oligomers of hemocyanin found in their hemolymph and in the protein's functional characteristics. Both these types of changes depend ultimately on the structure of the types of monomers constituting the hemocyanin aggregates. Since the aggregates contain large numbers of monomeric subunits (six to twelve, usually), examination of differences between the structure of the types of monomers may elucidate further the flexibility in functional parameters seen in the crustacean hemocyanins. Also, changes in the number of types of monomers may reflect the documented developmental shift from almost exclusively hexameric hemocyanin to exclusively dodecameric hemocyanin.

Monomers such as those which comprise the oligomers of hemocyanin can differ in size or side chain charge or both. A number of types of electrophoresis can therefore be used to separate the types of monomers from one another and determine their heterogeneity. Alkaline dissociation gels, isoelectric focusing gels, SDS gels, and even gel filtration (under denaturing conditions) have been used (Markl *et al.*, 1979; Larson *et al.*, 1981; Jeffrey and Treacy, 1982; Makino and Kimura, 1988 as examples). SDS gels, which sort monomers on the basis of size, seem to be the most popular method for studying hemocyanin types of monomers.

A number of studies have compared the number of types of monomers occurring in the hemocyanin of various species, most often from types of monomers determined using immunoelectrophoresis (Markl, 1986) or SDS-PAGE. The number of SDS-PAGE types of monomers attributed to some common crustacean species, along with the aggregate types found in those same species, is presented in Table 1. The number of types of monomers is quite variable, especially considering that the molecular weights of all the types of monomers fall between 75,000 and 85,000 Daltons. Markl *et al.* (1986) described the immunological types of monomers and aggregation state of a number of crustacean species (Figure 1.3). It appears from this figure that species containing a single type of monomer do not possess aggregates larger than hexamers, but in species with multiple types of monomers (including all decapods studied to date) hemocyanins may exist as hexameric or dodecameric forms, with no clear relationship between number of types of monomers and aggregate state. Studies of reassociation of immunological types of monomers of hemocyanin from adult *H. americanus* showed that the presence of two types of monomers was sufficient for the majority of hemocyanin to spontaneously reaggregate to the hexameric level *in vitro*, but all three types of monomers were required for a majority of the hemocyanin to reassociate to the dodecameric form (Stocker *et al.*, 1990). Stocker *et al.* (1990) suggested that the purpose of monomer heterogeneity may be to ensure proper assembly into the aggregate form, but *in vivo* folding may involve other regulatory mechanisms such as maintenance of

types of monomers in a particular conformation by enzymes, spatial or temporal separation of types of monomers in the cells where hemocyanin is produced. Less heterogeneity might therefore be required for assembly to larger aggregates within a living organism. So far, no conclusive relationship between monomer heterogeneity and aggregation state has been demonstrated.

Once the types of monomers have been separated from each other on the basis of size or charge using the techniques previously mentioned, protease digestion patterns can be used to examine the intrasubunit structure and obtain some information on the relatedness of the types of monomers to one another. This technique cleaves the polypeptide chain of the monomer into fragments at specific residues so that the pattern of fragments generated can be used to compare the types of monomers. Protease digestion patterns have been used in the past to compare hemocyanin types of monomers of larval *C. magister* with those of the adult (Terwiliger and Terwilliger, 1982).

The present study is an evaluation of subunit composition of hemocyanin from all larval stages, juveniles, and adults of *H. americanus*. As outlined in previous chapters, following hemocyanin development in a single species holds promise for eliminating confounding details such as differences in evolutionary history between species. Relationships between the number of types of monomers and aggregation state may be much clearer in a single species, where multiple monomer types probably evolved from a common ancestor.

A shift in the size of the hemocyanin oligomer from a hexamer to a dodecamer has been demonstrated in the larval stages and juvenile of the lobster in the previous chapter. SDS-PAGE gels and protease digestion patterns of the aggregates were used to elucidate the changes in structure that result in the ontogenic change in hemocyanin oligomer size.

