Physicochemical Properties of *Planorbis corneus* Erythrocrucorin

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The erythrocrucorin from the snail *Planorbis corneus* had a sedimentation coefficient, $s_{20,w}^0$, of $33.5 \pm 0.31S$, and a molecular weight of $1.65 \times 10^6 \pm 0.04 \times 10^6$ by high-speed sedimentation-equilibrium ultracentrifugation. The amino acid composition and absorption spectrum of the protein are reported. A very low number of half-cystine residues was found, corresponding to 0.4 residue per haem group. The haem content was $2.76 \pm 0.22\%$, corresponding to a protein molecular weight of about 22300. Under both acid and alkaline conditions partial dissociation took place to yield mixtures of products that could not be identified. A subunit corresponding to that containing one haem group was not obtained under any of the dissociating conditions tried. Electron microscopy revealed a ring-shaped molecule about 12.2 ± 0.5 nm in diameter. The native erythrocrucorin bound O_2 co-operatively, the intermediate value of h in Hill plots having values between 1.7 and 3.4 depending on the conditions.

The erythrocrucorins are respiratory proteins found in certain invertebrates and contain iron co-ordinated in a protoporphyrin IX system. The pigments are sometimes referred to as haemoglobins, though they differ from vertebrate haemoglobins in having molecular weights of over 1×10^6 and in having 50–100 O_2 -binding sites. The erythrocrucorins of *Lumbricus* (Rossi Fanelli *et al.*, 1970; Wiechelman & Parkhurst, 1972; David & Daniel, 1974), *Arenicola* (Waxman, 1971), *Cirraformia* (Swaney & Klotz, 1971) and *Marphysa* (Chew *et al.*, 1965) have sedimentation coefficients of 55–60S, believed to correspond to a molecular weight of about 3×10^6 . The above-mentioned species are all worms, and in contrast with these, the snails *Planorbis* (Svedberg & Pedersen, 1940) and *Biomphalaria* (Almeida & Neves, 1974) possess erythrocrucorins of sedimentation coefficient about 35S, corresponding to a molecular weight in the region of 1.5×10^6 . Most of the other gastropods, including snails and whelks, have a copper-containing haemocyanin, mol.wt. 9×10^6 , as their respiratory pigment. In both worm erythrocrucorins and snail haemocyanins there is a characteristic arrangement of subunits, the most notable feature being that the subunits, or groups of subunits, are arranged in the form of rings (Levin, 1963; Rossi Fanelli *et al.*, 1970; Fernández-Morán *et al.*, 1966). Further, both types of pigment bind O_2 co-operatively. Recent evidence has suggested that the possession of ring structures may be essential for the manifestation of co-operativity (Pearson & Wood, 1974; Van Driel & Van Bruggen, 1974). Some reports had suggested that *Planorbis* erythrocrucorin was capable of binding

O_2 co-operatively (Zaaijer & Wolvekamp, 1958), but little appeared to be known about its quaternary structure. It was therefore decided to re-examine the O_2 -binding properties of this protein and to investigate its structure. In the present work we have studied the behaviour of *Planorbis* erythrocrucorin in the analytical ultracentrifuge under a variety of conditions, and we also report its absorption spectrum and electron-microscopic appearance. Part of this work was presented at the 9th FEBS Meeting in Budapest (Wood, 1974).

Materials and Methods

Preparation of Planorbis erythrocrucorin

Specimens of *Planorbis corneus* were obtained locally. The shells were cleaned and dried with tissue, part of the shell was cut away, and the foot was cut in several places with a scalpel. Blood flowed freely and was filtered through glass wool. The volume of the combined blood from about 20 animals was approx. 4ml, containing 10–15mg of protein/ml, and had a pH of approx. 8.0. The blood was combined with the more dilute erythrocrucorin solution obtained by washing the animals with a few ml of 0.15M-NaCl. An equal volume of 0.1M-sodium phosphate buffer, pH7.0, was added and the mixture was centrifuged for 10min in a bench centrifuge to remove shell and body debris. The supernatant was centrifuged for 3.5h at 4°C and 133500g (r_{av} , 5.9cm). The resulting red pellet was resuspended in 0.1M-sodium phosphate buffer, pH7.0, and spun down and resuspended twice more. After this treatment the ratio E_{417}/E_{280} (cf. Chew *et al.*, 1965) of the CO-

