3. *d*-Chaulmoogramide is converted into the *d*-chaulmoogronitrile by means of thionyl chloride.

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[CONTRIBUTION FROM THE LABORATORY OF PHYSICAL CHEMISTRY OF THE UNIVERSITY OF UPSALA]

# The Molecular Weight of Erythrocruorin

BY THE SVEDBERG AND INGA-BRITTA ERIKSSON

The stable native proteins so far studied in this Laboratory by means of the ultracentrifugal methods can with regard to molecular weight be divided into two large groups: the hemocyanins with molecular weights of the order of millions and all other proteins with molecular weights from about 35,000 to about 200,000. A few preliminary determinations which we had the opportunity to carry out some time ago on the respiratory protein chlorocruorin from the blood of the marine worms *Spirographis spallanzanii* and *Pomatoceros triqueter* indicated that chlorocruorin, like the hemocyanins, has a molecular weight of the order of millions. Unfortunately, owing to the difficulty of procuring enough material of this comparatively rare and very unstable protein, a complete investigation has not yet been possible.

The very high molecular weight of chlorocruorin shows that proteins of molecular mass of the order of millions are not restricted to the hemocyanins as was previously believed. The enormous weight, therefore, is not conditioned by the presence of the copper-containing active hemocyanin group in the protein molecule. As a working hypothesis we adopted the idea that a very high molecular weight might be a characteristic of the respiratory proteins dissolved in the blood of the invertebrates. From this point of view an ultracentrifugal study of the red respiratory pigment in solution in the blood of many invertebrates suggested itself. This protein has hitherto been considered as identical, or almost identical, with the hemoglobin in the erythrocytes of the blood of the vertebrates. As a matter of fact it closely resembles hemoglobin with regard to its absorption spectrum and its oxygen-binding capacity.[1] These two properties, however, are connected with the hemin group and not with the protein part of the molecule. It is not impossible that there might exist respiratory pigments resembling hemoglobin very closely as far as light absorption and oxygen-binding go but differing completely in the chemical and mass-properties of the molecule. A few preliminary runs on the red blood proteins of *Arenicola marina* and *Lumbricus terrestris* fully confirmed this supposition.[2] The molecular weight is of the order of millions and the

(1) Barcroft, *Proc. Roy. Soc.* (London), **B96**, 28 (1924).
(2) Svedberg and Eriksson, *Nature*, **130**, 434 (1932).

sedimentation constant more than ten times that of hemoglobin. The $P_H$ stability range and the position of the isoelectric point are also quite different.

The detailed ultracentrifugal investigation has shown that the red respiratory pigment dissolved in the blood of these worms is, with regard to the properties of the protein part of the molecule, more allied to chlorocruorin and to hemocyanin than to the hemoglobin of the vertebrates. It therefore seems inappropriate to call it a modification of hemoglobin. Now in the article of 1868 where Ray Lankester proposed the term chlorocruorin for the green blood pigment of the polychaete worms he suggested the term erythrocruorin for the red blood pigment of the invertebrates.[3] In England the name cruorin at that time stood for the respiratory blood pigment in general (suggested by G. G. Stokes[4] in 1864). In his next paper[5] Ray Lankester, however, abandoned the term erythrocruorin and called the red blood pigment of the invertebrates hemoglobin, the latter name, suggested by Hoppe-Seyler in 1864, having in the meantime come into general use for the blood pigment of the vertebrates. It seems to us appropriate to revive Ray Lankester's term and we therefore propose the name erythrocruorin for the red respiratory proteins of the invertebrates.[6]

## The Sedimentation Constant and Molecular Weight of Erythrocruorin from the Blood of Arenicola Marina

The material used was collected at Havstensund and at Kristineberg on the west coast of Sweden. It was brought to Upsala partly as iced blood and partly as living worms. Even in a well-aired aquarium it was not possible to keep the worms living for more than a few days. The blood pigment deteriorates very rapidly. Even when kept in ice it is partly decomposed after a few days, the red color turning brown. The stability seems to depend on the purity of the blood. If contaminated with coelemic fluid it is less stable than when drawn in a very pure state out of one of the large vessels of the worm. An attempt was made to purify the protein by precipitation with ammonium sulfate but the product did not seem to keep any better than the blood. The sedimentation constant was the same for the protein after this precipitation and redissolution. In order to shorten the time between drawing the blood and studying the respiratory protein the determinations were carried out directly on the blood diluted with 1% sodium chloride or with suitable buffer solutions. The sedimentation pictures were taken on panchromatic plates by illuminating the rotating solution with the mercury lines 577–579 and 546 m$\mu$ using a Wratten K3 filter to remove the violet and ultraviolet of the mercury arc. Now the absorption bands of the *Arenicola* blood in the yel-

