Isolation, Characterization and Oxygen Equilibrium of an Extracellular Haemoglobin from *Eunice aphroditois* (Pallas)

By JOSEPH V. BANNISTER, WILLIAM H. BANNISTER and ANGELA ANASTASI
Department of Physiology and Biochemistry, Royal University of Malta, Msida, Malta

and EDWARD J. WOOD*

Department of Biochemistry, University of Leeds, 9 Hyde Terrace, Leeds LS2 9LS, U.K.

(Received 23 April 1976)

The extracellular haemoglobin from the polychaete, *Eunice aphroditois*, existed as a mixture of a heavy major component ($s_{20,w}^0 = 56.96 \pm 0.125$) and a light minor component ($s_{20,w}^0 = 10.00 \pm 0.13$ S), the latter probably being a dissociation product of the former. The molecular weight of the purified heavier component, as determined by sedimentation equilibrium, was $3.44 \times 10^6 \pm 0.04 \times 10^6$. The molecule had the electron-microscopic appearance typical of annelid haemoglobins, consisting of a stack of two hexagonal plates, with dimensions 26.32 ± 0.27 nm across the flats of the hexagon, height of stack 17.86 ± 0.34 nm. The sugar composition is reported, and the isoelectric point was approx. pH 7.8. The haem content was $2.31 \pm 0.01\%$, corresponding to a minimal mol.wt. of 26700. Detergent/gel electrophoresis revealed the presence of at least four bands with molecular weights in the range 14600–31000. Five *N*-terminal amino acids were found. In addition to the 10S component, which co-existed with the 57S component at all pH values in the range 4.0–10.6, at low pH values (<pH 5.0) a 16S and a 1.9S component were found. The absorption and circular-dichroic spectra are reported, and the α -helical content, calculated from the ellipticity at 222 nm, was about 40%. The molecule bound O₂ co-operatively with a maximum value of the Hill coefficient, *h*, of 3.9. Over the pH range 7.0–8.0 there was a positive Bohr effect.

Respiratory function in annelids is closely associated with the properties of the respiratory pigments, but in spite of much recent work on the subunit structures of the annelid haemoglobins, there is little information on how the molecules are constructed (Weber, 1975; Garlick & Terwilliger, 1975; Vinogradov *et al.*, 1975; Wood *et al.*, 1976a). Electron microscopy reveals that almost all of the annelid haemoglobins that have been examined, and which have originated from worms from diverse habitats, have the same quaternary structure, consisting of two rings of six subunits opposed to one another (see Van Bruggen & Weber, 1974). Nevertheless, there are striking differences in the O₂-binding properties of the pigments. At one extreme, some of them (e.g. *Eupolymania*) have a very low O₂ affinity, no Bohr effect and show no significant co-operativity, whereas others (e.g. *Arenicola*) have a high O₂ affinity, a pronounced Bohr effect, and show a high co-operativity, with values of the Hill coefficient (*h*) of 5 (Waxman, 1971, 1975; Weber, 1975).

The other main feature of these characteristically shaped multisubunit molecules, of mol.wt. in the

native state approaching 4×10^6 , is that they are composed of a number of polypeptide chains with mol.wts. in the region 14000–30000 as observed by detergent/gel electrophoresis. However, so far no simple correlation has been observed between the molecular weights of the chains and the molecular weights per haem moiety (Wood *et al.*, 1976a). Unlike the myoglobins and haemoglobins of higher animals, there is no minimal functional subunit of mol.wt. approx. 17000.

In the present work we have examined the physical and chemical properties of an extracellular haemoglobin from the polychaete *Eunice aphroditois* (Pallas), which is in the same family as *Marphysa sanguinea*, whose haemoglobin was investigated by Chew *et al.* (1965). *Eunice* haemoglobin differs in a number of respects from other haemoglobins that have been investigated, although the overall picture is similar. Unfortunately, Chew *et al.* (1965) did not report the O₂-binding properties of *Marphysa* haemoglobin, and Weber (1975) in his work on the O₂-binding properties of three haemoglobins from Eunicid polychaetes gives no physicochemical data. A preliminary report of some of the present results has appeared (Wood *et al.*, 1976b).

