THE STRUCTURE AND FUNCTION OF HEMOCYANIN FROM *CANCER MAGISTER*

by

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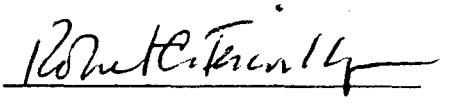
APPROVED: 

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Hemocyanins are copper-containing proteins which transport oxygen in some arthropods and molluscs. Arthropod hemocyanins are polymers assembled from subunits with molecular weights of about 75,000. The quaternary structure of arthropod hemocyanin is based on a hexameric assembly of these subunits; one, two, four, or eight of these hexamers are further assembled to form the native hemocyanins.

Arthropod hemocyanins show extensive subunit heterogeneity, which has been shown to be related to the quaternary structure of the complex four- and eight-hexameric chelicerate hemocyanins. The role of subunit heterogeneity in the simpler two-hexamer crustacean hemocyanins is less clear. One question addressed in this thesis is what is the structural role of subunit heterogeneity in the two-hexamer crustacean hemocyanins. A second question is how do the different subunits contribute to the overall oxygen binding properties of native hemocyanin.

The two-hexameric hemocyanin of Cancer magister consists of heterogeneous subunits and exhibits extensive homeotropic and heterotropic allostery. Therefore, a study of the structure and function of this hemocyanin should contribute to the understanding of the role of subunit heterogeneity in the crustacean hemocyanins.

The oxygen binding behavior of native C. magister hemocyanin was measured in the presence of L-lactate and its structural analogs. L-lactate increases the oxygen binding affinity by binding to a specific allosteric site on the protein. A model of the interaction of L-lactate with this site which accounts for the effector activity of L-lactate and its structural analogs is proposed. Investigation of the L-lactate effect in vivo shows that L-lactate functions to increase oxygen affinity during exercise.

Numerous chromatographic and electrophoretic techniques resolve only one two-hexamer component. Therefore, unlike other two-hexamer hemocyanins studied, C. magister 25S hemocyanin appears to be homogeneous. This implies specificity in the structural roles of the different subunits in this hemocyanin. Preliminary evidence is presented which suggests the subunit stoichiometry is 2:1:2:4:2:1.

An artificial hemocyanin, constructed from dissociated subunits and having a unique subunit composition, has a different oxygen binding behavior from the native hemocyanin. Thus different subunits may make different contributions to the oxygen binding behavior of the assembled hemocyanin.

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INTRODUCTION

The respiratory proteins have long attracted the interests of biochemists and physiologists. The study of these proteins, in particular the hemoglobins and myoglobins, has made fundamental contributions to our understanding of the structure of proteins (e.g. Svedberg and Pederson, 1940; Perutz et al., 1960; Kendrew et al., 1960) and of the mechanisms of control of protein function (e.g. Monod et al., 1965; Perutz, 1970; Baldwin and Clothia, 1979). Recently, the hemocyanins have come under considerable scrutiny as models of the study of the self assembly of multi-subunit proteins (Antonini and Chiancone, 1977) and for the study of allostery (e.g. Colosimo et al., 1977; Bonaventura and Bonaventura, 1980; Bonaventura, et al., 1981; Decker et al., 1983). The hemocyanins are also of interest to respiratory physiologists as adaptable components of the oxygen delivery system (Mangum, 1983a).

Arthropod hemocyanin structure

The hemocyanins are blue copper containing proteins which serve as the oxygen carriers in the blood or hemolymph of some arthropods and molluscs. Unlike the hemoglobins, these proteins are always found dissolved in the blood, never in cells. The structure and function of the hemocyanins have been extensively reviewed (van Holde and van Bruggen, 1971; Lonti and Witters, 1973; Bonaventura et al., 1975;

Antonini and Chianconi, 1977; Wood, 1980; Mangum, 1980, 1981, 1983b; van Bruggen et al., 1981; van Holde and Miller, 1982). Therefore this introduction will focus on the more recent findings relevant to the structure and function of the arthorpod hemocyanins in general and the crustacean hemocyanins in particular.

In response to selection pressures to increase the oxygen carrying capacity of the blood and to maintain the blood colloidal osmotic pressure and viscosity within workable limits, the hemocyanins have evolved into very large multisubunit proteins (Snyder and Mangum, 1982). The arthropod hemocyanins have molecular weights of 450,000 to 3,100,000 (van Holde and Miller, 1982). The subunit polypeptide chain contains a pair of copper atoms capable of binding one molecule of oxygen (Redfield et al., 1928), and have molecular weights of 65,000 to 90,000 (van Holde and Miller, 1982; Jeffrey and Treacy, 1982). This makes a single peptide subunit of a hemocyanin about the same size as a vertebrate tetrameric hemoglobin, which is capable of binding four molecules of oxygen.

The basic unit of organization of the arthropod hemocyanins is an assembly of six subunits with a molecular wieght of about 450,000. The circulating hemocyanin may be composed of one, two, four, or eight of these hexamers, which have molecular weights of about 450,000, 900,000, 1,800,000, or 3,200,000 and sediment with coefficients of about 16S, 25S, 37S or 60S respectively. The aggregation state of the circulating hemocyanin molecule is species specific and is loosely correlated with taxonomic groupings.

The hemocyanins are sporadically distributed in the arthropods.

At present they are known to occur in certain Crustacea, and in the Chelicerata. There are reports of a hemocyanin in a centipede (Rajulu, 1969; 1974). The crustacean hemocyanins are usually in the hemolymph as the 25S species, but very often a minor 16S component is also present. The thalassinid shrimp have a 37S hemocyanin circulating with a minor 16S component (Roxby et al., 1974). In some shrimp, spiny lobsters and isopods only 16S molecules have been reported (e.g. Kuiper et al., 1975; Ellerton and Anderson, 1981; van Holde and Brenowitz, 1981). Other isopods have both 16S and 25S molecules, which are found in the blood in almost equal amounts (Terwilliger, 1982) or with the 25S species predominating (Terwilliger et al., 1979). The Chelicerata generally have four-hexamer hemocyanins, yet many spiders have 25S, two-hexamers (Markl et al., 1983). The Merostomata, the horseshoe crabs, have an eight-hexamer which is unique to this group (Johnson and Yphantis, 1978; Sugita and Sekiguchi, 1980). Thus the arthropod hemocyanins show an interesting hierarchy of quaternary structure apparently based on the successive dimerization of hexameric units. The hierarchal nature of the structures of these proteins has greatly simplified the problems associated with the elucidation of the structures of these huge molecules.

The structures of a few hemocyanins are known in some detail. The three dimensional structure of the hexameric hemocyanin from Panulirus interruptus, a spiny lobster, has been solved to 0.5 nm (van Schaick et al., 1981) and 0.4 nm (Gaykema et al., 1983) by X-ray diffraction. The kidney-shaped subunits are arranged in a trigonal antiprism which results in 32 point group symmetry. Purified subunit II

of Limulus polyphemus, the horseshoe crab, and subunits 2 and 4 of Androctonus australis, a scorpion, hemocyanin show identical structures by X-ray diffraction at lower resolution (Magnus and Love, 1983; Fearon et al., 1983). Projections of this molecule show striking similarity to the square and hexagonal profiles of hemocyanins seen by electron microscopy (van Bruggen et al., 1981). Most of the EM profiles of two-hexamer hemocyanins, including the hemocyanin of C. magister (Terwilliger and Terwilliger, 1982), appear as a square and a hexagon joined side by side (van Bruggen et al., 1981). The square and hexagonal profiles very closely resemble the square and hexagonal profiles of the 16S hemocyanin of P. interruptus. Thus the structure of the 25S hemocyanin is thought to be two hexameric units each with an antiprismatic arrangement of kidney-shaped subunits. The two hexamers are joined with their three fold axis turned 90° to one another and to the longest dimension of the molecule (van Bruggen et al., 1981; van Holde and Miller, 1982).

This may not be the only structure for the 25S hemocyanins. Pilz et al. (1980) found that the structure that best fit their low-angle X-ray scattering data for the hemocyanin of Astacus leptodactylus, a crayfish, was a structure with the trigonal axis parallel. Electron micrographs of this hemocyanin, however, give typical profiles with most of the molecules showing a square and a hexagon (van Bruggen et al., 1981). The two-hexamer hemocyanin of the stomatopod Squilla mantis show mainly two squares touching each other along one edge (Schonenberger et al., 1980), which would be a profile expected from the structure proposed by Pilz et al. (1980).

The four-hexamer hemocyanins of the chelicerates and thalassinid shrimps appear to be dimers of the two-hexamer structures described above (van Holde and Miller, 1982). In the models of the hemocyanin of the scorpion A. australis by Lamy et al. (1981a) and Sizaret et al. (1982) and the bird eating tarantula Eurypelma (Digesiella) californicum by Markl et al. (1981d), the two two-hexamers lie roughly antiparallel to one another connected along one long corner. The long axes are not quite antiparallel but are slightly tipped with respect to one another generating a slightly rhombic structure (Bijlholt et al., 1982). The 37S hemocyanin of the thalassinids has a different structure (van Bruggen, 1983). The two dimers lie across one another (van Holde and Miller, 1982).

The eight hexamer of the horseshoe crab appears to be, in turn, a dimer of spider- or scorpion-like four-hexamers (van Holde and Miller, 1982). Image analysis of the EM profiles of L. polyphemus hemocyanin reveals that the four-hexamers lie atop another (Bijlholt et al., 1982, van Bruggen et al., 1981) and rotated by 90° (Lamy et al., 1983).

The quaternary structures described here are made more complicated by the fact that the subunits of the arthropod hemocyanins are heterogeneous (Markl et al., 1979a, 1979b; van Holde and Miller, 1982; Linzen, 1983). The number of the different subunits that have been resolved is very loosely correlated with the native aggregation state (Markl et al., 1979a, 1979b; Linzen, 1983). The eight-hexamer of L. polyphemus has at least fifteen different subunits (Brenowitz et al., 1981); the four-hexamers from four to eight different subunits; the two-hexamers from two to seven; and the hexamers from one to six (van Holde and Miller, 1982; Terwilliger, 1982; Linzen, 1983). The different subunits of a

hemocyanin from a single species have been shown to have different peptide maps (e.g. Sullivan et al., 1976; Markl et al., 1979c; Larson et al., 1981), amino acid compositions (e.g. Sullivan et al., 1976; Markl et al., 1979c) and N-terminal sequences (Jolles et al., 1981). In vitro translation of mRNA from L. polyphemus (Wood and Bonaventura, 1981; Siggens and Wood, 1983) and Leirus quinquestriatus, a scorpion, (Avissar et al., 1981) using the rabbit reticulocyte lysate system, have resulted in the synthesis of most if not all of the different polypeptides. The subunit heterogeneity in these animals does not appear to be the result of post-translational events and very likely represents the expression of different genes (Avissar et al., 1981; Siggens and Wood, 1983). Subunit heterogeneity is not due to genetic polymorphism. The subunit patterns are species specific (e.g. Markl et al., 1979a, 1979b) and show little if any variation from animal to animal (e.g. Sullivan and Tentori, 1981). On the other hand, changes in the subunit composition of the 25S hemocyanin with development have been demonstrated in C. magister (Terwilliger and Terwilliger, 1982) and in other brachyurans (Terwilliger and Terwilliger, 1983; S. Wache pers. comm.).

Except for the four-hexamers from the chelicerates, the number of antigenically discernable subunits in a species is less than the number resolved by other techniques. For example, Markl and Kempter (1981a) found only three immunologically distinct classes of subunits in the 25S hemocyanins and two classes in the 16S hemocyanins of several species of Crustacea. Subunits in two of the classes were immunologically related to each other and to subunits in the same two classes from other species. A single subunit in each species belonged to a third

class (in the 25S hemocyanins) which were not immunologically related to subunits in the other two classes or to any subunit in other species. The four-hexamer hemocyanins from several spiders have seven immunological types of subunits, which are related to the seven subunits of E. californicum (Markl et al., 1983). Antibodies specific for each of the seven subunits of E. californicum and eight subunits of A. australis can be raised (Lamy et al., 1977, 1979). Each of the seven antibodies to the subunits of E. californicum cross reacts with a particular subunit of L. polyphemus (Markl et al., 1983).

The two-hexamers of the spiders only have two subunit types by immunological criteria. The two types show immunological relatedness to each other and to a particular subunit of E. californicum hemocyanin (Markl et al., 1983). Markl et al. (1983) conclude from the immunological data that the crustacean and chelicerate hemocyanins represent two lines of evolution. The remarkable similarity of structure between the two lines of evolution implies that the two lines share a common ancester. In the chelicerates the two-hexamers seem to have evolved once from the four-hexamers (Markl et al., 1983). The two-hexamers of the Crustacea seem to have evolved from the one-hexameric hemocyanins, and to have done so independently several times (Markl et al., 1983). These conclusions, based on immunological considerations, are supported by the observations that there are several possible structures for the crustacean two-hexamers as has been discussed above, and several mechanisms by which the two-hexamers may be linked together, as will be discussed below. Analysis of the amino acide composition of hemocyanins leads to a similar scheme for the evolution of the arthropod hemocyanins

(Ghiretti-Magaldi and Tamino, 1977).

Arthropod hemocyanins can be dissociated into their constituent peptides by high pH, low ionic strength, and the removal of Ca^{++} and/or Mg^{++} (Lamy et al., 1977; Decker et al., 1980; van Holde and Miller, 1982). In some of the chelicerate hemocyanins urea is also required for complete dissociation (Lamy et al., 1977; Decker et al., 1980). In some hemocyanins dissociation by high pH, low salt, and the removal of divalent ions results in a mixture of mostly 5S subunits of about 75,000 molecular weight and a 7S subunit fraction of about 150,000 molecular weight (e.g. Murray and Jeffrey, 1974). These 7S subunits further dissociate to 5S peptides when exposed to reducing agents and/or urea; therefore, they are believed to be dimers of 5S subunits (e.g. Markl et al., 1981c). The specific roles of these dimeric subunits in organizing the quaternary structure of the hemocyanins will be discussed later in this chapter.

Decreasing the pH and/or restoring the divalent ions or ionic strength results in the reassociation of the hemocyanin but often the reassociation stops at the level of the hexamer (e.g. Carpenter and van Holde, 1973; Hamlin and Fish, 1977; Herskovitz et al., 1981a; Jeffrey et al., 1978; and Terwilliger et al., 1979). This result is usually explained by subunit heterogeneity. It is argued that there is a correct and specific arrangement of subunits in the hexamer that is necessary for the hexamer-hexamer associations. The failure to make higher ordered 25S structures represents the low probability of achieving such a specific arrangement by chance associations (van Holde and Miller, 1982). The failure of the subunits of the isopod Ligia

pallasii hemocyanin to reassemble to the two-hexamer has been explained by the preferential formation of an anomalous 16S hemocyanin in the reassembly process (Terwilliger, 1982). Also the different subunits of Cherax destructor, a crayfish, hemocyanin have been shown to reassemble to 16S structures at different rates and under different conditions (Marlborough et al., 1981). This phenomenon, if consistent for all crustacean hemocyanins, would further lower the probability of making oligomers with the proper proportions of all of the constituent subunits found in the native molecule.

It is, therefore, unexpected to find that it is the most complex hemocyanins that have been successfully reassembled to native or near-native structures. The subunits of E. californicum hemocyanin have been reassembled to the four-hexamer state (Decker et al., 1980). The reassembled four-hexamer contained all subunits in the same proportion as found in the native hemocyanin. Similar results were obtained by Lamy et al. (1980) for A. australis hemocyanin except one of the eight subunits is not incorporated in the reassembled four-hexamer and another is included in an extra copy. An eight-hexamer can also be reassembled from a mixture of subunits from L. polyphemus (Bijlholt et al., 1979).

The reassembly experiments using the chelicerate hemocyanins actually support the hypothesis of van Holde and Miller (1982) that the difficulty with reassembling crustacean hemocyanins to two-hexamer structures is that assembly is blocked by the formation of incorrect hexamers. Such hexamers are formed by subunits from chelicerate hemocyanins, but they can disassemble in the presence of free subunits to eventually form the correct four-hexamer structures (Decker et al., 1980).

The hypothesis is further supported by the careful study of the roles of the different subunits in the reassembly process in the chelicerate hemocyanin. All of the seven subunits of E. californicum hemocyanin, all but one of the eight subunits of A. australis hemocyanin and four of the five fractions of subunits of L. polyphemus hemocyanin are required to make the four- (or eight-) hexamer structures. Specific subunits have specific roles at each level of organization (van Bruggen et al., 1980). Specific models of the quaternary structure of the native hemocyanins of both E. californicum and A. australis have been constructed based on the reassociation behavior of the individual subunits and the EM profiles of the hemocyanins tagged with FAB fragments of antibodies specific to each subunit (Markl et al., 1981d; Lamy et al., 1981a; Sizaret et al., 1982). Each subunit has been assigned a specific location in the four-hexamer structure. It appears that in the 37S hemocyanins of the chelicerates each of the different subunits has a specific location in the quaternary structure, and a specific role in the assembly of the hemocyanin.

The structural roles of the different subunits in the other hemocyanins is less well established. One or two of the different subunits appear to be involved in linking the hexamers at each level of organization of the quaternary structure (Markl et al., 1981c). The hexamers are joined via a linkage between one subunit in each of the hexamers. In the case of the four- and eight-hexamers a different subunit pair is involved at each level of organization (Bijlholt et al., 1982). The nature of the linkage is quite variable. In some hemocyanins the pair of subunits are disulfide linked across the hexamers (e.g.

Jeffrey et al., 1978; Markl, 1980) and in others the linkage is broken under the influence of urea (e.g. Decker et al., 1980). These kinds of linker subunits are often seen as 7S dimers in alkaline dissociated hemocyanins (e.g. Markl et al., 1981b). The proposed disulfide linked subunits also remain linked as dimers in SDS dissociated hemocyanin (e.g. Murray and Jeffrey, 1974). No dimers have been demonstrated in other hemocyanins, but the hexamer-hexamer linkage can be Ca^{++} and/or Mg^{++} dependent (e.g. Terwilliger et al., 1979; Terwilliger, 1982; Arisaka and van Holde, 1979). In many other cases the nature of the interhexamer linkages are not known.

A general model for the structure of arthropod hemocyanin has been presented by Klarman and Daniel (1981). In this model, which will be discussed in more detail in Chapter Four, the authors propose that subunit heterogeneity is the result of a requirement for different kinds of subunits at structurally inequivalent positions in the two-, four-, or eight-hexamer hemocyanins. For example, in the two-hexamer there are three kinds of positions in each of the hexamers: the linker subunit, its four neighbors, and the one subunit not in contact with the linker. Thus there is a structural requirement for at least three kinds but not more than six kinds of subunits. However, only two kinds of subunits are found in Ligia pallasii two-hexamers (Terwilliger, 1982), and two-hexamer hemocyanins can be assembled in low yield from binary mixtures of pure subunits if one of the two is the linker subunit (Markl and Kempter, 1981b; Marlborough et al., 1981). Thus it appears that subunit heterogeneity beyond two kinds of subunits is not a structural necessity in all hemocyanins.

The fact that subunit heterogeneity is not a requirement for structure in some hemocyanins raises the possibility that there are several ways to arrange the different subunits in a given hemocyanin. In other words, subunit heterogeneity may be reflected in heterogeneity at the level of the one-, two- etc. hexamer protein. Surprisingly the question of the possibility of heterogeneity at the level of the native hemocyanin has not been directly addressed experimentally in the literature except in the case of E. californicum hemocyanin. Markl et al. (1981b) attempted to fractionate this four-hexamer by methods which fractionate proteins by charge and by molecular weight. They could not fractionate the protein; therefore, they conclude that there is only one kind of four-hexamer structure. Since all the subunits are required to assemble the four-hexamer and the assembled four-hexamer is not detectably different from the native (Decker et al., 1980), it is not surprising that this hemocyanin is homogeneous. Of course the model of the structure of the hemocyanin from A. australis proposed by Lamy et al. (1981a) and Sizaret et al. (1982) is based on the assumption that the native four-hexamer is homogeneous; yet, if homogeneity has been assessed, it is not reported in the literature. Reassembly studies also do not regenerate the native protein exactly (Lamy et al., 1980).

Homogeneity at the level of the native hemocyanin is not the rule for all hemocyanins. Lamy (1983) and Lamy et al. (1983) report that the eight-hexamer of L. polyphemus is heterogeneous by immunological criteria. Jeffrey et al. (1978) and Robinson and Ellerton (1977) saw multiple bands when the 25S and 16S hemocyanins of the crayfish Cherax destructor, spiny lobster Jasus edwarsii, and crab Ovalipes catharsis

were electrophoresed at neutral pH. Jeffrey et al. (1978) attribute the multiple bands in the C. destructor hemocyanin to the presence of hemocyanins with different subunit compositions. Purified subunits from C. destructor hemocyanin can be assembled into 25S hemocyanins containing the linker subunit and only one other kind of subunit. In fact, given the limitation of having one copy of the linker dimer per two-hexamer, all possible combinations of subunits appear to be possible in this hemocyanin and all possible combinations may appear in vivo (Marlborough et al., 1981). Two-hexamers can also be assembled in low yield from binary mixtures of purified subunits from the two-hexamer of Cupiennius salei, a spider (Markl and Kempter, 1981b).

In summary, the arthropod hemocyanins are found dissolved in the blood of certain arthropods as 16S hexamers, 25S two-hexamers, 37S four-hexamers, and 60S eight-hexamers depending on the species and taxonomic group. The subunits are typically heterogeneous, but have similar molecular weights of around 75,000. The subunits are arranged as a trigonal antiprism in the hexamer, and the higher ordered structures are built by successive dimerization of the 16S hexamer.

In some hemocyanins, in particular the 37S four-hexamers of spiders and scorpions, the different subunits have specific roles in the structure of the native hemocyanin. These hemocyanin molecules are very likely to be homogeneous. On the other hand, a few hemocyanins, in particular some of the 25S two-hexamers, have been shown to be heterogeneous. Subunit heterogeneity beyond two kinds of subunits is not generally required for structure in the two-hexamers. However, the difficulty by which some two-hexamers can be assembled from subunits implies

some specificity of subunit interaction is required for the assembly to two-hexamer structures. At present few generalizations are possible as to the structural roles of the subunit heterogeneity which is so characteristic of the arthropod hemocyanins.

Arthropod hemocyanin function

Functionally, the arthropod hemocyanins are characterized by showing striking homotropic and heterotropic effects. The oxygen binding behavior of any hemocyanin is quite dependent on its ionic environment and temperature; therefore, the comments here will be confined to discussing the oxygen binding behavior of the hemocyanin under conditions of pH and/or ionic composition that is relevant to the behavior of the hemocyanin *in vivo*. Discussion of specific values for the binding parameters, P_{50} and n_H, will be minimized since these values are so dependent on experimental conditions that direct comparisons between experiments in different laboratories, almost always done under different conditions, are difficult and/or meaningless.

The binding of oxygen to arthropod hemocyanins is highly co-operative. Hill coefficients (n_H) of the crustacean hexamers and two-hexamers are typically from 3 to 4 (Mangum, 1983b), and values as high as 5.7 have been reported under non-physiological conditions (Decker *et al.*, 1983). For the larger four-hexamer chelicerate hemocyanins Hill coefficients of around 9 are typical (van Holde and Miller, 1982). Careful analysis of the binding curves have shown that the co-operative unit in the crustacean two-hexamer may be the hexamer, while the coperative unit in the four-hexamers appears to be larger than the

hexamer but smaller than the two-hexamer (e.g. Decker et al., 1983).

Typically the oxygen binding behavior of arthropod hemocyanins is highly sensitive to pH. The Bohr shifts of the crustacean hemocyanins are remarkably uniform, $\Delta \log P_{50} / \Delta pH = -0.9$ to -1.2, for most of the hemocyanins that have been studied (Mangum, 1983b). The chelicerate hemocyanins can also show very large normal Bohr shifts (e.g. Linzen et al., 1977; Angersbach, 1978; Lamy et al., 1981c). However the eight-hexamer of L. polyphemus shows a Bohr shift in the opposite direction (Brouwer et al., 1977). Co-operativity is also highly sensitive to pH in both the crustacean and chelicerate hemocyanins (van Holde and Miller, 1982; Mangum, 1983b).

In addition to H^+, arthropod hemocyanins have been shown to respond to other inorganic ions. Ca^{++} and/or Mg^{++} have been shown to increase the oxygen affinity and co-operativity of C. magister hemocyanin (Wajcman, 1977) and most of the other crustacean and chelicerate hemocyanins that have been tested (van Holde and Miller, 1982; Mangum, 1983b). Most of the hemocyanins that have been tested respond to changes in ionic strength. In the chelicerate hemocyanins, Cl^- decreases oxygen affinity (Brouwer et al., 1977; Lamy et al., 1980); in the Crustacea, Na^+ (Kuiper et al., 1979; Mason et al., 1983) or Cl^- (van Holde and Brenowitz, 1981; Brouwer et al., 1978) increase oxygen affinity. As a rule the effects of Na^+ or Cl^- are small in comparison to the effects of divalent cations of H^+ (e.g. Mason et al., 1983).

Remarkably, the effect of CO_2 or HCO_3^- on hemocyanin oxygen affinity is not well studied. Truchot (1973) showed that the O_2

affinity of the hemocyanin of Carcinus maenas, a crab, is increased in the presence of CO_2 at a constant pH. This is a curious response for it is in the opposite direction to the CO_2 effect of vertebrate hemoglobins and not the response expected from classic principles of respiratory physiology.

Recently Truchot (1980) made the exciting discovery that the presence of L-lactate increases the oxygen affinity of the hemocyanin of two crabs, C. maenas and Cancer pagurus. This is the first known report of a respiratory protein, other than the tetrameric vertebrate hemoglobins, that responds to an organic effector. It is the only known report of an organic effector of an extracellular respiratory protein. As in the case of the effect of CO_2, (also reported by Truchot) the presence of L-lactate produces a response that is opposite in sign to the effects of the comparable organophosphate effectors of vertebrate hemoglobins, and the physiological function of the L-lactate effect is not readily apparent.

The role of subunit heterogeneity in the function of hemocyanin is unknown. There is limited evidence that different subunits may contribute differently to the overall oxygen binding behavior of the hemocyanin. The subunits of L. polyphemus hemocyanin, separated into five chromatographic zones, show differences in P_{50}, in sensitivities to Cl^- (Sullivan et al., 1974), and in the oxygen dissociation constant (k_{off}) (Bonaventura et al., 1977). However, the purified monomeric subunits of E. californicum show only small differences in P_{50} (Decker et al., 1979), which are reflected by small differences in the k_{off} (Markl et al., 1981a). All the subunits showed similar responses to

Cl^- (Decker et al., 1979). Similar studies on the isolated monomeric subunits of A. australis hemocyanin gave similar results to the results from the study of Limulus subunits (Lamy et al., 1980).

The oxygen binding behavior of the hemocyanin subunits does not resemble that of the assembled hemocyanin. The typical O_2 binding characteristics of the native hemocyanin arise solely by subunit interactions (Markl et al., 1981c). A description of the binding properties of the individual subunits so far seems to offer little help in analysing the role of subunit heterogeneity in expression of the native hemocyanin function (Linzen, 1983). Therefore, the contribution of the different subunits to the overall binding behavior of the hemocyanin will have to be studied in higher ordered structures.

One such study has been reported. Jeffrey and Treacy (1980) were able to compare the oxygen binding behaviors of two C. destructor hexamers made from purified subunits. These two hexameric hemocyanins had different values for P_{50} and n_H under the same conditions, and only one of the two showed a response to Ca^{++}. The native hexamers and two-hexamers showed slight differences in their responses to Ca^{++} and H^+. Thus at least in one case different subunit arrangements can make different contributions to the binding behavior of the hemocyanin. In C. destructor the differences in binding behavior of the two different hexamers may occur in vivo since the hexamer species studied probably occur as ciculating hemocyanin molecules (Jeffrey and Treacy, 1980).

The mechanisms by which these allosteric effectors act to change the oxygen binding behavior of the hemocyanins is unknown. In contrast, the mechanism of action of the modifiers of the vertebrate hemoglobins

is known to atomic detail (e.g. Baldwin and Clothis, 1979). The hierarchal structure of the various hemocyanins, the presence of a high degree of subunit heterogeneity, and the striking homotropic and heterotropic effects, including the possibility of an organic allosteric effector, make the hemocyanins intriguing molecules for the study of allostery (Bonaventura et al., 1981). Similarly the complex heterotropic interactions raise some interesting questions as to how these respiratory proteins function in vivo (e.g. Mason et al., 1983; Mangum, 1983a).

Accordingly this thesis addresses the question of the functional and structural relationships in the two-hexamer hemocyanin of Cancer magister, the Dungeness crab.

