

Erythrocrucorin from the water-flea *Daphnia magna*

Quaternary structure and arrangement of subunits

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The subunit structure of erythrocrucorin from the cladoceran *Daphnia magna* was studied. The native protein was found to have a sedimentation coefficient ($s_{20,w}^0$) of $17.9 \pm 0.2S$ and a molecular weight, as determined by sedimentation equilibrium, of $494\,000 \pm 33\,000$. Iron and haem determinations gave $0.312 \pm 0.011\%$ and $3.84 \pm 0.04\%$, corresponding to minimal molecular weights of $17\,900 \pm 600$ and $16\,100 \pm 200$ respectively. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis gave one band with mobility corresponding to a molecular weight of $31\,000 \pm 1500$. The molecular weight of the polypeptide chain determined by sedimentation equilibrium in 6M-guanidinium chloride and 0.1M-2-mercaptoethanol is $31\,100 \pm 1300$. On a molecular-weight basis, *Daphnia* erythrocrucorin is composed of 16 identical polypeptide chains carrying two haem groups each. The native structure is stable between pH 5 and 8.5. At alkaline and acidic pH, a gradual decrease in the sedimentation coefficient down to 9.8S occurs. Above pH 10 and below pH 4, a slow component with $s_{20,w}$ between 2.7S and 4.0S is observed. The 2.7S, 4.0S and 9.8S species are identified as single-chain subunits, subunit dimers and half-molecules respectively. We propose a model for the molecule composed of 16 2.7S subunits grouped in two layers stacked in an eclipsed orientation, the eight subunits of each layer occupying the vertices of a regular eight-sided polygon. Support for this arrangement is provided from electron microscopy and from analysis of the pH-dissociation pattern.

Erythrocrucorins are high-molecular-weight haem containing respiratory proteins found in the haemolymph of many species from the Phyla Annelida, Mollusca and Arthropoda. In the arthropods, their occurrence is mainly in few species of insect larvae and in the four Orders of the Subclass Branchiopoda: Anostraca, Notostraca, Cladocera and Conchostraca (Fox, 1957; Redmond, 1971). Erythrocrucorin from the water-flea *Daphnia pulex*, a cladoceran, was one of the first erythrocrucorins studied by Svedberg and co-workers after the development of the analytical ultracentrifuge (Svedberg & Eriksson-Quensel, 1934). Almost 40 years elapsed, however, before the next structural studies of a daphnid erythrocrucorin were undertaken (Sugano & Hoshi, 1971; Dangott & Terwilliger, 1980). The cladocerans are widespread, and the reason for the scarcity of structural studies on cladoceran erythrocrucorin is to be attributed to the difficulties of isolation of a pure protein from these small animals. The present paper reports the results of a physicochemical study of purified erythrocrucorin from *Daphnia magna*.

Materials and methods

Preparation of erythrocrucorin

Water-fleas identified as *Daphnia magna* were brought from a natural pond in the vicinity of Tel-Aviv and reared in 150-litre tanks, close to the laboratory. Filtered air was bubbled in continuously and nutrients were added daily. When the *Daphnia* population was judged to be sufficiently dense, aeration was stopped. A few days later the animals developed a pink colour, indicating that the haemolymph had become rich in erythrocrucorin. The small size of *Daphnia magna* (approx. 5 mm at most) makes it impracticable to collect haemolymph directly from the heart. As a starting material for the preparation of erythrocrucorin, use was made of the crude extract obtained by squeezing. The preparation of erythrocrucorin from the extract involved high-speed ultracentrifugation and ion-exchange chromatography. Following is the protocol of a typical preparation.