METHODS

SDS-PAGE

Hemolymph samples from all stages were run on the non-denaturing gels described in Chapter 4. After a brief staining and destaining in Coomassie Brilliant Blue, slices were cut from the hemocyanin containing bands and frozen individually in 1 ml of SDS reducing buffer. This Tris-SDS buffer (which contained B-mercaptoethanol) is and the recipes for the gels and running buffers are described in chapter 2. The SDS-PAGE gels consisted of a 2.5 cm long 4% T stacking gel and a 7.5% T resolving gel. These gels were run at 90 V constant for 2 h (at which point the dye front had passed through the stacking gel into the resolving gel), then at 150 V constant for 5 h. Gels were then stained and destained with Coomassie Brilliant Blue.

PROTEASE DIGESTION PATTERN GELS

Protease digestion pattern gels of the slices of aggregates from nondenaturing gels were done using the protocol described in chapter 2. The hemocyanin in the slices was digested with *Staphylococcus V8* protease in the wells of the SDS-PAGE slab gel used to separate the polypeptide fragments. Protease (0.05 ug) was suspended in 30 ul of buffer instead of 10 ul so that the large, slow-moving hemocyanin proteins would remain within the protease containing buffer during electrophoresis. The gel consisted of a 0.5 cm 4% T stacking gel with a 15% T resolving gel. After the slices and protease were loaded into the wells, electrophoresis proceeded at 4 mAmps for 2.5 h (at which point the samples and protease had entered the resolving gel), then proceeded at 15 mAmps constant for 7 h. These gels were silver stained to identify the location of the peptide fragments.

RESULTS

SDS GELS

The SDS band patterns represent the different types of monomers. The band patterns for the dodecameric hemocyanin of the adult and the hexameric forms

from the first three larval stages are presented in Figure 1a. It is readily apparent that the adult form contains three types of monomers which are quite close in molecular weight, whereas the hexamers from stages I-III have a single type of monomer, designated monomer M since it appears to be the same as the middle monomer band from the adult. Hexameric hemocyanin from Stage IV larvae, on the other hand, contains 2 types of monomers, as does dodecameric hemocyanin from juvenile lobsters. These two types are monomer M and a faster migrating type (monomer F). Monomer composition of these two stages and adult dodecameric hemocyanin are presented in Figure 1b. Although the banding pattern of the adult is less distinct here, it is clear that the Stage IV hexameric and juvenile dodecameric forms correspond to the bottom two bands (M and F) of the three in the adult. The two bands from the hexamer of the stage IV animals also correspond to the M and F monomers (Figure 2a). Types of monomers of all the larval hexamers in comparison to the adult dodecamer are presented in Figure 2b. The middle band of the adult form appears first ontogenetically as the sole band (monomer M) in the hexamers from stages I-III, with monomer F being added in the hexamer from stage IV larvae. The third, slowest migrating band seen in the adult dodecamer (designated monomer S) appears sometime after the first juvenile.

Types of monomers of larval dodecameric hemocyanin are presented in Figures 3a and 3b. The types of monomers of all the larval dodecamers appear the same: they each contain 2 bands which migrate the same distance on the gel

for stages III, IV, and the juvenile. These two bands from the larval dodecamers also correspond to the M and F bands of the adult dodecamer, as did the bands from the larval hexamer. Comparison of Figures 1b (Stage IV hexamer and juvenile dodecamer) and 3a (Stage IV dodecamer and juvenile dodecamer) reveals that the hexamer and dodecamer for stage IV animals contain the same two types of monomers (M and F).

The F monomer was first seen in the dodecamer of the third stage larvae (dodecameric forms from stages I and II was too scarce to analyze) and is present in all subsequent dodecameric forms. However, it is also present in the hexamer of the fourth larval stage. It is important to note that the F and M monomers appear to be present in approximately equal concentrations in the dodecamers from stages II, IV, and the juvenile. In the hexamer from stage IV the M monomer (corresponding to the one found in hexamers from larval stages) predominates. This is most clearly shown in Figures 1b and 4b, and may have important implications for the role of the F monomer in the aggregation state of the hemocyanin. The F monomer is also the predominant subunit in the adult. The substantial difference in density of this type of monomer compared to the two above it in the adult are shown in Figures 1a and 2a.

The SDS gels of the monomers making up the oligomers reveal not only a progression of changes with development, but also some interesting differences in the adult form between the dodecamer of hemocyanin and the putative hexamer.