C. magister hemocyanin was chosen for this project for several reasons. The hemocyanin is known to be a two-hexamer, and it is well characterized as to its molecular wieght, the molecular weights of the subunits, its copper content, N-terminal amino acids, and subunit heterogeneity (Ellerton et al., 1970; Carpenter and van Holde, 1973; Larson et al., 1981). Extensive O_2 binding studies show that the protein exhibits both homotropic and heterotropic effects (Wajcman, 1977), and its close relationship to Cancer pagurus make it likely that L-lactate would be an effector. The respiratory physiology of the animal is well described (Johansen et al., 1970; McDonald et al., 1979; McMahon et al., 1979; McDonald et al., 1980); thus any data from this study would be directly applicable to the function of the hemocyanin in vivo. Finally, the animal has very large with a large blood volume, and is readily available on a year round basis.

This thesis addresses three specific questions. First, the mechanism of action of L-lactate is investigated: Is the mechanism of action of L-lactate specific, and how does the L-lactate effect function *in vivo*? Second, the structure of the two-hexamer hemocyanin is investigated: Is the two-hexamer of *C. magister* heterogeneous, and what are some of the characteristics of its subunit arrangement? Third, the O_2 binding behavior of an artificial two-hexamer is investigated: Do different subunits make different contributions to the oxygen binding behavior of the assembled 25S hemocyanin?

Results from this study will show that: 1) L-lactate does indeed affect the oxygen binding behavior of *C. magister* hemocyanin, and the effect is quite specific for L-lactate. A model of how L-lactate binds to the hemocyanin is presented. It will be proposed that the function of the L-lactate response is to counter the combined effects of the large Bohr shift of the hemocyanin and a large drop in blood pH that occurs during exercise. 2) The two-hexamer hemocyanin of *C. magister* appears to be homogeneous, like the four-hexamers of the chelicerates. At present there appear to be a diverse set of rules for the construction of crustacean hemocyanins. 3) *C. magister* hemocyanins with different subunit compositions do have different oxygen binding behaviors. However, no specific function can be as yet assigned to any specific subunit.

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CHAPTER TWO

THE EFFECT OF ORGANIC ACIDS ON OXYGEN BINDING OF HEMOCYANIN FROM THE CRAB *CANCER MAGISTER*

Introduction

The effect of organic and inorganic molecules on the oxygenation properties of vertebrate hemoglobins has been investigated widely at both the physiological and molecular levels (reviewed by Wood, 1980 and Isaaks and Harkness, 1980). However, modulation of the oxygenation properties of other oxygen carriers, such as the copper-containing protein hemocyanin (Hc), has been demonstrated only recently, and the molecular basis of these effects is incompletely understood. In 1975 Truchot showed that hemolymph of the crab *Carcinus maenas* contains both dialysable and non-dialysable factors which influence Hc oxygen affinity. More recently he identified L-lactate, a major end product of anaerobic metabolism in crustaceans, as a dialysable factor that influences Hc oxygen affinity in *C. maenas* and *Cancer pagurus* (Truchot, 1980). He also found that lactate alone could not fully explain the change in oxygen affinity of dialysed Hc; there must be additional dialysable factors in *Cancer pagurus* hemolymph which remain to be identified (Truchot, 1980).

In order to elucidate the effect of lactate on crustacean Hc, we have studied the response of serum and purified Hc of the Dungeness crab, *Cancer magister* Dana. We have compared the effect of L-lactate

with those of a number of its structural analogues to ascertain the specificity of this modulation process. Because lactate has been reported to interact with divalent cations (Jackson and Heisler, 1981), and inorganic ions are known to affect Hc oxygen affinity (cf. Truchot, 1975; Wajcman et al., 1977; Mason and Mangum, unpublished data), we have also assayed for changes in inorganic ion activity when lactate is added to serum. In the course of our investigation we found that the copper from hemocyanin interfered with the widely used enzymatic assay for L-lactate. This finding led us to reexamine the in vivo production of lactate in C. magister. Finally, to elucidate the physiological and possible evolutionary implications of modulation by an anaerobic metabolite, we determined the effect of small organic acids that are either potential or actual end products of anaerobic metabolism in the crustaceans or in other taxa.

Materials and Methods

Male crabs were obtained near the mouth of Coos Bay, Oregon, and maintained in running seawater at 31-33 o/oo and 11-14^{o}C. They were fed about twice weekly and discarded after two weeks in captivity. Blood was taken into a syringe from an infrabranchial sinus, allowed to clot on ice, and then centrifuged at low speed to remove the clot. In some experiments the serum was passed through a Sephadex G-25 column (1.5 x 30 cm) equilibrated with a saline consisting of 455 mM NaCl, 11 mM KCl, 13 mM $CaCl_2$, 18 mM $MgCl_2$ and 22 mM Na_2SO_2 buffered with 50 mM Tris, adjusted to experimental pH with HCl (Holliday et al., 1980).

Hemocyanin treated in this way is designated stripped hemocyanin. Hemocyanin samples were generally used for O_2 equilibrium measurements on the same day. Oxygen equilibrium curves of the stripped hemocyanin were determined at 20°C by a tonometric method (Benesch et al., 1965). The air-equilibrated sample was first diluted with buffered saline to an absorbance of 1.0 at 337 nm prior to analysis by this method.

For the experiments on hemocyanin serum with no exogenous buffer, the hemolymph supernatant was used without further modification. Oxygen equilibrium curves were determined by the cell respiration method, described in detail by Johansen et al. (1978). Two aliquots of the same sample were used, one to yield P_{50} and n_H and one to be terminated at P_{50} so that a subsample could be withdrawn for the measurement of pH. For experiments on the effects of a test substance on Hc, the serum was diluted 10:1 or 5:1 with Tris-maleate (final concentration 50-70 mM) buffered saline which contained a test analogue or metabolite. The linearity of the rate of oxygen depletion by the yeast cells in the presence of each test compound alone (with no hemocyanin) was ascertained. The pH of the reoxygenated sample was measured at the end of the experiment with a Radiometer BMS1 blood gas apparatus. The pH and P_{O_2} of anaerobically taken blood samples were measured with the same apparatus. A few measurements were made with this method using stripped, undiluted Hc. To avoid dilution of the Hc in these experiments, the Sephadex G-25 column was overloaded with serum and only the center of the void volume collected.

The concentration of L-lactate in resting and exercised animals and in the test solutions was measured enzymatically (Sigma Chemical

Co. Tech. Bull. No. 826 UV), using the Sigma Chemical Co. reagents. After an initial difficulty in obtaining a stable reaction end point, we modified the procedure as described in results.

Inorganic ion activities were measured with ion selective electrodes, using buffered (Tris-maleate, pH 7.85), stirred preparations of the standards (five step-wise dilutions of IAPSO seawater) and the unknowns (diluted 1:99). Details of the procedure are described in full by Mangum and Lykkeboe (1979). The effects of the analogues and metabolites on the inorganic ion activities in serum were determined by measuring the difference before and after the addition of a test solution. The same volume of a blank was added to the standards.

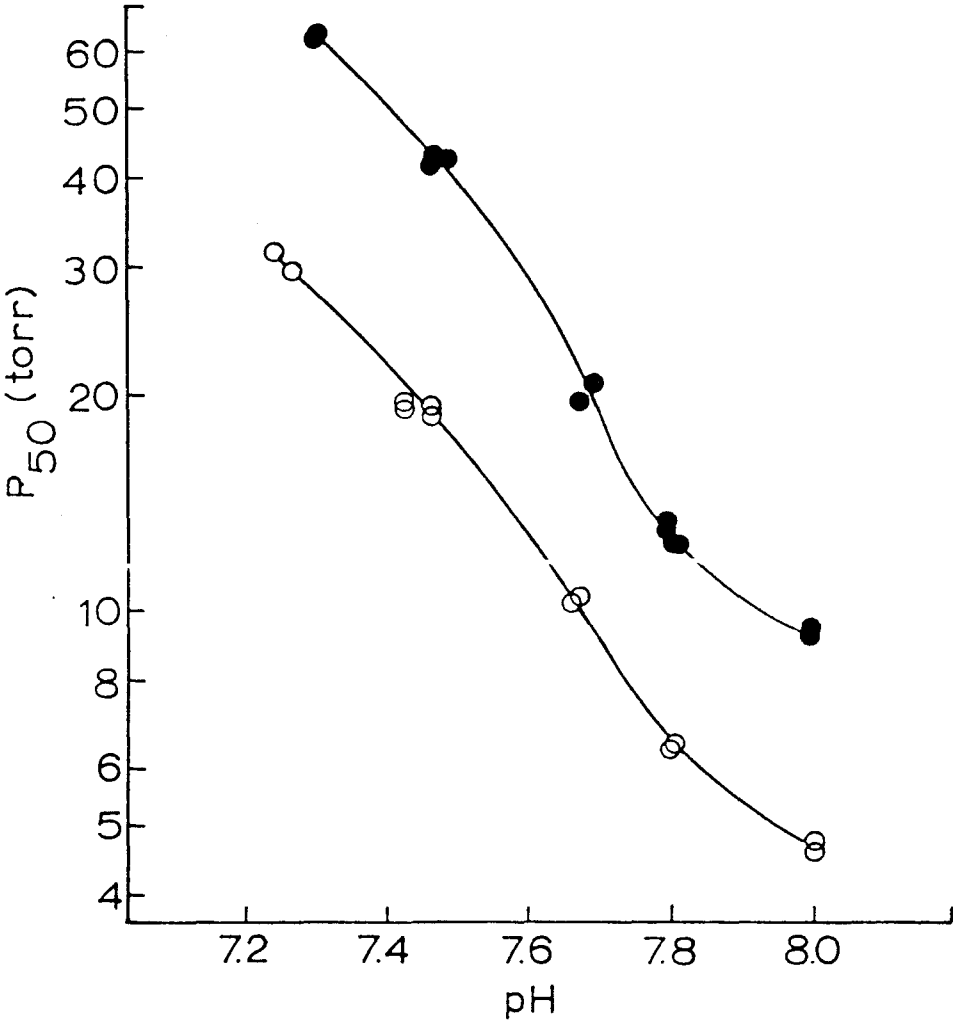
L-lactic acid, D-Li lactate, Na-2-methyl-lactate, DL- α -Na hydroxybutyrate, L-alanine, DL-alanine, Na succinate, Na propionate, L-aspartic acid, octopine, glycolic acid and Na pyruvate were added as concentrated solutions of the test substance in crab saline buffered with Tris-HCl and Tris-maleate. The reagents were purchased from Sigma Chemical Co.

Results

Direct Effect of Organic Acids on C. Magister Hc

At 20°C the oxygen affinity of stripped C. magister hemocyanin increases by about 50% in the presence of 10 mM lactate between pH 7.2 and 8.0 (i.e., log P_{50} decreases by 0.325 $\pm$ 0.025 SD). This response is independent of pH (Fig. 1). The effect of L-lactate on C. magister Hc approaches an apparent maximum at 5 mM (Fig. 2); the logarithmic

Figure 1: The effect of L-lactate on the O_2 affinity of stripped Hc, 50 mM Tris-HCl buffered saline, (●) + 10 mM NaCl; (○) + 10 mM L-lactic acid. 20°C, tonometric method.


60
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P_{50} (torr)
7.2
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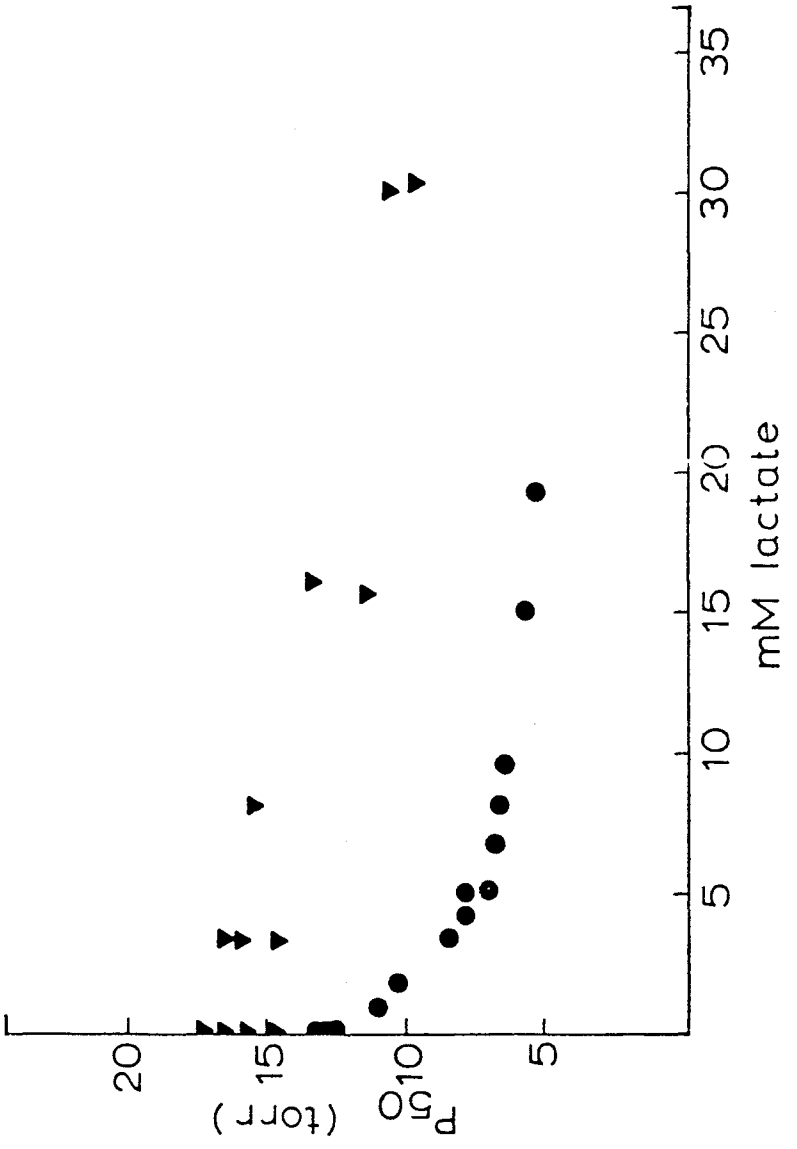
pH

Figure 2: The relationship between the O_2 affinity of stripped Hc and the concentration of lactate. 50 mM Tris-HCl buffered saline. (●) L-lactate, pH 7.81; (▼) D-Li lactate, pH 7.75. 20°C, tonometric method.


mM lactate
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25
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P_{50} (torr)
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regression line describing the data for L-lactate (Fig. 2) appears to be monophasic, with a slope of -0.247. D-li lactate also increases Hc oxygen affinity (Fig. 2), but the effect is smaller (Table 1). The effect of D-lactate is also independent of pH. Raising the ionic strength of the Hc solution with 5, 10 or 15 mM NaCl or LiCl instead of Na or Li lactate causes no detectable change in affinity.

The response of C. magister Hc to lactate appears to be specific to a particular stereoisomer. Of the other structural analogues examined (Table 1), glycolate (2-hydroxyacetic acid) and 2-methyl-lactate (2-methyl-2-hydroxypropanoate) had an effect, the magnitude of which was similar to that of D-lactate (Table 1). Pyruvate (2-oxo-propanoate) had a very small effect (Table 1). The small effect of pyruvate can be explained by the presence of contaminating L-lactate, which we measured to be 0.5%.

We continued these studies under more physiological conditions using serum at the temperature to which the crabs were acclimatized. First the effects of L-lactate, D-lactate, and glycolate on serum were confirmed (Fig. 3A); the magnitude of the response was indistinguishable from that of stripped hemocyanin. None of the other analogues had an appreciable effect on oxygen binding (Fig. 3B). Lactate had a small effect on the cooperativity of oxygen binding (Fig. 4).

Possible Indirect Effects of Organic Acids on O_2 Affinity

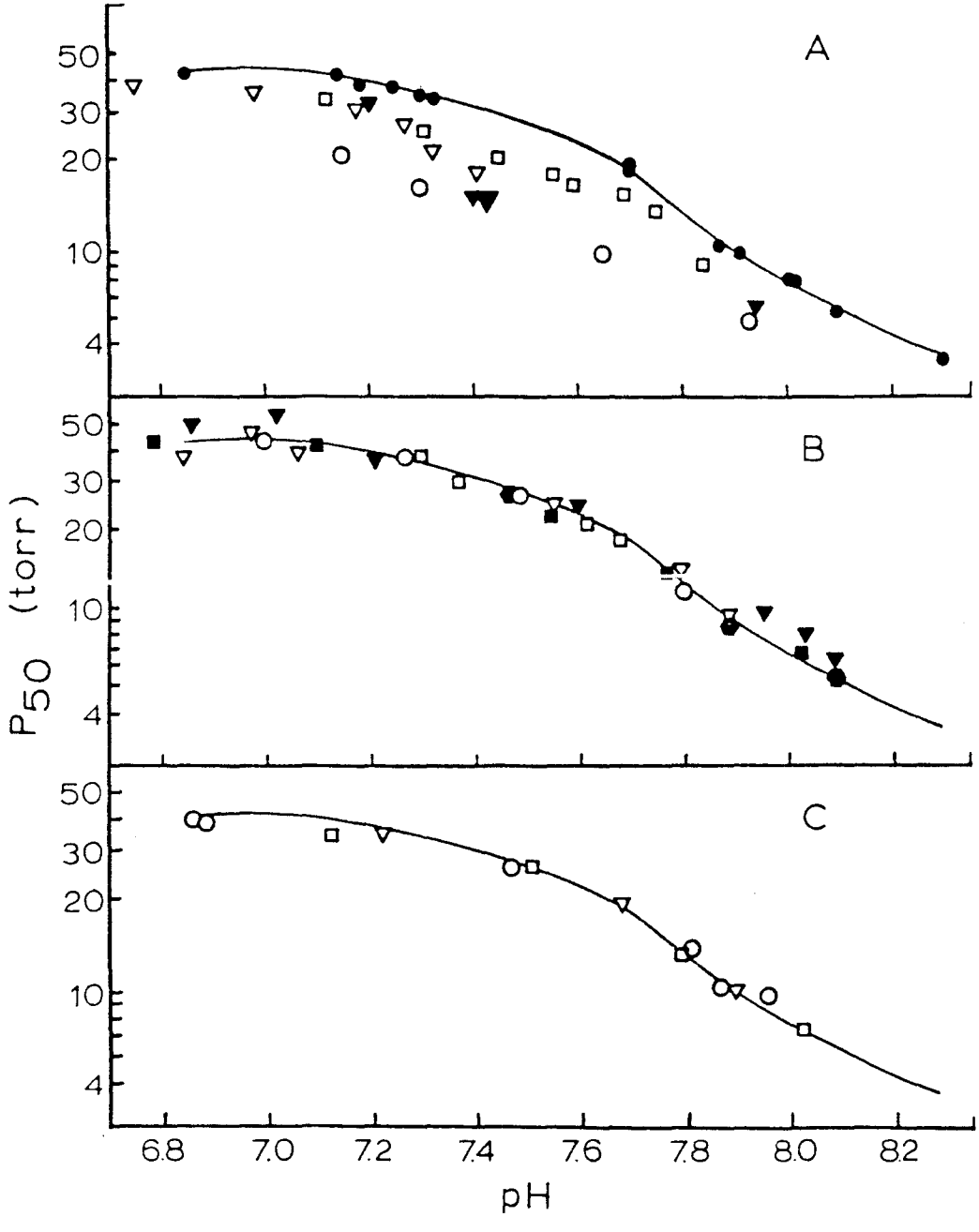
The concentrations of free ions in C. magister serum are: 458 ($\pm$ 1 S.E.) mM Na^+, 9.51 ($\pm$ 0.12) mM K^+, 9.23 ($\pm$ 0.05) mM Ca^{2+}, 22.2

TABLE 1. The Effect of L-Lactate and Its Analogues on Log P_{50} of Stripped Cancer Hemolymph.

Compound	pH	$\frac{\log P_{50}}{\log \text{mM effector}}$	r^2	log P_{50} (control) − log P_{50} (10 mM effector)	species
L-lactate	7.81	-0.247	0.971	0.307	C. magister
	7.90	-0.290	0.984	0.313	
	7.81	-0.211	-	-	C. pagurus*
	7.41	-0.237	-	-	
D-lactate	7.75	-0.192	0.839	-	C. magister
	7.72	-0.191	0.979	0.142	
glycolate	7.67	-0.126	0.991	0.127	C. magister
2-methyl-lactate	7.75	-0.148	0.998	0.135	C. magister
	7.70	-0.169	0.996	-	
pyruvate	7.68	-0.053	0.842	0.048	C. magister
propionate	7.72	-4.5×10^{-4}	0.979	0.001	C. magister
	7.71	1.0×10^{-3}	0.531	0.006	

* C. pagurus from Truchot (1980)

Figure 3: The effects of structural analogues and anaerobic metabolites on the oxygen affinity of buffered C. magister serum. 60-70 mM Tris-maleate, 13.5°C, cell respiration method. A: (●) control, no metabolites added; (O) L-lactic acid, 27-33 mM; (▼) D-Li lactate, 27-33 mM; (▽) D-Li lactate, 7.7-8.3 mM; (□) Glycolic acid, 9-20 mM. B-C: Solid line represents control curve from A.
B: (O) Na acetate, (□) Na pyruvate, (▽) L-alanine, (▼) DL-alanine, (■) Dl-α-hydroxybutyrate, 6.7-8.3 mM. (●) Na propionate, 45 mM.
C: (O) octopine, (□) L-aspartic acid, (▽) Na succinate, 6.7-8.3 mM.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P_{50} (torr)
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7.0
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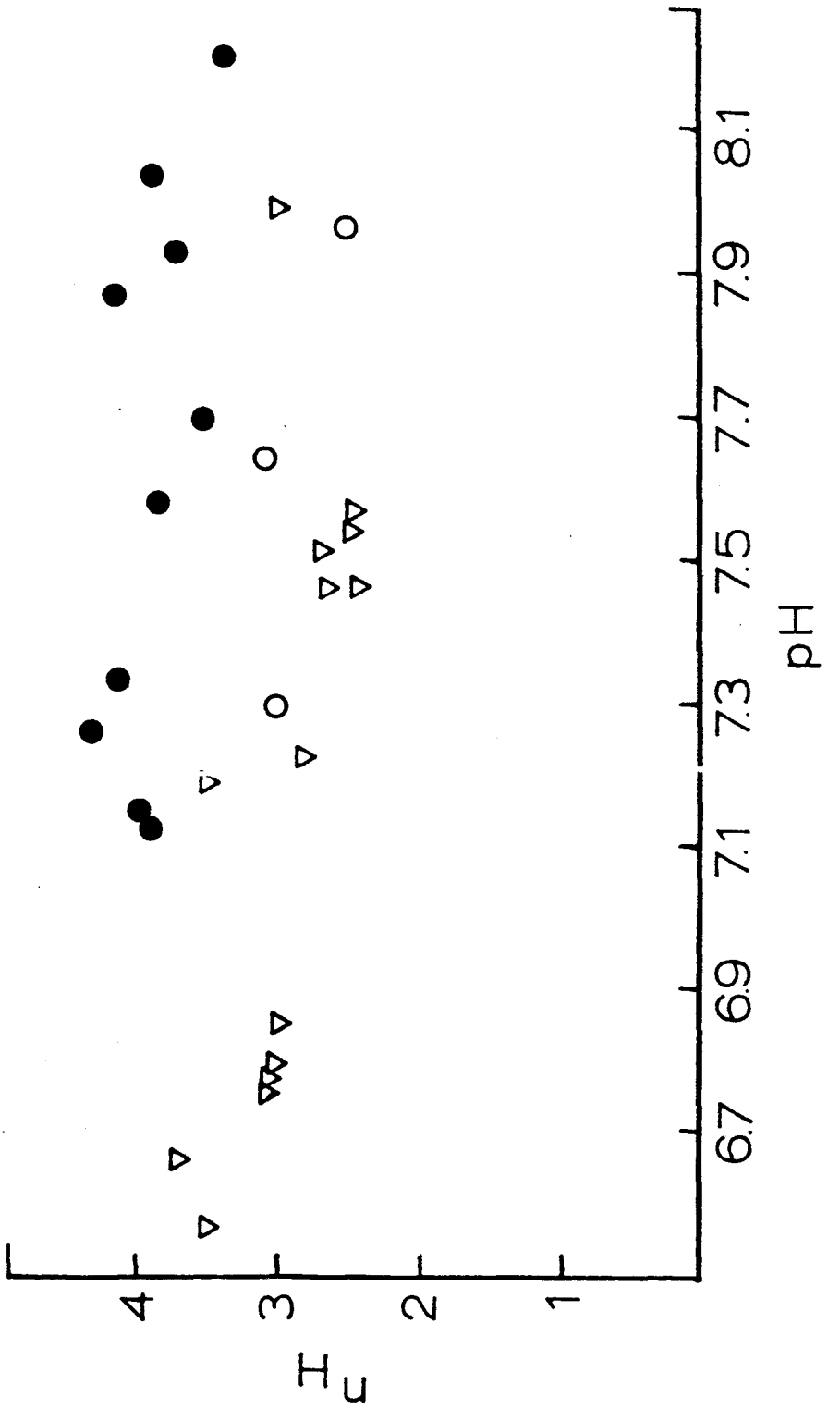
pH

Figure 4: The effect of lactate on the cooperativity of oxygen binding by buffered C. magister serum. (●) control, no metabolites added; (O) L-lactic acid, 27-33 mM; (∇) D-Li lactate, 31-33 mM. 60-70 mM Tris-maleate, 13.5°C, cell respiration method.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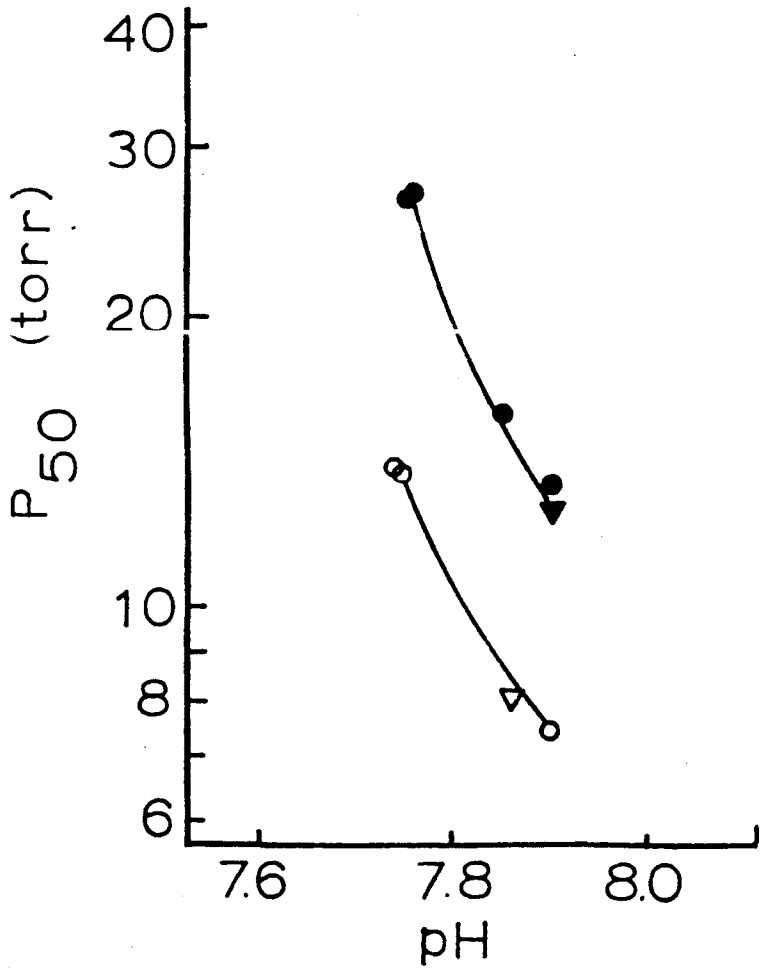
($\pm$ 0.5) mM Mg^{2+} and 531 ($\pm$ 2) mM Cl^- (N = 8). Following the addition of L-lactic acid to the serum at a final concentration of 9.1 mM, neither free Ca^{+2} nor free Mg^{+2} changed significantly ($P < .05$, according to Student's t test for paired observations). We should add that the evidence for Ca^{+2} binding by lactate reported by Jackson and Heisler (1981) was obtained in the presence of much higher (145 mM) lactate concentrations. None of the other test substances listed in Fig. 3 changed divalent cation activity significantly.

The Role of Lactate as a Physiological Modulator

To determine the quantitative role of lactate as a modulator in the blood, we first compared the O_2 affinity of an aliquot of undiluted but stripped Hc with that of an aliquot of undiluted, unstripped serum using the cell respiration method. Both samples were prepared from the same resting animal. At 20°C the stripped Hc had a P_{50} of 12.5 torr (pH 7.90 at P_{50}) (Fig. 5), a value consistent with our data for animals obtained at the same time of year (the affinity of stripped Hc decreases in the winter, unpublished observations). The unstripped aliquot had a P_{50} of 8.1 torr at pH 7.86. If the difference in O_2 affinities were due entirely to the removal of lactate, then the data in Fig. 5 would predict a lactate level in the resting animal of about 10 mM in the unstripped aliquot. In fact, the measured lactate level in this crab was only 0.06 mM. This observation suggests that effectors other than lactate and inorganic ions also occur in the blood. We therefore tested the effect of several other substances which are structurally less similar to lactate than the analogues, but which

Figure 5: The effect of stripping on the oxygen affinity of C. magister hemocyanin. (●) diluted stripped Hc, tonometric method; (O) diluted stripped Hc + 10 mM L-lactate, tonometric method; (▼) undiluted stripped Hc, cell respiration method; (▽) undiluted, unstripped serum, made 50 mM in Tris-HCl, cell respiration method. 20°C.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pH

concentration of which had been adjusted to 4.8 mM (Fig. 6).

