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Quaternary structure of erythrocruorin from the nematode *Ascaris suum*

Evidence for unsaturated haem-binding sites

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The quaternary structure of erythrocruorin from the nematode *Ascaris suum* was studied. The native protein had a sedimentation coefficient, at a protein concentration of 1 mg/ml, of 11.6 ± 0.3 S and an M_r, as determined by sedimentation equilibrium, of 332000 ± 17000. SDS/polyacrylamide-gel electrophoresis gave one band with a mobility corresponding to an M_r of 43000 ± 2000. The M_r of the polypeptide chain was determined to be 41600 ± 1500 by sedimentation equilibrium in 6 M-guanidinium chloride and 0.1 M-2-mercaptoethanol. Cross-linking with glutaraldehyde followed by SDS/polyacrylamide-gel electrophoresis yielded a maximal number of eight bands. The haem content of *Ascaris* erythrocruorin was observed to vary from one preparation to another. This finding was shown to be due to non-realization of the full binding capacity for haem. By titration with haemin, the haem content was found to attain a maximal value of 2.86 ± 0.14%, corresponding to a minimal M_r per haem group of 21600 ± 1000. Our findings indicate that *Ascaris suum* erythrocruorin is composed of eight identical polypeptide chains, carrying two haem sites each.

INTRODUCTION

Erythrocruorin is the name given to extracellular haemoglobin found in species of the phyla Annelida, Arthropoda, Mollusca and Nematoda (Hendrickson, 1977; Chung & Ellerton, 1979; Terwilliger, 1980; Wood, 1980; Vinogradov, 1985). Nematode erythrocruorins (Lee & Smith, 1965), perhaps with the exception of erythrocruorin from the species *Ascaris suum*, have not been studied to the same extent as erythrocruorins from other phyla. In the past, there has been confusion in the literature regarding the names of the nematodes *Ascaris lumbricoides* and *Ascaris suum*. According to current nomenclature (e.g. Marshall & Williams, 1972), the parasite of man is called *A. lumbricoides*, and that of the pig is named *A. suum*. Erythrocruorin from *A. suum* has been the subject of a number of investigations with respect to its spectral, O_2-binding and structural properties (Davenport, 1949; Hamada *et al.*, 1963; Smith & Lee, 1963; Wittenberg *et al.*, 1965; Okazaki *et al.*, 1965; Okazaki & Wittenberg, 1965). The study by Okazaki *et al.* (1965) indicated that a molecule of *A. suum* erythrocruorin has an M_r of 328000 and is composed of eight polypeptide chains. A minimal M_r per haem group of 40600 was also determined. This value is considerably higher than the values for the minimal M_r per haem group of about 15000–25000 commonly found for erythrocruorins from other invertebrate species.

The present paper is concerned with a structural study of *Ascaris suum* erythrocruorin. Our results show that, in the native erythrocruorin isolated from the worm, the full binding capacity for haem is not realized. This finding affords an explanation for the uniquely high minimal M_r per haem group previously reported for this erythrocruorin.

EXPERIMENTAL

Preparation of erythrocruorin

Ascaris suum worms were brought live to the laboratory, rinsed with distilled water and dried in the air. Each worm was separately transferred to a Petri dish and a longitudinal cut was made along the fore part of the body. Pooled haemolymph from 30–60 worms, made 0.1 mM in phenylmethanesulphonyl fluoride, was repeatedly centrifuged for 20 min at low speed to remove particulate matter. The resulting clear fluid was centrifuged at 153000 g ($r_{av.}$ 5.9 cm) for 6 h and the supernatant was discarded. The tube containing the pellet and 1 ml of added cold distilled water was left for 24 h at 4 °C. The contents of the tube were centrifuged at 60000 g ($r_{av.}$ 5.9 cm) for 30 min to separate the red solution of erythrocruorin from white material insoluble in distilled water. The erythrocruorin solution was mixed with an equal volume of 50 mM-NaCl/25 mM-Tris/HCl buffer, pH 7.5, referred to below as 'loading buffer', and centrifuged at 153000 g for 6 h. The red pellet was dissolved in 0.2 ml of loading buffer and the solution was applied to a DEAE-Sephadex A-50 column (1.5 cm × 16 cm) equilibrated with loading buffer. Elution was performed with 250 mM-NaCl/25 mM-Tris/HCl buffer, pH 7.5. The coloured fractions containing erythrocruorin were centrifuged at 153000 g for 6 h and the supernatant was discarded. Dissolution of the pellet in about 0.1 ml of loading buffer gave a concentrated stock solution of purified erythrocruorin. All work was done at 4 °C.