SDS banding patterns for the two adult hemolymph proteins are shown in Figure 4a with a lane of pre-stained SDS standards between them. The molecular weights of the standards (from the top down) are 205,000 Daltons, 116,500 D, 77,000 D, and 46,500 D. The dodecameric hemocyanin, which is composed of three closely spaced types of monomers, migrates almost the same distance as the 77,000 Dalton standard band. The putative hexamer is composed of two types of monomers which are larger and have a greater difference in molecular weight between them. This difference in monomer size is seen between the adult hexamer and the types of monomers of hexamers from other stages as well. The adult hexamer next to the types of monomers for the stage IV hexamer is shown in Figure 4b. Hexamer from Stage IV has only two bands, yet those bands clearly differ from the two comprising the adult hexamer.

Finally, in Figure 5 one can see the adult hexamer in comparison with the hexamer from the first two larval stages. It is clear that the monomers from the larval stages differ in size from those of the adult hexamer. An overall comparison between the types of monomers of the larval hemocyanins, the adult dodecamer, and the adult hexamer is presented in Figures 6 and 7. The monomer from the early larval hexamers clearly has a molecular weight equal to that of the middle type of monomer of the adult dodecamer. The hexamer from the adult has a subunit composition quite distinct from all the other forms found in any of the developmental stages.

PROTEASE DIGESTION PATTERN GELS

From examination of Figures 8 and 9 it is clear that there is substantial difference in the peptide fragments making up the adult dodecamer and the putative hexamer from the adult. At least five areas of heavy staining differ between the two forms; these are marked with arrows on the two figures. Protease digestion patterns from all the larval hemocyanins, on the other hand, produce maps strikingly similar to that of the adult dodecamer. Maps from the stage I hexamer (which contains one type of monomer), the juvenile dodecamer (which contains two types of monomers), and the adult dodecamer (which possesses all three types of monomers) appear virtually identical.

Figure 5.1.a (top). SDS-PAGE of adult hemocyanin dodecamer compared with hexameric fraction from each of the first three stages.
b(bottom). Fourth larval stage hexamer compared with dodecamers from juvenile and adult.

Figure 5.2.a. SDS-PAGE of all larval hexamers in comparison with dodecamer from the adult. b. magnified view of two lanes on right end of gel in 2.a.

Figure 5.3. SDS-PAGE of larval and juvenile dodecamers of hemocyanin.
Irregular area across left lane of bottom photo is a crack in the gel.

Figure 5.4.a. The two fractions, dodecamer and putative hexamer, from adult lobsters with a lane of m.w. standards between them. The standard migrating closest to the hemocyanin monomers weighs 77,000 D.
b. putative adult hexamer, compared with hexamer from oldest larval stage.

Figure 5.5. Hexamer from first two larval stages compared with putative adult hexamer.

Figure 5.6. Overviews of two gels from which enlargements used in figures 1 through 5 were made.

Figure 5.7. Overviews of other gels used for enlargements.

Figure 5.8. Protease digestion pattern of aggregates of hemocyanin from different stages. D = dodecameric form, H = hexameric form, I - IV = larval stages, VI = juvenile, A = adult, and BSA = lane with bovine serum albumin digested using same protocol as a standard.

Figure 5.9. Protease digestion pattern of hemocyanin aggregates. This figure uses the same symbols as figure 8, plus V8 = lane with only protease (no substrate).

DISCUSSION

Before tackling an analysis of the ontogenic changes in the structure of lobster hemocyanin, it is essential to characterize the hemolymph proteins of the adult. The dodecameric oligomer of hemocyanin has been clearly established in previous chapters as an active crustacean hemocyanin: it migrates the appropriate distance on native gels and in chromatographic columns, shows a form consistent with hemocyanin in electron micrographs, contains copper, and (most importantly) reversibly binds oxygen to produce the characteristic oxygenated and deoxygenated spectra for a crustacean hemocyanin. The putative hexameric form of hemocyanin occurring in adult hemolymph shares these characteristics - except for the ability to reversibly bind oxygen. Markl *et al.* (1979) also described a hexamer in lobster hemolymph which showed no absorption at the characteristic wavelength for oxygenated hemocyanin; they therefore designated it a nonrespiratory protein. As discussed in Chapter 3, the same result was seen in our studies on the adult serum proteins; for the remainder of the discussion, the inactive hexamer will be referred to as the 16S protein. The structural investigations conducted in the present study further elucidate the relationship between the 16S protein and the dodecamer.

