cules, since the properties of active molecules are obscured when present in a mixture.[37]

(37) R. D. Hotchkiss, in "The Chemical Basis of Heredity," McElroy and Glass, eds., Johns Hopkins Press, Baltimore, Md., 1957, p. 391.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOPHYSICS, UNIVERSITY OF PITTSBURGH]

Specific Reactions of Hydrogen Peroxide with the Active Site of Hemocyanin. The Formation of "Methemocyanin"[1]

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The effect of hydrogen peroxide on the hemocyanin of *Limulus polyphemus* and *Busycon canaliculatum* has been studied. In the case of *Limulus* it is found that the deoxygenated hemocyanin is much more sensitive to attack than is the oxygenated hemocyanin, that one equivalent of peroxide per mole of copper is sufficient to destroy most of the oxygen-carrying capacity of the deoxygenated hemocyanin and that peroxide acts by oxidizing the cuprous ion of deoxygenated hemocyanin to cupric ion. It has so far not been possible to regenerate hemocyanin from the attacked material by the use of reducing agents. *Busycon* hemocyanin behaves similarly except that it is possible to regenerate hemocyanin by the use of reducing agents, notably by use of an excess of peroxide. In both species the product of attack has a sufficiently strong affinity for the oxidized copper ions to prevent their removal by an IR-120 cationic exchange resin. It is proposed that the properties of the product of peroxide oxidation on hemocyanin for the first time justify the use of the name "methemocyanin." The remarkable effectiveness of peroxide in the oxidation of hemocyanin and in the reduction of *Busycon* methemocyanin, and the correlation between cuprous ion oxidation and active site destruction, provide important information about the active site structure of hemocyanin.

Introduction

In 1933, Conant and his collaborators[2] reported that they had been able to prepare a material which they termed "methemocyanin" by the action of potassium permanganate or potassium molybdicyanide upon the hemocyanin of the horseshoe crab. The fact that this "methemocyanin" had the same oxygen-carrying properties as native hemocyanin and that there was no other evidence of oxidation of copper led Rawlinson[3] to conclude that Conant had not succeeded in attacking the active site of the molecule and that his product was not methemocyanin, but merely hemocyanin in which certain groups unrelated to physiological activity had been oxidized.

Our more recent knowledge of the nature of the active site in the hemocyanins permits us to understand why oxidizing agents strong enough to oxidize cuprous ion to cupric ion might not be able to attack the active site copper of hemocyanin, even though in deoxygenated hemocyanin this copper is entirely in the cuprous state.[4] The cuprous ions of hemocyanin, which are probably bound to the sulfur atoms of cysteine side chains,[4] have an equilibrium constant of about 10^{18} for associat on with the protein.[5] This equilibrium constant (which is nearly the same as that of the cuprous–cysteine complex)[6] is much larger than any of the equilibrium constants for association of cupric ion with the usual amino acid side chains of proteins; any difference of this kind would alter the oxidation potential of hemocyanin-bound cuprous ion in such a way as to reduce its susceptibility to oxidation.[7] It is also possible that the low dielectric constant of the protein may result in decreased oxidation rates if charged reactants or products are involved despite an equilibrium position favorable to oxidation.[9] Finally, steric factors may decrease the rate of oxidation sufficiently so that reagents which are capable of oxidizing free cuprous ion to cupric ion do not affect the active site.

In an attempt to find an effective oxidant, we have studied the action of hydrogen peroxide upon the cuprous ion of hemocyanin. We will show that the hemocyanin obtained from two different species, the arthropod *Limulus polyphemus* and the mollusc *Busycon canaliculatum*, is attacked by hydrogen peroxide. The attack is specifically upon the active site; it involves stoichiometric amounts of peroxide, and it results in specific oxidation of the cuprous ion with attendant loss of oxygen-carrying properties. In the case of *Busycon*, the oxidized material can be reduced with a suitable reducing agent, with a full restoration of ability to carry oxygen. We will propose that these properties justify the designation of the product of peroxide attack on hemocyanin as "methemocyanin."

(1) Contribution No. 63 from the Department of Biophysics. This research was supported in part by a grant (G5728) from the National Science Foundation. Presented in part at the 136th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept. 13-18, 1959.

(2) J. B. Conant, B. F. Chow and E. B. Schoenbach, *J. Biol. Chem.*, **101**, 463 (1933).

(3) W. A. Rawlinson, *Australian J. Exp. Biol. Med. Sci.*, **19**, 137 (1941).