Amino acid analysis

Amino acid analysis was carried out as described by Spackman *et al.* (1958) in a Durrum 500 amino acid analyser. Samples were hydrolysed with 6 M-HCl in evacuated sealed tubes for 24, 48 and 72 h at 110 °C. For the determination of tryptophan, hydrolysis was carried out in 4 M-methanesulphonic acid containing 0.2% 3-(2-aminoethyl)indole as described by Simpson *et al.* (1976). Cysteine and cystine were determined as cysteic acid (Hirs, 1956).

Protein content and haem determination

Protein content was determined by dry-weight measurement (Ilan & Daniel, 1979). Haem determination was

performed by the pyridine haemochromogen difference-spectrum method (Falk, 1964).

Titration with haemin

Titration of erythrocruorin with haem [iron(III) haem] was monitored spectrophotometrically in a Cary 118C spectrophotometer at 20 °C (Yonetani, 1967). Small volumes of haemin solution, in 0.1 M-NaOH, were added to a measured volume of erythrocruorin solution in a quartz cuvette of 1 cm light-path. Readings of the A_{408} were taken 4 min after each addition. The haem content of the erythrocruorin at the end of the titration was calculated from the equation:

$$\%(\mathrm{w/w})\ \mathrm{haem} = 100(w_h + VmM)/(w_e + VmM) \quad (1)$$

where w_e is the weight of erythrocruorin, w_h is the weight of constituent haem, V is the volume of haemin solution, of molarity m, added up to the end point of the titration, and M is the M_r of a haem group.

SDS/polyacrylamide-gel electrophoresis

This was performed on 5%-polyacrylamide gels as described by Weber *et al.* (1972) in the presence of 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol. M_r values corresponding to band mobilities were estimated from a calibration curve obtained with protein markers of known M_r.

Cross-linking

Cross-linking was carried out by treating erythrocruorin with the bifunctional reagent glutaraldehyde. At various times samples were withdrawn from the reaction mixture and treated with hot SDS solution to stop the reaction. The cross-linked species were analysed by SDS/polyacrylamide-gel electrophoresis.

A 20 μl portion of 1% (v/v) glutaraldehyde in water was added to 200 μl of 2 mg/ml solution of erythrocruorin in 0.01 M-phosphate buffer, pH 7.0, and the mixture was incubated at 20 °C. After 1 min, and every 2 min thereafter, a 20 μl sample was removed to an Eppendorf tube containing 90 μl of 0.01 M-phosphate buffer containing 1% SDS and 1% 2-mercaptoethanol. The mixture was heated at 100 °C for 2 min, and a 50 μl sample was used for SDS/3.3%-polyacrylamide-gel electrophoresis.

Ultracentrifugation

Ultracentrifugation was performed with a Beckman model E analytical ultracentrifuge. Sedimentation velocity was carried out with schlieren phase-plate optics. Sedimentation equilibrium was 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, $\mathrm{d}(\ln c)/\mathrm{d}r^2$, 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 M_r at r, $\overline{M}_{w,r}$, according to the relation:

$$\overline{M}_{w,r} = [2\boldsymbol{R}T/\omega^2(1-\bar{v}\rho)]\cdot \mathrm{d}(\ln c)/\mathrm{d}r^2 \quad (2)$$

where $\boldsymbol{R}$ 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 M_r in 6 M-guanidinium chloride solution, $\bar{v}$ in the expression for $\overline{M}_{w,r}$ 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 (for details see Ilan & Daniel, 1979). The value of ϕ' in guanidinium chloride solution was calculated as described by Lee & Timasheff (1979).

