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Autoxidation of Oxymyoglobins*

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SUMMARY

During the autoxidation of oxymyoglobin to metmyoglobin, there is an evolution of oxygen. The experimental results support the conclusion that 0.25 mole of the oxygen in oxymyoglobin is used for the oxidation and 0.75 mole is released. This finding differs significantly from earlier reports of oxygen consumption during the oxidation. The latter results are shown to be attributable to interference by oxidation products of sodium hydrosulfite, which is used as a reducing agent. In the present system, we remove such products completely by means of an ion exchange column. This paper includes a design for a completely sealed cell for oxygen electrode measurements.

In view of the finding of interference by hydrosulfite, we have reexamined the effects of temperature, pH, and oxygen level on rates of oxidation of oxymyoglobin. Absolute rates in systems free of hydrosulfite differ substantially from those previously reported, but the relative effects are the same, i.e. there is a direct dependency on hydrogen ion concentration, particularly over the pH range from 5 to 7, and an indirect dependency on oxygen level. There is a marked effect of temperature on the reaction, with Q_{10} values approximating 5. Activation energies obtained with myoglobins from different sources and at different pH values range from 24,000 to 27,800 calories. Carboxymethylation of available histidine residues in undenatured sperm whale and yellowfin tuna myoglobins has no effect on the oxidation rate. The presence of diphosphoglycerate increases the reaction rate. Under equivalent conditions, the tuna oxymyoglobin oxidizes much faster than those from sperm whale or bovine muscle.

Because ferric forms of hemoproteins cannot be oxygenated and are therefore biologically inactive, there has long been interest in the nature of the autoxidation reactions of oxyhemoglobins and oxymyoglobins. Following the early observations of Brooks (1), there have been numerous studies of factors influencing the autoxidation of HbO_2. More recent analogous work has been done with MbO_2. George and Stratmann (2, 3) found that the autoxidation of MbO_2 to MetMb was similar to that undergone by hemoglobins, being first order with respect to unoxidized MbO and with a rate maximum at low oxygen pressures; in addition, they reported the utilization of 2.5 moles of oxygen per mole of MbO_2 oxidized. These same workers later reported that the rate of oxidation increased with increasing hydrogen ion concentration (4). These studies were done with horse myoglobin; similar findings have been reported by others with a variety of mammalian and fish myoglobins (5–8). However, different values have been obtained for the magnitude of the hydrogen ion effect, and for the amount of oxygen used in the oxidation reaction (2, 7). Several mechanistic interpretations of the reaction have been offered; none has been compatible with all experimental findings. A useful review has appeared recently (9).

We report here in contrast to earlier work that there is a net evolution of oxygen from MbO_2 during its oxidation. We show that the previously reported oxygen consumption is attributable to interference in the earlier studies by oxidation products of sodium hydrosulfite, which is used as a reducing agent. Thorough removal of such products as described herein allows the measurement, in a specially designed oxygen electrode cell, of the evolution of stoichiometric amounts of oxygen. Under these new conditions, we have reexamined the effects of temperature, hydrogen ion, and oxygen level on the oxidation rates of mammalian and fish myoglobins. A mechanism for the autoxidation reaction is proposed.

EXPERIMENTAL PROCEDURE

Materials—Myoglobins were prepared from aqueous extracts of yellowfin tuna red muscle (supplied by Star-Kist Laboratories, Terminal Island, California) and bovine muscle (purchased locally) by ammonium sulfate fractionation and chromatography on DEAE-cellulose (10). Commercially prepared sperm whale myoglobin (Calbiochem) was further purified by the chromatography step. Tris buffer used for chromatography was removed by dialysis. Myoglobins so prepared were inevitably in the Met-form. For preparation of MbO_2, 2 ml of a concentrated stock solution of MetMb (15 to 30 mg per ml) were reduced by the addition of 2 mg of sodium hydrosulfite. The solution was immediately passed down a column (1 × 40 cm) of mixed bed ion exchange resin (Bio-Rad AG 501-X8) in the cold (4°). The sample eluted as MbO_2 and was essentially completely deionized with conductivity of 2 to 3 μmhos (Radiometer type CDM 2c conductivity meter).

This means of preparation of MbO_2 is highly critical, because, as discussed later in this paper, if MbO_2 is prepared with the hydrosulfite and breakdown products of the latter substance are not

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removed by such a column, the experimental results are substantially affected.