The activity of Ca^{2+} in the blood before exercise was 9.55 mM and that of Mg^{2+} 19.7 mM; after exercise the values were 9.61 mM Ca^{2+} and 20.6 mM Mg^{2+}, within the error of the measurements (see above).

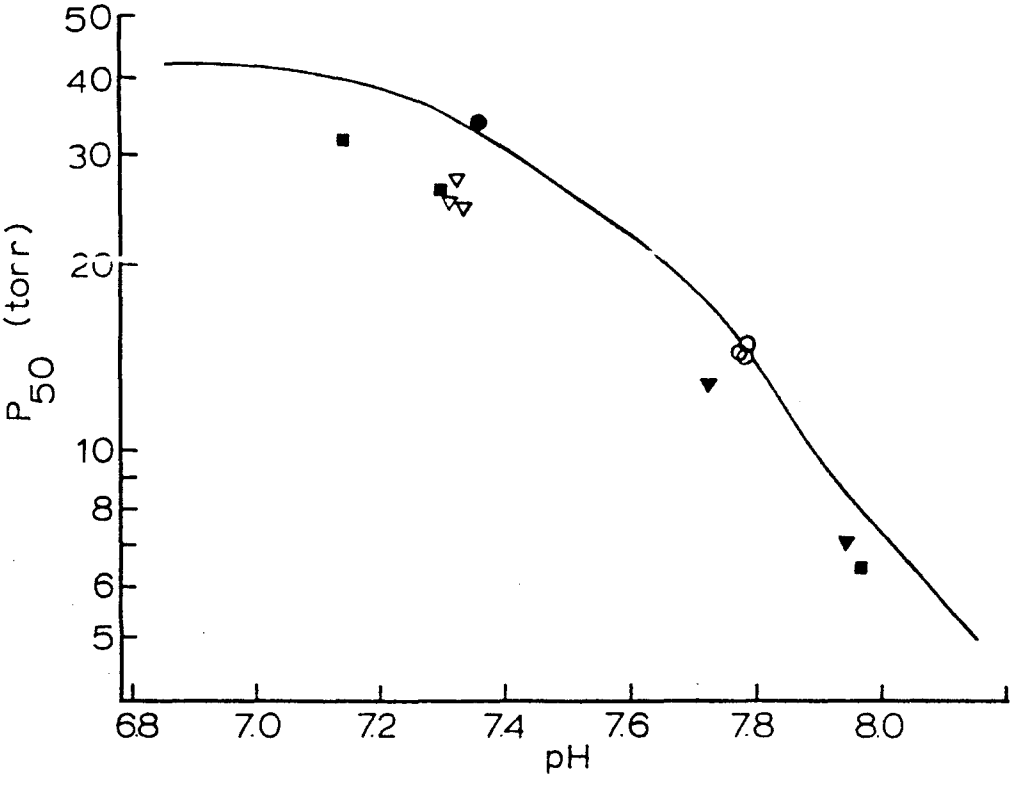
Blood P_{O_2} and pH

Extensive data on the respiratory properties of the blood before and after a similar exercise regime at 8°C were reported by McMahon et al. (1979). In order to illustrate the physiological importance of the lactate effect we made a few observations at the higher acclimation temperature (13.5°C) used here. In three animals, prebranchial blood P_{O_2} changed from an average of 11 torr before exercise to 3.5 torr following exercise, and the pH changed from 7.97 to 7.46. A single sample of postbranchial (pericardial) blood taken after exercise had a P_{O_2} of 40 torr and a pH of 7.48. As expected, the P_{O_2} values are slightly lower that those found at 8°C, although they lie within the error reported by McMahon et al. (1979).

A Note on the Use of Hydrazine Buffers in the Enzymatic Assay for Lactate in Hemocyanin-Containing Bloods

In studies of lactate in blood from the crab Callinectes sapidus, one of us (C.P.M.) recently noticed that the presumptive end point of the enzyme analysis, viz. the absorbance of NADH at 340 nm after 45 min. incubation at room temperature, was not stable. Instead it continued to increase for at least 32 hr., finally reaching values that were entirely unrealistic. We encountered the same phenomenon in our

Figure 6: The effect of L-lactate and exercise on O_2 binding by C. magister serum. (O, ●) before exercise, no lactate added; (Δ, ▲) before exercise, L-lactate added to 4.8 mM. (■) after 20 min. of high levels of locomotor activity. Open symbols, no exogenous buffer; closed symbols, 60-70 mM Tris-maleate. Solid line represents control curve from Fig. 3A. Cell respiration method, 13.5°C.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P_{50} (torr)
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pH

are known to be end products of anaerobic metabolism in other taxa. None of these compounds had a clear effect on oxygen binding (Fig. 3C).

The role of lactate in shifting the O_2 affinity of whole serum before and after maximal locomotor activity was determined. After a sample of prebranchial blood was taken, the animal was exercised to exhaustion. The animal was forced by various mechanical stimulations to walk continuously and then to perform repeated righting responses. When the animal would no longer right itself, it was removed from the water and left on its back in air until the legs stopped flailing. Finally, the "apron" (the ventrally recurved abdominal segments) was stimulated, inducing more leg flailing. As reported by McMahon et al (1979), the animal becomes refractory to any stimulation in about 20 minutes. A second sample of prebranchial blood was taken at the end of the "exercise" period. Blood pH was lowered by about 0.5 and lactate concentration was elevated by about 5 mM.

The value for O_2 affinity of an unbuffered aliquot of the blood (pH 7.79) taken before exercise (Fig. 6) agrees closely with the other control data. Similarly, the value for the same sample buffered to 7.35 with 60 mM Tris-maleate saline does not differ from the other control values. This pre-exercise aliquot contained 0.05 mM endogenous L-lactate. When L-lactate was added to raise the concentration to 5.1 mM (actual measured value, 4.8 mM), the O_2 affinity of the Hc increased to a level indistinguishable from that of unbuffered blood taken immediately after exercise, which contained 5.1 mM endogenous lactate (Fig. 6). The O_2 affinity of post-exercise blood buffered to pH 7.94 is also very similar to that of pre-exercise blood buffered to 7.97, the lactate

studies of C. magister hemolymph. In the absence of perchlorate extracted blood, the change in absorbance with time was zero in about 45 minutes.

We suggest that the interference is caused by the liberation of Cu ions into the solvent when Hc is precipitated by perchlorate. The presence of heavy metals, including Cu, in a deproteinized extract has the same effect on this assay (Engel and Jones, 1978). As suggested by Engel and Jones (1978), lowering the pH of the glycine-hydrazine buffer (Sigma Chemical Co.) to 9.0 with HCl and adding EDTA (12 mM) completely eliminates the interference and produces a stable end point in 45 min.

The importance of the modification in measurements on Hc-containing bloods should be emphasized. For example, when measured by the unmodified procedure, the apparent level of lactate in the blood of resting crabs was 0.5-0.6 mM, a concentration which has an effect on the O_2 affinity of C. magister Hc (Fig. 2). When the same sample was remeasured using the modification suggested above, the lactate level measured only 0-0.05 mM. In replicate samples with higher lactate levels the mean value also decreased somewhat and the error decreased considerably.

Discussion

The Nature of the Lactate Effect

The simplest explanation of our findings is that L-lactate exerts a direct allosteric effect on the oxygen binding site of C. magister Hc. The effect of lactate on oxygen affinity is quite specific, and

it is distinct from changes in H^+ or divalent cation activity. It is somewhat surprising that the protein incompletely distinguishes the two optical isomers. The effect of D-lactate cannot be due to the presence of L-lactate as a contaminant since L-lactate contributes less than 0.02% of the D-lactate (according to Sigma Chemical Co.). The amount of L-lactate in our test solutions of D-lactate is too low to be measured by our methods.

We propose the following model to describe the interaction of L-lactate at a specific allosteric site on C. magister Hc. The L-lactate binding site on the Hc appears to interact with at least three of the groups occupying the four positions around the chiral carbon of L-lactate. This hypothesis would explain the ability of the protein to distinguish between L- and D-lactate. It is likely that the hydroxyl group is required for lactate binding since substitution at this position with a hydrogen atom, amino or keto group (e.g. acetate, propionate, alanine, pyruvate) results in a complete loss of activity. We did not substitute for the carboxyl group since this group profoundly influences the chemistry of these compounds and it is likely that the charged carboxyl group participates in the lactate/glycolate-protein interaction.

The protein does not appear to completely distinguish between the methyl group and H atom of the lactate which accounts for the partial activity of D-lactate. Substitution of the methyl group of L-lactate with a hydrogen atom (glycolate) lowers the activity of the effector to the level of D-lactate; conversely, substituting the hydrogen atom of L-lactate with a methyl group (2-methyl-lactate) also lowers the activity of the effector to that of D-lactate. However, substitution of the

methyl group or hydrogen atom of L-lactate with a larger ethyl group (DL- α-hydroxybutyrate) results in a loss of activity, presumably due to steric hindrance of binding by the bulkier ethyl group. Whether the protein specifically interacts with the H atom or methyl group of L-lactate or both cannot be decided from our data, but the interaction of the protein with lactate via one or both of these positions must lack a degree of precision that allows four ligands (L-lactate, D-lactate, glycolate, and 2-methyl-lactate) to act as effectors.

We should emphasize that our data, though consistent with this hypothesis, do not provide the evidence necessary to eliminate the more complex hypothesis of separate sites for the binding of L-lactate, D-lactate, glycolate and 2-methyl-lactate. We do not have direct evidence that these molecules either: 1) actually bind to the protein, 2) bind differently to the oxy- and deoxy-hemocyanins, or 3) compete with one another for the same binding site. Further investigation of these points is necessary to provide full support for an allosteric model.

The Physiological Significance of the Lactate Effect

The effect of L-lactate on the hemocyanin of C. magister is very similar in magnitude to that on Cancer pagurus Hc (Truchot, 1980) and Callinectes sapidus Hc (Johnson and Becker, 1981). The effect of L-lactate on Carcinus maenas hemocyanin (Truchot, 1980) is much smaller than for either of the Cancer crabs, and is even smaller than the effect of D-lactate on C. magister hemocyanin.

Our data confirm the suggestion by Truchot (1975, 1980) that divalent cations and L-lactate are not the only effectors present in

brachyuran hemolymph. Since the lactate levels in the blood of resting C. magister are neglibile, the observed decrease in Hc oxygen affinity that accompanies stripping must be due almost entirely to the removal of other unidentified effectors. The stripping procedure replaces hemolymph molecules smaller than about 100-1000 daltons with buffered saline, which suggests that the unidentified factors have low molecular weights.

The increase in O_2 affinity due to lactate serves to oppose the Bohr shift that would otherwise accompany the large drop in blood pH following heavy exercise. If there were no lactate effect, the change in pH following exercise would lower the O_2 affinity of the blood at the gill by 7-8 torr, or 24-28%. This large change in pH would result in oxygenation of only about 81% of the Hc at the gill (P_{O_2} = 40 torr and pH 7.48). The increase in O_2 affinity of Hc due to lactate, however, raises the oxygenation state at the gill to about 95%. Thus, Hc is almost completely oxygenated even after these extreme levels of locomotor performance. The venous reserve, however, is relatively unaltered by the lactate effect. An unopposed Bohr shift would leave a venous reserve of 2.5% HcO_2 at P_{O_2} = 3.5 torr and pH 7.46; the lactate effect increases this value to only 3.0% HcO_2. Thus it appears that, at least after the high levels of motor activity studied here, the function of the lactate effect is to facilitate oxygen uptake at the gill rather than to conserve the venous reserve.

Of the end products of anaerobic metabolism found in different kinds of animals, only L-lactate and alanine (Trausch, 1976) are known to be made by decapod crustaceans. No activity of D-lactate dehydrogenase is found in several tissues of C. magister (Holzinger, 1974), and the

available evidence suggests no appreciable accumulation of alanine, aspartate or succinate in the hypoxic midgut gland or cardiac and locomotor muscles of intermolt C. sapidus (J.H.A. Fields, N.A. Mauro and C.P. Mangum, preliminary obs). With the possible exception of octopine, the other end products of anaerobic metabolism found in various kinds of animals usually follow periods of hypoxia. There is little evidence that they are important products of exercise metabolism. Thus, it is not entirely surprising that the changes in Hc oxygen affinity induced by exercising C. magister can be explained in full by the observed changes in pH and lactate alone. Perhaps more important, our evidence indicates that the as yet unidentified effector in hemolymph is not a product of exercise metabolism.

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CHAPTER THREE

A MODEL FOR L-LACTATE BINDING TO *CANCER MAGISTER* HEMOCYANIN

Introduction

L-lactate, which accumulates in the blood of crabs during hypoxia, has been shown to raise the oxygen affinity of hemocyanins (Hc) from two species of crab (Truchot, 1980). Recent studies of other crustacean hemocyanins have shown that this effect is likely to be widespread amongst the Crustacea (Johnson and Becker, 1981; Booth *et al.*, 1982; Graham *et al.*, 1982, 1983; Mangum, 1983). In two species of crab, *Cancer magister* (Graham *et al.*, 1982, 1983) and *Callinectes sapidus* (Johnson *et al.*, 1982), it was found that L-lactate is the most effective organic modulator of Hc oxygen affinity; glycolate, D-lactate and pyruvate increased the O_2 affinity, but to a lesser degree than L-lactate. The effect of pyruvate can be best explained by contamination of pyruvate samples with some L-lactate (Graham *et al.*, 1983). The specific modulation of the HcO_2 affinity by L-lactate (Johnson *et al.*, 1982; Graham *et al.*, 1982, 1983) and the proposal that the L-lactate binds to the oxy Hc-state (Johnson *et al.*, 1982) strongly suggests that the modulation is allosteric.

Graham *et al.* (1983) presented a list of L-lactate analogues and other organic acids that showed no effect on *C. magister* HcO_2 binding. However, 2-methyl-lactate (2-hydroxy-2-methyl-propionate) modulated HcO_2 binding to a degree similar to glycolate and D-lactate. From

these series of experiments, it was concluded that a hydroxyl group is required for successful allosteric interaction, and a carboxyl group is likely to be required. Similar conclusions were reached by Johnson et al. (1982). An explanation for the effectiveness of the weaker modulators might be that C. magister Hc cannot completely distinguish between hydrogen atoms and methyl groups (Graham et al., 1983).

Allosteric modification of respiratory proteins by organic molecules has been extensively studied for the vertebrate hemoglobins (cf. Perutz, 1970). Detailed models of these interactions were first proposed from studies of allosteric effectors with different affinities for Hb as well as different effectiveness as modulators of O_2 affinity (Benesch and Benesch, 1969). Since the relatively recent discovery of the lactate effect, no comparable work has been carried out on the hemocyanins until the work of Johnson et al. (1982) and Graham et al. (1982, 1983). In this paper, we attempt to provide a tentative model for the binding of L-lactate to the 25S C. magister hemocyanin which would account for the effect of L-lactate and the effects of its structural analogues on raising the respiratory protein's oxygen affinity.

Materials and Methods

The experimental procedures are described in full in Graham et al. (1983).

Results and Discussion

Projections of the structures of organic molecules which raise

the O_2 binding affinity of C. magister hemocyanin and some related analogues which do not are shown in Fig. 1. The relative effectiveness of these compounds were reported in Graham et al. (1983). β-hydroxybutyrate was tested more recently. The orientation of the molecules in the figure do not necessarily conform to traditional Fischer projections, but should be useful in interpreting the model proposed below. A model of how L-lactate might interact with the hemocyanin is presented in Fig. 2. In this model, it is assumed that all four positions around the chiral carbon are involved in the ligand binding.

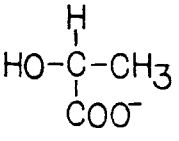
It was reported by Graham et al. (1983) and Johnson et al. (1982) that the hydroxyl group is required for activity. In Fig. 1, it can be seen that substitution of the hydroxyl group with an H atom (e.g. propionate) results in a loss of effector activity. In the model the hydroxyl group is proposed to interact with the Hc via a hydrogen bond.

The carboxyl group is also probably required since lengthening the distance between the carboxyl group and chiral carbon by one carbon (e.g. β-hydroxybutyrate) completely eliminates effector activity. The interaction between the carboxyl group and the protein is presumed to be electrostatic.

A specific arrangement of the H atom and methyl group appears to be required for full activity. A substitution of either group with an ethyl group (e.g. DL- α-hydroxybutyrate) results in the loss of activity. The Hc apparently can only partially distinguish between the H atom and the methyl group. Compounds with the H atom and methyl groups substituted for one another (e.g. D-lactate, glycolate, 2-methyllactate) all have similar effector activity; they raise the O_2 affinity

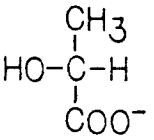
Fig. 1 Projections of the structure of L-lactate and its structural analogues. The orientation of the projections do not necessarily conform to the traditional Fischer structures. (eg. D-lactate is traditionally drawn with the OH on the right). The conventions regarding the three dimensional relationships of the bonds around the chiral carbon are retained.

Strong Effect

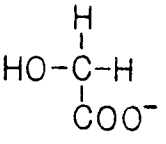


L-Lactate

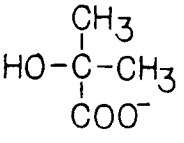
Weak Effect



D-Lactate

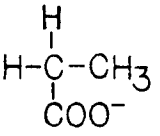


Glycolate

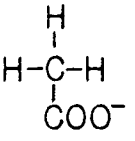


2-Methyllactate

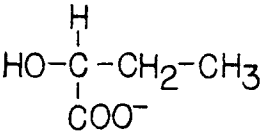
No Effect



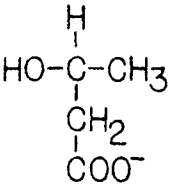
Propionate



Acetate

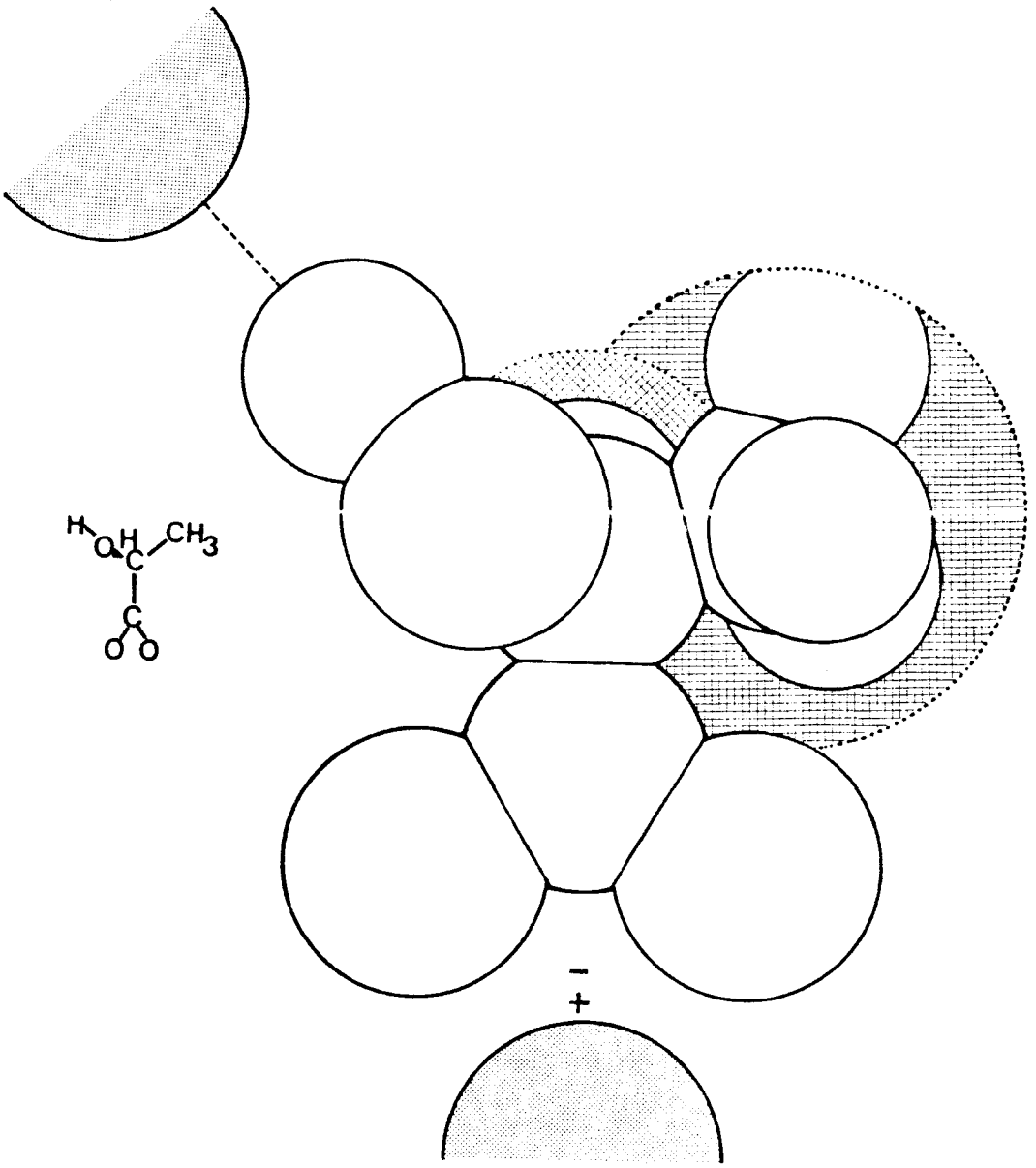


α - Hydroxybutyrate



β - Hydroxybutyrate

Fig. 2 Proposed interaction between L-lactate and C. magister Hc. The open circles represent atoms of L-lactate, identified at left; the protein is shown stippled or hatched. The effector binds to the protein at all four positions around the chiral carbon. The COOH interacts by means of electrostatic forces, and the OH by an H bond. The H atom and CH_3 group fit in hydrophobic sites (hatched) which incompletely distinguish between them, thus permitting low activity of the weak effectors.


H
O
H
C
CH3
C
O
O
−
+

of the Hc to a lesser degree than L-lactate.

The H atom and methyl group of L-lactate are proposed to interact with the protein via hydrophobic interactions. However, either H atoms or methyl groups can occupy the hydrophobic sites on the hemocyanin; ethyl groups appear to be too large to fit into these sites. For example, when glycolate interacts with the protein, a hydrogen atom is in the methyl site; when 2-methyl-lactate interacts with the protein a methyl group is present in the H atom site; and with D-lactate bound to the protein, there is a hydrogen atom in the methyl site and a methyl group in the H atom site. It is proposed that activity of the weak effectors reflects the lower affinity of the methyl site for H atoms and the H site for methyl groups.

This model represents one of the simplest we could propose based on the present data. Other more complex models for lactate binding will accommodate the data. For example, since it has not been shown that the effectors in Fig. 1 compete with one another for the same site, it is possible that there are separate binding sites for the different effectors. On the other hand, it is unlikely that there are selection pressures for separate weak effector sites, since the weak effectors probably do not appear in the blood of C. magister (Graham et al., 1983). Another possible model is one in which only three positions around the chiral carbon are involved in lactate binding. However, in this model it is difficult to explain the similar effector activity of glycolate and 2-methyl-lactate.

Many, but not all, crustacean hemocyanins are modulated by L-lactate. The L-lactate responsive hemocyanins include both two-hexamer

and one-hexamer types, and as yet do not include the hemocyanins found in chelicerates and molluscs (Mangum, 1983). The magnitude of the modulation effect of L-lactate is remarkably similar in most of the L-lactate responsive hemocyanins (cf. Truchot, 1980, Johnson and Becker, 1981; Booth et al., 1982; Graham et al., 1983; Mangum, 1983). Therefore the model presented here, developed using data from C. magister Hc, is very likely to be applicable to all the hemocyanins which respond to L-lactate.

This is the only binding site for an organic effector of oxygen binding known for an oxygen transporting protein other than the tetrameric vertebrate hemoglobins, and is the only organic effector binding site known for an extracellular oxygen transporting protein. The organophosphate effectors of vertebrate hemoglobins act to lower the oxygen binding affinity by binding to the low affinity, deoxy, form of the hemoglobin (Benesch et al., 1968b). L-lactate presumably binds to the oxy form of hemocyanin to increase oxygen affinity (Johnson and Becker, 1981). Therefore, the modulation of the O_2 binding affinity of hemocyanin by lactate is a fundamentally different process from the modulation of the organophosphates on the oxygen affinity of hemoglobins.

There is only one organophosphate binding site per hemoglobin molecule and two of the four chains participate in the binding (Benesch et al., 1968a). The location of the lactate binding site on the much larger and structurally more complex hemocyanins is, of course, unknown. The binding site could be a property of a single subunit or a property of the oligomer as is the hemoglobin organophosphate binding site. The fact that the lactate binding site is not uniformly distributed among the crustacean hemocyanins raises the interesting possibility that the

lactate binding site is associated with particular kinds of subunits which appear in some species and not others. Presently the functional significance of the subunit heterogeneity seen in the arthropod hemocyanins is unknown.

The study of the allosteric interactions of L-lactate and crustacean hemocyanins, now in its infancy, should provide an interesting contrast to the extremely well studied allosteric interactions of the vertebrate hemoglobins.

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CHAPTER FOUR

HOMOGENEITY, SUBUNIT HETEROGENEITY, AND THE QUATERNARY STRUCTURE OF THE TWO-HEXAMER HEMOCYANIN FROM CANCER MAGISTER

Introduction

The hemocyanins (Hc) are copper-containing, oxygen transport proteins found dissolved in the blood of some molluscs and arthropods. Arthropod hemocyanins exist as multimeric assemblies with molecular weights in the range of 450,000 to 3,100,000. The polypeptide subunit of arthropod Hc contains one oxygen binding site per molecular weight of about 75,000 and is assembled into the functional protein in hexameric units. The native Hc may be composed of one, two, four, or eight hexamers with sedimentation coefficients of about 16S, 25S, 37S and 60S respectively (for review see Wood, 1980; van Holde and Miller, 1982). Most Crustacea contain one- and two-hexameric Hc with the two-hexamer present in high concentration (Markl et al., 1979b). A few crustaceans have only one-hexamer Hc, and the thalassinid shrimp contain four-hexamer Hc. Two-, four-, and eight-hexamers are found in the chelicerates with eight-hexamer Hc restricted to the Merostomata (van Holde and Miller, 1982).

The subunits of a particular Hc are typically heterogeneous (Markl et al., 1979a,b; van Holde and Miller, 1982; Linzen, 1983). The number of different subunits in an arthropod Hc ranges from one in the one-hexamer and two in the two-hexamer of the isopod Ligia pallasii (Terwilliger, 1982) to perhaps fifteen different subunits in the eight-hex-

amer of the horseshoe crab Limulus polyhemus (Brenowitz, et al., 1981). In the scorpion Androctonus australis, the Hc peptides have different N-terminal sequences (Jolles et al., 1979, 1981). In vitro translation of mRNA from the scorpion Leiurus quinquestriatus and L. polyphemus indicates the different polypeptides are the products of different genes (Siggens and Wood, 1983; Avissar et al., 1981).

The best studied arthropod Hc's are the four-hexamer molecules from A. australis and the bird eating tarantula Eurypelma (Dugesiella) californicum. In these structurally complex Hc's, subunit heterogeneity appears to play an important role in assembly of the native molecule (van Bruggen et al., 1980). In building the models of the quaternary structures of these proteins, it has been assumed that the native Hc is homogeneous, that is, there is only one four-hexamer molecule which contains all subunits arranged in a precise way (Lamy et al., 1981a,b). Only in the case of E. californicum Hc has the question of possible heterogeneity at the level of the four-hexamer been addressed in the literature (Markl et al., 1981a).

The role of subunit heterogeneity in the apparently simpler quaternary structure of the 25S crustacean Hc is not well understood. There may well be fundamental differences between 25S two-hexamer and 37S four-hexamer Hc with respect to the way different subunits are organized (van Holde and Miller, 1982). One important difference for example is that the 25S Hc's of some crustaceans have been shown to be electrophoretically heterogeneous (Jeffrey et al., 1978; Robinson and Ellerton, 1977). On the other hand, there are probably basic similarities between the two which may shed light on some general rules for Hc assembly.

Accordingly we are studying the quaternary structure of the two-

hexamer Hc of the crustacean, Cancer magister, in order to begin to understand the role of subunit heterogeneity in crustacean 25S hemocyanins. We chose this Hc because it has been extensively studied with respect to molecular weights of the native molecule and its subunits, copper content, N-terminal amino acids and subunit heterogeneity (Ellerton et al., 1970; Carpenter and van Holde, 1973; Larson et al., 1981). Extensive O_2 binding studies on the Hc show the protein exhibits both homotropic and heterotropic allosteric interactions. Furthermore, recent studies show changes in Hc subunit composition during development in this crab (Terwilliger and Terwilliger, 1982). The present study could help our understanding of the relevance of these structural changes to functional properties. We have attempted to answer the following question in this study: Is the two-hexamer Hc of C. magister heterogeneous and if so, is this related to its subunit composition? Our approach was to first look for heterogeneity in the molecular weight and/or charge of the 25S Hc by gel filtration, electrophoresis, and isoelectric focusing. We then examined the nature and numbers of subunits. Finally, we have attempted to put these data together in a tentative model for the structure for this protein.

