Biochem. J. (1981) **193**, 353–359
Printed in Great Britain

Biophysical characterization of *Artemia salina* (L.) extracellular haemoglobins

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(*Received 8 May 1980/Accepted 1 September 1980*)

Sedimentation coefficients ($s^0_{20,w}$) of 11.57 ± 0.10S and 11.52 ± 0.09S were assigned for *Artemia salina* (L.) extracellular haemoglobins II and III respectively. These values are not significantly different. The molecular weights, M^0_w and M^0_z, of the native haemoglobins as determined by the high-speed sedimentation-equilibrium method were for haemoglobin II 239400 ± 7200 and 240400 ± 2600 respectively, and for haemoglobin III 216300 ± 6500 and 219300 ± 4500 respectively. The observed increase of $M_{app.}$ with concentration suggested that association was occurring over the concentration range investigated. Exposure of haemoglobin II to either 6 M-guanidinium chloride or to low pH (pH 4) resulted in dissociation to units of approximately half the size of the native protein, with molecular weights approx. 115000. Electron-microscopic observations indicated a molecular structure composed of two stacked lobed discs. These results strongly support the dimeric model for *Artemia* haemoglobins proposed by Moens & Kondo [(1978) *Eur. J. Biochem.* **82**, 65–72].

Haemoglobin molecules having molecular weights much higher than those of vertebrate haemoglobins have been characterized in several invertebrate animals, including annelids (Waxman, 1971; Shlom & Vinogradov, 1973; Wood *et al.*, 1976), molluscs (Waxman, 1971; Wood & Mosby, 1975) and crustaceans (Hoshi *et al.*, 1976; Moens & Kondo, 1977, 1978; Ilan & Daniel, 1979*a*,*b*). Annelid haemoglobin molecules are generally made up of a stack of two hexagonal plates, each being composed of six submultiples with a molecular weight of about 250000 (Levin, 1963; Yamagishi *et al.*, 1966; Waxman, 1971; Shlom & Vinogradov, 1973; Wood *et al.*, 1976). However, the stoicheiometry of subunit proteins is not clear and species with molecular weights ranging between 10000 and 30000 have been identified by SDS/polyacrylamide-gel electrophoresis (Waxman, 1971; Shlom & Vinogradov, 1973; Wood *et al.*, 1976).

Only few studies have been reported for molluscan haemoglobins, which also indicate a possible hexagonal structure (Wood & Mosby, 1975), composed of high-molecular-weight polypeptides of mol.wt. above 200000 (Waxman, 1975). Terwilliger *et al.* (1976) have shown a ring structure with tenfold symmetry consisting of ten polypeptides of mol.wt. 175000–200000 for the planorbid mollusc *Helisoma trivolvis*.

On the other hand, the haemoglobin from the carapaced crustacean *Cyzicus hierosolymitanus*, the clam shrimp, possibly has a pentagonal structure (David *et al.*, 1977), consisting of ten subunits with mol.wt. about 30000 (Ilan & Daniel, 1979*a*). The haemoglobins from other carapaced branchiopods, such as the tadpole shrimp (*Lepidurus apus*), and the water-flea (*Daphnia* sp.), seem to contain about 20 similar-sized subunits with mol.wt. between 31000 and 34000 (Ilan & Daniel, 1979*a*,*b*).

We have been studying the molecular structure of the extracellular haemoglobins from the carapaceless branchiopod *Artemia salina*. We have already presented biochemical evidence that all three *Artemia* haemoglobins (I, II and III) are constructed in a dimeric form, being composed of two similar-sized polypeptides of mol.wt. approx. 125000 (Moens & Kondo, 1977, 1978). In the present paper we are able to support our earlier

Abbreviation used: SDS, sodium dodecyl sulphate.

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0306-3275/81/010353-08$01.50/1

proposal on the structure of *Artemia* haemoglobins by physicochemical and electron-microscopic means.

Experimental

Preparation of Artemia haemoglobins

The encysted gastrulae of the brine shrimp were obtained from Metaframe (San Francisco Bay Brand, Newark, CA, U.S.A.) and cultivated to nauplius or young adult stages as described by Heip *et al.* (1978). Individual haemoglobins were purified by repeated chromatography on DEAE-Sephadex A-50 columns as described elsewhere (Moens & Kondo, 1978).

