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THE SUBUNIT STRUCTURE OF THE HEMOCYANIN FROM THE CRAYFISH *JASUS EDWARDSII*

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The hemocyanin from the crayfish *Jasus edwardsii* (= lalandii) has been studied using ultracentrifugation, viscosity, circular dichroism and oxygen binding techniques. Sedimentation velocity experiments at pH 7.0 indicated the presence of a principal species with $S_{20,w}$ = 16.4 S, and at higher pH the presence of a species with $S_{20,w}$ = 5.2 S. Sedimentation equilibrium experiments yielded molecular weights of 490 000 and 81 000 respectively, indicating that the larger unit is a hexamer of the monomer unit. However, preliminary experiments with gel filtration and electrophoresis under denaturing conditions indicate that more than one monomer species may be present with molecular weight in the range 76–100 000.

Circular dichroism (CD) spectra are presented at pH 7.0, 8.6, 10.0 and 11.0 for oxy-, deoxy- and apo-hemocyanins. Slight differences were observed in the magnitude of the bands in the presence or absence of Mg^{++}.

Oxygen binding studies have been made at pH 6.1, 7.0, 8.8 and 10.6, in the presence of 0.01 M $MgCl_2$. The extent of cooperative binding was indicated by a maximum value of n = 3.7, and a pronounced Bohr effect was observed.

1. Introduction

The hemocyanins are respiratory proteins found in certain Arthropods and Molluscs. The oxygen-carrying metal is copper in a non-heme protein environment, and this imparts a blue colour to solutions of the protein. A remarkable feature of the hemocyanins is the variety of subunit structures – for arthropods alone, five different levels of aggregation have been reported in all [1].

Jasus lalandii is a marine crayfish, or rock lobster, which is common to continental or island waters of the Southern Ocean. Six species in the *Jasus lalandii* group are recognised [2]. The hemocyanin of two of these, *Jasus novaehollandiae* from Australia, and *Jasus lalandii* from South Africa, have been studied before [3–6], but the overall picture of the structure and behaviour of this protein was still sketchy, with even the exact molecular weight of the species, particularly on dissociation, not clearly resolved [5]. Another tantalising question arises as to why the main association observed with *Jasus* is the 16S species, whereas 24S and 39S species are observed with other Crustacea. We have therefore made a fresh investigation of the structure and function of *Jasus* hemocyanin, and preliminary details have already been reported [7]. In this paper we present the results of physical studies on the subunit structure of the hemocyanin from the species found in New Zealand coastal waters, *Jasus edwardsii*, using sedimentation and spectroscopic techniques. Later papers will deal with the nature of the subunit, and with protein titrations.

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2. Experimental

The crayfish were obtained from commercial fishermen from catches in the coastal vicinity of Wellington. The hemocyanin was extracted by insertion of a hypodermic syringe or Pasteur pipette in the first tail segment, and then allowed to clot. The clotted material was homogenised for a few seconds, 0.1 I tris buffer pH 7.0 was added, and the total sample was centrifuged for 10 min at 10 000 rpm and 4°C.

The supernatant was separated and kept aside, and the clot was washed again with buffer and centrifuged. The pooled supernatants were centrifuged for 18 hrs at 30 000 rpm (78 500 g) in a Beckman Model L2 preparative centrifuge. The blue pellet of hemocyanin was redissolved in 0.1 I tris, pH 7.0, and this solution was used as a starting point for further purification in a column containing agarose (Biogel A-1.5), as required.

Reagents used were "analytical reagent" grade wherever possible. Buffers used were 0.1 I tris (pH 7–9) and 0.1 I bicarbonate (pH 9–11) made up by standard procedures [8].

Sedimentation experiments were performed using a Beckman Model E analytical ultracentrifuge equipped with electronic speed control. Velocity studies were performed using schlieren optics, and the photographic plates (Kodak Metallographic) were measured with a Nikon model 6C Profile Projector. The data were corrected to water at 20°C. Sedimentation equilibrium experiments were performed to determine molecular weights, and the high speed technique of Yphantis [7] was used. Solutions with initial concentrations of 0.3 to 0.9 mg/ml were run in a 6-channel centre-piece in an An-D rotor. The 16S particle was studied in pH 7.0 buffer at 12 000 rpm, and 30 000 rpm was used for the 5 S particle in pH 10.6 buffer. Photographs were taken after equilibrium had been established (within 16–18 hrs) using Rayleigh optics, and Kodak IIG Spectroscopic plates. Fringes were measured at 0.05 mm intervals starting near the meniscus. The data were then analysed by computer for both weight-average molecular weight averaged over the whole solution column and for weight-average molecular weights at each point. The computer programme was written by Dr. R.D. Dyson of Oregon State University.

Densities were determined at 25.000 ± 0.005°C using 25 ml pycnometers of the single stem type whose volumes were calibrated using distilled water. Partial specific volumes, $\bar{v}$, were calculated from densities according to the relation

$$\bar{v} = \frac{1}{\rho_0}\left(1 - \frac{\Delta\rho}{c}\right),$$

where ρ_0 is the density of the buffer, $\Delta\rho$ is the difference in densities of protein solution and dialysate, and c is the protein concentration in g ml^{-1}. Relative viscosities were measured using a low shear modified Ubbelohde viscometer based on the design of Reichmann et al. [8], in which the fine capillary of the usual Ubbelohde instrument was replaced by about 1 metre of 1 mm diameter glass tubing, wound in a coil. This modification results in sheer rates of at least 10 times less than those of the normal viscometer [9].

Protein concentrations were measured routinely by observing the optical density at 280 nm. Samples of known optical density were freeze-dried and the dry weight of protein was determined against a buffer blank. This yielded $E^{1\%}_{1\,cm} = 12.2$ for the 16 S hemocyanin and $E^{1\%}_{1\,cm} = 12.7$ for the 5 S particle.

Quantitative tests for carbohydrates yielded an estimated 0.9% hexoses, and a scarcely detectable amount of hexosamines. Although the error at such low concentrations is considerable, it would appear that the carbohydrate content in *J. edwardsii* hemocyanin is only of the order of 1%.