(4) I. M. Klotz and T. A. Klotz, *Science*, **121**, 477 (1955).

(5) G. Felsenfeld, *J. Cell. and Comp. Physiol.*, **43**, 23 (1954).

(6) W. Stricks and I. M. Kolthoff, THIS JOURNAL, **73**, 1723 (1951).

(7) This is contrary to the situation in hemoglobin and in the more highly coördinated complexes of copper, in which the higher oxidation state of the metal is usually stabilized (Ref. 8). In the case of copper ion which has only one coördination site occupied by a ligand other than water, the lower oxidation state is stabilized, since the first association constant of a copper complex with a given ligand is generally greater for cuprous ion than for cupric ion. (See, for example, F. R. N. Gurd and P. E. Wilcox, in "Advances in Protein Chemistry," Vol. XI, ed. M. L. Anson, K. Bailey and J. T. Edsall, Academic Press, Inc., New York, N. Y., 1956, p. 351.)

(8) A. E. Martell and M. Calvin, "Chemistry of the Metal Chelate Compounds," Prentice–Hall, Inc., New York, N. Y., 1952, pp. 57, 373.

(9) J. H. Wang, THIS JOURNAL, **80**, 3168 (1958).

Experimental

Serum obtained from the horseshoe crab, *Limulus polyphemus*, and the whelk, *Busycon canaliculatum*, was stored under toluene until needed. Small amounts were purified shortly before use by centrifugation at 40,000 r.p.m. in the Spinco Model L ultracentrifuge for 5 hr. The pellet was redissolved in the desired buffer and small amounts of insoluble material, if present, removed by low speed centrifugation. The optical absorption maximum at 340 mμ was used as a measure of oxygen-carrying capacity, since it disappears upon deoxygenation[10] or destruction of oxygen-carrying ability, and is about twenty times more intense than the absorption maximum in the visible region. In each case, disappearance of the 340 mμ maximum is accompanied by loss of the characteristic blue hemocyanin color. Unless otherwise noted, solutions for ultraviolet absorption studies were obtained by diluting 0.2 ml. of the working hemocyanin solutions, after peroxide attack, with 4 ml. of 0.2 *M* potassium phosphate buffer at *p*H 7, and filtering the diluted solution through a coarse sintered glass filter to remove any small undissolved particles present. Spectra were obtained with the Cary Model 14 Recording Spectrophotometer.

Deoxygenation was accomplished by bubbling Air Reduction Co. specially purified nitrogen (O_2 <0.002%) through the hemocyanin solution, to which was usually added a small quantity of a dilute suspension of Dow antifoam A, to permit rapid gas flow without foaming. Controls in which the antifoam agent was omitted showed no differences in response to deoxygenation or to hydrogen peroxide. Analysis of total protein copper was carried out by wet digestion of the protein (sulfuric acid–30% H_2O_2) and the use of the reagent sodium diethyldithiocarbamate.[11] Standard reference solutions of cupric ion were analyzed by the usual iodometric method.[12]

Analysis for cuprous ion was carried out by a modification of previous methods[4] for measuring cuprous ion in hemocyanin. To an aliquot of hemocyanin was added 0.2 ml. of 0.2 *M* ethylenediaminetetraacetic acid disodium salt (EDTA). The solution was made up to a total volume of 2 ml. with water and 2 ml. of a solution (0.5 mg./ml.) of 2,2′-biquinoline (Eastman Kodak Co.) in glacial acetic acid was added. The effect of the EDTA is to protect cupric ions from possible reduction by sulfhydryl groups when the protein is denatured by the glacial acetic acid.[13] The optical density of the final solution was read with a Beckman DU spectrophotometer at 540 mμ. The combination of this method with the absorption studies of oxyhemocyanin gives a molar optical density for the chromatic group (O.D. of oxyhemocyanin minus O.D. of reduced hemocyanin) at 340 mμ of 13,900 for *Limulus* (serum) and 10,500 for *Busycon*, ±10%.

The hydrogen peroxide used was Fisher 3% analytical reagent. It was analyzed frequently either by iodometric titration[12] or by measurement in the Warburg apparatus of the volume of oxygen liberated at 20° by the addition of catalase (Nutritional Biochemicals, lyophilized). The two methods gave results which agreed within 2%.

The Spinco Model E ultracentrifuge and Model H electrophoresis apparatus were used for physical characterization.

