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THE PROPERTIES OF HEMERYTHRIN

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INTRODUCTION

Hemerythrin is the name given by KRUKENBERG[1] to a pigment of the cells of the coelomic fluid of sipunculids and certain other animals. It was originally discovered by DELLE CHIAIE[2] who, working with *Sipunculus nudus*, also found a connection between colour change and oxygenation. However DELLE CHIAIE thought that the dark-coloured pigment was typical of the "venous" and the clear pigment of the "arterialized" blood, while just the reverse is true. SCHWALBE[3] working on *Phascolosoma elongatum* found that the pigment is contained in cells and becomes "burgundy-red" when exposed to the air, whereas RAY-LANKESTER[4] first succeeded in extracting the pigment by hemolysis. This pigment was found by ANDREWS[5] to be a globulin containing iron, but the iron is not bound in a hematin group (GRIFFITHS[6].) MARRIAN gave[7] the first oxygen dissociation curve of the pigment. Hemerythrin from *Phascolosoma elongatum* was crystallized by FLORKIN[8], who reported a maximal binding capacity of one molecule of oxygen for each three iron atoms. Further physical characterization of the pigment comes from the work of ROCHE[9] and ROCHE AND ROCHE[10]. They found that hemerythrin from *Sipunculus nudus* has an isoelectric point of 5.85. They also reported the spectroscopic properties of the pigment and

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References p. 493.

determined a molecular weight of 66,500 from measurements of osmotic pressure. KUBO[11] found that the magnetic susceptibility of hemerythrin decreases with its oxygenation. He believes that the iron atoms in the reduced and the oxygenated hemerythrin are at a valence state of two. The oxidized ferrihemerythrin obtained by treatment with ferricyanide by MARRIAN[7] is a different product.

Recently there have been conflicting results and opinions concerning the biochemistry of hemerythrin. KLOTZ AND KLOTZ[12] believe that two out of three iron atoms of deoxygenated hemerythrin are bivalent and one is trivalent, while in oxyhemerythrin all are trivalent. This opinion is based on results obtained by treating either oxygenated or deoxygenated hemerythrin solutions with dilute sulphuric acid and *o*-phenanthroline. However, their experiments were criticized by WILLIAMS[13].

Our results indicate a maximal binding of oxygen in a ratio of one molecule for each two iron atoms in the hemerythrin from *Sipunculus nudus*. In addition the present work gives some information on the effect of the addition of acid and ferricyanide to hemerythrin solutions. During the course of our experiments we learned that Dr. W. E. LOVE[14], working with crystallized hemerythrin from *Phascolosoma gouldi*, had obtained results similar to those we have obtained and we agreed to publish our results simultaneously.

METHODS

Fresh hemerythrin solutions were prepared in the following way. The coelomic fluid from *Sipunculus nudus* was centrifuged at 3,000 r.p.m. in a small centrifuge for few minutes. Such a centrifugation permitted complete sedimentation of both pigmented and nonpigmented cells, the latter on top of the pigmented ones. Most of the white cells were removed by gentle suction, while the bottom sediment was resuspended in filtered sea water. By repeating this procedure six or seven times the nonpigmented cells were almost completely removed. The red cells were then hemolyzed by addition of the smallest possible volume of 1/150 *M* phosphate buffer pH 7.0. Cell debris and residual white cells were removed by high speed centrifugation. A clear dark-red solution of hemerythrin was obtained.

The oxygen capacity was measured by the micromethod of VAN SLYKE AND NEILL[15] on 0.2 ml samples in a manometric Van Slyke blood gas apparatus. Correction for the oxygen in solution was included in the blank, for which 0.2 ml of the solvent was used. The amount of oxygen in the blank was generally 10–20% of the total oxygen in the sample.

The spectral properties of hemerythrin were measured in a Beckmann DU spectrophotometer. Deoxygenated hemerythrin was obtained in Thunberg shaped cuvettes by repeated evacuation and admission of hydrogen. So-called metahemerythrin was obtained by treatment with 1/150 *M* potassium ferricyanide in 0.2 *M* tris buffer, followed by prolonged dialysis.

