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Characterization of the Extracellular Haemoglobin of *Haemopsis sanguisuga* (L.)

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The haemoglobin from the blood of the horseleech, *Haemopsis sanguisuga* (L.), had a sedimentation coefficient, $s^0_{20,w}$, of 59.11±0.55S, and a molecular weight as determined by sedimentation equilibrium of $3.71\times10^6\pm0.04\times10^6$. In the electron microscope the molecule appeared to be made up of two hexagonal plates, as is found with other worm haemoglobins, with dimensions 24.4±2.0nm (across the hexagon) and 15.2±1.4nm (height). The amino acid composition and spectrum were closely similar to those of the haemoglobins of other annelids (e.g. *Lumbricus*). The α-helical content, calculated from circular-dichroism measurements in the far-u.v. region, was 56–63%. The haem content was 2.49%, corresponding to a minimum molecular weight per haem group of 24800, but detergent-gel electrophoresis indicated the presence of polypeptide chains of mol.wts. 12600, 14800, 15500 and 25100. The pH-induced dissociation of the native molecule yielded components of approximate sedimentation coefficients 4.2S and 2.2S. The native molecule bound O_2 co-operatively at pH values in the range 7.46–8.49, the Hill coefficient (h) having a maximum value of about 2. Over this pH range there was a normal Bohr effect.

There has been much interest in the giant haemoglobins of invertebrates because they appear to be quite distinct from vertebrate haemoglobins in structure but have similar O_2-binding properties, and show co-operativity and Bohr effects (Giardina *et al.*, 1975; Weber, 1975). The gross structure of annelid haemoglobin is clearly discernible in the electron microscope (Roche *et al.*, 1960; Roche, 1965; Van Bruggen & Weber, 1974), and this picture is broadly supported by ultracentrifugal, light-scattering and X-ray-scattering evidence (Russell & Osborn, 1968; Rossi-Fanelli *et al.*, 1970; Stöckel *et al.*, 1973; David & Daniel, 1974). There is confusion however, as to what constitutes the subunit structure of these molecules. It is becoming clear that the molecular weight per haem group is probably in the region 22000–24000, rather than about 17000 as in vertebrate haemoglobins and myoglobin (Waxman, 1975). This is difficult to reconcile with values for the molecular weight of the smallest polypeptide chain(s) as determined by detergent-gel electrophoresis of 12000–17000, many haemoglobins apparently having several polypeptide chains with molecular weights in this region. *Lumbricus* haemoglobin, for example, is reported to have polypeptide chains of mol.wts. 12000 14000, 16000, 19000, 31000 and 33000 (Shlom & Vinogradov, 1973), and *Arenicola* haemoglobin similarly had chains of mol.wts. 13000 and 14000 (Waxman, 1971).

Though much attention has been given to the haemoglobins from *Lumbricus* (Rossi-Fanelli *et al.*, 1970; Harrington *et al.*, 1973; Shlom & Vinogradov, 1973; David & Daniel, 1974; Giardina *et al.*, 1975) and from a number of marine worms such as *Arenicola* (Waxman, 1971), *Cirraformia grandis* (Swaney & Klotz, 1971), *Thelapus crispus* (Garlick & Terwilliger, 1975) and *Pista pacifica* (Terwilliger *et al.*, 1975), comparatively little information seems to be available on the haemoglobins of the freshwater leeches. Svedberg & Pedersen (1940) reported that both *Hirudo* and *Haemopsis* haemoglobins had sedimentation coefficients of 57S–58S. Needham (1966) briefly reported the absorption spectra of some leech haemoglobins, and Roche *et al.* (1960) reported an electron micrograph of the haemoglobin of *Hirudo*. A paper by Waxman (1975) has given some information on the subunit structure of leech haemoglobin, though it is not quite clear what species of leech was investigated, and a preliminary communication by Shlom *et al.* (1975) deals with the subunit structure of the haemoglobin from the leech *Placobdella*.

In the present work the haemoglobin of the horseleech, *Haemopsis sanguisuga* (L.), has been purified and characterized by physicochemical methods. In addition its O_2-binding properties and subunit structure have been investigated.