saturated solution of the pigment had increased from 1.78 to 2.41. This preparation sedimented as a single boundary in the analytical ultracentrifuge, and filtration through a column (120 cm $\times$ 2 cm) of Sepharose 4B did not improve the purity. Svedberg & Eriksson-Quensel (1934) reported that the protein was stable for several months if stored frozen, and the protein was therefore stored as a concentrated solution in water at -20°C . However, it was found that material stored in this way eventually contained considerable amounts of met-erythrocrucorin, and therefore O_2 -equilibrium curves were only determined with fresh material.

The protein concentration was determined from the dry weight. Samples of erythrocrucorin solution were dialysed against several changes of water and then known volumes were evaporated to dryness *in vacuo* over P_2O_5 at 105°C to constant weight.

Analytical ultracentrifugation

Sedimentation-velocity and sedimentation-equilibrium experiments were performed in a Beckman model E instrument equipped with schlieren and interference optical systems and a rotor temperature-indicating and -control system. Sedimentation-velocity experiments were done in cells with 4 or 12 mm aluminium centre-pieces in an An-D rotor. Boundaries were observed with the schlieren optical system and were photographed with Ilford R-30 plates and a red filter. Plates were measured in a projectorscope (Precision Grinding Ltd.) and sedimentation coefficients were calculated from the rate of movement of the peak maxima. Sedimentation coefficients were simplified to $s_{20,w}$ values in the usual way (Svedberg & Pedersen, 1940).

Sedimentation-equilibrium experiments were performed with the Rayleigh interference optical system. As the speeds required, even for the so-called high-speed method, were very low, an An-H (titanium) rotor was used in a model E having an electronic speed-control system to achieve maximum stability. Protein solutions were dialysed against an appropriate buffer, and the protein solution and diffusate were then placed in separate channels of a 12 mm double-sector cell. A protein concentration of 0.25–0.35 mg/ml was used (Yphantis, 1964). Photographs were taken and measured until no further change occurred in the disposition of the fringes. Buffer densities were determined by means of a 10 ml pycnometer, and buffer viscosities by means of an Ostwald viscometer. For the 0.1 M-Tris-HCl buffer, pH 8.0, used in the examination of the native erythrocrucorin, $\rho_{20} = 1.0028 \text{ g} \cdot \text{ml}^{-1}$ and $\eta/\eta_0 = 1.0096_4$.

Spectrophotometry

Extinctions for the estimation of protein concentrations were determined on a Unicam SP.500

instrument, and spectra were obtained with a Unicam SP.1800 instrument fitted with a chart recorder. A single stock solution of purified erythrocrucorin was used in this latter case, portions of which were bubbled with O_2 or CO or treated with a small amount of solid $\text{Na}_2\text{S}_2\text{O}_4$ in order to obtain the oxy, carbonmonoxy or deoxy derivatives respectively.

Haem determination

The haem content of erythrocrucorin in water was determined by the pyridine haemochromogen method (Falk, 1964) with Aristar-grade pyridine (BDH Chemicals, Poole, Dorset, U.K.).

Electrophoresis

Electrophoresis was performed on polyacrylamide gels as described (Bannister *et al.*, 1971), and detergent-gel electrophoresis was carried out on samples that had been heated at 90°C for 3 min (Waxman, 1971) in the presence of sodium dodecyl sulphate and 2-mercaptoethanol. Bovine serum albumin, ovalbumin and myoglobin [all obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.] were treated similarly for use as molecular-weight markers. Isoelectric focusing was carried out with a Pharmacia 110 ml column and a sucrose gradient as described by Bannister & Wood (1970).

Succinylation of erythrocrucorin

Various amounts of solid succinic anhydride were added slowly to 2 ml of 0.01 M-sodium phosphate buffer, pH 8.0, containing 20 mg of erythrocrucorin. The pH was maintained in the range 7.5–8.0 throughout by means of dilute NaOH until no further reaction took place. The resulting solution was dialysed against several changes of 0.01 M-sodium phosphate buffer, pH 8.0, to remove succinate.