Electron microscopy

Adhesion of purified haemoglobin II molecules in aqueous solution to carbon-coated 300-mesh copper grids was completed within 30s. Carbon films were prepared by floating off a thin carbon layer, which was evaporated on freshly cleaned mica. Staining was for 30s with aq. 1% (w/v) uranyl acetate. Electron-microscopic observation was performed with a J.E.M. 100 B transmission electron microscope.

Dimensions of haemoglobin molecules were determined by using crystalline catalase as a standard. Image analysis was performed with a Kontron MOP-AM02 semi-automatic image analyser, and a number of molecules was classified by their shape factor.

Analytical ultracentrifugation

Sedimentation-velocity and sedimentation-equilibrium experiments were performed in a Beckman model E analytical ultracentrifuge fitted with a rotor-temperature-indicating and control system. All experiments performed were with an An-H rotor (titanium). Sedimentation-velocity experiments were performed in single-sector cells (12mm), with the schlieren optical system. Protein samples were dialysed against 50mM-Tris/HCl buffer, pH 7.5, containing 100mM-NaCl for at least 24h before use. Photographs were taken of the schlieren trace at 4min intervals, and the plates were measured for calculation of sedimentation coefficients with a projectorscope (Precision Grinding Ltd.). Runs were performed at a number of protein concentrations in the range 0.5–2.0mg/ml at temperatures near 20°C. Values for $s_{20,w}$ were calculated in the usual way (Svedberg & Pedersen, 1940), and values of $s^0_{20,w}$ were obtained by linear regression analysis.

Sedimentation-equilibrium experiments were performed by the meniscus-depletion method of Yphantis (1964), with six-sector centre-pieces in the cells, and both interference and schlieren optical systems. Photographs were taken after 24h of centrifugation, and the run was continued until equilibrium was attained. Rotor speed was calculated from the odometer reading. Plates were measured in the projectorscope. Molecular weights were calculated for interference measurements by using:

$$\overline{M}_w = \frac{2RT}{(1-\overline{v}_\rho)\,\omega^2} \cdot \frac{\mathrm{d}(\ln \Delta y)}{\mathrm{d}r^2}$$

where Δy is the fringe shift compared with the meniscus position (i.e. $y-y_0$) and r is the radial position in the cell, and for schlieren measurements by using the logarithmic schlieren plot (Lamm plot):

$$\overline{M}_z = \frac{2RT}{(1-\overline{v}_\rho)\,\omega^2} \cdot \frac{\mathrm{d}\left[\ln\left(\frac{1}{r}\cdot\frac{\mathrm{d}y}{\mathrm{d}r}\right)\right]}{\mathrm{d}r^2}$$

Experiments were conducted at a number of protein concentrations, and the molecular weight at infinite dilution was obtained by linear-regression analysis of plots of $1/M$ versus concentration.

SDS/polyacrylamide-gel electrophoresis

Protein samples were denatured by heating at 95°C for 3min in 5mM-Tris/glycine buffer, pH 7.5, containing 1% (w/v) SDS, 1% (w/v) 2-mercaptoethanol and 0.01% Bromophenol Blue and analysed by electrophoresis in 7.5% slab gels with a current of 25mA at room temperature (Kamen *et al.*, 1972). The molecular-weight-reference proteins used were Combitek (Boehringer, Mannheim, Germany) and the HMW Calibration Kit (Pharmacia Fine Chemicals, Uppsala, Sweden).

Cross-linking with imido-esters

Haemoglobin samples (0.25mg/ml) were cross-linked with dimethyl suberimidate (6mg/ml) in 0.02M-triethanolamine/HCl buffer, pH 8.3, for 20min at room temperature as described by Young & Blumenthal (1975). The reaction was stopped by the addition of 1% SDS and 4% 2-mercaptoethanol and heating at 90°C for 5min.

Reversible cross-linking was done by incubating haemoglobin samples (0.25mg/ml) in 0.2M-triethanolamine/HCl buffer, pH 8.3, containing 0.4M-2-mercaptoethanol and 30mM-methyl 4-mercaptobutyrimidate at 0°C for 20min. After dialysis against 0.2M-triethanolamine/HCl buffer, pH 8.3, samples were oxidized with 5mM-H_2O_2 for 30min at room temperature. The reaction mixture was then made 1% with respect to SDS and 4% with respect to 2-mercaptoethanol and heated at 90°C for 5min. SDS/polyacrylamide-gel electrophoresis was performed as described above.