Materials and Methods

Preparation of Haemopsis haemoglobin

Specimens of *Haemopsis sanguisuga* (L.) were obtained from T. Gerrard and Co., East Preston, Sussex, U.K. They were washed and put into 5% (v/v)

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ethanol for about 5min, blotted dry and then cut in half. The head and the tail end were then impaled on a long syringe needle which was supported over a centrifuge tube and held in place by means of a rubber band. A few drops of 0.1M-Tris/HCl buffer, pH8.0 (Long, 1961), were added to the tube, which was then spun at very low speed in a bench centrifuge (setting no. 1, MSE Minor). After about 5min, between 0.2 and 0.3ml of blood was found in the tube along with some debris. The contents of several tubes were pooled and spun in the bench centrifuge to remove debris, and the supernatant was carefully removed and centrifuged for 2h at 4°C and 133500*g* ($r_{av.}$ 5.9cm) in a Beckman model L2-65B ultracentrifuge. The deep-red pellet so obtained was resuspended in the 0.1M-Tris/HCl buffer, pH8.0, and spun down again, and finally resuspended in the same Tris buffer. After this treatment the material sedimented as a single boundary in the analytical ultracentrifuge. For comparison, a small sample of *Lumbricus* haemoglobin was prepared by the same technique.

Only freshly prepared material was used for the investigation of spectra and O_2-binding properties. Otherwise the material was stored frozen at −20°C.

The protein concentration was determined from the dry weight obtained by evaporating known volumes of aqueous haemoglobin solution to dryness *in vacuo* over P_2O_5 at 105°C to constant weight.

Analytical ultracentrifugation

Sedimentation-velocity and sedimentation-equilibrium experiments were performed in a Beckman model E instrument equipped with schlieren and interference optical systems and a rotor-temperature indicating and control system. Sedimentation-velocity runs were performed in cells with 3, 12 or 30mm aluminium centre-pieces in An-D or An-B rotors. Boundaries were observed with the schlieren optical system and were photographed with Ilford R-30 plates and a red filter. Plates were measured and sedimentation coefficients calculated as described previously (Wood & Mosby, 1975). Sedimentation-equilibrium experiments were also performed as described by Wood & Mosby (1975).

Spectrophotometry and circular dichroism

Absorption spectra were obtained on a Unicam SP.1800 instrument fitted with a chart recorder. C.d. (circular dichroism) spectra were obtained on a Jasco model J-40C spectropolarimeter. This instrument gives a reading directly in ellipticity, and this was converted into mean residue weight ellipticity, $[\theta]_\lambda$, by means of the expression:

$$[\theta]_\lambda = \theta/c \cdot l$$

where θ is the observed ellipticity, c is the concentration and l is the light-path.

Stock solutions of the haemoglobin were bubbled with O_2 or CO, or treated with a small amount of $Na_2S_2O_4$, to obtain the oxy, carbonmonoxy or deoxy derivatives respectively.

Electron microscopy

Electron microscopy was performed in a Philips 300 instrument on samples negatively stained with uranyl acetate.

O_2-binding curves

O_2-equilibrium curves were determined by the spectrophotometric method of Rossi-Fanelli & Antonini (1958), by using the shift of the Soret band as a measure of the extent of oxygenation.

Analytical methods

The haem content was determined by the method described by Antonini & Brunori (1971). Amino acid analysis was performed on the globin, which was prepared by the acid–acetone method of Rossi-Fanelli *et al.* (1958). The acetone-dried material thus obtained was dissolved in ice-cold water. Samples of this were hydrolysed with 6M-HCl in evacuated sealed tubes for up to 96h at 110°C. Amino acid analysis was performed on the resulting hydrolysates with a Biocal amino acid analyser. Tryptophan was determined in samples of the globin by means of the *N*-bromosuccinimide method (Spande & Witkop, 1967), and cysteine was determined as cysteic acid in performic acid-oxidized samples.

Subunit structure

The native haemoglobin was investigated by the sedimentation-velocity method in the analytical ultracentrifuge after treatment at pH values up to pH10.6 in the buffers used by Wood & Mosby (1975) in the presence and the absence of 0.01M-EDTA, to see if dissociation to smaller units took place.

The globin was reduced and carboxymethylated with iodoacetamide as described by Waxman (1971) to dissociate it into subunits. The untreated globin and the carboxymethylated globin were investigated by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate and 2-mercaptoethanol after heating to 100°C for 2min to destroy any proteases present, essentially as described by Weber & Osborn (1969).