Electron microscopy

Electron microscopy was performed in a Phillips 300 instrument. Samples were negatively stained with either uranyl oxalate or phosphotungstic acid (Levin, 1963). Negatives were measured in the projectorscope to determine the size of the molecules.

O_2 -binding curves

O_2 -equilibrium curves were determined by means of a 100 ml tonometer attached to a 10 mm-light-path cuvette. The tonometer bulb was fitted with two taps through which air could be admitted. Approx. 3 ml of erythrocrucorin solution was placed in the tonometer bulb, which was then cooled while the tonometer was gently evacuated. N_2 was then drawn in and the tonometer was re-evacuated. This very gentle procedure at low temperatures was essential to prevent precipitation and denaturation of the erythrocrucorin. The evacuated tonometer was then equilibrated

in a water bath at the desired temperature, and the absorption spectrum was recorded. Finally, portions of air were admitted, with equilibration between each addition, until the sample was saturated with O_2 . Partial O_2 pressures and percentage saturation were calculated in the usual way.

Preparation of globin

Haem was removed from erythrocrucorin in water at low temperature by the acid-acetone method (Rossi Fanelli *et al.*, 1958) or by the butan-2-one method (Teale, 1959). Despite very careful temperature control throughout, the product obtained in each case was discoloured, and was insoluble in water but soluble in 0.1 M-acetate buffers, pH below 4.8, or in 6–8 M-urea.

Reduction and carboxymethylation of globin

The globin was taken up in 8 M-urea and was treated with iodoacetamide (Waxman, 1971). After exhaustive dialysis against water the product precipitated.

Amino acid analysis

Samples of globin or carboxymethylglobin were hydrolysed with 6 M-HCl in evacuated sealed tubes for 24, 48 and 96 h at 110°C. Amino acid analysis was performed with a Biocal amino acid analyser. Tryptophan was determined by the method of Spies & Chambers (1949) and by the *N*-bromosuccinimide method (Spande & Witkop, 1967). Cysteine was determined (a) in the normal acid hydrolysates, (b) as carboxymethylcysteine in hydrolysates of the carboxymethylated globin, and (c) as cysteic acid in performic acid-oxidized samples of the globin.

Fig. 1. Sedimentation of purified *Planorbis erythrocrucorin* in the analytical ultracentrifuge

Protein concentration: 1.8 mg/ml in 0.1 M-Tris-HCl buffer, pH 8.0. Conditions of run: speed, 37020 rev./min; temperature, 20°C; schlieren phase-plate angle, 70°; picture taken approx. 10 min after rotor had reached full speed.

Fig. 2. Dependence of sedimentation coefficient of *Planorbis erythrocrucorin* on protein concentration

Protein was dissolved in 0.1 M-Tris-HCl buffer, pH 8.0. *s* values were corrected to *s*_{20,w} in the usual way. Value for *s*_{20,w}⁰: 33.5 ± 0.31 S.

Fig. 3. High-speed sedimentation-equilibrium experiment with *Planorbis erythrocrucorin*

Protein concentration was initially 0.3 mg/ml in 0.1 M-Tris-HCl, pH 8.0. Conditions of run: speed, 5200 rev./min; temperature, 22°C; interference photograph taken after 50 h. Molecular weight of *Planorbis erythrocrucorin* (*M*_{w,app.}) 1.65 × 10⁶ ± 0.04 × 10⁶.

Results

Physical parameters of the native erythrocrucorin

Fresh *Planorbis* blood showed one major boundary and several minor boundaries in sedimentation-velocity experiments, with approximate sedimentation coefficients 14S, 28S and 50S. The 28S component was the major one. The purified material sedimented as a single sharp boundary (Fig. 1), and it was clear from the schlieren pictures that all the red colour was associated with the protein. The sedimentation properties were examined over the concentration range 0.3–6.0 mg/ml (Fig. 2), and a linear extra-

potation yielded a value for $s_{20,w}^0$ of 33.50 ± 0.31 S. Over this concentration range the coefficient, K , in the equation

$$s_{20,w} = s_{20,w}^0(1 - Kc)$$

was $30.1 \pm 3.1 \text{ ml} \cdot \text{g}^{-1}$, where c is the protein concentration in $\text{g} \cdot \text{ml}^{-1}$.