Conventional Warburg apparatus (Bronwill) was used. Measurements were made at high shaking rates (120 per minute). Ion-exchange studies were carried out with Amberlite IR-120(H) resin, using preparative methods which have been described.[14]

All peroxide–hemocyanin reactions were carried out at 25°, except where noted. Analytical or C.P. grade inorganic chemicals were used throughout.

Results

Limulus **Hemocyanin.**—When small amounts of hydrogen peroxide, of the order of one equivalent of peroxide per mole of copper, are added to *Limulus* oxyhemocyanin, there is no apparent effect upon the absorption at 340 mμ. It is not until a ten-fold excess of peroxide has been added that a loss of blue color, and of the 340 mμ absorption peak, is at all observable. On the other hand, if the hemocyanin is first deoxygenated, the peroxide added while the protein is in the deoxygenated state, and the solution is then reoxygenated, an equivalent of peroxide per mole of copper is sufficient to destroy nearly all of the absorption at 340 mμ (Fig. 1). The product of this attack is not an oxygen carrier; there is no evolution of gas when it is mixed with a cyanide solution in the Warburg apparatus.

It is possible to titrate the active sites[15] with peroxide, as shown in Fig. 2. The end-point of the titration can be extrapolated to about 1.2 equivalents of peroxide per mole of copper, and there is a linear relationship between number of equivalents of peroxide added and number of sites destroyed. If, in addition, the concentration of cuprous ion is measured at each point in the titration (Fig. 2) it is found that one mole of cuprous ion is oxidized for each equivalent of peroxide added[17] and therefore that every cuprous ion has been oxidized in the completely inactive material. Oxygenated hemocyanin, which is protected from destruction of the oxygen-carrying property, is also protected from oxidation of cuprous ion. An equivalent of peroxide added to oxyhemocyanin causes no change in cuprous ion concentration.

The kinetics of this reaction have not yet been studied, but the reaction is 50% completed within two minutes of addition of the peroxide. We have permitted the reaction to continue in a nitrogen atmosphere for 30 minutes, beyond which no further change is observed. The results shown in Fig. 2 were obtained at *p*H 7.0, but there is no significant difference in results obtained either at *p*H 6.0 or *p*H 8.5.

Despite the known tendency of the hemocyanins to aggregate or disaggregate under mild changes of condition,[18] ultracentrifuge and electrophoretic studies of the purified material suggest that the peroxide attack has no effect upon the size and shape of the protein molecule. The sedimentation

(10) C. R. Dawson and M. F. Mallette in "Advances in Protein Chemistry," Vol. II, ed. M. L. Anson and J. T. Edsall, Academic Press, Inc., New York, N. Y., 1945, p. 207.

(11) T. D. Price and R. E. Telford, "Analytical Chemistry of the Manhattan Project," McGraw-Hill, Inc., New York, N. Y., 1950, p. 408.

(12) I. M. Kolthoff and E. B. Sandell, "Textbook of Quantitative Inorganic Analysis," Revised Edition, the Macmillan Co., New York, N. Y., 1948.

(13) Details of this method will be given in another publication.

(14) I. H. Scheinberg and A. G. Morell, *J. Clin. Invest.*, **36**, 1193 (1957).

(15) The results shown in Figs. 1 and 2 were obtained with *Limulus* serum, rather than purified *Limulus* hemocyanin. Results with serum have been chosen for illustration because purification of *Limulus* hemocyanin, either by our method or the method of Redfield (ref. 16), causes some loss in oxygen-carrying capacity per mole of copper. In addition, the purified material, unlike either *Limulus* serum or *Busycon* hemocyanin (native or attacked), does not show a simple relationship between cuprous ion in the oxygenated state and in the deoxygenated state (ref. 3, 13 and 17). This makes the measurement of oxidized cuprous ion more difficult. However, the inactivation curve obtained is the same as that shown in Fig. 2, and every statement made regarding the serum–peroxide reaction is equally true of reaction of peroxide with the purified hemocyanin.

(16) A. C. Redfield, *Biol. Bull.*, **58**, 150 (1930).

(17) Since the biquinoline reagent is added after the solution is reoxygenated, only half the copper of the unattacked oxyhemocyanin gives the cuprous reaction (ref. 3, 13). The measured cuprous ion decreases linearly as peroxide is added and is always equal to one half the copper bound to unattacked hemocyanin. Therefore, in the course of the reaction between peroxide and deoxygenated hemocyanin two atoms of copper must have been oxidized by each peroxide molecule. This is confirmed by measurement of total Cu^+ in selected samples of deoxygenated attacked material.