Results

Purification and physical properties of Haemopsis haemoglobin

When the blood of *Haemopsis* was centrifuged at 133500*g* as described a deep-red pellet was obtained and the supernatant remained faintly coloured. The

pellet, resuspended in 0.1M-Tris/HCl buffer, pH8.0, sedimented as a single boundary in the analytical ultracentrifuge with sedimentation coefficient at infinite dilution, $s^0_{20,w}$, of 59.11±0.55S. Over the concentration range investigated (0.5–2.0mg/ml) the coefficient, K, in the equation:

$$s_{20,w} = s^0_{20,w}(1-Kc)$$

was 27ml·g^{-1}, where c is the protein concentration in g·ml^{-1}. Waxman (1975) claimed to have found two heavy components in his leech blood. However, it seems likely that this was due to his using a mixture of blood from two species of leech. We found no evidence for two heavy components in *Haemopsis* haemoglobin.

Waxman (1975) also claimed that the supernatant obtained after the heavy material had been spun down contained a third component of sedimentation coefficient 8.1S and apparent molecular weight in detergent-gel electrophoresis of 80000–90000. Ultracentrifugal analysis of the pale-coloured supernatant obtained in the present work revealed the presence of at least two components. One, of uncorrected sedimentation coefficient approx. 6.8S, was associated with the red colour, whereas the other, of sedimentation coefficient approx. 1.8S, did not appear to be coloured. Examination of the supernatant on detergent-gel electrophoresis produced a strong band corresponding to a mol.wt. of 30000–33000, and several weaker bands corresponding to lower molecular weights. No evidence was obtained for a component of mol.wt. 80000–90000. The material present in the supernatant liquid may be degradation or dissociation products of the haemoglobin or other non-haem proteins in the blood, and has not been investigated further in the present work. In what follows, purified haemoglobin should be taken to mean the material sedimenting from *Haemopsis* blood after centrifuging for 2h under the stated conditions.

The molecular weight of this material was determined by sedimentation equilibrium (Yphantis, 1964). Because of the very low rotor speeds required even for a so-called high-speed experiment (e.g. in the region of 3000rev./min) an An-H Ti rotor was used in a model E ultracentrifuge having an electronic speed control. Table 1 summarizes the results obtained at a number of different protein concentrations, and Fig. 1 shows a plot of the natural logarithm of the fringe displacement at equilibrium ($\ln \Delta Y$) against the square of the radial distance (r^2). At the concentrations indicated in Table 1 straight-line plots were obtained, indicating that the material was homogeneous. Results obtained at lower protein concentration showed more scatter and there was some evidence to indicate a lower molecular weight at these concentrations. The scatter may be due to the inherent inaccuracy in trying to measure very small fringe shifts. It is also possible that at these low concentrations (remembering that these were all meniscus-depletion experiments) the haemoglobin molecule

Table 1. *Determination of the molecular weight ($\bar{M}_w$) of Haemopsis haemoglobin by sedimentation equilibrium*

The protein was dissolved in 0.1M-Tris/HCl buffer, pH8.0, and dialysed against the same buffer, the diffusate being used in one compartment of the double-sector ultracentrifuge cell. The molecular weights were calculated from:

$$\bar{M}_w = \frac{2RT}{(1-\bar{v}\rho)\omega^2}\cdot\frac{d(\ln \Delta Y)}{dr^2}$$

The value taken for $\bar{v}$, 0.729ml·g^{-1}, was calculated from the amino acid composition (Cohn & Edsall, 1943). The selected speed was 3400rev./min, in each case, though the actual speed as determined from the odometer was used in the calculation. All experiments were high-speed or meniscus-depletion runs (Yphantis, 1964).

Initial protein concentration (mg/ml)	Duration of experiment (h)	$10^{-6}\times\bar{M}_w$
0.50	66	3.79
0.48	90	3.86
0.40	30*	3.67
0.30	24*	3.61
0.24	90	3.67
	116	3.70

* Including 1h periods of overspeeding.

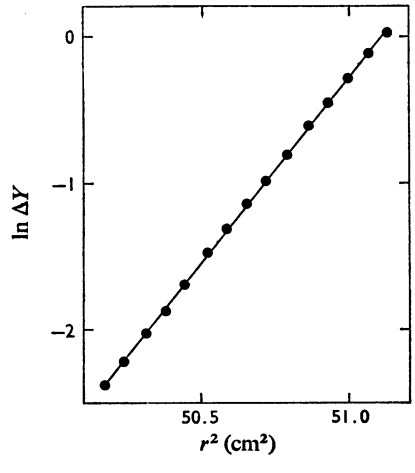


Fig. 1. *Sedimentation-equilibrium experiment with purified Haemopsis haemoglobin*

Initial protein concentration was 0.5mg/ml in 0.1M-Tris/HCl buffer, pH8.0. Conditions of run: speed, 3397rev./min; temperature, 22°C; interference photograph taken after 66h. Molecular weight ($\bar{M}_{w,app.}$) 3.79×10^6.

is partially dissociated. The average value for the molecular weight of native *Haemopsis* haemoglobin was $3.71\times10^6\pm0.04\times10^6$ (S.E.M.). In experiments with *Lumbricus* haemoglobin an average value of $3.86\times10^6\pm0.09\times10^6$ was obtained (mean of four determinations).

