Magnetic Susceptibility of Erythrocruorin from the Blood of an Ark-Shell

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Since Pauling and Coryell[1] studied the magnetic properties of hemoglobin and oxyhemoglobin from bovine blood, it has become clear that a hemoglobin molecule undergoes a change in electronic structure on combination with oxygen molecules. The conversion of hemoglobin to oxyhemoglobin is accompanied by a change in the molecule from the paramagnetic to the diamagnetic condition and also the oxygen molecule, which in its free state is paramagnetic, is involved in the change of the magnetic condition. Following this, one of the present writers[2] carried out a similar investigation on another iron-containing respiratory pigment called hemerythrin. He found that the change from the paramagnetic to the diamagnetic condition on oxygenation is so profound that the effect of the combining of an oxygen molecule extends to all of the three iron atoms of a magnetic group in the molecule of hemerythrin. On the other hand, in hemoglobin, which combines with oxygen in the definite proportion of one molecule of oxygen per one atom of iron, the effect is naturally limited to a single iron atom.

The present investigation deals with erythrocruorin, another respiratory pigment containing ferroheme. It can be found in the blood and tissue fluids of invertebrates. Erythrocruorin (or hemoglobin) occurs even in the root nodules of certain legumes[3]. Although erythrocruorins bear resemblance to hemoglobins from vertebrates in many respects, these two types are by no means identical. As a class, the erythrocruorins have more acid isoelectric points than the hemoglobins. In some cases the protein is present in solution, but in others the pigment is found in cells. In the former, most of the erythrocruorins have very high molecular weights of the order of a million or more,

1) L. Pauling and C.D. Coryell, *Proc. Natl. Acad. Sci. U.S.*, 22, 159 (1936); 22, 210 (1936). C.D. Coryell, F. Stitt and L. Pauling, *J. Am. Chem. Soc.*, 59, 633 (1937).

2) M. Kubo, This Bulletin, 26, 244 (1953); *Monograph Series of Research Inst. Applied Electricity*, 5, 37 (1955).

3) H. Kubo, *Acta Phytochim.* (Japan), 11, 195 (1939).

and presumably contain a very large number of hemes. In view of these facts, it seemed worth while to make magnetic measurements on erythrocruorin and to compare the results with findings on hemoglobin. The probable reason why erythrocruorins have eluded magnetic investigation is that this respiratory pigment in any great amount is difficult to obtain, whereas measurement by a Gouy magnetic balance reguires a fairly large amount of sample solution. Fortunately an ark-shell known as *Anadara* (=*Arca*) *inflata* (Reeve) is commonly found at various places along the coast of Japan. Ample blood is obtainable from this shell-fish[4]. The present writers prepared a solution of erythrocruorin using the blood of this shell-fish and measured the magnetic susceptibility of the solution in both deoxygenated and oxygenated states. A control experiment was made on horse hemoglobin using the same apparatus and experimental technique.

Material

Anadara inflata (Reeve) used in the present experiment was procured in a fish market in Nagoya. Presumably the shell-fish were taken from a nearby bay. All precautions were taken in the preparation of erythrocruorin solution. The umbonal part of each shell was broken by a blow with the base of a glass bottle. Then the heart was carefully incised with the sharp point of a glass rod in order to let the blood flow into a beaker. These precautions were taken because the use of an iron hammer and a steel knife might possibly introduce ferromagnetic impurities into the sample. To remove the fragments of tissues, the blood was filtered through several layers of antiseptic gauze. When centrifuged, the dark red corpuscles settled as sediment on the bottom of the tube. To completely remove the plasma, the sediment was washed several times with 3 percent sodium chloride solution. After the solution was aerated for about one hour, it was left to stand in a refrigerator for 1–1.5 hours. Finally these blood cells were put into distilled water to subject them to hemolysis. Immediately before use, the stroma was removed by centrifuging. The clear solution which was pipetted out assumed a dark red color. Blood cells occupying approximately 25 cc. were obtained from about 4 kg. of this material.