(18) T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Oxford, 1940 p. 366.

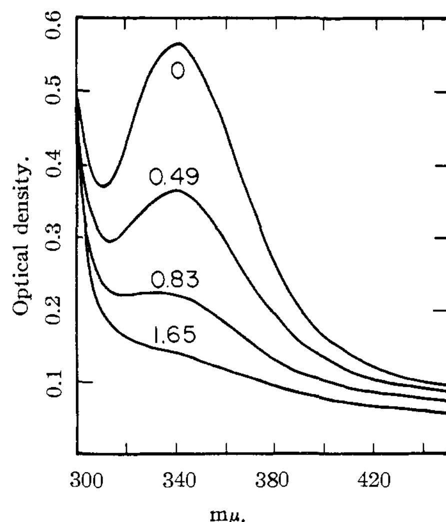

Fig. 1.—The change in the ultraviolet absorption spectrum of *Limulus* serum with addition of hydrogen peroxide. Numbers near each spectrum give the ratio of equivalents of peroxide to moles of copper.

coefficients of a 0.3% solution in 0.05 *M* phosphate buffer, *p*H 7, (s_{20}) before and after attack are 35 and 37.2, which are the same within experimental error. The schlieren pattern when the boundary is near the bottom of the cell consists in both native and attacked hemocyanin of one peak containing about 95% of the sedimenting material, and a smaller peak, which separates from the bigger peak only near the end of the run, comprising the remainder. No other components are observed under these conditions. The numbers given above are the sedimentation coefficients of the large peak. Electrophoretic studies (0.05 *M* phosphate buffer, *p*H 6.75, 0.3% protein) show a single skewed peak in both native and peroxide-attacked material, with mobilities of 3.32 and 3.44 $cm.^2 sec.^{-1} volt^{-1} \times 10^{-5}$, respectively, which are again the same within experimental error.

The oxidized copper ions are still strongly bound to the protein, since dialysis of attacked material 10^{-3} *M* in bound copper against 0.1 *M* EDTA at *p*H 7 results in no change in the total amount of copper bound. If inorganic cupric ion is added to attacked *Limulus* hemocyanin and the mixture passed through a column containing Amberlite IR-120(H) cationic exchange resin, the effluent contains an amount of copper equal to that bound to the protein before addition of the inorganic cupric ion.

We have not found it possible to restore oxygen-carrying properties of *Limulus* hemocyanin by reduction with ferrocyanide or ascorbic acid. In agreement with the results of Conant[2] we have not been able to destroy the oxygen-carrying properties of the native hemocyanin with excesses of weaker oxidants, such as permanganate (*p*H 7) or ferricyanide, even when the system is first deoxygenated.

Busycon **Hemocyanin.**—If a large excess of hydrogen peroxide is added to purified *Busycon* oxyhemocyanin,[19] there is no destruction of active

(19) All experiments with *Busycon* hemocyanin were performed on purified material, since there is no loss of activity on purification.

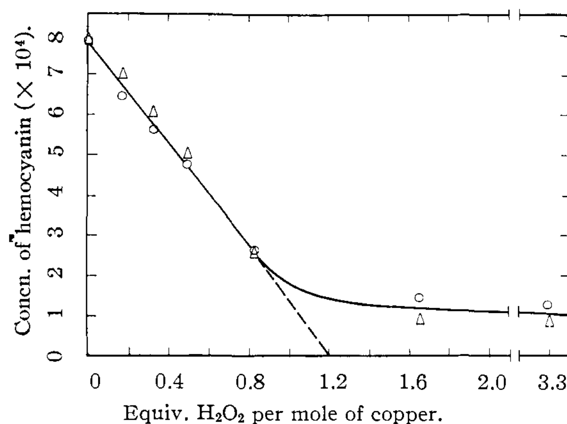

Fig. 2.—The titration of *Limulus* serum with hydrogen peroxide: O, active site concentration from absorption spectrum; Δ, cuprous ion concentration from biquinoline reaction. Concentration of hemocyanin in all figures is given in terms of copper concentration.

sites, but the peroxide is rapidly decomposed to oxygen and water. If the protein is deoxygenated and an excess of peroxide is added, there is again a rapid liberation of oxygen, and total restoration of the blue color and 340 mμ absorption maximum. If small quantities of peroxide are used to perform a titration on deoxygenated *Busycon* hemocyanin in a manner analogous to that shown in Fig. 2 for *Limulus*, the result shown in Fig. 3 is obtained upon reoxygenation. The initial portion of the inactivation curve has the same slope as that of