Circular dichroism experiments were performed at 25°C using a Jasco J-20 instrument with cells of 1 and 10 mm path length. The molecular ellipticity, (θ), in deg cm^2 decimole^{-1}, was calculated from

$$(\theta) = \theta^\circ M/10\,lc,$$

where θ° is the observed ellipticity in degrees, l is the path length of the cell in cm, c is the concentration of protein in g ml^{-1}, and M is the molecular weight. We have used a value of $M = 81\,000$ corresponding to the average monomer molecular weight (containing two copper atoms).

Absorption spectra were measured using 1 mm or 10 mm silica cells in a Perkin-Elmer 402 spectrophotometer. Oxygen binding studies were made using a tonometer based on the design of Johnston et al. [12], but modified so that it fitted totally within the sample compartment of the spectrophotometer. The oxygen sensor was a Clark electrode, and the oxygen tension was read from a Beckman 121 recorder. The tonometer was thermostatted at 20°C.

3. Results

3.1. pH dependence of hemocyanin structure in neutral and alkaline region

The behaviour of the hemocyanin as a function of

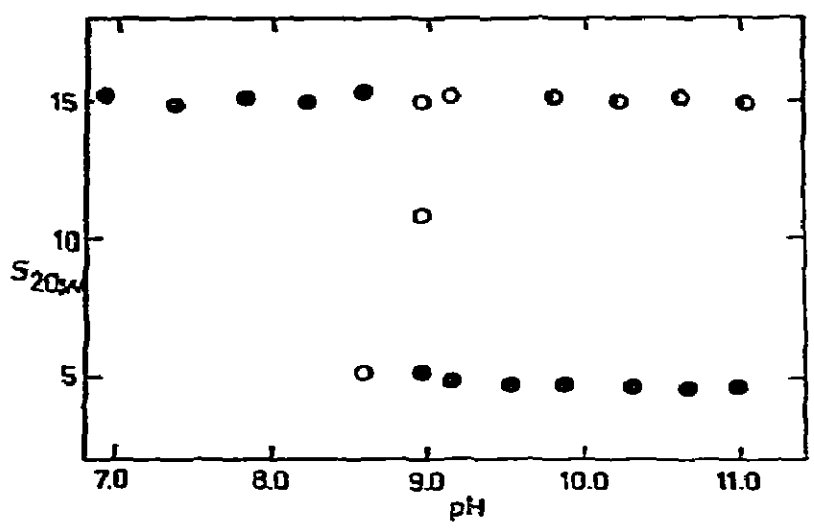

Fig. 1. Sedimentation coefficients of the hemocyanin at 20°C as a function of pH. Filled circles and unfilled circles represent major and minor sedimenting components respectively in the absence of added $MgCl_2$. Half filled circles indicate major component between pH 9.8 and 11 in the presence of 0.01 M $MgCl_2$. Protein concentrations in each run were 5 mg ml^{-1}.

pH was studied by sedimentation velocity experiments. Portions of concentrated stock hemocyanin were run at pH intervals between 7 and 11.

The sedimentation coefficients obtained are presented in fig. 1. At pH 7, all the hemocyanin is present as a "16S" particle, and this trend continues until pH 8.6, when a particle with a sedimentation coefficient of approximately 5S is observed as well as the 16S particle, though the latter is still the major species. At pH 9, more dissociation has taken place, and the 5S particle is the major species. A trace of an intermediate peak was also observed at this pH, which was assumed to be a p tly broken down 16S unit. Above pH 9.2, the hemocyanin has entirely dissociated into 5S particles.

The sedimentation velocity patterns at pH 7, 8.6, 9.0 and 10.6 are shown in fig. 2.

3.2. Sedimentation velocities of the 16S and 5S components

Sedimentation velocity experiments on both the 16S and 5S particles were studied as a function of solute concentration using hemocyanin samples purified from the agarose column. The results are plotted in fig. 3. Extrapolation to zero solute concentration yields values of 16.4 S and 5.2 S for $S^0_{20,w}$, at pH 7.0 and 10.6 respectively.

Moore et al. [5] studied sedimentation at pH 8.0 and 8.7 and found a value of $S^0_{20,w}$ of 15.6 S for the larger particle. The smaller particle gave $S^0_{20,w}$ = 5.2 S at pH 8.9. Thus there is good agreement between the two laboratories for the 5S particle, in spite of the pH difference, but the larger particle appears to sediment faster at pH 7 than at pH 8.0 or 8.7.

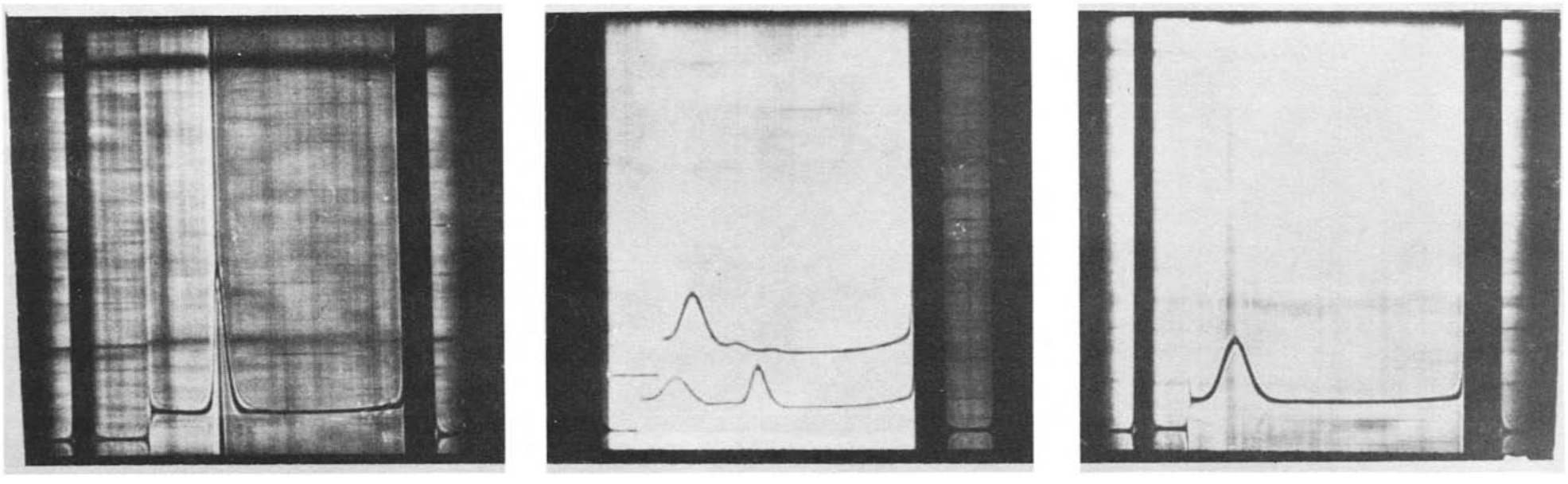

Fig. 2. Sedimentation velocity patterns of *J. edwardsii* hemocyanin at 20°C and various pH (no $MgCl_2$ present). (a) At pH 7 after 16 min at 48 075 rpm; (b) lower pattern, pH 8.6, upper pattern pH 9.0, after 40 min at 48 429 rpm; (c) at pH 10.6, after 32 min at 56 094 rpm.

