

## Haemoglobin from the Tadpole Shrimp, *Lepidurus apus lubbocki*

### CHARACTERIZATION OF THE MOLECULE AND DETERMINATION OF THE NUMBER OF POLYPEPTIDE CHAINS

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Haemoglobin from the tadpole shrimp, *Lepidurus apus lubbocki*, was found to have a sedimentation coefficient ( $s_{20,w}^0$ ) of  $19.3 \pm 0.2$  S and a molecular weight, as determined by sedimentation equilibrium, of  $798\,000 \pm 20\,000$ . The amino acid composition showed the lack of cysteine and cystine residues. A haem content of  $3.55 \pm 0.03\%$  was determined, corresponding to a minimal mol.wt. of  $17\,400 \pm 200$ . The pH-independence in the range pH 5–11 of the sedimentation coefficient indicates a relatively high stability of the native molecule. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis gave one band with mobility corresponding to a mol.wt. of  $34\,000 \pm 1500$ . The molecular weight of the polypeptide chain was determined to be  $32\,800 \pm 800$  by sedimentation equilibrium in 6M-guanidinium chloride and 0.1M-2-mercaptoethanol. The findings indicate that *Lepidurus* haemoglobin is composed of 24 identical polypeptide chains, carrying two haem groups each.

Arthropod extracellular haemoglobins exhibit diversity of molecular size (Ilan & Daniel, 1979). This diversity is reflected in the sedimentation coefficients: values of 1.7–3.1 S have been reported for insect larval haemoglobins, 11.3–11.7 S for anostracan and conchostracan, and 16.3–17.8 S for cladoceran crustacean haemoglobins. Much progress has been achieved in the elucidation of the structure of insect larval haemoglobins (Svedberg & Eriksson-Quensel, 1934; Behlke & Scheler, 1967; Thompson *et al.*, 1968; Huber *et al.*, 1968; Behlke *et al.*, 1975; Kleinschmidt & Braunitzer, 1976). The structural information on haemoglobins from crustacea is less complete, haemoglobins from the anostracan *Artemia salina* (Bowen *et al.*, 1976; Moens & Kondo, 1977), the conchostracan *Cyzicus hierosolymitanus* (Ar & Schejter, 1970; David *et al.*, 1977) and the cladoceran *Moina macrocopa* (Sugano & Hoshi, 1971) being the most thoroughly investigated. Haemoglobin was also found in notostracan crustaceans (Fox, 1949). However, no structural work has been done, to our knowledge, on this haemoglobin.

The present paper describes an initial study of the structural properties of extracellular haemoglobin from a notostracan crustacean, the tadpole shrimp *Lepidurus apus lubbocki*.

### Materials and Methods

#### Preparation of haemoglobin

*Lepidurus apus lubbocki* specimens were gathered during the winter and early spring of 1978 from a temporary pond at Migdal Sedek, Sharon Valley, Israel. Animals were transferred live to the laboratory, washed with cold distilled water and dried in air. The heart cavity was punctured with a fine glass capillary and about 0.1 ml of haemolymph was collected from each animal. Special care was taken to avoid damaging the surrounding tissue. Pooled blood from 20–40 shrimps was diluted with an equal volume of 0.1M-sodium phosphate buffer, pH 6.8, centrifuged for 20 min at low speed, and the minute precipitate discarded. The supernatant was then centrifuged for 3 h at  $232\,000g$  ( $r_{av}$ , 5.7 cm). The precipitate was dissolved in the same buffer and centrifuged again. The resulting red pellet was redissolved in about 0.1 ml of the buffer and the concentrated haemoglobin solution (100–200 mg/ml) stored in the cold until use. All work was done at 4°C. The purified oxyhaemoglobin exhibited an absorbance ratio,  $A_{416}/A_{280}$ , of 3.2. Haemoglobin that had been subjected to a third high-speed-centrifugation step showed the same  $A_{416}/A_{280}$  ratio. Protein prepared in this manner could be stored for 2 weeks with no discernible changes in its spectral properties.

\* Abbreviation used: SDS, sodium dodecyl sulphate.