Pink animals, about 50g wet weight, were transferred to a small volume of water in a 2.5-litre

beaker. Contaminating worms, mosquito larvae, ostracoda etc. were removed with a pair of forceps. The *Daphnia* were then transferred to a fine-mesh nylon net and repeatedly rinsed with distilled water. The animals were introduced into 10 ml disposable plastic syringes that had their needles removed. Gentle pressure was applied to the piston, and the fluid that came out was passed through cotton gauze into a test tube that contained about 1 ml of extraction buffer (50 mM-NaCl/20 mM-Tris/HCl buffer, pH 7.70) containing 1 mM-phenylmethanesulphonyl fluoride, a proteinase inhibitor. The solution was centrifuged twice at low speed to remove particulate matter (tissue debris, carapace fragments etc.). The clear supernatant was centrifuged at 232 000 g (r_{av} , 5.7 cm) for 4.5 h. The precipitate was dissolved in the extraction buffer and centrifuged again. The resulting red pellet was redissolved in 0.2 ml of the buffer, and the solution was applied to a DEAE-Sephadex column (3 cm × 15 cm) equilibrated with the extraction buffer. Elution was performed with 200 mM-NaCl/45 mM-Tris/HCl buffer, pH 7.70. The coloured fractions containing erythrocrucorin were centrifuged at 232 000 g and the supernatant was discarded. The red precipitate was dissolved in extraction buffer and subjected to a second passage through DEAE-Sephadex under the same conditions as before. The red fraction that came out contained pure erythrocrucorin. Centrifugation at 232 000 g and dissolution of the pellet in about 0.1 ml of 0.1 M-sodium phosphate buffer, pH 6.8, gave a concentrated (approx. 50 mg/ml) stock solution of purified erythrocrucorin ($A_{416}/A_{280} = 2.8$). All work was done at 4°C.

Concentration determinations

Erythrocrucorin concentrations were measured by absorption spectroscopy with a Cary 118 spectrophotometer. Absorption coefficients were determined as described elsewhere (Ilan & Daniel, 1979a). At 280 nm, a value for $A_{1\text{cm}}^{1\%}$ of 19.6 litre · g⁻¹ · cm⁻¹ in 0.1 M-phosphate buffer, pH 6.8, was obtained.

Haem and iron determinations

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

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

This was performed on 7.5% polyacrylamide gels as described by Weber *et al.* (1972), in the presence of 1% (w/v) sodium dodecyl sulphate and 1% (v/v) 2-mercaptoethanol. Molecular weights corresponding to band mobilities were estimated from a

calibration curve obtained with protein markers of known molecular weight.

Ultracentrifugation

Ultracentrifugation was performed with a Beckman model E ultracentrifuge. Sedimentation-velocity studies were performed at 20°C or at a temperature close to it, with schlieren phase-plate or absorption scanning optics. Sedimentation coefficients were corrected to $s_{20,w}$ in the usual way (Svedberg & Pedersen, 1940). Sedimentation-equilibrium studies were performed by using the short-column meniscus-depletion technique (Yphantis, 1964), with interference optics. The fringe pattern was read at 0.1 mm intervals on photographs taken immediately on reaching speed and after attainment of equilibrium. Local slopes of the plot of $\ln c$ versus r^2 , $d(\ln c)/dr^2$, c being the concentration and r the distance from the axis of rotation, were determined by least-squares fitting of the baseline-corrected concentration distribution at equilibrium and used to calculate the weight-average molecular weight at r , $\bar{M}_{w,r}$, according to the relation:

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

where R is the gas constant, T is the absolute temperature, ω is the angular velocity, \bar{v} is the partial specific volume and ρ is the density of the solution. For the calculation of the molecular weight in concentrated guanidinium chloride solution, \bar{v} in the expression for $\bar{M}_{w,r}$ has to be replaced by ϕ' , the apparent specific volume of the protein in dialysis equilibrium with the solvent (Casassa & Eisenberg, 1964). The value of \bar{v} in water was determined experimentally from density measurements made with a Digital Densimeter DMA-02 (Anton Paar K.G., Graz, Austria). The value of ϕ' in guanidinium chloride solution was calculated as described by Lee & Timasheff (1979).

Electron microscopy

Negative staining was done with 1% uranyl acetate. Observation and photography were made with a JEOL-JEM 100B electron microscope. Contrast enhancement was performed by the procedure of Markham *et al.* (1963).