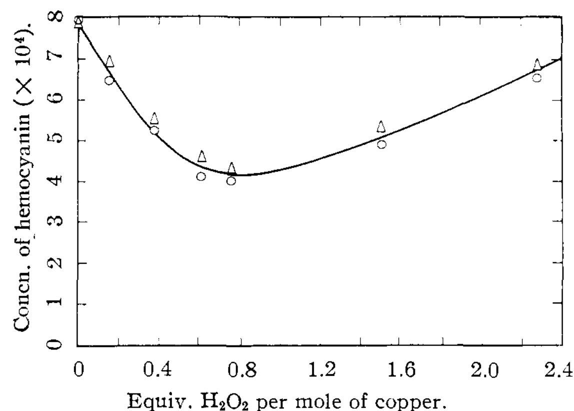

Fig. 3.—The titration of *Busycon* hemocyanin with hydrogen peroxide. No nitrogen bubbled through solutions after addition of peroxide (see text): O, active site concentration from absorption spectrum; Δ, cuprous ion concentration from biquinoline reaction.

Limulus.[20] However unlike *Limulus* the activity does not reach a value near zero at one equivalent and remain there, but goes through a minimum at 0.7 equivalent and then rises again. The concentration of cuprous ion, shown in Fig. 3, behaves in the same manner as the 340 mμ absorption. If an excess of peroxide is added in the presence of oxygen to

(20) The fact that with both *Limulus* and *Busycon* hemocyanins the extrapolated end-point occurs at 1.2 equivalent rather than 1 equivalent can be attributed at least partly to the catalase-like activity of the hemocyanins and their oxidation products. A small amount of attack on groups unrelated to physiological activity is not excluded.

any one of the solutions containing some attacked material, the optical density at 340 mμ rapidly rises to that of the original completely native material. The regenerated hemocyanin is physiologically active, since deoxygenation causes disappearance of all of the 340 mμ peak, and reoxygenation causes its restoration.

It is clear from these results that hydrogen peroxide can act both as an oxidant and reductant for the copper of *Busycon*. Oxygen is generated during the reduction step, and since oxyhemocyanin is protected against attack by peroxide, the "trapping" of liberated oxygen by regenerated hemocyanin results in the observed rise in optical density and cuprous ion concentration beyond 0.7 equivalent. This is possible under the conditions of the experiment, in which the bubbling of nitrogen through the solution ceases immediately after addition of the peroxide, and the solution remains under a nitrogen atmosphere for 30 minutes. If bubbling of nitrogen is continued for five minutes or more after addition of peroxide, so that any oxygen generated is removed, a different result is obtained. Figure 4 shows that under these conditions the amount of unattacked hemocyanin approaches a

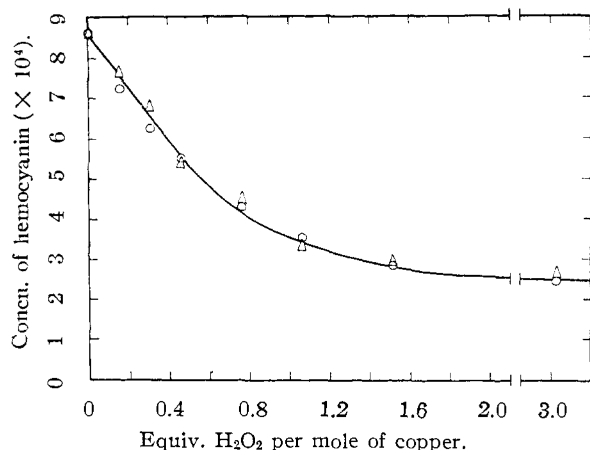

Fig. 4.—The titration of *Busycon* hemocyanin with hydrogen peroxide. Nitrogen bubbled through solutions for five minutes after addition of peroxide (see text): ○, active site concentration from absorption spectrum; Δ, cuprous ion concentration from biquinoline reaction.

minimum and remains there as further peroxide is added. The minimum concentration of unattacked material which can be obtained is about 30% of the original hemocyanin concentration. Further additions of peroxide do not alter this result, presumably because at these relative concentrations of attacked and unattacked hemocyanin the oxidation and reduction rates are equal. There is no significant change in this result if the experiment is carried out at *p*H 5 (0.1 *M* acetate buffer) or *p*H 10 (0.2 *M* glycine buffer) instead of *p*H 7.