* To whom requests for reprints should be sent.

Materials and Methods

Preparation of *Eunice haemoglobin*

The worms were anaesthetized by prolonged immersion in sea water containing 0.1% (w/v) MS-222 (ethyl *m*-aminobenzoate; Sandoz Products, 208 Acton Lane, London N.W.10, U.K.). All procedures were carried out at 0–4°C. The dorsal skin of the worms was excised and blood was collected from multiple punctures of blood vessels. The blood was diluted with 2–3 vol. of 0.1 M-Tris/HCl buffer, pH 8.0, or 0.1 M-glycine/NaOH buffer, pH 9.0, to which 0.01 M-MgCl₂ had been added. The glycine buffer appeared to improve the keeping quality of the oxy-haemoglobin. The blood was centrifuged for 15 min at 10000g (*r*_{av}, 6.5 cm) in an MSE Super Speed 65 centrifuge to remove debris, then for 2–3 h at 260000g (*r*_{av}, 6.5 cm) to sediment the haemoglobin. The pellet was allowed to dissolve in the glycine buffer and the haemoglobin was spun down again two or three times under the same conditions.

Methaemoglobin was prepared by the procedure of Benesch *et al.* (1964).

Analytical ultracentrifugation

Sedimentation-velocity and sedimentation-equilibrium experiments were performed in a Beckman model E instrument fitted with an electronic speed-control system, schlieren and interference optical systems, and a rotor temperature-indicating and control system. An An-H rotor was used throughout and photographs were taken on Ilford R-30 plates, with the use of a red filter where necessary. Sedimentation-velocity experiments were performed in cells with 12 mm centrepieces, and the schlieren photographs were measured, and sedimentation coefficients calculated, as described by Wood & Mosby (1975). Sedimentation-equilibrium experiments were also performed as described by Wood & Mosby (1975). Solutions of haemoglobin were dialysed against an appropriate buffer, and the protein solution and diffusate were placed in separate channels of a 12 mm double-sector ultracentrifuge cell. Initial protein concentrations were determined spectrophotometrically.

Electron microscopy

Electron microscopy was performed in a Philips 300 instrument on samples negatively stained with uranyl acetate. Photographs were sandwiched between two glass plates and measured in a projector-scope (Precision Grinding Ltd., Mitcham, Surrey, U.K.).

Spectrophotometry and circular dichroism

Absorption spectra were obtained on a Pye-Unicam SP.700A instrument, calibrated with a holmium filter. The acid dissociation of the haem-

bound water molecule in the met (aquoferric) form of the haemoglobin was followed spectrophotometrically at 400 nm. The haemoglobin was dissolved in buffers of *I* = 0.1 mol/litre and pH in the range 6.0–10.5 at 25°C. The degree of dissociation of the haem-bound water molecule was measured as $(E_a - E_x)/(E_a - E_b)$, where E_a is the absorbance (at 400 nm) of the acidic ferric form of the haemoglobin (pH 6), E_b is the absorbance of the basic ferric form (pH 10.5) and E_x is the absorbance of the ferric form at intermediate pH.

C.d. (circular-dichroism) spectra were obtained on a Jasco model J-40C spectropolarimeter as described previously (Wood *et al.*, 1976a). In the far-u.v. region, results were expressed in terms of the mean residue weight ellipticity, and the fraction of the protein estimated to be in the α -helical form, f_H , was calculated from:

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

(Harrington *et al.*, 1973). In the near-u.v. and visible region, results were expressed on the basis of the minimal mol.wt. per haem group (26700).

O₂-binding curves

O₂-equilibrium curves were determined by a modification of the spectrophotometric method of Rossi-Fanelli & Antonini (1958) by using the shift of the Soret-band absorption (at several wavelengths) as a measure of the extent of oxygenation. All measurements were performed in a temperature-controlled room at 20 ± 1°C.

Analytical methods

The haem content of the protein was determined by the method described by Antonini & Brunori (1971). The globin was prepared by the method of Rossi-Fanelli *et al.* (1958). The globin was soluble in cold water and was used for amino acid analysis as described by Wood *et al.* (1976a). *N*-Terminal amino acids in the globin were determined by the dansyl method (Percy & Buchwald, 1972).