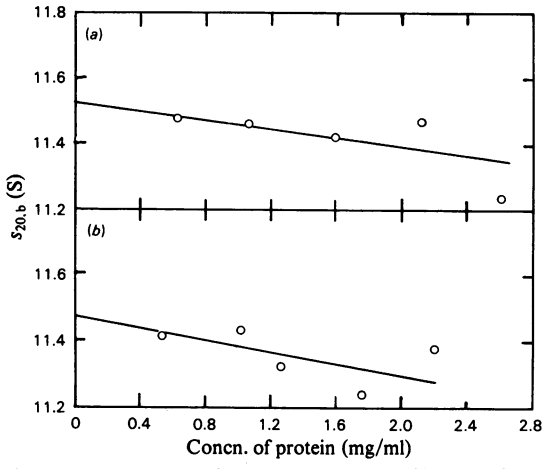


Fig. 1. *Dependence of sedimentation coefficient of Artemia haemoglobins on protein concentration*
The haemoglobin was dissolved in 50mM-Tris/HCl buffer, pH 7.5, containing 100mM-NaCl. Sedimentation was performed at 52640rev./min and 20°C. The values of $s^0_{20,b}$ in this buffer were 11.53 ± 0.09S (*a*, haemoglobin II) and 11.48 ± 0.09S (*b*, haemoglobin III) respectively. The extrapolation to zero concentration was performed by linear regression.

Results

Molecular weights of native Artemia haemoglobins

The sedimentation coefficients ($s^0_{20,w}$) of *Artemia* haemoglobins II and III, purified as described by Moens & Kondo (1978) and dissolved in 50mM-Tris/HCl buffer, pH 7.5, containing 100mM-NaCl, were 11.57 ± 0.10S and 11.52 ± 0.09S respectively (Fig. 1). These values are not significantly different by Student's *t* test, and the correlation between sedimentation coefficient and concentration was very poor (r^2, the coefficient of correlation, <0.6). Although the dependence of $s_{20,w}$ on sugar concentration appears to be consistent with what would be expected for the sedimentation of non-interacting spheres, the statistical tests applied show that this conclusion is not justified.

The molecular weights were determined by sedimentation-equilibrium experiments (Yphantis, 1964). The homogeneity of the haemoglobin samples investigated was indicated by the fact that straight-line plots of the natural logarithm of the fringe displacement ($\ln \Delta y$) versus the square of the radial distance (r^2) were obtained for haemoglobin II (Fig. 2*a*) and haemoglobin III (Fig. 2*b*). Fig. 3 presents

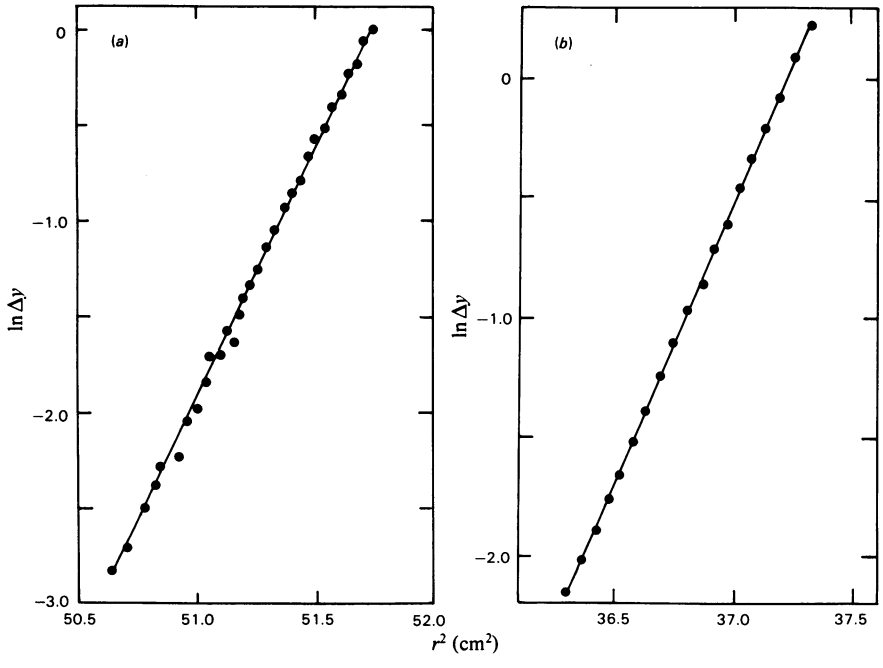


Fig. 2. *Sedimentation-equilibrium experiment with Artemia haemoglobins*
Initial protein concentrations of haemoglobin II (*a*) and haemoglobin III (*b*) were 0.4mg/ml and 0.71mg/ml in 50mM-Tris/HCl buffer, pH 7.5, containing 100mM-NaCl. Conditions of run were 13000rev./min and 19.8°C. Interference photograph was taken after 24h of centrifugation. Molecular weights ($\overline{M}_{w,app.}$) of haemoglobins II (*a*) and III (*b*) were 257900 and 228900 respectively.