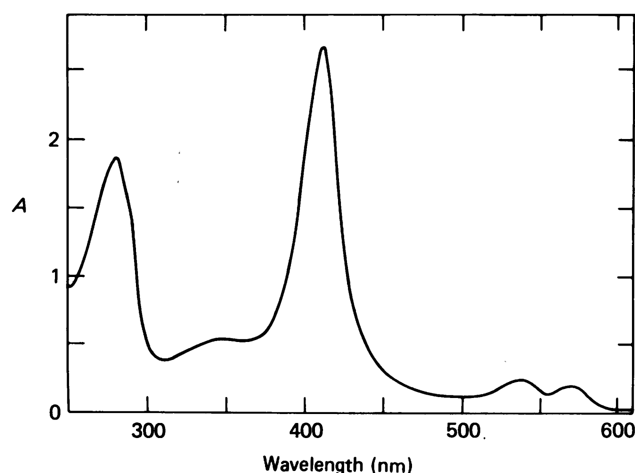

Fig. 1. Absorption spectrum of *Ascaris suum* erythrocruorin

Protein concentration was 1 mg/ml. Conditions: solvent, 250 mM-NaCl/25 mM-Tris/HCl buffer, pH 7.5; light-path, 1 cm.

Table 1. Amino acid composition of *Ascaris suum* erythrocruorin

The values are given as number of mol of residue per 21600, the minimal M_r per haem group. Values for all amino acids are averages for 24 h, 48 h and 72 h hydrolysis, except valine and isoleucine, for which values of maximal recovery are given.

	Composition	
Amino acid	(%, by wt.)	(mol of residue/ 21600 g)
Aspartic acid	9.54	17.9
Threonine	4.21	9.0
Serine	1.84	4.6
Glutamic acid	18.16	30.4
Proline	2.28	5.1
Glycine	1.74	6.6
Alanine	4.05	12.3
Valine	2.52	5.5
Methionine	2.59	4.3
Isoleucine	2.35	4.5
Leucine	6.80	13.0
Tyrosine	4.98	6.6
Phenylalanine	6.25	9.2
Histidine	9.04	14.2
Lysine	8.79	14.8
Arginine	7.99	11.0
Half-cystine	1.92	4.0
Tryptophan	2.15	2.5
Amide NH_3	—	21.5
Haem	2.86	1.0

RESULTS

Characterization of *Ascaris* erythrocruorin

Erythrocruorin prepared by the method described gave a single symmetrical boundary on sedimentation in the analytical ultracentrifuge. The red colour characteristic of haemoglobin always migrated with the schlieren peak, as expected for a pure protein. The various preparations showed uniformity in their sedimentation coefficients. At a concentration of 1 mg/ml in 0.1 M-phosphate buffer, pH 6.8, an $s_{20,w}$ value of 11.6 ± 0.3 S was determined.

The absorption spectrum of *Ascaris* erythrocruorin was of an oxy derivative type. Absorption maxima occur at 280 (protein), ~ 345, 412 (Soret), 538 and 573 nm. The ratio of the absorbances at 412 and 280 nm, A_{412}/A_{280}, varied from one preparation to another: the maximal value was 1.43 (Fig. 1). Preparations with much lower ratios (as low as about 1) were, however, not uncommon.

The amino acid composition is presented in Table 1. Noticeable is the high content of acidic amino acids, aspartic acid and glutamic acid.

The M_r was determined by meniscus-depletion sedimentation equilibrium. The reciprocal of the weight-average M_r, $1/\overline{M}_{w,r}$, was found to be independent of protein concentration (Fig. 2*a*). From the plot, a value of 332000 ± 17000 for the M_r of *Ascaris* erythrocruorin was obtained. In this calculation, a value of $\bar{v} = 0.717$ ml/g experimentally measured by us in water at 20.1 °C was used.