Unless otherwise specified, biochemicals were from Sigma and chemicals were reagent grade. Glass-distilled water was used throughout.

Oxygen Utilization—During the oxidation reaction, oxygen utilization was measured by conventional Warburg manometry and by use of an oxygen electrode. For the latter measurements, we designed and constructed a cell that could be tightly sealed and in which contents are visible (Fig. 1). The cell, milled from a Plexiglas block, has a double O-ring seal holding an oxygen electrode (Yellow Springs Instrument, model 5331) inside the reaction chamber (volume about 5 ml). The latter contains a small, round, magnetic stirrer bar (about 1 cm in diameter), and has two valve-regulated ports for injection and removal of samples. Commercially available cells tested were unsatisfactory for our purposes, because the gas leakage rate was too high. For determination of utilization, the appropriate buffer was equilibrated to 30° in a water bath and, at the same time, was fully saturated with air. The cell and electrode were similarly equilibrated by immersion in the same water bath. The cell was then filled with the air-saturated buffer, the electrode was inserted, and the cell was sealed, with care being taken that no air bubbles were trapped inside. The cell was kept in a 30° bath, and the contents stirred with a magnetic stirrer. The oxygen monitor (Yellow Springs Instrument, model 53, with a Gilford model 242 strip chart recorder) was then set to a 100% value. Nitrogen gas was bubbled for 3 to 5 min through an aliquot of the same buffer held in a small flask at 30°. Then, 5 ml of this nitrogen-saturated buffer were injected into the cell, displacing the buffer originally present. This procedure usually resulted in a change in the oxygen monitor reading to a value of about 30%. The base-line recorded from the monitor does not level at a constant value because of continuous consumption of oxygen by the electrode. Consequently, a stable base-line is one that decreases at a constant rate (in our case, about 5% per hour). When such a base-line was reached, 1 ml of MbO_2 solution that previously had been equilibrated to 30° in air was injected into the cell. The oxygen monitor read about 40% immediately after such injection. The subsequent change in percentage of oxygen was continuously recorded until the reaction was complete, *i.e.* when the initial base-line rate was again reached. The MetMb solution was then removed, its volume was measured, and the myoglobin concentration was determined from the absorbance of the Soret peak. The fact that there was complete conversion to MetMb was also ascertained spectrophotometrically.

Oxidation Rate Measurements—Freshly prepared stock MbO_2 solution, 1 ml, and buffer (0.4 M phosphate-0.2 M citrate), 4 ml, were separately equilibrated to the experimental temperature. After equilibration, the MbO_2 solution was added to the buffer and oxidation rate measurements, based on change in absorbance of the MbO_2 peak at (approximately) 580 nm, were begun. In practice, a recording was made of the region from 620 to 560 nm in a Cary model 11 spectrophotometer, and the peak absorbance was taken from the trace. Stoppered cuvettes were used for reaction vessels; a gas bubble was left at the top of the cuvettes.

pH was controlled to 0.01 unit as determined in a Radiometer model TTT1c pH meter. Temperature control was achieved by use of a water bath maintained to within ±0.1°. Temperature control was not attempted during the brief periods required for spectrophotometric recording.