plots of the reciprocal of the weight-average molecular weight calculated by using a partial specific volume of 0.7272 cm³/g, obtained from the amino acid composition (Moens & Kondo, 1978), versus concentration. Extrapolation to zero concentration gave M_w^0 values of 239400 ± 4900 and 216300 ± 1700 for haemoglobins II and III respectively. Lamm plots of $\ln \Delta y/r$ versus r^2 were used to calculate z-average molecular weights, and extrapolation to zero concentration gave the M_z^0 values of 240400 ± 2600 and 219300 ± 4500 for haemoglobins II and III respectively.

The values for M^0 for haemoglobins II and III were significantly different ($P > 5\%$ by Student's t test), implying that haemoglobin II is larger than haemoglobin III by some 20000 daltons. However, this conclusion is not necessarily justified, as it will be noted from Fig. 3 that in each case the molecular weight increased with initial protein concentration. Thus it is highly likely that dissociation of the dimeric native molecule occurs towards infinite dilution. Since these were meniscus-depletion experiments the concentration in the ultracentrifuge cell at equilibrium at the meniscus region is zero or very close to zero. There is no reason to suppose, however, that the dissociation constant for haemoglobin II is the same as that for haemoglobin III, and therefore at any given protein concentration the extents of dissociation may be different, yielding different values for $M_{app.}$ for haemoglobins II and III. Much more work, including experiments at higher protein concentrations, would be needed to investigate this effect.

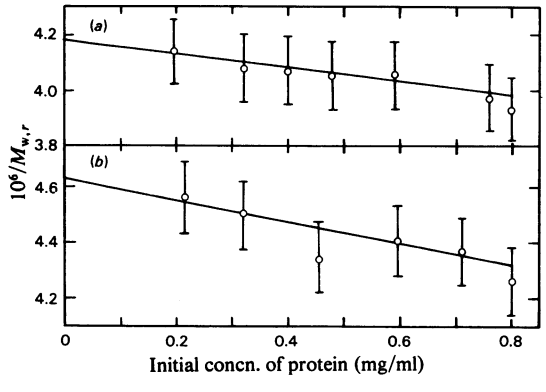


Fig. 3. *Reciprocal of the weight-average molecular weight of Artemia haemoglobins as a function of protein concentration*

Molecular weights at zero concentration (M_w^0) of haemoglobin II (*a*) and haemoglobin III (*b*) were 239400 ± 7200 and 216300 ± 6500 respectively. The extrapolations were performed by linear regression.

Molecular weight of dissociated Artemia haemoglobin II

Artemia haemoglobin II was dissociated by dialysis against either 50mM-Tris/HCl buffer, pH 7.5, containing 6M-guanidinium chloride and 20mM-2-mercaptoethanol or 100mM-sodium acetate buffer (pH 4). The molecular weight of the dissociated subunits was determined by sedimentation equilibrium. The plots of $\ln \Delta y$ versus r^2 (not shown) were reasonably linear in both conditions, suggesting that dissociated polypeptide chains of haemoglobin II were uniform in molecular size. The estimated molecular weights ($\overline{M}_w$) were 113300 and 115000 for denaturant- and low-pH-treated haemoglobin II preparations respectively (Table 1), indicating that dissociation into half-molecules had occurred. The sedimentation coefficient of this haemoglobin was concomitantly decreased to 6.17S at low pH (not shown).

From studies by SDS/polyacrylamide-gel electrophoresis as well as by gel filtration in the presence of guanidinium chloride (Moens & Kondo, 1978), the dissociated subunits of *Artemia* haemoglobin II (and also haemoglobins I and III) were homogeneous, having a molecular weight of 122000–126000. The molecular weight of native *Artemia* haemoglobins was estimated by gel filtration and sucrose-density-gradient centrifugation to be twice of that of subunit chains (Moens & Kondo, 1976, 1977, 1978). The present results obtained by analytical-ultracentrifugation experiments are in excellent agreement with these results.