Materials and Methods

Preparation of the Hemocyanin

Cancer magister were trapped near the mouth of Coos Bay, Charleston, OR, and were maintained in running sea water at 31-32 o/oo and 11-14°C. Blood was removed by syringe from an infrabrachial sinus, allowed to clot on ice for 1 hour and centrifuged at low speed to remove

the clot and debris. The supernatant was then applied to a 1.5 x 100 cm Bio-Gel A-5m (200-400 mesh) column, equilibrated with 0.05 M Tris-HCl, 0.5 M NaCl, and 1 mM EDTA (pH 7.5). The hemocyanin chromatographed as a major fraction, designated 25S or two-hexamer Hc, and a minor 16S or one-hexamer Hc fraction as reported by Carpenter and van Holde (1973) and Larson et al. (1981). The Hc concentration was estimated by the absorbance at 280 nm using the value $E^{1\%}_{1\ cm} = 15.0$ (Nickerson and van Holde, 1971).

In some experiments 5S Hc was used as a standard. To prepare 5S Hc, the 25S was dissociated by dialysis against 0.052 M Tris/glycine, 0.01 M EDTA (pH = 8.9). The 5S material was separated from the 16S material by chromatography on a 2.4 x 50 cm on Bio-Gel A-5m column, equilibrated with the above buffer.

Electrophoresis

All electrophoresis experiments were done on 1.5 x 100 x 140 mm slabs using the apparatus described by Studier (1973).

The discontinuous buffer system of Laemmli (1970) was used for SDS PAGE. Hemocyanin samples were treated and electrophoresed as in Larson et al. (1981) except that dithiothreitol (0.05M) was substituted for 5% 2-mercaptoethanol in some experiments. When samples were first electrophoresed in a native gel and then alayzed by SDS PAGE, the gel slices were handled as described in Larson et al. (1981).

Regular gel electrophesis without SDS was carried out near neutral pH in two buffer systems. System I separated the proteins at pH 7.5, and was modified from the procedure of Davis (1964). The gel buffer

was 0.05 M Tris-HCl, 1 mM EDTA, pH 7.5. The chamber buffers were 0.05 M tris-maleate, 10 mM EDTA, pH 7.5. System II was the gel buffer system #6 of Maurer (1971) used without a stacking gel. 0.01 M $CaCl_2$ was added to the buffers to stabilize the quaternary structure of the Hc's at the slightly higher running pH (pH 8.0) of this system. The hemocyanin samples, prepared in 10% glycerol and 0.005% bromphenol blue, were electrophoresed on 4-5% polyacrylamide gels at 35 mA until the tracking dye reached the bottom of the gel.

Gels were stained with Coomassie Brilliant blue R (Fairbanks, 1971). For some experiments Fast green FCF was used (Bertolini et al., 1976). In this case gels were first soaked in 10% acetic acid and 25% propanol to fix the protein and remove the SDS. All gels were destained in 10% acetic acid.

The amount of Coomassie blue and Fast green dye bound to the separated proteins in SDS gels was measured by the method of Fenner et al. (1975). The gels were destained until there was no measurable background absorbance from slices cut from a region of the gel with no protein. All the slices were eluted in 0.8 ml of the 25% pyridine for 48 hr. with vigorous shaking. The volume of each gel slice was measured by the amount of distilled water displaced by the slice in a marked 1 ml tube. All OD's were then corrected to the same final total volume of 0.83 mls. The absorbance of the eluted Fast green was read at 630 nm.

Isoelectric Focusing

The hemocyanins were focused on 1.5 mm thick 4% polyacrylamide gels using a LKB Multiphore apparatus and 4% LKB Ampholines, pH 4-6. The protocol suggested by LKB (Application Note 250) was used. Samples were first dialyzed against 1% glycine or 4% Ampholines and a[illegible] to the gel in plastic cups made by slicing polyethylene rings from 0.4 ml microcentrifuge tubes. The proteins were focused at 5°C for 5 hr at 4.25 watt/cm^2. Gels were stained with Coomassie Brilliant blue R according to the LKB protocol. The pH gradient was measured by dividing a 1 cm wide strip of the focused gel into 0.5 cm sections, and the ampholytes in each section eluted in 0.5 ml of degassed 0.05 M KCl. The pH of the eluate was measured at 5°C with an Orion Ross electrode equilibrated and calibrated at 5°C.

Coomassie Brilliant blue R, sodium dodecyl sulfate, and acrylamade were purchased from Bio-Rad. Fast green FCF, glycine and horse spleen ferritin were supplied by Sigma. Bis-acrylamide was purchased from Eastman.

Results

Heterogeneity Based on Molecular Weight

The subunits of C. magister Hc have apparent molecular weights by SDS PAGE ranging from 67,000 to 82,000 (Larson et al., 1981). The six values are consistant with the range of values obtained by sedimentation equilibrium for alkaline dissociated subunits and subunits in guanidine

HCl (Ellerton et al., 1970; Carpenter and van Holde, 1973). This suggests that if 25S Hc's of different subunit compositions exist, they should have different molecular weights. These differences are likely to be too small to be resolved into separate peaks by gel filtration; however, the larger hemocyanins might be expected to predominate in the leading portion of a 25S gel filtration peak and the smaller ones in the trailing portion.

We therefore sampled the leading, middle, and trailing fractions of the 25S Hc peak from a Bio-Gel A-5M column (Fig. 1). Since the trailing portion of the 25S peak might be contaminated with traces of the 16S hemocyanin, each aliquot was first electrophoresed at pH 7.5 (system I) to separate this contaminant. To further test for any molecular weight differences that might be present, each of the 25S bands in the pH 7.5 electrophoretic gel were subdivided into slices containing the slow, middle, and fast portions of the band. The Hc in each slice was then analyzed for subunit composition by SDS PAGE (Fig. 2). There do not appear to be any qualitative differences in subunit compositions in any of the gel slices.

Heterogeneity Based on Charge

The subunits of C. magister Hc show different mobilities when electrophoresed at pH 8.9 in a non-denaturing gel (Larson et al., 1981). However, the subunits do not migrate in the pH 8.9 gels in the same order as they do in SDS. For example, one of the two subunits which migrate in the slowest electrophoresing band in the non-denaturing gels

Fig. 1. Elution profile of C. magister Hc chromatographed on a Bio-Gel A-5M column (1.5 x 100 cm) equilibrated to 0.05 M Tris-HCl, 0.5 M NaCl, 10 mM EDTA, pH 7.5. Arrows mark fractions analyzed for subunit composition. Absorbance at 280 (●) and 337 (▲) nm.

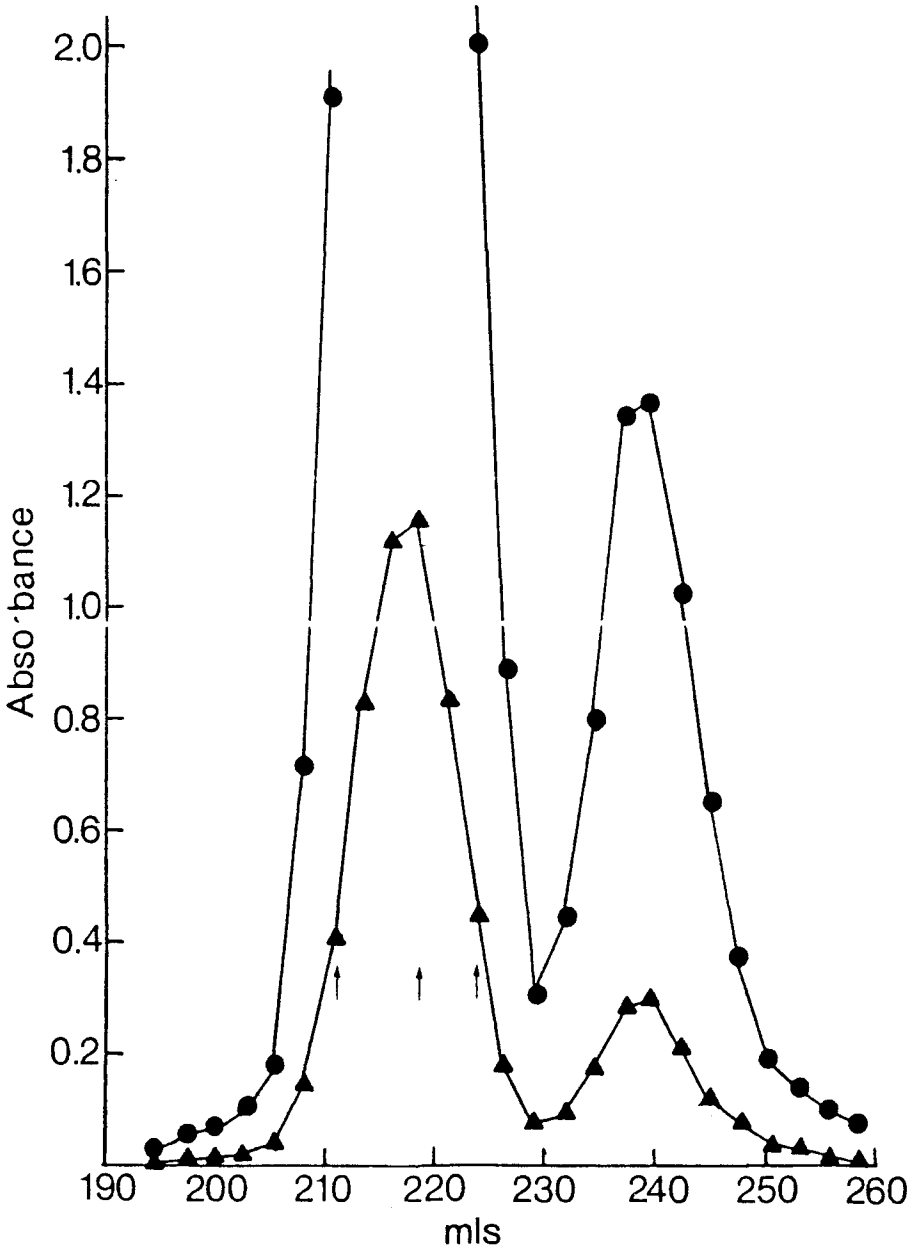
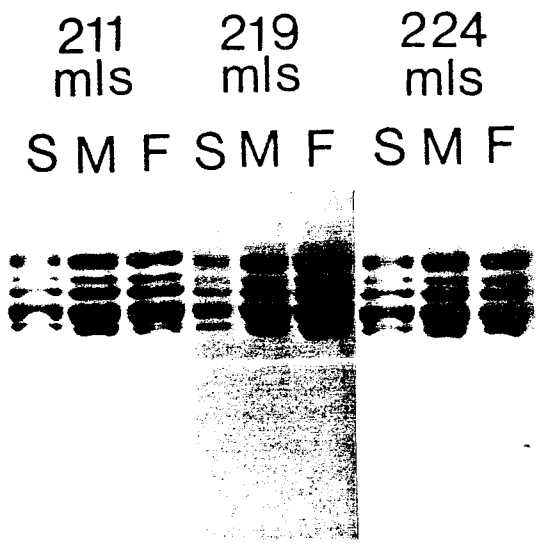

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Abso·bance
190
200
210
220
230
240
250
260
mls

Fig. 2. SDS PAGE of samples from the A-5m column in Fig. 1 after electrophoresis at pH 7.5. 10 ug of Hc from each fraction marked in Fig. 1 was electrophoresed in system I, the 25S bands divided, and the gel slices subjected to SDS PAGE. (S) slow, (M) middle, and (F) fast portions of the 25S band. The elution volume of the A-5m column sampled is indicated.

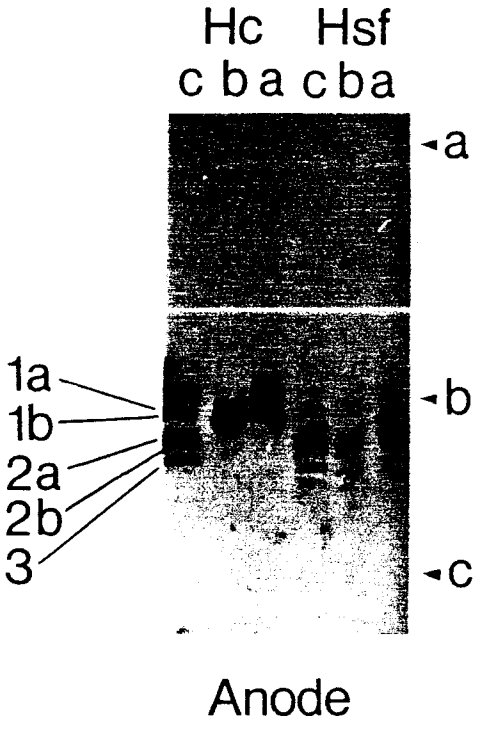

211
mls
219
mls
224
mls
S M F S M F S M F

at pH 8.9 migrates as the fastest band in SDS gels (Larson et al., 1981). It would appear, then, that the subunit pattern seen on pH 8.9 gels is due at least in part to charge differences among the subunits. Thus, if there are different kinds of 25S molecules, composed of different subunits, it is likely that they also will differ by charge.

To test this hypothesis, the 25S hemocyanin from C. magister was isoelectrically focused in a linear pH gradient (pH 4-5.5). The results, shown in Fig. 3, depend on where the Hc is applied to the gel. If the Hc is applied anodal to its eventual focused position, at least five bands are resolved with different isoelectric points between 4.4 and 4.6. If the protein is applied at the site where it will eventually focus or cathodal to that point, no sharp bands are resolved. Instead the protein forms a broad zone spanning the gradient from pH 4.5 to 4.6. Horse spleen ferritin, run as a control, focuses to a complex pattern of bands with isoelectric points between 4.3 and 4.7. The pattern of the focused ferritin is independent of its site of application. The results were the same for Hc or ferritin first dialyzed against either 4% Ampholytes or 1% glycine.

There are several explanations for the hemocyanin's particular banding pattern: 1) The bands could represent different aggregation states of the Hc, since C. magister Hc is known to change aggregation state in response to changes in pH and ionic strength (Ellerton et al., 1970). 2) The bands could be an artifact, since banding patterns of focused proteins that are dependent on the site of loading have been interpreted to be the result of artifacts generated as the protein migrates through the ampholyte gradient (Righetti and Drysdale, 1976), or

Fig. 3. Isoelectric focusing gel of 10 ug of 25S C. magister Hc and horse spleen ferritin (Hsf). The letters refer to the site of application of the proteins.


Hc
Hsf
c b a c b a
a
b
c
1a
1b
2a
2b
3
Anode

3) The bands could be 25S hemocyanins with different subunit compositions and therefore different isoelectric points.

To determine the aggregation state of the Hc bands at their pI, the focused bands, visible in the unstained gel by oblique lighting, were sliced from the focused gel. Four of the bands numbered 1a, 1b, 2a and 2b from the cathode (Fig. 3), were removed in two slices, 1 and 2, since these pairs of bands were too close together to be completely separated in the very soft gels. The slices were immediately placed in the wells of a pH 8.0 gel (system II) and electrophoresed along with 25S, 16S, and 5S Hc standards. The pre-focused proteins all migrated in this electrophoresis system identically to one another and to the 25S hemocyanin standard (Fig. 4).

The bands in Fig. 3 were also analysed for differences in subunit composition. The slices were placed in SDS incubation buffer and analyzed by SDS PAGE. As can be seen in Fig. 5, all of the 3 slices appear to contain Hc with the same subunit composition.

To test for the possibility that the isoelectric focusing pattern was artifactual, the bands were sliced from the isoelectric focusing gel as in the previous experiments and immediately refocused (Fig. 6). Five bands corresponding to the original five IEF bands were generated from each of the two anodal slices. The most cathodal slice (bands 1a and 1b) produced two bands, 1a and 2b.

Fig. 4. Native gel electrophoresis at pH 8.0 of focused 25S Hc and 2.5 ug of 25S and 16S Hc in 1% glycine and 5S Hc in tris/glycine, 10 mM EDTA, pH 8.9 as standards. 10 ug of 25S Hc was focused, the bands cut out, and the gel slices subjected to PAGE in system II. (S) standards; (1) focused bands 1a, 1b; (2) focused bands 2a, 2b; (3) focused band 3.

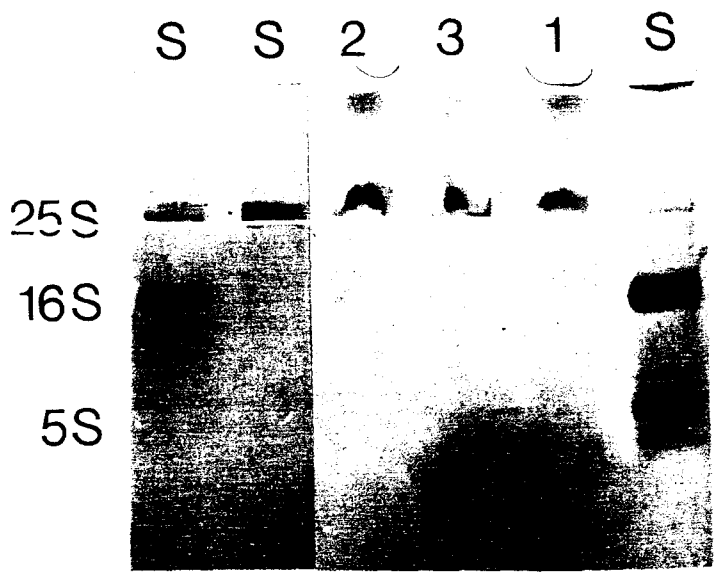

S
S
2
3
1
S
25S
16S
5S

Fig. 5. SDS PAGE of focused 25S Hc. 10 ug of 25S Hc was focused, the bands cut out and the gel slices subjected to SDS PAGE. (S) 25S Hc standard, 2.5 ug; (1) focused bands 1a, 1b; (2) focused bands 2a, 2b; (3) focused bands 3.

S 3 2 1

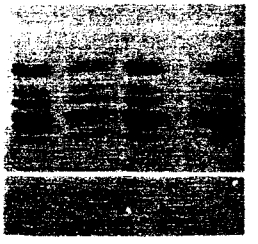
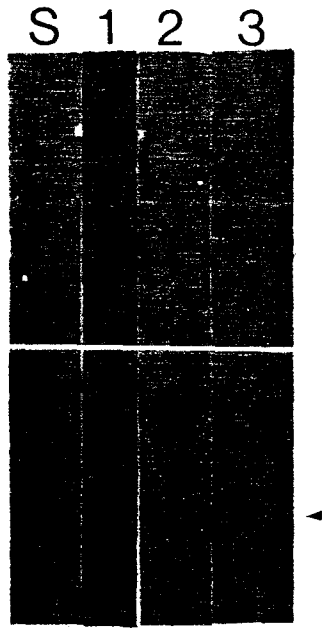


Fig. 6. Isoelectric focusing gel of previously focused Hc. 20 ug of Hc was isoelectrically focused, the bands cut out and immediately refocused. (S) 10 ug of 25S Hc in 1% glycine (1) bands 1a, 1b; (2) focused bands 2a, 2b; (3) focused band 3; (◄) site of application of the samples.


S 1 2 3

Other Tests for Heterogeneity

Electrophoresis of the 25S hemocyanin in system I or II at gel concentrations between 4 and 5% and at different hemocyanin concentrations results in single sharp bands. However, if there are slight differences in charge and/or molecular weights in the 25S molecules, differences in subunit composition in the hemocyanin from the leading or trailing parts of the electrophoresed band might be detected.

The results of an experiment in which the 25S bands from a system I gel were sliced into slow, middle, and fast portions and analyzed by SDS-PAGE were shown in Fig. 2. Fig. 7 shows the result from a similar experiment from a system II gel. In this case a sample from the pooled fractions containing the 25S Hc from the A-5m column in Fig. 1 were electrophoresed. Again no qualitative differences in banding patterns are seen.

In order to test the ability of the electrophoretic systems to detect heterogeneity, an artificial C. magister 25S Hc was reassembled from dissociated subunits as described elsewhere (Chapter 5). It differs in subunit composition by having less of subunit VI and more subunit I. A 50/50 mixture of this protein and the native protein migrates as a single sharp band in both system I and system II. If, however, this band is divided into slow and fast portions and analyzed by SDS PAGE, subtle differences in subunit patterns are evident. Fig. 8 shows the results of the SDS PAGE of the slow and fast slices from a system I gel. There appears to be less subunit VI relative to the other bands in the fast portion of the 25S band than in the slow

Fig. 7. SDS PAGE of the 25S bands from a system II gel. 10 and 15 ug of 25S Hc were electrophoresed at pH 8.0, the bands divided, and the gel slices subjected to SDS PAGE. (S) slow, (M) middle and (F) fast portions of the 25S band.

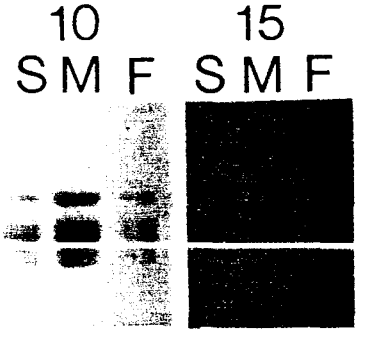
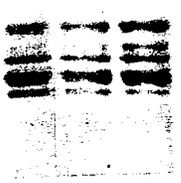

10
15
S M F S M F

Fig. 8. SDS PAGE of the 25S band from a system I gel of a 50/50 mixture of native and artificial 25S Hc. 5 ug of a 50/50 mixture of an artificial Hc and the native Hc were electrophoresed at pH 7.5, the 25S band divided, and the gel slices subjected to SDS PAGE. (S) slow; (F) fast portions of the 25S band of the 50/50 mixture; (N) slice S, fraction 219 of Fig. 2.

N S F



portion. Similar results were obtained using system II gels.

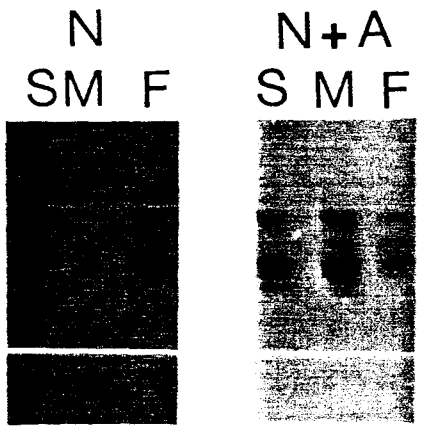
If the 16S band from a system I or system II gel is separated into slow, middle, and fast portions substantial differences in subunit composition are seen. For example, there is little or no subunit II (16S C. magister Hc lacks subunit III) in the slow portion of the the 16S bands (Fig. 9). When a 50/50 mixture of an aritifical 16S Hc, also reduced in subunit VI (Chapter 5), and the native 16S hemocyanin is electrophoresed and the broad band divided, the slow portion of the band is clearly enriched in subunit VI in comparison to the fast portion (Fig. 9).

Further Characterization of Quaternary Structure

The results described above indicate that the two-hexamer population of C. magister is homogeneous. Assuming this is so, the different subunits should be present in whole number molar ratios. The first step to determine subunit stoichiometry would be to isolate each of 5S subunits and to determine its specific absorbance, etc. Attempts were made to purify individual subunits by numerous methods such as ion exchange chromatography in several buffers, including urea-containing buffers, and preparative gel electrophoresis. Our attempts were frustrated by the tendency of certain subunits to reassemble under the conditions of these experiments (Chapter 5).

An estimation of the ratio of subunits within the 25S two-hexamer was obtained by staining the protein in gels after the subunits were separated by SDS PAGE, and measuring the amount of dye bound to each band. Two dyes were used, Fast green FCF and Coomassie Brilliant Blue

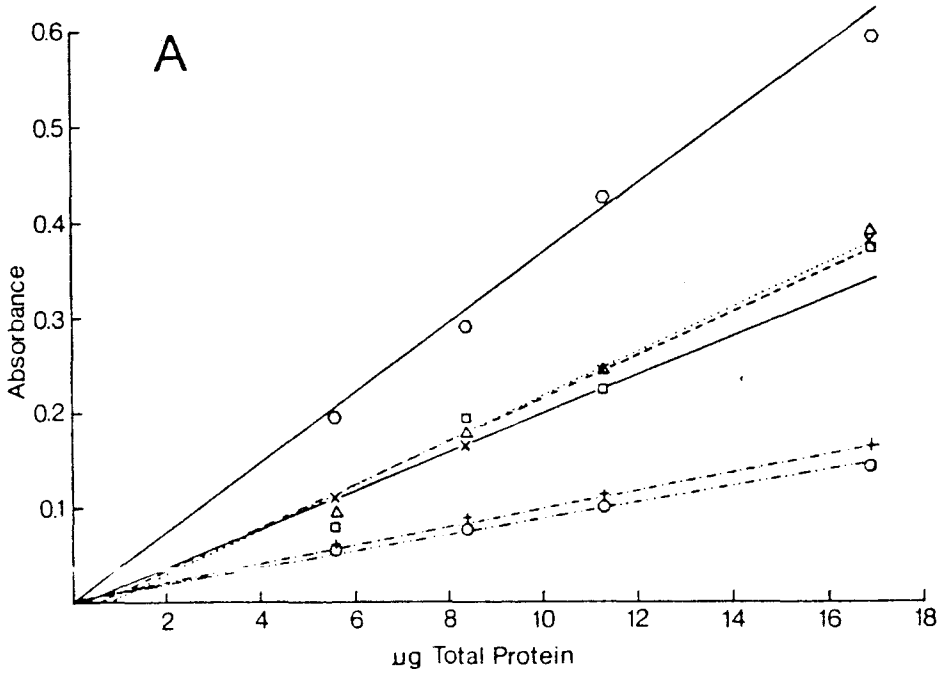
Fig. 9. SDS PAGE of the 16S bands from a system II gel. 10 ug of 16S Hc was electrophoresed at pH 8.0, the bands divided, and subjected to SDS PAGE. (N) native Hc; (N+A) native Hc and artificial Hc 1:1; (S) slow, (M) middle, and (F) fast portions of the bands.


N
SM F
N+A
S M F

R. These two dyes bind to proteins by different mechanisms and thus show different variations in dye binding with different proteins (Wilson, 1979). Fig. 10a shows the amount of dye eluted from gels stained with Coomassie blue and Fig. 10b shows Fast green. Coomassie blue stains all the bands more intensely than the Fast green, but both gels show the same general pattern of the amount of stain bound in the bands. There is a small and equal amount of dye bound in bands II and VI, an intermediate and equal amount in bands I, II, and V; band IV bound the most dye.

The slopes and standard error of the slopes of the regression lines drawn in Fig. 10 are expressed as a fraction of the total slope and are normalized to parts in twelve in Table I. There are slight differences in the results from the experiment using Fast green versus those using Coomassie Brilliant blue. There was relatively more dye bound to Band IV and less to Band V and I, in the Fast green stained gels. However, these results are not significantly different from one another using student's test (95% confidence interval). The amount of either dye in Band I through VI can be expressed by the ratio 2:1:2:4:2:1. Only in the case of Band V, stained by Fast green, does the whole number ratio (2) lie outside the 95% confidence interval (1.67 ± 0.28) for the data. The predicted fraction, based on the above stoichiometry and apparent molecular weights of the subunits (Larson et al., 1981), are also shown in Table 1. Only in the case of subunit II for Coomassie blue is the predicted value (0.087) outside the confidence interval (0.070 ± 0.011) for the data.

Fig. 10. Absorbance of Coomassie Brilliant blue (A) and Fast green FCF (B) eluted from gel slices from SDS PAGE, 25S Hc. The lines drawn are the least squares regression lines. (✕---) band I, (○-·-·-) band II, (△···) band III, (○ ——) band IV, (□——) band V, (+-·-·) band VI.