Sedimentation-equilibrium experiments resulted in linear plots of $\ln \Delta Y$ against r^2 , indicating that the material was homogeneous. Calculation of the molecular weight of the protein from the slope of such

Fig. 4. Isoelectric focusing of *Planorbis* erythrocrucorin

Equipment: LKB 110ml column, sucrose gradient, 3 ml fractions collected at the end of a 3-day run at 4°C . ●, E_{280} ; ▲, pH. Isoionic point: pH4.72.

plots, by using a value for partial specific volume ($\bar{v}$) of $0.733 \text{ ml} \cdot \text{g}^{-1}$ calculated from the amino acid composition, yielded a value for the weight-average molecular weight ($\bar{M}_w$) of $1.65 \times 10^6 \pm 0.04 \times 10^6$ (Fig. 3).

In polyacrylamide-gel electrophoresis, *Planorbis* erythrocrucorin did not penetrate 4% (w/v) gels, but did penetrate 3% gels, to give a single red band. No other bands were observed after staining with Coomassie Blue. The isoelectric point as determined by isoelectric focusing was pH4.72 (Fig. 4). Svedberg & Pedersen (1940) reported a value of 4.77.

It proved difficult to obtain electron micrographs of *Planorbis* erythrocrucorin, and usually only a few intact molecules were observed per frame (Plate 1). The photographs reveal several noteworthy features. The subunits of the protein appear to be arranged in the form of a ring, probably a hexagon, the distance across the hexagon being 12.2 ± 0.5 (30) nm. By comparison the diameter of the hexagon observed with worm erythrocrucorins is about 26 nm (Levin, 1963). Often with worm erythrocrucorins one can observe some of the molecules from the side-on aspect and this has led to the conclusion that the molecule is composed of two hexagonal plates. In the electron micrographs of *Planorbis* erythrocrucorin no such structures were identified and so little can be said about the overall shape of the molecule. It also appears that the subunits of *Planorbis* erythrocrucorin, like those of worm erythrocrucorins, are possibly arranged in the form of six small rings joined in a hexagon. It is dangerous to make any other interpretation of the structure until further electron micrographs or hydrodynamic data become available.

The haem content of *Planorbis* erythrocrucorin was

Fig. 5. Absorption spectra of *Planorbis* erythrocrucorin (---), oxyerythrocrucorin (—) and carbonmonoxyerythrocrucorin (···), dissolved in water

Right-hand scale applies to wavelengths > 500nm: read in a 10mm-light-path cell.

EXPLANATION OF PLATE I

Electron micrographs of negatively stained Planorbis corneus erythrocrucrin

Stain: uranyl oxalate; length of bar, $0.1\ \mu\text{m}$. Smaller pictures show molecules taken from large fields and assembled into a montage, with the same stain; length of bar, $0.05\ \mu\text{m}$.

Table 1. *Amino acid composition of Planorbis erythrocrucorin compared with published data for other erythrocrucorins, snail haemocyanin and human haemoglobin*

All were put on an arbitrary basis of mol of residue per 22300 g. *Planorbis* erythrocrucorin: all amino acids, average of value after 24, 48 and 96 h hydrolysis, except threonine and serine, which were extrapolated to zero time, and valine, isoleucine and histidine for which the 96 h values are given. Half-cystine was determined as cysteic acid, and tryptophan by *N*-bromosuccinimide titration.

