Haemoglobins of Invertebrate Tissues

NERVE HAEMOGLOBINS OF *APHRODITE*, *APLYSIA* AND *HALOSYDNA*

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1. The occurrence of haemoglobin in invertebrate nerves is surveyed. Haemoglobin was observed in the nerves and ganglia of the marine nematode *Amphiporus* sp. and of the polychaet annelid *Halosydna* sp. 2. Haemoglobins from the nerve and ganglia of the polychaet annelid *Aphrodite aculeata* L. and from the nerve of the gastropod mollusc *Aplysia californica* have been partially purified. The haem in each case was identified as iron protoporphyrin IX. 3. The minimum molecular weight of *Aphrodite* nerve haemoglobin deduced from the haem content and amino acid analysis is 17090, in agreement with the molecular weight 15600 ± 1000 determined by sedimentation equilibrium. 4. The molecular weight of *Aplysia* nerve haemoglobin was determined by sedimentation equilibrium to be 16400 ± 1000. 5. The oxygen dissociation curves are hyperbolic. Half-saturation is achieved at 1·1mm. Hg for *Aphrodite* nerve haemoglobin and at 4·0mm. Hg for *Aplysia* nerve haemoglobin. The coefficients for partition between carbon monoxide and oxygen are: *Aphrodite* nerve haemoglobin, 167; *Aplysia* nerve haemoglobin, 116. 6. The ferrous haemoglobins combine with cyanide. 7. We conclude that the intracellular haemoglobins of muscle and nerve are similar.

The present study expands an observation of Lankester (1872): 'The chain of nerve ganglia of the annelid *Aphrodite aculeata* possesses a bright crimson tint. The colour is particularly marked in the ganglia themselves, and is most intense in the supraoesophageal ganglion, which has as intense a colour as a drop of fresh human blood. When examined with the microscope, the colour is found to impregnate the nerve tissue itself, and not to be held in any liquid bathing the tissue.' Roche & Raphaël (1936) have extracted this pigment into water and illustrated the absorption spectra.

Although it is by no means of general occurrence, haemoglobin has been noted also in the nerves of many animals scattered among several phyla, e.g. nemerteans (Lankester, 1872; Hubrecht, 1874; Nicol, 1960; this paper) and the nematode *Ascaris* (Smith & Lee, 1963). Haemoglobin occurs in the nerves of several annelids including *Thallasema* (Fox, 1955; Fox & Vevers, 1960), *Glycera* (C. Manwell, personal communication), *Urechis* (Baumberger & Michaelis, 1931), *Halosydna* (Manwell, 1960), and a number of aphroditeans (Raphaël, 1936; Bloch-Raphaël, 1939). Haemoglobin occurs in the nerves of several crustaceans (Fox, 1955, 1957), an insect (Fox, 1955), the lamellibranch mollusc *Tivela* (Fox, 1953), the gastropod mollusc *Busycon* and the gastropod mollusc *Aplysia*, which has been the subject of the important studies by Arvanitaki & Chalazonitis (1960). Strittmatter & Burch (1963) have purified a haemoglobin-like pigment from the ganglia of the lamellibranch mollusc *Spisula*. When deoxygenated in solution (Strittmatter & Burch, 1963) or *in situ* (Kennedy, 1960) the pigment has the spectrum of a *b*-type cytochrome; when oxygenated it displays the spectrum of oxyhaemoglobin. The haemoglobin concentration in the ganglia of crustaceans (e.g. *Daphnia*) increases and decreases, like that of muscle and blood, in response to diminished and augmented concentrations of dissolved oxygen (Fox, 1955).

The present paper describes the properties of the haemoglobins contained in cytoplasm from the nerves of two polychaet annelids, *Aphrodite aculeata* L. and *Halosydna* sp., and the gastropod mollusc *Aplysia californica*.

METHODS

Animals. Aphrodite aculeata L., the closely related *Hermione hystrix*, *Aplysia punctata* and a nemertean, *Amphiporus* sp., were collected at Plymouth. *Aplysia californica*, *Aplysia vaccaria* and *Halosydna* sp. were

purchased from Dr Rimmon Fay, Pacific Bio-Marine Supply Co., P.O. Box 285, Venice, Calif., U.S.A.

Extraction of nerve haemoglobins. Method 1. The haemoglobin of the *Aphrodite* ventral nerve chain could be leached from the nerve by dilute salt solutions. However, such extracts contained a mixture of haemoglobin that was rapidly oxidized by air to the ferric form and haemoglobin that resisted oxidation. The easily oxidized and oxidation-resistant fractions could be obtained separately. Preliminary leachings of the nerves for 1hr. at 20° in 0·01M-potassium phosphate buffer, pH 7·4, removed a fraction of the haemoglobin that was easily air-oxidized. Subsequent leaching for 1hr. in 0·15M-potassium phosphate buffer, pH 7·4, extracted the bulk of the haemoglobin in a form that resisted air-oxidation.

Method 2. *Aphrodite* nerve haemoglobin in dispersions of cytoplasm extruded into a medium known to be suitable for the perfusion of the squid axon (Baker, Hodgkin & Shaw, 1962) resisted air-oxidation and was stable on storage for several hours. The cytoplasm of dissected nerve chains was extracted by grinding by hand in a conical glass homogenizer into a solution containing potassium isethionate (potassium β-hydroxyethylsulphonate) (0·56M) and potassium phosphate buffer, pH 7·4 (0·05M) (Baker *et al.* 1962). The resulting solutions were clarified by centrifugation. The nerve sheaths were recovered as a haemoglobin-free fibrous bundle.