Results

Characterization of *Daphnia erythrocrucorin*

Erythrocrucorin prepared by the method described gave a single symmetrical peak on sedimentation in the ultracentrifuge. The sedimentation coefficient was determined over the concentration range $c = 0.5$ – 5.0 mg/ml. The results were found to fit the relation $s_{20,w} = s_{20,w}^0(1 - Kc)$, where $K = 0.0145$ ml/mg and $s_{20,w}^0 = 17.9 \pm 0.2$ S. Purified *Daphnia*

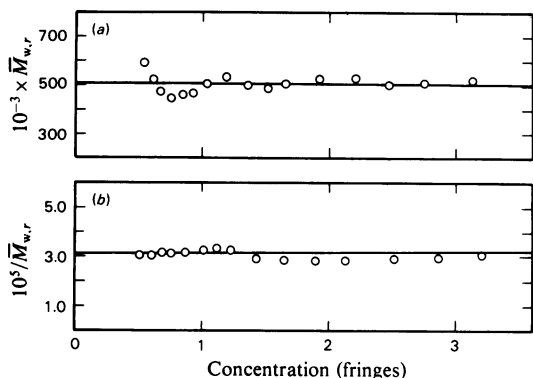


Fig. 1. Sedimentation equilibrium of *Daphnia erythrocrucorin*

(a) Weight-average molecular weight of native erythrocrucorin as a function of protein concentration. Protein concentration was initially 1mg/ml in 0.1M-phosphate buffer, pH 6.7. Conditions: speed, 8103 rev./min; temperature 20.4°C. (b) Reciprocal weight-average molecular weight of erythrocrucorin in guanidinium chloride as a function of protein concentration. Protein concentration was initially 1mg/ml in 6M-guanidinium chloride and 0.1M-2-mercaptoethanol. Conditions: speed, 40 422 rev./min; temperature, 20.8°C. For further experimental details see the text.

erythrocrucorin showed a typical oxyhaemoglobin absorption spectrum, with peaks at 345, 416 (Soret), 537 and 573 nm. The protein peak occurs at 280 nm. Determination of the iron and haem content gave $0.312 \pm 0.011\%$ and $3.84 \pm 0.04\%$ (w/w), values corresponding to minimal molecular weights of 17900 ± 600 and 16100 ± 200 respectively. A value $\bar{v} = 0.749$ ml/g was measured for the partial specific volume in water at 20°C.

The molecular weight was obtained by sedimentation equilibrium. Point-by-point weight-average molecular weights were found to be independent of protein concentration. Two experiments gave (\pm s.d.) $\bar{M}_w = 505\,000 \pm 35\,000$ (Fig. 1a) and $483\,000 \pm 27\,000$. Taken together, the data from the two experiments give for the molecular weight of *Daphnia erythrocrucorin* $\bar{M}_w = 494\,000 \pm 33\,000$.

Dependence of the sedimentation coefficient on pH

Fig. 2 shows the effect of pH on the sedimentation coefficient. At pH values in the range 5.0–8.5 a single peak, 17.1S, corresponding to the native molecule is observed. Outside the stability range, at more acidic and at more alkaline pH values, a decrease in the sedimentation coefficient occurs. On the alkaline side, the decrease in sedimentation coefficient is gradual. At about pH 10, an additional slow boundary, approx. 3.5S, is observed. As the

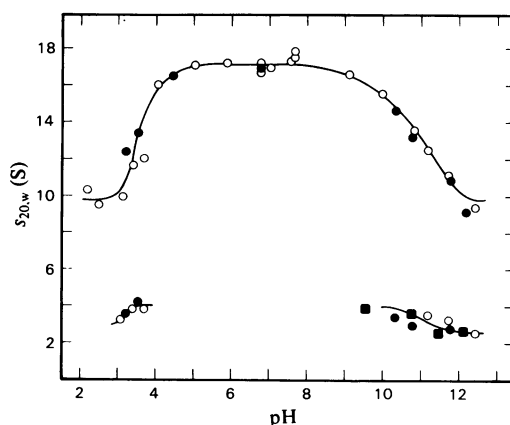


Fig. 2. Dependence of sedimentation coefficient of *Daphnia erythrocrucorin* on pH