(3) Lankester, *J. Anat. Physiol.*, **2**, 114 (1868).

(4) Stokes, *Phil. Mag.*, [4] **28**, 394 (1864).

(5) Lankester, *J. Anat. Physiol.*, **4**, 119 (1870).

(6) A comparative ultracentrifugal study of the respiratory proteins throughout the animal kingdom now being carried out in this Laboratory by Mrs. Astrid Hedenius has shown that there exist several varieties of erythrocruorin in the blood of the invertebrates. Most of them have very high sedimentation constants and therefore very high molecular weights, but examples of comparatively low molecular weights have also been found. The hemoglobin, however, characterized by its molecular weight 68,000 and its isoelectric point $P_H$ 6.7 has, so far, not been met with in the blood of the invertebrates [*cf. Nature*, **131**, 325 (1933)].

low and the green almost coincide with these mercury lines and it is therefore possible to dilute the blood considerably (about 20 times) so that a quantity of 0.05 cc. of blood is sufficient for an ultracentrifugal run. Each worm gave from 0.05 to 0.30 cc. of blood according to its size.

The isoelectric point of the *Arenicola* erythrocruorin was determined by Dr. Kai O. Pedersen of this Laboratory by means of the electrophoresis method and was found to be situated at $P_H$ 4.56. The protein is quite insoluble at its isoelectric point. Chemically, therefore, erythrocruorin is entirely different from hemoglobin. Owing to the difficulty of procuring large quantities of quite fresh *Arenicola* blood no attempt to determine the specific volume was made. For calculation of the molecular weight the value 0.740 obtained for the more stable erythrocruorin of *Lumbricus* (see p. 2838) was used. The specific volumes of the proteins vary very little. For those of high molecular weight they are about 0.740 and for those of lower molecular weight about 0.748.

The sedimentation constant was determined in the $P_H$ range 2.6 to 11.1. In Table I and Fig. 1 the measurements are summarized.[7]

TABLE I

ERYTHROCRUORIN OF ARENICOLA, SEDIMENTATION VELOCITY MEASUREMENTS

Dilution of blood 20 times, corresponding to a concn. of erythrocruorin of about 0.15%; centrifugal force 56,000 times gravity; thickness of column of soln., 0.8 cm.; source of light, mercury arc; light filter, Wratten K3; aperture of lens, F:25; plates, Ilford rapid panchromatic process; time of exposure, 15 sec.; metol developer, 1 min.

| Solvent | Total molar | $P_H$ of soln. | $s_{20} \times 10^{13}$ |
|---|---|---|---|
| HCl, KCl | 0.2 | 2.6 | 57.2 |
| HCl, KCl | .2 | 2.6 | Aggregation |
| HCl, KCl | .2 | 3.1 | 58.3 |
| HAc, NaAc | .2 | 3.8 | 58.3 |
| HAc, NaAc | .2 | 4.8 | 59.1 |
| HAc, NaAc | .2 | 5.0 | 58.7 |
| NaCl | .171 | About 5 | 60.3 |
| NaCl | .171 | About 5 | 56.2 |
| NaCl | .171 | About 5 | 55.9 |
| NaCl | .171 | About 5 | 58.2 |
| NaCl | .171 | About 5 | 56.9 |
| NaCl | .171 | About 5 | 58.5 |
| $Na_2HPO_4$, $KH_2PO_4$ | .2 | 6.0 | 57.2 |
| $Na_2HPO_4$, $KH_2PO_4$ | .2 | 6.5 | 54.3 |
| $Na_2HPO_4$, $KH_2PO_4$ | .2 | 7.4 | 57.7 |
| $Na_2HPO_4$, $KH_2PO_4$ | .2 | 8.0 | 55.9[a] |
| $Na_2HPO_4$, $KH_2PO_4$ | .2 | 8.0 | 55.7[a] |
| $Na_2HPO_4$, NaOH | .2 | 8.9 | 52.5[a] |
| $Na_2HPO_4$, NaOH | .2 | 8.9 | 2 compn.: 50.2 and 11.0 |
| $Na_2HPO_4$, NaOH | .2 | 10.0 | 2 compn.: 53.3 and 10.0 |
| $Na_2HPO_4$, NaOH | .2 | 10.0 | 2 compn.: 55.4 and .. |
| $Na_2HPO_4$, NaOH | .2 | 11.1 | .. 5.9 |