	<i>Planorbis</i>	<i>Biomphalaria</i> *	<i>Arenicola</i> †	<i>Lumbricus</i> ‡	<i>Cirraformia</i> §	Human haemoglobin	<i>Helix</i> haemocyanin
Aspartic acid	24.9	31.4	24.4	22.3	27.6	15.3	22.4
Threonine	5.0	5.5	8.2	6.6	5.9	9.9	11.5
Serine	9.9	14.1	9.7	9.4	10.4	10.7	10.0
Glutamic acid	16.9	22.1	22.8	17.7	19.8	10.7	21.7
Proline	3.6	6.2	5.7	6.3	6.7	8.6	10.5
Glycine	10.8	12.3	13.9	11.0	15.6	13.3	11.7
Alanine	15.2	17.2	16.3	13.7	21.6	21.9	13.2
Half-cystine	0.4	0.7	5.7	6.1	6.3	1.3	2.1
Valine	11.2	16.6	15.5	10.0	13.7	18.6	10.7
Methionine	2.5	1.9	4.9	0.7	0	2.0	1.0
Isoleucine	6.9	7.4	6.5	9.9	7.9	0	8.6
Leucine	19.2	22.1	15.2	18.4	17.8	21.9	18.0
Tyrosine	1.7	2.5	4.0	2.8	2.5	2.6	9.4
Phenylalanine	7.0	9.2	9.7	8.3	11.2	9.3	10.8
Lysine	10.4	11.0	9.7	10.1	7.7	13.3	7.6
Histidine	3.3	2.5	7.3	10.9	2.5	10.0	9.7
Arginine	8.4	9.8	11.4	10.9	12.4	3.9	7.8
Tryptophan	3.5	3.1	4.0	3.0	—	2.6	4.8

* Taken from Almeida & Neves (1974).

† Taken from Waxman (1971).

‡ Taken from Shlom & Vinogradov (1973).

§ Taken from Swaney & Klotz (1971).

|| Taken from Witters & Lontie (1968).

$2.76 \pm 0.22\%$, which would correspond to about 74 haem groups per molecule, or 9–12 protomers per one-sixth molecule.

Fig. 5 shows the absorption spectrum of *Planorbis* erythrocrucorin and its oxy and carbonmonoxy derivatives. The band extinctions for each of the forms of the pigment have maxima at wavelengths conforming broadly with those for haemoglobins of diverse origin (Lemberg & Legge, 1949), and in particular with those for worm erythrocrucorin (Chew *et al.*, 1965; Harrington *et al.*, 1973; Swaney & Klotz, 1971; Rossi Fanelli *et al.*, 1970). The identity of the absorption spectrum of pyridine haemochromogens prepared from the pigment and from haemin confirms the protohaem nature of the prosthetic group. Both had maxima at 554, 523 and 474 nm.

Amino acid analysis of *Planorbis* erythrocrucorin globin revealed that the protein had a composition somewhat similar to that of the worm erythrocrucorins (Table 1), except that the amino acid cysteine was either present in extremely small amounts or absent altogether. In the analysis of normal acid hydrolysates, the column buffer change was delayed to try to expand the region of the chart where cysteine appears: no cysteine peak was found. Analysis of

the reduced carboxymethylated globin revealed no carboxymethylcysteine and analysis of performic acid-oxidized globin revealed the presence of a little cysteic acid, representing less than one-half of a cysteine molecule per haem group. It is of course possible that there are some cysteine-containing subunits in the molecule, whereas most of the subunits contain none.

O₂ equilibrium

O₂-binding curves were determined under a number of experimental conditions and Fig. 6 shows the type of result obtained. The binding was co-operative, the Hill plots having intermediate slope (*h*) of 1.7–3.4. The form of the Hill plots was typical of those for other multi-subunit O₂-binding proteins (Wyman, 1964) in that there was a high intermediate slope, but a slope approaching unity at high and at low partial pressures of O₂. The O₂ affinity is comparatively high ($p_{50} = 4\text{--}5\text{ mmHg}$; 533–667 Pa), and over the pH range 7.0–8.4 and at temperatures between 16° and 25°C no Bohr effect was observed (Table 2).

Fig. 6. Oxygen equilibrium (Hill plot) for *Planorbis erythrocrucruorin* dissolved in 0.1 M-Tris-HCl, pH 8.0, at 16°C

The straight line has a slope of 1. Intermediate slope (h) approx. 3.4.