By adding peroxide to such a mixture of active and inactive material and observing the rate of increase in optical density at 340 mμ, it is possible to study the kinetics of reactivation. The reaction is carried out in air and the reactivation produces a molecule of oxygen for each hemocyanin "site" regenerated, so that any hemocyanin produced is oxygenated.[21] The kinetics are complicated by the catalase-like activity of the native hemocyanin, which causes a decrease in peroxide concentration with time. However, it is possible to measure the initial reaction rate as a function of starting peroxide and protein concentration. The variation in this rate (Fig. 5) indicates that the reactivation is a second-order reaction in the range of concentrations studied, with a rate proportional to peroxide and attacked hemocyanin concentrations.

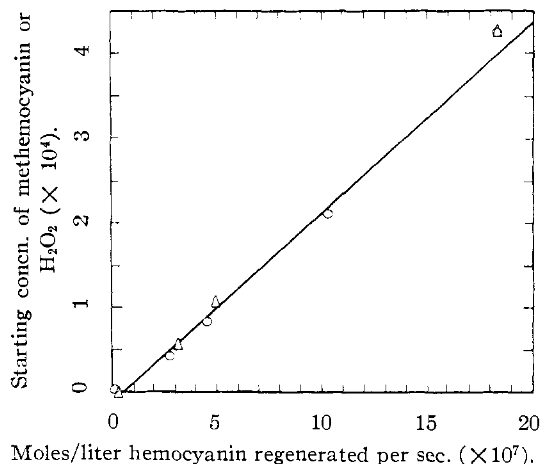

Fig. 5.—Kinetics of reduction of *Busycon* methemocyanin by hydrogen peroxide. Initial rate of hemocyanin formation as a function of reactant concentration: ○, H_2O_2 concentration varied, methemocyanin concentration held constant at 4.25×10^{-4}; Δ, methemocyanin concentration varied, H_2O_2 concentration held constant at 4.24×10^{-4}.

Busycon hemocyanin also appears to be regenerated by the action of other reducing agents. Addition of potassium ferrocyanide at *p*H 7 results in an increase in the 340 mμ maximum, though the rate of the reaction is more than 100 times slower than that found with comparable concentrations of peroxide.[22]

Attacked *Busycon* hemocyanin, like that of *Limulus*, does not surrender its active site copper to an IR-120 ion-exchange column.

Catalase-like Activity.—Ghiretti[23] has made a study of the ability of hemocyanin to catalytically decompose hydrogen peroxide, liberating oxygen. The catalytic efficiency varies markedly from one species to another. It is greatest in the hemocyanin of *Octopus*, which shows two activity peaks at *p*H 6.8 and *p*H 10.

We have made a similar study of the activity of *Busycon* and *Limulus* hemocyanins. *Busycon* hemocyanin has marked catalytic activity both at *p*H 7 and *p*H 10 (Table I) as might be expected from the kinetic behavior described in the previous section. *Limulus* hemocyanin (or serum) also is capable of decomposing peroxide, though the rate at *p*H 10 is considerably greater than that at *p*H 7. Figure

(21) At these partial pressures of oxygen *Busycon* hemocyanin is nearly 100% oxygenated. A. C. Redfield and E. N. Ingalls, *J. Cell. and Comp. Physiol.*, **1**, 253 (1932).

(22) In the case of reductants other than peroxide, it is necessary to mix the solution thoroughly before each reading to prevent diffusion of oxygen from becoming the rate determining factor in appearance of the 340 mμ peak.

(23) F. Ghiretti, *Arch. Biochem. and Biophys.*, **63**, 165 (1956).

TABLE I

TYPICAL DECOMPOSITION RATES OF H_2O_2 BY HEMOCYANINS

H_2O_2 concentration = 1.6×10^{-2}; total volume = 0.9 ml.

	μmoles H_2O_2 dec. per μmole Cu per min. (initial rate)
Busycon hemocyanin, *p*H 7 (25°)	0.86
Busycon hemocyanin, *p*H 10 (25°)	1.1
Limulus serum, *p*H 7 (25°)	0.050
Limulus serum, *p*H 10 (25°)	.62
Limulus hemocyanin, *p*H 10 (24°)	.50

6 shows the liberation of oxygen by *Limulus* hemocyanin as a function of time, measured in the Warburg apparatus. The very large excesses of peroxide involved in the manometric studies completely destroy the *Limulus* hemocyanin, despite the protection afforded by oxygenation. Destruction takes place rapidly, and it is clear that the catalytically active material in this case is not native hemocyanin. The rate of oxygen production is directly proportional to the attacked protein concentration. Addition of more peroxide to the flask after reaction is complete results in duplication of the first oxygen liberation curve. Material which has been previously destroyed by deoxygenation and addition of one equivalent of peroxide per mole of copper is as active as native hemocyanin. Though *Limulus* and *Busycon* hemocyanins both have catalase-like activity, the mechanism of this activity is apparently different in the two cases.

