THE OXIDATION OF HEMOCYANIN

By JAMES B. CONANT, B. F. CHOW, and E. B. SCHOENBACH

(From the Converse Memorial Laboratory of Harvard University, Cambridge)

Reprinted from
THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 101, No. 2, July, 1933

Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 101, No. 2, July, 1933

THE OXIDATION OF HEMOCYANIN

BY JAMES B. CONANT, B. F. CHOW, AND E. B. SCHOENBACH

(From the Converse Memorial Laboratory of Harvard University, Cambridge)

(Received for publication, May 5, 1933)

Hemocyanin is a copper-containing protein which occurs in the blood of a number of species of arthropods and molusks. The extent of the reversible combination with molecular oxygen is a function of the partial pressure of oxygen, and like the formation of oxyhemoglobin is affected by changes in acidity and salt concentration. The ratio of combined molecular oxygen to copper is O_2:2Cu in all the hemocyanins studied from a variety of different bloods. The deoxygenated protein is colorless, while the oxygenated compound (oxyhemocyanin) has an intense blue color.

No evidence has hitherto been available as to whether the copper in hemocyanin was in the cupric or cuprous state. The usual oxidizing agents which oxidize the ferrous compounds, hemoglobin and oxyhemoglobin, to the ferric compound, methemoglobin, appear to be without effect on hemocyanin or oxyhemocyanin. We have now found that by the use of the two very powerful oxidizing agents, potassium molybdicyanide or potassium permanganate, it is possible to oxidize the hemocyanin (or oxyhemocyanin) of *Limulus polyphemus*. In this way two new proteins are formed in which the copper is in the cupric state. One of these, prepared from hemocyanin in the absence of oxygen, is colorless and we shall designate it as *methemocyanin*. The other, *oxymethemocyanin*, is formed when a solution of methemocyanin is shaken with air or oxygen; the deoxygenation of methemocyanin like that of hemocyanin may be brought about by diminishing the partial pressure of the oxygen above the solution. It is evident that, unlike methemoglobin, methemocyanin combines reversibly with oxygen.

The cupric compounds, methemocyanin and oxymethemocyanin, are reduced by the action of a variety of reducing agents,

but two particular reagents have proved of special value in this work. These are potassium ferrocyanide and 1,4-naphthohydroquinone, which are oxidized only very slowly by atmospheric oxygen at a pH value of 7. Because of this fact they may be used for titrating oxidizing agents in the presence of air. The end-point may be determined electrometrically in the usual type of oxidation-reduction cell, or in the case of naphthohydroquinone phenolindophenol may be used as an indicator. In a phosphate buffer of pH 7, this indophenol is reduced by naphthohydroquinone and reoxidized by methemocyanin (or oxymethemocyanin) but only slowly by air. The titration of the cupric compound is therefore complete when the indophenol color (pink) has disappeared. In the use of potassium ferrocyanide an excess of the reducing agent is added and then the solution back titrated rapidly with molybdicyanide, the end-point being determined electrometrically. The reoxidation of the cuprous compound is sufficiently slow as compared with that of the ferrocyanide so that a satisfactory end-point may be obtained.

Oxymethemocyanin

In Table I are summarized a number of experiments in which oxyhemocyanin in a phosphate buffer (pH 7) was treated with varying quantities of dilute potassium permanganate. The color of the permanganate solution disappears rapidly; any excess is probably consumed by attacking some organic grouping in the protein molecule. As will be seen, the oxidizing power of the resulting solution is essentially independent of the amount of permanganate used, if more than 3.5 equivalents are added.

By adding sodium hydrosulfite to an oxymethemocyanin solution, the greenish blue color is discharged and on now being shaken with oxygen the blue oxyhemocyanin is formed. Colorimetric comparison of such regenerated oxyhemocyanin provides evidence as to whether or not the characteristic structure of the protein has been destroyed by this cycle of oxidation, reduction, and oxygenation reactions. In a number of experiments it was shown that no appreciable decomposition had occurred as judged by this criterion.

Oxymethemocyanin may be purified by precipitation with ammonium sulfate. The preparation of purified samples by two different methods is given below.