In the electron microscope *Haemopsis* haemoglobin presented the typical appearance of an annelid haemoglobin (Plate 1). The molecule appeared to be a hexagon composed of six sub-multiples when viewed from one direction, but in several instances 'side-on' views were obtained, indicating that the whole molecule is in fact made up of a stack of two hexagonal plates. The dimensions of the molecule were 24.4±2.0 (25) nm between two sides of the hexagon, with 15.2±1.4 (15) nm as the height of the stack.

Spectra

The absorption spectrum of *Haemopsis* haemoglobin and some of its derivatives is reported in Fig. 2. The band intensities and wavelengths are closely similar to those for other haemoglobins, and especially annelid haemoglobins (Rossi-Fanelli *et al.*, 1970; Swaney & Klotz, 1971; Harrington *et al.*, 1973). The extinction coefficient $E^{0.1\%}_{1cm,280}$ was 2.22.

Only the far-u.v. region of the c.d. spectrum of the oxy form of *Haemopsis* haemoglobin is reported in the present work (Fig. 3). Calculation of the fraction of the protein estimated to be in the α-helical form, f_H, from:

$$[\theta]_{222} = -30000f_H - 2340$$

(see Chen *et al.*, 1972; Harrington *et al.*, 1973) yielded a value of 56–63%. Saturating the solution under investigation with CO did not produce a significant change in either the shape or the intensities of the bands in the far-u.v. c.d. spectrum.

Chemical properties of Haemopsis haemoglobin

Two samples of purified *Haemopsis* haemoglobins had haem contents of 2.44 and 2.54% respectively, on the basis of the dry-weight value for $E^{1\%}_{280}$. The mean value for the haem content of 2.49% corresponds to a minimum mol.wt. of 24800. The whole native molecule would thus appear to contain about 150 haem groups.

The amino acid composition of *Haemopsis* haemoglobin is given in Table 2, and is closely similar to that of the haemoglobins of *Lumbricus*, *Arenicola* and *Cirraformia* (see Wood & Mosby, 1975).

The amino acid compositions of several haemoglobins were compared by using the formula of Harris & Teller (1973):

$$D = \sqrt{[\Sigma(X_A - X_B)^2]}$$

where X_A and X_B are the mole fractions of an amino acid in proteins A and B respectively. The lower the value of D, the more similar are the amino acid compositions. On this basis *Lumbricus* ($D = 0.069$) and *Arenicola* haemoglobin ($D = 0.077$) were the most similar to *Haemopsis* haemoglobin, compared with *Planorbis* erythrocruorin ($D = 0.102$) and human haemoglobin ($D = 0.129$). Harris & Teller

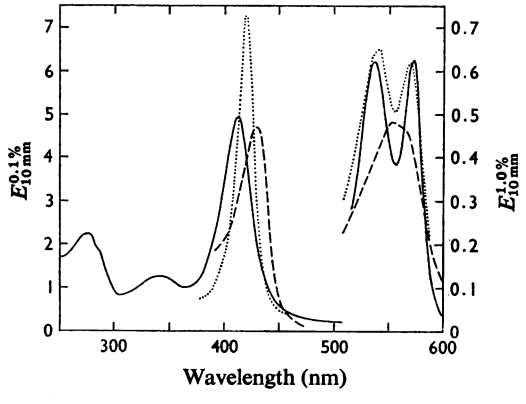


Fig. 2. *Absorption spectrum of Haemopsis haemoglobin* (----), *oxyhaemoglobin* (——) *and carbonmonoxyhaemoglobin* (····)

Protein concentration was 1 mg/ml and light-path 10 mm. The right-hand scale applies to wavelengths >500 nm.