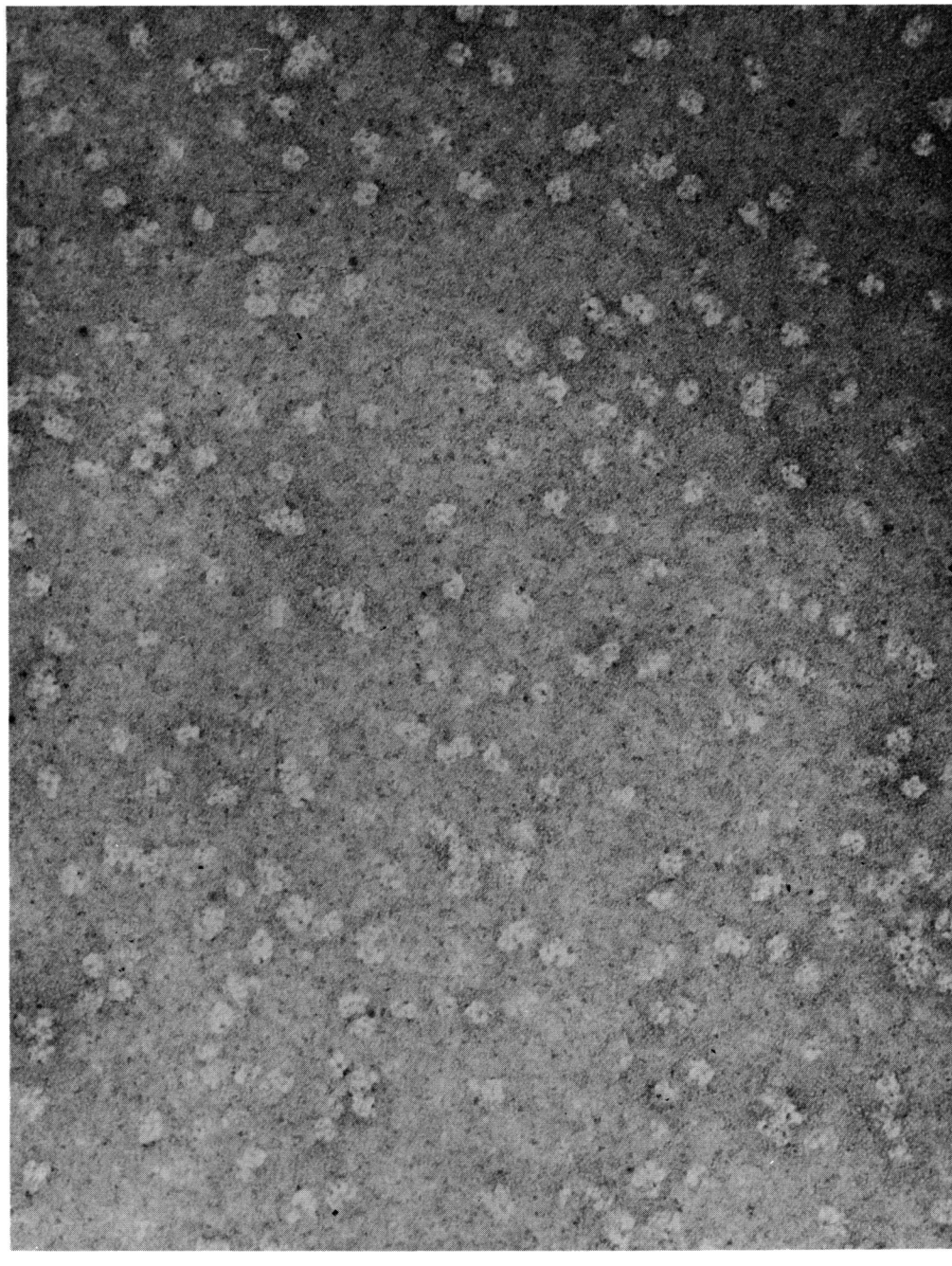
Cross-linking of Artemia haemoglobins

The molecular weights of cross-linked haemoglobins estimated by SDS/polyacrylamide-gel electrophoresis are summarized in Table 2. The

Table 1. *Determination of the molecular weight ($\overline{M}_{w,app.}$) of dissociated Artemia haemoglobin II by sedimentation equilibrium*