Preparation of Purified Oxymethemocyanin—The oxyhemocyanin used was obtained from the serum of *Limulus polyphemus* and purified by dialysis and precipitation at the isoelectric point

TABLE I

Oxidizing Power of Crude Oxymethemocyanin Prepared by Means of Permanganate at pH 7

$KMnO_4$ employed*	Total copper†	Cupric copper		Source of oxyhemocyanin
		By $K_4Fe(CN)_6$	By $C_{10}H_8O_2$	
equivalents	*m.-eq. per cc.*	*m.-eq. per cc.*	*m.-eq. per cc.*	
1	1.10		0.42	Crude *Limulus polyphemus* blood
1.8	1.10		0.66	
3.5	1.10		0.94	
5.3	1.10		1.01	
7.0	1.10		1.01	
5.0	0.77	0.86	0.76	Dialyzed and precipitated hemocyanin from *Limulus*
5.0	0.77	0.78	0.75	

* Calculated on the assumption that a manganous salt is the product of reduction.

† Determined after decomposition with H_2SO_4-HNO_3 (the digestion must be complete) by the method of colorimetric comparison, with $K_4Fe(CN)_6$; (see Yoe (1)).

according to the method of Redfield, Coolidge, and Shotts (2). It was dissolved in a 0.1 M phosphate buffer of pH 7, so that the concentration corresponded to about 1×10^{-3} milli-equivalents of copper per cc. 50 cc. of such a solution were treated with either 10 cc. of 0.0077 M $KMnO_4$ or 20 cc. of 0.01 M potassium molybdicyanide. The solution was diluted with about 35 cc. of phosphate buffer and allowed to stand at ice temperature until the reaction was complete. Preliminary experiments had shown that this required less than 20 minutes in the case of potassium permanganate and 1 to 3 hours in the case of molybdicyanide; the course of the reaction could be followed by withdrawing samples (5 cc.) and titrating electrometrically with naphthohydroquinone (0.002 M). The naphthohydroquinone solution was prepared by the catalytic reduction of a naphthoquinone solution in phosphate buffer with palladium-asbestos and hydrogen; it was stored in an atmosphere of nitrogen. It was standardized against a carefully prepared solution of potassium ferricyanide. If potas-

sium permanganate is used in the preparation, a titration may also be carried out with potassium ferrocyanide (0.005 M) as the reducing agent. In this case 5 cc. are treated with 1 cc. of ferrocyanide solution and 3 cc. of buffer solution; the mixture is then titrated rapidly with 0.003 M $K_3Mo(CN)_8$, the end-point being determined electrometrically. The total copper was determined by digestion of a 3 cc. portion. 15 cc. were reduced with hydrosulfite and oxygenated with air; the blue color which resulted was compared in a colorimeter with a sample of the original oxyhemocyanin solution. If molybdicyanide is used, the blue color is attended by the presence of the yellow molybdocyanide and this procedure cannot be used. The reproducibility of the results is indicated by the typical analyses given at the end of this description.

After the reaction mixture had been analyzed as described above, it was centrifuged for 10 to 15 minutes if permanganate was employed. This removed a small amount of brownish precipitate. 2 volumes of saturated ammonium sulfate solution were now added (the temperature was kept at 0°), and the mixture centrifuged for 2 hours in a cold room. The precipitate of purified oxymethemocyanin was dissolved in 25 cc. of buffer solution. The analyses of some typical solutions thus prepared are given in Table II.

The course of a typical preparation is indicated by the following analytical figures, all expressed in terms of milli-equivalents $\times 10^3$ per cc.

(*a*) *With $KMnO_4$*—Crude reaction mixture (total volume 95 cc.) Cu^{++} = 0.25, 0.26 (by $C_{10}H_8O_2$), total Cu = 0.24; after centrifuging, Cu^{++} = 0.24, 0.23, total Cu = 0.25; after precipitating and redissolving in 25 cc. of buffer, Cu^{++} = 0.24, 0.26, total Cu = 0.23.

(*b*) *With Molybdicyanide*—Crude reaction mixture, Cu^{++} = 0.36, 0.39, total Cu = 0.44; after precipitating and redissolving in 25 cc., Cu^{++} = 0.18, 0.14, total Cu = 0.14.

The quantitative data in regard to the purified samples are given in Table II. The last column records the colorimetric results obtained according to the procedure outlined above in which the oxyhemocyanin is regenerated. An inspection of Table II shows that within the limits of experimental error (± 10 per cent) the total copper in the purified oxymethemocyanin is present as

cupric copper determined by either of the two methods employed. The last column affords evidence that on reduction and oxygenation the full color of oxyhemocyanin resulted from each of the samples. The facts presented in Table II, as well as those in Table I, demonstrate that in oxymethemocyanin we are dealing with a cupric compound.