Characterization of the polypeptide chain

Fig. 3 presents typical results of SDS/polyacrylamide-gel electrophoresis. The protein migrates in virtually one band, with a mobility corresponding to an M_r of 43000 ± 2000. Faint minor bands with mobilities corresponding to M_r values of 87000 and 130000, twice and thrice the value for the main band, could be observed at high protein loading.

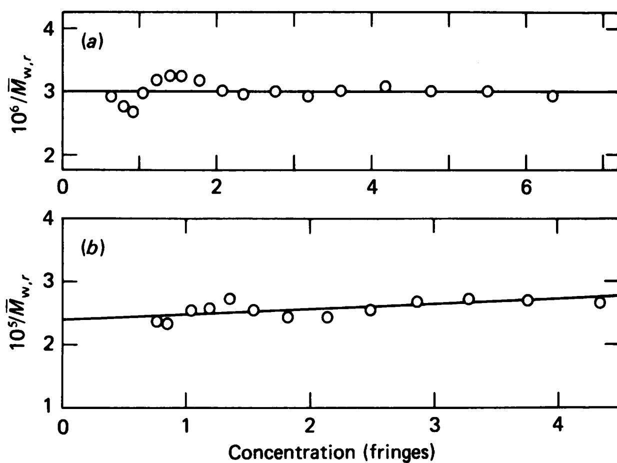

Fig. 2. Sedimentation equilibrium of *Ascaris suum* erythrocruorin

(*a*) Reciprocal M_r of native erythrocruorin as a function of protein concentration. Protein concentration was initially approx. 1 mg/ml in 0.1 M-phosphate buffer, pH 6.8. Conditions: speed, 10086 rev./min; temperature, 17.8 °C. (*b*) Reciprocal M_r of erythrocruorin in guanidinium chloride as a function of protein concentration. Protein concentration was initially approx. 1 mg/ml in solution containing 6 M-guanidinium chloride and 0.1 M-2-mercaptoethanol, pH 8.8. Conditions: speed, 36085 rev./min; temperature, 19.8 °C.

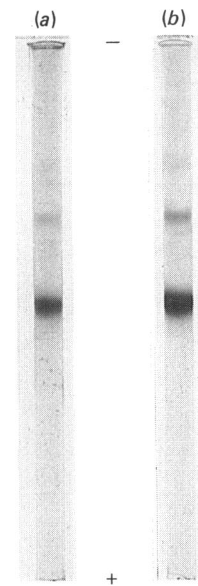

Fig. 3. SDS/polyacrylamide-gel electrophoresis of *Ascaris suum* erythrocruorin

Samples contained 2 μg (*a*) and 5 μg (*b*) of protein.

The M_r of the polypeptide chain was determined by sedimentation equilibrium in solution containing 6 M-guanidinium chloride and 0.1 M-2-mercaptoethanol. A linear extrapolation to zero concentration of a plot of the reciprocal of the weight-average M_r against protein concentration (Fig. 2*b*) gave $\overline{M}_w = 41600 \pm 1500$. In this determination, we used a value for the specific volume $\phi' = 0.705$ ml/g, calculated from the amino acid composition.

Cross-linking

Ascaris erythrocruorin was exposed to glutaraldehyde at pH 7.0, and cross-linked products were analysed by SDS/polyacrylamide-gel electrophoresis. Before exposure to the cross-linking agent, the typical electrophoretic pattern showing essentially one band was observed. At progressively increasing times of reaction, bands of lower mobility appeared. A maximal number of eight bands was found after 9 min of reaction (Fig. 4*a*). At still longer times, the relative intensity of the low-mobility bands increased, but no new bands could be detected.

