Erythrocrucorin of *Marphysa sanguinea*

ISOLATION AND SOME PHYSICAL, PHYSICOCHEMICAL AND OTHER PROPERTIES

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1. The polychaete worm *Marphysa sanguinea* has a circulating erythrocrucorin of mol.wt. about 2.4×10^6 ($S_{20,w}^0$ 58.2 s, $D_{20,w}$ 2.06×10^{-7} cm.²/sec). This is the predominant form existing at pH 6–8 and (non-protein) I 0.10–0.21, and also at approx. pH 6.7 and I 0.15–3.00. 2. The pigment contains 2.24% of protohaem. 3. The 58 s protein has an electrophoretic mobility of 8.08×10^{-5} cm.²/v/sec. at pH 8.12, I 0.21 and 0°. The isoelectric point of suspended particles is 4.63 at I 0.16 and 21.5°. 4. At very low ionic strength and pH 6.7 (unbuffered) the 58 s pigment associates reversibly to 97 s and 150 s forms, which are probably dimer and tetramer species. 5. At pH 10.0 and I 0.025, it dissociates irreversibly to give a small amount of 2–4 s non-haem-containing protein and much 9 s haem-enriched protein. These and the 58 s pigment may correspond to structures found in Levin's (1963) electron-microscope studies of other erythrocrucorins. 6. Absorption spectra of the 58 s oxygenated erythrocrucorin and the deoxygenated and carbon monoxide derivatives have been obtained.

The polychaete worm *Marphysa sanguinea* (common name, 'bloodworm') frequents the estuarine mud banks of the Swan River, Western Australia. The adult forms in this habitat rarely exceed 15 cm. in length in repose. Freshly withdrawn from the dark mud they are greyish pink, changing rapidly to deep brownish red on exposure. Laceration leads to the prompt and copious exudation of a fluid containing a red pigment that is the subject of the present study.

The circulating red respiratory pigments of many worms have long been known to exist in free solution in the blood plasma. Svedberg & Eriksson (1933) and Svedberg & Eriksson-Quensel (1934), who studied these pigments in the ultracentrifuge, gave the name 'erythrocrucorin' to the group of pigments that occurred in solution in the plasma, had high molecular weights (4×10^5 to 3×10^6) and had low isoelectric points, to distinguish them from the vertebrate haemoglobins. The prosthetic groups are of the ferrohaem type (Svedberg & Eriksson, 1933). The pigment of *M. sanguinea* appears not to have been studied in detail hitherto. Properties described below justify its classification as an 'erythrocrucorin' in the sense used by Svedberg & Eriksson (1933).

MATERIALS AND METHODS

The protein and other solutions had generally been equilibrated with air at atmospheric pressure, except when conditions were deliberately changed (e.g. formation of deoxygenated pigment). Where not otherwise stipulated work was performed at room temperature (20–25°).

All values for ionic strength ignore the protein contribution.

Preparation of crude extract. Adult worms were washed free from mud with distilled water, blotted with filter paper, cooled to 0–5° and ground in a mortar. The mash was squeezed in muslin and washed with 0.165 M-NaCl solution. The mixed filtrate and washings were centrifuged at 0° for 30 min. at 12000 *g* (at tip of tube in rotor 296, International refrigerated centrifuge model PR2), and the liquid was decanted from the residue and centrifuged as before. The resulting liquid was the 'crude extract'. Samples were freeze-dried, stored for up to 2 months at 4°, and redissolved for examination.

Zone electrophoresis of the crude extract (first buffered at pH 8.6) on cellulose acetate strips indicated the presence of one minor and three major protein constituents, all negatively charged, of which the least mobile alone contained the pigment. Ultracentrifugal analysis of the crude extract (pH 6.7, unbuffered) confirmed the presence of three major protein constituents, with observed sedimentation coefficients, *S*, of 48, 26 and 3 s, the red colour being associated

only with the first, which represented considerably more than half of the total protein. Total protein in the crude extract (approx. 20 mg./ml., representing a yield of about 10 mg./g. of wet worm tissue) was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). From this information conditions were devised for purification of the pigment.