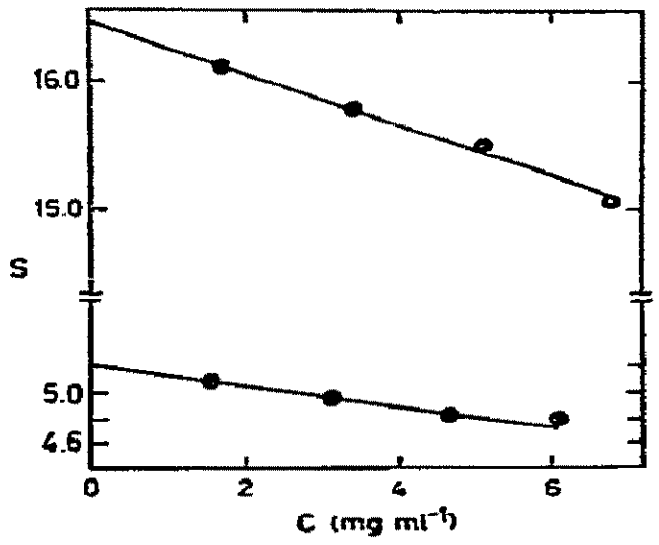

Fig. 3. Sedimentation coefficients of the hemocyanin at 20°C as a function of concentration of protein, for the 16S and 5S particles.

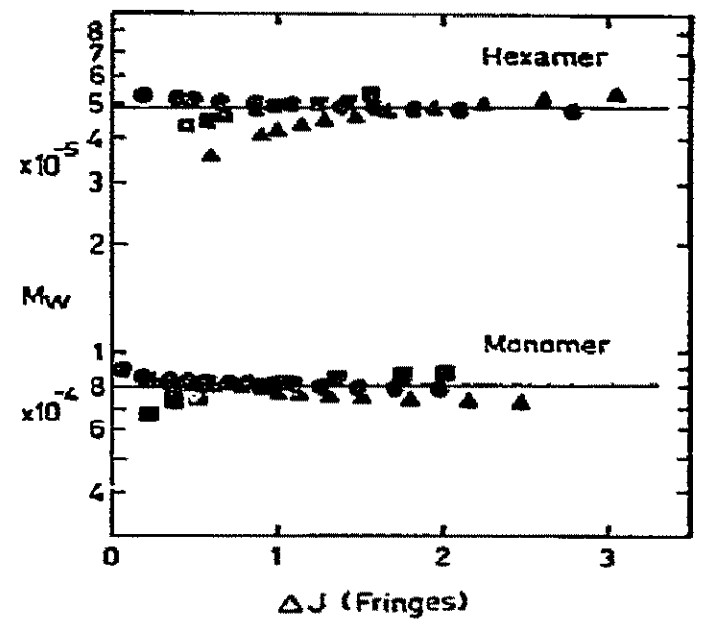

Fig. 4. Weight average molecular weights as a function of concentration in the cell (expressed as fringe displacement) computed from the sedimentation equilibrium data.

3.3. Molecular weights of the components

Plots of ln C (with concentration C expressed as fringe displacement ΔJ) versus Δr^2 (where r is the position of the point relative to the meniscus of the solution) were made, and the slopes of the linear fits of these plots were used to yield an average molecular weight over the whole cell.

For the 16S particle, the average of three high speed equilibrium experiments gave an average molecular weight over the whole of the cell of 490 000 ± 25 000.

In the case of the 5S particle, average molecular weights from six such experiments yielded a molecular weight of 81 000 ± 3 000.

Point average M_w computed from the data are plotted in fig. 4. It may be seen that the results are in agreement with the averages indicated above. Values of M_n and M_z gave satisfactory agreement with M_w and indicated that the samples under study were homogeneous within the sensitivity of the sedimentation equilibrium technique.

The density data required in determination of molecular weights by sedimentation equilibrium are summarised in table 1, which also includes viscosity measurements made on the same solutions. A summary of the final data for *Jasus* hemocyanin is presented in table 2, which includes intrinsic viscosity $[\eta]$, frictional ratios, f/f_0, and an estimate of the molecular weights of both particles from sedimentation and intrinsic viscosity measurements from the approach of Schera-ga and Mandelkern [13] using the relation

Table 1
Densities, partial specific volumes and viscosities of hemocyanin from *Jasus edwardsii*

Species	Solvent	Protein conc. (mg ml^{-1})	Solution density (g cm^{-3})	ϕ (mg ml^{-1})	η_{sp}/C (ml g^{-1})
16S	0.1 I Tris, pH 7.0	15.8	1.00647	0.73_6	5.3_9
	ρ = 1.00233	23.7	1.00863	0.73_3	5.2_9
		31.6	1.01074	0.73_2	5.3_1
		39.5	1.01307	0.72_7	5.4_0
5S	0.1 I Bicarbonate, pH 10.6	7.3	1.00307	0.71_1	12.0_0
	ρ = 1.00096	14.7	1.00519	0.71_1	12.5_3
		36.7	1.01153	0.71_1	14.3_8

Table 2
Summary of the physical data for hemocyanin from *Jasus edwardsii*

Component	$S_{20,w}$ (S)	Mol. wt. (from sed. eq.)	$\bar{v}$ (ml g^{-1})	η (ml g^{-1})	f/f_0	Mol. wt. (from S and $[\eta]$)
16S	16.4	490 000	0.73_1	5.4	1.46	524 000
5S	5.2	81 000	0.71_1	11.3	1.54	121 000

$$M = 4690(S_{20,w})^{3/2}[\eta]^{1/2}/(1 - \bar{v}\rho)^{3/2}.$$

We measured relative viscosities of the hemocyanin in the presence of guanidine hydrochloride, and by extrapolation of reduced viscosity to zero concentration obtained an intrinsic viscosity of 57 ml g^{-1}. Applying the relation of Tanford et al. [14]

$$[\eta] = 0.716\, n^{0.66},$$

where n is the number of amino acids in the polypeptide chain, we obtained a value of $n = 759$. If we assume the mean residue weight per amino acid is 110, this yields an estimated molecular weight of 83 500 for the polypeptide, in good agreement with the sedimentation equilibrium data on the intact 5S particle.

