Magnetic Susceptibilities of Reduced and Oxygenated Hemerythrins

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(Received November 13, 1952)

The molecular weight of hemerythrin, an iron-containing respiratory pigment, determined by Adair's osmotic pressure method is 66,000.(1) Since hemerythrin contains 1.01% of iron, one molecule of hemerythrin contains twelve iron atoms. The molecule combines with oxygen in the proportion of one oxygen molecule to three atoms of iron,(2) hence the number of oxygen molecules fully combined with a hemerythrin molecule is four. This seems to be very peculiar in view of the fact that in two other iron-containing respiratory pigments, hemoglobin and chlorocruorin, the number of iron atoms in a molecule is equal to the number of oxygen molecules which can combine with it. To elucidate the problem more closely, I have undertaken magnetic susceptibility measurements on hemerythrin. Similar measurements, adopted by Pauling and

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(1) A. Roche and J. Roche, *Bull. soc. chim. biol.*, **17**, 1494 (1935).

(2) M. Florkin, *Arch. intern. physiol.*, **36**, 247 (1933).

Coryell,(3) have led to a great extension of the structural knowledge of hemoglobin and related compounds.

Apparatus.—For the determination of magnetic susceptibility, a simple Gouy magnetic balance was employed. A test tube, filled with solution to a definite level, was suspended from a balance between the poles of a magnet. The test tube was 25 cm. long and 8.9 mm. in outer diameter, and the magnetic pole pieces were 7.2 cm. in vertical length and separated by 1.2 cm. The liquid meniscus was at the center of a strong homogeneous field of intensity H. The top and bottom ends of the tube were in a region of weak field H'. The current through the coil of the electromagnet was adjusted exactly to 9.7 amp; with this current magnetic saturation was almost attained. H and H' amounted to 1.03×10^4 and 2.2×10^2 oersteds respectively, hence H'^2 is entirely negligible compared with H^2. The effect of the residual magnetic field after the current was switched off was also small and need not be considered.

The magnitude of downward force f experienced by the tube along its length is given by the expression,

$$f=\frac{1}{2}(\kappa_{\mathrm{gas}}-\kappa_{\mathrm{liq}})H^2A=g\Delta w.$$

where κ_{gas}, κ_{liq} are the volume susceptibilities of the gas and liquid in the tube, A is the cross-sectional area of the liquid column having the value of $\pi(6.9/2)^2$ mm.^{2}, g the gravitational constant, and Δw the apparent increase in weight of the tube on application of the magnetic field.

The magnetic balance was operated at room temperature (25°), and was calibrated with distilled water saturated with air and its magnetic susceptibility per unit volume was set equal to -0.719×10^{-6}. The susceptibility per unit volume of the solution under investigation was calculated from

$$\frac{\kappa_{\mathrm{gas}}-\kappa_{\mathrm{sol}}}{\kappa_{\mathrm{air}}-\kappa_{\mathrm{water}}}=\frac{\Delta w_{\mathrm{sol}}}{\Delta w_{\mathrm{water}}}$$

To fill the upper part of the tube, 99.9% pure commercial nitrogen was used for reduced hemerythrin and air for oxygenated hemerythrin and water. It would be more desirable to use nitrogen in place of air to saturate the water and to remove dissolved oxygen completely from it. However, we had to use air for oxygenated hemerythrin and hence the effect of dissolved air is worth our attention. On the assumption of the additivity of magnetic susceptibility, the increase of susceptibility due to the paramagnetic contribution of dissolved oxygen can be calculated from the known solubility of the gas in water and the molecular susceptibility of oxygen, with the aid of Curie's law. The correction amounted to $+0.0009\times10^{-6}$, which is entirely negligible in comparison to -0.719×10^{-6} of pure water. It was of course ascertained that no bubbles of air came out of water or solution on the wall of the container during measurement. The value of π_{gas} is -0.00040×10^{-6} for nitrogen, which is entirely negligible compared with κ_{sol}, and $+0.0294\times10^{-6}$ for air. Two calibrated test tubes were used; one for reduced hemerythrin and the other for the oxygenated preparation in order to carry out the magnetic measurements at the same time.