A
Absorbance
0.6
0.5
0.4
0.3
0.2
0.1
2
4
6
8
10
12
14
16
18
µg Total Protein

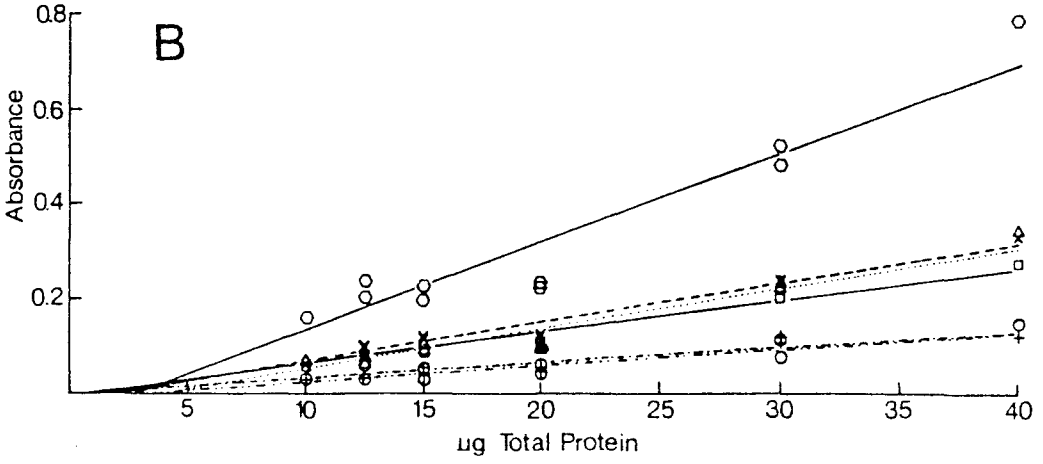

B
Absorbance
0.8
0.6
0.4
0.2
5
10
15
20
25
30
35
40
µg Total Protein

TABLE 1. The Relative Amounts of Dye Bound to SDS PAGE Separated Subunits of C. magister 25S Hc.

Subunit	Fraction of total slope		Predicted fraction*	Number per twelve	
	Coomassie blue	Fast green		Coomassie blue	Fast green
I	0.190 ± 0.011	0.170 ± 0.011	0.192	2.28 ± 0.13	2.04 ± 0.12
II	0.070 ± 0.004	0.073 ± 0.008	0.087	0.83 ± 0.04	0.87 ± 0.10
III	0.175 ± 0.004	0.174 ± 0.016	0.170	2.10 ± 0.04	2.08 ± 0.19
IV	0.325 ± 0.009	0.376 ± 0.034	0.325	3.89 ± 0.11	4.51 ± 0.41
V	0.165 ± 0.011	0.139 ± 0.010	0.156	1.97 ± 0.13	1.67 ± 0.12
VI	0.077 ± 0.004	0.069 ± 0.006	0.076	0.92 ± 0.04	0.82 ± 0.07

* Predicted fraction calculated from a stoichiometry of 2:1:2:4:2:1 and the apparent molecular weights of the subunits as reported by Larson et al. (1981).

Discussion

The evidence reported above supports the hypothesis that there is only one 25S C. magister Hc molecule. The 25S Hc cannot be further fractionated by methods that distinguish proteins based on charge nor molecular weight.

If the 25S hemocyanin were heterogeneous, it is likely that the different two-hexamers would have different molecular weights. The subunits of C. magister hemocyanin have apparent molecular weights ranging from 67,000 to 82,000 as determined by SDS PAGE (Larson et al., 1981). If the apparent molecular weights measured in SDS gels reflect actual molecular weights, and if there is more than one kind of 25S hemocyanin with respect to its subunit composition, these 25S molecules should also have slightly different molecular weights. For example, the largest and smallest subunits differ in apparent molecular weight by 18%. Therefore, hemocyanins made exclusively of the largest or smallest subunit will also differ in molecular weight by 18%. Although these different oligomers would not fractionate as separate peaks by gel filtration, the larger hemocyanin might be expected to dominate in the leading portion of the 25S peak and the smaller one in the trailing portions. When this hypothesis was tested, no differences could be found between the trailing and leading edges of the 25S peak. When this experiment was further refined by looking for charge and/or mol. wt. differences by gel electrophoresis, no heterogeneity was evident. Our data are consistent with the earlier studies of Ellerton et al. (1970) who reported that C. magister Hc is homogeneous in the ultra-

centrifuge to within 2% (M_r = 938,000 ± 20,000). Both this data and our data suggest that if the subunits vary in size by up to 16,000 mol. wt., only a limited number of subunit combinations are possible.

One possible problem with the above reasoning might be that the values obtained for subunit molecular weights in SDS are unreliable and that they overestimate the actual variation in subunit size. We think that this is unlikely. The range of values for subunit molecular weights of this Hc as determined by sedimentation equilibrium at alkaline pH and in guanidine HCl (Ellerton et al., 1970; Carpenter and van Holde, 1973) are consistent with those determined in SDS by Larson et al. (1981). Furthermore, the molecular weights of purified Hc subunits from Eurypelma californicum, a tarantula, and Cherax destructor, a freshwater crayfish, show good agreement between M_r values determined by SDS PAGE and those determined by sedimentation equilibrium (Schneider et al., 1977; Markl et al., 1979a, 1979b; Jeffrey and Treacy, 1982). Molecular weight values for arthropod hemocyanins as determined by the two methods are in general very similar (Jeffrey and Treacy, 1982). However, until there is some sequence data to support these subunit molecular weights, we shall be unsure of their precise values. Nevertheless, we and Ellerton et al. (1970) were unable to detect molecular weight heterogeneity in this protein.

If the hemocyanin were heterogeneous, it is likely that the different oligomers would have different charges. The first attempt to fractionate the hemocyanin based on charge utilized isoelectric focusing.

The hemocyanin can be focused isoelectrically to five bands; this could be interpreted as evidence for charge heterogeneity. However, the specific banding pattern of the focused Hc is dependent on the site of application of the protein to the gel. Therefore, we believe the banding pattern represents artifacts and not charge isomers of the 25S hemocyanin. Our focusing technique appears to be satisfactory since horse spleen ferritin, run on the same gels, focuses to the same complex pattern of bands no matter how or where it is applied to the gel. All five bands can be produced by refocusing any one of three of the bands. Thus it is likely that the bands represent an artifact or conformation change introduced as the protein migrates through the pH gradient. The two bands which do not refractionate may have undergone a more irreversable change. The different bands do not represent different aggregation states of the hemocyanin as shown by the electrophoresis experiments. The pH dependent dissociation seen by Ellerton et al. (1970) at alkaline pH apparently does not occur under the more acid conditions of the isoelectric focusing experiments. Furthermore, all of the bands had indistinguishable subunit compositions, which supports the argument that the bands do not represent charge isomers.

The behavior of C. magister Hc on isoelectric focusing gels does not appear to be unusual for arthropod hemocyanins. Markl et al. (1981a) found that the Hc of E. californicum focused to several different patterns depending on the support medium. For example, the Hc focused to a broad zone in silicone rubber tubing, and, as in our study, no differences in subunit composition were detected across that zone.

Our failure to fractionate the Hc at two higher pH's by PAGE

further supports the conclusion that there is only one kind of 25S Hc with respect to its charge distribution.

The above experiments are predicated on the assumption that the subunits differ in charge and that their charge differences will be evident when assembled into the two-hexamer. The five subunit bands of C. magister, resolved by alkaline disc electrophoresis (Larson et al., 1981), are likely to be charge isomers. The order of mobilities are different from those seen for the same subunits in SDS (Larson et al., 1981). Furthermore, the order of elution of subunit fractions from a DEAE sephadex column is consistent with the order of migration of the subunits in alkaline PAGE (Chapter 5). Other investigators working with both decapod and chelicerate hemocyanins have shown that the separation of subunits with different charges but similar molecular weights (Murray and Jeffrey, 1974; Lamy et al., 1977; Robinson and Ellerton, 1977; Schneider et al., 1977; van den Berg et al., 1977; Markl et al., 1979a; Brenowitz et al., 1981; Ellerton and Anderson, 1981; and van Eerd and Folkerts, 1981).

Although there are charge differences among the subunits, are these differences apparent in the native 25S protein? It is possible that any charge differences among the subunits could be masked in the assembled 25S Hc. We have some evidence that the charge of the subunits is not completely masked when they are assembled in the 25S molecule. If the native Hc is co-electrophoresed with a reassembled C. magister Hc with a slightly different subunit composition, they can be resolved from one another. The separation of the two kinds of hemocyanin appears to be based on charge. The artificial Hc runs slightly faster

than the native Hc, as would be predicted from the assumed charges of the constituent subunits. The artifical Hc has less subunit VI, the slowest moving subunit in alkaline PAGE, and more subunit I, which just precedes subunit VI in alkaline PAGE (Chapter 5). Results from similar experiments on the 16S hemocyanin of C. magister also support our argument that different charges of the subunits are not completely masked in the aggregated state. Furthermore, Jeffrey et al. (1976, 1978) and Marlborough et al. (1981) have shown that Cherax destructor hexamers and two-hexamers produced by the assembly of different subunits with charge differences can be separated electropheretically. The relative mobility of the assembled Hc is predictable from the relative mobilities of its constituent subunits.

The two-hexamer hemocyanin of C. magister behaves as if it were homogeneous with respect to charge and molecular weight. The data leave some room for the existence of a limited level of 25S heterogeneity; there are a small number of subunit substitutions possible that would result in different hemocyanins with molecular weights which would differ by less than 2%. The most sensitive technique for resolving proteins by charge, isoelectric focusing, failed to resolve the 25S Hc into fractions with different subunit compositions, but the presence of the multiple bands and broad zones in the focusing gels is troubling.

If we assume from the above analysis that C. magister 25S Hc is homogeneous, the six different subunits of the Hc should be found in stoichoimetric amounts. We attempted to get an idea of the ratios of the subunits by measuring the amount of dye bound to the subunits separated by SDS PAGE. We used two different dyes, Fast green FCF

and Coomassie Brilliant blue R, each of which binds to proteins by a different mechanism (Wilson, 1979). Both dyes gave almost identical estimates of subunit stoichiometry. For almost all of the subunits the calculated ratios are close to whole number values for both dyes.

There are limitations to this technique. One cannot be sure that each polypeptide is binding the same amount of dye. We have not successfully purified the subunits, therefore, we cannot determine the amount of dye bound to a known quantity of each pure subunit. However, some indirect evidence suggests that the data may be useful as a first approximation of subunit stoichiometry in this Hc. The Fast green and Coomassie blue data agree remarkably well even though the dyes bind to proteins by different mechanisms and in strikingly different amounts. A protein can bind four or five times more Coomassie blue than Fast green (Wilson, 1979). Different proteins do not show the same variations in dye binding when stained with Fast green and Coomassie blue (Wilson, 1979). If there are significant differences in the dye binding properties of the C. magister Hc subunits, the differences are very similar for both dyes, and are such that the relative amounts of the subunit appear to be in whiole number ratios. Furthermore, it is not surprising that the subunits might show similar dye binding properties. Markl and Kempter (1981a) studies the immunological relatedness of the different subunits of three different crabs including C. pagurus from the same genus as C. magister. They found three kinds of subunits with respect to their immunological relatedness. Two of the four different subunits in each case were immunologically identical, one was immunologically deficient to these two and one was unrelated to

the other three. At least the three immunologically related subunits would be expected to have considerable sequence homology and therefore may bind similar amounts of dye.

We propose that the 25S Hc of C. magister is homogeneous or very nearly so and have reported a possible stoichiometry for its subunit composition. The fact that the native 25S Hc of Cancer magister is homogeneous implies that there is some specificity of subunit interactions. Presumably it is the specificity of these interactions that determines the structure of the hemocyanin. In other words, homogeneity at the level of the 25S protein implies that the subunits have specific roles in determining the structure of the two-hexamer hemocyanin. However, the native 25S hemocyanin from the megalopa and 1st instar juvenile crabs has only five of the six subunits seen in the adult hemocyanin, and the relative amounts of the five subunits are different from the adult as judged by their staining properties on SDS PAGE (Terwilliger and Terwilliger, 1982). Therefore, if specific subunits do have specific roles in determining structure, some substitutions must be allowed in some positionsto allow for the assembly of the larval Hc and the Hc assembled in vitro (Chapter 5). Therefore there seems to be more kinds of subunits than can be accounted for by structural arguments alone.

The other hemocyanins that are probably homogeneous are the 37S Hc's of the chelicerates. In these Hc's the subunit-subunit interactions can be highly specific. Most of the subunits of the 37S four-hexamers and 60S eight-hexamers of three chelicerates have been assigned specific structural roles (van Bruggen et al., 1980). For

example, each of the seven subunits of E. californicum has a specific structural role, and the four-hexamer Hc has been shown to be homogeneous with respect to charge and molecular weight (Markl et al., 1981b). The presence of all seven subunits is required to assemble the 37S protein and only a molecule identical to the native is made (Markl et al., 1981a). The subunit interactions are not quite as specific in the four-hexamer of Androctonus australis as they are in E. californicum Hc. When the four-hexamer of A. australis is reassembled, one of the eight subunits is left out and is replaced by another (Lamy et al., 1981a). Specific models of the quaternary structure of these two proteins have been proposed in which each of the different subunits is assigned a specific location in the four-hexamers (Lamy et al., 1981a; Markl et al., 1981c; Sizaret et al., 1982). The models are based on reassembly experiments and the EM profiles of the Hc tagged with FAB fragments specific to each subunit.

In the 25S hemocyanins for which data is available, there does not seem to be a structural requirement for more than two kinds of subunits. One of the two serves as the linker subunit, a specific subunit that has been proposed by Markl et al. (1981b) to hold the 16S units into the higher ordered structures. The simplest case is that of the Hc of Ligia pallasii, a semiterrestrial isopod. The two-hexamer Hc of this animal has only two kinds of subunits one of which is proposed to be the linker (Terwilliger, 1982). So subunit heterogeneity beyond two kinds of subunits is not required to build a 25S hemocyanin. Also each of the purified monomeric subunits of the 25S Hc of Cupiennius salei, a spider, will assemble to a 25S Hc in low yield when incubated

with the dimeric linker subunit (Markl and Kempter, 1981b). Two-hexamer hemocyanins can also be made from binary mixtures of purified subunits of Cherax destructor (Jeffrey et al., 1978). However, unlike the Hc of C. magister, the Cherax two-hexamer can be resolved into multiple bands by PAGE at near neutral pH (Jeffrey et al., 1978). By comparing these bands with Hc assembled from mixtures of purified subunits Jeffrey et al. (1978) conclude that these bands represent two-hexamers with one copy of the homodimer (the pair of linking subunits) and varying numbers of copies of the different monomers. The results of the reassociation experiments of Jeffrey et al. (1978) and Marlborough et al. (1981) indicate that all of the different subunits may be able to occupy all of the positions in the reassociated Hc. Since some of these possibilities include multiple copies of the homodimer, structures larger than 25S are generated and are seen in their gels as a continuum of bands running to the top of the gel. The 25S Hc of Ovalipes cartharus, a brachyuran crab, and Jasus edwardsii, a spiny lobster, contain three kinds of subunits each and can be resolved into multiple bands with PAGE at near neutral pH (Robinson and Ellerton, 1977). Presumably the bands represent 25S hemocyanins with different proportions of the three subunits.

In the cases of the 25S hemocyanins of C. destructor, C. salei and perhaps also O. cartharus and J. edwardsii, subunit heterogeneity does not appear to be related to any requirement for structure. It would appear that the subunit-subunit interactions are not specific with the exception of the inter-hexamer linkage. The Hc of C. magister is different from these other two-hexamer hemocyanins in that there is no

detectable heterogeneity at the level of the two-hexamer, and therefore there appears to be some specificity among the subunit-subunit interactions. The specificity is such that some substitutions are allowed over the course of development, or when the protein is assembled in vitro.

The way the 25S and 37S hemocyanins are organized appears to fall into two groups. One group, consisting of the 25S hemocyanins of the crustaceans C. destructor, L. pallasii, and perhaps O. cartharus and J. edwardsii, and the chelicerate C. salei, have varying amounts of subunit heterogeneity; however, the heterogeneity is not necessary for structure. All the subunits can apparently occupy all positions in the 25S molecule with the exception of the position of the linker. These hemocyanins usually show detectable heterogeneity. The second group includes the 25S Hc of C. magister and the 37S hemocyanins of E. californicum and A. australis. Heterogeneity at the level of the two-hexamer or four-hexamer is not evident and there is considerable specificity of subunit-subunit interactions. Within this latter group there seems to be some spectrum of the specificity of the subunit interactions. At one extreme is the Hc of E. californicum in which each of the subunits has a unique structural role. At the other extreme is the 25S Hc of C. magister where subunit interactions are specific enough to maintain a high level of homogeneity in the 25S Hc of the crab, but a limited number of different hemocyanins are possible; e.g., over the course of development or when the Hc is assembled in vitro.

The apparent diversity of the rules which govern the way the subunits of arthropod hemocyanins interact structurally raise interesting questions as to the evolution of the quaternary structure of these

proteins, the specific selective advantage of the subunit heterogeneity, and the mechanisms by which these heterogeneous subunits interact functionally to produce the striking allosteric behavior characteristic of these very large proteins.

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CHAPTER FIVE

STRUCTURE, FUNCTION, AND ASSEMBLY IN THE 25S TWO-HEXAMER HEMOCYANIN OF CANCER MAGISTER: EVIDENCE THAT SUBUNIT COMPOSITION DETERMINES OXYGEN BINDING BEHAVIOR

Introduction

The arthropod hemocyanins are characterized by showing striking subunit heterogeneity (Markl et al., 1979a; 1979b; van Holde and Miller, 1982; Linzen, 1983). The function of this heterogeneity is not well understood.

It has been determined that subunit heterogeneity does play an important role in the assembly of the structurally complex 37S four-hexamer and 60S eight-hexamer hemocyanins (Hc's) of the chelicerates (eg. van Bruggen et al., 1980). In the 37S Hc's of Eurypelma californicum, a tarantula, and Androctonus australis, a scorpion, each of seven kinds of subunits has a specific role in the assembly of the Hc (Decker et al., 1980; Lamy et al., 1980). A subunit with a particular role in a hemocyanin from one species can perform the same role in a mixture of subunits from another species to produce hybred four-hexamer Hc's (van Bruggen et al., 1980). Models showing the specific location of each of the seven and eight different subunits of the 37S Hc's from Eurypelma and Androctonus have been proposed (Lamy et al., 1981a; Markl et al., 1981d; Sizaret et al., 1982).

The role of subunit heterogeneity in the apparently simpler quaternary structure of the crustacean 25S two-hexamer Hc's is less well

understood. These Hc's have not been as amenable to experimental manipulation as the four-hexameric Hc's of the chelicerates. To our knowledge the only two-hexamer crustacean Hc from which subunits have been purified is the Hc of Cherax destructor, a freshwater crayfish, (Murray and Jeffrey, 1974; Jeffrey et al., 1976; Marlborough et al., 1981). Also the dissociation of the 25S hemocyanins is not as reversible as it is in the chelicerate Hc's. Reassociation from dissociated subunits usually results in hexameric hemocyanins with only small amounts of 25S Hc being reassembled (Carpenter and van Holde, 1973; Hamlin and Fish, 1977; Jeffrey et al., 1978; Herskovitz et al., 1981; Terwilliger et al., 1979; Terwilliger, 1982).

There may well be fundamental differences between 25S two-hexamer and 37S four-hexamer Hc's with respect to the way different subunits are organized. One important difference, for example, is that the 25S Hc's of some crustaceans have been shown to be electrophoretically heterogeneous (Jeffrey et al., 1978; and Robinson and Ellerton, 1977). On the other hand, there are also basic similarities between the two. For example, no heterogeneity was detected in the 25S Hc of Cancer magister, the Dungeness crab, (Chapter 4). Also, as in the chelicerate Hc's, a specific subunit pair seems to be involved in holding the hexamers together in two-hexamer structures (eg. Jeffrey et al., 1978; Markl et al., 1981c).

The contribution of the different subunits to the function of the assembled hemocyanin is even less well understood. There is some evidence that different monomeric subunits of Limulus polyphemus,

Eurypelma, and Androctonus Hc's have different oxygen binding behaviors (Bonaventura et al., 1977; Decker et al., 1979; Lamy et al., 1980; Markl et al., 1981a). Jeffrey and Treacy (1980) have shown that two hexameric Hc's made from different subunits from Cherax destructor have different oxygen binding behaviors; one responds to Ca^{++}, the other is insensitive.

Accordingly this study was undertaken to elucidate the structural and functional role of the subunit heterogeneity in the two-hexamer Hc of the brachyuran crab, Cancer magister. Previous studies in our laboratory have established that the subunits of this Hc are heterogeneous (Larson et al., 1981). The 25S Hc is probably homogeneous (Chapter 4); and the subunit composition and function of the 25S Hc changes with development (Terwilliger and Terwilliger, 1982, Terwilliger et al., 1982).

In the course of attempting to isolate the six different subunits of C. magister Hc by ion exchange chromatography, we reconstituted a 25S Hc that is slightly different in subunit composition from the native Hc. Previous attempts to reconstitute the 25S Hc had yielded mostly 16S Hc and very little 25S material (Carpenter and van Holde, 1973). In addition we have available to us a native 25S Hc from first instar C. magister crabs that also has a different subunit composition (Terwilliger and Terwilliger, 1982). We therefore used the opportunity presented by the availability of these three Hc's with different subunit compositions to investigate the contribution of different subunits to the oxygen binding behavior of a crustacean 25S two-hexamer hemocyanin.

We report, here, some aspects of the reassociation behavior of the hemocyanin subunits which provide further support for the hypothesis, advanced in our earlier report (Chapter 4), that the subunit interactions show some specificity. Results form the oxygen binding experiments on an artificial 25S Hc, and two native 25S Hc's indicate that different subunits do contribute differently to the oxygen binding behavior of the assembled Hc.

Materials and Methods

Preparation of the Hemocyanin

Mature male Cancer magister were trapped near the mouth of Coos Bay, Charleston, OR, and were maintained in running sea water at 31-32 o/oo and 11-15°C. Blood was removed by syringe from an infrabranchial sinus, allowed to clot on ice for 1 hr, and centrifuged to remove the clot and debris. If the whole Hc was to be stored the serum was dialyzed against 0.05 M Tris-HCl, 0.5 M NaCl, 10 mM EDTA, pH 7.5 and stored at 5°C.

The 25S and 16S Hc's were purified on a Bio-Gel A-5m (200-400 mesh) column (2.2 x 100 cm) equilibrated with 0.05 M Tris-HCl, 0.5 M NaCl, and 10 mM EDTA (pH 7.5). The hemocyanin chromatographed as a major fraction, designated 25S or two-hexamer Hc, and a minor 16S or one-hexamer Hc fraction as reported by Carpenter and van HOlde (1973) and Larson et al. (1981).

The Hc concentration of the purified Hc was estimated by the absorbance at 280 nm using the value $E^{1\%}_{1\ cm}$ = 15.0 (Nickerson and van Holde, 1971). The Hc concentration of whole Hc was estimated by the absorbance at 337 nm using the value $E^{1\%}_{1\ cm}$ = 3 which is based on the experimentally determined value of 0.20 for the 337/280 absorbance ratio of purified Hc.

The 25S Hc from first instar juvenile crabs was prepared as described by Terwilliger and Terwilliger, (1982). A sample of 25S Hc from adult crabs was also purified on the same column in the same buffer to compare with the juvenile 25S Hc.

Ion Exchange Chromatography

C. magister serum dialyzed, against a 0.052 M Tris-glycine, 10 mM EDTA, pH 8.9 dissociating buffer, was chromatographed on a DEAE Sephadex A-50 column (1.5 x 25 cm) equilibrated to the dissociation buffer. The columns were developed as described in results. Hc's to be used in binding experiments were prepared on 2.5 x 25 cm ion exchange columns. They were developed in the same way as the analytical columns with the amount of buffers scaled for the larger diameter column. Flow rates were kept as low as practicable to maximize the yields of the subunit VI fraction and the 25S fraction.

Electrophoresis

All electrophoresis experiments were done on 1.5 x 100 x 140 mm slabs using the apparatus described by Studier (1973).

The discontinuous buffer system of Laemmli (1970) was used for SDS PAGE. Hemocyanin samples were treated and electrophoresed as in Larson et al. (1981) except that dithiothreitol (0.05 M) was substituted for 5% 2-mercaptoethanol in some experiments. PAGE at pH 8.9 in the absence of denaturants followed the procedure of Davis (1964) without a stacking gel. Samples were treated and electrophoresed as in Larson (1981). Electrophoresis at near neutral pH was carried out using the buffer system I described in Chapter 4. Gels were stained with Coomassie Brilliant blue R (Fairbanks, 1971), and destained in 10% acetic acid.

The amount of Coomassie blue bound to the separated proteins in SDS gels was measured by the method of Fenner et al. (1975). The gels were destained until there was no measurable background absorbance from slices cut from a region of the gel with no protein. All the slices were eluted in 0.8 ml of the 25% pyridine and the absorbances corrected for the gel slice volume as in Chapter 4.

Sedimentation Velocity

Sedimentation velocity analyses were performed on the Beckman-Spinco Model E analytical ultracentrifuge equipped with a photoelectric scanner. Double sector cells were used in an An-D rotor, and the sample scanned at 280 nm. Samples at the higher concentrations were also scanned at 337 nm.

Oxygen Binding Equilibrium

Oxygen binding equilibrium curves of the Hc were determined at 20°C by a tonometric method (Benesch et al., 1965). The air equilibrated sample was first diluted to an absorbance of about 1.0 at 337 nm prior to analysis by this method. Some experiments were done at lower concentrations of Hc with no change in the results. For examples that did not saturate in 100% oxygen at 1 atm, the absorbance at infinite oxygen pressure was estimated from the y intercept of a plot of reciprocal absorbance vs reciprocal P_{O_2} for the air and 100% oxygen equilibrated samples. The pH of the air equilibrated samples was measured at 20°C at the end of the binding experiment with an Orion Ross combination electrode calibrated at 20°C against precision standard buffers.

For binding experiments on whole fresh Hc the serum was immediately passed through a Sephadex G-25 column equilibrated to a crab saline (Graham et al., 1983). The binding experiments were done on the same day the blood was collected. For binding experiments on stored whole Hc or purified Hc's the samples were dialyzed against the crab saline prior to the binding experiments. Some binding experiments were performed in a less complex buffer described in results.

The effect of L-lactate on the oxygen binding equilibrium was determined as in Graham et al., (1983).

Results

Ion Exchange Chromatography

An example of an elution profile of the ion exchange chromatography of C. magister serum dialyzed against dissociation buffer is shown in Fig. 1. The hemocyanin elutes in two peaks. Peak I is almost pure subunit VI (Fig. 2) and migrates as a 5S monomer in PAGE at pH 7.5 (Fig. 3). Peak II is typically asymetrical and contains the rest of the subunits plus some subunit VI especially near the front of the peak (Fig. 2). The Hc in the front part of Peak II migrates mostly as 16S Hc in PAGE at pH 7.5 with some Hc migrating as a monomer (Fig. 3). The trailing portion of Peak II is dominated by material that migrates as 25S, and has less material migrating as 5S Hc.

When the portion of Hc Peak II containing the 25S Hc is dialyzed against dissociation buffer and rechromatographed, the Hc elutes in two peaks (Fig. 4). The first peak contains Hc which migrates as 16S Hc in PAGE at pH 7.5 (Fig. 5) and contains subunits I, II, IV, V, and a trace of VI, except the leading edge which also has subunit III (Fig. 6). Band VI is visible in the gels but not readily apparent in the photographs. The second peak contains a Hc which migrates as 25S Hc on PAGE at pH 7.5 (Fig. 5) and contains a normal complement of subunits except VI is reduced (Fig. 6). Up to 40% of the material eluting from some of these columns is in the form of a 25S Hc.

When the second "25S" peak is concentrated by vacuum dialysis and chromatographed on A-5m the Hc elutes in the same volume as the native

Fig. 1. Elution profile of the DEAE Sephadex chromatography of C. magister serum dialyzed against dissociation buffer. The 1.5 x 25 cm column was loaded with 90 mg of Hc and developed with 20 mls of 0.25 M NaCl in dissociation buffer followed by 300 mls of a 0.25 to 0.50 M linear gradient of NaCl in dissociation buffer. The fractions sampled for analysis by electrophoresis are marked by letters. The bars denote fractions pooled for further experiments. (●) absorbance at 280 nm, (▲) absorbance at 337 nm.