The neutral sugar content of *Eunice* haemoglobin was determined by g.l.c. of the trimethylsilyl derivatives of the sugars as follows. Methyl glycosides were prepared from 1–4 mg of globin by the method of Bhatti *et al.* (1970), mannitol being added as an internal standard before methanolysis. Trimethylsilyl derivatives were prepared by addition of 50 μ l of trimethylsilylating reagent (pyridine/trimethylchlorosilane/hexamethyldisilazane, 5:1:1, by vol.) and desiccated over P₂O₅ before chromatography. G.l.c. was carried out on a Pye 104 gas chromatograph fitted with a dual-flame-ionization detector. A 75 m open tubular column of internal diameter 0.25 mm was used, with OV 101 as liquid phase and He as carrier

gas at a flow rate of 5 ml/min. Sample volumes of 0.1–1.0 μ l were injected into the gas chromatograph. The temperature programme was as follows: 5 min at 160°C, increase from 160° to 200°C at 4°C/min, and finally 10 min at 200°C. Carbohydrate contents were determined from the areas under the recorder trace by comparison with known monosaccharides. The response of this method is poor for amino sugars, which in any case produce asymmetric peaks. Amino sugar determinations were therefore carried out by ion-exchange chromatography on samples that had been hydrolysed with 4M-HCl in evacuated sealed tubes for 4 h at 105°C. Sialic acids were determined by the method of Warren (1959).

Electrophoresis

Electrophoresis on cellulose acetate strips was performed in a standard Shandon-Kohn tank, and strips were stained in 0.1% (w/v) Ponceau S dissolved in 5% (w/v) trichloroacetic acid. Isoelectric focusing was carried out with a Pharmacia 400 ml column and sucrose gradient by using pH range 3.5–10.0 Ampholines (1%, v/v) as described in the manufacturer's instructions. Electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulphate and 2-mercaptoethanol was performed essentially as described by Weber & Osborn (1969) with samples of globin. Gels were stained with Coomassie Blue and scanned by means of a Helena Laboratories Flur-Vis Auto Scanner. The following proteins, all obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K., were used as mol. wt. markers: lysozyme (14400), sperm-whale myoglobin (17600), ovalbumin (46000), bovine serum albumin (66000) and β -galactosidase (136000). Relative mobility (R_m) was measured with respect to chymotrypsinogen A. The regression of R_m on the logarithm of molecular weight for the markers was used for molecular-weight estimations.

Results

Purification and physical properties of *Eunice* haemoglobin

Freshly taken blood and purified blood at certain pH values (see below) sedimented as two boundaries in the analytical ultracentrifuge, both of which were associated with red colour. The sedimentation coefficients of these two species were approx. 57S and approx. 10S. The two components were separated by repeated preparative ultracentrifugation. The concentration-dependence of the sedimentation coefficients of the two components was investigated in sodium phosphate buffer, $I = 0.1$ mol/litre, pH 6.8, containing 0.01 M-MgCl₂ (Fig. 1). The values obtained for $s_{20,w}^0$ were 56.96 ± 0.42 (S.E. of intercept at zero

Fig. 1. Dependence of sedimentation coefficient of *Eunice* haemoglobin and dissociation product on protein concentration

The haemoglobin was dissolved in sodium phosphate buffer, $I = 0.1$ mol/litre, pH 6.8, containing 0.01 M-MgCl₂. The straight lines were fitted by least squares. The values for the sedimentation coefficients at zero concn. in this buffer, $s_{20,b}^0$, were calculated to be 56.79 ± 0.42 S (O) and 9.97 ± 0.13 S (Δ).

Fig. 2. Dependence of the logarithm of fringe displacement (Δy) on the square of position in the cell (r) for the 57S component of *Eunice* haemoglobin at sedimentation equilibrium

The straight lines were fitted by least squares. The values of the slopes were calculated to be 3.35 ± 0.04 (O, after 24 h) and 3.30 ± 0.02 (Δ, after 48 h).

concentration from fitted line) S and 10.00 ± 0.13 (S.E.) S. Over the concentration range 0–6 mg/ml the values for k in the equation $s_{20,w} = s_{20,w}^0 (1 - kc)$, where c is the protein concentration, were calculated to be 16.4 ± 2.1 ml/g and 17.8 ± 3.3 ml/g for the 57S and 10S components respectively.