On the basis of their mobilities, the fastest-moving band and the band migrating next to it (Fig. 4*a*) can be identified respectively with single polypeptide chains of M_r value M_1 and cross-linked pairs of polypeptide chains with M_r value $M_2 = 2M_1$. In the order of decreasing mobilities, one may assume that the next band corresponds to three cross-linked polypeptide chains, with $M_3 = 3\,M_1$. Generalizing, the *i*th band ($1 \leqslant i \leqslant 8$) would correspond to *i* cross-linked polypeptide chains, with M_r value $M_i = iM_1$. Fig. 4(*b*) presents a plot of $\log(M_i/M_1)$ versus band mobility u_i, where the relationship $M_i = iM_1$ is assumed. The linearity of the plot affords a confirmation of the correctness of

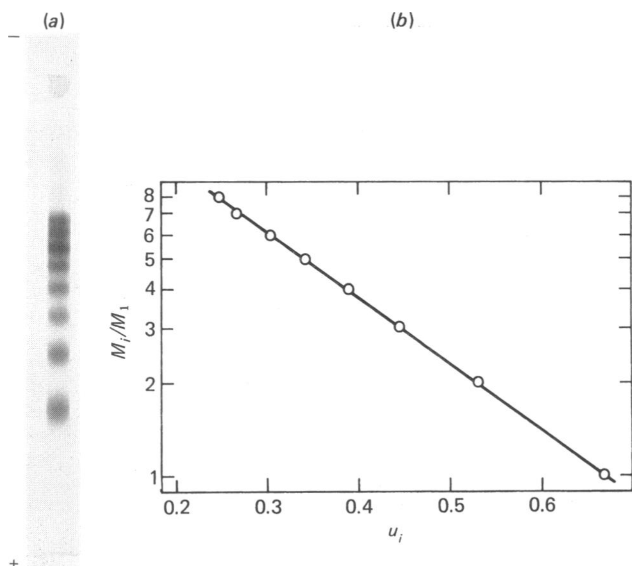

Fig. 4. Cross-linking of *Ascaris suum* erythrocruorin by glutaraldehyde

(*a*) SDS/polyacrylamide-gel-electrophoretic pattern of cross-linked species after 13 min of cross-linking. For experimental details see the text. (*b*) Graphical demonstration of the linear logarithmic dependence of the ratio M_i/M_1, where M_1 and M_i are the M_r values corresponding to the monomeric and *i*th band, on band mobility, u_i, assuming the relation $M_i = iM_1$ $(1 \leqslant i \leqslant 8)$.

the assumption made, indicating that the *i*th band may indeed be associated with *i* cross-linked polypeptide chains.

Haem content and haem-binding capacity

The haem content of a number of *Ascaris* erythrocruorin preparations was determined. As with the absorbance ratio A_{412}/A_{280}, the haem content was found to be variable. A direct proportionality was found between the percentage of haem and the absorbance ratio:

$$\%\ (\text{w/w})\ \text{haem} = k \cdot A_{412}/A_{280} \qquad (3)$$

where $k = 1.03$.

The capacity of native *Ascaris* erythrocruorin for binding extraneous haem was determined by titration with haemin. The results of a typical experiment are presented in Fig. 5(*a*) as a plot of the A_{408} against haemin added. Two intersecting straight lines can be fitted to the data at low and high titrant additions. The slope of the line corresponding to high additions of titrant is close to that obtained on addition of haemin to buffer. This behaviour is indicative of haemin binding to erythrocruorin. To obtain unequivocal evidence for the binding, the reaction mixture at the end of haemin addition was dialysed against buffer. The spectrum of the dialysed solution and, for comparison, the spectrum of the solution before titration are presented in Fig. 5(*b*). A remarkable increase in the Soret absorbance of the dialysed solution is evident. Moreover, the A_{408} at the end of titration is practically equal to that of the solution after dialysis. This result shows that the end point of the spectrophotometric titration corresponds to the point where the take-up of non-diffusible haemin is completed.