Purification of pigment. The crude extract was centrifuged at 0° for 5.5 hr. at 30 000 *g* (max.) in 10 ml. portions/50 ml. tube in the 9RA rotor of a Lourdes refrigerated centrifuge. The liquids were discarded and the sedimented red pellets were each dissolved in 10 ml. of 0.65 M-NaCl solution. The centrifugation cycle was twice repeated, the pellets finally being taken up in the NaCl solution. The progress of purification was followed spectrophotometrically by observing the change in ratio of the extinctions (*E*) of dissolved pellet material saturated with carbon monoxide, at two wavelengths: 417 $m\mu$ as characteristic of haem substances, and 280 $m\mu$ as characteristic of protein. The solutions were stored at -25°. Approximate values were obtained of the protein concentration in these solutions by the method of Lowry *et al.* (1951), and more accurate ones by evaporating measured volumes *in vacuo* over conc. H₂SO₄ at room temperature, heating at 105°, weighing the residues and subtracting the weight of the NaCl. To estimate NaCl the protein was flocculated by adding 7-15 vol. of 0.02 M-sodium acetate buffer, pH 4.63, coagulated at 100° for 2 min., centrifuged and washed twice with buffer. Chloride was determined in the combined liquid and washings by a micro-modification of the Volhard method.

Sedimentation and diffusion studies. At pH 6.60 and *I* 0.10, values consistent with the existence of the purified pigment as virtually a single peak, measurements were made of the sedimentation and diffusion coefficients. A Spinco model E ultracentrifuge (Beckman Instruments Inc., Palo Alto, Calif., U.S.A.) was employed with phase-plate schlieren optics, the rotor temperature indicator and control unit, the An-D rotor, the 12 mm. aluminium centre-piece cells (4° sector) for sedimentation, or the 12 mm. double-sector cells (2° sector) for diffusion runs. Boundaries were photographed on Ilford HP3 film, usually with a red filter (maximum transmission 640 $m\mu$ at the light-source).

The sedimentation coefficient at zero protein concentration was obtained by extrapolation in the standard manner. The diffusion coefficient was determined from boundary-spreading measurements. The boundary was formed at 29 980 rev./min. (65 320 *g*) and the rotor coasted back to 4 908 rev./min. (1 750 *g*), at which speed movement of the boundary was very slow. Ten photographs at this speed, at 16 min. intervals, provided the necessary data. Values at protein concentrations of 2.8 and 5.6 mg./ml. differed by less than 1%.

Reversible association-dissociation relationships, prevailing in the pH range 6-8 and *I* range 0.005-3.0, were also briefly examined by sedimentation technique.

Irreversible dissociation occurring at higher pH values was similarly studied by ultracentrifugal sedimentation methods. For this purpose pigment solution was dialysed overnight on a rocking machine at 0-2° against 200 vol. of buffer. The resulting solution was diluted with the same buffer to give a solution containing 5.6 mg. of protein/ml. Ultracentrifugal analysis was carried out as described above.

Zone ultracentrifugation in sucrose concentration gradients. For zone centrifugation 15 ml. portions of sucrose solution

in the glycine buffer, pH 10.0, were delivered to nitrocellulose tubes fitting the SW 39E rotor of the Spinco model E ultracentrifuge. The sucrose concentration ranged from 20% (w/v) at the bottom to 5% (w/v) at the top of the tube. The gradient was prepared as described by Britten & Roberts (1960). Portions (0.2 ml.) of the stock solution of the pigment (23 mg./ml.) at pH 10.0 were layered on the top of the sucrose solutions and the tubes were centrifuged for 316 min. at 39 460 rev./min. (170 600 *g* at tip) at 18°. At the end of the run, a hole was pierced in the bottom of each tube and about 2.5 ml. of the colourless bottom fraction was run out. Seven further fractions were collected. Each fraction was suitably diluted with the glycine buffer and the extinctions were determined at 417 and 280 $m\mu$.

Zone electrophoresis on cellulose acetate strips. Oxoid-brand strips (18 cm. $\times$ 2.5 cm.) were soaked in 0.07 M-veronal buffer, pH 8.6 and *I* 0.06, and 5 μ l. of protein solution (approx. 27 mg./ml.) was applied as a stripe. After 2 hr. electrophoresis at 14 v/cm. the strips were dried, stained with Lissamine Green (see Smith, 1960) and photometrically scanned for reflected light with a Zeiss scanning densitometer (model ERI-10), the 600 $m\mu$ filter being used.

Crude extracts revealed four bands, of which only one contained ferrohaem, as judged from original colour and from pseudo-peroxidase activity (King & Wootton, 1956). Densitometric measurements of stained bands showed this band to represent about 70% of the total. The purified material displayed this band only.

Starch-gel electrophoresis. Gels were prepared from hydrolysed starch (supplied by Connaught Medical Laboratories, Toronto, Canada), as described by Wise, Ballard & Ezekiel (1963). For vertical electrophoresis purified pigment was applied in slots in the gel and a potential gradient of 9-16 v/cm. was applied. The gels were stained with Naphthalene Black as described by Rubinstein, Owen & Larsen (1960). Haem-protein bands were identified as described in the preceding sub-section.