Table 2. Oxygen-binding data for *Planorbis erythrocrucruorin* dissolved in 0.1 M-Tris-HCl at the stated pH compared with published data for *Planorbis* blood

Data from Zaaijer & Wolvckamp (1958) were taken from the plots of percentage saturation against pO_2 given and replotted in the form of Hill plots to estimate h_{max} .

Temperature (°C)	p_{50}			
	pH	mmHg (Pa)	h_{max}	
Present work				
20.1	6.5	4.6 (613)	1.7	
21.6	7.0	4.3 (573)	2.1	
21.3	7.3	5.0 (667)	2.3	
23.0	7.6	4.2 (560)	1.9	
21.0	8.5	4.5 (600)	2.0	
Zaaijer & Wolvckamp (1958)				
20	7.7	6.5 (867)	2.2	
20	8.1	3.9 (520)	2.1	
20	8.6	3.0 (400)	1.6	

pH-induced dissociation

Planorbis erythrocrucruorin was stable over a very wide pH range. However, at pH values less than 2.2 and greater than 9.9 dissociation occurred. At pH values near pH 2.0 dissociation took place, resulting in the appearance in sedimentation-velocity experiments of three poorly separated boundaries. These corresponded to species with approximate sedimentation coefficients 12S, 16S and 24S (Fig. 7a), and might represent one-sixth, one-third and one-half molecules. However, the separation was extremely poor and precipitation took place on longer standing at the low pH, making it likely that denaturation was occurring.

At high pH values (pH > 9.9) dissociation took place, resulting in the appearance of two boundaries in sedimentation-velocity experiments with sedimentation coefficients 20–21S and 6–8S (Fig. 7b). Attempts were made to separate these components on a column of Sephadex G-200, but the fractions obtained were not homogeneous on polyacrylamide-gel electrophoresis nor in sedimentation-equilibrium experiments. It was likely that slow dissociation was occurring or that the system was one involving rapid equilibrium between components. In view of the good separation between the peaks observed in sedimentation-velocity experiments, the former seems more likely.

Preparations of globin dissolved in 0.1 M-sodium acetate buffer, pH 4.4, were also examined in the ultracentrifuge (Fig. 7c). Three poorly separated components were observed with sedimentation coefficients of approx. 10, 13.8 and 19.5S (uncorrected values). The presence of a major component with sedimentation coefficient 13S suggested that dissociation into very small subunits has not taken place in this solvent. However, as the boundaries were poorly resolved and the baseline appeared to be displaced, it was possible that the protein was undergoing reversible dissociation under these conditions. If this is the case then a qualitative examination of the record is unlikely to indicate the size of the smallest component in the mixture, and it would be necessary to investigate the system over a range of protein concentrations to obtain further information.

Size of the smallest subunit

The haem content of *Planorbis erythrocrucruorin* might indicate a molecular weight for a smallest subunit of about 22000. Attempts were made to dissociate the protein with 8M-urea, 6M-guanidinium chloride and sodium dodecyl sulphate to provide information on this point. With sodium dodecyl sulphate-polyacrylamide gels only part of the protein penetrated the gels appreciably, and the protein that did penetrate had a mobility less than that of the

Fig. 7. Sedimentation of *Planorbis erythrocrucorin* at low (a) and high (b) pH, and of globin derived from *Planorbis erythrocrucorin* at low pH (c)

(a) Erythrocrucorin was dialysed against 0.1M-glycine, pH2.0, containing 0.1M-NaCl. Conditions of run: speed, 37020 rev./min; temperature, 19°C; schlieren phase-plate angle, 70°; picture taken approx. 12 min after rotor had reached full speed. Sedimentation coefficients of peaks from left to right: 12S, 16S and 24S. (b) Erythrocrucorin was dissolved in pH9.9 buffer made by mixing equal volumes of 0.1M-Na₂CO₃ and 0.1M-NaHCO₃. Conditions of run: speed, 37020 rev./min; temperature, 19°C; schlieren phase-plate angle, 70°; picture taken approx. 12 min after rotor had reached full speed. Sedimentation coefficients of peaks: 7.0S and 19.8S. (c) Globin was dissolved in 0.1M-sodium acetate buffer, pH4.4. Conditions of run: speed, 44770 rev./min; 30mm ultracentrifuge cell; temperature, 22°C; schlieren phase-plate angle, 70°; picture taken 29 min after rotor had reached full speed. Sedimentation coefficients of peaks: approx. 10S, 13.8S and 19.5S.

dimer of bovine serum albumin, indicating a molecular weight of greater than 134000. It made no difference to these results whether globin or carboxymethylated globin was used.