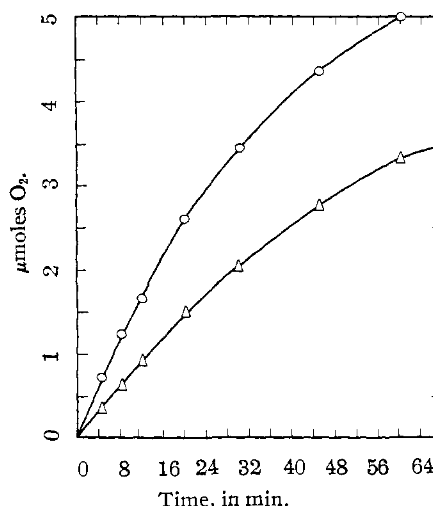

Fig. 6.—Oxygen liberated from H_2O_2 in the presence of *Limulus* hemocyanin, *p*H 9.9. Warburg flasks contained 14.4 μmoles H_2O_2 (0.2 ml. of 0.072 *M*), *Limulus* hemocyanin (6.52×10^{-4} *M* in copper, dissolved in 0.05 *M* *p*H 7 phosphate buffer), and sufficient 2 *M* glycine buffer (*p*H 10) to make a total volume of 0.9 ml.: O, 0.5 ml. of hemocyanin; Δ, 0.25 ml. of hemocyanin.

Discussion

The results obtained with *Limulus* hemocyanin show that peroxide is capable of a selective attack upon the active site. The single fact that oxygenated hemocyanin is insensitive to attack, while the deoxygenated protein is inactivated by an equivalent of peroxide, is sufficient evidence of this. The decrease in cuprous ion with addition of peroxide shows that inactivation involves oxidation of the cuprous ions of deoxygenated hemocyanin to the cupric state.

The generally accepted though poorly substantiated conception of the active site structure[4] involves a pair of copper ions oriented in such a manner that an oxygen molecule can form a bridge between them. Of particular interest in this connection is the correlation between amount of cuprous ion destroyed by peroxide and loss of activity. If the paired-copper structure is correct, it should only be necessary to oxidize one of each pair of cuprous ions in the deoxygenated active site in order to destroy the ability to carry oxygen. This effect should be particularly noticeable at low peroxide/copper ratios, where one equivalent of peroxide, in oxidizing one mole of cuprous ion, should destroy one mole of active site (*i.e.*, the oxygen carrying capacity of two moles of copper ions). As more peroxide is added, the probability of a "double hit" on the same site should become greater, but even at the point at which half the cuprous ions are oxidized, considerably more than half the activity should be lost. Figure 2 shows that this is not the mechanism of inactivation. For every active site destroyed two cuprous ions are oxidized. This could be taken to mean that half the copper ions of hemocyanin are in no way related to the active site structure. As a matter of fact, Klotz's results[4] with biquinoline–acetic acid suggest that only half the cuprous ions change oxidation state upon oxygenation. However, the fact that oxygenation protects both copper ions of hemocyanin from oxidation by peroxide shows that both are involved in the active site. The most reasonable explanation of the result shown in Fig. 2 is that the copper ions are located in pairs (or multiples of two) and that because of this proximity, a peroxide molecule always oxidizes both cuprous ions of the pair.

The second-order kinetics for restoration of activity to the inactive material are also consistent with the paired structure, and it is reasonable to speculate that the much more rapid restoration rate of peroxide as compared with other reducing agents is related to the ability of peroxide to "fit" the active site structure, resulting in a lower activation energy. The same may be true of the role of peroxide as an oxidant of native hemocyanin. The explanation of the inability of as strong an oxidant as molybdicyanide[2] to oxidize the active site may lie in the rate of the reaction (relative to oxidation of other groups on the protein) rather than in the equilibrium position. In this connection it should be pointed out that hydrogen peroxide is capable of oxidizing the cysteine side chains of proteins,[24] and on the basis of the inactivation studies alone it would not be possible to conclude that cuprous ion is being oxidized, since oxidation of some other essential group, ordinarily protected by oxygenation, might explain the results. The biquinoline reaction resolves this difficulty.[25]

(24) H. S. Olcott and H. Fraenkel-Conrat, *Chem. Revs.*, **41**, 151 (1947).