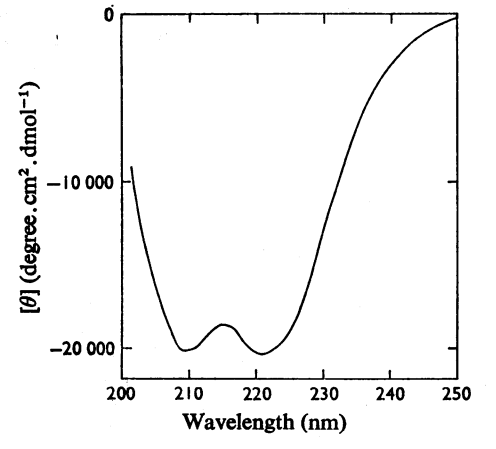
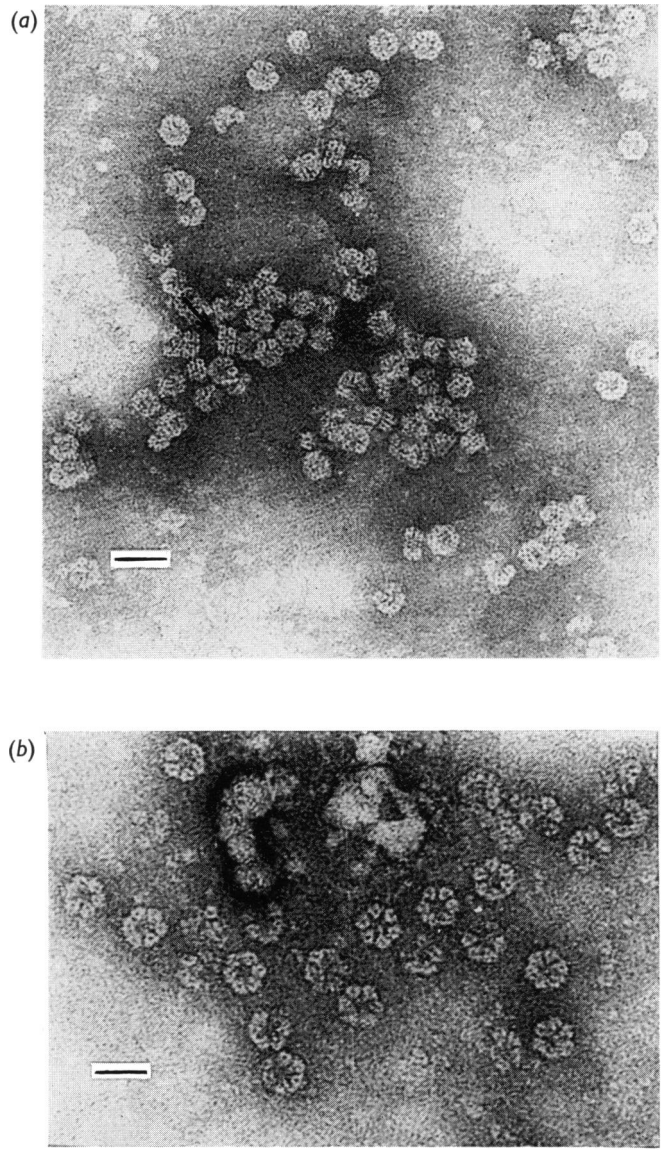


Fig. 3. *Far-u.v. c.d. spectrum of Haemopsis oxyhaemoglobin*

The spectrum was recorded in duplicate by using a solution of protein concentration 0.053 mg/ml in 0.1 M-Tris/HCl buffer, pH 8.0, and a cell of light-path 1 mm. The spectrum of the carbonmonoxy derivative was identical within experimental error.



EXPLANATION OF PLATE 1

Electron micrographs of negatively stained Haemopsis sanguisuga haemoglobin

Stain: uranyl acetate. Length of bar: (*a*) 40nm, (*b*) 30nm. Most of the molecules are seen as hexagons, which appear to be composed of six smaller ring-shaped subunits. Some molecules are seen 'side-on' (arrowed), revealing that the whole molecule is in fact a stack of two hexagonal plates.

Table 2. *Amino acid composition of Haemopsis haemoglobin*

The values are given on the basis of mol of residue per 24800g, corresponding to one mol of haem. Values for all amino acids are averages of 24, 48 and 96h hydrolysis, except threonine and serine, which were extrapolated to zero time, and valine, isoleucine and histidine, for which 96h values are given. Half-cystine was determined as cysteic acid (Leggett Bailey, 1962), and tryptophan by *N*-bromosuccinimide titration (Spande & Witkop, 1967).