The spectra of haemoglobin derivatives were the same in solutions prepared by the two methods. The rate of deoxygenation of oxyhaemoglobin in solutions prepared by method 2 and equilibrated with nitrogen was, however, much slower than in solutions prepared by method 1. Most of the present work was carried out with solutions prepared by method 2.

Aplysia nerve haemoglobin was extracted by method 2. The ganglia of the particular batches of *Aplysia* studied contained much yellow pigment (most probably carotenoid) and relatively little haemoglobin. Accordingly, the ganglia were rejected and only the haemoglobin-containing portions of the nerve were used.

The ventral nerve chain of *Halosydna* is not easily dissected from the very tough connective tissue in which it lies. The entire mid-ventral portion of the body wall containing the nerve chain was homogenized to give a solution that remained turbid after centrifugation.

Purification of nerve haemoglobins. All operations were carried out at 0–4°. Dialysis sacs (Visking Corp.) were boiled in water before use. The weight of cytoplasm was taken as the difference between the weights of the tissues and the extracted nerve sheath bundles.

Aphrodite nerve haemoglobin was prepared as follows. A 1·31g. sample of nerve yields 1·05g. of cytoplasm. Cytoplasm was extracted by method 2 from the pooled ventral nerve chains of 49 animals into 12ml. of potassium isethionate–potassium phosphate buffer solution. The homogenate was clarified by centrifugation and fractionated by the addition of solid $(NH_4)_2SO_4$. The fraction that precipitated between 50% and 65% saturation was retained. It contained most of the haemoglobin and formed a very dark-red compact pellet when collected in the centrifuge. The haemoglobin was further purified by starch-granule electrophoresis in sodium barbitone buffer, *I* 0·05, when the haemoglobin moved toward the cathode as a sharp band. This band was eluted in 0·01M-potassium phosphate buffer, pH 7·5, and dialysed against two changes of this buffer and finally against three changes of 0·1M-potassium phosphate buffer, pH 7·5. The overall recovery of haemoglobin, based on the homogenate, was about 50%. The particulate fraction, separated from the homogenate by centrifugation, was washed three times by suspension in 6ml. portions of isethionate–phosphate solution and was finally suspended in 5·0ml. of isethionate–phosphate solution for spectroscopic examination.

Aplysia californica nerve haemoglobin was prepared as follows. A 4·24g. sample of nerve yields 3·33g. of cytoplasm. Cytoplasm was extracted by method 2 from the pooled red portions of the nerves from 90 animals into 14ml. of isethionate–phosphate solution. The small red fraction that precipitated between 60% and 100% saturation with $(NH_4)_2SO_4$ was collected by filtration under pressure on a pad of analytical-grade Filter-Cel (Johns–Manville Products). The haemoglobin was eluted from the filter with 0·1M-tris–HCl buffer, pH 7·5, and portions were dialysed against appropriate buffers. The particulate fraction separated from the homogenate in the centrifuge was washed as before and suspended in 6·0ml. of isethionate–phosphate solution.

Electrophoretic evidence for the homogeneity of Aphrodite *nerve haemoglobin.* Phosphate-buffer-leached extracts and homogenates of *Aphrodite* nerves each revealed only a single haemoglobin component when subjected to electrophoresis on cellulose acetate strips in phosphate buffer, pH 7·4, barbitone buffer, pH 8·8, borate buffer, pH 9·5, or glycine–NaOH buffer, pH 9·8. *Aphrodite* haemoglobin purified by $(NH_4)_2SO_4$ fractionation migrated to the cathode as a single component in barbitone buffer, pH 8·6, on paper and on starch block.

Amino acid analyses. Amino acid analyses were performed with a Technicon Amino Acid Analyser.

Preparation of other annelid haemoglobins. Blood was drawn from the large vessels of the marine annelids *Nereis* and *Arenicola* and diluted with sea water. Earthworms (*Lumbricus*) and the fresh-water annelid *Tubifex* were ground in 0·05M-tris–HCl buffer, pH 7·5, and the haemoglobins were purified by fractionation with $(NH_4)_2SO_4$ followed by electrophoresis on starch granules. Haemoglobin was purified from the radula muscle of the gastropod *Busycon* in the same way. The iron content of these preparations was determined by the Lorber (1927) sulphosalicylic acid method after digestion with perchloric acid (Paul, 1948). Spectra of the deoxygenated haemoglobin were determined under nitrogen both before and after the addition of solid $Na_2S_2O_4$.

Preparation of ferric derivatives. The haemoglobin in the crude extracts of *Aphrodite* nerve was not completely oxidized by the addition of 10mol.prop. of $K_3Fe(CN)_6$, but was oxidized by the addition of ferricyanide to a final concentration of 0·01M. Dialysis of the extract removed materials interfering with the oxidation of the haemoglobin by ferricyanide. The spectra presented were obtained with purified haemoglobin oxidized by the addition of 10mol.prop. of potassium ferricyanide, and dialysed against buffer to remove excess of ferricyanide.

Spectroscopic methods. Spectra were determined in the Beckman DK2 recording spectrophotometer or in the Cary model 11 recording spectrophotometer. Turbid suspensions were examined by the opal-glass technique of Shibata (1959). Unless otherwise stated 1cm. light-path cuvettes were used.

Haemoglobin concentrations were estimated as pyridine haemochromogen and are expressed as m-moles of haem/l.