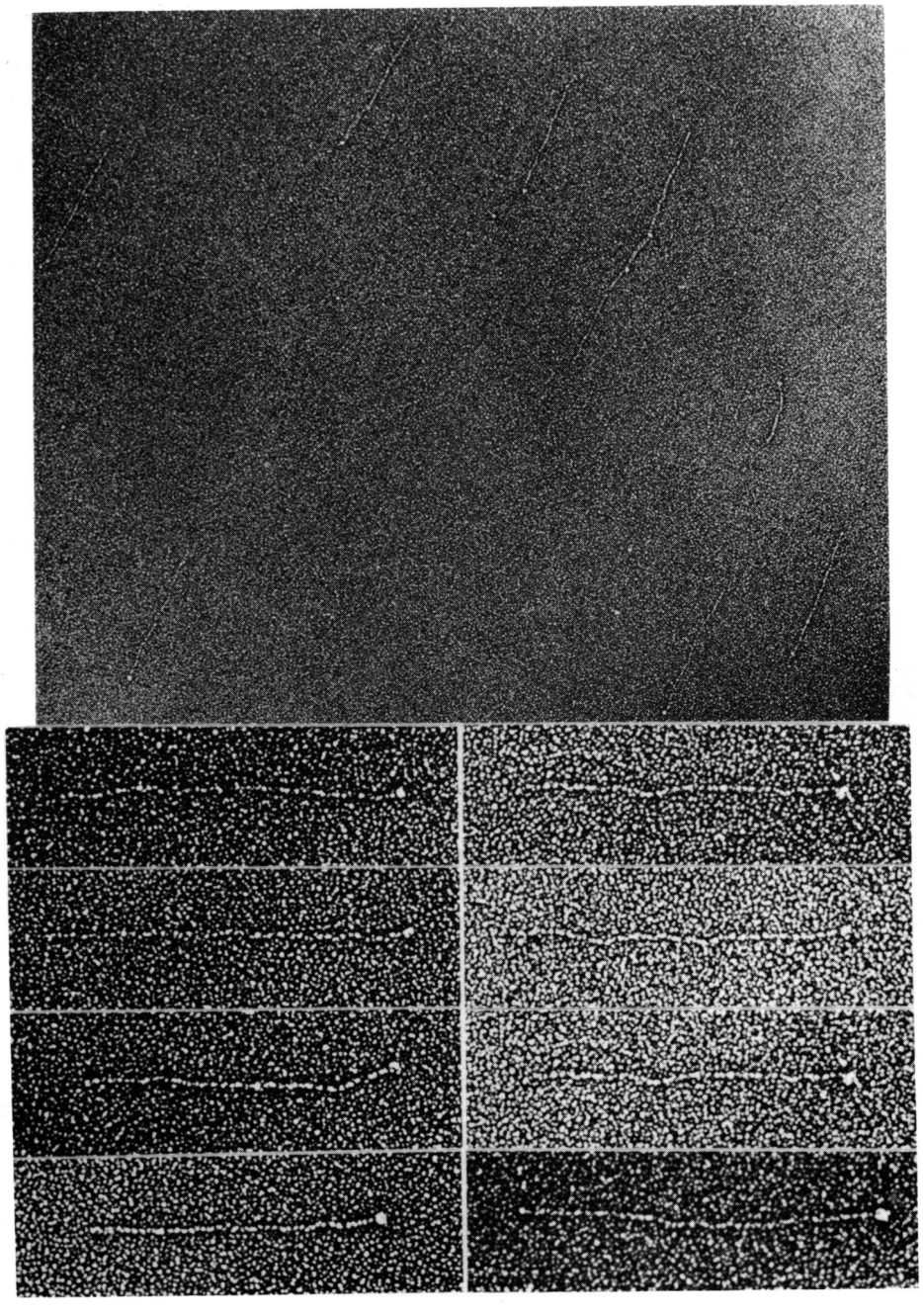
Haemoglobin II was dissociated by dialysis against either 6M-guanidinium chloride in 50mM-Tris/HCl buffer, pH 7.5, containing 100mM-NaCl and 20mM-2-mercaptoethanol or 100mM-sodium acetate buffer, pH 4. Conditions of run were 24630rev./min and 18.9°C.