Oxymethemocyanin is of a greenish blue color, the shade being distinctly different from that of oxyhemocyanin itself but of about the same degree of intensity. The presence of cupric copper in the compound can be shown by a number of qualitative tests as

TABLE II

Properties of Purified Oxymethemocyanin

Source	Total copper	Cupric copper		Oxyhemocyanin; colorimetric after reduction and oxygenation
		By $K_4Fe(CN)_6$	By $C_{10}H_8O_2$	
	m.-eq. per cc. $\times 10^3$	*m.-eq. per cc.* $\times 10^3$	*m.-eq. per cc.* $\times 10^3$	
By $KMnO_4$ from purified oxyhemocyanin	*1.92	2.29	1.91	1.95
	*1.99	2.32	2.00	
	0.226	0.236	0.260	0.222
	0.340		0.306	
	0.420		0.390	
By $K_3Mo(CN)_8$ from purified oxyhemocyanin	0.156		0.144	
	0.137		0.144	
	*0.486		0.442	
	*0.480		0.440	

* Duplicate experiments.

well as by the quantitative titrations mentioned above. Oxymethemocyanin oxidizes reduced indophenol (colorless) to the colored indophenol. It oxidizes gum guaiac (made by Eimer and Amend) and benzidine sulfate with the formation of the characteristic colors which, however, are only transient because of further oxidation. When treated with potassium cyanide the color of oxymethemocyanin is destroyed and oxygen is evolved as in the case of the cuprous compound, oxyhemocyanin. Preliminary results indicate that a colorless cyanide complex is formed in the case of both the cuprous and cupric compounds.

Methemocyanin

The oxygen combined with the protein in methemocyanin may be removed by reducing the partial pressure of the oxygen above the solution of the compound. Repeated evacuations of a tonometer containing oxymethemocyanin followed by the introduction of oxygen-free nitrogen are necessary. After repeated evacuations and fillings of the apparatus the color of the solution fades almost completely. On readmission of oxygen, the color is restored.

Another method of preparing the colorless cupric compound, methemocyanin, is to start with deoxygenated hemocyanin (colorless) in a tonometer filled with nitrogen. 3 to 5 equivalents of dilute permanganate solution (all at pH 7) are then added. The permanganate color is rapidly destroyed and an almost colorless solution results. There is a small amount of brownish color, perhaps due to a trace of colloidal manganese compound. The colorless methemocyanin thus prepared or prepared by deoxygenation of oxymethemocyanin shows all the characteristic oxidation reactions of oxymethemocyanin. It oxidizes reduced indophenol and potassium ferrocyanide, naphthohydroquinone, and benzidene sulfate. The quantitative determinations must, of course, be carried out in the absence of oxygen, as otherwise one is dealing with oxymethemocyanin and not methemocyanin.

The result of the titration in nitrogen of a sample of methemocyanin made by deoxygenation of a sample of purified oxymethemocyanin was as follows: milli-equivalents per cc. of oxidizing power by naphthohydroquinone (electrometric end-point) 0.295×10^{-3} and 0.306×10^{-3}, total copper 0.340×10^{-3} and 0.323×10^{-3}; ratio of Cu^{++} to total Cu = 0.88, 0.95. It is evident that the colorless methemocyanin is a cupric compound.

Both oxymethemocyanin and methemocyanin itself slowly decompose on standing and are less stable than the corresponding cuprous compound. The solutions must therefore be kept at ice temperature and worked with within a day of their preparation.

Methemocyanin absorbs oxygen, forming the blue-green oxymethemocyanin previously described. The absorption may be qualitatively demonstrated by the admission of a solution to a given volume of air in a constant volume apparatus. To determine more accurately the amount of combined oxygen in oxymet-

hemocyanin we have used the method of Redfield, Coolidge, and Shotts (2) which was developed for oxyhemocyanin. Like the cuprous compound, oxymethemocyanin forms a colorless cyanide complex which has no affinity for oxygen; the addition of potassium cyanide to an oxymethemocyanin solution therefore evolves oxygen. A constant volume Van Slyke apparatus was employed. A sample of oxymethemocyanin prepared by permanganate in the usual way (but not purified by precipitation) gave the following figures: total Cu 0.95×10^{-3} mg. per cc.; cupric Cu 0.91×10^{-3}; O_2 evolved 0.46×10^{-3} (0.49, 0.43) mM per cc. Ratio of Cu to O_2 = 2.1, 2.0, 2.2.