Aspartic acid	16.0
Threonine	5.3
Serine	8.1
Glutamic acid	19.6
Proline	6.6
Glycine	8.0
Alanine	13.1
Half-cystine	2.7
Valine	16.9
Methionine	2.1
Isoleucine	5.9
Leucine	18.7
Tyrosine	4.9
Phenylalanine	10.7
Lysine	12.3
Histidine	14.0
Arginine	8.5
Tryptophan	3.1

(1973) note that values for D of less than 0.07 would indicate a non-fortuitous similarity of composition.

Subunit structure

Both the haem content and the electron micrographs of *Haemopsis* haemoglobin suggest that, like many other invertebrate and especially annelid haemoglobins, the molecule is built up of a large number of subunits. Attempts were therefore made to obtain information on the size of the subunits and in particular on the relationship of the haem group to the smallest polypeptide chain.

Analysis of the globin by detergent-gel electrophoresis yielded the results shown in Table 3. The globin obtained from *Lumbricus* haemoglobin was analysed simultaneously and the results obtained agreed closely with those given by Shlom & Vinogradov (1973). Although it seems that there is a component in *Haemopsis* globin of mol.wt. 25100 which would correspond very closely to the weight containing one haem group (24800), the other bands (except for that corresponding to mol.wt. 52500) were all of approximately equal intensity. Thus the band corresponding to mol.wt. 25100 accounts for only about one-quarter of the protein applied to the gel (assuming that all the components bind the locating dye to approximately the same extent). It is not

Table 3. *Analysis of the subunit structure of Haemopsis and Lumbricus globin by detergent-gel electrophoresis*

The 10% gels were calibrated with marker proteins of known molecular weight: cytochrome *c* (11700), ribonuclease (13700), myoglobin (17000), chymotrypsinogen (25700) and ovalbumin (43000). Each value is the mean of at least three determinations.

Mol.wt.	
Haemopsis	*Lumbricus*
51500 (very faint)	33900
25100	27500
	18400
15500	15500
14800	
	13600
12600	12700
	11750

immediately apparent how a combination of any of the other components can give rise to the observed haem–protein stoicheiometry. The series 12500, 25100, 52500 would be a possibility but leaves the components of apparent mol.wt. 14800 and 15500 unaccounted for.

Another way of finding out about the quaternary structure of annelid haemoglobins is to cause them to dissociate partially by means of a pH change. From an examination of the electron micrographs it might for example be expected that the molecule would dissociate to one-half molecules and then to one-twelfth molecules. When the pH of a solution of *Haemopsis* haemoglobin was raised in steps from pH8.0 to 8.6, 9.0, 10.0 and finally 10.6 by dialysis against the appropriate buffers, dissociation took place. The extent of the dissociation and the approximate size of the products of dissociation was estimated by analytical ultracentrifugation. At pH9.0, whole molecules (59S) co-existed with a species with approximate sedimentation coefficient 4.2S. This dissociation step was to some extent influenced by the presence of 0.01M-EDTA. For example in 0.05M-sodium borate buffer, pH9.0, containing 0.01M-EDTA and 0.04M-NaCl, about 70% of the protein existed in the 4.2S form and the remainder sedimented at approx. 60S. In the absence of EDTA only about 35% of the protein existed in the 4.2S form. At higher pH values the predominant species had a sedimentation coefficient of about 2.2S, and at pH10.6 this species accounted for over 95% of the protein. When the solution at pH10.6 was dialysed overnight against buffer of pH8.0, the major species found had a sedimentation coefficient of 4.16S, almost no faster-sedimenting material being found. Thus the two species of dissociation products that result from pH-induced dissociation have sedimentation coefficients

Table 4. *O_2-binding data for Haemopsis haemoglobin*

The haemoglobin was dissolved in 0.1 M-Tris/HCl buffer at the stated pH and 26–27°C. Each value is the mean from at least two separate experiments. Protein concentration: 0.2–0.4 mg/ml.

pH	p_{50} (mmHg)	p_{50} (Pa)	$h_{max.}$
7.46	12.3	1640	2.05
7.76	8.2	1093	2.15
8.10	7.2	960	2.25
8.49	5.8	773	2.07