3.4. Absorption spectra, circular dichroism and oxygen binding

The absorption spectrum of *J. edwardsii* respiratory protein is characteristic of hemocyanins, and is shown in fig. 5. At pH 7, when the hemocyanin is present almost entirely as 16S hexamers, maxima are located at 218, 279, 337 and 579 nm, and the extinction ratios E_{279}/E_{579}, E_{280}/E_{337}, and E_{218}/E_{280} are 75.7, 4.00 and 12.3 respectively.

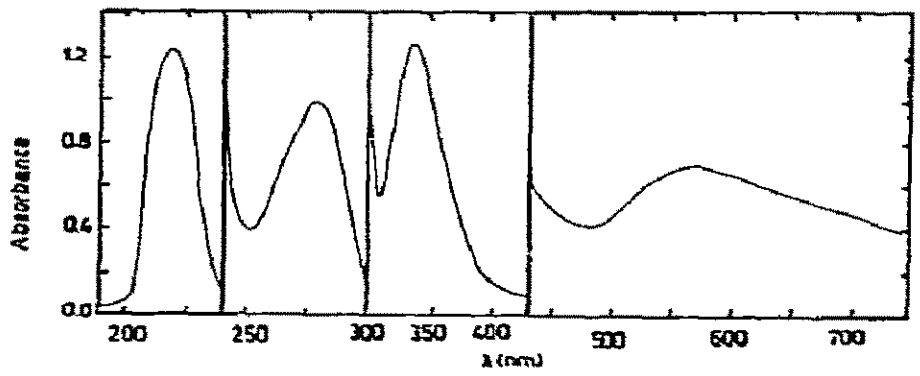

Fig. 5. Ultraviolet and visible absorption spectra of *J. edwardsii* oxyhemocyanin at pH 7.0 in 0.1 I tris buffer containing 0.01 M $CaCl_2$. Concentration of protein is 39.9 mg ml^{-1} in the range 750–300 nm, 0.82 mg ml^{-1} for 300–240 nm, and 0.081 mg ml^{-1} for 240–190 nm. 10 mm path length cell used in the ranges 750–430 and 300–190 nm; 1 mm cell in the range 430–300 nm.

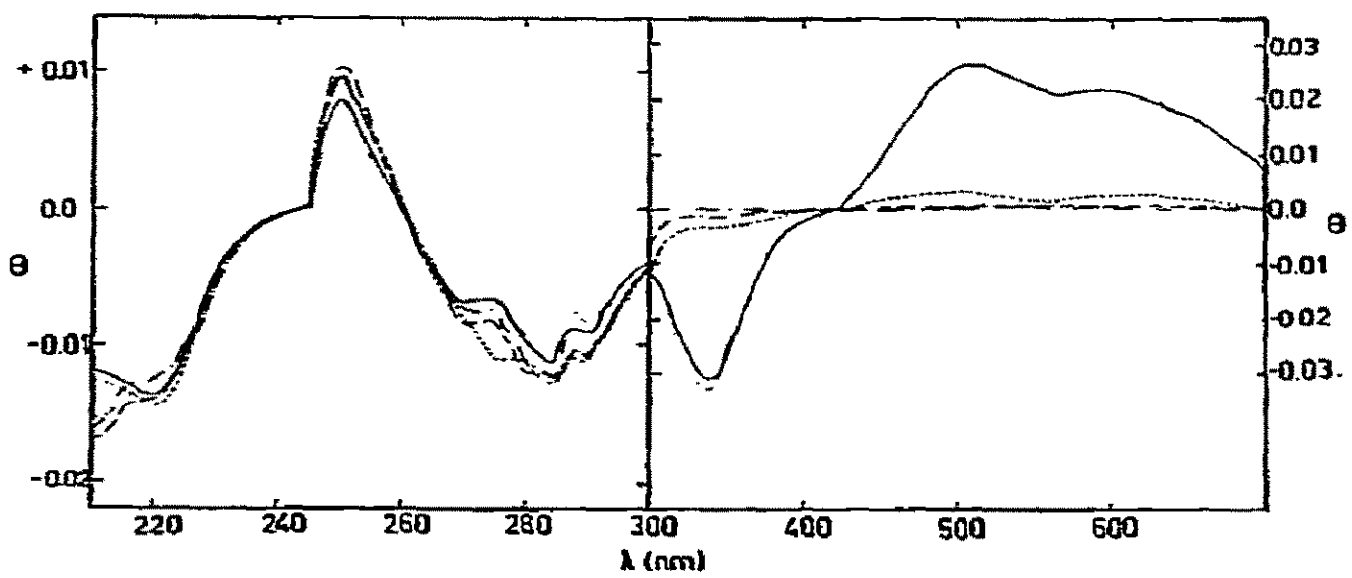

Fig. 6. UV and visible CD spectra of *Jasus edwardsii* hemocyanin at pH 7.0 in the presence or absence of 0.01 M $MgCl_2$. Concentrations of protein: 25.0 mg ml^{-1} for the range 700–300 nm, 1.25 mg ml^{-1} for 300–245 nm, and 0.25 mg ml^{-1} for 245–210 nm. 10 mm cells used in ranges 700–400 and 300–245 nm; 1 mm cell used for 400–300 and 245–210 nm. Legend: —— oxyhemocyanin, no Mg^{++}; – – – deoxyhemocyanin, no Mg^{++}; ····· oxyhemocyanin in presence of 0.01 M Mg^{++}; –.–.– deoxyhemocyanin in presence of 0.01 M Mg^{++}; –...– apohemocyanin.