Iron analyses were performed by LORBER'S[16] method as modified by THEORELL *et al.*[17].

RESULTS AND DISCUSSION

1. *The iron to oxygen ratio*

Table I shows that the ratio of iron to oxygen in freshly prepared solutions of hemerythrin from *Sipunculus nudus* is of two to one. This finding modifies previous knowledge. In fact, as we have already stated, FLORKIN in 1933[8] found a value of three to one for this ratio in hemerythrin from *Phascolosoma elongatum*. FLORKIN'S results have never been repeated and have generally been accepted as such in the literature (*e.g.* by HAUROWITZ AND HARDIN[18]). However FLORKIN himself was aware of the need for further experiments as he clearly stated in the following sentence: "Avant d'avoir une certitude complète sur le point que nous occupe, il faudra attendre les résultats de longues séries de déterminations faites sur des solutions trés concentrées d'hemerythrine avec des méthodes diverses, par des expérimentateurs différents".

References p. 493.

Recently W. E. LOVE[14] found for hemerythrin from *Phascolosoma gouldi* a ratio of iron to oxygen of 2.4. We believe that the three to one ratio is erroneous and that it is due to the use of solutions in which there was nearly as much oxygen dissolved as combined, and also to denaturation from prolonged aeration and excessive dilution.

TABLE I

Preparation number	O_2 *Mmoles/l*	*Fe g atoms/l*	Fe/O_2
1	3.40	6.80	2.00
2	2.96	6.30	2.12
3	2.65	4.87	1.84
4	3.02	7.20	2.38
5	2.78	6.04	2.17
6	3.39	7.12	2.10
7*	4.00	7.47	1.87
8	2.09	4.39	2.10
9*	1.80	3.58	1.99
10	1.48	3.08	2.08

* Preparations 7 and 9 were suspensions of cells in plasma, concentrated by centrifugation and partial removal of the supernatant.

The value of 2.4 given by LOVE might be due either to some inactivation during the crystallization procedure or to the different origin of the biological material.

It seems worthwhile to mention that red cell suspensions of *Sipunculus nudus* gave an Fe/O_2 ratio close to 2 (see Table I), thus showing that non-hemerythrin iron is negligible in comparison to hemerythrin iron.

2. The effect of ferricyanide

FLORKIN[8] found that extraction *in vacuo* in the presence of ferricyanide releases rapidly all the oxygen bound to hemerythrin, which behaves therefore like hemoglobin. HALDANE[19] found that even in air ferricyanide is able to displace all the oxygen bound to oxygenated hemoglobin. We have seen that the same happens with hemerythrin. When ferricyanide is added to a solution of oxygenated hemerythrin in a

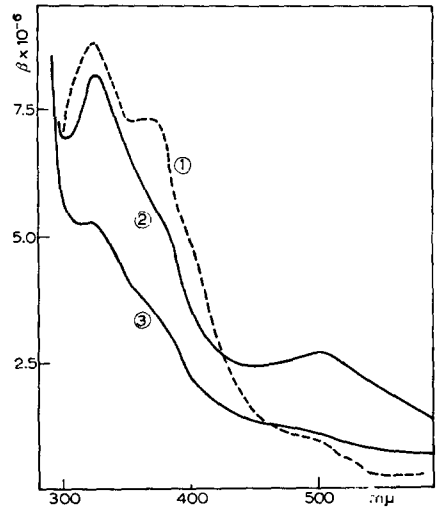

Fig. 1. Absorption spectra of hemerythrin, oxyhemerythrin and metahemerythrin from *Sipunculus nudus*. Abscissa: wavelength in millimicrons. Ordinate: extinction in natural logarithms, for a concentration of pigment of 1 g atom Fe per ml. Curve 1: metahemerythrin; curve 2: oxyhemerythrin; curve 3: deoxygenated hemerythrin. The ordinate value for oxyhemerythrin was $42 \cdot 10^6$ at 278 mμ, giving a ratio of 15.7 for E_{278}/E_{500}.

References p. 493.

Warburg vessel, oxygen is released in an amount exactly equal to the oxygen capacity, as measured in the Van Slyke blood gas apparatus by VAN SLYKE AND NEILL's technique. For instance, 1.65 mmoles/l of bound oxygen were measured by VAN SLYKE AND NEILL's method and 1.65 mmoles/l with ferricyanide addition in air.