Formation of haemochromogens. These were prepared by adding NaOH to 0·2N final concentration, allowing the solution to stand at room temperature for 20min., and adding either solid NaCN followed by $Na_2S_2O_4$, or 0·6vol. of pyridine followed by $Na_2S_2O_4$. Recrystallized haemin hydrochloride served as reference standard.

Reaction of haemoglobins with oxygen and carbon monoxide. Oxygen equilibria of haemoglobin in homogenates diluted with isethionate–phosphate solution were determined spectrophotometrically at 20–21° and pH7·43 essentially as described by Allen, Guthe & Wyman (1950). Measurements were made at 540, 560 and 577mμ. The gas volume of the cuvette was about 500ml., and the haemoglobin concentration approx. 0·1mM.

The extracts of *Aplysia* nerves were too turbid to permit direct readings of extinction. The opal-glass technique of Shibata (1959) was used to study the oxygen equilibria of haemoglobin in these solutions.

The results are presented graphically (Fig. 7) by using the logarithmic form of Hill's equation:

$$\log\frac{y}{100-y} = \log K + n\log p \qquad (1)$$

where y is the percentage saturation of the haemoglobin with oxygen, p is the partial pressure of oxygen in mm.Hg, K is an equilibrium constant and n (Hill's constant) expresses the degree of haem–haem interaction.

The equilibrium between haemoglobin, oxygen and carbon monoxide was investigated spectrophotometrically at 20–21° and pH7·43 in homogenates diluted with isethionate–phosphate solution. The greatest difference between the absorption spectra of oxyhaemoglobin and carbon monoxide–haemoglobin is found in the Soret region. To take advantage of this difference and at the same time avoid the surface denaturation that occurs when the protein is excessively diluted, the light-path was shortened to 2mm. and solutions with extinctions approx. 2 at the Soret maximum were used. The gas volume of the cuvette was about 500ml. and the concentration was approx. 0·05mM. Measurements were made near the Soret maximum of carbon monoxide–haemoglobin: 420 and 423mμ for *Aphrodite* and *Aplysia* respectively. Initially the cuvette contained either water-saturated air or water-saturated oxygen at 1atm. Successive increments of carbon monoxide were injected and the spectral changes recorded.

Since under the conditions of the experiment the haemoglobin is always saturated with gases, one may write an expression analogous to Hill's equation:

$$\log\frac{[HbCO]}{[HbO_2]} = \log\alpha + \log\frac{p_{CO}}{p_{O_2}} \qquad (2)$$

The partition coefficient, α, is defined as the ratio of the equilibrium constants, K_{CO}/K_{O_2}. The partition coefficient may be estimated graphically (Fig. 8), since at equal concentrations of oxyhaemoglobin and carbon monoxide–haemoglobin:

$$\frac{p_{CO}}{p_{O_2}} = \frac{1}{\alpha} \qquad (3)$$

Sedimentation coefficients and equilibria. Sedimentation velocities were carried out in a Spinco model E analytical ultracentrifuge run at 59780rev./min. by using schlieren optics with a Corning 2–61 red filter and Kodak 1–D spectroscopic plate.

Molecular weights of *Aphrodite* and *Aplysia* haemoglobin were determined by sedimentation equilibrium in the analytical ultracentrifuge by the method of Yphantis (1964). A solution of approx. 0·03% protein (estimated spectrophotometrically, assuming a mol.wt. of 20000) in a column approx. 3mm. long was centrifuged overnight to equilibrium at a speed sufficient to make the protein concentration at the meniscus substantially zero. Determinations were performed at 42040 and 39460rev./min. at 15–20°. Rayleigh interference optics were employed and Kodak 1–D plates were again used with red filters. Sapphire cell windows were used to minimize window distortion, and both sides of a double-sector cell were loaded with fluorocarbon oil (FC43; Spinco). The sample side of the cell was then filled with approx. 100μl. of haemoglobin solution, and the reference side with the same volume of the buffer solution against which the haemoglobin had been dialysed. Protein concentrations at equilibrium in the cell were measured from photographs of the interference pattern by using a Nikon model VI comparator. Molecular weights were calculated from the equation:

$$M_{app.} = \frac{2RT}{(1-\bar{v}\rho)\omega^2}\frac{d\ln C}{d(r^2)} \qquad (4)$$

where $M_{app.}$ is the apparent weight-average molecular weight, R the gas constant, T the absolute temperature, $\bar{v}$ the partial specific volume of the protein, ρ the density of the solution, ω the angular velocity, C the protein concentration in arbitrary units and r the distance from the centre of rotation.

The partial specific volume of *Aphrodite* haemoglobin was calculated as 0·741 from the amino acid composition, neglecting tryptophan and the haem group, according to the method of Cohn & Edsall (1943), by using the values given by Schachman (1957) for the partial specific volumes of the individual residues. McMeekin & Marshall (1952) measured and calculated $\bar{v}$ for horse haemoglobin and obtained the values 0·749 and 0·741 respectively. This suggests that molecular weights based on calculated values of $\bar{v}$ are subject to an error of about 5%.

The partial specific volume of *Aplysia* muscle haemoglobin, calculated from the amino acid composition (Rossi-Fanelli, Antoni & Povoledo, 1958), is 0·736. This is slightly lower than, but in the same general range as, the known values for other haemoglobins (Svedberg & Pederson, 1940), which fall between 0·741 and 0·751. For calculation of the molecular weight of *Aplysia* nerve haemoglobin, its partial specific volume was assumed to be the same as that of the muscle haemoglobin, i.e. 0·736.