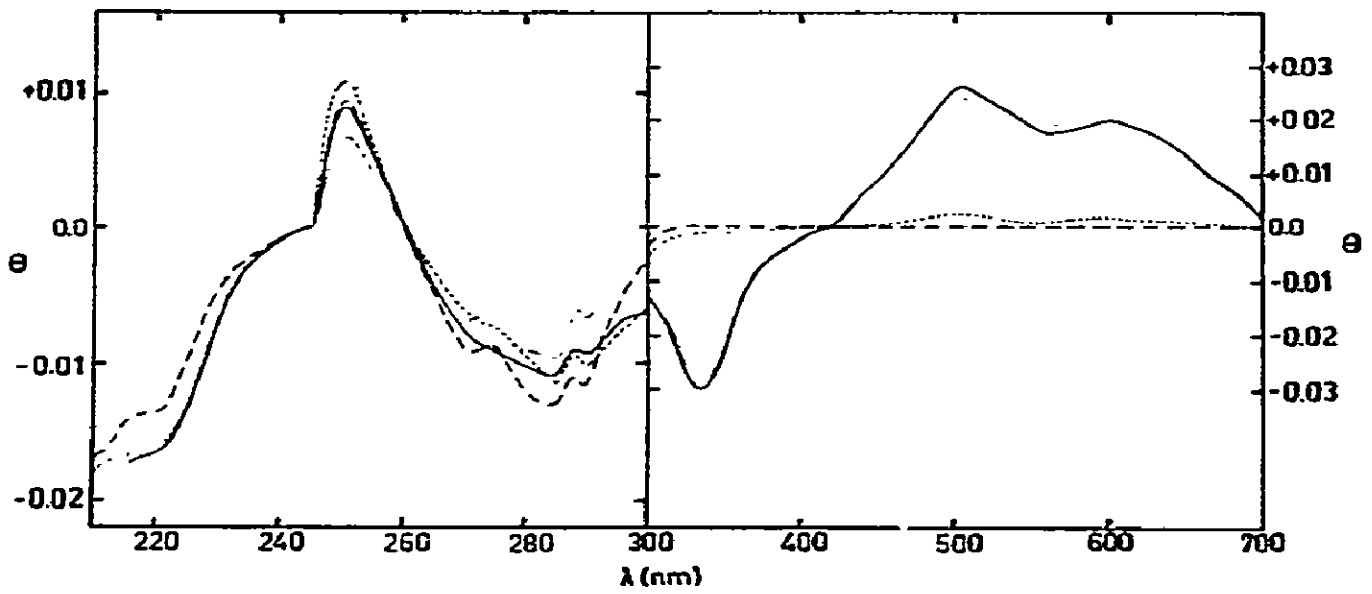

Fig. 7. UV and visible CD spectra of the hemocyanin at pH 8.6. Other details as in fig. 6.

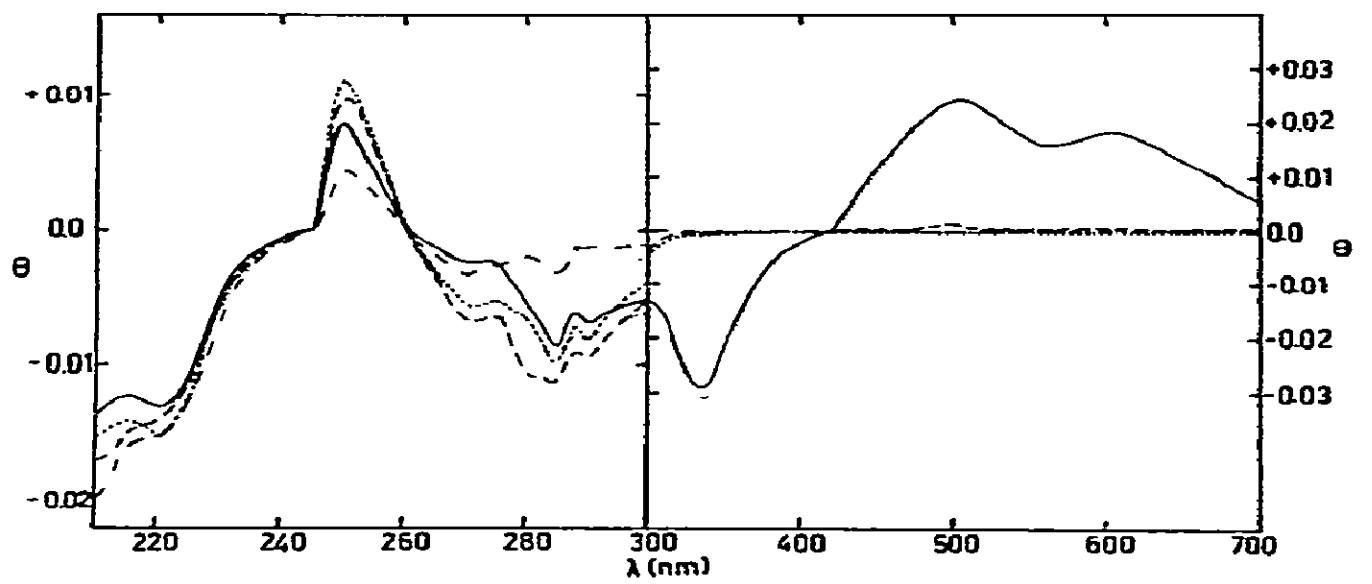

Fig. 8. UV and CD spectra of the hemocyanin at pH 10.0. Other details as in fig. 6.

Circular dichroism spectra were measured at pH 7.0, 8.6, 10.0 and 11.0. Figs. 6–9 present the spectra at these pH values, for hemocyanin in the oxy, deoxy and apo forms, both in the presence or absence of 0.01 M $MgCl_2$, as indicated. Table 3 summarises the molar ellipticities at peak values of the spectra.

Oxygen binding studies were made at pH 6.1, 7.0, 8.8 and 10.6, in the presence of 0.01 M $MgCl_2$. The oxygen binding curves were sigmoidal at pH 6.1, 7.0 and 8.8, but became almost hyperbolic at pH 10.6.