The haem content of haemin fully saturated *Ascaris*

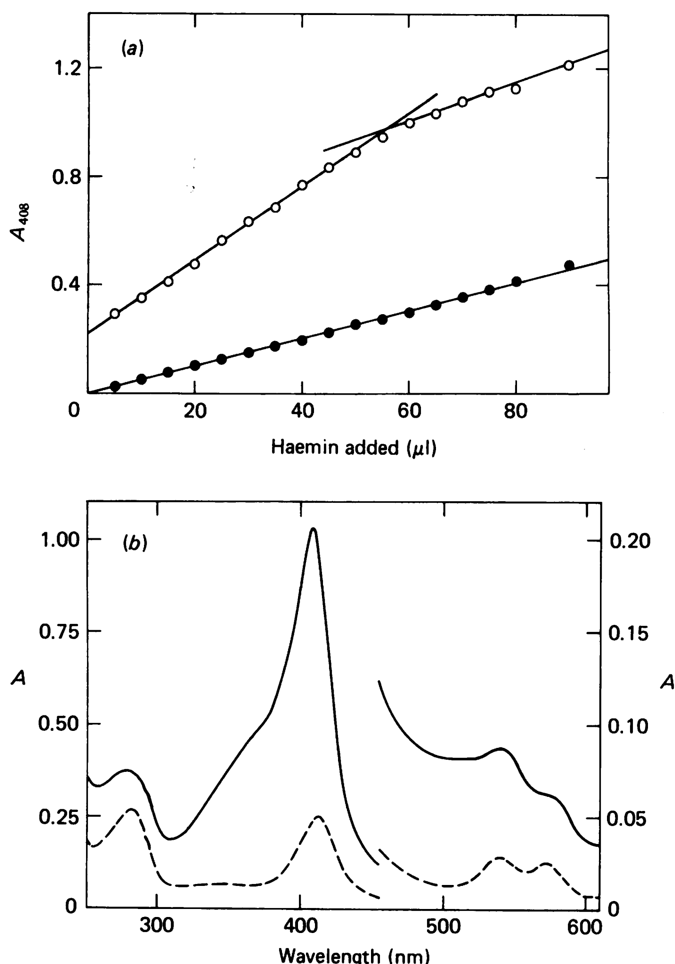

Fig. 5. Spectrophotometric titration of *Ascaris suum* erythrocruorin with haemin

(*a*) A_{408} of erythrocruorin (0.14 mg/ml) in 3 ml of 0.1 M-phosphate buffer, pH 6.8 (○), and of 3 ml of the same buffer (●) after successive additions of the indicated volumes of 0.25 mM-haemin in 0.1 M-NaOH. (*b*) Absorption spectra of erythrocruorin solution at start of titration (----) and after haemin addition and dialysis against 0.1 M-Tris/HCl buffer, pH 7.5 (——). The right-hand scale applies to wavelengths greater than 450 nm.

erythrocruorin was calculated from the end points of titration experiments according to eqn. (1). Three determinations with different erythrocruorin preparations gave $2.86 \pm 0.14\%$ (w/w) haem. This value corresponds to an M_r per haem group of $21\,600 \pm 1000$.

DISCUSSION

One of the criteria used in the purification of a haemoprotein is the haem/protein absorbance ratio. A ratio that does not increase on inclusion of further purification steps is usually taken as an indication for purity. Difficulties were encountered in applying this criterion to *Ascaris* erythrocruorin. Here, values of A_{412}/A_{280} ranging from about 1 to 1.43 were obtained, suggesting that the *Ascaris* erythrocruorin preparations, maybe with the exception of that with the highest A_{412}/A_{280} ratio, contain a protein contaminant. Sedimentation profiles and SDS/polyacrylamide-gel-electrophor-

Table 2. Summary of M_r data for *Ascaris suum* erythrocruorin

M_r of the native molecule	332000±17000
M_r of the polypeptide chain from:	
SDS/polyacrylamide-gel electrophoresis	43000±2000
Sedimentation equilibrium in guanidinium chloride	41600±1500
Minimal M_r from haem content	21600±1000
No. of haem sites per chain	1.96±0.12
No. of polypeptide chains per molecule	7.85±0.52

etic patterns of the various erythrocruorin preparations showed, however, no indication of impurity. We were therefore led to the conclusion that the variable absorbance ratio is to be attributed to a variable haem content due to incomplete realization of the haem-binding capacity of the protein. This conclusion was confirmed in the haemin-titration experiments.