It is evident from the figures just given that the ratio of combined oxygen to copper is the same in oxymethemocyanin as in oxyhemocyanin itself. For this reason, no gas is evolved when oxymethemocyanin is reduced with ferrocyanide in a Warburg apparatus and no gas is absorbed when oxyhemocyanin (in air) is oxidized with permanganate or molybdicyanide.

Oxidation-Reduction Potential of the Hemocyanin System

Potassium ferrocyanide is oxidized by the cupric compounds, methemocyanin and oxymethemocyanin. If the former is used, the experiment must be performed in nitrogen, of course. If 1 equivalent of oxymethemocyanin is added to 1 equivalent of ferrocyanide (in a phosphate buffer at pH 7), 10 minutes after adding the oxymethemocyanin, the potential rose to +0.542 from the initial potential of the ferrocyanide of +0.365 volt; after 1 hour the potential was +0.545. (The potential is expressed in terms of the normal hydrogen electrode, the European convention in regard to sign being employed.) The fact that the equilibrium may be approached from both sides is shown by the behavior of a mixture of ferricyanide and oxyhemocyanin. The initial potential of ferricyanide is +0.615 volt and is lowered to +0.565 volt 10 minutes after adding 1 equivalent of oxyhemocyanin; in 1 hour the potential was practically constant at +0.545 volt. In duplicate experiments, the final equilibrium potential was found to be $+540 \pm 5$ millivolts. When methemocyanin or hemocyanin was used, and the electrochemical cell filled with nitrogen, very similar results were obtained. When the equilibrium was approached from the methemocyanin side, the final value was +563

± 10 millivolts and from the other side +525 ± 15 millivolts. If tungsti- and tungstocyanides are used in place of ferri- and ferrocyanides, equilibrium may also be approached from both sides with oxygenated or deoxygenated compounds. The equilibrium potential with 1 equivalent each of reagent and protein is +0.540 ± 0.010 volt both in nitrogen and in air. The fact that the oxygenated and deoxygenated compounds give essentially the same results is to be expected since both the cuprous and cupric compounds combine with oxygen.

The experiments just described provide convincing evidence of the reversibility of the hemocyanin-methemocyanin system and the oxyhemocyanin-oxymethemocyanin system. At first sight it is surprising that the equilibrium potentials are essentially the same whether the ferricyanide system ($E_h^0 = 0.440$) or the tungsticyanide system ($E_h^0 = 0.530$) is employed. The explanation is undoubtedly due to the large value of n in the usual electrochemical equation.

$$E_h = E_h^0 - \frac{RT}{nF} \ln \frac{[\text{reduced}]}{[\text{oxidized}]}$$

If the hemocyanin molecule had a molecular weight corresponding to the minimum value of 73,400 which corresponds to 2 copper atoms or 1 oxygen molecule, the value of n in the electrochemical equation should be 2. Using this value and the results given above, we calculate E_h^0 from the ferricyanide experiment as +0.596 and from the tungsticyanide experiment as 0.558. The discrepancy between these numbers might be due to experimental error but is more probably an indication that the value of n in the electrochemical equation is much larger than the minimum value. For example, if the value of 6 is taken, the two results become 0.561 and 0.537 respectively, while if a value of 10 is taken, the two numbers are in fairly good agreement (0.554 and 0.544). While these measurements cannot be taken as conclusive proof of the large value of n, they are very suggestive that a value at least as large as 10 should be taken. The difficult problem connected with the value of n in the electrochemical equation governing the oxidation of hemoglobin has been discussed in papers from this laboratory (3) and need not be considered again at this point. It is interesting, however, that if we take the molecular weight of

Limulus hemocyanin as determined by Svedberg (4) as 2,040,000 the value of n in the electrochemical equation should be approximately 56, provided there are no intermediates. Such a large value would put the two determinations of the oxidation-reduction potential from the tungsticyanide and ferricyanide experiments very close together (0.546 and 0.540 volt).