### Concentration determinations

Haemoglobin concentrations were measured by absorption spectroscopy, with a Cary 118 spectrophotometer. Absorption coefficients were determined by reference to dry-weight measurements. Solutions of haemoglobin were exhaustively dialysed at 4°C against deionized distilled water. After dialysis, the solution was usually clear. However, sometimes a slight turbidity developed and was removed by low-speed centrifugation. The absorbance of the clear solution was determined by carrying out measurements on a series of samples volumetrically diluted with buffer. The protein content was obtained by drying to constant weight under vacuum at 40°C of about 1 ml of solution; the exact volume was determined from density and weight measurements. At 280 nm, a value for  $A_{1\text{cm}}^{1\%}$  of 18.65 litre·g<sup>-1</sup>·cm<sup>-1</sup> in 0.1 M-phosphate buffer, pH 6.8, was obtained.

The absorption coefficient in 6 M-guanidinium chloride was determined in the following manner. Portions of a protein stock solution, 20–30 mg/ml, were diluted to identical extents with low-salt buffer and with 6 M-guanidinium chloride. The spectra were determined about 4 h later. Knowledge of the absorption coefficient of the protein in buffer and of the absorbance ratio in buffer and 6 M-guanidinium chloride allowed a determination of the absorption coefficient in the latter solvent. At 276 nm, a value for  $A_{1\text{cm}}^{1\%}$  of 15.95 litre·g<sup>-1</sup>·cm<sup>-1</sup> in 6 M-guanidinium chloride was obtained.

### Amino acid analysis

Amino acid analysis was carried out as described by Spackman *et al.* (1958) in a Beckman Unicrom amino acid analyser. Samples were hydrolysed with 6 M-HCl in evacuated sealed tubes for 24, 48 and 72 h at 110°C. Cysteine and cystine were determined as cysteic acid in performic acid-oxidized samples of the protein (Hirs, 1956). Methionine was determined in the hydrolysate of unoxidized protein and methionine sulphoxide in the hydrolysate of the oxidized material. Tryptophan was determined as described by Matsubara & Sasaki (1969).

### Haem and iron determination

Haem determination was performed by the pyridine haemochromogen difference-spectrum method (Falk, 1964). Iron determination was carried out by atomic-absorption spectroscopy at 248.3 nm with a Varian Techtron model AA-5 spectrophotometer.

### SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed as described by Weber *et al.* (1972). Gels (0.5 cm × 10 cm) contained 7.5% acrylamide, 0.20% methylenebisacrylamide, and 0.15% NNN'N'-

tetramethylethylenediamine. Haemoglobin samples were made 1% (w/v) in SDS, 1% (v/v) in 2-mercaptoethanol and 0.01 M in sodium phosphate buffer, pH 7.0. The samples were heated for 5 min at 90°C before their application to the gels. A constant current, 4 mA per gel for the first 30 min and 8 mA for the following 5 h, was applied. Molecular weights were determined from band mobilities with a calibration curve obtained with six protein markers. The markers (Pharmacia, Uppsala, Sweden) were: rabbit muscle phosphorylase *b* (mol. wt. 94000), bovine serum albumin (67000), egg-white ovalbumin (43000), bovine erythrocyte carbonic anhydrase (30000), soya-bean trypsin inhibitor (20100) and bovine milk  $\alpha$ -lactalbumin (14400).

### Ultracentrifugation

Ultracentrifugation was performed in a Beckman model E analytical ultracentrifuge. Sedimentation-velocity experiments were carried out at 20°C. Schlieren phase-plate optics were used, and the sedimentation coefficients were corrected to  $s_{20,w}$  values in the usual way (Svedberg & Pedersen, 1940). Wherever necessary, buffer densities and viscosities were determined by means of a 25 ml pycnometer and an Ostwald viscometer.