The 16S protein possesses two types of monomers compared to three found in the adult dodecamer. At least one of the 16S protein bands is distinctly larger

than any of the dodecamer's types of monomers. The second type of monomer from the 16S protein is close in size to the S monomer of the dodecamer, although this does not necessarily mean that they are the same monomer. As shown in Figure 4b, both types of monomers of the adult 16S protein are quite distinct from the stage IV larval hemocyanin hexamer types of monomers, or from those of any larval hemocyanins (see figures 6-8). The protease digestion pattern also show major differences between the 16S protein and dodecamer in the adult. The large differences in banding patterns are particularly striking in light of the similarity of the protease digestion patterns for all the larval stages and the adult dodecamer. The pattern of peptide fragments from the 16S protein is not characteristic of a breakdown product from the dodecamer. The 16S protein may consist of types of monomers which have been extensively modified by attachment of carbohydrate moieties, which would decrease the electrophoretic mobility and alter the protease digestion pattern (Dunbar, 1987). The results of the structural studies and the lack of oxygen binding indicate that the adult 16S protein is not hemocyanin.

The dodecameric hemocyanin of lobsters is known to be an active hemocyanin, but some controversy still exists regarding the number of monomer types comprising this hemocyanin. The present study demonstrated three types of monomers in the purified dodecamer of hemocynain from adults. Markl *et al.* (1979) describes five types of monomers, whereas a later paper from the same research group lists 6 types of monomers (Stocker *et al.*, 1990). In both cases, the

purification technique consisted of pelleting the hemocyanin through centrifugation to remove other hemolymph proteins, then dissociation into monomers at high pH and separation of the types of monomers from all other proteins by gel filtration. This technique does not separate the dodecamer from the 16S protein, since both will appear in the pellet and then dissociate into types of monomers close enough in size that gel filtration would not separate them. SDS gels of fractions of undissociated lobster serum from a BioGel A5M column have shown that the pure dodecamer has three types of monomers, the pure 16S protein has two, and fractions containing both material have five bands. This was verified by SDS gels of whole serum from adults - serum from adults with only dodecameric hemocyanin had three bands; serum from adults with both forms had five bands. It seems reasonable to conclude, therefore, that the difference between the results presented in this thesis and the published studies of Markl *et al.* (1979) and Stocker *et al.* (1990) is due to the difference in preparative technique resulting in both the dodecameric hemocyanin and the 16S protein being present in samples from the papers cited. No other reports of SDS subunit composition for *H. americanus* are available. For comparative purposes the number of types of monomers in hemocyanin from other crustacean species are reported in Table 1.

Markl, Stocker and colleagues from the University of Munich have determined that there are three immunologically distinct types of monomers (alpha, beta, and gamma) in adult lobster hemocyanin (Markl and Kempter, 1981; Markl *et al.*,

1986; and Stocker *et al.*, 1990). If we assume that the types of monomers from the dodecamer correspond to the lowest three bands on the SDS gel (as demonstrated in Figure 1 where the types of monomers from the 16S protein lie above those for the dodecamer), then only two immunological types of monomers (alpha and gamma) occur in the dodecameric hemocyanin. Subunit beta would correspond only to the very top SDS band, and would have to be assigned to the 16S protein. This is particularly interesting since homologous beta types of monomers appear in hemocyanin from a number of other species, particularly the brachyuran crabs (Markl, 1986). Subunit beta may be part of the active hemocyanin in other species, since this subunit may have modifications at sites other than the one to which the antibody was constructed. These additional modifications may render that subunit inactive for oxygen binding in the lobster.

In general the number of types of monomers of hemocyanin in adults is fairly constant for a given species. *Uca pugilator* has genetically diverse populations in terms of the number of types of monomers of hemocyanin (pers. comm. by Sullivan *et al.* reported in Van Holde and Miller, 1982). There are also reports of differences between members of the same species inhabiting different environments. *Callinectes sapidus* from estuaries contain six types of monomers, whereas those from intertidal areas possess primarily four types of monomers, with only traces of the last two (Mason *et al.*, 1983). Transfer experiments suggested that this difference resulted from environmental differences in salinity; animals transferred from full strength seawater to dilute seawater (and the

reverse) synthesized hemocyanin characteristic of animals from their new salinity (Mangum and Rainer, 1988). The results of this study, however, should be considered preliminary since only one crab survived the acclimation experiment in each test group.