RESULTS

Distribution and concentration of haemoglobins. All of the ganglia and the ventral nerve cord of *Aphrodite* are intensely red. The lateral segmental nerves arising from the ganglia are, however, colourless. The haemoglobin concentration was 0·7m-mole of haem/kg. of nerve or 0·9m-mole of haem/kg. of cytoplasm. The concentration of haem in human blood is about ten times as great.

No haemoglobin could be detected in the ventral nerve chain of the closely related species *Hermione*.

The particulate fraction from *Aphrodite* cytoplasm contained only 4–6% of the total haemoglobin of the nerve. This haemoglobin is probably adsorbed on to the particles. No cytochrome was detected in the suspension of particles when these were examined under nitrogen and in the presence of dithionite by the opal-glass technique.

The nerve cord of *Halosydna* appeared even more intensely red than that of *Aphrodite*. These nerves were not easily dissected from the body wall, and were not weighed.

The haemoglobin concentration in the nerves of *Aplysia californica* varies widely among individuals collected together and also among groups of individuals collected at different times or places. Within about 2–3 cm. of the ganglia the nerves contain little haemoglobin and appear white; the remainder of the length is red. The ganglia themselves contain haemoglobin but at lower concentration than in peripheral nerve. The average haemoglobin concentration of the red portions of the nerves was estimated to be 0·1 m-mole of haem/kg. of nerve or 0·15 m-mole of haem/kg. of cytoplasm.

The nerves of *Aplysia vaccaria* from Southern California contain about the same concentration of haemoglobin as those of *Aplysia californica*. Arvanitaki & Chalazonitis (1960) reported that nerves of *Aplysia depilans* from the Mediterranean contain about 0·1 mM-haemoglobin. No haemoglobin could be detected in the nerves of *Aplysia punctata* collected at Plymouth.

A substantial amount of pigment identified as haemoglobin by the absorption maxima of the oxyhaemoglobin, deoxygenated haemoglobin and carbon monoxide–haemoglobin forms was found in the particulate fraction sedimented from extracted *Aplysia* nerve cytoplasm. In three experiments particle-bound haemoglobin made up 20, 18 and 14% of the total. No cytochrome was detected in these particles. Haemoglobin in the neuron cell bodies of *Aplysia* ganglia (Arvanitaki & Chalazonitis, 1960) and in the gill of the clam *Phacoides pectinatus* (Read, 1962) is located in granules of about 1 μ diameter.

The anterior ganglia of the nemertean *Amphiporus* were examined under the microscope. Some areas and the tracts arising from them were red; other portions of the ganglia and the main nerve trunks were unpigmented. The red parts of the ganglia,

Table 1. *Absorption maxima of pyridine and cyanide haemochromogens of nerve haemoglobins and reference compounds*

	$\lambda_{max.}$ (mμ)		
Pyridine haemochromogen			
Aphrodite	419	524	557
Aplysia	418	526	557
Ferroprotoporphyrin IX	419	526	557
Cytochrome *c*	414	521	551
Cyanide haemochromogen			
Aphrodite	433	533	562
Aplysia	431	533	562
Ferroprotoporphyrin IX	433	536	562
Cytochrome *c*	421	525	553

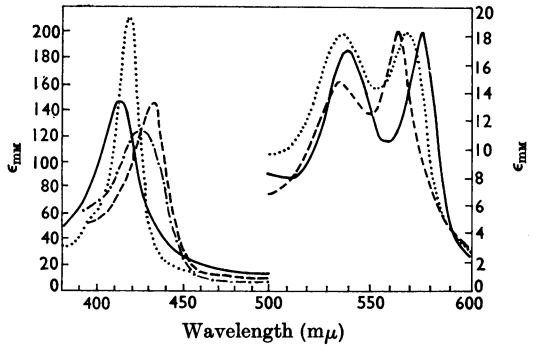

Fig. 1. Absolute absorption spectra of purified *Aphrodite* nerve haemoglobin. The left ordinate refers to the Soret region, the right ordinate to the visible region. ——, Oxyhaemoglobin; - - - - - -, carbon monoxide–haemoglobin; – – – –, ferrous haemoglobin–cyanide; – · – · –, deoxygenated haemoglobin.

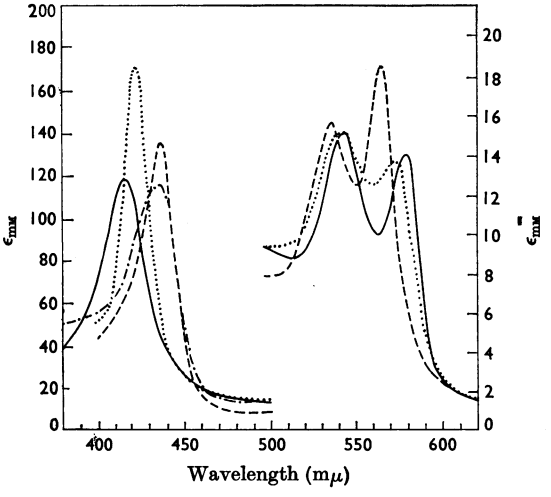

Fig. 2. Absolute absorption spectra of purified *Aplysia* nerve haemoglobin. The left ordinate refers to the Soret region, the right ordinate to the visible region. ——, Oxyhaemoglobin; - - - - - -, carbon monoxide–haemoglobin; – – – –, ferrous haemoglobin–cyanide; – · – · –, deoxygenated haemoglobin.

examined with a Zeiss microspectroscope ocular, exhibited the intense absorption bands at 540 and 578mμ of oxyhaemoglobin. As expected, these bands faded when the animal was placed under a coverslip and thus denied air.