Sedimentation equilibrium was carried out by using the short-column meniscus-depletion technique (Yphantis, 1964). Rayleigh interference optics were used to obtain the concentration distribution at equilibrium, and the photographic plates were analysed with a Nikon model 6 C microcomparator. Readings of the fringe pattern were taken at 0.1 mm intervals, and fringe displacements less than 100  $\mu$ m were ignored. Local slopes of the  $\ln c$ -versus- $r^2$  plot, where  $c$  is the concentration and  $r$  is the distance from the axis of rotation, were determined at each concentration by least-squares straight-line fitting of five points, two points on either side of the central point with which the slope is associated. The weight-average mol. wt. at  $r$ ,  $\bar{M}_{w,r}$ , was calculated from the equation:

$$\bar{M}_{w,r} = [2RT/\omega^2(1 - \bar{v}\rho)] d \ln c/dr^2 \quad (1)$$

where  $R$  is the gas constant,  $T$  is the absolute temperature,  $\omega$  is the angular velocity,  $\bar{v}$  is the partial specific volume,  $\rho$  is the density of the solution and  $d \ln c/dr^2$  is the local slope of the  $\ln c$ -versus- $r^2$  plot. A difficulty in obtaining absolute-molecular-weight values from sedimentation-equilibrium experiments in concentrated guanidinium chloride solutions arises from the preferential interaction of solvent components with the protein. The problem can formally be handled (Casassa & Eisenberg, 1964; Reisler & Eisenberg, 1969) by replacing  $\bar{v}$  in eqn. (1) with an apparent specific volume,  $\phi'$ , defined as:

$$\phi' = \frac{1}{\rho_0} \left( 1 - \frac{\Delta\rho}{c} \right) \quad (2)$$

where  $\Delta\rho = \rho - \rho_0$  is the difference in density between the solution and the solvent with which it is in dialysis equilibrium.

#### Density measurements

The determination of the specific volume in water and especially in concentrated guanidinium chloride poses severe demands on the precision of the density measurements. Density measurements were performed with the Digital precision density meter DMA02 C manufactured by Anton Paar K.G., Graz, Austria. General procedures for exact density measurements with this instrument are discussed in detail in several publications (Kratky *et al.*, 1969; Reisler & Eisenberg, 1969; Lee & Timasheff, 1974). Determination of densities with the exactness needed for the purpose of the present study required special consideration be given to the following points.

(a) *Temperature stability.* The temperature of the measuring-cell compartment had to be kept constant ( $\pm 0.01^\circ\text{C}$ ) for the period of the time needed to carry out a density measurement of the sample and calibration fluids. An ultrathermostat (bath, Haake KT33; thermoregulator, Haake T52) with a flow rate of about 6 litres/min, placed close to the density meter, was found satisfactory for this purpose. In order to improve the performance of the thermostat, room temperature was maintained  $0.5\text{--}2^\circ\text{C}$  above that of the measuring-cell compartment.

(b) *Exclusion of contaminants from sample solution and calibration fluids.* Air was dried and freed from dust by passage through silica gel and cotton-wool. Sample solutions clarified by low-speed centrifugation and deionized distilled water were kept free from dust in closed glass vessels. To prevent bubble formation, water and sample solution were allowed, before being used to fill the cell, to equilibrate with air at  $0.5\text{--}2^\circ\text{C}$  above that of the measuring cell.

(c) *Cleanness of the measuring cell.* It is very important to have the measuring cell thoroughly clean and dry before each filling. This was achieved by a series of rinsings with mild detergent, tapwater, deionized distilled water and absolute dust-free ethanol (ten rinsings with each solvent) followed by streaming dry dust-free air until a constant reading of the density meter was obtained.

(d) *Attainment of thermal equilibrium within the measuring cell.* Successive readings of the density meter were taken after each filling of the measuring cell. During equilibration, the readings changed asymptotically, attaining a constant value (at a pre-set count of  $1 \times 10^4$ ) when thermal equilibrium had been reached. The fact that the sample solution and water had been maintained before filling at a temperature close to that of the measuring cell helped to decrease the time needed for the attainment of thermal equilibrium.

Density determinations for specific-volume measurements were performed on solutions containing 20–30 mg of protein/ml. First, the calibration constant of the instrument was determined by carrying out a series of alternate fillings with air and water until the readings of each fluid were identical (at a pre-set count of  $1 \times 10^4$ ). Repeated series of fillings with air, water, protein solution or diffusate were then carried out until the readings of each fluid were identical. All density measurements were carried out at  $20^\circ\text{C}$ .