The value of the parameter n of the Hill equation [1, 15] was determined at each pH by plotting $\log_{10}[y/(1-y)]$ against $\log_{10} pO_2$ (where y is the fraction of

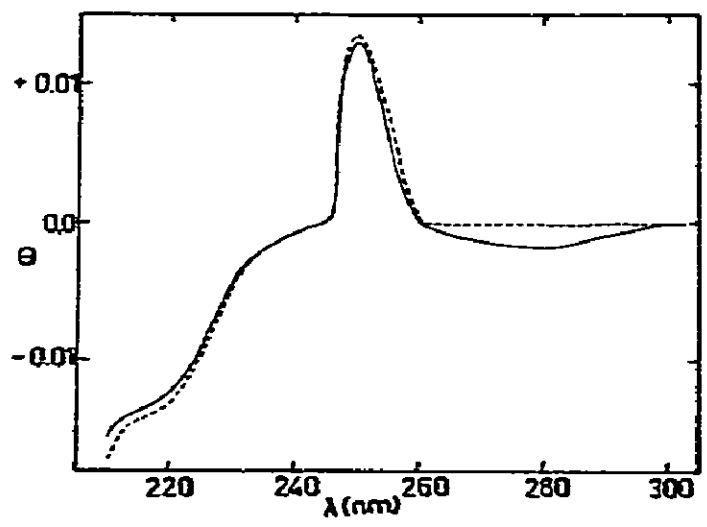

Fig. 9. UV CD spectra of the hemocyanin at pH 11. Other details as in fig. 6.

Table 3
Circular dichroism in the ultraviolet and visible: Molecular ellipticities in deg cm^2 decimole^{-1} for hemocyanin from *Jasus edwardsii*, assuming a subunit of molecular weight 81 000

λ_{max} (nm)	pH	Molecular ellipticity (θ)				
		No Mg^{++}		0.01 M Mg^{++}		Apo-
		Oxy-	Deoxy-	Oxy-	Deoxy-	
600	7.0	7.00		7.67		
	8.6	6.54		6.52		
	10.0	5.79		7.11		
	11.0	0				
502	7.0	8.66		8.88		
504	8.6	8.51		7.84		
506	10.0	8.01		8.75		
	11	0				
336	7.0	−103.0		−108.9		
	8.6	−98.1		−93.1		
	10.0	−95.5		−102.1		
	11.0	0				
290	7.0	−59.8	−74.5	−54.2	−72.6	−60.5
	8.6	−62.1	−66.7	−39.1	−77.1	
	10.0	−46.0	−54.4	−52.3	−63.5	−4.32
	11.0	−6.70	0			
285	7.0	−74.5	−83.4	−64.4	−82.5	−79.9
	8.6	−71.9	−72.4	−64.2	−85.1	
	10.0	−57.8	−64.6	−59.2	−73.7	−10.8
	11.0	−13.2	0			
280	7.0	−63.5		−61.1	−79.1	−76.0
(275)	7.0		−72.6			
280	8.6	−68.6	−62.8	−60.0	−79.3	
280	10.0	−35.7	−40.9	−55.9	−70.2	−6.48
270	7.0	−45.4	−50.8	−44.1	−52.7	−50.8
	8.6	−52.3	−43.2	−44.1	−59.4	
	10.0	−17.0	−37.4	−41.9	−43.4	−10.8
	11.0	−8.64	0			
250	7.0	52.5	59.8	40.6	65.9	60.3
	8.6	58.8	70.6	44.1	59.4	
	10.0	51.0	71.4	52.3	63.5	15.1
	11.0	88.6	87.7			
220	7.0	−4519	−4972	−4411	−4568	−4108
	8.6	−5363	−5249	−3912	−4441	
	10.0	−4417	−5063	−4499	−4545	−4981
	11.0	−4028	−4251			

hemocyanin in the oxygenated form at equilibrium) and taking the slope, n, of each line. The results are plotted in fig. 10, and yielded values of $n = 3.6, 3.7, 3.5, 2.0$ at pH values 6.1, 7.0, 8.8 and 10.6 respectively.

4. Discussion

4.1. Sedimentation and viscosity studies

The variation in dissociation of *J. edwardsii* hemo-

cyanin as a function of pH is similar to that observed for several other arthropod hemocyanins [1]. Our observations differ somewhat from the observations of Moore et al. [5], who found a significant degree of dissociation even at pH 8. Perhaps it is possible that the precipitation technique used in their preparation resulted in their sample being more readiy dissociable.

The average value of 490 000 for the molecular weight of the 16S particle is in very good agreement with values of 492 000 and 506 000 reported by Joubert [6] from light scattering measurements at pH 6.5 and 7.5 respectively on *Jasus lalandii*, and a value of 480 000 at pH 8.0 by Moore et al. [5] from sedimentation measurements by the Archibald procedure, using *Jasus novaehollandiae*.

The value of 81 000 for the average of molecular weight measurements on the 5S particle is significantly different from the values of 86 000 and 90 000 reported by Moore et al. [5]. Their values gave rise to some uncertainty as to whether the 16S particle was a pentamer or hexamer, but our results suggest that this particle is definitely a hexamer. This conclusion would indicate a close correlation in structure and size between *Jasus* hemocyanins and the hexamers of other arthropod hemocyanins, such as, for example, the 16S particle from the crab *Cancer magister* whose monomeric unit is believed to have a molecular weight of about 80 000 [16].

Considerable interest with the hemocyanins has centred around the nature of the monomer, particularly with regard to the number of polypeptide chains in the unit. In particular, the question has arisen as to whether there are 2 or 3 polypeptide chains in the 80 000 molecular weight subunit, or even perhaps just one chain, although until comparatively recently it was felt less likely that the latter was the case in view of the comparatively high molecular weight that would result from only one chain. However, the molecular weight determined from viscosity measurements in guanidine hydrochloride gives credence to the belief that our 81 000 molecular weight subunit contains no shorter polypeptide chains within the subunit.