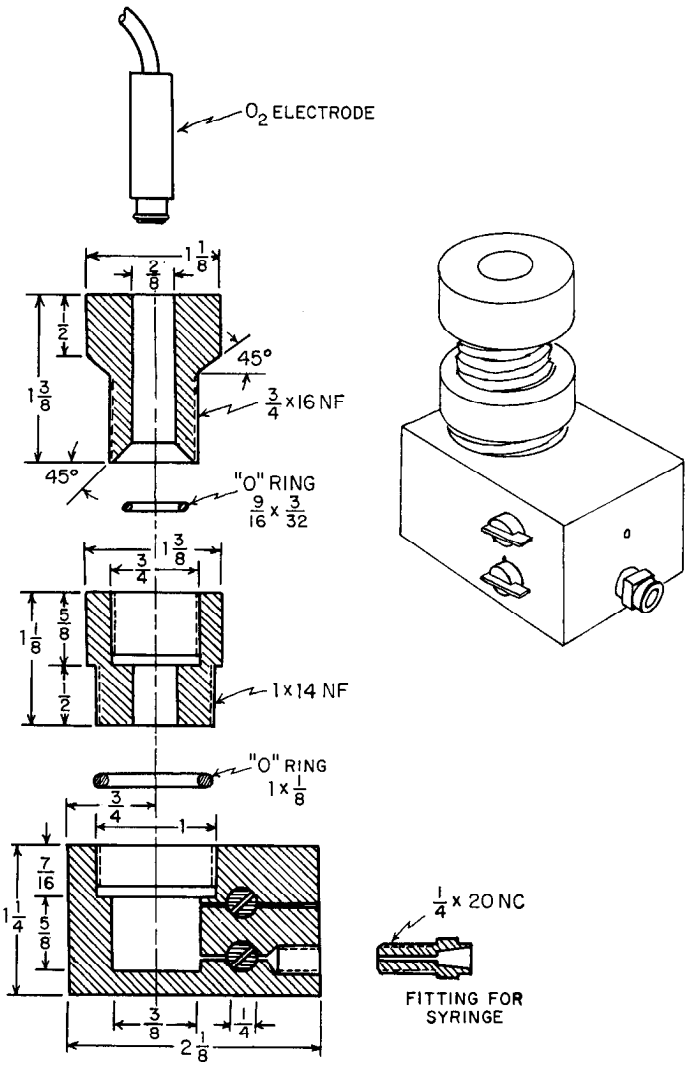

FIG. 1. Cell for measuring oxygen levels in solutions in a sealed system. *Top*, an oxygen electrode (Yellow Springs Instrument, model 5331) if fitted through a milled Plexiglas coupling. The fitting is sealed by means of an O-ring in the upper half of the interlocking screw connector (*center*). The lower half of the connector is in turn sealed by another O-ring in the cell compartment (*bottom*). Two ports are drilled through the right hand side of the cell, as shown, and are regulated by valves to which small plastic handles are attached. Teflon tubing is sealed into the upper port and used for removal of cell contents. To the lower port is affixed a fitting for a hypodermic syringe which can be used for injecting cell contents. During use, a round, magnetic stirrer bar is placed in the cell compartment. The cell volume is approximately 5 ml.

Oxygen concentration, measured with the instruments described in the preceding section, was varied by saturating solutions with different known mixtures of N_2 and O_2 (Matheson-Coleman-Bell, East Rutherford, New Jersey). Solutions were transferred under a stream of the same mixture into a cuvette that previously had been flushed with the same gas. Cuvettes were then stoppered.

For studies of the effect of carboxymethylation on oxidation rates, myoglobins were treated with recrystallized bromoacetic acid according to the procedure of Clark and Gurd (11). Final concentrations of 0.2 M bromoacetate and 1 M phosphate were used, and the reaction was allowed to proceed for 2 days and 9

days, at pH 6.9 to 7.0 and at 23°. Both yellowfin tuna and sperm whale myoglobins were so treated and then used in the oxidation rate system just described. To ascertain the extent of carboxymethylation, aliquots of each treated myoglobin were hydrolyzed in 6 N HCl at 110° for 24 hours, and amino acid composition was determined with a Spinco model 120 B analyzer. Duplicate hydrolysis and analysis were done. In addition, the known alterations of spectra of carboxymethylated derivatives held at high pH (11) were observed with a recording spectrophotometer.

For studies of the effect of diphosphoglycerate on the oxidation rate, the material was added to the systems described above without preincubation. This reagent was purchased as the pentacyclohexylammonium salt and converted to the free acid by mixing a solution with excess Dowex 50W-X8 (H^+) (12). Because at the highest levels of diphosphoglycerate some precipitation eventually occurred, we attempted to determine parameters of denaturation. For this purpose, difference spectra in the far ultraviolet, recording of Soret peak maxima, and estimation of turbidity by absorbance in the transparent region at 700 nm were done in a Cary model 11 spectrophotometer. Fluorescence determinations were made in a Perkin-Elmer (Hitachi) model 203 spectrophotometer with a xenon light source.

RESULTS

Evolution of Oxygen during Oxidation of Oxymyoglobins—Our findings, which indicate a net evolution of oxygen during the oxidation of MbO_2, are summarized in Table I, together with the results of earlier investigators. Our results with the manometric apparatus were poorly reproducible for reasons we never clarified. When the cell described under "Experimental Procedure" became available, we were able to obtain reproducible results with the oxygen electrode. At this point, we did no additional manometric experiments.