[a] Traces of low molecular component noticeable.

From the values in Table I and Fig. 1 it follows that erythrocruorin is stable from $P_H$ 2.6 to about 8, the sedimentation constant being quite

(7) In cases where more than one determination was carried out at the same $P_H$ the mean value has been used for the diagram.

independent of $P_H$ within this range[8] with a mean value of $57.4 \times 10^{-13}$. Very near $P_H$ 2.6 denaturation with aggregation sets in. In solutions more alkaline than $P_H$ 8 a low molecular component begins to appear and at the same time the values of the high molecular component seem to fall slightly. At a $P_H$ of about 10.5 the high molecular component suddenly disappears, leaving the low component with a value of $5.9 \times 10^{-13}$. This behavior of erythrocruorin resembles very much that of the Octopus hemocyanin at the alkaline limit of the stability range.

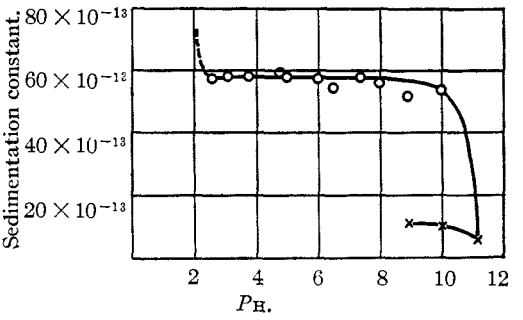

Fig. 1.

The determination of the molecular weight of erythrocruorin by means

TABLE II

ERYTHROCRUORIN OF ARENICOLA, SUMMARY OF SEDIMENTATION EQUILIBRIUM RUNS

Solvent, 0.2 *M* phosphate buffer; $P_H$ of soln., 5.3; concn. of protein about 0.15% at start; centrifugal force 140 times gravity (speed, 1380–1420 r. p. m.); time of centrifuging 122–124 hours.

| No. of run | Mol. wt. |
|---|---|
| 1 | 2,710,000 |
| 2 | 2,800,000 |
| 3 | 3,030,000 |
| 4 | 2,860,000 |
| | Mean 2,850,000 |

TABLE III

ERYTHROCRUORIN OF ARENICOLA, SEDIMENTATION EQUILIBRIUM RUN NO. 2

Spec. volume, 0.740; density of solvent, 1.018; abs. temp., 293°; length of column of soln., 0.48 cm.; thickness of column of soln., 1.20 cm.; dist. of outer end of soln. from center of rotation, 5.95 cm.; speed, 1420 r. p. m.; source of light, mercury lamp; light filter, Wratten K3; aperture of objective, F:25; plates, Ilford rap. proc. panchromatic; time of exp., 15, 30, 60 sec.; exp. made after 100, 113, 124 hours of centrifuging.

| Distances, cm. $x_2$ | $x_1$ | Concn. ratio $c_2/c_1$ | Number of exposures | Mol. wt. |
|---|---|---|---|---|
| 5.80 | 5.75 | 1.191 | 7 | 2,700,000 |
| 5.75 | 5.70 | 1.236 | 12 | 3,310,000 |
| 5.70 | 5.65 | 1.204 | 12 | 2,920,000 |
| 5.65 | 5.60 | 1.191 | 12 | 2,780,000 |
| 5.60 | 5.55 | 1.154 | 12 | 2,290,000 |
| | | | | Mean 2,800,000 |

(8) The values for the sedimentation constant have been corrected for the influence of the density and the viscosity of the salts present. If this is not done the stability line becomes slightly inclined toward the alkaline side. The slope of the stability line for the high molecular component of the hemocyanin of Octopus blood as borne out by the data given in a previous communication [THIS JOURNAL, **54**, 4730 (1932)] likewise vanishes if such corrections are introduced.

of sedimentation equilibrium measurements is rather difficult because of the instability of the protein and the considerable time necessary for attaining equilibrium. Four successful runs, however, were carried out on *Arenicola* blood which had been drawn with special care so as to avoid contamination by coelemic fluid. Table II summarizes the results and Table III gives the complete data of run No. 2.