Apparatus and Experimental Procedure

For the determination of magnetic susceptibility, a Gouy magnetic balance was used at room temperature (ca. 15°C) as described in a paper[5] by two of the present writers and their collaborators.

An erythrocruorin solution prepared in accordance with the foregoing procedure was in the state of practically complete oxygenation. For magnetic measurement, the solution was placed in a cell. In order to prepare a deoxygenated specimen, the air in a vessel which contained the solution (about 15 cc.*) was evacuated and nitrogen bubbled through alkaline pyrogallol solutions in advance was admitted into the vessel. The equilibration was repeated several times until deoxygenation was complete, as indicated by the red color of the solution becoming tinged with violet. Finally, the solution was transferred from the vessel into the cell which had been connected to the vessel by means of a rubber tube. The intrusion of air into the cell was prevented by keeping the pressure of nitrogen in the vessel higher than the atmospheric pressure.

It was of prime importance that in spite of repeated partial evacuation and equilibration with nitrogen, the concentration of the deoxygenated solution was the same as that of the oxygenated solution. Therefore use was made of nitrogen saturated with water. Moreover, the fact that no appreciable loss of water took place during manipulation was confirmed by carrying out measurements both on the original oxygenated solution and on the solution subjected to deoxygenation followed by oxygenation. The results of these two measurements were in good agreement with each other within the allowable limit of experimental errors.

Determination of Iron Content

The solution of erythrocruorin (4 cc.) was subjected to the wet-ashing procedure and the amount of iron brought into solution as an inorganic salt was determined colorimetrically by the use α,α'-dipyridyl. The method employed was essentially the same as those given by Eden and Green[6] and by Thorp[7]. A color comparison was made using a Coleman spectrophotometer with green light of wavelength 525 mμ. A blank test was carried out in exactly the same manner as above with an equal volume of water in place of the solution under investigation.

Hemoglobin Solution

Fresh horse blood was defibrinated and centrifuged. The sediment was washed six times with 0.85 percent solution of sodium chloride. The blood cells, after being aerated, were hemolysed with approximately a half volume of water. The hemoglobin solution was centrifuged before use. The experimental procedures for magnetic measurements and for iron content determination were the same as those employed for erythrocruorin solution.

Experimental Results and Discussion

The results are summarized in Table I.

The difference, $\kappa_{\text{deoxy.}}-\kappa_{\text{oxy.}}$, divided by the iron concentration and multiplied by the

4) T. Sato, *Z. vergleich. Physiol.*, **14**, 763 (1931).

5) M. Kondo, M. Kishita, M. Kimura and M. Kubo, This Bulletin, **29**, 305 (1956).

* A single measurement required only about 3.5 cc. of solution.

6) A. Eden and H.H. Green, *Biochem. J.*, **34**, 1202 (1940).

7) R.H. Thorp, *Biochem. J.*, **35**, 672 (1941).

atomic weight of iron yields the difference ($\chi_{deoxy.}-\chi_{oxy.}$) between the susceptibility of deoxygenated erythrocruorin (or hemoglobin) and that of oxygenated preparation per gram atom of iron, as given in Table I. The

TABLE I

MAGNETIC SUSCEPTIBILITIES, κ, PER UNIT VOLUME OF ERYTHROCRUORIN SOLUTION AND HEMOGLOBIN SOLUTION IN DEOXYGENATED AND OXYGENATED STATES

	Erythrocruorin	Hemoglobin
Deoxygenated	$-0.691_4\times10^{-6}$	$-0.599_1\times10^{-6}$
Oxygenated	$-0.724_0\times10^{-6}$	$-0.727_0\times10^{-6}$
Density	1.023 g./cc.	1.043 g./cc.
Iron Concentration	170.9 γ/cc.	558.1 γ/cc.
$\chi_{deoxy.}-\chi_{oxy.}$	0.01064cc./mole	0.01279cc./mole

difference between molecular susceptibilities is some simple multiple of $\Delta\chi$ depending upon how many atoms of iron are contained in a molecule. Let $\Delta\chi$ be used rather than the difference between molecular susceptibilities simply because this does not involve making an initial assumption on the molecular weight of erythrocruorin. The use of $\Delta\chi$ presupposes that the magnetic coupling between different hemes is inappreciable, or in other words that the different magnetic groups in a molecule are practically indepedent of each other as if they were located in separate molecules. This condition is satisfied to a fair degree of exactness for hemoglobin and presumably also for erythrocruorin.