In a sedimentation-equilibrium experiment with the purified 57S component performed at 4000 rev./min and 21°C in 0.1 M-Tris/HCl buffer, pH 8.0, with an initial protein concentration of 0.3 mg/ml, a straight-line plot of $\ln(\text{fringe displacement})$ versus

Table 1. *Sedimenting boundaries in Eunice haemoglobin as a function of pH*

The following buffers ($I = 0.1$ mol/litre) were used: sodium acetate (pH 4.0, 4.6, 5.0, 5.6); sodium phosphate (pH 6.0, 7.0); Tris/HCl (pH 8.0); glycine/NaOH (pH 9.0, 10.6). The values for species with 1.9S and 15.6S are those observed in the buffers at 20°C.

pH	Proportion of species (%)			
	1.9±0.2S	10S	15.6±0.6S	57S
(a) With 0.01 M-Mg ²⁺ (protein concn. 4.6–5.8 mg/ml)				
10.6	—	23.5	—	76.5
9.0	—	33.1	—	66.9
8.0	—	11.6	—	88.4
7.0	—	20.5	—	79.5
6.0	—	13.6	—	86.4
5.6	—	32.6	—	67.4
5.0	23.2	44.8	11.3	20.7
4.6	54.6	34.5	10.9	—
4.0	64.6	35.4	—	—
(b) With 0.01 M-EDTA (protein concn. 3 mg/ml)				
10.6	—	35.4	14.2	50.4
9.0	—	41.0	—	59.0
8.0	—	28.9	—	71.1
7.0	—	13.8	—	86.2
6.0	—	18.2	—	81.8

(radial position in the cell)² was obtained (Fig. 2). This indicated that the material was indeed homogeneous, and a mol.wt. $\bar{M}_w$, of $3.47 \times 10^6 \pm 0.04 \times 10^6$ was calculated from the slope of the line obtained after 24 h (including overspeeding), and $3.42 \times 10^6 \pm 0.03 \times 10^6$ after 48 h.

The dissociation of the haemoglobin was investigated as a function of pH in the presence and absence of Mg²⁺. The experiments were conducted by dialysing a neutral solution of the haemoglobin against the appropriate buffer. The components were observed with schlieren optics in the analytical ultracentrifuge and their relative abundance is given in Table 1. In the presence of 0.01 M-MgCl₂, 57S and 10S components were observed in the pH range 5.6–10.6, with little systematic change in their relative abundance, except for a small increase in 10S component (at the expense of the 57S) at the extremes of the pH range. This trend was accentuated in the pH range 4.0–5.0. The relative concentrations of 57S component decreased markedly at pH 5.0 and was not evident at pH 4.6. In addition there was the appearance of new components of approx. 16S and 2S, both of which gave red sedimenting boundaries. The former was not evident at pH 4.0, whereas the latter had increased markedly. At this pH, about two-thirds of the haemoglobin was in the 2S and about one-third was in 10S form.

In the absence of MgCl₂, in the pH range 6.0–10.6, there was a tendency for the 57S component to de-

crease and the 10S component to increase in abundance with increase in pH. Some 16S component was seen at pH 10.6.

In detergent/gel electrophoresis, the haemoglobin showed four components with estimated mol.wts. of 14600, 20200, 28000 and 33100 and approx. relative proportions of 5.9, 12.4, 35.1 and 46.6%, respectively.

In the electron microscope, *Eunice* haemoglobin had the characteristic appearance of annelid haemoglobins (Plate 1), and the dimensions of the molecule were, across the flats of the hexagon 26.32 ± 0.27 (15) (S.E.M.) nm, and height of stack 17.86 ± 0.34 (7) (S.E.M.) nm. Plate 1 provides some evidence that the molecules may stack to even larger aggregates of up to six molecules. It is not known whether or not this is an artifact.