TABLE I

Oxygen utilization in oxidation of oxymyoglobins

Investigator(s)	Source of MbO_2	Oxygen absorbed (+) or evolved (−)	Net utilization[a]
		moles O_2/mole MbO_2	
George and Stratmann (2)	Horse	+ (1.50 ± 0.3)[b]	2.50 ± 0.3
Matsuura *et al.* (7)	Tuna[c]	+ (0.34 ± 0.1)	1.34 ± 0.1
Our manometric data[d]	Yellowfin tuna	− (0.64 ± 0.3)	0.36 ± 0.3
	Sperm whale	− (0.67 ± 0.3)	0.33 ± 0.3
Our oxygen electrode data[e]	Yellowfin tuna	− (0.71 ± 0.02)	0.29 ± 0.02
	Sperm whale	− (0.78 ± 0.02)	0.22 ± 0.02
	Bovine	− (0.70 ± 0.06)	0.30 ± 0.06

[a] Net values take into account the mole of O_2 contributed by MbO_2 during oxidation.

[b] Plus-minus figures indicate range of values.

[c] Oxygen absorbed is based on an average of 23 values, including 16 with bluefin tuna (*Thunnus orientalis*), 3 with bigeye tuna (*Parathunnus sibi*), 2 with rorqual whale (*Balaenoptera physalus*), and 2 with horse oxymyoglobin.

[d] Manometric data are based on 8 determinations with yellowfin tuna and 11 determinations with sperm whale oxymyoglobins.

[e] All oxygen electrode data are based on duplicate runs. Values are uncorrected; see text.

The results show good agreement among the different myoglobins tested. As indicated in Table I, Footnote *e*, the oxygen evolution values determined by the oxygen electrode are uncorrected. That is, they are based on the assumption that the solubility of oxygen in the buffer is the same as in water. Clearly, this is not strictly true. We have determined in limited control experiments that the solubility in the buffer is approximately 0.9 times that in water. If the average evolution figure for the three myoglobins of 0.73 mole per mole of MbO_2 is corrected for this, it becomes 0.66.

We believe that the true value is 0.75 mole of oxygen evolved per mole of MbO_2 oxidized for the following reasons. Because the oxidation reaction is logarithmic and because the reaction rate under the conditions used is quite high, some oxidation of MbO_2 and some evolution of oxygen will take place during the period required for equilibration of the final system. We have not attempted to extrapolate back to correct for this loss of oxygen. The net effect, however, would be to increase the corrected experimental value of 0.66 mole of oxygen. Additionally, inasmuch as the oxidation must occur in one-electron transfers, the oxygen utilized as an oxidant must be 0.25 mole or a multiple thereof. If this reasoning is correct, there is a net utilization of 0.25 mole per mole of MbO_2, the theoretical amount required for the oxidation reaction. The total oxygen would then account for the 1.00 mole of oxygen that is released from MbO_2.

The primary difference in our technique and those of earlier workers is our use of an ion exchange column to remove sodium hydrosulfite from the MbO_2 solutions. We attribute the fact that we find net oxygen evolution, not absorption, to this difference. George and Stratmann (2) reported using a "small quantity" of hydrosulfite; they do not report any effort to remove it or its products from the MbO_2 solutions. Under such conditions, they found an absorption of about 1.50 moles of oxygen per mole of MbO_2 oxidized. In control experiments, when we omitted the ion exchange step from our procedure, we too noted oxygen absorption of similar magnitude. Matsuura *et al.* (7) removed hydrosulfite by dialysis and obtained a considerably lower average value for oxygen absorption (0.34 mole). However, we found that this dialysis did not effectively remove all the hydrosulfite. In an approximation of Matsuura's procedure, we took a 2-ml sample of MbO_2, prepared from MetMb by using 1 mg of sodium hydrosulfite, and dialyzed it overnight with constant stirring against 200 ml of glass-distilled water. At the end of this time, the solution had a relatively high conductivity of 142 μmhos, compared to an initial conductivity of 512 μmhos. In comparison, when we use the ion exchange column to remove hydrosulfite, the emergent MbO_2 fractions have conductivities of only 2 to 3 μmhos and can be considered totally free of hydrosulfite. Using the MbO_2 prepared by the dialysis procedure, we obtained oxygen absorption values similar to those of Matsuura *et al.* As previously indicated, using MbO_2 which has been passed through the ion exchange column, we note evolution rather than absorption of oxygen. In consideration of these various observations, we feel that incomplete removal of hydrosulfite is responsible for the high values of oxygen consumption previously reported.