Initial concn. of protein (mg/ml)	Dissociation by	$\overline{M}_{w,app.}$
0.40	Guanidinium chloride	113300
0.23	Low pH	120800
0.34	Low pH	113900
0.45	Low pH	110400
	Average ...	115000



EXPLANATION OF PLATE 1

Electron micrograph of negatively stained Artemia haemoglobin II
Stain: uranyl acetate. Magnification × 280000.



EXPLANATION OF PLATE 2

Electron micrograph of epiglycanin complexed with concanavalin A
Magnification 38000×. Insets are selected complexes at 90000×.

Table 2. *Molecular weights of cross-linked Artemia haemoglobins*

The 7.5% gels were calibrated with marker proteins of known molecular weight: trypsin inhibitor (20100), lactate dehydrogenase (36000), catalase (60000), serum albumin (68000), ferritin (220000) and thyroglobulin (330000). For details see the text. Each value was the mean of at least four determinations. Abbreviation: N.D., not determined.

		Mol.wt. of haemoglobin cross-linked by		
			Methyl 4-mercaptobutyrimidate	
Haemoglobin	Subunit	Dimethyl suberimidate	Oxidized	Reduced
I	Monomer	135000	N.D.	N.D.
	Dimer	297000	N.D.	N.D.
	Tetramer	631000	N.D.	N.D.
II	Monomer	147000	135000	135000
	Dimer	287000	226000	—
	Tetramer	627000	562000	—
III	Monomer	141000	138000	138000
	Dimer	292000	229000	—
	Tetramer	631000	562000	—

apparent molecular weight of the monomer subunit was higher by 10000–20000 daltons after the reaction, but this probably is an artifact caused by conformational change. The quantity of tetramers was dependent on haemoglobin concentrations in the reaction mixture, indicating the formation of intramolecular cross-linking. Reduction by 2-mercaptoethanol of haemoglobins II and III that had previously been cross-linked with a reversible cross-linker, methyl 4-mercaptobutyrimidate, gave rise to only one polypeptide chain of 135000–138000 daltons (Table 2). No smaller polypeptides were observed, and this is strong evidence against the concept that *Artemia haemoglobins* are composed of 12 subunits of 17000–19000 daltons (Bowen *et al.*, 1976).

Electron microscopy of Artemia haemoglobin II

Haemoglobin II was negatively stained with 1% uranyl acetate on carbon-coated copper grids. Although the observed morphology of *Artemia* haemoglobin II molecules was not clear-cut (Plate 1), we were able to identify two distinct morphological features that occurred consistently in electron micrographs. One was a relatively circular lobed structure with a diameter of 12 ± 1 nm, and the other was a rectangular one with dimensions 7 ± 1 nm $\times$ 12 ± 1 nm, composed of two equal parts divided by a dark line. We interpret these structures in terms of the former being a top view of the haemoglobin molecule, the latter being a side view of two stacked lobed discs. It appears likely that each disc represents a morphological unit for one haemoglobin subunit.

Discussion

The results of analytical-ultracentrifuge studies shown in the present paper confirm the dimeric model of the *Artemia* haemoglobin molecule proposed on the basis of biochemical data (Moens & Kondo, 1978), namely each haemoglobin is made up of two similar-sized globin subunits with a mol.wt. of 125000. Both cross-linking experiments and electron-microscopic observations seem to support this model.

There was some evidence from the analytical-ultracentrifuge data of the existence of an association–dissociation processes. Since the two subunits in the proposed dimeric native haemoglobins are not linked covalently, one would expect that at comparatively low concentrations a significant proportion of monomers would be present. As the experiments were of the meniscus-depletion type, there will indeed be regions in the ultracentrifuge cell of zero solute concentration, and therefore some dissociation must occur. It seems likely that the association between the monomer units in the dimer is quite strong, as the degree of dissociation, even at these low concentrations, was apparently small. Since the haemoglobins exist in free solution in the haemolymph of *Artemia* and since at some stages in the life cycle the haemoglobin concentration will be extremely low, dissociation of the haemoglobin may well be disadvantageous to the animal. On this basis one would expect the monomers to bind together tightly to form the dimer, and furthermore it may be more appropriate to take the molecular weight of the native molecule to be higher than the extrapolated apparent M^0 value, i.e. in the region 240000–250000 rather than 220000–240000. Experiments at higher protein concentrations would help to resolve this problem.