○, Protein concentration 1–2 mg/ml, follow-up by schlieren optics; ●, protein concentration 0.2 mg/ml, follow-up by absorption optics. Buffers (0.1M) used in the pH ranges indicated were: glycine/HCl (pH 2.2–3.6); acetate (pH 3.6–5.7); phosphate (pH 5.7–7.3); Tris/HCl (pH 7.3–9.0); glycine/NaOH (pH 9.0–11.8); NaOH was used for pH above 11.8. For further experimental details see the text. Also shown (■) are four determinations on *Daphnia pulex* erythrocrucorin taken from Svedberg & Eriksson-Quensel (1934).

pH is made more alkaline, there is an increase in the relative abundance of the slower-sedimenting material and a concomitant decrease in its sedimentation coefficient. At pH 12 the slower-sedimenting component is the principal species and its sedimentation coefficient has levelled off to 2.7S. At the acidic side of the stability range, the pH profile of the sedimentation coefficient resembles that at the alkaline side. As the pH is decreased below 5.0, there is a decrease in the sedimentation coefficient and, at about pH 4, an appearance of a slower-sedimenting boundary, $s_{20,w} = 4.0$ S. As the pH is made still more acid, there is a gradual decrease in the sedimentation coefficient of the slower-sedimenting material. The $s_{20,w}$ of the fast-sedimenting component levels off at about pH 2, reaching a limiting value of 9.8S.

Characterization of the polypeptide chain

Fig. 3 presents typical results of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Virtually all the protein migrates in two bands, a major band with a mobility corresponding to a molecular weight (\pm s.d.) of $31\,000 \pm 1\,500$ (three determinations), and a minor band with a mobility corresponding to a molecular weight of 63 000, twice the molecular weight of the major band component.

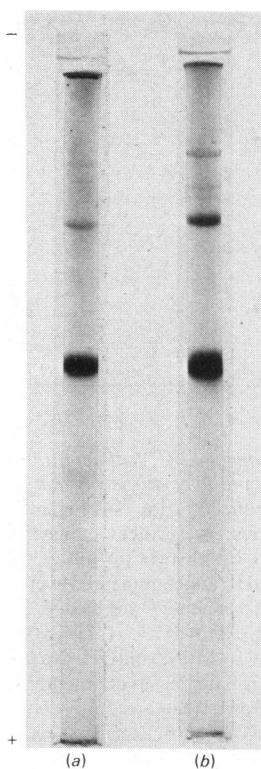


Fig. 3. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of *Daphnia erythrocrucorin*. Samples contained 30 μg (a) and 50 μg (b) of protein. For experimental details see the text.

High protein loading caused an increase in the relative intensity of the band corresponding to 63 000 molecular weight.

The molecular weight of the polypeptide chain was determined by sedimentation equilibrium in solution containing 6M-guanidinium chloride and 0.1M-2-mercaptoethanol. A linear extrapolation to zero concentration of a plot of the reciprocal of the weight-average molecular weight against concentration (Fig. 1b) gave (\pm S.D.) $\bar{M}_w = 31100 \pm 1300$. For this calculation, a value for the specific volume $\phi' = 0.730$ ml/g was used, lower by 0.019 ml/g than the partial specific volume of the protein in water, 0.749 ml/g. A decrease of 0.011 ml/g in partial specific volume resulting from binding of water and guanidinium chloride was estimated (Lee & Timasheff, 1979) from amino acid composition, taken to be identical with that of *Daphnia pulex* erythrocrucorin (Dangott & Terwilliger, 1980). A further minimal decrease of 0.008 ml/g was assumed to take account of the volume change accompanying the loss of haem, on the basis of the observation that for haemoglobins the partial

specific volume calculated from amino acid composition always falls short of the value experimentally measured: $\bar{v}_{\text{exp.}} - \bar{v}_{\text{calc.}}$ equals 0.008 ml/g for horse haemoglobin (McMeekin & Marshall, 1952), 0.009 ml/g for erythrocrucorin from *Lumbricus terrestris* [$\bar{v}_{\text{exp.}} = 0.740$ ml/g (Svedberg & Eriksson, 1933); $\bar{v}_{\text{calc.}} = 0.731$ ml/g (Shlom & Vinogradov, 1973)], 0.012 ml/g for erythrocrucorin from *Planorbis corneus* [$\bar{v}_{\text{exp.}} = 0.745$ ml/g (Svedberg & Eriksson-Quensel, 1934); $\bar{v}_{\text{calc.}} = 0.733$ ml/g (Wood & Mosby, 1975)].