## Results

### Characterization of *Lepidurus native haemoglobin*

*Lepidurus* haemoglobin sedimented as a single boundary in the ultracentrifuge. Fig. 1 presents a plot of the sedimentation coefficient against protein concentration. Extrapolation to zero concentration gave  $s_{20,w}^0 = 19.3 \pm 0.2\text{S}$ . The amino acid composition is given in Table 1. Noticeable is the absence of cysteine and cystine residues. The minimal molecular weight based on the methionine content is  $16900 \pm 500$ . Determination of the iron and haem content gave  $0.296 \pm 0.009$  and  $3.55 \pm 0.03\%$  (w/w), values corresponding to minimal mol.wts. of  $18900 \pm 600$  and  $17400 \pm 200$  respectively.

The molecular weight was determined by sedimentation equilibrium. The  $\ln c$ -versus- $r^2$  plot was linear. Point-by-point weight-average molecular weights were found to be practically independent of concentration. Two experiments gave  $\bar{M}_w = 789000 \pm 21000$  (Fig. 2) and  $806000 \pm 19000$ , taking the experimentally determined value  $\bar{v} = 0.745\text{ ml/g}$ , measured in water. The molecular weight of *Lepidurus* haemoglobin will hereafter be taken as 798000.

### Dependence of the sedimentation coefficient on pH

Fig. 3 shows the effect of pH on the sedimentation coefficient. It is seen that the structure of the native molecule is stable over a wide range of pH values

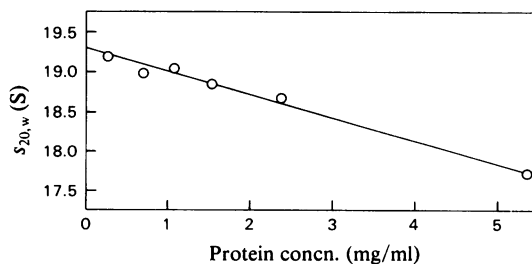


Fig. 1. Dependence of sedimentation coefficient of *Lepidurus* haemoglobin on protein concentration. Sedimentation was carried out in 0.1M-Tris/HCl buffer, pH 7.4, at 52000 rev./min and  $20^\circ\text{C}$ .

Table 1. *Amino acid composition of Lepidurus haemoglobin*

The entries for threonine and serine were calculated by back-extrapolation to zero time of the values obtained for 24, 48 and 72h hydrolysates; those for valine, isoleucine and leucine are values of maximal recovery; other entries are average values obtained for 24 (three determinations), 48 and 72h hydrolysates. The maximal deviation from the average of an individual determination did not exceed 3.5%.

Amino acid	Composition (% by weight)	No. of residues per formula weight*
Lysine	5.15	6.8
Histidine	3.09	3.8
Arginine	6.93	7.5
Aspartic acid	10.96	16.1
Threonine	5.98	10.0
Serine	5.61	10.9
Glutamic acid	11.91	15.6
Proline	1.95	3.4
Glycine	4.49	13.3
Alanine	7.48	17.8
Half-cystine	0	0
Valine	6.80	11.6
Methionine	0.77	1.0
Isoleucine	3.88	5.8
Leucine	11.98	17.9
Tyrosine	2.22	2.3
Phenylalanine	5.74	6.6
Tryptophan	1.32	1.2

\* On the basis of methionine content, the formula weight was calculated as  $16900 \pm 500$ ; this value includes the molecular weight of one haem group.

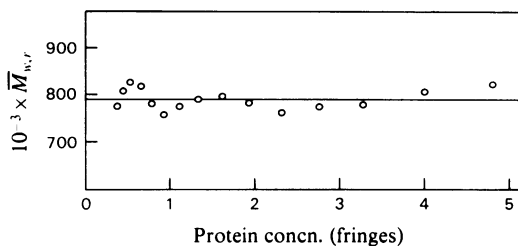


Fig. 2. *Weight-average molecular weight of Lepidurus haemoglobin as a function of protein concentration* Protein concentration was initially 1.0mg/ml in 0.1M-Tris/HCl, pH7.4. Conditions: speed, 7797 rev./min; temperature, 13°C.