Isoelectric focusing on a sucrose gradient revealed that the haemoglobin had an isoionic point at approx. pH 7.8, but the material was somewhat heterogeneous (Wood *et al.*, 1976b). This value is high compared with the value of pH 4.0 reported for *Marphysa* haemoglobin by Chew *et al.* (1965). In electrophoresis on cellulose acetate in 0.06 M-barbitone/sodium barbitone buffer, pH 8.6, *Eunice* haemoglobin moved slowly towards the cathode as at least two very poorly separated bands. In the same run a sample of *Haemopsis* haemoglobin (see Wood *et al.*, 1976a) moved approximately 6 times as fast, as a single band, in an anodic direction.

Chemical properties

Preliminary results indicated that the amino acid composition of *Eunice* haemoglobin was similar to those of related invertebrate haemoglobins. However, there was poor agreement from batch to batch for unknown reasons. The sugars present were those typically found in glycoproteins (Table 2). No sialic acid was found. The haem content was 2.31 ± 0.01 (S.E.M.)% by weight, giving a minimum mol.wt. of 26700. Five free N-terminal groups (alanine, valine,

Table 2. *Carbohydrate composition of Eunice haemoglobin*

Neutral sugars were determined by g.l.c. of their trimethylsilyl derivatives, amino sugars were determined by means of an amino acid analyser after acid hydrolysis and sialic acids were determined by the method of Warren (1959) (see the Materials and Methods section for details). The values are given on the basis of mol of residue per 26700 g, corresponding to 1 mol of haem.

Glucosamine	3.6
Galactosamine	2.1
Fucose	0.39
Mannose	0.92
Galactose	0.46
Glucose	0.21
Sialic acids	0

EXPLANATION OF PLATE I

Electron micrograph of negatively stained Eunice haemoglobin

Stain: uranyl acetate. Length of bar approx. 100nm.

Table 3. Absorption maxima and corresponding millimolar extinction coefficients of *Eunice* haemoglobin derivatives

The haemoglobin was dissolved in sodium phosphate buffer, $I = 0.1$ mol/litre, pH 7.7. (In this buffer the values for $E_{280\text{nm}, 1\text{cm}}^{0.1\%}$ were: oxyhaemoglobin, 1.95; methaemoglobin, 1.87; cyanmethaemoglobin, 1.90; globin, 1.13.) The deoxy form was obtained by adding dithionite to the oxy form, and the cyanmet form by adding KCN to the met form. The values of ϵ_{mM} are based on a mol.wt. per haem group of 26700.

Haemoglobin	$\lambda_{\text{max.}}$ (nm)	ϵ_{mM}
Oxy(oxyferrous)	345	32.9
	418	118.9
	541	14.9
	576	14.7
Deoxy(ferrous)	431	119.7
	555	12.5
Met(aquoferric)	397	108.5
	504	13
	637*	3
Cyanmet(cyanoferric)	357	41.7
	421	120.2
	545	13.5

* Shoulder.

Fig. 3. Degree of dissociation of haem-bound water molecule with increase in pH of *Eunice* methaemoglobin

The dissociation (α) was measured spectrophotometrically at 400 nm in buffers of $I = 0.1$ mol/litre. The buffers were: phosphate (pH 6.0–8.0), Tris/HCl (pH 8.0–9.0) and glycine/NaOH (pH 9.0–10.5) as appropriate. The curve was fitted by inspection.

Fig. 4. Visible-region c.d. spectra of *Eunice* haemoglobin derivatives

(—) Oxy-, (····) deoxy-, (----) met- and (-.-.-) cyanmet-haemoglobin in phosphate buffer, $I = 0.1$ mol/litre, pH 7.7. Protein concn. was 2.74–3.07 mg/ml in a 1 cm cell.

Circular dichroism

The visible-region c.d. spectrum of the haemoglobin (Fig. 4) shows several bands whose positions and magnitudes vary with the oxidation state of the iron and the nature of the ligand. The oxy(oxyferrous) and deoxy(ferrous) forms have visible c.d. spectra with both positive and negative bands. The c.d. spectra for ferric haemoglobin derivatives in the same region are in the main positive. The deoxy form has a broad positive band at 545–550 nm, and a negative extremum at 575–580 nm. The oxy form has larger extrema of the same sign at 565 and 578 nm respectively, with an additional negative band at 525 nm

isoleucine, leucine and phenylalanine) were found by dansylation.