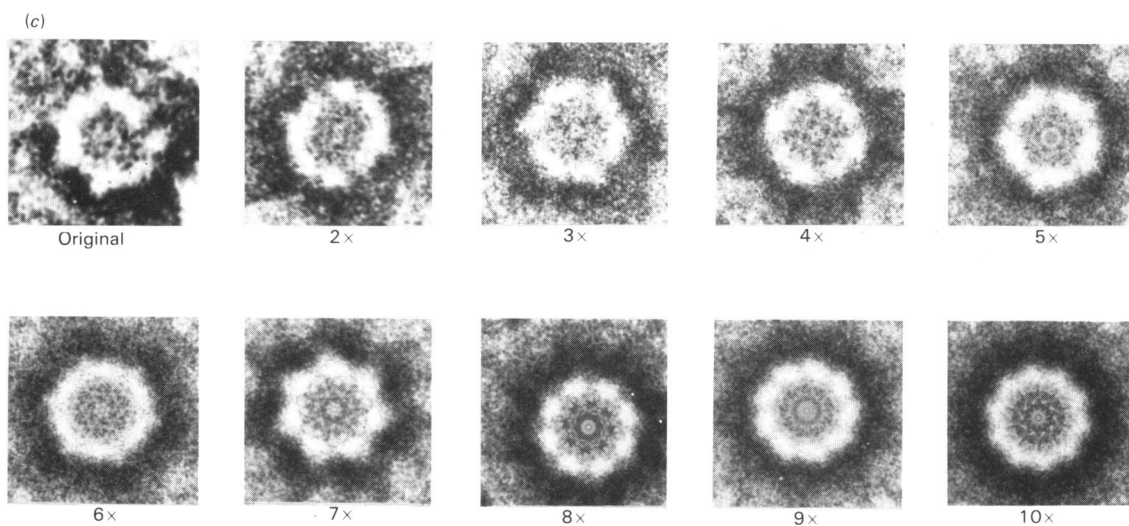
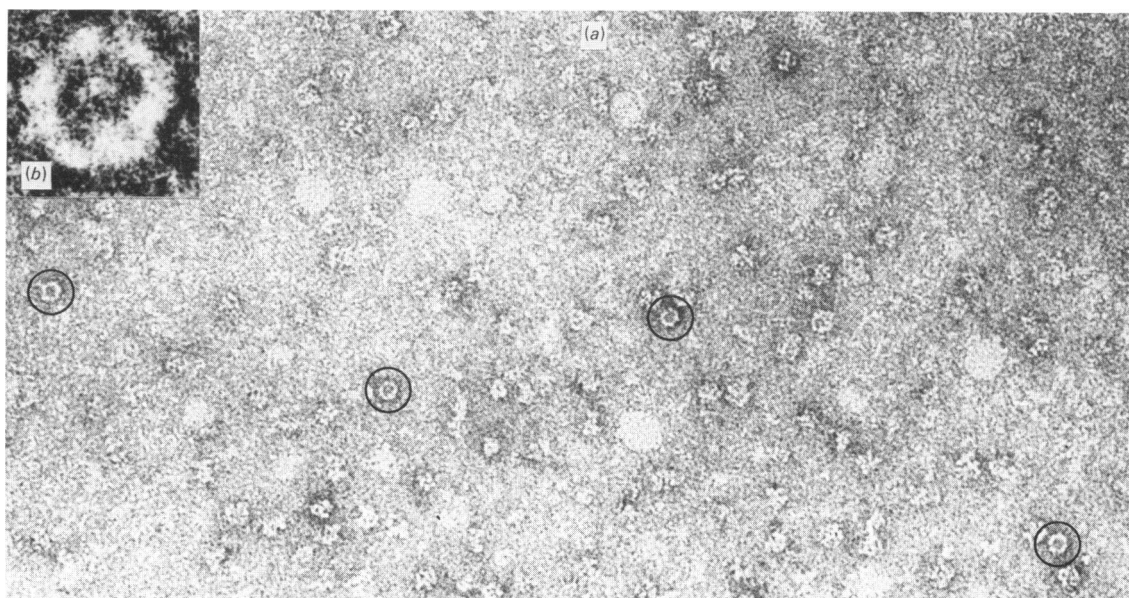
Electron microscopy