A criterion for the specificity of binding of haem to globin, and in general of a ligand to a protein, is the sharpness of the end point in the titration curve. The absence of a sharp end point indicates the presence of different binding sites of different affinities, i.e. non-specific sites (Chiancone *et al.*, 1978). A sharp end point indicates, in contrast, specific binding, as is illustrated in the titration of *Scapharca* apohaemoglobin with haemin (Verzili *et al.*, 1982). The sharp end point observed in the titration curve of *Ascaris* erythrocruorin with haemin (Fig. 5*a*) demonstrates that the binding of extraneous haem is specific. The spectrum of the fully haemin-saturated erythrocruorin (Fig. 5*b*) resembles that of cytochrome *c* peroxidase reconstituted from apoenzyme by recombinaiton with haemin (Yonetani, 1967). In both spectra a shoulder at 370–380 nm, indicative of the presence of a met derivative, can be seen.

As mentioned in the Introduction, a minimal M_r per haem group of 40600 was determined for *Ascaris* erythrocruorin by Okazaki *et al.* (1965). Their determination was based on the haem content of erythrocruorin as prepared from the haemolymph of the animal. Our study shows that the haem content of such preparations is variable and thus cannot serve as a basis for the calculation of the minimal M_r value. The minimal M_r of 21600 determined in the present study was derived from the haem content of fully haem-saturated erythrocruorin, which is constant.

The haem contents of erythrocruorins from arthropod, mollusc and annelid origin examined to date correspond to minimal M_r values per haem group of about 15000–25000. The minimal M_r per haem group determined in the present study for *Ascaris* erythrocruorin falls within this range. This finding emphasizes the resemblance of nematode erythrocruorin to other invertebrate erythrocruorins.

The results from sedimentation equilibrium in 6 M-guanidinium chloride show the presence of polypeptide chains with an M_r of 41600. The fact that the bands obtained in SDS/polyacrylamide-gel electrophoresis correspond to this M_r value and multiples thereof indicates that *Ascaris* erythrocruorin is composed of identical, or nearly identical, polypeptide chains. On a weight basis (Table 2), each polypeptide chain carries two haem groups.

The sedimentation coefficient and M_r determined in the present work for *Ascaris suum* erythrocruorin, 11.6 S and 332000, are practically identical with the values, 11.8 S and 328000, reported for erythrocruorin from the same species by Okazaki *et al.* (1965). The number of polypeptide chains determined from the M_r values of the whole molecule and of the constituent polypeptide chain is eight (Table 2), in complete agreement with the value reported by Okazaki *et al.* (1965). The homo-octameric structure of *Ascaris* erythrocruorin is vividly demonstrated in this study by our obtaining, in cross-linking experiments with glutaraldehyde, cross-linked species with the M_r relationships predicted for an eight-chain molecule.

From the present study, *Ascaris* erythrocruorin emerges as a molecule composed of eight polypeptide chains carrying two haem sites each. The occurrence of polypeptide chains carrying two or more haem groups in arthropod and mollusc erythrocruorins is known (Terwilliger *et al.*, 1976; Ilan & Daniel, 1979; Dangott & Terwilliger, 1979, 1980; Ilan *et al.*, 1981, 1982). It has been suggested that the multi-haem polypeptide chains of invertebrate erythrocruorins are composed of repeating covalently linked single-haem sequences (Ilan *et al.*, 1981). The existence of two-haem polypeptide chains in *Ascaris* erythrocruorin may likewise be due to the occurrence of a repetition in the polypeptide chain sequence of this nematode erythrocruorin.

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