Spectra

The absorption spectrum of *Eunice* haemoglobin was typical, and the positions and intensities of the observed bands are reported in Table 3.

The degree of dissociation of the haem-bound water molecule in the met (aquoferric) form of the haemoglobin, as measured by the decrease in E_{400} with increase in pH, is shown in Fig. 3, which indicates 50% dissociation at pH 8.8–8.9. The curve represents a forward titration. Reversibility was not studied. The asymmetry of the curve suggests heterogeneity, which probably reflects multiple equilibria, so that the observed pH for half-dissociation is at best a guide to the region of dissociation. Other ferric haemoglobins and myoglobins have pK' values in the same region (Antonini & Brunori, 1971), but meaningful comparison cannot be made. [pK' is defined as the apparent pK for the ionization of the haem-linked water molecule:

$$pK' = \text{pH} + n \cdot \log \frac{[\text{Fe}^+(\text{H}_2\text{O})]}{[\text{Fe}(\text{OH})]}$$

in which n is unity for a simple ionization process (Brunori *et al.*, 1968).]

Fig. 5. Soret- and u.v.-region c.d. spectra of *Eunice* haemoglobin derivatives

(—) Oxy-, (····) deoxy-, (----) met- and (-.-.) cyanmet-haemoglobin in phosphate buffer, $I=0.1$ mol/litre, pH 7.7. Protein concn. was 0.38–0.91 mg/ml in a 1 cm cell.

Fig. 6. Far-u.v.-region c.d. spectra of *Eunice* haemoglobin derivatives

(—) Oxy- and (----) met-haemoglobin in phosphate buffer, $I=0.1$ mol/litre, pH 7.7. Protein concn. was 0.08 (oxy) and 0.12 (met) mg/ml in a 0.1 cm cell.

and a positive band at 597 nm. The met(aquoferri)-haemoglobin has a large positive band at 540 nm and a smaller positive band at 640–645 nm. The cyanmet-(cyanoferri)haemoglobin has a small positive band at 568–570 nm, a minor negative extremum at 470–475 nm and minor positive contributions at 495 and 525–530 nm.

Fig. 7. Hill plots of O_2 -equilibrium of *Eunice* haemoglobin

The fractional saturation, Y , at each partial pressure of O_2 was measured by taking readings at several wavelengths in the Soret region and averaging calculated values of Y . The protein concn. was 0.1–0.3 mg/ml. The lines were fitted by inspection. $\circ$, pH 8.5; Δ , pH 7.0; 0.1 M-Tris/HCl buffer containing 0.01 M-MgCl₂.

The Soret-region c.d. spectrum (Fig. 5) is also complex for each form of the haemoglobin. Oxy-, cyanmet- and deoxy-haemoglobins have marked positive peaks at 408 nm, 423 nm and 436 nm respectively. The c.d. of the methaemoglobin does not show prominent peaks in this region. It is weakly negative immediately below 425 nm and weakly positive immediately above this wavelength. Oxyhaemoglobin has a positive contribution centred at 380–385 nm, but the c.d. of the deoxy-, met- and cyanmet-haemoglobins is negative in this region. Oxyhaemoglobin shows a negative extremum at 330–333 nm. Cyanmethaemoglobin also has a negative extremum at 330–335 nm. Methaemoglobin has a weakly positive band in this region (Fig. 5).

Oxy-, met- and cyanmet-haemoglobins have small negative extrema at about 300 nm, small positive peaks at 292 and 282–285 nm and larger positive bands at 262 nm. The far-u.v. spectra of the oxy-, met- and cyanmet-haemoglobins are virtually indistinguishable (Fig. 6). The ellipticity at 222 nm indicates the following helical content: oxy, 40.1%; met, 36.8%; cyanmet, 36.2%.

O_2 binding

Some O_2 -equilibrium curves are shown in Fig. 7. It is immediately apparent that O_2 binding is co-operative, the Hill plots having high intermediate slopes, and slopes of 1.0 at the extremes. Table 4 summarizes the data over the pH range 7.0–8.5. Over this range the haemoglobin shows a normal Bohr effect.