Difficulties were encountered in the electron microscopy of *Daphnia* erythrocrucorin. Plate 1 is an electron micrograph of a negatively stained preparation. The field shows a small number of ring-like projections (diameter approx. 14 nm). Images obtained by the technique of rotational photography for contrast enhancement (Markham *et al.*, 1963) are consistent with the presence of rotational symmetry. Partial resolution occurs with 7- and 9-fold rotations. The best resolution is obtained with 8-fold rotation.

Discussion

The intrinsic sedimentation coefficient determined in the present work for *Daphnia magna* erythrocrucorin, $s_{20,w}^0 = 17.9$ S, is practically identical with the value, $s_{20,w}^0 = 17.8$ S, reported for erythrocrucorin from the same species (Hoshi & Kobayashi, 1971) and from the species *Moina macrocopa* (Sugano & Hoshi, 1971). For erythrocrucorin from *Daphnia pulex*, $s_{20,w}$ values of 16.3–16.9 S (Svedberg & Eriksson-Quensel, 1934) and 16.9–17.4 S (Dangott & Terwilliger, 1980) have been reported. The lower values for *Daphnia pulex* erythrocrucorin do not necessarily, however, indicate a real difference in $s_{20,w}^0$, since they were obtained at finite concentration and not extrapolated to infinite dilution.

The dependence of the sedimentation coefficient on pH indicates that dissociation of the native molecule occurs at both alkaline and acid pH values. On both sides of the stability range, outside of it, dissociation follows the same general pattern, leading to the formation of a 'fast' component ($s_{20,w}$ between 9.8 S and 17.1 S) and, at pH below 3.75 or above 10.25, a 'slow' component ($s_{20,w}$ between 2.7 S and 4.0 S). The limiting sedimentation coefficient of 2.7 S can be assigned, as has been shown by direct molecular-weight determination for the 30000-mol.wt. 2.7 S polypeptide chain of erythrocrucorin from the conchostracan *Caenestheria inopinata* (Ilan *et al.*, 1981), to single-polypeptide-chain subunits. By using the theoretical value of 1:1.50 calculated for the ratio of the sedimentation coeffi-



EXPLANATION OF PLATE 1

Electron microscopy of Daphnia erythrocrucorin

(a) Purified preparation negatively stained with uranyl acetate (magnification $\times 210000$). Molecules showing ring-like projections are circled. (b) Magnified ring-like projection. (c) Rotational photograph of a ring-like projection. Rotation steps: $2 \times 180^\circ$, $3 \times 120^\circ$, $4 \times 90^\circ$, $5 \times 72^\circ$, $6 \times 60^\circ$, $7 \times 51.4^\circ$, $8 \times 45^\circ$, $9 \times 40^\circ$, $10 \times 36^\circ$.

cients of a spherical particle and its dimer (Van Holde, 1975), the sedimentation coefficient of 4.0S can be assigned to a dimer of the 2.7S subunit ($2.70 \times 1.50 = 4.05$ S). With these assignments, the slow component may be accounted for by a chemical equilibrium involving single-polypeptide-chain monomers and their dimers. The fast component is attributed to an association-dissociation of the whole-to-halves type. Here the ratio of sedimentation coefficients of wholes to halves, $17.1:9.8 = 1.76$, exceeds the theoretical value of 1.50, but it should be remembered that the latter value represents a lower limit realized when the dimerizing particle is spherical, whereas the actual shape of the half-molecule deviates much from sphericity. Use of a realistic model for *Daphnia magna* erythrocrucorin, described in a following paragraph, leads, in a calculation like the one performed by Van Holde (1975), to a value of 1.71 for the ratio of the sedimentation coefficients of wholes and halves. With this latter value, one predicts for the half-molecule a sedimentation coefficient of $17.1:1.71 = 10.0$ S (10.5 S at infinite dilution), very close to the limiting sedimentation coefficient of the fast component attained at very acid and alkaline pH values. The existence of intermediate states in the acid and alkaline dissociations of *Daphnia pulex* erythrocrucorin has been reported by Svedberg & Eriksson-Quensel (1934). These authors observed components with sedimentation coefficients 2.5–2.8, 3.7–3.8 and 10.2 S, which may be identified with one-chain subunits, dimers of one-chain subunits and half-molecules discussed in the present study.