Nature of the haem group. The haem of *Aphrodite* and *Aplysia* nerve haemoglobin is identified as iron protoporphyrin IX by the spectra of the reduced alkaline cyanide and pyridine haemochromogens (Vernon & Kamen, 1954). The wavelength maxima of these derivatives are indistinguishable from those of the derivatives of authentic protoporphyrin IX (Table 1), and are different from those of cytochrome *c* haem, the only compound with which they might easily be confused.

Absorption spectra of haemoglobin derivatives. The absorption spectra of the oxyhaemoglobin, carbon monoxide–haemoglobin and ferrous haemoglobin–cyanide forms are presented in Figs. 1, 2 and 3, and their extinction coefficients are collected in Table 2. Both the wavelength maxima of the α-, β- and Soret bands and the molecular extinction coefficients are typical of other blood or tissue haemoglobins. An absorption maximum centred at about 400mμ is exhibited by the carbon monoxide–compound of both *Aphrodite* and *Aplysia* haemoglobin. This band is seen in the spectra of some other but not all haemoglobins.

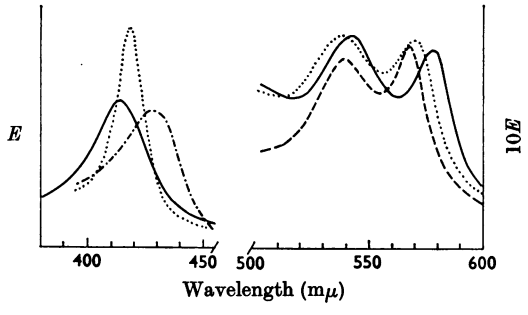

Fig. 3. Absorption spectra of extracts of *Halosydna* nerve. The ordinate scale in the visible region is 10 times the ordinate scale in the Soret region. ——, Oxyhaemoglobin; ------, carbon monoxide–haemoglobin; – – – –, ferrous haemoglobin–cyanide; – · – · –, deoxygenated haemoglobin.

The pigments differ in the spectra of the ferrous deoxygenated form (Fig. 4 and Table 2). *Aplysia* haemoglobin exhibits the single broad absorption band centred at 560mμ that is typical of many haemoglobins. Ferrous deoxygenated *Aphrodite* haemoglobin displays two absorption bands in the visible region with maxima at 549 and 566mμ. The two-banded spectrum is obtained when the haemoglobin solution is equilibrated with nitrogen and is not changed by the addition of sodium dithionite. Thus the nitrogen-equilibrated solution is fully deoxygenated and free from ferric haemoglobin. The two-banded spectrum is seen in the intact nerve (J. B. Wittenberg, P. K. Brown & B. A. Wittenberg, unpublished work), in extracts prepared by leaching the pigment into 0·15M-phosphate buffer, in extracts prepared by homogenizing the nerve in isethionate–phosphate solution or in iso-osmotic sucrose (0·98M) solution, and in solutions of the purified haemoglobin. The spectrum of the ferrous deoxygenated form is unchanged from pH 5 to pH 9·3, the limits of stability of the protein.

The absorption spectra of acid and alkaline ferric *Aphrodite* haemoglobin closely resemble those of

Table 2. *Millimolar extinction coefficients of ferrous haemoglobin derivatives*

Hb, Haemoglobin.

	$\lambda_{max.}$ (mμ)	ϵ_{mM}	$\lambda_{max.}$ (mμ)	ϵ_{mM}	$\lambda_{max.}$ (mμ)	ϵ_{mM}
Aphrodite						
Hb	425	126	549	14·1	566	14·8
HbO_2	414	147	541	17·0	577	18·4
HbCO	419	220	537	18·2	569	18·4
HbCN	434	146	536	15	564	19
Aplysia						
Hb	435	120	560	13		
HbO_2	416	130	543	15	578	14
HbCO	423	170	541	15	571	14
HbCN	437	150	539	16	568	18
Halosydna						
Hb	429		560			
HbO_2	414		542		576	
HbCO	418		537		568	
HbCN			538		567	

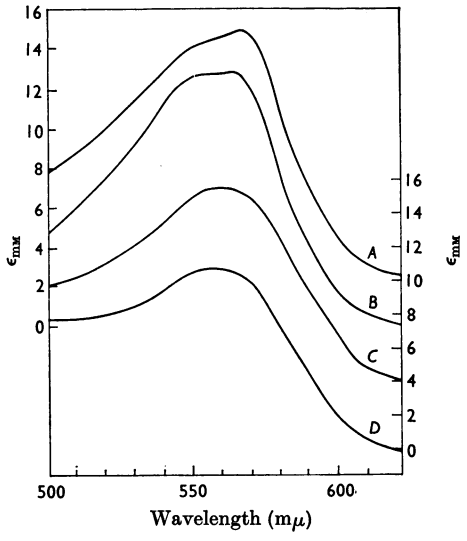

Fig. 4. Absorption spectra of ferrous deoxyhaemoglobins. The preparations are: *A*, purified *Aphrodite* nerve haemoglobin; *B*, purified *Tubifex* haemoglobin; *C*, purified *Aplysia* nerve haemoglobin; *D*, extract of *Halosydna* nerve. The left ordinate refers to *Aphrodite* nerve haemoglobin only; the right ordinate refers to *Aplysia* nerve haemoglobin only. The other spectra are arbitrarily displaced on the ordinate scale. *Tubifex* haemoglobin is included for comparison with *Aphrodite* nerve haemoglobin.