From the mean sedimentation constant $57.4 \times 10^{-13}$ and the mean molecular weight 2,850,000 we get for the molar frictional constant $f = M(1 - V\rho)/s$, where $M$ is the molecular weight, $V$ the specific volume and $\rho$ the density of the solvent, the value $12.3 \times 10^{16}$. A spherical molecule of the same specific volume would have the frictional constant $f_s = 10.7 \times 10^{16}$. This gives a dissymmetry number $f/f_s$ of 1.14. The molecule of *Arenicola* erythrocruorin is therefore slightly dissymmetrical in shape. The absence of drift in the molecular weight values with distance from center of rotation (see Table III) shows that the protein is monodisperse.

**The Sedimentation Constant and Molecular Weight of Erythrocruorin from the Blood of Lumbricus Terrestris.**—The blood of the earthworm is easy to procure in sufficient quantities and is more stable than that of the lug-worm, *Arenicola*. A big worm gives 0.1 cc. of blood with an erythrocruorin content of over 4%. For the ultracentrifugal runs the blood can be diluted 25–30 times. The specific volume was determined pycnometrically using 5 cc. of blood which had been dialyzed against distilled water for two days in the cold. From two determinations the mean value 0.740 was obtained. The isoelectric point was measured by Dr. Kai O. Pedersen of this Laboratory. He found the value $P_H$ 5.3

TABLE IV

ERYTHROCRUORIN OF LUMBRICUS, SEDIMENTATION VELOCITY MEASUREMENTS

Experimental conditions as in Table I; centr. force 56,000 times gravity

| Solvent | Total molar | $P_H$ of soln. | $s_{20} \times 10^{13}$ |
|---|---|---|---|
| HCl, KCl | 0.2 | 1.6 | 104.8 |
| HCl, KCl | .2 | 2.6 | 60.7 |
| HAc, NaAc | .2 | 3.8 | 56.4 |
| HAc, NaAc | .2 | 3.9 | 58.9 |
| HAc, NaAc | .2 | 5.0 | 58.8[a] |
| NaCl | .171 | About 5 | 61.0 |
| NaCl | .171 | About 5 | 60.0 |
| NaCl | .171 | About 5 | 61.5 |
| NaCl | .171 | About 5 | 62.6 |
| $Na_2HPO_4$, $KH_2PO_4$ | .2 | 5.5 | 60.1 |
| $Na_2HPO_4$, $KH_2PO_4$ | .2 | 6.0 | 62.1 |
| $Na_2HPO_4$, $KH_2PO_4$ | .2 | 7.1 | 61.1 |
| $Na_2HPO_4$, $KH_2PO_4$ | .1 | 8.0 | 59.6 |
| $Na_2HPO_4$, NaOH | .2 | 8.9 | 59.3 |
| $Na_2HPO_4$, NaOH | .2 | 10.0 | 64.3 |
| $Na_2HPO_4$, NaOH | .2 | 11.1 | 2.6[b] |

[a] Centr. force 11,250 times gravity. [b] Centr. force 180,000 times gravity.

against $P_H$ 4.56 for the *Arenicola* erythrocruorin. The protein is quite soluble at the isoelectric point. The erythrocruorin of *Lumbricus* is therefore not identical with the *Arenicola* erythrocruorin in chemical respect.

The sedimentation constant was determined in the $P_H$ range 1.6 to 11.1. Table IV and Fig. 2 give a summary of the results.