(5.0–10.6). At acidic (<5.0) or alkaline (>10.6) pH, a decrease in  $s_{20,w}$  is observed. In the acidic range, the  $s_{20,w}$  levels off at pH2, reaching a limiting value of  $\approx 12S$ . At still lower pH, a tendency for reassociation is observed. A single boundary was obtained over the entire range of pH studied.

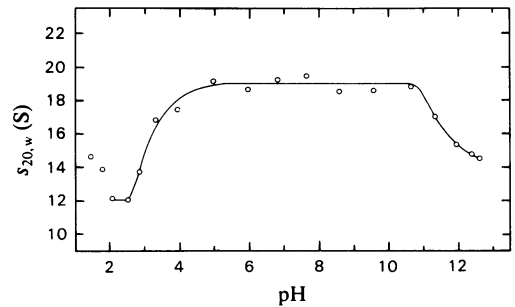


Fig. 3. *Dependence of sedimentation coefficient of Lepidurus haemoglobin on pH*

The protein concentration was 0.7mg/ml. Buffers, about 0.1M, containing 1mM-EDTA, were used in the pH ranges indicated: HCl/KCl (below 2.2); glycine/HCl (2.2–3.6); acetate (3.6–5.7); phosphate (5.7–8.0); glycine/NaOH (8.6–10.6); NaOH/Na<sub>2</sub>HPO<sub>4</sub> (above 10.6). Conditions: speed, 52000 rev./min; temperature, 20°C.

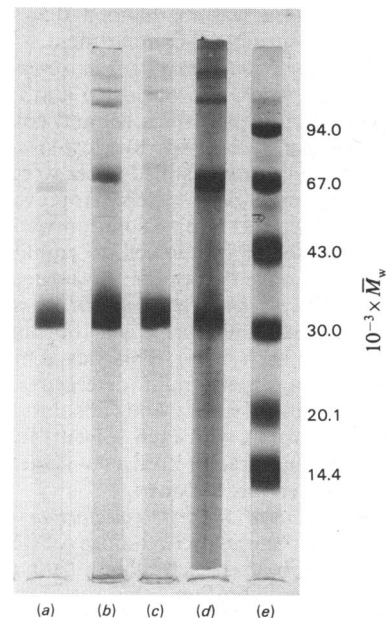


Fig. 4. *SDS/polyacrylamide-gel electrophoresis of Lepidurus haemoglobin*

Samples, prepared as described in the Materials and Methods section, contained: (a) 25  $\mu$ g of haemoglobin; (b) 50  $\mu$ g of haemoglobin; (c) haemolymph from live animal; (d) same as (a) except for omission of 2-mercaptoethanol; (e) mixture of protein markers.

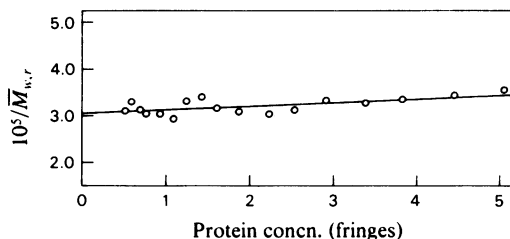


Fig. 5. Reciprocal weight-average molecular weight of *Lepidurus haemoglobin* in guanidinium chloride as a function of protein concentration

Protein concentration was initially 0.8mg/ml in 6M-guanidinium chloride and 0.1M-2-mercaptoethanol. Conditions: speed, 39778 rev./min.; temperature, 18.7°C.

#### Molecular weight of the polypeptide chain

Fig. 4 presents typical results of SDS/polyacrylamide-gel electrophoresis. The electrophoretic pattern of *Lepidurus haemoglobin* shows a single band with a mobility corresponding to a mol.wt. of 34000.

At relatively high protein loading ( $\sim 50 \mu\text{g/gel}$ ), additional faint bands of low mobility can be discerned. These correspond to mol.wts. of 68000 and about 100000, twice and thrice the molecular weight of the major-band component. The same pattern was obtained with haemolymph drawn from a live animal. Omission of 2-mercaptoethanol caused an increase in the relative intensity of the band corresponding to 68000 mol.wt.