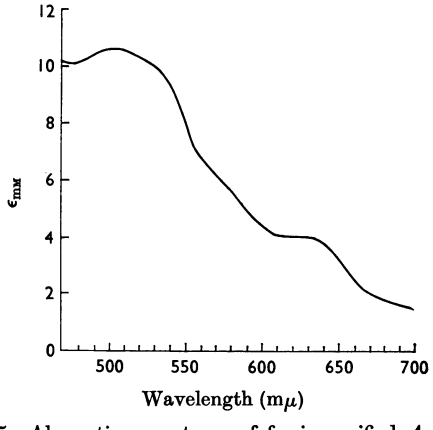

Fig. 5. Absorption spectrum of ferric purified *Aphrodite* nerve haemoglobin at pH 7·4.

Table 3. *Absorption maxima of ferrihaemoglobin and ferrihaemoglobin–cyanide*

	$\lambda_{max.}$ (mμ)				
Aphrodite					
Acid	406	505			630
Alkaline	408		537	575	
Cyanide	419		538		
Aplysia					
Acid	—	502			635
Alkaline	—	485	542	579	600
Cyanide	—		535		

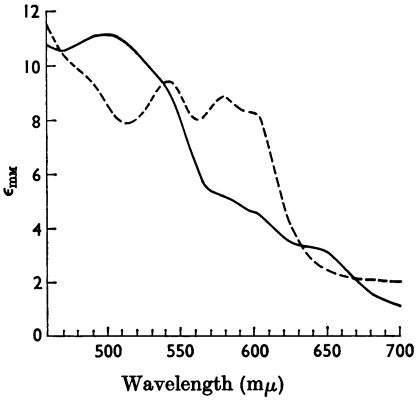

Fig. 6. Absorption spectra of ferric purified *Aplysia* nerve haemoglobin at pH 7·35 (——) and pH 9·22 (----).

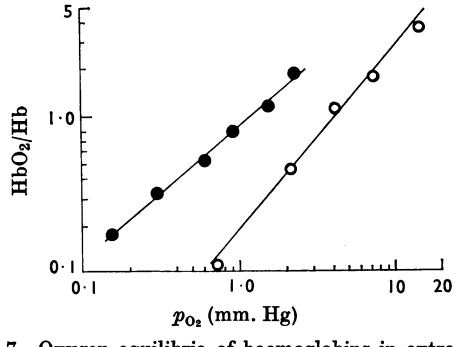

Fig. 7. Oxygen equilibria of haemoglobins in extracts of nerves. ●, *Aphrodite* haemoglobin; ○, *Aplysia* haemoglobin.

ferric human haemoglobin. The acid ferric haemoglobin spectrum (determined at pH 7·4, but unchanged in more acid solution) is presented in Fig. 5. The alkaline ferric form is unstable at pH 9·2. Absorption maxima are listed in Table 3. The absorption spectra of acid and alkaline ferric *Aplysia* haemoglobin are presented in Fig. 6 and the absorption maxima in Table 3. At pH 7·35 conversion into the acid form is largely complete. The alkaline ferric haemoglobin spectrum, in particular, is unusual in form, and resembles that of *Aplysia* muscle ferric haemoglobin (Rossi-Fanelli & Antonini, 1957).

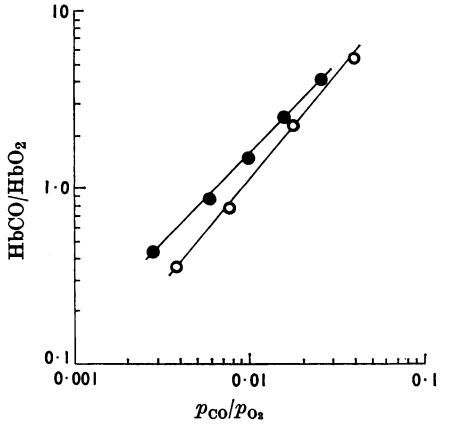

Fig. 8. Partition between oxygen and carbon monoxide of haemoglobins in extracts of nerves. The cuvette initially contained water-saturated oxygen at 1 atm. ●, *Aphrodite* haemoglobin; ○, *Aplysia* haemoglobin.

Reaction of haemoglobins with cyanide. Sodium cyanide (0·5M) does not displace oxygen from combination with *Aphrodite* or *Aplysia* nerve haemoglobin. However, cyanide does combine with each of the three deoxygenated proteins to give compounds with a two-banded visible spectrum (Figs. 1, 2 and 3 and Table 2). These absorption spectra are very similar to that of myoglobin–cyanide (Keillin & Hartree, 1955), which has absorption maxima at 434, 535 and 566mμ.

Equilibria with oxygen and carbon monoxide. The nerve haemoglobins could be repeatedly oxygenated and deoxygenated by equilibrating alternately with oxygen and nitrogen. At the end of several cycles the fraction of ferric haemoglobin was always less than 5%.