The direct titration of either hemocyanin or oxyhemocyanin with an oxidizing agent is not entirely satisfactory, although the results were sufficiently definite so that they provided us with the first definite clue that we were working with a cuprous-cupric reversible system. After each addition of oxidizing agent it is necessary to wait for a considerable time before the potentials become constant. If one waits until the change of potential with time is small and constant and records these points as equilibrium points, it is possible to obtain a titration curve. Such a titration curve shows a fairly definite "break" at a point corresponding to 1 equivalent of molybdicyanide per copper atom. The back titration with sodium hydrosulfite is more rapid but the potentials are considerably below those obtained with molybdicyanide. The number of equivalents, as before, corresponds to 1 reducing equivalent per copper atom within 10 per cent. The potentials determined by this titration method are much less satisfactory criteria of the reversibility of the system than the experiments mentioned previously. They indicate approximately the same value of the oxidation-reduction potential as obtained by the more accurate experiments with 1 equivalent of oxidizing or reducing agent. The difficulties with the titration method are undoubtedly due to the fact that each small increment of oxidizing agent reacts to some extent with the organic part of the protein molecule as well as with the copper. It is also probable that the cuprous-cupric protein compounds themselves do not impress a satisfactory potential upon the electrode. For all these reasons the direct titration procedure, which is so valuable in studying many oxidation-reduction systems, does not particularly lend itself to the case of hemocyanin.

In all these experiments we have confined our attention to the hemocyanin from *Limulus polyphemus*. A few experiments with the hemocyanin from the blood of the lobster, *Homarus americanus*, indicate that it also may exist in a cupric state and the

potential is not very different from that of the *Limulus* hemocyanin.

We have never succeeded in preparing methemocyanin or oxymethemocyanin by the action of any catalyst on oxyhemocyanin. The potential of the oxygen electrode is sufficiently high so that oxyhemocyanin has the possibility of decomposing to give oxymethemocyanin, but we have not as yet been able to bring this change about in the laboratory. Unless a catalyst could be found that would make the change rapid, there is little chance of success, since the slow autoreduction of the cupric compound would keep the protein in the cuprous condition.

In a preliminary paper from this laboratory (5), the isolation of a black material from the alkaline decomposition of *Limulus* hemocyanin was described. This substance contained sulfur and nitrogen and about 20 per cent of copper; we believed it to be a true prosthetic group. Further experiments, which will be published shortly, have shown that this material is a complex copper salt of a polypeptide and a sulfur-containing amino acid. It is almost certainly a true prosthetic group and probably bound to the protein by coordinate linkage of the copper (in a manner similar to the binding of heme in hemoglobin). The black prosthetic group is insoluble in 0.1 N aqueous alkali but in the presence of a third of the volume of pyridine dissolves, forming a greenish solution. Such a solution undoubtedly contains a complex soluble salt of the prosthetic group and pyridine; it is comparable to a parahematin solution (or in the reduced state to a hemochromogen). The titration of such a solution (0.0123 mM of Cu per cc.) with sodium hydrosulfite in nitrogen showed the presence of cupric copper and back titration with ferricyanide confirmed this (mM of Cu per cc. by $K_3Fe(CN)_6$, 0.0127). The oxidation-reduction potential from the mid-point of the not very satisfactory titration curves was +0.15 volt. This is much lower than the value for the protein compound but the comparison between these measurements was only possible in a strongly alkaline solution. It appears, however, that, as in the case of hemoglobin, a combination of the prosthetic group with the protein raises the oxidation-reduction potential.

In conclusion attention may be drawn to the fact that the oxidation-reduction potential of the hemocyanin system is extremely

high. With the exception of the complex cyanides of molybdenum and tungsten and potassium permanganate, methemocyanin and oxymethemocyanin are among the strongest reversible oxidizing agents known. Whether or not this has any biological significance further experiments alone can decide. It is clear that if the cupric compound functioned in any biological process it would not persist long in the cupric state but would be reduced by many of the substrates present in the biological fluids. We could therefore not expect to obtain direct evidence of the existence of the cupric compound in living tissue.

We wish to express our sincere appreciation of the invaluable cooperation of Professor A. C. Redfield, without which this work would not have been possible.

BIBLIOGRAPHY

1. Yoe, J. H., Photometric chemical analysis, New York, **1**, 182 (1928).
2. Redfield, A. C., Coolidge, T., and Shotts, M. A., *J. Biol. Chem.*, **76**, 185 (1928).
3. Conant, J. B., and Fieser, L. F., *J. Biol. Chem.*, **62**, 595 (1924–25).
4. Svedberg, T., and Heyroth, F. F., *J. Am. Chem. Soc.*, **51**, 539 (1929).
5. Conant, J. B., and Humphrey, W. G., *Proc. Nat. Acad. Sc.*, **16**, 543 (1930). See also Philippi, E., *Z. physiol. Chem.*, **104**, 88 (1919). Schmitz, A., *Z. physiol. Chem.*, **194**, 232 (1931); **196**, 71 (1931).