Electrophoretic mobility. The mobilities of purified pigment were measured by the moving-boundary method in a Perkin-Elmer model 38A electrophoreter. The pigment solution was dialysed overnight at 0° against buffer and diluted with the same buffer to a concentration of 9.2 mg./ml. for electrophoresis at 0 $\pm$ 0.5°.

This method could not be used at pH less than 6.0 because of flocculation of the protein. To measure mobilities at low pH, therefore, 0.1 ml. of the purified pigment solution (3.3 mg./ml.) was added with thorough mixing to 2 ml. of buffer. Movement of the flocculated particles in a 3 mm. diam. horizontal tube (potential gradient 5 v/cm.) at room temperature was observed through a light-microscope equipped with eye-piece graticule.

Spectrophotometry. Extinctions were measured with a Unicam SP.500 spectrophotometer (Cambridge Instrument Co.).

Determination of haem and iron. The haem contents of pigment solutions were estimated by Rimington's (1942) pyridine haemochromogen method, except that extinctions were measured spectrophotometrically at 525 $m\mu$. Purity of the recrystallized haem (from ox blood) employed as standard in the haem assays was checked by assay of its iron content [8.53 $\pm$ 0.08 (S.E.M.) (nine estimations); theoretical value, 8.567%]. Iron was estimated by densitometry of ferric thiocyanate after wet-ashing (Natelson, 1961).

RESULTS

Table 1 shows values of the ratios of extinction at 417 m μ and 280 m μ of carbon monoxide-saturated crude extract and solutions of sedimented pellets obtained in the purification procedure. Although a steady value was not reached, further sedimentation was not carried out because the third pellet was found electrophoretically and ultracentrifugally to contain a substantially pure product.

In the ultracentrifuge the purified pigment in buffer, pH 6.60 and I 0.10, at 17.9°, sedimented as a single species. The sedimentation coefficient corrected to standard conditions, $S_{20,w}^0$, was 58.2 s; and the diffusion coefficient, likewise corrected, $D_{20,w}$, was 2.06×10^{-7} cm.²/sec. From these data, assuming a partial specific volume of 0.73, the mol. wt. was calculated to be 2.4×10^6 .

Electrophoretic studies at pH 8.12 and I 0.21 likewise showed only single ascending and descending boundaries, considered to be those of the 58 s species.

Table 1. *Purification of the pigment by successive centrifugations*

Details are given in the text. Ratios of the extinctions of CO-saturated solution samples are recorded.

Steps in procedure	$E_{417m\mu}/E_{280m\mu}$
Crude extract	1.42
First pellet	1.83
Second pellet	2.24
Third pellet	2.44

Fig. 1. Linear regression of pH on electrophoretic mobilities of the purified pigment (●) and the pigment in freeze-dried crude extract (▲) (9.2 mg. of protein/ml.) in 0.15 M-NaCl—0.02 M-sodium phosphate buffers at $0 \pm 0.5^\circ$.

Fig. 1 shows the variation with pH at $0 \pm 0.5^\circ$ of the electrophoretic mobility (ascending boundary) of what was judged to be the 58 s protein. Extrapolation, assuming linear regression of pH on mobility, yields an isoelectric point of 3.93 ± 0.6 (S.E.M.). The protein begins to flocculate at a little below pH 6.0, redissolving at very low pH values but with change of colour from red to brown, suggestive of transformation of ferrohaem to ferrihaem or even of haem dissociation, and boundary mobilities were therefore not determined in this region. Particle electrophoresis in 0.15 M-sodium chloride—0.02 M-sodium acetate buffers, I 0.16, at 21.5° gave a value of 4.60–4.65 for freshly prepared floccula, a value apparently independent of particle size for the range 0.002–0.02 mm. mean dimension. This value is regarded as much more reliable than that from Fig. 1. Aging of the particles for several hours at 21.5° or for 20 hr. at 4° raised the isoelectric point to about 4.8.

In Fig. 2 are shown (a) the boundaries found on centrifuging a solution of the pigment at very low ionic strength, pH 6.7 (unbuffered), and (b) the return to virtually a single boundary when the ionic strength was raised. The runs were made simultaneously with a pair of cells in the rotor, one (a) containing a wedge window. The photograph was taken without the red filter, resulting in some obscuring of boundaries to the right but affording support for the view that the faster-sedimenting species in (a) were also red. The lightest species in (a) obviously corresponds to the predominant species in (b). It also corresponds with the single species seen at pH 6.60 and therefore is the 58 s protein. The corresponding values of S for the other two species were estimated to be 97 s and about 150 s respectively. They can be regarded as reversibly dissociable association complexes of the 58 s

Fig. 2. Sedimentation patterns showing (a) the partial association of the 58 s protein into 97 s and 150 s species at I 0.005 (NaCl) and (b) return to virtually the single 58 s species when I was raised to 0.13 (NaCl). The conditions used were: 2.9 mg. of protein/ml., pH 6.7 (unbuffered), at 25.4° . The photographs were taken (bar angle, 50°) 8 min. after the rotor had attained full speed, 39360 rev./min. (113150 g).

protein. In unbuffered solutions of I 1.5 and 3.0 (sodium chloride) the 58s species alone was discernible.