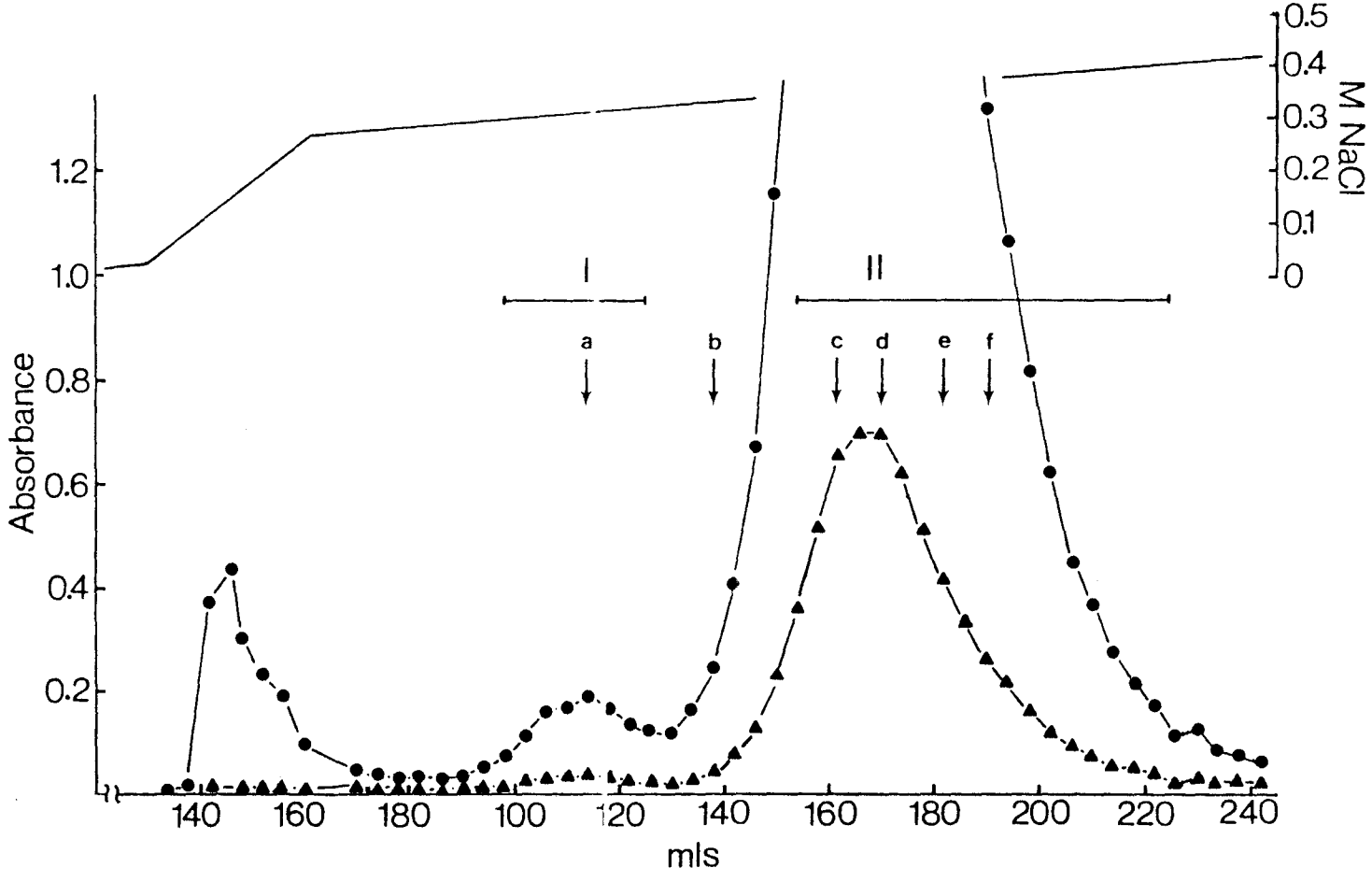

Absorbance
1.2
1.0
0.8
0.6
0.4
0.2
M NaCl
0.5
0.4
0.3
0.2
0.1
0
I
II
a
b
c
d
e
f
140
160
180
100
120
140
160
180
200
220
240
mls

Fig. 2. SDS PAGE of whole Hc and samples from the ion exchange in Fig 1. 2.5 ug of Hc from each sample was loaded onto the gel. (S) whole Hc; (a-f) samples from the ion exchange.

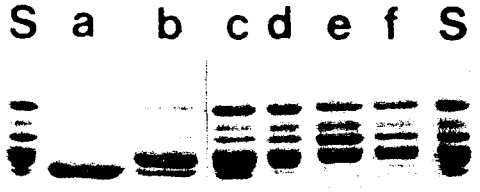

S a b c d e f S

Fig. 3. PAGE at pH 7.5 of whole Hc, dissociated Hc, and samples from the ion exchange in Fig. 1. 2.5 ug of Hc from each sample was loaded onto the gel. (S) whole Hc, (D) dissociated Hc, (a-f) samples from the ion exchange.

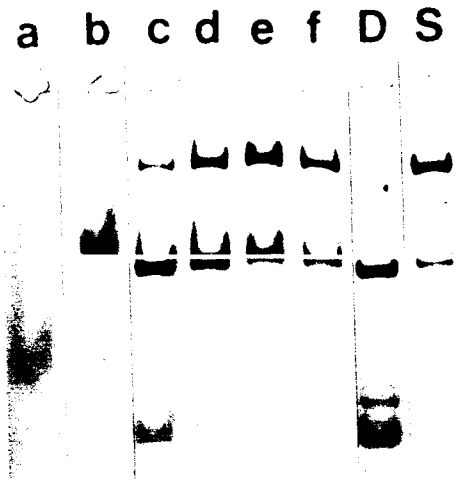

a
b
c
d
e
f
D
S

Fig. 4. Elution profile of the DEAE Sephadex chromatography of a portion of the pooled fractions, 151-226 mls, from the ion exchange chromatography shown in Fig. 1. 40 mg of the Hc, which had been dialyzed against dissociation buffer, was loaded onto the 1.5 x 25 cm column, and the column developed with 15 mls of 0.25 M NaCl, 25 mls of 0.30 M NaCl, and 300 mls of a 0.30 to 0.45 M NaCl linear gradient buffered with dissociation buffer. The fractions sampled for analysis by electrophoresis are denoted by letters. The bars mark fractions pooled for further experiments. (●) absorbance at 280 nm, (▲) absorbance at 337 nm.

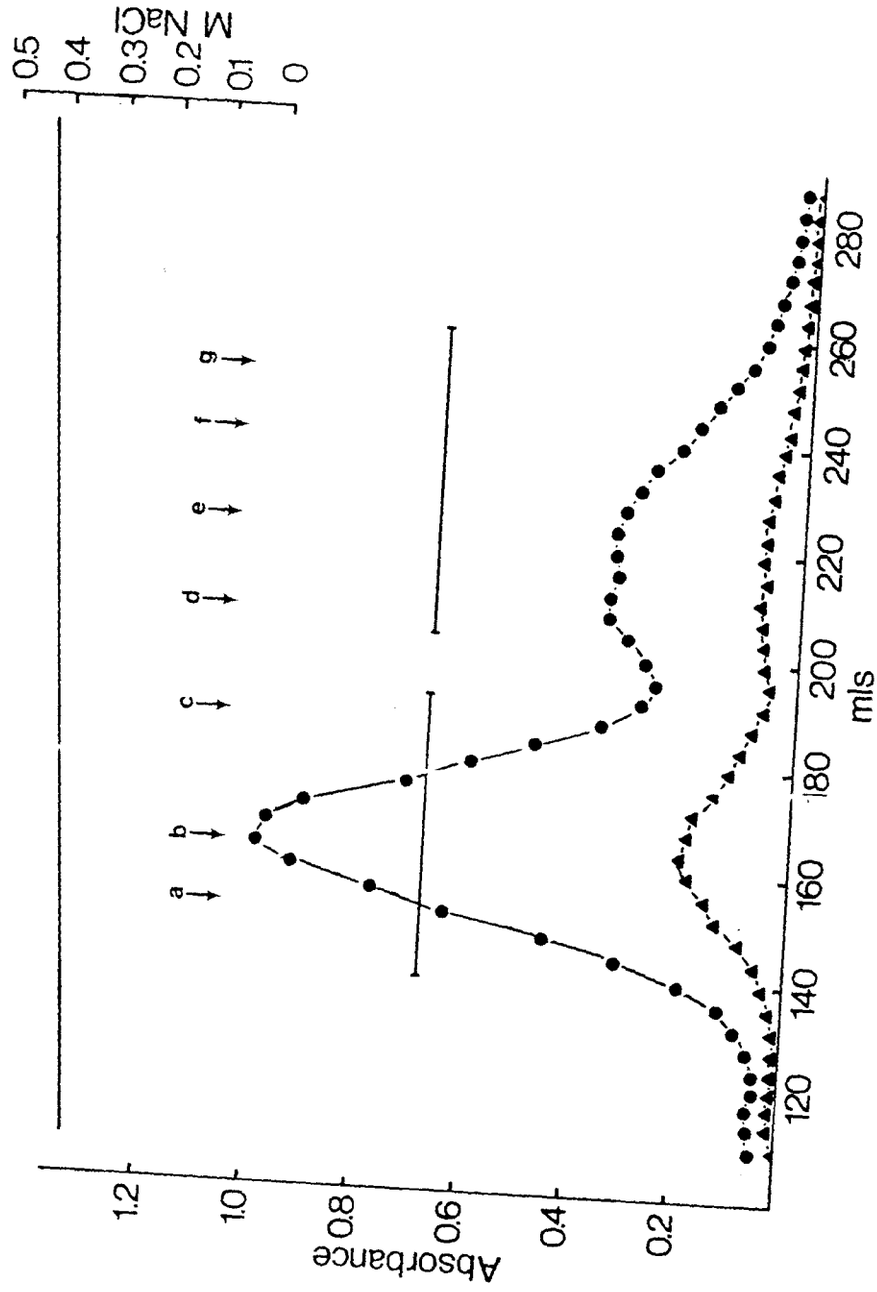

M NaCl
0.5
0.4
0.3
0.2
0.1
0
a
b
c
d
e
f
g
Absorbance
1.2
1.0
0.8
0.6
0.4
0.2
120
140
160
180
200
220
240
260
280
mls

Fig. 5. PAGE at pH 7.5 of whole Hc, dissociated Hc, and the samples from the ion exchange shown in Fig. 4. 2.5 ug of Hc from each sample was loaded onto the gel. (S) whole Hc, (D) dissociated Hc. (a-g) samples from the ion exchange.

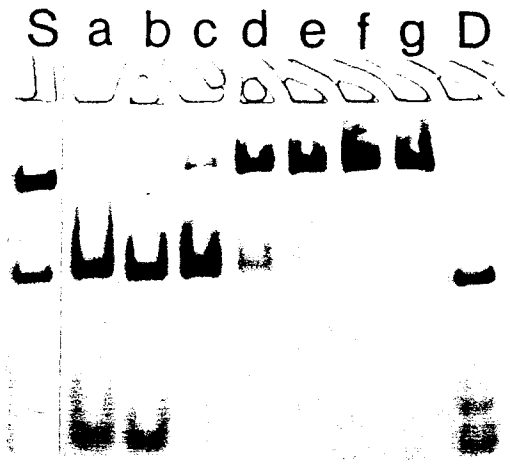
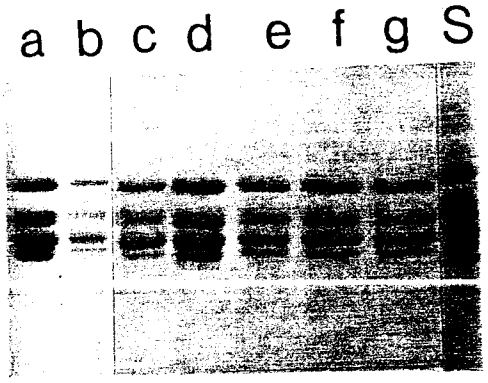

S a b c d e f g D

Fig. 6. SDS PAGE of whole Hc and the samples from the ion exchange in Fig. 4. 2.5 ug of Hc from each sample was loaded onto the gel. (S) whole Hc, (a-g) samples from the ion exchange.


a b c d e f g S

25S Hc (Fig. 7). A small amount of material elutes in this column as 16S Hc. Presumably this Hc is from the first, "16S", ion exchange peak that overlaps into the "25S" peak. The first, "16S", ion exchange peak elutes in the same volume as the native 16S Hc when chromatographed on this column; there is some material also eluting as 25S.

Larson et al. (1981) report a slow moving band in their pH 8.9 disc PAGE of subunits dissociated at pH 10, that is not seen when 25S Hc is electrophoresed. They speculate that this band represents reassociated Hc. We experienced the same phenomenon when whole Hc and subunits dissociated at pH 8.9 were electrophoresed at pH 8.9. Therefore, we were concerned as to the nature of the material represented by the slow moving band that was being loaded onto our ion exchange columns. We, therefore, chromatographed a sample of dissociated Hc on Bio-Gel A-5m. The Hc elutes in two peaks (Fig. 8). A sample of the first "aggregate," peak migrates as a 16S Hc in PAGE at pH 7.5; a sample from the second, "monomer," peak migrates as monomers, hexamers and a trace of two-hexamers in PAGE at pH 7.5 (Fig. 9). The first, "aggregate," peak contains subunits I, II, and IV; the second peak contains subunits I, III, IV, V, and VI (Fig. 10). The aggregate peak contains about 30% of the total protein eluted from the column.

Further Characterization of the Ion Exchange Products

Peak I from the first ion exchange chromatography contains mostly subunit VI and traces of subunit I and IV. Subunit VI was purified by taking advantage of the tendancy of subunits I and IV to form aggregates

Fig. 7. Elution profile of the gel permiation chromatography of 4 mg of Hc from the pooled 25S fractions (205-261 mls) of the ion exchange column shown in Fig. 4. The Hc was chromatographed on a 2.2 x 100 cm Bio-Gel A-5m column equilibrated to dissociation buffer made 0.35 M in NaCl. The elution volume of the native 25S and 16S Hc are marked. (●) absorbance at 280 nm, (▲) absorbance at 337 nm.

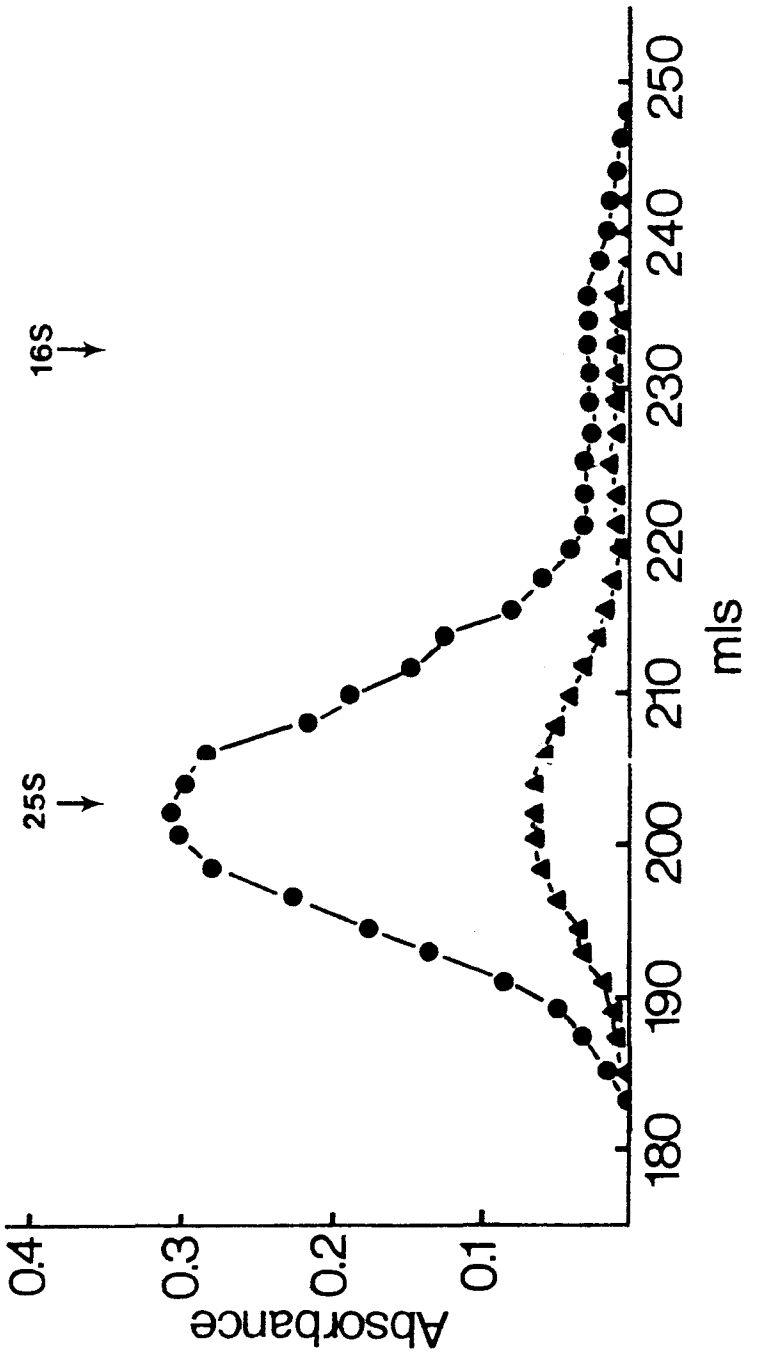

16S
25S
0.4
0.3
0.2
0.1
Absorbance
180
190
200
210
220
230
240
250
mls

Fig. 8. Elution profile of the chromatography of 33 mg of dissociated C. magister Hc on a Bio-Gel A-5m column (2.2 x 50 cm) equilibrated to dissociation buffer. The fractions analyzed by electrophoresis are marked by letters. (●) absorbance at 280 nm, (▲) absorbance at 337 nm.

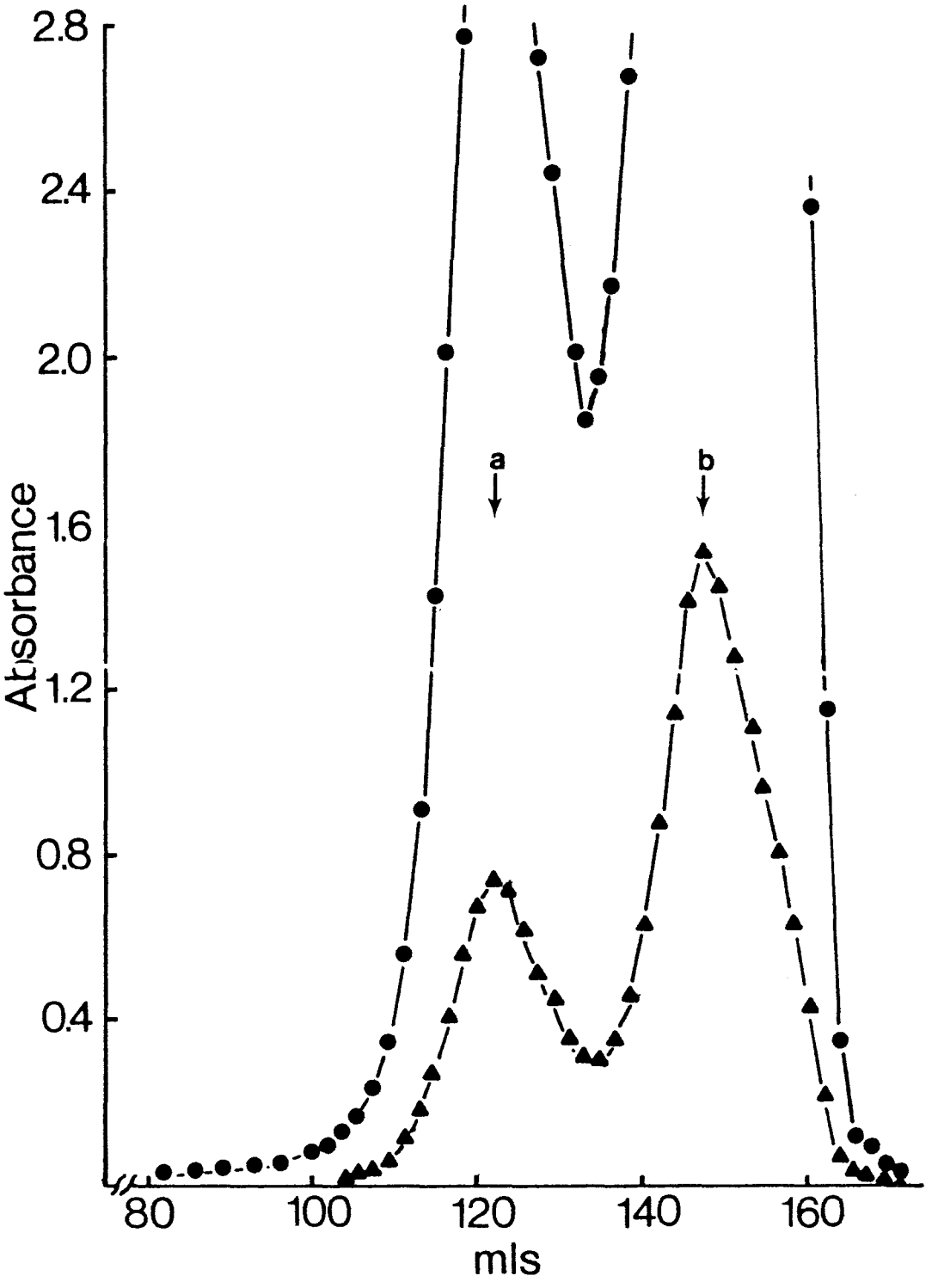

2.8
2.4
2.0
1.6
1.2
0.8
0.4
Absorbance
a
b
80
100
120
140
160
mls

Fig. 9. PAGE at pH 7.5 of whole Hc and samples from the Bio-Gel A-5m column shown in Fig. 8. 2.5 ug of Hc from each sample was loaded onto the gel. (S) whole Hc, (a,b) samples from the Bio-Gel column.


a b S

Fig. 10. SDS PAGE of whole Hc and samples from the Bio-Gel column shown in Fig. 8. 2.5 ug of Hc from each sample was loaded onto the gel. (S) whole Hc, (a,b) samples from the Bio-Gel column.


a b S

under conditions where much of subunit VI remains monomeric. The subunit VI peak from a 2.5 cm diameter ion exchange column was vacuum dialyzed to 1 mg/ml and dialyzed against 0.05 M Tris-HCl, 1 mM EDTA, pH 7.5. This material was then chromatographed on Bio-Gel A-5m (Fig. 11). The hemocyanin eluted in two peaks at the same elution volumes of the aggregate and monomer peaks shown in Fig. 8. The aggregate peak contained mostly subunit VI and traces of subunits I and IV. The monomer peak contained only subunit VI (Fig. 12).

To establish that the reassociated products from the second ion exchange step were indeed 25S and 16S Hc as indicated by the gel permiation chromatography and electrophoresis, the artificial 25S and 16S Hc's, the native 25S and 16S Hc's, and the purified subunit VI were investigated by sedimentation velocity. The native hemocyanin of C. magister has been well described by both sedimentation velocity and sedimentation equilibrium experiments (Ellerton et al., 1970; Carpenter and van Holde, 1973). We saw no need to duplicate this work. Our interest was to establish that the artificial Hc's that we had assembled from subunits are indeed hexameric and two-hexameric molecules with molecular weights, i.e. S values, similar to the native. We chose to do these experiments in 0.05 M Tris-HCl, 0.5 M NaCl, 10mM EDTA, pH 7.5 because the quaternary structures of the Hc's are stable in this buffer as judged from their behavior on Bio-Gel A-5m columns, and because the Hc's showed no evidence of degradation of either structure or function after long term storage in the cold in this buffer.

Fig. 11. Elution profile of the gel permiation chromatography of 5.5 mg of the subunit VI fraction from the ion exchange of dissociated Hc. The pooled subunit VI fraction from the ion exchange column, treated as in the text, was chromatographed on a 2.2 x 50 cm column of Bio-Gel A-5m equilibrated to 0.05 M Tris-HCl, 1 mM EDTA, pH 7.5. The fractions sampled for analysis by electrophoresis are marked by letters. The bar denotes the fractions containing pure subunit VI which were pooled for further experiments. (●) absorbance at 280, (▲) absorbance at 337 nm.

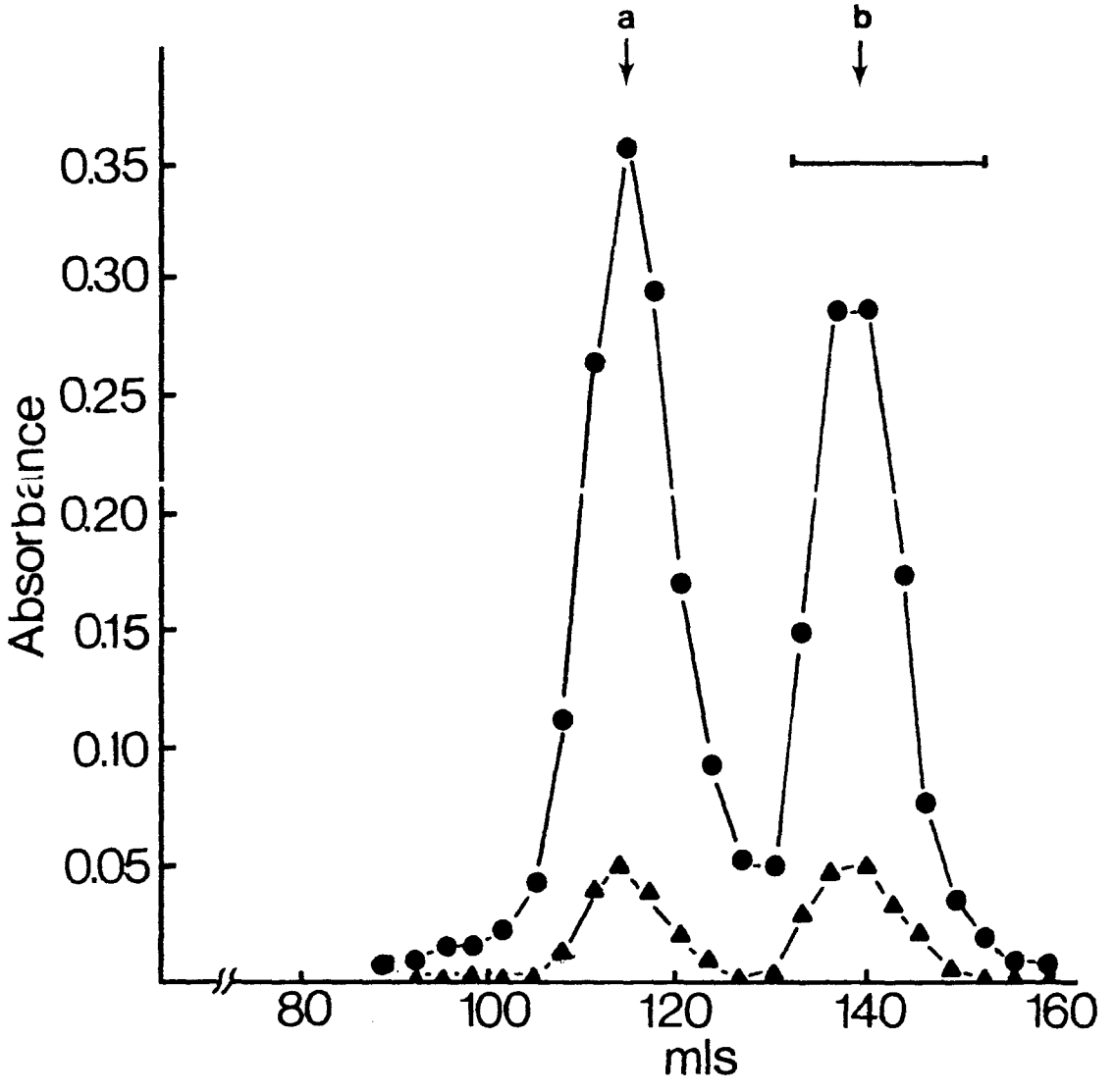
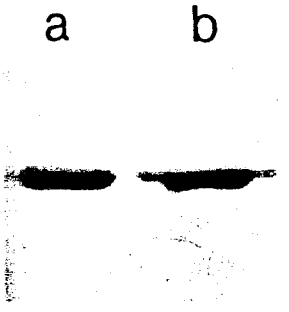

a
b
0.35
0.30
0.25
0.20
0.15
0.10
0.05
Absorbance
80
100
120
140
160
mls

Fig. 12. SDS PAGE of samples from the Bio-Gel column in Fig. 11. 5.0 ug of Hc from each sample was loaded onto each lane. (a,b) samples from the Bio-Gel column.


a
b

The 25S and 16S Hc's were purified by chromatography on Bio-Gel A-5m in the above buffer. The monomeric subunit VI fraction from the column in Fig. 11 was dialyzed against the above buffer. Preliminary experiments showed that this caused the subunits to aggregate, presumably to hexamers. The concentration of the proteins was adjusted to about 1 mg/ml by vacuum dialysis or dilution for experimentation in the ultracentrifuge. The results of the sedimentation experiments are shown in Fig. 13. The artificial Hc's all sediment similarly to the native Hcs. The S values, corrected for the density and viscosity of 0.5 M NaCl, are similar to those reported by Ellerton et al., 1970.

The subunit compositions of the purified artificial 25S and 16S Hcs are indistinguishable from the subunit compositions of the 25S and 16S peaks from the second ion exchange shown in Fig. 8. The quantitative difference in subunit composition between the native and artificial 25S Hc derived from the same batch of serum was determined by measuring the amount of dye eluted from the six stained subunits seperated by SDS PAGE. To control for any differences in dye binding among the subunits that might occur, a dilution series of equal amounts of native and artificial 25S Hc were electrophoresed on the same gel. The slopes and standard errors of the slope of plots of the absorbance of the eluted dye vs ug total protein (eg. Fig. 10A, Chapter 4) for two such gels are shown graphically in Fig. 14. The ratios of the two slopes for each subunit are shown in Table 1. The amount of dye bound to band VI in the artificial Hc is about one-half that bound to the band in the native Hc. The amount of dye bound to Band I of the artificial Hc is about 1.5

Fig. 13. The uncorrected sedimentation coefficients of the various Hc's in 0.05 M Tris-HCl, 0.5 M NaCl, 10 mM EDTA, pH 7.5. The Hc samples were treated as in the text and were serially diluted for the determination of S at various concentrations of Hc. (●) native 25S Hc, (○) artificial 25S, (▲) native 16S Hc, (△) artificial 16S Hc, (□) subunit VI.