Thus the molecular weight of native *Artemia* haemoglobin II is 240000–250000 from our present and previous data. Manwell (1977*a*) developed a

modified electrophoretic technique to determine molecular weights of various proteins, where a value of a hypothetical limiting polyacrylamide-gel pore size ($1/G_L$, the reciprocal of the gel concentration at which the migration of a protein molecule becomes limited) was experimentally determined. The $1/G_L$ value is then used to calculate a molecular weight by an empirical equation:

$$1/G_L = -3.034 + 0.1732\,(\text{mol.wt.})^{\frac{1}{2}}$$

The experimentally obtained limiting gel-pore size value of 7.90 for *Artemia* haemoglobin (Manwell, 1977*a*) gave the molecular weight of 250000. This value is in excellent agreement with our estimate.

Bucks & Bowen (1979) proposed a molecular weight of 306000 for three *Artemia* haemoglobins by using another empirical equation:

$$K_R = 4.08 + [(\text{mol.wt.}) \times 4.6 \times 10^{-5}]$$

where K_R is a retardation coefficient estimated by the negative slope of a Ferguson plot. This discrepancy might be due to the glycoprotein nature of *Artemia* haemoglobins (Moens & Kondo, 1976). It was, however, claimed that no significant difference was found between proteins and glycoproteins by Manwell's method, which employed an exponential-gradient gel (Manwell, 1977*a*).

Besides *Artemia* haemoglobins, to date, only two examples have been reported for the molecular weight of extracellular haemoglobins from carapaceless branchiopods (Manwell, 1977*b*; Ilan & Daniel, 1979*a*). The molecular weight of another brine shrimp (*Parartermia zietziana*) haemoglobin was estimated by electrophoretic retardation (Manwell, 1977*a*) to be 230000–250000 (Manwell, 1977*b*). The haemoglobin of the fresh-water fairy shrimp (*Streptocephalus torvicornis*) has a sedimentation coefficient of 11.4S by analytical ultracentrifugation and contains two similar-sized polypeptide chains of mol.wt. 100000–120000 by SDS/polyacrylamide-gel electrophoresis (Ilan & Daniel, 1979*a*). It appears likely that the *Streptocephalus* haemoglobin has an analogous molecular structure to that of *Artemia salina* haemoglobin II, but this point remains to be investigated further.

The haemoglobins of carapaced branchiopods on the other hand display more diversity in their molecular weight, which range from 280000 to 798000 (Hoshi & Kobayashi, 1971; Hoshi *et al.*, 1976; David *et al.*, 1977; Ilan & Daniel, 1979*a*,*b*). In contrast with *Artemia* haemoglobins, these haemoglobins are apparently constructed from 10–24 identical subunits of mol.wt. approx. 32000 (Ilan & Daniel, 1979*a*). Electron-microscopic studies on *Cyzicus* haemoglobin with mol.wt. of 280000 revealed a possible pentagonal structure with dimensions 13 ± 1 nm (diameter) and 8.5 ± 0.5 nm (height) (David *et al.*, 1977). Like *Artemia* haemoglobin, the *Cyzicus* haemoglobin also seems to be composed of two stacked discs. Since the molecular sizes of both haemoglobins are much smaller than those of annelid ones, it is difficult to attempt a more detailed description of the molecular architecture of crustacean haemoglobins at present.

Recently we have been able to isolate haem-containing functional units from the *Artemia* subunit chains by limited trypsinolysis (Heyligen *et al.*, 1980). The basic unit has an apparent molecular weight of 14000–16000 by SDS/polyacrylamide-gel electrophoresis (Heip *et al.*, 1980), corresponding to a minimal molecular weight of 17200 per haem-containing subunit (Moens & Kondo, 1977). Tryptic peptide 'mapping' of native haemoglobin as well as of isolated subunit molecules indicated that these basic functional units constituting the globin subunits are almost identical with one another (Heyligen *et al.*, 1980). A similar finding of haem-containing domains was also reported for *Helisoma trivolvis* haemoglobin (Terwilliger *et al.*, 1976).

The authors thank H. Heyligen, R. Hertsens, M.-J. Pollenus and G. Houben for their participitation in certain aspects of these experiments, and M.-L. Van Hauwaert for her technical assistance. L. M. and J. H. are an established investigator and a postdoctoral associate of the Belgian National Science Foundation respectively. This work was supported by grants 'Fonds voor Kollektief Fundamenteel Onderzoek' (no. 2.0021.75) and 'Fonds voor Geneeskundig Wetenschappelijk Onderzoek' (no. 3.0031.77).

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