Absorption spectra of the pigment (oxygenated) and some derivatives, at pH and I values that would ensure the existence of the former essentially as the 58s protein, are shown in Fig. 3. The deoxygenated pigment was prepared by adding a slight excess of solid sodium dithionite to solutions of the oxygenated one, and the carbon monoxide derivative by treatment with coal-gas that had been bubbled through concentrated sulphuric acid and then water. Patel & Spencer (1963) have drawn attention to specific effects of individual reducers like dithionite and ammonium sulphide on the spectra of haem-protein pigments. However, the band extinctions with all three forms of pigment had maxima at wavelengths conforming broadly with those for vertebrate haemoglobins of diverse origin (Lemberg & Legge, 1949).

Confirmation of the protohaem nature of the prosthetic group followed from the identity of the absorption spectra of pyridine haemochromogens prepared from the pigment and from recrystallized haemin (from ox blood). Both had band maximum E values at 557, 525 and 480 $m\mu$ under the conditions of preparation. Replicate haem assays on two batches of purified (heat-dried) pigment gave mean values 2.23 and 2.25%. The overall mean, 2.24%, had S.E.M. ± 0.008 (four estimations). The non-haem iron represented 16 and 28% respectively

of the total iron present in the two batches, and was regarded as a contaminant.

Electrophoresis of the 58s protein from slots in starch gel prepared with lithium borate-tris citrate solution, pH 8.20 (see Wise *et al.* 1963), yielded unexpected and confusing results attributable, perhaps, in part to some change in buffer composition resulting from migration of ions from the electrode compartments in this discontinuous system. Two non-haem-containing protein bands soon left the slots and migrated towards the anode. These were followed after some time by two haem-protein bands, the second of which largely exhausted the slots of haem-protein and, having emerged, migrated at about the same rate as its predecessor. Freezing, thawing and pressing of gel sections yielded insufficient material for profitable study.

These results suggested some dissociation of the pigment at alkaline pH. This was unexpected, the other electrophoretic studies having shown no such effect even at pH values up to 8.6 (on cellulose acetate strips). To clarify the position ultracentrifuge studies at alkaline pH values were made and evidence of dissociation was sought.

Fig. 4 demonstrates the dissociation of the protein at pH 10 as seen in the ultracentrifuge. A trace of the 58s species was still present with most of the protein now appearing in a form with S 9.2s.

Fig. 3. Absorption spectra of the erythrocrucorin and derivatives in 0.15 M-NaCl-0.05 M-sodium phosphate, pH 7.00. The concentrations of pigment used were: 32.9 $\mu\text{g./ml.}$ for wavelengths less than 450 $m\mu$, 329 $\mu\text{g./ml.}$ for wavelengths greater than 450 $m\mu$ —, Oxygenated form; — · —, deoxygenated form; — — —, carbon monoxide derivative.

Fig. 4. Sedimentation diagrams of the dissociated erythrocrucorin, 5.6 mg./ml., in 0.05 M-glycine-sodium glycinate, pH 10.0 and I 0.025, at 16.6°. The photographs were taken, with the red filter, at intervals after the rotor had attained full speed, 50 740 rev./min. (187 100 g): (a) after 8 min., 9s boundary and a trace of 58s visible (bar angle, 55°); (b) after 48 min., the emergence of a diffuse peak of 2-4s after 9s (bar angle, 45°).

Table 2. Zone centrifugation at pH 10.0 of 0.2 ml. of solution of erythrocrucorin layered on 15 ml. of sucrose-gradient medium

Details are given in the text. Extinctions at 417 and 280 m μ and their ratios are shown for each fraction.