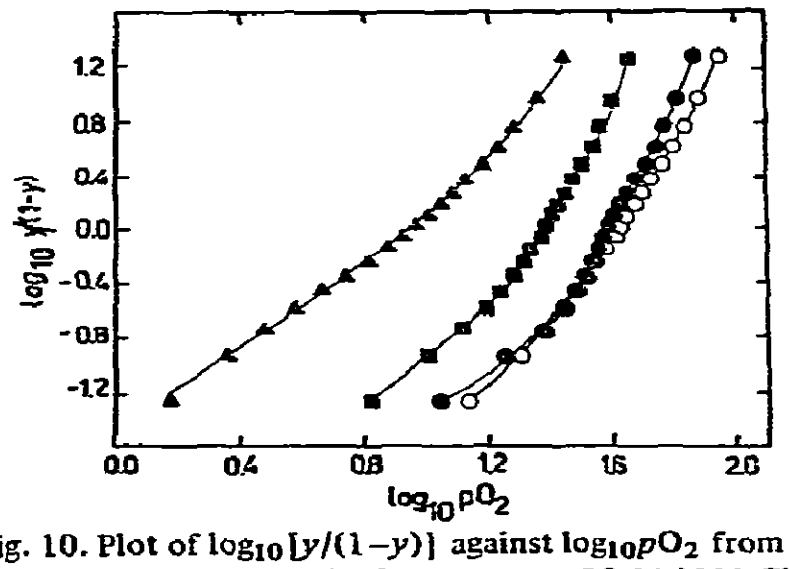

Fig. 10. Plot of $\log_{10}[y/(1-y)]$ against $\log_{10} pO_2$ from oxygen binding of hemocyanin in the presence of 0.01 M $MgCl_2$ at 20°C and pH 6.1, (o) 7.0, (•) 8.8 (■) and 10.6 (▲).

Much of the work described in this report was performed between 1971 and 1973, and at that time we made some preliminary studies on the molecular weight of the polypeptide chain of the hemocyanin, using gel filtration in 8 M urea and 0.1% β-mercaptoethanol, and polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS). The gel filtration experiments showed that most hemocyanin eluted in a band corresponding to a molecular weight of 76 000. A shoulder on this band also suggested the presence of a higher molecular weight of approximately 90 000. In addition, a smaller band of higher molecular weight eluted before either of these in the void volume. Electrophoresis in SDS gave similar results, with bands corresponding to molecular weights of 87 000, 100 000 and 170 000. Whilst there is some discrepancy between the two techniques for the molecular weights of the monomer bands, the important point here is that we could demonstrate with these preliminary experiments that: (1) two monomer species were present, not necessarily in a 1:1 ratio, and (2) a particle consistent in size with a dimer was observed.

Since the above experiments have been performed, considerable interest has been shown in the application of the polyacrylamide gel electrophoresis technique to hemocyanin. Loehr and Mason [17] have shown that the so-called 80 000 molecular weight monomer of *Cancer magister* hemocyanin actually consists of two different subunits of molecular weights 76 000 and 84 000. Carpenter and Van Holde [18] have studied *C. magister* hemocyanin by gel filtration and sedimentation equilibrium in guanidine hydrochloride, and have concluded that the polypeptide chains are no smaller than 70 000 to 80 000 in molecular weight. Murray and Jeffrey [19] report three different monomer units for the Australian freshwater crayfish *Cherax destructor*, all with mo-

lecular weights close to 75 000. In the case of the horseshoe crab, *Limulus polyphemus*, Sullivan et al. [20] have reported five electrophoretically different species of molecular weight about 66 000. We have also been making fresh detailed electrophoretic studies of hemocyanin from two species, one being *Jasus edwardsii*, and there are indications that subunit heterogeneity is indeed present with both these species. Our results, which will be discussed in detail shortly [21], support, in essence, our earlier observations – that there is more than one "monomer" unit with molecular weight in the range 76–100 000, and that a dimer of 160 000 is also present.

4.2. Circular dichroism

The presence of divalent cations is known to stabilize hemocyanin structures and prevent dissociation. Mg^{++} has been used successfully for this purpose with *Cancer magister* hemocyanin [22] and CD spectra were also run in this work to assess the influence, if any, of this cation.

From an inspection of the circular dichroism spectra of oxyhemocyanin in the 400–700 nm region at the 3 different pH values 7.0, 8.6 and 10.0 we see that the spectrum at pH 7 is greater in magnitude than those at either pH 8.6 or 10.0. The same trend is also observed at these pH values when the spectra of the oxyhemocyanin in the presence of 0.01 M $MgCl_2$ are compared. In addition, comparing the spectra at each pH, we find that the spectra are consistently slightly greater in magnitude when $MgCl_2$ is present.

In common with other hemocyanin species [1,23], the negative peak at 336 nm is the feature of spectra in the 300 to 400 nm region. This band also decreases in magnitude for oxyhemocyanin as pH increases, in common with the 400–700 nm region. Also, at pH 7 and 10, the band is slightly greater in magnitude in the presence of 0.01 M $MgCl_2$.

Upon deoxygenation, the CD spectra in the 300–700 nm region is observed to disappear, though small residual amounts of ellipticity are observed to remain unless the samples are very exhaustively deoxygenated. Apohemocyanin, in which the copper had been removed by dialysis against a buffered hemocyanin solution at pH 7 in the presence of 0.01 M KCN, as described by Cohen and Van Holde [24], also yields no CD spectra, indicating the relation of the observed spectra in this region to oxygen binding with the protein.

The spectrum in the 260–300 nm range has been attributed to contributions from aromatic amino acid side chains [20]. Three or four bands or shoulders, all negative, are observed under most conditions used, the peaks being located at 270, 280, 285 and 295 nm. Negative bands have also been observed in this region for other arthropod hemocyanins [22,23].

In the absence of $MgCl_2$, the spectra at pH 7 and 8.6 show only minor differences, but at pH 10 the magnitude of the spectrum in this range has diminished to a significant extent, presumably reflecting a change in environment of the side chains when the 16S particle breaks down into the 5S subunits. In general, the deoxygenated bands are of greater magnitude at a given pH than the corresponding bands in the oxygenated form, this difference being greatest in the 270–280 nm range.

In the presence of $MgCl_2$, the CD spectra at all three pH values show only small differences. Most notable is the close similarity of the CD bands at pH 10 with those at the lower pH values, compared with the diminished band at pH 10 in the absence of $MgCl_2$. Clearly, then, the Mg^{++} has stabilized the 16S structure, and this is borne out in the sedimentation behaviour, where 16S particles are still observed at pH 10.

The deoxyhemocyanin bands are also greater in magnitude in this region in the presence of $MgCl_2$ than the corresponding bands in the oxygenated form. Interestingly, though, the bands in the oxy- and deoxy-forms are closer together in the 270–280 nm region, and the 270–275 nm band is more finely resolved, when $MgCl_2$ is present. On the other hand, the differences between oxy- and deoxy-bands are greatest in the 280–290 nm region in the presence of $MgCl_2$. The differences in the CD spectrum in the presence and absence of Mg^{++} at different pH values clearly indicate that a perturbation of the aromatic side chains by $MgCl_2$ definitely takes place.