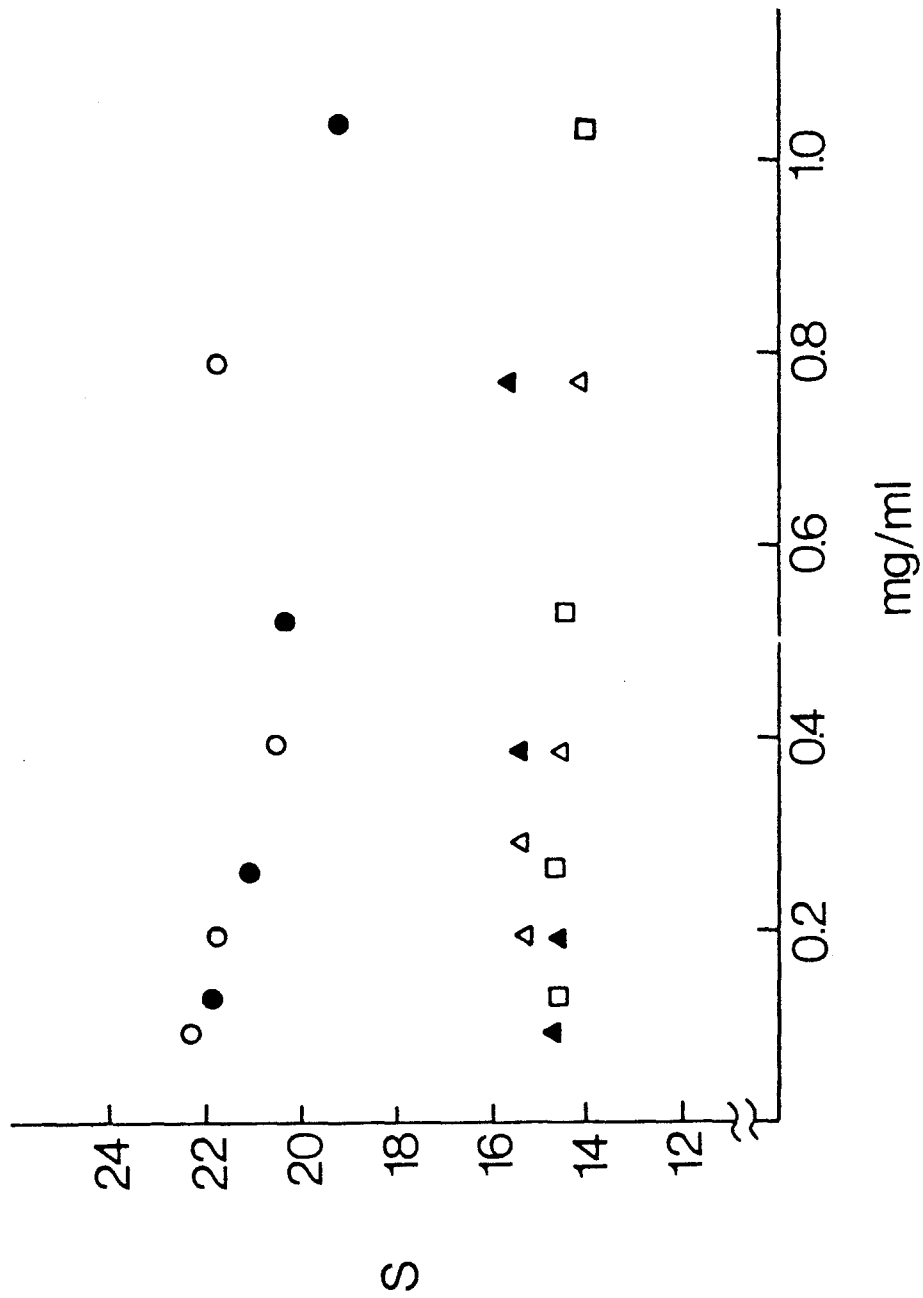

S
24
22
20
18
16
14
12
0.2
0.4
0.6
0.8
1.0
mg/ml

Fig. 14. Absorbance of Coomassie Brilliant blue eluted from gel slices containing the subunits separated by SDS PAGE. The slope of the regression line of plots of the absorbance of dye eluted vs ug Hc loaded on the gel is shown on the left vertical scale and the slope per average total slope times 12 is shown on the right vertical scale. Two paired experiments are shown. The amount of dye eluted from the subunits from the artificial Hc are shown hatched. The standard errors of the slopes of the regression lines are indicated.

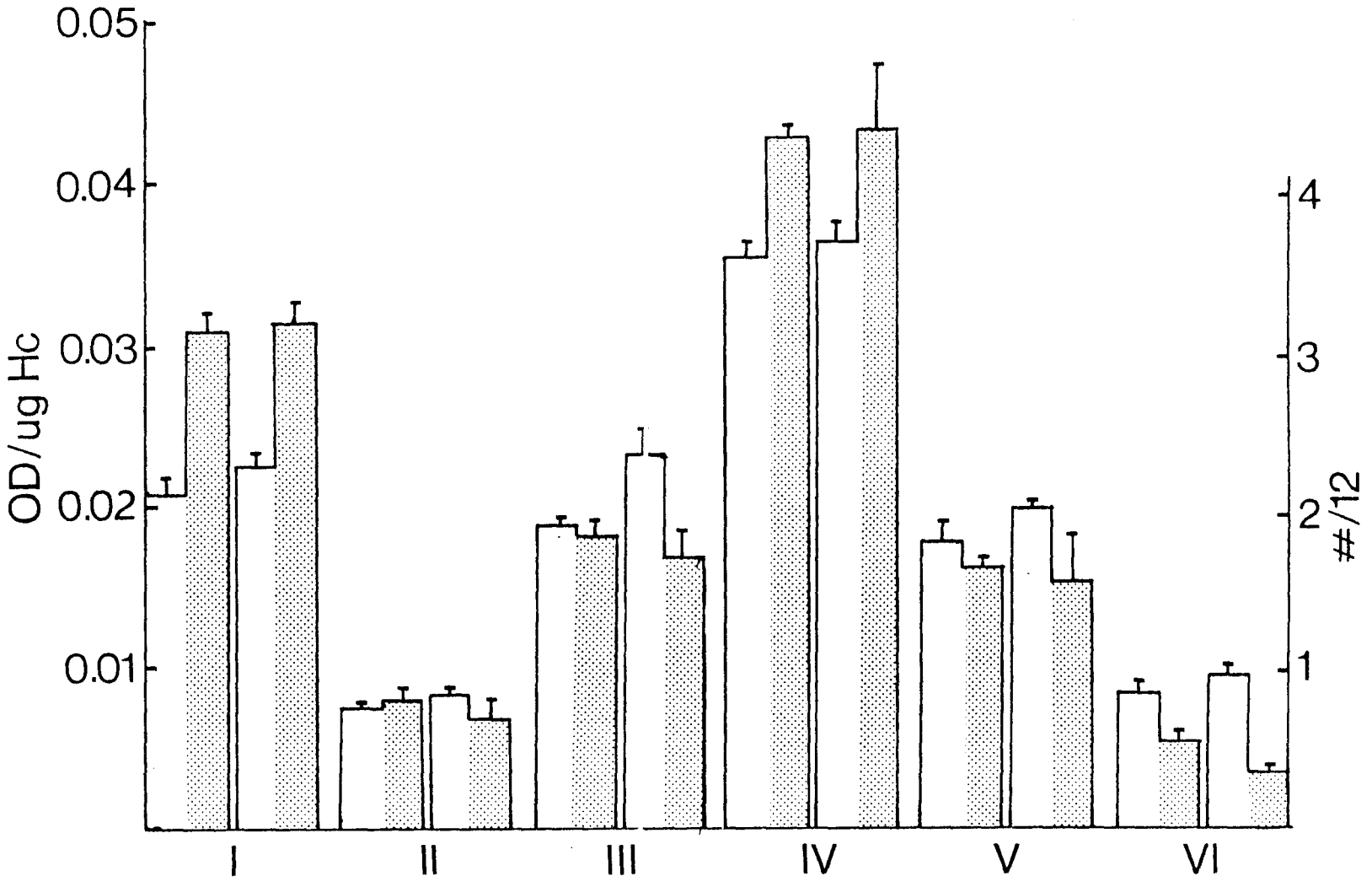

0.05
0.04
0.03
0.02
0.01
OD/ug Hc
4
3
2
1
#/12
I
II
III
IV
V
VI

TABLE 1. Ratio of Slopes of the Absorbance of Eluted Dye vs ug Total Hc from the Subunits of the Native and Artificial Hc Separated by SDS PAGE

Subunit	Slope, Artificial Hc / Slope, Native Hc Gel #1	Gel #2
I	1.50	1.39
II	1.05	0.82
III	0.98	0.73
IV	1.21	1.18
V	0.90	0.79
VI	0.65	0.39
Σ I-VI	1.12	0.98

times that bound to the band in the native Hc. There appears to be slightly more dye bound to band IV of the artificial Hc and slightly less dye bound to bands III and V of the artificial. The difference in the values of the slopes for bands I and VI in the native and artificial Hc are significant by Student's t test for the difference of two slopes ($P < .05$). The difference in the values of the slopes for subunit II and V in the two Hc's are not significant. In the case of band III, gel #2 the difference is significant (95% confidence interval for the difference = 0.0063 ± 0.0051) as it is for band IV in gel #1 (95% confidence interval = 0.073 ± 0.028).

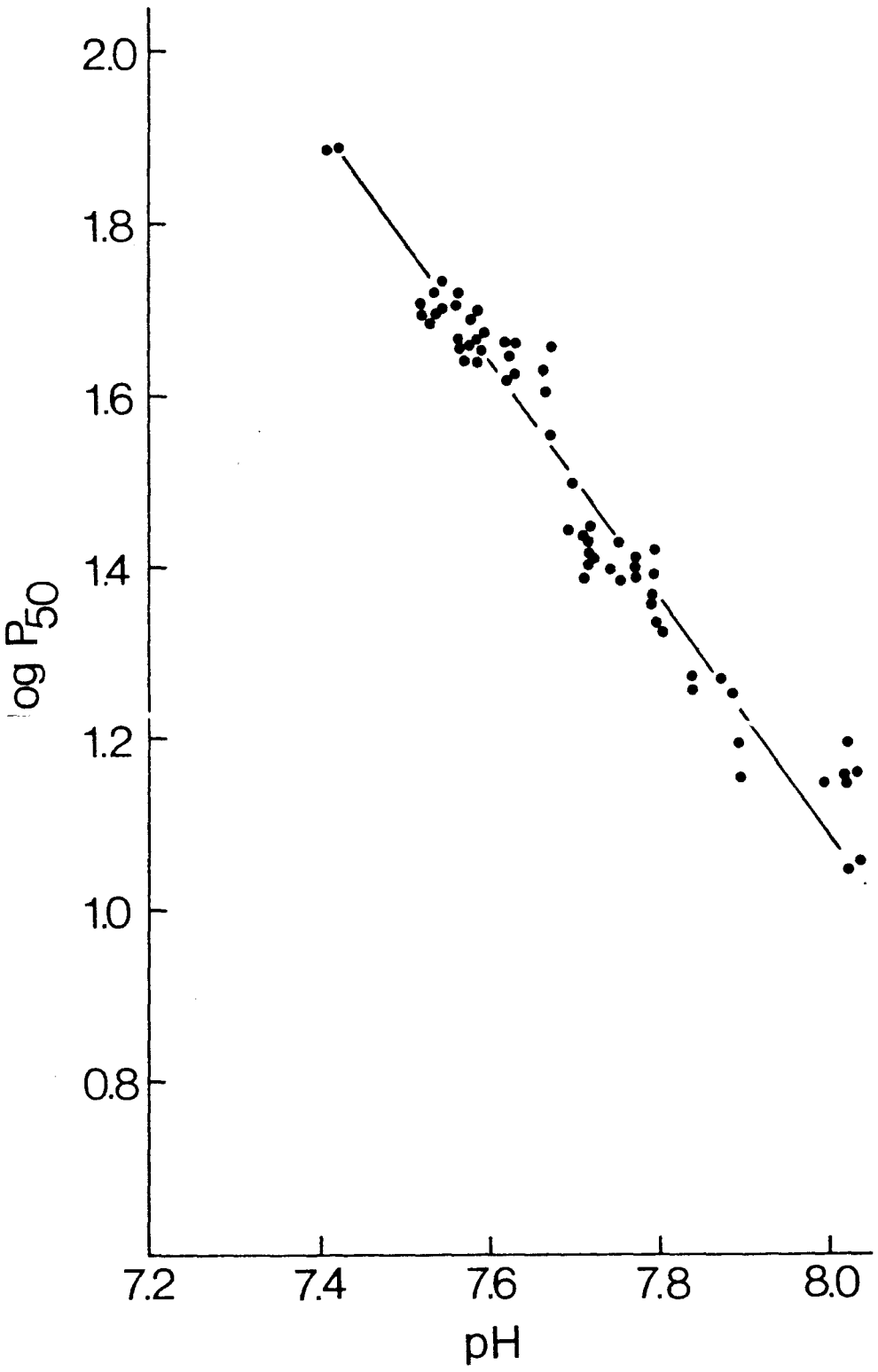
The Oxygen Binding Properties of the

25S Native and Artificial Hemocyanins

The oxygen binding affinity of C. magister whole Hc at 20°C in crab saline is shown in Fig. 15.

The oxygen binding behavior of purified native and artificial 25S Hc's in crab saline was compared by making paired observations of the binding behavior of the two proteins. The two Hc's, prepared from the same batch of serum and stored in 0.05 Tris-HCl, 0.5 M NaCl, 10mM EDTA, pH 7.5 were dialyzed against the same crab saline, and the oxygen binding equilibrium was measured at the same time. As controls for the possible effects of aging of the Hc, the O_2 binding properties of the original sample of unfractionated Hc was determined on the day the blood was drawn from the animal. A sample of the unfractionated serum was also dialyzed against the above buffer, stored in the cold,

Fig. 15. Oxygen binding affinity of C. magister unfractionated Hc in C. magister saline at 20°C.


2.0
1.8
1.6
1.4
1.2
1.0
0.8
log P50
7.2
7.4
7.6
7.8
8.0
pH

and its binding properties determined along with the purified 25S Hc's. A sample of fresh serum drawn from a different animal was also investigated along with the experimental samples. Fig. 16 shows the oxygen binding curves generated in two such experiments. Fig. 17 shows the oxygen binding affinity of the various Hc samples in five experiments at various pH's. The Hill coefficients at P_{50} are shown in Fig. 18.

The oxygen binding affinity of the native 25S Hc is indistinguishable from the stored unfractionated Hc, the unfractionated Hc before it was stored, fresh Hc drawn from a different animal, and the unfractionated Hc's shown in Fig. 15. The $n_{H_{50}}$ is also indistinguishable from the stored unfractionated Hc and the same Hc before it was stored. The fresh unfractionated Hc from a different animal has a slightly higher $n_{H_{50}}$ than the rest of the samples in Fig. 18. Therefore, storage or fractionation of the Hc does not affect the oxygen binding properties of the Hc.

The oxygen binding properties of the artificial Hc is distinguishable from the native. The artificial Hc has an O_2 binding affinity that is slightly lower than the native 25S Hc. The average difference in log P_{50} of 0.060 is significant according to students $\underline{t}$ test for paired observations ($P < .05$). The artificial Hc is also more cooperative than the native. The average difference in $n_{H_{50}}$ of 0.48 is highly significant ($P < 0.005$). The Bohr effects in the two proteins are indistinguishable.

The possibility that the two Hc's might respond differently to L-lactate was investigated. The results from these experiments at pH 7.8 are shown in Fig. 19. The slope of the least squares regression line for the artificial Hc (Δ log P_{50} / Δ log mM lactate = -0.242) is indis-

Fig. 16. Hill plots of the oxygen binding equilibrium of the purified artifical and native 25S Hc's and unfractionated Hc's at 20°C. (O) Native 25S, (Δ) artificial 25S, (●) unfractionated Hc from the same batch of serum and stored as in results, (■) fresh unfractionated Hc drawn from a different animal.

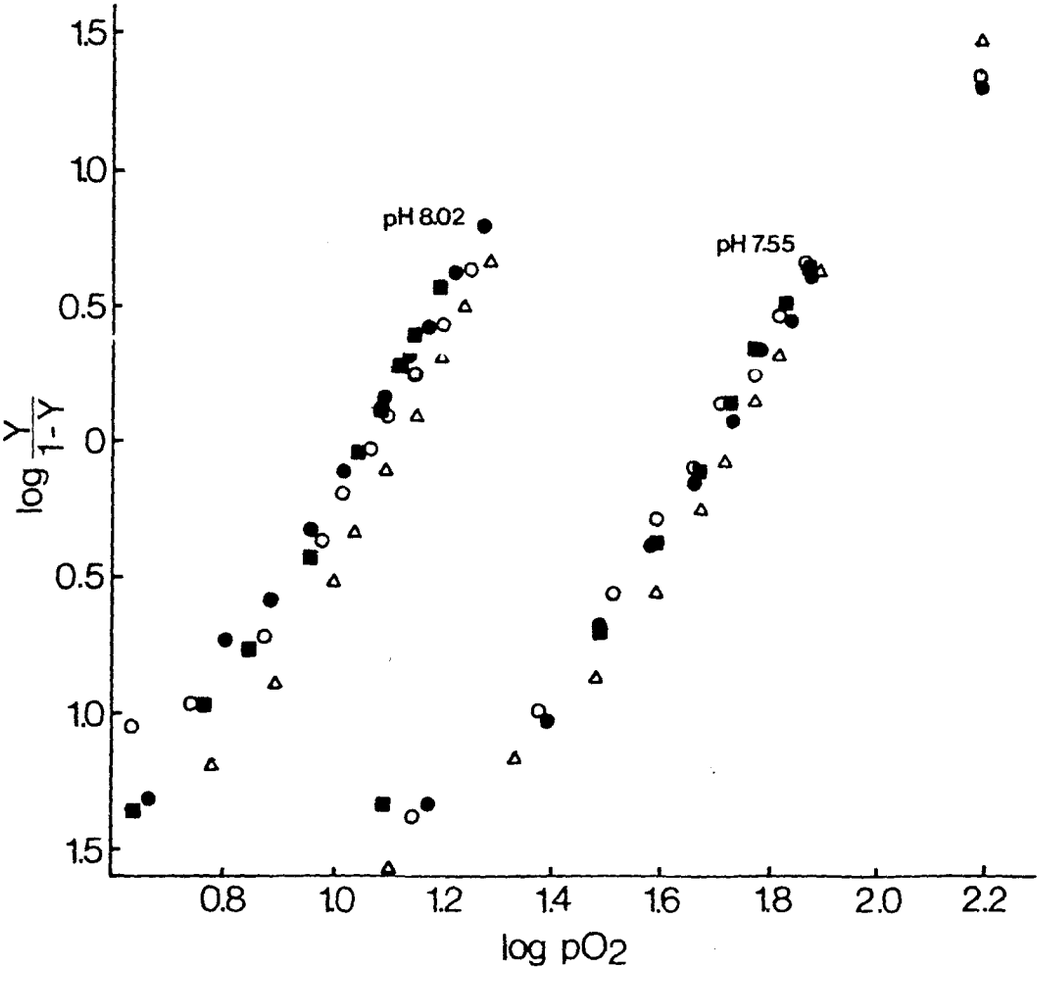

pH 8.02
pH 7.55
1.5
1.0
0.5
0
0.5
1.0
1.5
log Y/(1-Y)
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
log pO2

Fig. 17. The oxygen binding affinity at 20°C of various native Hc's and the artificial 25S Hc. The line drawn summarizes the data presented in Fig. 16. (O) native 25S Hc, (Δ) artificial 25S Hc, (●) unfractionated, stored Hc from the same batch of serum, (□) the same unfractionated Hc before it was stored, (■) fresh unfractionated Hc drawn from a different animal.

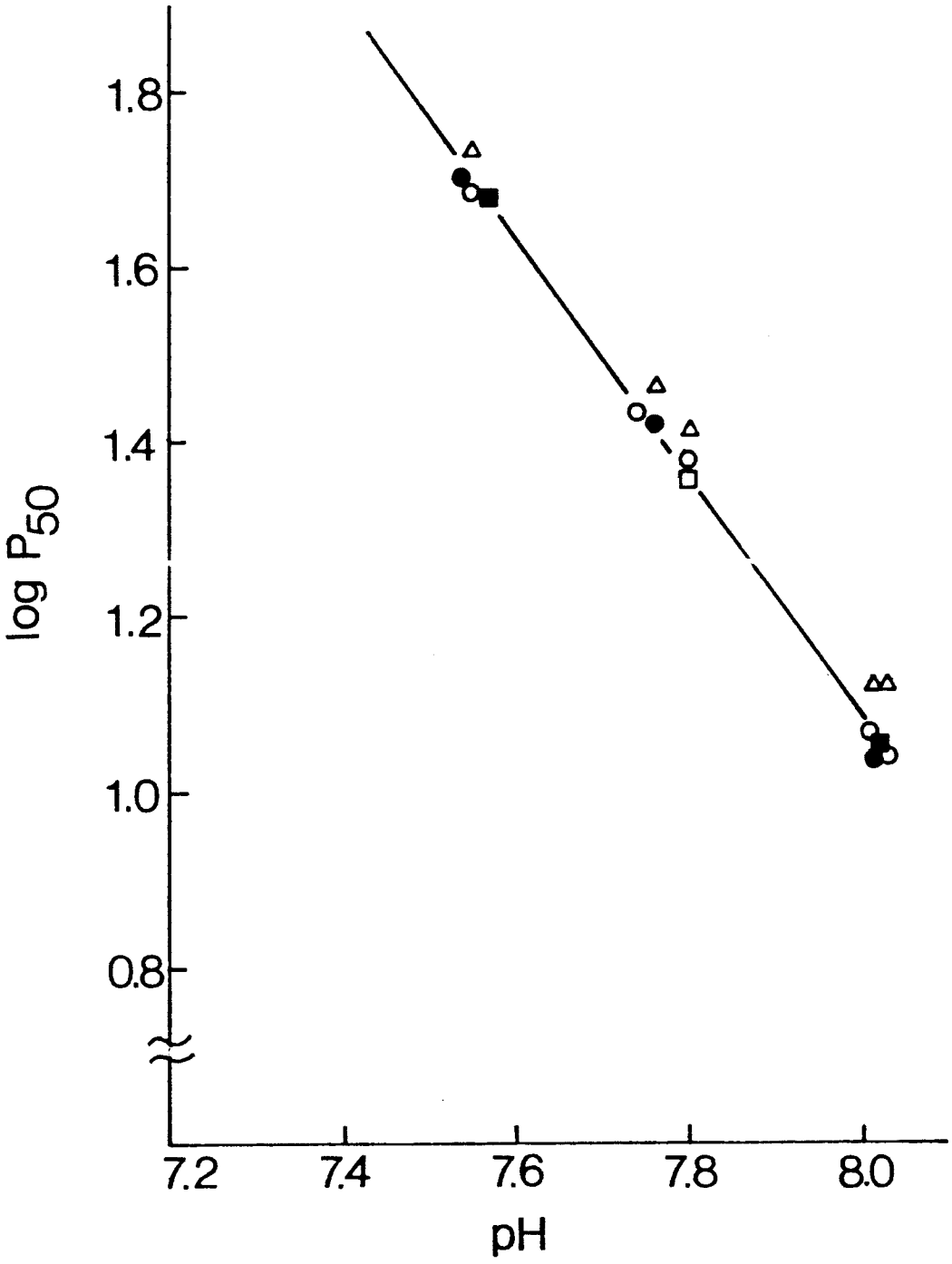

1.8
1.6
1.4
1.2
1.0
0.8
log P_{50}
7.2
7.4
7.6
7.8
8.0
pH

Fig. 18. The co-operativity of oxygen binding by various native Hc's and the artificial 25S Hc. (O) native 25S, (Δ) artificial 25S Hc, (●) unfractionated, stored Hc from the same batch of serum, (□) the same unfractionated Hc before it was stored, (■) fresh unfractionated Hc drawn from a different animal.

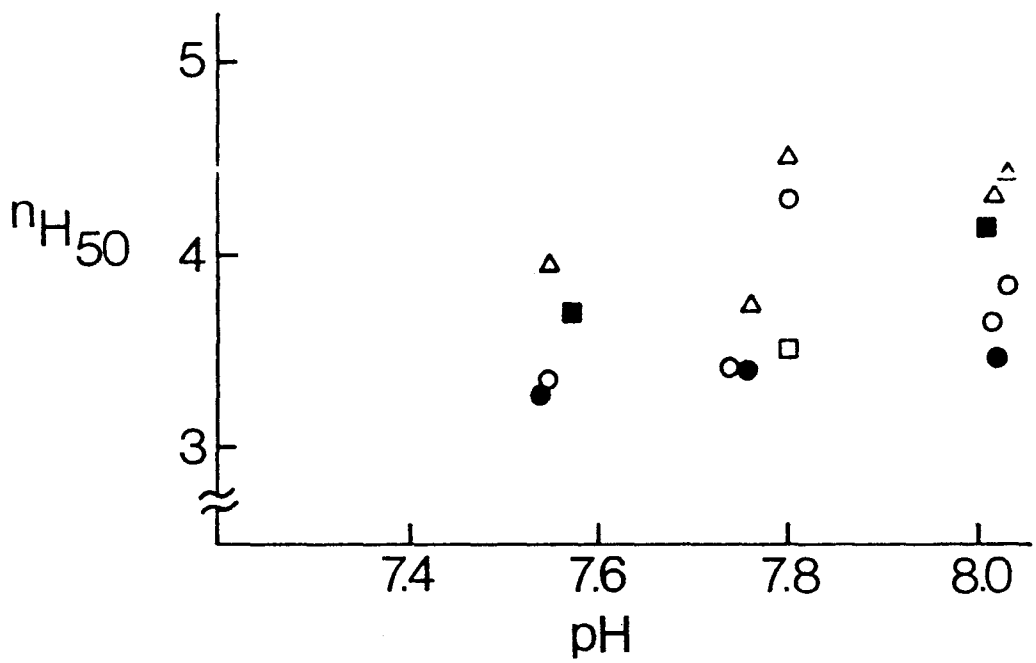
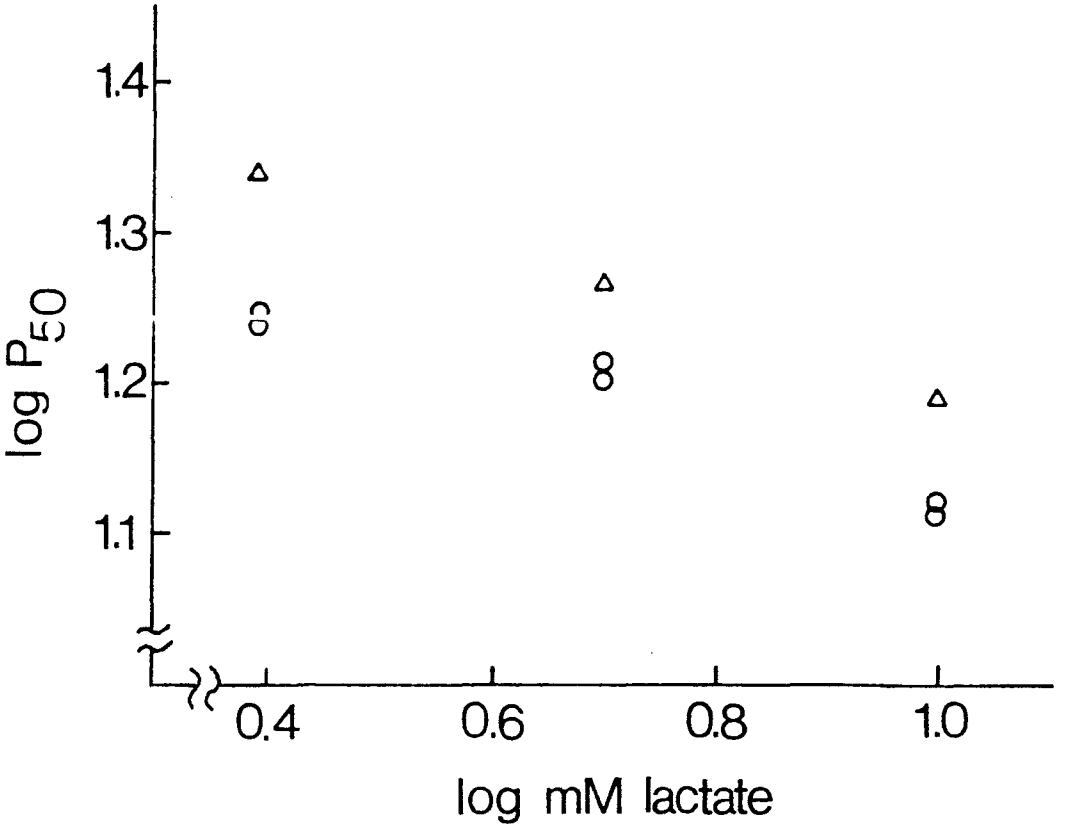

5
4
3
$n_{H_{50}}$
7.4
7.6
7.8
8.0
pH

Fig. 19. The effect of L-lactate on the oxygen binding affinity of the native and artificial 25S Hc's at pH 7.80. (O) native 25S, (Δ) artificial 25S Hc.