The diamagnetic term is cancelled in the difference, $\Delta\chi$. Therefore assuming that all oxygen combining centers are alike, it holds that

$$\Delta\chi=\frac{N}{3kT}(\mu^2_{deoxy.}-\mu^2_{oxy.}).$$

Here N and k denote Avogadro number and Boltzmann constant respectively, T the absolute temperature, and μ the magnetic moment associated with a heme. There are good reasons[1] for believing that oxyhemoglobin contains no unpaired electrons, i.e., $\mu_{oxy.}=0$. This leads directly to $\mu=5.43$ Bohr magnetons per heme of deoxygenated horse hemoglobin, which value is in good agreement with 5.46 and 5.43 Bohr magnetons given respectively by Pauling and Coryell[1] for cow hemoglobin and by Taylor and Coryell[8] for horse hemoglobin. In the case of erythrocruorin, one has no *ad hoc* reason to assign any particular value to $\mu_{oxy.}$. The assumption that oxygenated erythrocruorin contains no unpaired electrons gives the minimum value of 4.96 Bohr magnetons or the magnetic moment of deoxygenated erythrocruorin. If some iron atoms were not affected by oxygenation, and/or if there were some unpaired electrons in the oxygenated erythrocruorin, the figure would be larger. Since it is known that molecules of all kinds of respiratory pigments heretofore investigated contain no unpaired electrons, let it be taken for granted that this assumption is adequate. This leads to the conclusion that

$$\mu_{deoxy.}=4.96 \text{ Bohr magnetons.}$$

This value corresponds closely to the presence of four unpaired electrons per heme and to the almost complete quenching of orbital moments because the resultant of the spin moments of four unpaired electrons has the theoretical value of 4.90 Bohr magnetons, neglecting contributions due to ordital moments. Although extracted from the blood of an invertebrate, the erythrocruorin under investigation must be regarded as being similar to hemoglobins from vertebrates at least with regard to its magnetic properties.

Recently Yagi[9] and his collaborators in the Chemical Department of Nagoya University succeeded in isolating this erythrocruorin in crystalline state. They found that the minimum molecular weight of this compound is equal to 19,000. The molecular weight is 73,000, as determined by Ui[9] in Tokyo University from the sedimentation equilibrium in an ultracentrifuge. Therefore one molecule contains four hemes, and the molecular weight is equal to 73,000~76,000. These facts also confirm the view that the erythrocruorin under investigation has something in common with vertebrate hemoglobins which have molecular weights close to 67,000.

Summary

The magnetic susceptibility of an aqueous solution of erythrocruorin from an ark-shell known as *Anadara inflata* (Reeve) was measured by a Gouy magnetic balance at 15°C in deoxygenated and in oxygenated state. On the plausible assumption that oxygenated erythrocruorin contains no unpaired electrons, the magnetic moment per one iron atom of this respiratory pigment in deoxygenated state was calculated to be equal to 4.96 Bohr magnetons. This corresponds closely to the presence of four unpaired electrons per heme and to almost complete quenching of orbital moments. For this reason, the erythrocruorin bears a remarkable

8) D.S. Taylor and C.D. Coryell, *J. Am. Chem. Soc.*, **60**, 1177 (1938).

9) Y. Yagi, T. Mishima, T. Tsujimura, K. Sato, F. Egami and N. Ui, *Compt. rend.; J. Biochem.* (Japan), to be published shortly.

resemblance to hemoglobins of vertebrates in so far as its magnetic properties are concerned.

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