Material.—The oxygenated hemerythrin solution was prepared as described in the preceding paper.(4) All the measurements on magnetic susceptibility, optical density, density, and the chemical analysis of iron were made on one and the same solution. These measurements were made in one day, except the iron analysis which took a longer time. The blood cells were washed during the previous evening and allowed to hemolyse overnight. One test tube for magnetic measurement was filled with approximately 5 cc. of the solution. Another part of the solution was deoxygenated in a tonometer, to which a test tube was attached by means of a rubber tube, according to the procedure already given.(4) After deoxygenation was complete, a portion of the solution was transferred to the test tube which was filled to the desired level for the magnetic measurements. The rubber tube was clamped on and a rubber stopper was inserted through the rubber tube to seal the test tube. In this case a very small quantity of air remaining in the rubber tube between the clamped part and the stopper was driven out by a stream of nitrogen injected through the rubber wall by means of a syringe and hypodermic needle.

It was of prime importance in this experiment that the concentration of deoxygenated and oxygenated solutions remained equal in spite of the repeated partial evacuation with an aspirator and equilibration with water-saturated nitrogen. To determine whether appreciable loss of water occurred, measurements were made on the optical density of the original oxygenated solution D_{ox}, that of deoxygenated solution D_{red}, and that of the latter solution exposed to and equilibrated with atmospheric air D'_{ox}. $D_{\mathrm{ox}}-D_{\mathrm{red}}$ and $D'_{\mathrm{ox}}-D_{\mathrm{red}}$ agreed with each other within 0.8%. Incidentally, this can be used to convert the relative concentrations in terms of optical density given in the previous paper into concentrations in terms of the iron content. The specific gravity of the solution at room temperature (25°) was found to be 1.018.

Determination of Iron Content.—The solution (16.408 g.) was dried in air at 100° (dry protein: 0.0221 g. per cc. of solution), and the iron content was determined by the thiocyanate method. A Coleman spectrophotometer was used for color comparison with green light of wave-length

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(3) L. Pauling and C. D. Coryell, *Proc. Natl. Acad. Sci. U. S.*, **22**, 210 (1936); C. D. Coryell and F. Stitt, *J. Am. Chem. Soc.*, **62**, 2942 (1940).

(4) M. Kubo, This Bulletin, **26**, 189 (1953).

525 mμ. The concentration of the solution was found to be 7.88×10^{-6} g. atoms of iron per cc.

Results and Discussion

The results of magnetic measurements are summarized in Table 1. The susceptibility of reduced hemerythrin solution is higher while that of the oxygenated solution a little lower than that of water. It is obvious that although masked by the diamagnetic effect of the solvent and the protein part of hemerythrin, paramagnetic contribution attributable to iron is surely present in the reduced state, whereas such contribution is doubtful in oxygenated solution. The difference of molecular susceptibilities of reduced and oxygenated hemerythrin molecules is given by

Table 1

Magnetic Susceptibilities of Reduced and Oxygenated Hemerythrin Solutions at 25°.

	Δw(mg.)	Δw_{water}(mg.)*	$\kappa\times10^6$
Reduced	11.9	16.4	−0.543
Oxygenated	16.35	16.1	−0.731

* Two values do not agree with each other exactly, because different test tubes were used for them.

$$\chi_{\text{red}}-\chi_{\text{ox}}=12\times\frac{-0.543+0.731}{7.88}=0.286\,\text{cm.}^3,$$

in which the factor 12 is introduced to take account of the fact that one molecule of hemerythrin contains twelve iron stoms. The difference method is particularly elegant because of the automatic cancellation of the effect of minute quantities of impurities such as indifferent proteins and sodium chloride, which might have come from blood cells and the solution used to wash them respectively, and of the diamagnetic term χ_0 in

$$\chi=\chi_0+N\frac{\mu^2}{3kT}$$

where μ denotes magnetic moment and other notations have their usual significances.

The change in magnetic susceptibility is, to be sure, closely connected with an iron atom or atoms directly combined with oxygen and those affected by it. In a complex molecule such as hemerythrin containing many iron atoms, there is a certain ambiguity as to the interpretation of μ. Let us assume that the different magnetic groups acting at the same time as oxygen-combining centers are oriented entirely independently of one another in the external magnetic field, as if located in separate molecules. This assumption will be justified, since as revealed by the studies on oxygen equilibrium⁽⁴⁾ the mutual interaction between these centers is weaker, indicating greater distance between them, than in the case of hemoglobin, for which Pauling and Coryell⁽⁵⁾ have given ample reasons for believing that the different magnetic groups in the molecule are magnetostatically independent. The further assumption will be made that the four groups in question are equivalent. Otherwise we would have obtained a more complicated shape of the oxygen equilibrium curve. Then we have