The design of the cell used for oxygen measurements is somewhat critical. Some commercially available cells have an opening to air; presumably, it is assumed that limited oxygen diffusion through this small area may be neglected. Because such cells are frequently used for measurements of consumption, not evolution, and because the amount of oxygen involved may be rela-

tively large the assumption may be justified. However, with our cell we measure about 0.5 μl of oxygen consumption per hour by the electrode used, contrasted to the claimed consumption of 0.1 μl per hour. The difference may, perhaps, be accounted for by gas leakage in the commercial cells.

Oxygen Dependency of Oxidation Rate—Results showing the effect of oxygen level on the oxidation rate of the different MbO_2 preparations are summarized in Fig. 2. In accord with earlier work, we find that rates of oxidation are inversely related to oxygen levels. We determined, with the tuna MbO_2, the effect of the presence of hydrosulfite products on oxygen dependency. The general behavior was the same, but a plot similar to those in Fig. 2 gave a line with a slope of 0.15, contrasted to the 0.34 value obtained in our usual system with hydrosulfite products removed.

The tuna MbO_2 oxidizes at rates considerably more rapid than MbO_2 from either of the mammalian sources, at the same pH. Here, we elected to determine oxygen dependency of the tuna MbO_2 at a higher pH than used for the other myoglobins for experimental convenience.

pH Dependency of Oxidation Rate—Again, in substantiation of earlier work, we note a strong dependency on pH of oxidation rates of all the MbO_2 preparations tested (Fig. 3). There is a linear relationship over a range of about 2 pH units. There are indications of lowered dependency at lower pH values. Our attempts to determine rates at still lower pH values than those shown were not successful. Although such measurements did suggest a lowered dependency, values were not reproducible due to problems with denaturation. At the highest pH values tested, the dependency again seems reduced. As an approximation, the pH dependency has an operational pK of around 6 in the sense that the effect is most striking at this value, plus or minus about 1 pH unit.

These results were obtained in hydrosulfite-free systems. For determination of the effect of residual products of the reducing agent, we repeated the same rate determinations without incorporating the ion exchange column step. The dependencies on pH of both tuna and sperm whale MbO_2 are relatively unaffected; however, the absolute rates at a given pH are approximately doubled when hydrosulfite products are completely removed.

Effect of Temperature of Oxidation Rate—As expected, there is a marked effect of temperature on rates of oxidation. Most of our measurements were done at 22°. In addition, we obtained rates of oxidation of the various MbO_2 preparations at −2° (unfrozen). At either pH 5.00 or 6.00, lowering the temperature over this 24° range lowers the oxidation rate by 40- to 50-fold. Thus, this reaction shows a very high temperature dependence, with Q_{10} values approximating 5. Calculation of activation energies for the reaction with the different myoglobins and pH values over the indicated temperature range gives values ranging between 24,000 and 27,800 calories. These values do not differ greatly from those previously reported (3, 5).

Effect of Carboxymethylation of Oxymyoglobins on Oxidation Rate—Because of the evidence presented above, indicating the possibility that a group with a pK around 6 might be involved, we determined the effect of carboxymethylation of tuna and sperm whale myoglobins on their oxidation rate. Table II shows the effect of this treatment on oxidation rates, and also indicates the extent of reaction of histidine residues in these proteins with bromoacetate. This treatment appears to be without particular effect on oxidation rates.

The numbers of histidines that we find to react in sperm whale myoglobin under the conditions used are in reasonable agreement with those previously reported (11). To our knowledge, such measurements have not been made previously with tuna preparations. The carboxymethylated tuna myoglobin shows the same alteration in spectra at high pH values as that previously reported for sperm whale myoglobin (11).