The erythrocruorin of *Lumbricus* is stable from $P_H$ 2.6 to 10. The mean value of the sedimentation constant is $60.9 \times 10^{-13}$. As in the case of *Arenicola* erythrocruorin aggregation occurs at the acid end of the stability range and decomposition into a low molecular product at the alkaline end. Unlike the *Arenicola* erythrocruorin, however, the *Lumbricus* protein does not give two components in the alkaline region.

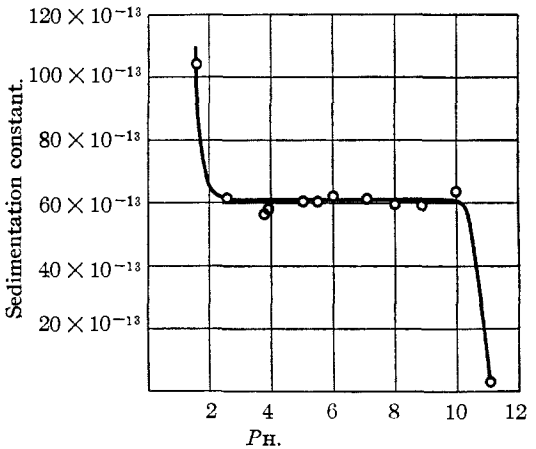

Fig. 2.

The values of the sedimentation constants for *Arenicola* and for *Lumbricus* erythrocruorin, *viz.*, $57.4 \times 10^{-13}$ and $60.9 \times 10^{-13}$, are rather close together but the difference is outside the limit of experimental error. We have been able to prove this conclusively by centrifuging a mixture of *Arenicola* and *Lumbricus* erythrocruorin solutions of equal concentration. In such a relative determination several sources of error, especially the uncertainty of the cell temperature during the run, are eliminated and a slight difference between two sedimentation constants can therefore be detected with a high degree of accuracy. The sedimentation pictures from a run carried out at a speed of 31,700 r. p. m. [centr. force 73,000 times gravity] showed very distinctly two boundaries. The sedimentation ratio was 1.07, while the ratio of the mean values from all determinations gives 1.06.

Three sedimentation equilibrium runs were made on *Lumbricus* erythrocruorin. In Table V the results are summarized and in Table VI is given detailed information about one of the runs.

The fact that the molecular weight is slightly lower for *Lumbricus* erythrocruorin than for *Arenicola* erythrocruorin while the sedimentation constant is higher indicates that the molecule of the *Lumbricus* protein deviates less from the spherical shape than does the molecule of the *Arenicola* protein. The calculation gives $f = 11.1 \times 10^{16}$, $f_s = 10.6 \times 10^{16}$

TABLE V

ERYTHROCRUORIN OF LUMBRICUS, SUMMARY OF SEDIMENTATION EQUILIBRIUM RUNS

Experimental conditions as in Table II

| Run No. | Mol. wt. |
|---|---|
| 1 | 2,540,000 |
| 2 | 2,690,000 |
| 3 | 3,070,000 |
| | Mean 2,730,000 |

TABLE VI

ERYTHROCRUORIN OF LUMBRICUS, SEDIMENTATION EQUILIBRIUM RUN NO. 1

Length of column of soln., 0.45 cm.; thickness of column of soln., 1.2 cm.; aperture of objective, F:36; times of exposure, 15, 30, 45 sec.; exposures made after 96, 102, 123 hours; other experimental conditions as in Table III.

| Distances, cm. $x_2$ | $x_1$ | Concn. ratio $c_2/c_1$ | Number of exposures | Mol. wt. |
|---|---|---|---|---|
| 5.81 | 5.76 | 1.195 | 12 | 2,750,000 |
| 5.76 | 5.71 | 1.174 | 12 | 2,500,000 |
| 5.71 | 5.66 | 1.147 | 12 | 2,160,000 |
| 5.66 | 5.61 | 1.199 | 9 | 2,870,000 |
| 5.61 | 5.56 | 1.157 | 9 | 2,330,000 |
| 5.56 | 5.51 | 1.176 | 9 | 2,610,000 |
| | | | | Mean 2,540,000 |

and therefore the dissymmetry number $f/f_s = 1.05$. The radius of the molecule if considered spherical would be 9.3 m$\mu$.