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

The haemoglobin was dissolved in 0.1M-Tris/HCl buffer containing 0.01M-MgCl₂ at the stated pH and 20°C. The protein concentration was 0.2–0.4mg/ml.

pH	P_{50}		$h_{max.}$
	(mmHg)	(Pa)	
7.0	18.2	2430	3.6
8.0	14.8	1970	3.9
8.5	12.9	1720	2.3

Discussion

The present work indicates that the physical properties and O_2 -binding capacity of *Eunice* haemoglobin are broadly similar to those of other annelid haemoglobins. The most marked difference in properties is the observation that the isoelectric point of *Eunice* haemoglobin was near pH7.8, compared with much lower values for other annelid haemoglobins, including those from *Marphysa* (pH4.0), *Arenicola* (pH4.56), *Lumbricus* (pH5.28) and *Nereis* (pH5.10) (see Svedberg & Pedersen, 1940). There are few marked differences in amino acid composition which would account for this, and neither *Eunice* nor *Haemopsis* haemoglobin (E. J. Wood, unpublished work) contained sialic acids.

The pronounced co-operativity of O_2 binding with $h_{max.}$ as high as 3.9 is also observed with *Lumbricus* (David & Daniel, 1973; Giardina *et al.*, 1975), *Lumbrineris* (Weber, 1975), *Terebella* (Wells & Dales, 1975) and *Arenicola* haemoglobins (Waxman, 1975). In addition, the O_2 affinity as measured by p_{50} , the partial pressure of O_2 at half-saturation, is in the medium range. One may compare the data in Table 4 with values for *Arenicola marina* haemoglobin, which has a high affinity [$p_{50} = 2.0$ mmHg (270 Pa) at 20°C and pH7.4], or with another polychaete, *Eupolyornia crescentis*, which has a low O_2 affinity [$p_{50} = 36$ mmHg (4800 Pa) at 10°C]. *Eunice* is a bottom-dwelling marine polychaete. The functional properties of its haemoglobin fit in with the idea that haemoglobin from annelids that are tube- or mud-dwelling should have very high O_2 affinities and that their haemoglobin may in some ways be looked on as functionally more analogous to myoglobins rather than a haemoglobin. Annelids which live on land or in open water would on the other hand be expected to have haemoglobins with properties similar to those of vertebrate haemoglobins. The possession by *Eunice* haemoglobin of a normal Bohr effect is in accord with this general idea.

It is more difficult to relate these properties of the haemoglobin to any feature of its structure and composition. The subunit structures of invertebrate haemoglobins as determined by detergent/gel electro-

phoresis give very little clue as to the architecture of the molecules. David & Daniel (1974), working with *Lumbricus* haemoglobin, claimed to be able to obtain a smallest haem-containing unit with sedimentation coefficient 2.3S and mol.wt. 22000 by treatment at pH11.3. The material appeared to be homogeneous in sedimentation-equilibrium experiments but contained material that had lost its haem. On the basis of an iron determination, the *Lumbricus* haemoglobin had a minimum mol.wt. of 25700, and in detergent/gel electrophoresis only two bands were observed, the majority of the protein being in the form of a 12000-mol.wt. unit. Another group of workers, on the other hand, obtained evidence for six polypeptide chains in *Lumbricus* haemoglobin (Shlom & Vinogradov, 1973), but in later work (Vinogradov *et al.*, 1975) suggested that the alkaline dissociation of this haemoglobin resulted in the formation of a dimer of the 12000-mol.wt. species. After an examination of haemoglobins from oligochaetes and polychaetes they proposed that all the invertebrate multisubunit haemoglobins may possess a subunit of mol.wt. about 12000. Certainly *Eunice* haemoglobin also possesses a component with a mol.wt. of about 14600 in detergent/gel electrophoresis. As shown in the present work, these proteins are in fact glycoproteins, and there is therefore some uncertainty in the molecular weights as determined by detergent/gel electrophoresis. It is more difficult to account for the finding of Vinogradov *et al.* (1976) that the 12000-mol.wt. subunit which they isolated by gel filtration at pH9 contained 0.53% iron, that is to say one haem group in a mol.wt. of about 11000. This must presumably mean that there is another, non-haem-containing subunit of approximately equal or slightly greater size which combines with this to form the minimal functional subunit of mol.wt. 22000. The present findings with *Eunice* haemoglobin throw little light on this problem. However, the species with sedimentation coefficient about 2S at low pH values presumably corresponds to the 2.3S haem-containing subunit of *Lumbricus* haemoglobin. On the basis of the haem content, this 2S species of *Eunice* haemoglobin would also contain a single haem moiety. It is not clear whether the 20200-mol.wt. species observed in detergent/gel electrophoresis corresponds to this or whether it is a dimer of the 14600-mol.wt. species.