$$\chi_{\text{red}}-\chi_{\text{ox}}=\frac{4N}{3kT}\left(\mu^{*2}_{\text{red}}-\mu^{*2}_{\text{ox}}\right)$$

in which μ^* denotes the magnetic moment of each group acting as an oxygen combining center. Quantum mechanical considerations give for a magnetic moment

$$\mu^2=g^2\beta^2J(J+1)$$

where g is the gyromagnetic ratio, β is the Bohr magneton, $eh/4\pi mc$ in Gaussian units, the numerical value of which is 9.2726×10^{-21} erg gauss^{-1} or erg$^{1/2}$ cm.$^{3/2}$, and J the resultant angular momentum in units $h/2\pi$. Neglecting contributions due to orbital moments, and putting $g=2$ and $J=n/2$ for electrons, where n denotes the number of unpaired electrons, we have

$$\mu=\sqrt{n(n+2)}\beta$$

for the effective moment. In connection with the assumption that the spins only are involved, it will be worthwhile to point out that the orbital moments are appreciable for rare earth elements but are probably not very large for other transition group elements.⁽⁶⁾ μ in this general treatment corresponds μ^* in this particular problem of a complex molecule. Hence

$$\chi_{\text{red}}-\chi_{\text{ox}}=\frac{4N\beta^2}{3kT}\left[n(n+2)_{\text{red}}-n(n+2)_{\text{ox}}\right]$$

Putting numerical values in the equation, we have

$$n(n+2)_{\text{red}}-n(n+2)_{\text{ox}}=171$$

There is no ad hoc reason to assign any particular value to n_{red} or n_{ox}. The assumption

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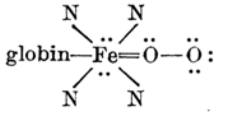
(5) L. Pauling and C. D. Coryell, *Proc. Natl Acad. Sci. U. S.*, **22**, 159, 210 (1936).

(6) Cf. for example P. W. Selwood, Magnetochemistry, Interscience Publ. Inc., New York, 1943, p. 144.

that the oxygenated group has no unpaired electrons, $n_{\mathrm{ox}}=0$, will give for the number of unpaired electrons in the deoxygenated group the value of 12.1 per three atoms of iron, which fixes the lower limit of n_{red}. This fact can hardly be explained if we suppose that only four of the twelve iron atoms of a hemerythrin molecule play a role in combining with four oxygen molecules and that other eight iron atoms remain indifferent. It is known that the iron in hemerythrin is in the ferrous state and that if it is oxidized to the ferric state, the resulting methemerythrin has a brownish color which no longer disappears when its solution is subjected to a vacuum or to the action of a reducing agent.(7) In an isolated ferrous ion or in a hydrated ferrous complex ion $[\mathrm{Fe(OH_2)_6}]^{++}$, in which the bonds between the iron atoms and water molecules are essentially ionic or the ion-dipole type, the six outer electrons in excess of those required to form a completed argon shell occupy five $3d$ orbitals, and give rise to a normal state in which four of the electron spins are unpaired. In a covalent ferrous complex ion such as $[\mathrm{Fe(CN)_6}]^{-\mathrm{IV}}$, on the other hand, the six outer electrons of iron occupy three $3d$ orbitals with their spins paired, the other two $3d$ orbitals forming octahedral bond orbitals with one $4s$ and three $4p$ orbitals, resulting in no unpaired electrons. Therefore irrespective of whether the bonds of iron atoms are ionic or covalent it is very improbable that three ferrous iron atoms contained in a reduced hemerythrin group can have more than twelve unpaired electrons. Hence we can conclude that the number of unpaired electrons in a group acting as an oxygen combining center in reduced state, n_{red}, is twelve.

The calculated value of n_{red} is rather insensitive to the assumed value of n_{ox} so long as the latter is less than three, the calculated value of n_{red} being $12.1\sim12.4$ for the assumed value of n_{ox} ranging from 0 to 2. Hence we have no means to decide from experimental data alone which of the three values $n_{\mathrm{ox}}=0, 1, 2$ is correct. In fact the neglect of orbital contributions to the moment overestimates the number of unpaired electrons, while the assumption of complete coupling between different iron atoms in a group underestimates the number. Theoretically $n_{\mathrm{ox}}=1$ or 2 is improbable and the most probable one among these three values will be $n_{\mathrm{ox}}=0$.