Treatment with 8M-urea yielded a product that was also heterogeneous on polyacrylamide gels and the anomalous results obtained by equilibrium sedimentation of the protein in 6M-guanidine hydrochloride containing 2-mercaptoethanol made it likely that a heterogeneous mixture of products was also obtained in this solvent.

Succinylation of *Planorbis erythrocrucorin* with a large excess of succinic anhydride yielded a product that was apparently homogeneous in sedimentation-velocity experiments, but which gave several bands on polyacrylamide gels. These bands might of course be due to charge isomers. The sedimentation coefficient of the peak observed in the analytical ultracentrifuge was 3–5S, indicating that considerable dissociation had taken place. However, sedimentation-equilibrium experiments gave anomalous results, making it impossible to obtain even an approximate guess of the molecular weight of the material. The anomalous effects observed in the ultracentrifuge may be due to the high negative change on the molecules resulting from succinylation in addition to the heterogeneity observed on polyacrylamide gels. The only conclusion reached from these studies was that the protein subunits are held together in such a way as to make them extremely resistant to dissociating reagents.

Discussion

Planorbis and certain related species of snail appear to be unique among the gastropods in possessing an erythrocrucorin rather than a copper-containing haemocyanin as their blood respiratory pigment. Both types of protein have very high molecular weights and characteristic subunit structures, and both bind O₂ in a co-operative manner. Svedberg & Eriksson-Quensel (1934) reported a sedimentation coefficient of 33.7S for *Planorbis erythrocrucorin*, and Almeida & Neves (1974) reported a value of 32.19S for *Biomphalaria erythrocrucorin*: the value of 33.5S obtained in the present work for *Planorbis erythrocrucorin* agrees well with these values. Svedberg & Pedersen (1940) recorded a molecular weight of 1.53×10^6 – 1.63×10^6 for the *Planorbis* protein. In the present work a value of $1.65 \times 10^6 \pm 0.04 \times 10^6$ was obtained by the high-speed sedimentation-equilibrium method, and this may be compared with the value of 1.75×10^6 obtained for *Biomphalaria erythrocrucorin* by Almeida & Neves (1974) from *s* and *D*. It is clear that the proteins from *Planorbis* and *Biomphalaria* are closely similar in size.

Many electron micrographs of worm erythrocrucorins have been published (Guerritore *et al.*,

1965; Yamagishi *et al.*, 1966; Van Bruggen & Weber, 1974) and all show a typical hexagonal structure, the whole molecule apparently being made up of two hexagonal plates. Often some molecules are seen from the side view, so that the two layers can be seen. In the present work electron micrographs were extremely difficult to obtain, and no side-on molecules could be distinguished. The most that can be said from an examination of the present electron micrographs is that the molecules are toroidal, probably being composed of six submultiples, the overall diameter being 12.2 ± 0.5 nm. This is less than one-half the diameter of worm erythrocrucorin molecules.

There is little doubt that O_2 is bound co-operatively, the slope of the Hill plot (h) having values in some instances as high as 3.4. There is some disagreement in the literature on the form of the O_2 -equilibrium curves, but the conclusions of Zaaier & Wolvekamp (1958) were that the O_2 affinity was high, p_{50} being between 133 and 933 Pa (1 and 7 mmHg) depending on the experimental conditions, that the O_2 binding was co-operative and that there were small positive and negative Bohr effects. The present work has largely confirmed these findings, except that we have failed to find a significant Bohr effect. It may be noted that Figueiredo *et al.* (1973) failed to find a Bohr effect with *Biomphalaria* erythrocrucorin, but also found values for h in the Hill equation of about 1.08, indicating a lack of co-operativity in this protein. Vertebrate haemoglobins have a tetrameric structure and show co-operative O_2 binding, whereas it appears to be a common feature of invertebrate multisubunit respiratory pigments (haemocyanins, erythrocrucorins and chlorocrucorins), which also show co-operative O_2 binding, that the subunits are arranged in the form of a ring. Pearson & Wood (1974) showed with *Colus gracilis* haemocyanin that whole molecules and half molecules were capable of co-operative binding, whereas one-tenth molecules, in which the ring structure was lost, bound O_2 non-co-operatively. *Planorbis* erythrocrucorin would seem to fit into this pattern.