Moreover the addition of ferricyanide changes the spectroscopic properties of a solution of oxyhemerythrin. Fig. 1 shows the spectra of oxygenated (2), deoxygenated (3) and ferricyanide-treated (1) hemerythrin. The latter state of the pigment was called "metahemerythrin" by MARRIAN[7].

3. *The effect of acidification on the release of oxygen*

In VAN SLYKE AND NEILL's technique oxygen is released *in vacuo* in the presence of ferricyanide at neutral pH. Acidification previous to ferricyanide addition and extraction *in vacuo* prevents the release of most of the oxygen.

The following experiment proves this point. 0.4 ml of fresh hemerythrin solution was introduced into the chamber of the Van Slyke apparatus and 2.5 ml of deaerated 0.3% ferricyanide solution were added and mixed. 1.0 ml of 6 *N* sulfuric acid was added and the gases were extracted under vacuum. The drop in pressure (reading at 0.5 ml) for oxygen absorption was of 64.6 mm of mercury (average of two determinations of 63.0 and 66.3 mm) corresponding to an oxygen content of 9.9 ml % in the original hemerythrin solution. This agrees well with the 10.0 ml % found by Van Slyke and Neill's method. The same experiment was then repeated, but the order of the reagent addition was changed: first the hemerythrin and the sulfuric acid and then the ferricyanide. The usual extraction *in vacuo* was performed. The drop in pressure after oxygen absorption was now only 6.0 mm of mercury.

Thus oxygen was fully released from an acid solution of hemerythrin if the solution was first oxidized with ferricyanide, but only a very small amount of oxygen (less than 10%) was released if the acidification was performed before the oxidation.

Evidently much of the oxygen is used at low pH for some oxidation reactions: probably oxidation of ferrous to ferric iron, if oxyhemerythrin contains ferrous iron, and/or of oxidizable groups of the protein moiety.

These results can be compared to those obtained on oxyhemoglobin solutions in which acidification to pH's lower than 3 or 4 causes oxidation of the ferrous iron to ferric (ROAF AND SMART[20]) as well as of oxidizable groups of the protein (LEMBERG AND LEGGE[21]). We believe that the 10% oxygen released in the above experiment results from a competition between the rate of release and the rate of disappearance in oxidation reactions.

These findings set a limitation to the conclusions drawn by KLOTZ AND KLOTZ[12] who used acidification of oxyhemerythrin solutions to liberate the iron from the protein in order to measure its valence with *o*-phenanthroline.

Addition of *o*-phenanthroline to the acid solution in order to complex the ferrous iron as soon as liberated failed to give different results. The experiment was performed using acid sodium sulfate containing 0.5% *o*-phenanthroline. The amount of acid sodium sulphate used was such that after addition of the hemerythrin solution a final pH of 2 was attained. This pH value still permits a rapid reaction between *o*-phenanthroline and ferrous iron (KOLTHOFF, LEE AND LEUSSING[22]). Less than 10% of the total bound oxygen was released when the acid solution was added before the

References p. 493.

ferricyanide, while all of it was released when the acid solution was added after the ferricyanide.

4. *Conclusions on the oxygen to iron bonds in hemerythrin*

Oxygen gas is bound in hemerythrin in a ratio of 1 mole for each two iron atoms. The bound oxygen is easily released by addition of ferricyanide both *in vacuo* and in air.

It seems at present difficult to decide whether the oxygen is bound to bivalent iron atoms, as is held by KUBO, or whether it is bound in a peroxy form to trivalent iron as is held by KLOTZ AND KLOTZ.

The decision between either alternative however is not a question of prime importance and is dependent on the point from which the matter is viewed.

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SUMMARY

One mole of oxygen is bound by each two iron atoms of oxyhemerythrin.

Ferricyanide liberates the oxygen from oxyhemerythrin solutions both *in vacuo* and in air.

Only a small part of the total bound oxygen is recovered from a solution of oxyhemerythrin following acidification and extraction *in vacuo*.

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