Changes in the number of types of monomers present in the hemolymph of larval versus adult crustaceans have also been reported (Terwilliger and Terwilliger, 1982). In *Cancer magister* megalopa, juveniles, and adults hemocyanin in all stages consists of six bands; megalopa and juveniles, however, contain one band unique from that of the adult, and possess less of another band which is common to all three (Terwilliger and Terwilliger, 1982). Results from analysis of the hexameric hemocyanins are less clear in this species due to the presence of a comigrating nonrespiratory protein in the hemolymph of the larvae.

The general pattern found in *C. magister* is similar to the pattern seen in *H. americanus* dodecameric hemocyanin, although the number of types of monomers involved differs. In the lobster the earliest dodecamer sampled (stage III) contains two types of monomers (M and F) in apparently equal proportions, and this remains constant through the first juvenile stage. Adults possess an additional type of monomer (S) and have more of monomer S relative to the other two types of monomers.

Ontogenic changes also occur in the monomer composition of the hexameric hemocyanin of lobsters. In the first three stages the hexamer contains only monomer M, while the fourth stage hexamer contains the same second monomer type as the larval dodecamers have (F). In the hexamer the ratios of the two types of monomers are not equal as they are in the dodecamers; monomer M predominates. This suggests a possibility not previously documented. The progression from hexamer to dodecamer in lobsters may require not only an additional type of monomer (which occurs in Stage III dodecamer and both forms of hemocyanin from Stage IV), but also for that monomer type to be present in substantial quantities, perhaps even in equal proportion to the other monomer. This phenomenon may occur in lobsters but not other species because, unlike many other species, lobsters possess no linking dimer to act as an interhexamer bridge in the formation of larger oligomers (Stocker *et al.*, 1990). Instead, multiple, noncovalent contact points involving calcium ions allow the formation of higher aggregates (Herskovits, 1988). A certain proportion of the second type of monomer may be required to provide enough contact points between the hexamers for formation of the higher oligomer. Markl (1986) observed that the second immunological subunit appears in juveniles of *Carcinus maenas* one stage prior to the change in aggregation state. This may be a similar phenomenon to the one documented in the present study in *H. americanus*.

Results from the protease digestion pattern experiments provide a less clear picture than the SDS studies. In lobsters very different maps are generated for

the two native gel bands from the adults. This lends credence to the suggestion that the 16S protein is not a hemocyanin, especially since the maps from all other hemocyanins are so similar. The similarity in maps of all the other aggregates is somewhat surprising. It seems strange that Stage I larvae with a single type of monomer should generate maps so similar to Stage VI larvae with two types of monomers and adult dedecamers comprised of three types of monomers. The types of monomers are quite close in molecular weight, and cutting with the V8 protease may not discern differences between the types of monomers if they occur at bases not clipped by that protease. Since the types of monomers are too close on the SDS gels to be separated, it may also be that the differences between them are obscured on digestion gels by overlapping of bands from different types of monomers. A previous study of protease digestion patterns from individual types of monomers in *C. magister* showed that the one monomer type unique to the megalopa and juvenile had a quite different protease digestion pattern (Terwilliger and Terwilliger, 1982). It is possible to see from that study how maps of aggregates could obscure differences between types of monomers, since all the banding patterns fall in the same area.

SUMMARY

Structural studies of the types of monomers of hemocyanin from lobsters reveal an interesting progression of changes which may explain the ontogenic shift in the size of the oligomer in the hemolymph. The middle band (M) of the adult

form appears first ontogenetically as the sole band in the hexamers from stages I-III, with the lowest adult band (F) being added in the hexamer of the stage IV larvae. The larval dodecamers appear the same - they each contain two bands which migrate the same distance on the gel for stages III, IV, and the juvenile. These two bands from the larval dodecamers also correspond to monomers M and F, as did the bands from the larval hexamer. The third, top band seen in the adult (S) dodecamer appears sometime after the first juvenile. The 16S protein in the adult is composed of two types of monomers which are substantially larger and have a greater difference in molecular weight between them. Protease digestion patterns support the contention that this protein is not a hexameric hemocyanin and is more appropriately referred to as a 16S protein. The results indicate that the shift in lobster hemocyanin from a hexameric form to a dodecameric form requires the addition of a second type of monomer in a quantity approximately equal to that of the other subunit.