The molecular weight of the polypeptide chain was determined by sedimentation equilibrium in solutions containing 6M-guanidinium chloride and 0.1M-2-mercaptoethanol. Fig. 5 presents a plot of the reciprocal of the weight-average molecular weight against concentration. A linear extrapolation to zero concentration gave  $\bar{M}_w = 32800$ . For this calculation a value for apparent specific volume ( $\phi'$ ) of 0.706ml/g, determined in 6M-guanidinium chloride, was used.

#### Discussion

The molecular weight of *Lepidurus haemoglobin* determined this study, 798000, reveals the notostracan crustaceans as the group with the highest molecular-weight haemoglobin among the arthropods. Nevertheless, it still remains true that arthropod haemoglobins are smaller in size than molluscan and annelid haemoglobins (Antonini & Chiancone, 1977).

The dependence of the sedimentation coefficient on pH indicates a relatively high stability of the native structure. The decrease in sedimentation coefficient observed at highly alkaline and acidic pH values indicates, however, that dissociation occurs under these conditions. The limiting sedimentation

coefficient attained at acidic pH,  $\sim 12S$ , suggests that the dissociation is of the 'whole-to-halves' type. Indeed, a value of  $19.3/1.50 = 12.9S$  is expected for the half-molecule, if one uses the theoretical value of  $1/1.50$  calculated for the ratio of the sedimentation coefficients of a spherical particle and its dimer (Van Holde, 1975).

Determination of the haem content, as well as the amino acid composition, indicates a minimal mol.wt. of 17000. A slightly higher value, 18900, is obtained from the iron content. SDS/polyacrylamide-gel electrophoresis under strongly dissociating conditions (heating at high temperature in the presence of a reducing agent) results in a single band with an estimated mol.wt. of 34000, twice the minimal molecular weight. One may thus conclude that *Lepidurus haemoglobin* is composed of identical, or nearly identical, polypeptide chains carrying two haem groups each. No satisfactory explanation is available for the finding (Fig. 4d) that a higher proportion of dimer is found in the absence of mercaptoethanol, taking into consideration that the protein appears to be devoid of cystine.

The exact molecular weight of the polypeptide chain was obtained by sedimentation equilibrium in 6M-guanidinium chloride and involved the determination of the isopotential apparent specific volume in this solvent. The measured value of  $\phi'$ , 0.706ml/g, is lower by 0.039ml/g than the partial specific volume ( $\bar{v}$ ) of the protein in water, 0.745ml/g. The difference in the two specific volumes found for *Lepidurus haemoglobin* is larger than usual for proteins, but by no means unprecedented, differences of 0.031 and 0.028ml/g having been reported for  $\beta$ -lactoglobulin (Lee & Timasheff, 1974) and haemocyanin of the mollusc *Busycon* (Quitter *et al.*, 1978) respectively. The results from sedimentation equilibrium in guanidinium chloride establish the value of 32800 as the molecular weight of the polypeptide chain in *Lepidurus haemoglobin*.

The present findings, summarized in Table 2, indicate a multi-chain structure for *Lepidurus haemoglobin*. The molecular weights of the native molecule and its constituent polypeptide chain have the ratio 24.3:1. Thus we may conclude that the

Table 2. Summary of molecular-weight data for *Lepidurus haemoglobin*

Molecular weight of native molecule	798000
Molecular weight of polypeptide chain	32800
Minimal molecular weight calculated from:	
Haem content	17400
Iron content	18900
Amino acid composition	16900
Number of haem groups per chain	2
Number of chains per molecule	24

molecule of *Lepidurus* haemoglobin is composed of 24 polypeptide chains.

The arrangement of the polypeptide chains in *Lepidurus* haemoglobin is not known. A clue to the possible structure is provided by the frictional ratio value ( $f/f_0$ ) of 1.50, calculated from the molecular weight and the sedimentation coefficient (Svedberg & Pedersen, 1940). This value excludes a compact spherical molecule, but is consistent with an asymmetrical particle (e.g. prolate or oblate ellipsoid of revolution with an axial ratio of about 10), or a spherical structure with a large fraction of void volume. Study of *Lepidurus* haemoglobin by electron microscopy could be of help in resolving its molecular structure.

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