The findings of the present study permit a determination of the subunit stoichiometry of *Daphnia* erythrocrucorin. From the molecular weights summarized in Table 1, the number of polypeptide chains per molecule is 15.9 and the number of haem groups per chain is 1.81, the nearest integers being 16 and 2 respectively. The 2.7S subunit obtained in the pH dissociation has been identified with a single polypeptide chain. We therefore conclude that a molecule of *Daphnia* erythrocrucorin is composed of 16 2.7S subunits. The

available evidence indicates that the 2.7S subunits are identical and carry two haem groups each.

On the basis of the number of subunits and the evidence from the electron microscope, we propose a working model for *Daphnia* erythrocrucorin. The model consists of 16 2.7S subunits grouped in two layers stacked in an eclipsed orientation, the eight subunits of each layer occupying the vertices of a regular eight-sided polygon. A projection of the model along the eightfold axis is consistent with the circular profiles seen by electron microscopy and their analysis by the contrast-enhancement technique.

Mention has been made of the difficulties encountered in obtaining good electron micrographs of *Daphnia* erythrocrucorin. In view of this, it seemed desirable to try to establish the arrangement of subunits within the molecule on independent evidence, without invoking electron microscopy. According to theory (Klotz *et al.*, 1970, 1975; Matthews & Bernhard, 1973), identical subunits can assemble to give a closed structure in three types of symmetries, cyclic, dihedral and cubic. Cubic symmetry is possible with 12, 24 and 60 subunits, dihedral symmetry with an even number of subunits and cyclic symmetry with any number of subunits. For *Daphnia* erythrocrucorin, with its 16 identical subunits, cyclic or dihedral structures are therefore possible *a priori*. These structures differ from each other with respect to the nature of the bonds linking the subunits together. Monod *et al.* (1965) introduced the classification according to which one may distinguish between isologous bonding, where each subunit contributes an identical binding domain, and heterologous bonding, where each subunit contributes a different binding domain. In the cyclic structure (A in Fig. 4), the intersubunit bonds are heterologous and all of one type. In the dihedral structures, in contrast, the bonds linking the subunits together cannot be all of the same type. Considering only those structures with minimum associations necessary for the integrity of the assembly, two dihedral structures are possible (Matthews & Bernhard, 1973), one (B1 in Fig. 4) containing both heterologous and isologous bonds,

Table 1. Summary of molecular-weight data for *Daphnia magna* erythrocrucorin

Molecular weight of the native molecule	494 000 ± 33 000
Molecular weight of the polypeptide chain from:	
Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis	31 000 ± 1500
Sedimentation equilibrium in guanidinium chloride	31 100 ± 1300
Minimal molecular weight from:	
Haem content	16 100 ± 200
Iron content	17 900 ± 600
Number of haem groups per chain	1.81 ± 0.16
Number of polypeptide chains per molecule	15.9 ± 1.3