Table 5.1. Number of types of monomers of hemocyanin in crustaceans

<u>SPECIES</u>	<u># SDS</u> <u>monomers</u>	<u>OLIGOMER</u> <u>SIZE*</u>	<u>REFERENCE</u>
<i>Callinectes sapidus</i>	6	12,6	Mason <i>et al.</i> , 1983
<i>Panulirus japonicus</i>	4	6	Makino & Kimuro, 1988
<i>Cancer magister</i>	6	12	Terwilliger and Terwilliger, 1982
<i>Panulirus interruptus</i>	4	6	
<i>P. vulgaris</i>	6	6	
<i>Homarus americanus</i>	5	12	Van Holde and Miller, 1982 (review table therein)
<i>Cancer pagurus</i>	4	12,6	
<i>Carcinus maenas</i>	4	12,6	
<i>Hyas araneus</i>	7	12	

*see Table 6 of chapter 4 for references for data in this column.

CHAPTER VI
SUMMARY AND CONCLUDING REMARKS

SUMMARY

Hemocyanin is found in the hemolymph of a substantial number of marine invertebrate species, particularly amongst the decapod crustaceans. The wide distribution of this protein, combined with its importance in the respiratory system, has resulted in a number of studies on hemocyanin. Although these investigations have revealed a great deal about the structural and functional properties of this respiratory pigment, a number of important questions remain unanswered. No strong correlation has yet been established between differences in the structure of the oligomer or the diversity of its subunits and the functional properties of the protein. The link between anatomy, habitat, and hemocyanin structure or function is also still unclear in many areas. Controversy still exists on the role played by this oxygen carrier in the respiratory system of the organisms in which it is found. This thesis was designed to provide evidence primarily to help answer the first two of these questions through examination of the structure and function of hemocyanin in the larval, juvenile, and adult stages of *Homarus americanus*.

Initial results from Chapter 3 demonstrate that hemocyanin is present in all larval stages of the American lobster. The concentration of hemocyanin appears to be fairly constant (between 8 and 12 mg/ml) for the four larval stages and rises gradually through the juvenile stages. The hemocyanin concentration in the

adult is about five times that of the larval stages. Calculations of cardiac output based on preliminary measurements of heart rate and heart size indicate that, for the first three larval stages, the oxygen bound to the hemocyanin may be required for routine respiration. No calculations could be made for stages beyond this, since the heart could not be seen to allow measurement of its dimensions.

Non-denaturing gels were used to separate hexameric and dodecameric forms of hemocyanin for each of the stages under study (Chapter 4). Stage I and II larvae possess almost exclusively hexameric hemocyanin, but stage III larvae have almost equal proportions of the two forms. The dodecamer predominates in the fourth stage larvae, and is the only form found in the juvenile lobster. Adult lobster hemolymph gives two bands on the native gels, but results from analysis of fractions separated by column chromatography indicate that (although both bands consist of copper-containing protein) only the fraction corresponding to the dodecameric hemocyanin has an absorption peak characteristic of oxygen binding at the hemocyanin active site. The putative hexamer does not reversibly bind oxygen, and does not occur in all adults, though its presence is not correlated with gender nor is it simply a separate phenotype.

Oxygen binding curves were constructed over a range of pHs for each stage at both 20 ° C and 25 ° C (Chapter 4). All stages showed sigmoid oxygen binding curves typical of hemocyanin. The P_{50} s and N_{50} s for all larval stages and the juvenile were similar to each other and showed no significant change between the

two temperatures. Cooperativity showed no trend with pH at any stage, except possibly in stage III. The Bohr shift over the pH range tested showed a significant increase at stage III, but the first two stages were not significantly different from one another, nor were the Bohr shifts for stages subsequent to stage III significantly different from that of stage III.