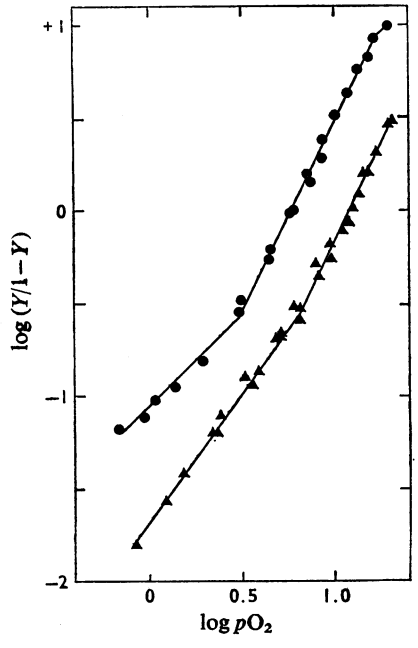


Fig. 4. *Oxygen-equilibrium (Hill) plots for Haemopsis haemoglobin*

Conditions: 0.1 M-Tris/HCl buffer, pH 7.46 (▲) and pH 8.49 (●), haemoglobin concentration about 0.2–0.3 mg/ml, 27°C. Each curve represents the data points from at least two duplicate runs. For values of p_{50} and h, see Table 4.

of approx. 4.2S and 2.2S. A change to acid pH produced a somewhat similar change. For example the globin, which had been prepared by exposing the native haemoglobins to acetone/HCl (Rossi-Fanelli *et al.*, 1958), was easily soluble in water and dilute buffers, and sedimented with a sedimentation coefficient of 2.15S. In the present work components with sedimentation coefficients corresponding to those expected of one-half molecules and of one-twelfth molecules (e.g. 35–40S and 11–13S) have not been encountered and possibly are not stable species. It is also possible to conclude that metal ions may be involved in forming the whole, native molecule, and that the dissociations produced by pH change appear to be to a large extent irreversible. It has been attempted to fit these observed patterns of dissociation into a scheme (see the Discussion section).

O_2 equilibrium

Determination of the O_2-binding curves for *Haemopsis* haemoglobin over the pH range 7.46–8.49 revealed that O_2 was bound co-operatively (Fig. 4). The maximum value for the Hill coefficient (h) was approximately 2. Over the pH range investigated there was a normal Bohr effect (Table 4).

Discussion

The physical and chemical properties of *Haemopsis sanguisuga* haemoglobin reported here are typical of those of annelid haemoglobins as a whole, and closely resemble those of *Lumbricus* haemoglobin. A mol.wt. of between 3000000 and 4000000 and a two-hexagonal plate structure are found in haemoglobins from terrestrial (e.g. *Lumbricus*), freshwater (e.g. *Haemopsis*) and marine (e.g. *Pista*) worms. In only one haemoglobin, that from the polychaete *Oenone fulgida* (Van Bruggen & Weber, 1974; Weber, 1975), has a different structure been reported, and here the double hexagon appears to have an additional subunit or subunits placed in the centre of the hexagon.

The amino acid composition of *Haemopsis* haemoglobin closely resembles that of *Lumbricus* haemoglobin by the rather coarse test, for such a large protein, of the Harris & Teller (1973) method. How this relates to the possession by both haemoglobins of multiple subunits remains to be seen. The spectral data obtained in the present work compare favourably with those reported for *Placobdella* haemoglobin by Shlom *et al.* (1975). These workers found that the absorption maxima were at 574, 538 and 413 nm. The corresponding values for *Haemopsis* haemoglobin were 573, 537 and 413 nm, and they are somewhat different from those reported by Needham (1966), namely 582, 544 and 417 nm. It may be noted that Needham (1966) also records that the peak corresponding to protein amino acid residues was at 273–274 nm rather than at 278 nm.

The O_2-binding properties of *Haemopsis* haemoglobin are similar in general to those of certain other invertebrate multisubunit haemoglobins. Only two values were quoted by Waxman (1975) for his leech haemoglobin (species uncertain) and both were determined in 0.025 M-KH_2PO_4/0.025 M-Na_2HPO_4, pH 7.0, at 27°C. At a concentration of 17 μM in haem, the partial pressure of O_2 required for half-saturation,

Table 5. *Dissociation and subunit structure of Haemopsis haemoglobin*

Entities at the top of the Table are suggested by electron-micrographic and sedimentation-velocity analysis. Entities at the bottom result largely from detergent-gel analysis.