Fraction	Approx. vol. (ml.)	$E_{280\text{m}\mu}$	$E_{417\text{m}\mu}$	$E_{417\text{m}\mu}/E_{280\text{m}\mu}$ ratio
1	2.5	0.192	0.258	1.34
2	1.9	1.12	3.52	2.90
3	1.9	3.59	10.95	3.06
4	1.9	5.87	18.0	3.01
5	1.3	2.70	7.67	2.84
6	1.9	0.840	1.23	1.40
7	1.9	0.998	0.757	0.76
8	1.9	1.08	0.650	0.60
Un-fractionated	—	—	—	2.55

There was a slowly-sedimenting diffuse peak of *S* 2–4 s, which was shown in a zone-ultracentrifugation run to be due to non-haem-containing protein. At pH values between 8.29 and 10 the dissociation was less complete and showed variation with the ionic strength and composition of the buffer.

The zone-ultracentrifugation results at pH 10 are shown in Table 2, extinctions being computed back to no dilution on an assumed validity of Beer's law. Two overlapping protein distributions are revealed: the major one, with a peak in the region of fractions 3–4, being haem-protein and the other, concentrated near the tip of the tube, being haem-free and corresponding probably to the fast-moving non-haem-containing protein bands encountered in starch-gel electrophoresis at pH 8.20. The 58 s protein could not be reconstituted from the products of dissociation at pH 10.0 by adjusting the pH to 6.6.

DISCUSSION

Svedberg & Eriksson (1933) applied the term 'erythrocrucorin' to the group of invertebrate haem-proteins occurring free in the blood plasma and having a high molecular weight (approx. 3×10^6) and a low isoelectric point [Svedberg (1933) cited values for five species of worms ranging from 4.56–5.28]. Keilin & Hartree (1951) criticized the use of this term on the grounds that when all haemoglobins are considered the values for the physical properties lie in continuous series and no line can reasonably be drawn between one group and another. Without prejudging this issue, which calls for a more detailed knowledge of these proteins than we so far possess, it is clear that the pigment of *M. sanguinea*, which has a protohaem prosthetic group, a molecular weight (for the 58 s species) of 2.4×10^6

Table 3. Absorption maxima and corresponding millimolar extinction coefficients in terms of haem of the erythrocrucorin and derivatives

The conditions were as given in Fig. 3.

	Absorption bands	
	Maxima (m μ)	ϵ_{mM}
Oxygenated form	576	14.2
	540	14.8
	414	122
Deoxygenated form	553	12.3
	428	122
Carbon monoxide derivative	570	16.0
	537	16.9
	417	170

and an isoelectric point of 4.63, is a typical 'erythrocrucorin' in the sense of Svedberg & Eriksson (1933). Svedberg & Eriksson-Quensel (1934) assumed all haemoglobins and erythrocrucorins to be built up of similar units, each of mol. wt. approx. 17 000 and each containing a single haem unit. This would imply a similar haem content, about 4%, for all such pigments; but the *M. sanguinea* pigment has considerably less than this, 2.24%. When calculated in terms of haem concentration, however, the extinctions correspond rather closely with those of vertebrate haemoglobins, except that values for the carbon monoxide derivative are somewhat higher. Values at the respective band maxima are shown in Table 3.

In solutions of ionic strength and pH values that are likely to be 'physiological' for the worm, the erythrocrucorin exists essentially as the 58 s species, tentatively accorded a molecular weight of 2.4×10^6 and thus computed to contain some 87 haem units. The dissociable association complexes, the 97 s and 150 s species, encountered at low ionic strength have not hitherto been described as forms of erythrocrucorins. It is suggested that they are respectively dimer and tetramer modifications of the 58 s protein.

The form of dissociation of the 58 s species itself is, at least at pH 10.0, irreversible. The haem-containing moiety has an *S* value (9.2 s) suggesting a size about one-sixteenth that of the 58 s species, but it is enriched in haem by the removal of non-haem material.

We have had no opportunity as yet of subjecting the materials to electron-microscopic examination. In the electron-microscopy of some 60 s erythrocrucorins of other origin and their split products, Levin (1963) has described as his largest species double 'pancakes', the one flatly superimposed a short distance above the other, each comprising

six wedge-shaped units in radial array, the two pancakes being linked axially by material that appears not to be identical with that of the wedge-shaped units. Among the split products he often encountered isolated wedge-shaped units.

If the double-pancake structure should prove to typify our 58 s species, our 9 s species might correspond to individual wedge-shaped units, the 2-4 s species perhaps representing the axial linking material. If the picture presented by our experiments with starch-gel electrophoresis is disregarded we have identified only one haem-containing dissociation product (9 s) of the 58 s species. However, in Levin's (1963) work there seemed to be several.

The pronounced colour change exhibited by *M. sanguinea* when withdrawn from its tunnels in dark mud and exposed to air appears to involve no pigment other than the one we have described. The change may be associated in part with changes in extinction values with degree of oxygenation of the erythrocruorin and in part with a redistribution of blood flow through the vascular system of the organism.

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