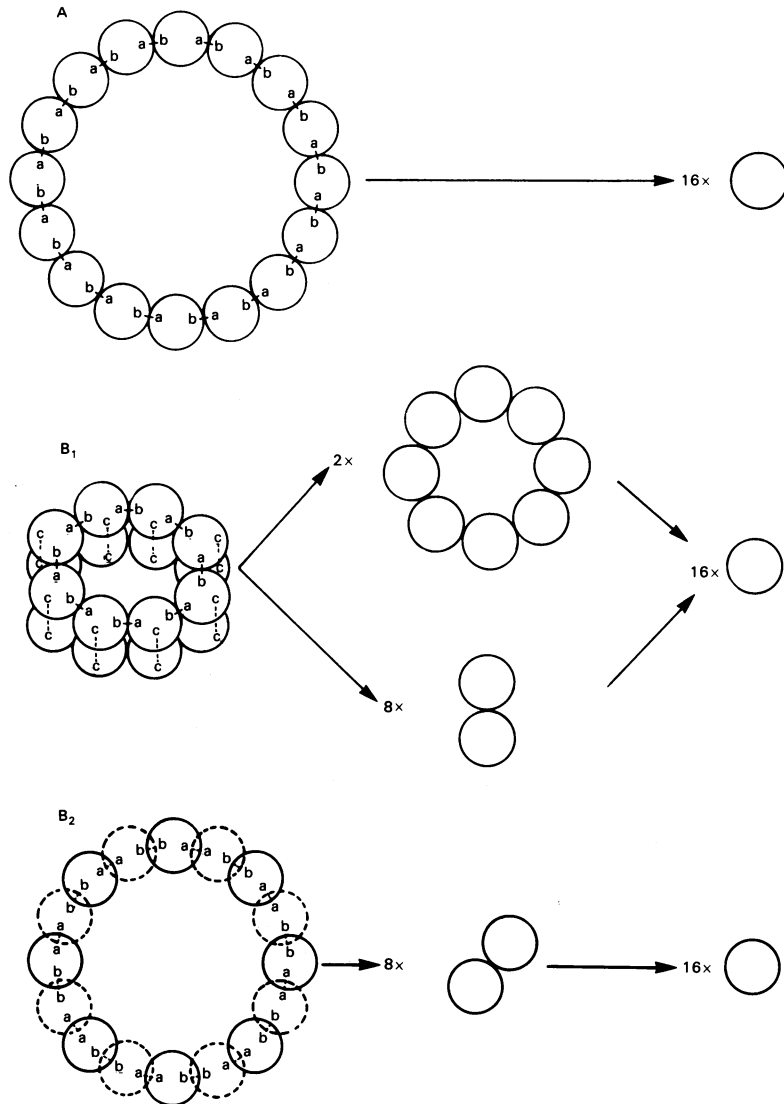


Fig. 4. Possible structural models for *Daphnia erythrocrucorin* and their modes of dissociation. A, Cyclic structure. B₁, Dihedral structure containing both heterologous and isologous bonds. B₂, Dihedral structure containing two types of isologous bonds.

the other (B₂ in Fig. 4) two kinds of isologous bonds. The three structures can be distinguished according to the different patterns of dissociation of the multisubunit assembly. Thus the cyclic structure yields only monomeric subunits (A in Fig. 4). Non-identity of the intersubunit bonds in the dihedral structures opens the possibility of obtaining intermediates, subunit dimers and half-molecules in one case (B₁ in Fig. 4), subunit dimers in the other (B₂ in Fig. 4), provided that the dissociating agent is sufficiently discriminative. The pH dissociation of

Daphnia erythrocrucorin has been shown to involve as intermediate states of aggregation half-molecules and dimers of 2.7S subunits, and is thus consistent with what is to be expected from a molecule with a dihedral symmetry, D_8 , containing both heterologous and isologous bonds. We are thus led to a molecule composed of two layers, containing eight subunits each, stacked one on top of the other. This conclusion is consistent with the model derived from the results of electron microscopy. One cannot distinguish, however, solely on the basis of the

dissociation pattern among the various ways of stacking of the two layers: eclipsed, staggered, or one in between.

Our results on *Daphnia magna*, and those of Dangott & Terwilliger (1980) on *Daphnia pulex*, establish the smallest subunit in cladoceran erythrocrucorins as a polypeptide chain carrying two haem groups. The number of subunits and their arrangement in the molecule, determined in the present study, places erythrocrucorin from Cladocera in a class of its own, different from other groups of branchiopod crustaceans. In a recent report (Ilan & Daniel, 1979b), we expressed the view that arthropod erythrocrucorins cannot be described as multiple aggregation states of a common structural unit, as is the case in mollusc or arthropod haemocyanins (Van Holde & Van Bruggen, 1971). As more and more different structures are revealed (Ilan & Daniel, 1979a; Ilan *et al.*, 1981; the present study), this view is gaining support, and it is becoming increasingly clear that the intraphyletic diversity of arthropod erythrocrucorins is a genuine one, being an expression of a structural diversity on the molecular level.

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