Effect of 2,3-Diphosphoglycerate on Oxidation Rate—We also

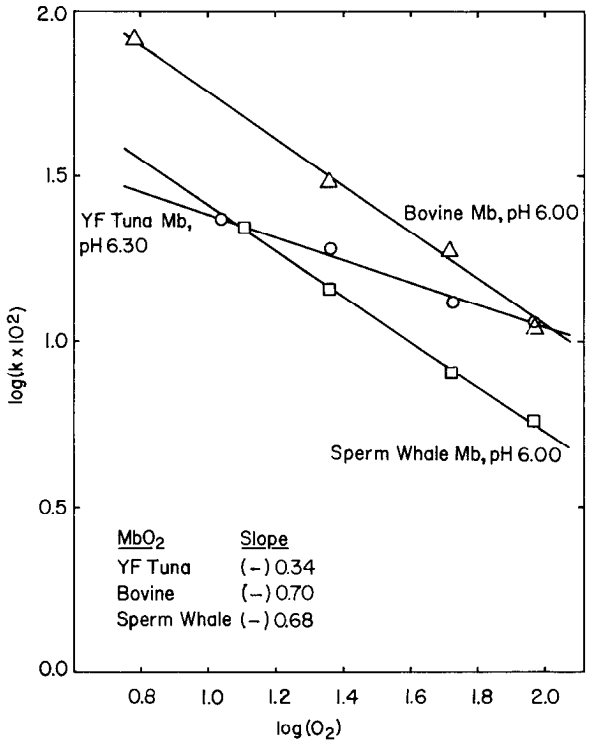

FIG. 2. Effect of oxygen level on oxidation rates of oxymyoglobins. Log (O_2) value of 2.0 corresponds to solutions saturated with air, or pO_2 of approximately 150 mm. All determinations were made at 22°; k values are in hr^{-1}. *YF*, yellowfin.

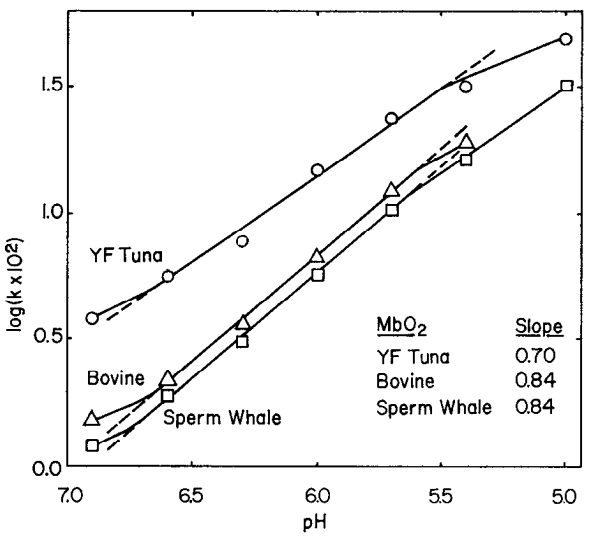

FIG. 3. Effect of hydrogen ion concentration on oxidation rate of oxymyoglobins. Oxygen level is that of air saturation. All determinations were made at 22°; k values are in hr^{-1}. *YF*, yellowfin.

TABLE II

Effect of carboxymethylation of myoglobins on oxidation rate

Rates are determined at pH 5.70. The times in days indicate length of reaction with bromoacetate. Histidine values determined by amino acid analyses are unreacted residues; no effort was made to determine quantitatively the derivatives of the reacted histidines.

Myoglobin	0 days		2 days		9 days	
	His residues	k	His residues	k	His residues	k
		hr^{-1}		hr^{-1}		hr^{-1}
Sperm whale.....	12	0.09	7	0.11	6	0.11
Yellowfin tuna.....	6	0.22	4	0.26	4	0.27

TABLE III

Effect of 2,3-diphosphoglyceric acid on oxidation rate

All determinations done at 22°.

MbO_2	pH	Rates at following ratios of diphosphoglycerate to oxymyoglobin:			
		None	2:1	10:1	20:1
		hr^{-1}	hr^{-1}	hr^{-1}	hr^{-1}
Yellowfin tuna	5.80	0.22	0.30		
	5.90	0.18		0.31	0.42
Sperm whale	5.62				0.36
	5.72	0.08		0.21	
	5.78	0.08	0.14		
Bovine	5.63	0.16			0.53
	5.70			0.32	
	5.77	0.13	0.19		

determined the effect of diphosphoglycerate on the rate of oxidation. Results are presented in Table III and indicate that the presence of diphosphoglycerate substantially increases oxidation rates. As indicated in the experimental procedure, there was no preincubation with diphosphoglycerate. We presume that its effect is similar to that now well known in hemoglobins of reducing oxygen affinity (12–16); this would be in accordance with the mechanism we propose under "Discussion."