The c.d. spectrum of *Eunice* haemoglobin may be compared with that of *Lumbricus terrestris* haemoglobin (mol.wt. 3.3×10^6). Harrington *et al.* (1973) have reported the c.d. of *Lumbricus* haemoglobin in oxy and cyanmet forms. In the visible, Soret and near-u.v. regions the spectra closely resemble the corresponding spectra of *Eunice* haemoglobin. This is shown in Table 5. The closeness of the c.d. spectra would indicate that the haem environment and interactions are very similar in the two macromolecular haemoglobins (erythrocrurins).

Table 5. *C.d. extrema and corresponding molar ellipticities of Eunice and Lumbricus haemoglobins*

Molar ellipticities are based on a mol.wt. per haem group of 26700 for *Eunice* haemoglobin and 23000 for *Lumbricus* haemoglobin. Data for *Lumbricus* haemoglobin are from Harrington *et al.* (1973).

Hb	Extremum (nm)		$10^{-1} \times$ Molar ellipticity (degree $\cdot$ cm 2 $\cdot$ dmol $^{-1}$)	
	<i>Eunice</i>	<i>Lumbricus</i>	<i>Eunice</i>	<i>Lumbricus</i>
Oxy (oxyferrous)	—	258	—	5.35
	262	262	7.82	4.82
	282–283*	—	1.07	—
	292	288	0.71	–1.70
	302	302	–0.85	–1.20
	330–333	330	–1.85	–2.86
	380–385	375	0.64	0.0
	408	412	3.06	3.12
	525	520	–0.43	–0.44
	565	560	0.68	0.54
	578	578	–0.45	–0.27
Cyanmet (cyanoferric)	596	—	0.27	—
	—	260	—	1.80
	262	265	4.04	1.74
	285	282	0.53	–1.80
	292	295	0.35	–2.22
	305	—	–0.64	—
	330–335	335	–1.23	–3.26
	423	422	3.17	4.30
	470–475	465	–0.02	–0.17
	495	490	0.05	0.06
	525–530	525	0.05	0.04
	568–570	575	0.21	0.28

In the u.v., both haemoglobins display a large c.d. envelope centred around 260 nm. *Eunice* haemoglobin has further positive bands at 282–285 nm and 292 nm in the spectra of oxy and cyanmet forms. In contrast, *Lumbricus* haemoglobin shows negative bands at 282 nm (cyanmet), 288 nm (oxy) and 295 nm (cyanmet). In the u.v., the c.d. of haem proteins is complicated by the overlap of aromatic and low-lying haem contributions. This may explain the different c.d., positive in *Eunice* haemoglobin and negative in *Lumbricus* haemoglobin, in the 280 nm and 290 nm regions in the two haemoglobins, but a reversal of aromatic contributions is not excluded.

In the far-u.v., the c.d. of *Eunice* haemoglobin is very similar to that of *Lumbricus* haemoglobin. The mean residue ellipticity at 222 nm indicates a helical content of about 40% for both haemoglobins, which is virtually unaffected by the oxidation state and ligand binding. The helical content is low relative to that of monomeric and tetrameric haemoglobins and

myoglobin. The value is probably not a low estimate owing to light-scattering distortion of the c.d. spectrum (Harrington *et al.*, 1973).

We are grateful to the Sequencing Unit, Department of Biochemistry, University of Leeds, for the amino acid analyses, to Mr. D. Kershaw for the electron micrography, and to Mr. R. L. Hall for the neutral sugar analysis. W. H. B. thanks the Wellcome Trust for financial support.

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