At pH 11, we observed only a small CD band in this region in the oxygenated form, and no measurable signal in the deoxyhemocyanin. Apparently, the asymmetric environment of the aromatic side chains has been almost totally lost at this pH. This effect was also observed with *Cancer magister* hemocyanin [22].

The CD spectra in the 260–300 nm region at pH 7 and 10 and in the presence of 0.01 M $MgCl_2$ showed quite a striking difference with apohemocyanin. At pH 7, the apohemocyanin spectra was observed to lie between the corresponding oxy- and deoxy-bands, but at pH 10 the apo spectrum was only about 25% of the magnitude of the corresponding oxy- and deoxy-bands. This observation implies that there are significant interactions between the copper and the aromatic side chain groups; perhaps it is these interactions that are stabilized by the presence of divalent cations such as Mg^{++}. Nevertheless, when the copper has been removed, the presence of Mg^{++} cannot by itself stabilize the aromatic amino acid conformation sufficiently at the higher pH to prevent diminution of the CD band.

The positive band observed at 250 nm is common to both molluscan and arthropod hemocyanins (Nickerson and Van Holde [23]). In our experiments, there was some variation in the magnitude of the peak under differing conditions of pH and $MgCl_2$ concentration. At pH values 7.0, 8.6 and 10.0, the 250 nm peak was smaller in magnitude in the presence of 0.01 M $MgCl_2$. At all four pHs studied the deoxyhemocyanin band was always greater in magnitude than in the oxy-form, in both the presence and absence of $MgCl_2$. At pH 11, where some change in the tertiary structure has taken place, (at least sufficient to virtually eliminate oxygen binding and change the conformation of amino acid side chain groups), the 250 nm band has increased in magnitude compared with the values observed at lower pH values. Apohemocyanin, at pH 7 in the presence of 0.01 M $MgCl_2$, displays a 250 nm band similar to deoxyhemocyanin under the same conditions. However, at pH 10 this band is considerably diminished in magnitude.

The origin of the 250 nm band has been discussed in some detail by Nickerson and Van Holde [23]. On the basis of experiments involving the addition of N-bromosuccinimide to *Busycon* hemocyanin, they suggest that histidine is involved. Furthermore, they suggest that although the band is strongly influenced by the state of the binding site, it does not arise from a chromophore which is part of the binding site. We believe this is also the case in *Jasus* hemocyanin. The influence of the state of the binding site can be seen in the differences in magnitudes of the band in the oxy- and deoxy-forms. The observation of the band even in apohemocyanin argues that it does not arise from the oxygen binding site itself. The presence of an apohemocyanin band at 250 nm has also been observed for *Loligo* hemocyanin by DePhillips et al. [25] and for *Cancer magister* hemocyanin by Ellerton [22]. Thus, although molluscan and arthropod hemocyanins have many physical differences, the band at 250 nm suggests at least one similarity in structure in the vicinity of the active site.

The negative band at 220 nm is regarded as a measure of secondary structure of the protein. There is a small but consistent difference between the oxy- and deoxy-forms at the four different pH values studied, indicating that oxygenation does cause some small change in the helical content of the polypeptide. The helical content does not drastically change on removal of copper either. It is particularly interesting that there is only a small change in magnitude of the 220 nm peak even at pH 11, where the changes in the bands between 250 and 300 nm were so pronounced. Thus, although the conformation of side chain groups has been influenced considerably at the higher pH, the helical regions of the protein's structure are largely unaffected.

4.3. Oxygen binding

Our oxygen binding studies show two effects. Firstly, there is a change in oxygen binding cooperativity at higher pH, as shown by the decrease in the value of the parameter n from the Hill equation. Between pH 6.1 and 8.8, the value of n changes very little from the maximum value of 3.7 observed, but at pH 10.6, $n = 1.95$, even though the 16S particle is still the main species in the presence of 0.01 M $MgCl_2$.

Secondly, there is a pronounced Bohr effect, resulting in a shift in the oxygen binding curve at different pH. The binding curve shifts to lower oxygen pressures at pH 8.8 and 10.6. At pH 8.8, the hemocyanin is still in the 16S form, and the value of $n = 3.5$, so the shift is due to the effect of pH as there is no dissociation of the protein. It is interesting to note that our oxygen binding curve at pH 7 is similar to that of Moore et al. [5] using whole *Jasus novaehollandiae* serum at pH 7.2, which they reported contained 0.01 M Mg^{++} on analysis.

It is of particular interest to compare our data with that of Johnston et al. [12] for the spiny lob-

ster *Panuliris interruptus*. This northern hemisphere species is similar to the *Jasus lalandii* group in that the hemocyanin is found mainly in the 16S form under physiological conditions, dissociating to a 5S subunit at higher pH. Values of $n = 3.29$ and 3.33 at pH 6.6 and 8.0 respectively were reported, with a Bohr shift over this range. It was concluded that the Bohr effect and dissociation into subunits are independent. These results and conclusions for *Panuliris* hemocyanin are in concurrence with our observations with *Jasus*.

The oxygen binding data for both *Jasus* and *Panuliris* hemocyanin may be contrasted with the recent study by Miller and Van Holde on the hemocyanin from the shrimp *Callianassa californiensis* [26] This species gives a very strong Bohr effect, shown by a wide variation of P_{50} over the range of pH 7 to 8.8. Miller and Van Holde discussed their observations in terms of the physiological significance that *Callianassa* spends much of its time buried in muddy sand. On the other hand, the maximum value of the Hill coefficient for *Callianassa* over this range is 3.57 at pH 7–8, and from their data they suggest that the cooperative unit is the 17S hexamer. Thus, although it may be concluded that differences in physiological environment of *Callianassa* and *Jasus* are reflected in differences in their respective Bohr effects, the comparable maximum values of the Hill coefficient in each species reflects the similarity of the two in having hexamers as the cooperative unit. It is yet to be understood why the hemocyanin of *Jasus* associates mainly to a 16S particle whereas higher aggregations are found with most other arthropod hemocyanins. It is hoped that work presently being carried out in our laboratory will provide additional insight into this problem.

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