At the higher levels of diphosphoglycerate we noticed the development of turbidity in the oxidizing systems. We explored this in a preliminary fashion. The Soret maxima of these proteins was not lowered or shifted to lower wave lengths, as it is under the influence of various denaturants (17). Difference spectra in the ultraviolet region between 300 and 240 nm showed that no peaks were generated with time in these systems. We were unable to obtain interpretable results with fluorescence spectrophotometry. One observation of interest is that the development of turbidity took place fairly rapidly, *i.e.* within 1 hour, in systems containing MbO_2 and diphosphoglycerate at higher levels. However, when we mixed diphosphoglycerate with MetMb under similar conditions, equivalent turbidity levels were not reached for 3 days.

Effect of EDTA on Oxidation Rate—Because of the known pronounced effects of metal ions on oxidation rates (18), we ran a series of controls in which EDTA was added in molar concentrations 1, 2, and 4 times those of MbO_2. The rates of oxidation were unaffected. Hence, we presume that our systems are sufficiently free of metal ions to avoid this complication.

DISCUSSION

On the basis of our findings and those previously reported, we propose the following mechanism for the autoxidation of MbO_2.

$$Mb^{+2}O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Mb^{+2} + O_2 \qquad (1)$$

$$H^+ + Mb^{+2} + \tfrac{1}{4}\,O_2 \xrightarrow{k_3} Mb^{+3} + \tfrac{1}{2}\,H_2O \qquad (2)$$

This simple mechanism seems to be in agreement with known experimental findings. It requires that there be a net evolution of 0.75 mole of oxygen per mole of MbO_2 oxidized, which we report herein. It allows for the known direct dependency on hydrogen ion concentration and the indirect dependency on oxygen level. Our results on hydrogen ion dependency (Fig. 3) show that the slopes of plots of log k against log (H^+) approach unity. The dependency is less with tuna MbO_2. Perhaps k_3 is greater for tuna myoglobin; if so, the autoxidation reaction, Equation 2, should be more rapid than with the mammalian preparations. Such is the case. The lesser indirect dependency on oxygen level of the tuna MbO_2 (Fig. 2) may be attributable to the same reason.

If the assumption is made that diphosphoglycerate reduces the oxygen affinity of myoglobin as it does hemoglobin, then the fact that this compound increases the rate of oxidation in our system may be ascribed to its influence on k_1 and k_2. The experimental results are in agreement with the proposed mechanism.

Our results showing lack of effect of carboxymethylation of histidine residues on oxidation rates are not conclusive. They do indicate, however, that histidine residues readily reactive with bromoacetate are not likely involved in the oxidation reaction. No inference is drawn from our results indicating that yellowfin tuna MbO_2 oxidizes much more rapidly than the mammalian myoglobins; the amino acid composition of the tuna myoglobin (19) is substantially different.

A number of other mechanisms have been proposed. George and Stratmann (3) suggested a free radical mechanism that involved an electron-accepting group in the protein acting as a catalyst. Later, they concluded that a bimolecular reaction between myoglobin and oxygen was involved (4), but they could not account for the consumption of oxygen they observed. Lemberg and Legge (20) had earlier proposed the participation of oxidizable groups in the protein. Weiss (21) envisioned that in HbO_2 the iron was in the ferric state, with oxygen present as O_2^-. Such a structure could have oxidizing effects from O_2^- or the HO_2 radical formed from the reaction of O_2^- with H^+. Hydrogen peroxide could be formed from the HO_2 radical. However, Keilin (22) had earlier reported evidence that no peroxides were formed during the autoxidation of hemoglobin.

This work indicates that the reducing agent hydrosulfite and its products must be completely removed to avoid a serious experimental artifact. Lack of removal of the reducing agent substantially affects pH and oxygen dependency of the oxidation reaction and results in oxygen absorption rather than evolution. Unfortunately, the previously reported consumption of 2.50 moles of oxygen per mole of MbO_2 oxidized (2) has been used as the basis for the proposal of an involved mechanism for MbO_2 autoxidation (23). This mechanism has, in turn, been used to support a hypothesis dealing with the general function of hemoprotein oxidases and peroxidases, the reduction of O_2 to water by cytochrome oxidase, and electron transfer within and between

hemoprotein molecules (23, 24). These hypotheses will, of course, require reevaluation in light of our present report.

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