Plotting oxygen dissociation curves for each stage at two pHs 0.7 units apart reveals the physiological significance of the functional changes described above. In the hemolymph of stage I and stage II larvae, the pH drop would result in the majority of hemocyanin-bound oxygen being released at a high oxygen tension (40 - 50 mm Hg). In stages III, IV, and juveniles a pH drop of the same magnitude results in the majority of hemocyanin-bound oxygen being released at 20 - 30 mm Hg. In adult lobsters no oxygen is released from the hemocyanin at these oxygen tensions, even with the 0.7 unit pH drop. Most of the bound oxygen would be released at 10 mm Hg oxygen partial pressure when the pH dropped as described. These calculations further illustrate that the hemocyanin-bound oxygen is probably used during routine respiration in the early stages, but functions as a venous reserve in adults.

Chapter 5 describes the results of investigations into the subunit composition of the hexamer and dodecamer of hemocyanin in each of the stages of the lobster. SDS-PAGE gels show a single subunit which migrates the same distance as the middle adult subunit for hexamers from the first three larval stages.

Dodecameric hemocyanin from stages III, IV and the juvenile have two subunits, these appear to be the same as the bottom two SDS subunits of the adult. Hexameric hemocyanin from stage IV larvae also has these two subunits, but the amount of the second subunit is significantly less than in that of the larval dodecameric hemocyanins. The top band of the adult three bands is the last to appear; it is first seen somewhere between the first juvenile stage and the market sized adult. The putative hexamer from the adult shows only two subunits, one or both of which are distinctly larger than the subunits found in the active hexamers. Peptide maps of the hexamers and dodecamers of the different stages show very similar patterns for the dodecamers and hexamers of all stages, except for the hexamer of the adult. The putative hexamer of the adult shows a pattern distinct from the other hemocyanins, further indicating that it may be an unrelated or highly modified form.

The developmental stages of *Homarus americanus* demonstrate an interesting pattern of structural and functional changes in hemocyanin. The larvae starts out with a hexameric hemocyanin consisting of a single subunit. At the third stage a second subunit is added which correlates with the appearance of a substantial proportion of dodecameric hemocyanin. These two structural changes are reflected in a significant increase in the slope of the Bohr shift. In market size adult lobsters a third subunit is present: there is no change in aggregate size or Bohr shift at this point, but the P_{50} is significantly lower. It appears that the SDS subunit composition changes during development, and that these structural

changes are reflected in alterations in oligomer size, response to pH changes, and the P_{50} of the respiratory pigment.

AREAS FOR FURTHER INVESTIGATION

Since this represents the first investigation into developmental changes in the structure and function of hemocyanin in lobsters as well as the first complete study of the structural and functional changes in hemocyanin in the development of a crustacean species, it opens up numerous avenues for further work. There are a few areas that present particularly exciting possibilities. Additional studies of the intrasubunit structure, particularly sequencing of the different subunits could yield some important insights into relationships between subunits. For example, are the three different subunits products from different, but related, genes? Or do they represent different posttranslationally modified products of the same gene? What are the structural differences between the three subunits that the addition of one results in changes in Bohr shift and aggregate state, whereas the addition of the other affects the P_{50} ?

Some interesting questions about larval lobster physiology have become apparent as the result of these studies as well. Several shifts in the hemolymph occur at stage III (appearance of the dodecamer, addition of an SDS subunit, drop in P_{50} , increase in Bohr shift, and change in *in vivo* pH); why do these changes occur at that stage? There seem to be more changes in the hemolymph

at the stage prior to metamorphosis than during it. Is this an example of pre-adaptation? Do larvae reach some critical point at this stage, so that they require the changes documented in this study? Perhaps the stage III animal has reached a size or exoskeleton thickness that prohibits cuticular respiration (presuming younger stages use this), or increase the energetic cost of swimming substantially. A great deal could be learned about the physiology of larval crustaceans by studies which concentrate on comparison of stage III lobsters to the previous and subsequent stages.

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BIOGRAPHIC NOTE

Kirby Olson grew up in the town of Harvard, MA, an area so far from the ocean that as a child she had to collect rocks instead of seashells. She moved to St. Petersburg, FL to attend Eckerd College. She received a B.S. in biology from Eckerd in 1984 and, after an unsuccessful attempt to join David Bowie on tour, returned to Massachusetts to enter the M.I.T./W.H.O.I. Joint Program in Biological Oceanography. She currently works as a postdoctoral fellow at Georgia State University investigating the mechanisms of chemoreception in spiny lobsters and enjoying the vastly superior climate. Did I mention it's spring here at the end of February?