The affinity of *Aphrodite* haemoglobin for oxygen is high; the partial pressure of oxygen at which the pigment is half-saturated ($p_{\frac{1}{2}}$) is 1·1mm. Hg (Fig. 7). The value of n is not significantly different from

Table 4. *Amino acid composition of purified* Aphrodite *nerve haemoglobin compared with* Aplysia *and sperm-whale muscle haemoglobin*

	Aphrodite haemoglobin			*Aplysia* haemoglobin§	Sperm-whale myoglobin‖
Amino acid	(μmole of amino acids/sample)	(no. of residues/haem)	(no. of residues/haem to nearest integer)	(no. of residues/haem to nearest integer)	(no. of residues/haem to nearest integer)
Cysteic acid	0·002	0·1	0		
Aspartic acid	0·275	13·7	14	17	8
Methionine sulphoxide	0·000	0·0	0		
Threonine	0·268	13·3	13	3	5
Serine	0·171	8·5	8	14	6
Glutamic acid	0·218	10·8	11	12	19
Proline	0·117	5·8	6	2	4
Glycine	0·374	18·6	19	10	11
Alanine	0·497	24·7	25	24	17
Valine	0·156	7·8	8	9	8
Cystine (half)	0·009	0·45	1		0
Methionine	0·041	2·04	2	2	2
Isoleucine	0·171	8·5	8	7	9
Leucine	0·293	14·6	15	12	18
Tyrosine	0·060	3·0	3	1	3
Phenylalanine	0·159	7·9	8	13	6
Lysine	0·220	10·9	11	9	19
Histidine	0·066	3·3	3	1	12
Arginine	0·085	4·2	5†	5	4
Hydroxyproline	0·000	0·0	0		0
Ornithine	0·008	0·4	0		
Amide NH_3	0·267	13·3	13‡		7·2‡
Haem*	0·0201	1·00	1		
Total			160	141	153
Calc. mol.wt.			17090	17550	17816

* Determined before hydrolysis as the pyridine haemochromogen.

† Includes arginine plus ornithine.

‡ The amide groups and the haem are not included in the summation of amino acid residues.

§ Recalculated from Rossi-Fanelli *et al.* (1958).

‖ Edmundson & Hirs (1961).

unity, indicating the absence of haem–haem interactions, i.e. the oxygen dissociation curve is hyperbolic. Manwell (1960) found $p_{\frac{1}{2}}$ of the nerve haemoglobin of a Pacific-coast species of *Aphrodite* to be 3·0mm. Hg. The value of $p_{\frac{1}{2}}$ for *Aplysia* nerve haemoglobin is 4·0mm. Hg (Fig. 7) and there is little evidence of haem–haem interaction: the value of 1·2 for n may not be significantly different from 1·0. Rossi-Fanelli *et al.* (1958) found $p_{\frac{1}{2}}$ of *Aplysia* buccal muscle haemoglobin to be 2·7mm. Hg, with no haem–haem interaction. Manwell (1960) has studied the oxygen equilibrium of *Halosydna* nerve haemoglobin and found $p_{\frac{1}{2}}$ to be about 15mm. Hg.

Partition coefficients, measuring the relative affinities of the haemoglobin for carbon monoxide and oxygen, are 167 for *Aphrodite* haemoglobin and 116 for *Aplysia* haemoglobin (Fig. 8). This is larger than the partition coefficient of horse-heart myoglobin ($\alpha = 20$) but is similar to that of *Aplysia* buccal muscle myoglobin ($\alpha = 106$) (Rossi-Fanelli *et al.* 1958).

Amino acid analyses. The amino acid composition of *Aphrodite* haemoglobin (Table 4) shows some similarity to that of *Aplysia depilans* muscle haemoglobin (Rossi-Fanelli *et al.* 1958) and to sperm-whale myoglobin (Edmundson & Hirs, 1961). The low histidine content is noteworthy. It is uncertain whether the low cystine value arises from an impurity or whether 1 cysteine residue is present in the molecule.

The minimum molecular weight deduced from the haem content and the sum of the amino acid residues is 17090. Since the protein may not have been quite pure, this value is probably too high.

Sedimentation coefficient and molecular weights. The sedimentation coefficient, $S_{20,w}$ of *Aphrodite* haemoglobin, measured at 20° in 0·2M-potassium phosphate buffer, pH 7·5, was 1·7s. Assuming $\bar{v}$ to be 0·741, the molecular weight of *Aphrodite* haemoglobin would be 15600 ± 1000. The molecular weight of *Aplysia* nerve haemoglobin, determined at about 20° in 0·1M-potassium phosphate buffer, pH 7·35, was 16400 ± 1000, assuming $\bar{v}$ to be 0·736.

DISCUSSION

The red pigments of invertebrate nerves may reside in the neurons or in the highly developed glial cells (Coggeshall & Fawcett, 1964) closely associated with the neurons. The large neuron cell bodies of the ganglia of *Aphrodite* appear in the microscope as clear areas against a red background, suggesting that the pigment is largely in the glial cells. The haemoglobin of the giant neurons of the *Aplysia* ganglion is certainly in the neuron cell bodies because Arvanitaki & Chalazonitis (1960) have been able to dissect haemoglobin-containing granules from these cells. We have no evidence for the cellular location of haemoglobin in nerve trunks.

The pigments of the invertebrate nerves exhibit spectra characteristic of haemoglobins. In addition to those described above, H. B. Burch & P. Strittmatter (personal communication) have shown that haemoglobin extracted from the ganglia and nerve trunks of the mollusc *Busycon* resembles the haemoglobin of *Busycon* muscle. Deoxygenated *Aphrodite* nerve haemoglobin displays two absorption maxima (549 and 566mμ) in the visible region. In this it differs from most haemoglobins, which have a single broad symmetric absorption band at about 560mμ. This property of *Aphrodite* nerve haemoglobin is not specific for nerve haemoglobin, but is encountered commonly in annelid blood haemoglobins (Vles, 1923; Kobayashi, 1936; Scheler, 1960). We have confirmed the observation of two-banded visible spectra of the deoxygenated forms of the blood haemoglobins of *Lumbricus* (549 and 563mμ), *Arenicola* (552 and 563mμ), *Nereis* (550 and 565mμ approximately) and *Tubifex* (548 and 563mμ). Muscle haemoglobin from the mollusc *Busycon* also displays a spectrum of this type (550 and 563mμ). The spectrum of deoxygenated purified *Tubifex* haemoglobin is included for comparison in Fig. 4. We cannot agree with the claim (Scheler, 1960) that these double-banded spectra are related to the presence of non-haem iron in the molecule. The iron/haem ratio for electrophoretically purified *Tubifex* and *Lumbricus* haemoglobin has been found to be unity (J. B. Wittenberg, unpublished work).