The expenses connected with this investigation have been defrayed by grants from the Nobel Fund of Chemistry, from the Rockefeller Foundation and from the Foundation "Therese och Johan Anderssons Minne." Part of the *Arenicola* material used was kindly put at our disposal by Dr. G. Gustafson of the Marine Zoölogical Station at Kristineberg and by Mr. B. Dahlberg of Havstensund.

## Summary

1. The sedimentation constant, the $P_H$ stability range and the molecular weight of the respiratory blood proteins of *Arenicola marina* and of *Lumbricus terrestris* have been determined by means of the ultracentrifugal methods. The name erythrocruorin, formerly used by Ray Lankester, has been proposed for the red blood-proteins of the invertebrates.

2. The erythrocruorins of *Arenicola* and of *Lumbricus* have been found to be similar with regard to sedimentation constant, $P_H$ stability and molecular weight. The *Arenicola* protein is stable from $P_H$ 2.6 to 8 with a sedimentation constant of $57.4 \times 10^{-13}$ and a molecular weight of 2,850,000. The *Lumbricus* erythrocruorin has the stability range $P_H$ 2.6–10, the sedimentation constant $60.9 \times 10^{-13}$ and the molecular weight 2,730,000.

3. Not only with regard to molecular weight but also in chemical respect is erythrocruorin widely different from the hemoglobin of the

vertebrates, the isoelectric point of hemoglobin being 6.7 and that of *Arenicola* and *Lumbricus* erythrocruorin being 4.56 and 5.3, respectively.

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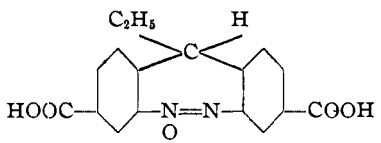
[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE UNIVERSITY OF ILLINOIS]

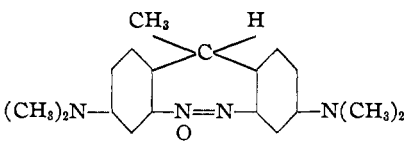
# A Proof of the Unsymmetrical Structure of the Azoxy Group[1]

BY TSE-TSING CHU[2] AND C. S. MARVEL

The isolation of optically active isomers of certain types of azoxy compounds would furnish evidence to establish the unsymmetrical structure of the azoxy group which was advanced by Angeli.[3] The first compound selected for resolution in order to obtain this evidence was prepared by the reduction of an *o,o'*-dinitrodiphenylmethane derivative.[4] In view of previous work[5] on closely related compounds, the substance was assigned a cyclic structure (I). King[6] has undertaken to solve the problem in a similar fashion and for this purpose has prepared a related compound to which he has assigned formula II.



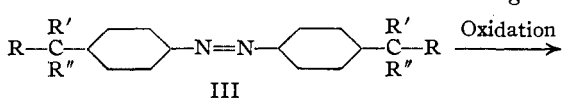

I



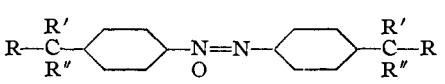

II

The azoxy compound (I) which was prepared in this Laboratory was not successfully resolved. A further study of this substance has shown that it is really not a simple cyclic compound since it has a molecular weight of 2000–3000. Obviously such a polymeric type of molecule is not suited to the work at hand and a new attack on the problem was undertaken.

An azo compound such as is represented in formula III should exist in one racemic and one meso form. If the Angeli formula of the azoxy group




III




IV

(1) This communication is a portion of a thesis submitted by Tse-Tsing Chu in partial fulfilment of the requirements for the Degree of Doctor of Philosophy in Chemistry at the University of Illinois.

(2) Research fellow of the China Foundation for the Promotion of Education and Culture, 1932–1933.

(3) Angeli, *Gazz. chim. ital.*, **46**, II, 67 (1916).

(4) Hussey, Marvel and Hager, THIS JOURNAL, **52**, 1122 (1930).

(5) Täuber, *Ber.*, **24**, 3083 (1891); Ullmann and Dieterle, *ibid.*, **37**, 24 (1904); Duval, *Compt. rend.*, **141**, 198 (1905); *Bull. soc. chim.*, [4] **7**, 527, 681 (1910); King, *J. Chem. Soc.*, **117**, 988 (1920).

(6) King, *J. Soc. Chem. Ind.*, **49**, 281 (1930).