The amino acid composition of *Planorbis* erythrocrucorin is very similar to that reported for *Biomphalaria* erythrocrucorin (Almeida & Neves, 1974), and somewhat similar to those of worm erythrocrucorins (Table 1). The major difference is the lack or very low number, in the snail erythrocrucorins, of cysteine residues. Though the molecules are very resistant to procedures designed to dissociate them into subunits, this resistance does not depend on the presence of disulphide bonds. It is also clear that the snail erythrocrucorins differ markedly in amino acid composition from snail haemocyanins on the one hand and vertebrate haemoglobins on the other.

Experiments designed to reveal the subunit structure of *Planorbis* erythrocrucorin have so far

yielded little useful information. The haem content of the protein was 2.76%, giving a minimum molecular weight of about 22300. The haem content may be compared with that reported for the erythrocrucorin of *Marphysa* (2.24%), *Cirraformia* (3.08%) and *Lumbricus* (2.67 and 2.78%). Figueiredo *et al.* (1973) did not determine the haem content, but did determine the iron content of *Biomphalaria* erythrocrucorin, finding a value of 0.315%, which corresponds to a minimum molecular weight of 17700. This of course compares very well with vertebrate haemoglobins and myoglobins, but it seems to be generally accepted now that there is no good reason why the subunits or protomers of erythrocrucorins should have the same molecular weights as those of vertebrate haemoglobins.

On the basis of a molecular weight of 1.65×10^6 for *Planorbis* erythrocrucorin, there appear to be about 74 haem groups, though on the basis of a possible sixfold symmetry it is probable that 72 would be a more appropriate number. However, dissociation experiments do not result in the production of any species as small as one with molecular weight 22300, and there was some indication that at pH 4.4 (Fig. 7c) the globin might behave as a reversibly dissociating system. Such behaviour makes it difficult to obtain an estimate of the size of the smallest subunit present in a mixture. The resistance of *Planorbis* erythrocrucorin to dissociating reagents is reminiscent of the behaviour of snail haemocyanins. Brouwer & Kuiper (1973) found that nothing of molecular weight smaller than about 300000 could be obtained from *Helix pomatia* haemocyanin by treatment with dissociating reagents. The smallest component observed with *Planorbis* erythrocrucorin was the approximately 5S component resulting from succinic anhydride treatment. Figueiredo *et al.* (1973), on the other hand, were able to identify a 3S component in *Biomphalaria* erythrocrucorin at pH 2.3. However, the results from experiments in which succinic anhydride was used were not completely satisfactory. It is noteworthy that both Swaney & Klotz (1971), working with *Cirraformia* erythrocrucorin, and Waxman (1971), working with *Arenicola* erythrocrucorin, tried succinylation as a dissociating procedure and in neither case did any satisfactory conclusion result. In contrast with this apparent resistance to dissociation to protomers, which may indicate the possession by snail haemocyanins and erythrocrucorins of a large protomer with multiple active sites, the worm erythrocrucorins have been dissociated into very small polypeptide chains. On the basis of his results Waxman (1971) considered that *Arenicola* erythrocrucorin had a basic unit of one haem attached to two polypeptide chains of mol.wt. 13000 and 14000, and Shlom & Vinogradov (1973), working with *Lumbricus* erythrocrucorin, found bands in detergent-polyacrylamide-gel electrophoresis corresponding to

molecular weights of 14000, 16000 and 19000, in addition to some higher-molecular-weight bands.

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