The minimum molecular weight of *Aphrodite* nerve haemoglobin, deduced from the haem content and the amino acid composition, is 17090. The molecular weight found by sedimentation equilibrium is 15600 ± 1000. The agreement between these values establishes that there is 1 haem group/molecule. This low molecular weight is common among tissue haemoglobins.

The molecular weight of *Aplysia* nerve haemoglobin, deduced from the sedimentation equilibrium, is 16400 ± 1000. *Aplysia californica* nerve haemoblogin closely resembles *Aplysia depilans* muscle haemoglobin (Rossi-Fanelli *et al.* 1958; Rossi-Fanelli & Antonini, 1957) in each of the properties that have been examined. The spectra, particularly the characteristic acid and alkaline ferric haemoglobin spectra, are virtually superimposable. The molecular weights in each case are about 16000–17000. The oxygen affinities are similar, $p_{\frac{1}{2}}$ being 4·0mm. Hg for nerve and 2·7mm. Hg for muscle, and the coefficients for partition between carbon monoxide and oxygen are also similar, being 116 for nerve haemoglobin and 106 for muscle haemoglobin.

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REFERENCES

Allen, D. W., Guthe, K. F. & Wyman, J., jun. (1950). *J. biol. Chem.* **187**, 393.

Arvanitaki, A. & Chalazonitis, N. (1960). *Bull. Inst. oceanogr. Monaco*, no. 1164.

Baker, P. F., Hodgkin, A. L. & Shaw, T. I. (1962). *J. Physiol.* **164**, 330.

Baumberger, J. P. & Michaelis, L. (1931). *Biol. Bull., Wood's Hole*, **61**, 417.

Bloch-Raphaël, C. (1939). Ph.D. Thesis: University of Paris.

Cohn, E. J. & Edsall, J. T. (1943). *Proteins, Amino Acids and Peptides*, p. 374. New York: Reinhold Inc.

Coggeshall, R. E. & Fawcett, D. W. (1964). *J. Neurophysiol.* **27**, 229.

Edmundson, A. B. & Hirs, C. H. W. (1961). *Nature, Lond.*, **190**, 663.

Fox, D. L. (1953). *Animal Biochromes and Structural Colours*, p. 254. Cambridge University Press.

Fox, H. M. (1955). *Proc. Roy. Soc.* B, **143**, 203.

Fox, H. M. (1957). *Nature, Lond.*, **179**, 148.

Fox, H. M. & Vevers, G. (1960). *The Nature of Animal Colours*, p. 127. New York: Macmillan.

Hubrecht, A. A. W. (1874). *Arch. Zool. exp. gén.* **2**, 99.

Keilin, D. & Hartree, E. F. (1955). *Biochem. J.* **61**, 153.

Kennedy, D. (1960). *J. gen. Physiol.* **44**, 277.

Kobayashi, S. (1936). *Sci. Rep. Tohoku Univ., Ser.* 4, **10**, 733.

Lankester, E. R. (1872). *Proc. Roy. Soc.* **21**, 70.

Lorber, L. (1927). *Biochem. Z.* **181**, 391.

McKeekin, T. L. & Marshall, K. (1952). *Science*, **116**, 142.

Manwell, C. (1960). *Arch. Biochem. Biophys.* **89**, 194.

Nicol, J. A. C. (1960). *The Biology of Marine Animals*, p. 177. London: Isaac Pitman and Sons.

Paul, K. (1948). *Acta chem. scand.* **2**, 430.

Raphaël, C. (1936). *C.R. Acad. Sci., Paris*, **202**, 588.

Read, K. R. H. (1962). *Biol. Bull., Wood's Hole*, **123**, 605.

Roche, J. & Raphaël, C. (1936). *C.R. Soc. Biol., Paris*, **121**, 1024.

Rossi-Fanelli, A. & Antonini, E. (1957). *Biokhimiya*, **22**, 336.

Rossi-Fanelli, A., Antonini, E. & Povoledo, D. (1958). In *Symposium on Protein Structure*, p. 144. Ed. by Neuberger, A. London: Methuen and Co. Ltd.

Schachman, H. K. (1957). In *Methods in Enzymology*, vol. 4, p. 70. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Scheler, W. (1960). *Biochem. Z.* **332**, 366.

Shibata, K. (1959). *Meth. biochem. Anal.* **7**, 77.

Smith, M. H. & Lee, D. L. (1963). *Proc. Roy. Soc.* B, **157**, 234.

Strittmatter, P. & Burch, H. B. (1963). *Biochim. biophys. Acta*, **78**, 562.

Svedberg, T. & Pederson, K. O. (1940). *The Ultracentrifuge*, p. 406. Oxford University Press.

Vernon, L. P. & Kamen, M. D. (1954). *J. biol. Chem.* **211**, 643.

Vles, F. (1923). *Arch. Phys. biol.* **2**, 1.

Yphantis, D. A. (1964). *Biochemistry*, **3**, 297.