(25) We exclude the possibility that there is some mechanism by which sulfhydryl groups, adjacent to cupric ions in native deoxygenated hemocyanin, are capable of instantaneously reducing the cupric ions on addition of biquinoline–glacial acetic acid. In that event, peroxide might oxidize the sulfhydryl groups and prevent reduction of the cupric ion. Such a structure and mechanism seem extremely unlikely, since

In the case of *Limulus* hemocyanin there appear to be irreversible effects associated with the oxidation. These perhaps involve minor shape changes which distort the active site structure sufficiently to prevent both rapid reduction by peroxide and combination of any reduced sites with oxygen. Conceivably there are some secondary changes, such as those reported by Klotz[26] for cupric–sulfhydryl bonding in bovine serum albumin. *Busycon* hemocyanin shows no such irreversible changes. The results of Fig. 4 are readily explained in terms of the competing oxidative and reductive powers of peroxide, assuming that the forward and backward rates are in the ratio of about 2:1 and that an odd number of "hits" inactivates, while an even number activates. The results of Fig. 3 are consistent with the idea that some of the oxygen liberated becomes bound to hemocyanin, which is then not subject to attack. If every oxygen molecule had remained bound to hemocyanin, the curve would be symmetric about the one equivalent point, and full activity would be restored at two equivalents of peroxide per mole of copper.

On the basis of these results we feel that the product of peroxide attack on hemocyanin may justifiably be named "methemocyanin," since the analogy to methemoglobin appears to be complete, involving specific oxidation of the metal ion with loss of physiological activity. We do not think it wise at the moment to provide a name other than methemocyanin for the *Limulus* oxidation product merely because of the apparent irreversibility of the attack. We shall therefore refer to *Limulus* and *Busycon* methemocyanins in subsequent publications.

The catalase-like activity of *Busycon* hemocyanin is understandable in terms of the hemocyanin–methemocyanin cycle. The high efficiency is of course related to the high reaction rates of peroxide with *Busycon* hemocyanin and methemocyanin. The activity of *Limulus* methemocyanin cannot be explained in this way, but catalytic behavior is to be expected if there is sufficient stabilization of the cuprous state by binding to protein so that the cuprous–cupric half reaction falls between the two half reactions of peroxide.

The strong binding of the oxidized copper ions to methemocyanin is evident from the inability of EDTA to remove the copper of *Limulus* methemocyanin. Both *Limulus* and *Busycon* methemocyanins retain their native copper when passed through ion-exchange columns which remove all added copper. This suggests that the copper of methemocyanin is not bound to single amino, carboxyl or imidazole groups, but to some unique site on the protein. The possibility of secondary structural changes in *Limulus* methemocyanin has been mentioned. In the case of *Busycon*, this seems unlikely. The ease with which physiological activity can be restored to *Busycon* methemocyanin makes it appear likely that cupric ion is located close to the active site of the native hemocyanin and that there are no major structural changes.

It is possible that the cupric ion is bound at the same place as its cuprous ion predecessor.[27] On the other hand, there may be a change to some other binding group, intimately connected with the active site structure, when hemocyanin is oxidized to methemocyanin. Whether the transition from hemocyanin to methemocyanin involves changes in the copper–protein bond or not, a study of the structure of methemocyanin and the mechanism of its formation will provide much evidence concerning the active site structure of hemocyanin and conceivably concerning the mechanism of action of a large class of enzymes which employ copper ions in their active site.

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it would be difficult to explain oxygen-carrying ability if deoxygenated hemocyanin were a cupric compound. Further evidence against this structure is the fact that incubation (30 minutes, *p*H 7) of *Limulus* serum with 6 moles of sodium *p*-chloromercuribenzoate per mole of copper has no effect upon the amount of cuprous ion observed in the deoxygenated material (Ref. 13).

(26) I. M. Klotz, J. Urquhart, T. A. Klotz and J. Ayers, THIS JOURNAL, **77**, 1919 (1955).

(27) Klotz (ref. 26) suggests that the 340 mμ maximum only appears when a disulfide structure is properly oriented with respect to a cupric–sulfhydryl bond. The oxygen molecule of oxyhemocyanin could play a role analogous to that of disulfide, resulting in an intensification of absorption in the ultraviolet similar to its effect upon the visible spectrum.