Mol.wt.	Fraction	Expected sedimentation coefficient
3.72×10^6	1	60S
↓		
1.86×10^6	1/2	~40S
↓		
3.10×10^5	1/12	~13S
↓		
5.25×10^4	1/72	~4S
↓		
2.48×10^4	1/144	~2.5S
↓		
1.26×10^4 + 1.48×10^4 + 1.64×10^4	—	—

p_{50}, was 13.2mmHg, and the maximum value of the Hill coefficient, h, was 24: at 48μM in haem the corresponding values were 10.5mmHg and 2.9. The present results are clearly in the same range, though it might be noted that the physiological pH of the blood is probably near to pH8.0. The origin of the variation in p_{50} and in h with concentration is not clear from Waxman's (1975) work.

Two features of the O_2 binding may be significant. First, *Haemopsis* haemoglobin did not show the extremely high values for the Hill coefficient ($h>4.5$) observed with *Arenicola* and *Lumbricus* haemoglobins. In our work h (at p_{50}) seldom exceeded 2.3 over the pH range investigated. In certain other annelids, such as *Neanthes* (Economides & Wells, 1975) and *Cirraformia* (Swaney & Klotz, 1971), h was considerably less than 2.0 and in some cases near to 1.0, indicating weak or zero co-operativity. Secondly, *Haemopsis* haemoglobin exhibited a Bohr effect. Here again some annelid haemoglobins appear to possess a Bohr effect, e.g. *Thelepus* haemoglobin (Garlick & Terwilliger, 1975), whereas others do not, e.g. *Neanthes* haemoglobin (Economides & Wells, 1975). Thus within the framework of the double-hexagon structure possessed by the multisubunit annelid haemoglobins, many functional dissimilarities have evolved (zero, moderate, strong co-operativity; the presence or absence of a Bohr effect), presumably to enable the different animals to cope with their varied life-styles. It will be interesting to discover how far these dissimilarities originate in the arrangement of the fundamental polypeptide chains of the molecules and how far in the chemistry of the relationship of the haem group to the protein.

The subunit structure of *Haemopsis* haemoglobin seems to be very similar to that of other annelid haemoglobins in general. On the one hand, the electron micrographs and the pH-induced dissociation behaviour as observed by sedimentation-velocity experiments suggest how the whole molecule is built up from smaller submultiples. On the other hand, detergent-gel electrophoresis suggests that the smallest hypothetical functional subunit (i.e. that containing one haem group) is made up of more than one polypeptide chain. This of course is very different from the situation in vertebrate haemoglobins and myoglobins. Waxman (1975) surveyed a range of annelid haemoglobins and concluded that all were composed of polypeptide chains of mol.wt. 12000–15000 which formed small aggregates of four to six chains stabilized by disulphide bonds. The possible stages in the dissociation of *Haemopsis* haemoglobin are set out in Table 5, though it should be noted that not all the possible intermediates appear to be stable. It is perhaps most surprising that the one-half molecule is not formed, as might be expected from the electron micrographs. Also, unlike *Lumbricus* haemoglobin (David & Daniel, 1974) where a stable 10.1S species was identified, no evidence for the existence of such a species was found with *Haemopsis* haemoglobin. David & Daniel (1974) considered this species to be a one-twenty-fourth molecule and were able to obtain it in a sufficiently pure state to determine its molecular weight by sedimentation equilibrium. They also showed that it bound O_2 co-operatively (David & Daniel, 1973).

The next dissociation products that David & Daniel (1974) observed were a 3.5S and a 2.3S species, which they considered contained two and one haem groups respectively. In the present work with *Haemopsis* haemoglobin, components with approximate sedimentation coefficients of 4.2S and 2.2S were identified at high pH, which might be expected to correspond to the 52500- and 25100-mol.wt. species observed in detergent-gel electrophoresis. Until such species are isolated and characterized, Table 5 must be looked on as presenting a very tentative picture of the structure of *Haemopsis* haemoglobin. It might be argued with justification that if the 52500- and 25100-mol.wt. species observed in detergent-gel electrophoresis are indeed composed of several polypeptide chains, then the molecular weights determined by this method are likely to be incorrect, as the protein is not completely dissociated. A final point having a bearing on this problem of subunit structure is the question of the reliability of the haem determination. If the determination is reliable, then it is possible that the apparent lack of stoicheiometry between the molecular weights of the polypeptide chains and the haem content could be an

artifact of isolation. This seems unlikely in the present work, as the only treatment that the protein had undergone after collection from the animals was two centrifugations. However, as Waxman (1975) has pointed out, it is also conceivable that not all the subunits bind haem with equal affinity, or even that the mechanism in the animal that inserts the haem groups into the newly synthesized protein is inefficient.

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