Reduced hemerythrin, like ferrohemoglobin, must therefore be regarded as containing an ionic type of bonding of all iron atoms, whereas in oxygenated hemerythrin all the bonds are essentially covalent in nature. It is a very remarkable fact that oxygen molecules combining with the pigment molecule exert such a profound influence on the electronic structure of all iron atoms present. Pauling and Coryell(8) suggest as the most probable linkage of an oxygen molecule or carbon monoxide molecule coordinated with an iron atom in hemoglobin the following formulas:



$$\text{globin}-\overset{\mathrm{N}\ \ \mathrm{N}}{\underset{\mathrm{N}\ \ \mathrm{N}}{\ddot{\underset{..}{\mathrm{Fe}}}}}=\ddot{\mathrm{O}}-\underset{..}{\ddot{\mathrm{O}}}:$$

$$\text{globin}-\overset{\mathrm{N}\ \ \mathrm{N}}{\underset{\mathrm{N}\ \ \mathrm{N}}{\ddot{\underset{..}{\mathrm{Fe}}}}}=\mathrm{C}=\ddot{\mathrm{O}}:$$

resonating between other possible electronic structures. In this connection it is important to point out that the similarity of hemerythrin and hemoglobin lies in their physiological function as oxygen-transporters rather than in their chemical properties. Unlike hemoglobin, hemerythrin does not combine with carbon monoxide. In fact Florkin(9) found that hemerythrin combined with the same amount of oxygen whether the partial pressure of carbon monoxide was 4 or 150 mm. Hg., and Roche(10) found that bubbling carbon monoxide through a hemerythrin solution brought about no change in its spectrum. This fact makes it very likely that the combination of oxygen with hemerythrin takes place in a quite different manner from that of oxygen with hemoglobin. At present our knowledge on the structure of hemerythrin molecule is so limited that it is almost impossible to propose any definite idea to explain how an oxygen molecule can affect the electronic states of all the three iron atoms in the group. Possibly these iron atoms are coupled with each other in a polynuclear complex so as to be able to transmit the effect of the change of electronic state directly to others. We may have in the diamagnetic state of oxygenated hemerythrin a situation similar to that in certain polynuclear complexes such as iron enneacarbonyl, in which two iron atoms, with an odd number of electrons, coexist in the same molecule, and yet the molecule is diagmagnetic. A possible explanation is that there is some kind of electron pairing taking place, because of the proximity of the iron atoms to one another.

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(7) G. F. Marrian, *Brit. J. Exptl. Biol.*, **4**, 357 (1927); M. Florkin, loc. cit.; J. Roche, *Bull. soc. chim. biol.*, **15**, 1415 (1933).

(8) L. Pauling and C. D. Coryell, loc. cit.

(9) M. Florkin, loc. cit.

(10) J. Roche, loc. cit.

Summary

The magnetic susceptibilities of reduced and oxygenated hemerythrin solutions of the same concentration were measured by a Gouy magnetic balance at 25°. The susceptibility of the reduced solution was found to be higher than that of the oxygenated one, indicating a paramagnetic contribution of iron atoms in the reduced molecule. With the assumptions that the four groups acting as oxygen combining centers in the molecule are equivalent to one another and that they are magnetostatically independent, the number of unpaired electrons in the group was calculated to be twelve in the reduced state and zero in the oxygenated preparation. In the reduced state the bonds of the iron atoms are ionic or ion-dipole type in nature. A profound change in the electronic structure takes place when oxygen molecules are combined with the hemerythrin molecule. An oxygen molecule not only loses its own paramagnetism, but also affects all three iron atoms in the group in such a way that the bond type is essentially covalent. These iron atoms are supposed to be coupled with each other in a polynuclear complex so as to be able to transmit the effect of the change of electronic state directly to the others.

The present experiments were made at the Biological Laboratories, Harvard University and the magnetic measurement at the Department of Metallurgy, Massachusetts Institute of Technology. I wish to express my sincere thanks to Prof. Jeffries Wyman, Jr. for his interest and suggestions and to Prof. Albert R. Kaufmann of MIT for giving me facilities to use his magnetic balance.

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