1.4
1.3
1.2
1.1
log P_{50}
0.4
0.6
0.8
1.0
log mM lactate

tinguishable from the slope for the native 25S Hc (Δ log P_{50}/ Δlog mM lactate = 0.200) and the whole unfractionated Hc reported by Graham et al., 1983. The differences in log P_{50} of the two Hc's at each concentration of L-lactate in Fig. 19 average 0.079 which is not significantly different from the values of the differences reported in the paired experiments in Fig. 18.

As we reported earlier (Graham et al., 1983) L-lactate decreases the co-operativity of oxygen binding somewhat (Fig. 20). L-lactate decreased the co-operativity of the artificial Hc, to a slightly greater degree than the native 25S Hc though the difference is not significant. In the presence of L-lactate the $n_{H_{50}}$ of the artificial 25S Hc is no longer distinguishable from the native.

The results of the oxygen binding studies of the native and artificial 25S Hc's indicate that differences in subunit composition may be reflected by differences in oxygen binding properties. In addition to the artificial Hc there is another C. magister 25S Hc which has a different subunit composition from the native 25S Hc isolated from mature crabs. The C. magister megalops and first instar juvenile crabs contain a 25S Hc which has no subunit VI, has more subunit V, and less subunit IV than the adult 25S Hc (Terwilliger and Terwilliger, 1982). We, therefore, investigated the oxygen binding behavior of this Hc.

The oxygen binding affinity of the 25S Hc from juvenile crabs, the native adult 25S Hc, and the artificial Hc in 0.05 M Tris-HCl, 0.1 M NaCl, 0.01 M $MgCl_2$, 0. 01 M $CaCl_2$ are shown in Fig. 21. The co-operativity of binding is shown in Fig. 22. The difference in the

Fig. 20. The effect of L-lactate on the co-operativity of oxygen binding of the native and artificial 25S Hc at 20°C and pH 7.80. (O) native 25S Hc, (.Δ) artificial 25S Hc.

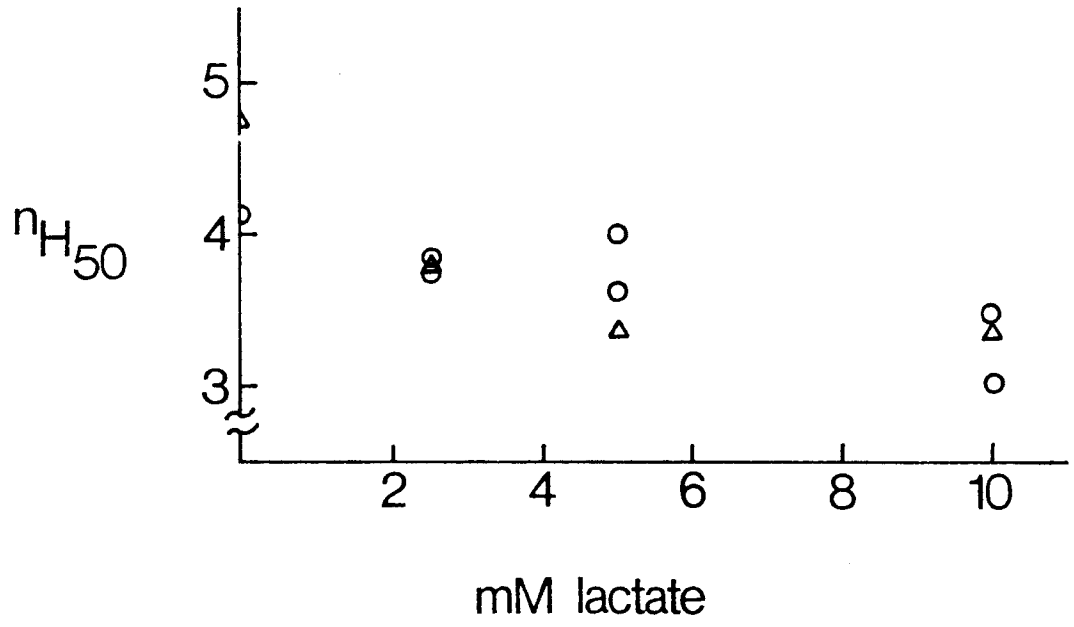

$n_{H_{50}}$
5
4
3
2
4
6
8
10
mM lactate

Fig. 21. The oxygen binding affinity of the juvenile C. magister 25S Hc, the adult 25S Hc, and the artificial 25S Hc at 20°C. The buffer was 0.05 M Tris-HCl, 0.1 M $CaCl_2$, 0.01 M $MgCl_2$. (□) 25S Hc from juvenile crabs, (○) 25S Hc from an adult crab, (△) artificial 25S Hc.

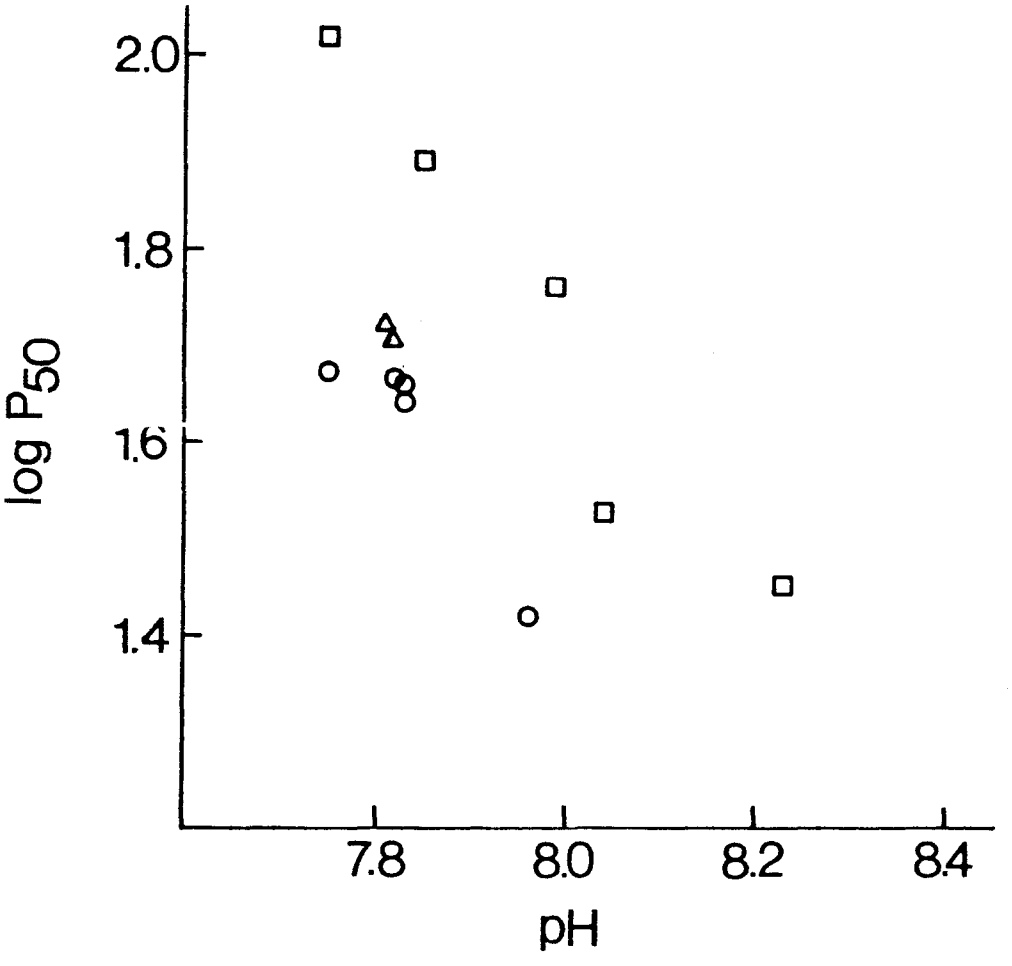
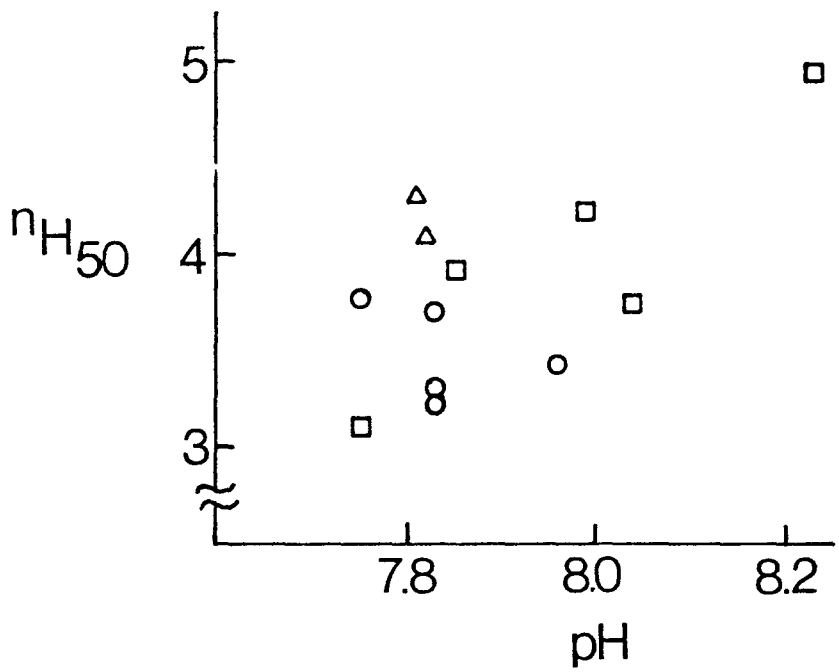

2.0
1.8
1.6
1.4
log P_{50}
7.8
8.0
8.2
8.4
pH

Fig. 22. The co-operativity of oxygen binding by the juvenile C. magister 25S Hc, adult 25S Hc, and the artificial 25S Hc. The buffer and temperature are as in Fig. 21. (□) 25S Hc from juvenile crabs, (O) 25S Hc from an adult crab, (Δ) artificial 25S Hc.


5
4
3
$n_{H_{50}}$
8
7.8
8.0
8.2
pH

log P_{50} of the adult native 25S and artificial 25S Hc is about 0.06, which is the same value we saw in the crab saline (Fig. 17). The difference in $n_{H_{50}}$ (Fig. 22) is also similar to the difference measured in crab saline (Fig. 18). The oxygen binding affinity of these two proteins is considerably lower in this buffer than in the crab saline.

The 25S Hc of the juvenile crab has a considerably lower oxygen affinity than the 25S adult Hc. The difference in log P_{50} is about 0.30. The juvenile Hc is also more co-operative than the native (Fig. 20) except at pH 7.75 where the affinity is very low. The effect of L-lactate on the binding affinity of the adult and juvenile 25S Hc's are similar (Δ log P_{50}/Δ log mM lactate = -0.287 and -0.291 respectively).

Discussion

We have successfully reassembled the dissociated Hc of C. magister to a 25S Hc by means of ion exchange chromatography. Increasing concentration of NaCl apparently promotes the reassociation; changes in pH are not involved, and Ca^{++} is not required for assembly. The reassembly behavior on ion exchange columns contrasts sharply with the reassembly behavior of the subunits in solution. C. magister hemocyanin is a typical 25S Hc in that reassociation of the Hc in solution yields mostly hexamers and only small amounts of 25S Hc (Carpenter and van Holde, 1973). The reassociated 16S and 25S Hc's are seen when dissociated HC is electrophoresed at pH 7.5 (e.g. Fig. 9).

Consideration of the reassociation behavior of the Hc subunits suggests that subunit heterogeneity plays a role in determining structure of the 25S Hc as we have hypothesized in a previous publication

(Chapter 4). In addition the results of the binding experiments on the reassembled Hc and on the Hc from juvenile crabs shows that subunit heterogeneity also plays a functional role in the 25S Hc.

Van Holde and Miller (1982) have suggested that it is the specificity of the structural roles of the different subunits that block the reassembly of 25S Hc's at the level of the hexamer. They postulate that there is a correct arrangement of subunits that leads to the formation of two-hexamer Hc's. The probability of achieving such a correct combination of subunits by chance association of the different subunits is low. Our data are consistent with this hypothesis. In addition, the probability of achieving the correct combination of C. magister subunits is further reduced by the fact that certain subunits are more likely to appear in hexamers than others. These hexamers contain only a few of the subunits and do not go on to form two-hexamers.

For example, subunits I, II, and IV are found in hexamers in a pH 8.9, low ionic strength buffer. Under these conditions most of the subunits are monomeric and no two-hexamers are seen (Figs. 8 and 9, and Ellerton et al., 1970). Since this hexameric Hc is not seen on disc PAGE of the 25S Hc at pH 8.9, but is seen after dialysis of the 25S Hc against pH 10 dissociating buffer (Larson et al., 1981) or pH 8.9 dissociating buffer, it is very likely a product of reassociation. Subunit II appears to play a special role in the formation of this hexamer since it is found exclusively in the hexamer fraction and not seen in the monomer fraction (Figs. 8 and 10). We did not investigate the structure of this Hc further, but it behaved identically to the other hexamers, verified to be 16S by sedimentation velocity, in pH 7.5

PAGE and Bio-Gel A-5m chromatography.

In contrast to subunits I, II, and IV, which form hexamers at high pH and low ionic strength, subunit VI is seen as a monomer at pH 7.5 and low ionic strength, conditions in which the native 16 S and 25S Hc's are stable (Ellerton et al., 1970) as are the artificial 16S and 25S Hc's as judged by their behavior in PAGE at pH 7.5. Stable homohexamers of subunit VI are seen at pH 7.5 in the presence of 0.5 M NaCl.

Therefore, the different subunits of C. magister Hc require different conditions to form oligomeric structures. The failure to reassemble C. magister Hc 25S structures in solution appears to be due to the preferential formation of particular anomolous hexamers before conditions are reached where all of the subunits can participate in the reassembly process. Terwilliger (1982) gave a similar explanation for her failure to reassemble the subunits of Ligia pallasii Hc to two-hexamers. In her case the anomolous hexamers contained the correct subunits but in the wrong proportions. Marlborough et al. (1981) reported that the different subunits of Cherax destructor Hc also require different conditions to reaggregate, and they reaggregate at different rates in a given condition. Dead-end hexamers also form when the four-hexamer of Eurypelma is reassembled, but apparently these hexamers once formed can dissociate such that the correct four-hexamer is eventually built (Decker et al., 1980).

We did reassociate the dissociated Hc to a 25S Hc by means of ion exchange chromatography. In some columns, particularly those loaded with small amounts of Hc and developed at low flow rates, up to 40% of the eluted Hc from the second ion exchange step was 25S Hc. The reasso-

ciated Hc behaved like the native 25S Hc on PAGE at pH 7.5, in Bio-Gel A-5m, and in the ultracentrifuge. Therefore there is little doubt that the reassociated Hc is a dodecamer of 75,000 Mr subunits like the native Hc.

Why the subunits reassociate to 25S in higher yield in the ion exchange columns is not clear. The reassociation by ion exchange is different from reassociation by dialysis against a buffer in several ways. 1) The NaCl concentration is raised slowly and gradually. 2) The subunits are not free in solution, but are bound to a solid matrix. 3) Which subunits are free to associate is not random, but is determined by their relative affinities for the ion exchange material and for each other. 4) Both of these latter properties are specific for each subunit. The latter two points need some elaboration. For example, subunit VI has a low affinity for the DEAE as expected; it migrates in the slowest band in disc PAGE at pH 8.9 (Larson et al., 1981). It also does not reassociate as readily as the other subunits. Subunit II, on the other hand, also migrates in the slowest band in disc PAGE at pH 8.9. It would be expected to co-elute with subunit VI in ion exchange chromatography. However, this subunit seems to have a high affinity for other subunits at pH 8.9 since it is not seen as a monomer at this pH. Therefore, subunit VI elutes early in the ion exchange as a monomer; subunit II elutes later in hexamers and dodecamers.

The two-hexamer that results from the ion exchange chromatography is remarkably similar to the native Hc. If the subunits reassembled randomly and all the possibilities make two-hexamers, we would expect to see the subunit composition of the reassembled Hc to resemble the native

Hc. However, as we have discussed, the reassociation is by ion exchange not random; random association in solution promotes the formation of dead-end hexamers. Also the native 25S Hc is not a random array of the six subunits; it appears to be homogeneous protein with a specific arrangement of subunits (Chapter 4). Therefore, the resemblance of the reassembled Hc to the native Hc cannot be due to random association, but must be a reflection of some specificity of subunit interactions.

In this sense the 25S Hc of C. magister is similar to the 37S Hc's of the chelicerates. The subunits of the two 37S Hc's that have been studied either reassemble to a 37S Hc identical to the native (Decker et al., 1980), or to a 37S Hc in which a single subunit substitution is made (Lamy et al., 1980). The Hc of C. magister is not all like the Hc of another crustacean. Cherax destructor, which reassembles to a large array of products including a variety of 25S Hc's (Marlborough et al., 1981). A specific arrangement of Cherax Hc subunits is not required in order to make a 25S Hc.

Since we have not been able to purify all of the subunits of C. magister Hc, we cannot systematically investigate the structural roles of each of the six subunits. We can make some inferences, however, from the data that we have presented here. In other systems the subunits appear to be divided among hexamer formers and linkers. The hexamer formers can assemble to hexamers, and the linkers function to hold the hexamers together in the various higher ordered structures (Schutter et al., 1977; Jeffrey and Treacy, 1978; Markl, 1980; van Bruggen et al., 1980; Lamy et al., 1981a, Markl et al., 1981c; Marlborough et al., 1981). Subunit VI will form homohexamers, and subunits I, II, and IV form a

hexamer or hexamers. Our data supports the hypothesis of Terwilliger and Terwilliger (1982) that the linker subunit in this Hc is subunit III. Subunit III is found in the 25S Hc of both the adult and juvenile Hc, but is not found in either 16S Hc (Terwilliger and Terwilliger, 1982). It is also not found in any of the purified artificial 16S Hc's that result from reassociation in solution or by ion exchange. It is seen in about two copies per two-hexamer in both the native and artificial 25S Hc (Fig. 14).

Though the reassembled 25S Hc of C. magister closely resembles the native Hc it is not identical to it. The same amount of artificial Hc has less subunit VI and more subunit I and perhaps IV. Subunits III and V might be slightly reduced. The data does not suggest a simple substitution of one subunit for one other when the Hc reassembles, as is the case for Androctonus Hc (Lamy et al., 1980). Several different substitutions seem to be involved. The close resemblance of the artificial Hc to the native Hc implies specificity of subunit interactions. The fact that the artificial Hc is somewhat different implies that the specificity is not absolute; there is a preferred subunit arrangement, but some subunit substitutions do occur when the Hc is reassembled.

The close structural resemblance of the artificial 25S to the native Hc is reflected in its oxygen binding behavior. The oxygen binding properties of the reassembled Hc are very similar to the native, but are not identical to it. The affinity of the artificial Hc is slightly lower, and the co-operativity of binding is higher. The differences in these two parameters are the same in a complex crab saline and in a simpler buffer in which the binding affinity of the two proteins

is much lower. The Bohr effect and L-lactate effect are the same in both Hc's. Storage of the native Hc at neutral pH, high salt, and EDTA has no measurable effect on oxygen binding. Our control Hc's had not been exposed to alkaline pH or low ionic strength, however. Thus it is possible that the change in the binding properties of the artificial Hc is due to the effect of exposure to the alkaline buffer and/or the dissociation/reassociation and not due to the small difference in subunit composition. Though possible, this seems to be unlikely. The co-operativity of oxygen binding of the reassembled Hc is significantly higher than the native. It is unlikely that dissociating and reassociating the protein would cause an increase in co-operativity. If anything the exposure to alkaline pH and dossociation of the Hc might be expected to disrupt secondary or tertiary structure of the subunits and thus lower their ability to interact functionally. Also the binding properties of the juvenile 25S Hc are quite different from the adult native Hc. The affinity for oxygen is considerably lower; the P_{50} of the juvenile Hc is almost twice as large as the P_{50} of the adult Hc at the same pH. The co-operativity is somewhat higher. This Hc was not exposed to alkaline pH nor was it ever dissociated. Both the adult Hc and juvenile Hc in these experiments were purified by the same methods in the same buffers, stored in the same buffers, and the binding equilibria were measured in the same way. The difference in the binding properties can only be attributed to the difference in the subunit composition of the two Hc's, which is striking. Subunit VI is not present in the 25S juvenile Hc; subunit V is the dominant band in SDS PAGE; and subunit IV is considerably reduced (Terwilliger and Terwilliger,

1982). The distinctly different subunit composition of the juvenile Hc is reflected by a distinctly different oxygen binding behavior.

This study has shown that the different subunits of C. magister Hc have different roles in determing the overall binding behavior of the Hc. Two hemocyanins which have different subunit compositions from the adult native Hc have distinct oxygen binding behaviors. The degree of difference in the oxygen binding behavior is correlated with the degree of difference of the subunit composition. We cannot attribute any specific oxygen binding function to a specific subunit, however.

This study contributes to the growing body of evidence that one of the functions of subunit heterogeneity is to provide specific functions to the Hc by providing different subunits which contribute differently to the oxygen binding behavior. The reassembled 37S Hc from Androctonus, which has a slightly different subunit composition from the native, also shows differences in oxygen binding behavior from the native. In this case the co-operativity is much lower, and the affinity is higher in the reassembled Hc (Lamy et al., 1980, 1981c). Unfortunately the antigenic properties of the subunits are also altered by the dissociation procedure. Thus, in this case, the difference in the O_2 binding behavior of the reassociated Hc cannot be unambiguously attributed to changes in subunit composition (Lamy et al., 1981c). Jeffrey and Treacy (1980) investigated the oxygen binding behavior of two hexameric Hc's assembled from purified subunits. These two hexamers had different values for P_{50} and n_H under the same conditions. Only one of the two showed a response to Ca^{++}.

This study is the only report to our knowledge that provides

evidence that different subunits have specific roles in determining oxygen binding behavior when they are associated in the 25S two-hexamer Hc. We have also provided evidence in this report and a previous report (Chapter 4) that the different subunits have specific roles in determining the structure of the 25S Hc of C. magister. The next question to be addressed is what are the specific contributions of each of the different subunits in determining the structure and function of the 25S Hc. The answer to this question will have to wait until the six different subunits are purified.

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CHAPTER SIX

CONCLUSION

The goal of the research presented in this thesis was to elucidate the structural and functional relationships in the two-hexamer Hc of Cancer magister. In particular I was interested in the possible allosteric modification of the oxygen binding properties by an organic compound, and the relationship of subunit heterogeneity to the structure and function of the native two-hexamer Hc.

Prior to this study, L-lactate had been known to affect the oxygen binding of two crustacean Hc's but the mechanism of action was unknown (Truchot, 1980). It was also not known how the lactate effect worked in vivo. The structural roles of the different subunits of some four-hexamer and eight-hexamer chelicerate Hc's were well understood (e.g. van Bruggen et al., 1980), but there was little date available regarding crustacean two-hexamers. The data that was available (e.g. Jeffrey et al., 1976) implied that some of the rules governing the role of subunit heterogeneity in the assembly of chelicerate Hc's might not apply to the crustacean two-hexamer Hc's. The role of subunit heterogeneity in determining the function of the 25S Hc's was even less well understood. There is evidence that different monomeric subunits have different oxygen binding behaviors (Bonaventura et al., 1977; Decker et al., 1979; Lamy et al., 1980; Markl et al., 1981), and two different hexameric Hc's made from two purified subunits have different oxygen

binding behaviors (Jeffrey and Treacy, 1980). However, how the different subunits function when assembled in a two-hexamer structure had not been investigated.

The research presented in this thesis establishes that L-lactate is a specific modulator of the oxygen binding of C. magister hemocyanin, the 25S hemocyanin appears to be homogeneous, and subunit heterogeneity appears to be necessary for both the quaternary structure and function of the 25S two-hexamer hemocyanin.

The mode of action of L-lactate is by binding to a proposed specific allosteric site on the protein. All four of the positions around the chiral carbon of L-lactate appear to be involved in the binding. The L-lactate effect appears to function to counter the large decrease in oxygen affinity of the Hc that accompanies exercise. The decrease in oxygen affinity is caused by the combined effects of the large decrease in blood pH which accompanies exercise and the large Bohr shift of the hemocyanin.

Since this work was published (i.e. Graham et al., 1983) the L-lactate effect has been reported to be relatively widespread among the crustacean hemocyanins, but is not ubiquitous. The lactate effect is seen in both hexameric and two-hexameric hemocyanins. It is restricted to those animals that have hemocyanins with large Bohr shifts and also show metabolic acidosis as a response to hypoxia. The particular selection pressures that I proposed to have selected for the L-lactate binding site of C. magister Hc appear to have operated in all of the Hc's with lactate effects (Mangum, 1983).

At this point it is not known whether the L-lactate binding site

is associated with a particular subunit or is a property of all of the subunits of L-lactate responsive Hc's. An important question to be addressed is how many L-lactate binding sites are there per hexamer or two-hexamer. Unfortunately equilibrium studies of L-lactate binding to hemocyanin may prove difficult to perform. L-lactate exerts its influence at millimolar concentrations which means the equilibrium binding experiments will need to be done at nearly millimolar concentrations of Hc. If one is able to do such experiments, the data may prove difficult to interpret, as there could be twelve or more binding sites with several equilibrium constants.

The data presented here do not indicate that particular subunits are involved in the lactate response, not do the data rule out this possibility. All three of the 25S Hc's that I tested, the native adult Hc, the native juvenile Hc, and a reassembled Hc, have indistinguishable response to L-lactate. Thus either the L-lactate binding site is common to all the subunits and is similar in its properties, or it is confined to one or more of the subunits that are common to all the Hc's that we tested. I had hoped to test the effect of L-lactate on the homohexamer of subunit VI, but preliminary experiments show that the oxygen affinity of this Hc is already so high as to make the binding experiments difficult with our techniques.

Even if the L-lactate effect is not related to subunit heterogeneity, the overall oxygen binding behavior is. Both the co-operativity of oxygen binding and the oxygen binding affinity of the 25S Hc's are influenced by the subunit composition. This is the only reported case of the binding behavior of two-hexamer Hc's which differ only

in subunit composition have been compared. One of the Hc's was an artificial Hc reconstructed from dissociated subunits and two were native Hc's purified from the same species of crab at different stages of development. The degree of difference in the oxygen binding properties is correlated with the degree of difference of Hc subunit composition.

The data strongly imply that the subunit heterogeneity might function to provide functional plasticity as the animal develops and its metabolism and environment change. Unfortunately we do not know the ionic composition of the juvenile crab blood nor can we measure the binding affinity of the whole blood as we did for the adult crab (Graham et al., 1983). How the difference in binding affinity between the adult and juvenile hemocyanin that we measured relate to any differences in binding affinity in vivo is unknown.

The data from the structural studies strongly suggest that the adult 25S Hc is homogeneous. The data also indicate that there is considerable specificity of subunit interaction, but the specificity is not absolute. This makes this Hc different from the 25S Hc of another crustacean, Cherax destructor, and similar to the chelicerate, 37S, four-hexamer Hc's. The problem still exists that the rules which govern the role of the non-linker subunits in the quaternary structure of the 25S Hc's seem to show several patterns.

Another interesting question is how are these 25S hemocyanins made in vivo. None of the two-hexamer hemocyanins that have been studied self-assemble to a product or products that resembles the native hemocyanin (Carpenter and van Holde, 1973; Hamlin and Fish, 1977; Jeffrey et al., Herskovitz et al., 1981; Terwilliger et al.,

1979; Terwilliger, 1982). The hemocyanin of Cherax comes close; the 16S and 25S products that are made resemble the native 16S and 25S hemocyanins, but other hemocyanins are made that do not appear in vivo (Jeffrey et al., 1978; Malborough et al., 1981). In Cherax these 16S Hc's represent all possible combinations of the different monomers and the 25S Hc's represent all possible combinations of one linker-dimer and ten copies of the different monomers (Jeffrey et al., 1978; Marlborough et al., 1981). In adult C. magister one specific two-hexamer Hc appears in the blood. In the juvenile crabs another 25S Hc appears in the blood. Some of the information for the correct assembly of the adult C. magister Hc appears to reside in the subunits themselves (as it does in the chelicerate 37S Hc's), since the subunits will reassemble in ion exchange to a product that closely resembles the adult native Hc. However, there is the question of where is the rest of the information for the construction of the specific hemocyanin. In other words, how is the subunit composition of the hemocyanin controlled as the Hc is being synthesized.

For the protein biochemist there are some interesting problems associated with these large and structurally complex proteins that now can be addressed. What are the mechanisms by which the different subunits interact to produce the different heterotropic and homotropic effects seen in the various hierarchal structures of the hemocyanins? How is the synthesis of these complex proteins controlled?

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