A STUDY OF SOME REDOX REACTIONS OF MYOGLOBIN AND HEMOGLOBIN

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ABSTRACT

A STUDY OF SOME REDOX REACTIONS OF

MYOGLOBIN AND HEMOGLOBIN

By

George Gosselin Giddings

Results of studies of selected redox reactions of myoglobin and hemoglobin in aqueous solution support the following conclusions:

The red pigment produced by gamma irradiation of ferrimyoglobin and ferrihemoglobin (+3 oxidation state) is the ferryl derivative (+4 oxidation state) when oxygen is present. In solutions deoxygenated prior to irradiation, oxymyoglobin and oxyhemoglobin (+2 oxidation state) are produced. The oxidation to the ferryl state in the presence of oxygen results from reaction with radiation-generated H_2O_2. Reductive oxygenation under anoxic conditions probably is due to reduction of the heme iron by hydrated electrons followed by oxygenation with O_2 generated by free radical recombination reactions.

Ferrimyoglobin and ferrihemoglobin are oxidized to the ferryl state by superoxide anion radicals (O_2^-) generated by xanthine oxidase reduction of oxygen following the aerobic oxidation of xanthine. By reducing superoxide anion to H_2O_2 in the process, ferrimyoglobin and ferrihemoglobin competitively inhibit reduction of ferricytochrome c by the anion.

The univalent reduction of oxygen to form freely-diffusible superoxide anion could not be detected during autooxidation of oxymyoglobin or oxyhemoglobin by oxygen. The detection system employed was ferricytochrome c which is reduced by superoxide anion in a diffusion-controlled bimolecular reaction, and Tiron which inhibits the cytochrome c reduction.

Ferricytochrome c was reduced in the process; however Tiron did not inhibit the reduction. This negative result suggests, but does not prove, that superoxide anion production does not occur as a result of autooxidation because the reduction of ferricytochrome c during oxidation of oxy- and deoxy-myoglobin and hemoglobin, plus supporting evidence strongly indicate that a direct intermolecular redox reaction (electron transfer) takes place between ferricytochrome c and the reduced forms of the other hemoproteins.

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George Gosselin Giddings

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To Thomas and Erika

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INTRODUCTION

The heme prosthetic chromophore of myoglobin and hemoglobin is responsible for the color of the hemoprotein pigments as well as for their in-vivo function of oxygen transport and storage. The oxidation state of the heme iron and the nature of its sixth coordination position ligand influence the electronic configuration of the chromophore, and thus affect the magnetic and light-absorbing properties of the pigments. As with all hemoproteins, the heme iron of myoglobin and hemoglobin can engage in redox reactions with a variety of inorganic, organic and biochemical electron donors and acceptors (e.g., ferricyanide, phenazine methosulfate, "methemoglobin reductase"), and can be made to undergo oxidation or reduction under the influence of applied perturbation (e.g., thermal and electromagnetic energy, lowering pH or oxygen concentration).

While the iron of the cytochromes functions by cycling between the +2 and +3 oxidation states, and that of catalase and peroxidases cycles between the +3 and +4 oxidation states when engaging in catalysis, myoglobin and hemoglobin function normally (i.e., bind oxygen) only when the heme iron has a +2 valence state and the entire macromolecule is in its native configuration. In relation to food science, the desirable cherry red color of fresh red meat requires that the heme iron of myoglobin, and any hemoglobin present, be in the ferrous (+2 oxidation) state and have oxygen coordinated to it. However myoglobin and hemoglobin readily oxidize to the non-oxygen binding +3 (ferric, or "met") oxidation state when in meat tissue, or in aqueous solution. The characteristic brown color of this oxidation state is most undesirable in fresh red meat cuts and frequently limits the saleable life of the product.

Because the redox and ligand binding chemistry of myoglobin and hemoglobin ultimately determines the color of meat, the food scientist has shared and continues to share with members of other scientific disciplines a common interest in the structure, function and physical and chemical properties of the hemoproteins. The aspects of myoglobin and hemoglobin chemistry which are the subject matter of this thesis are (a) the radiation chemistry of myoglobin and hemoglobin and (b) the mechanism of autooxidation of these two hemoproteins, and, in connection with these two aspects, (c) the possibility of a superoxide anion involvement in their redox and ligand binding chemistry. Uncertainty regarding the oxidation state and ligand of the iron of irradiated myoglobin and hemoglobin arose during the 1950's and continues to persist. Also the exact mechanism(s) of autooxidation of the two oxygen-binding hemoproteins by oxygen, particularly the all-important initial event, is an open question of long standing. The mode of interaction of myoglobin and hemoglobin with the recently discovered superoxide anion had not been investigated, and it occurred to the author that this entity may be involved with both the radiation chemistry and the autooxidation of the hemoproteins. Thus the subject investigations were undertaken, employing model systems containing the isolated, purified pigments with the aim of generating new information bearing on these questions. Hemoglobin was included in the studies along with myoglobin because (a) the chemistry of the two oxygen-carrying hemoproteins is very similar, and (b) hemoglobin has been found to contribute up to one-third of the hemoprotein pigmentation of red meat from well bled animals (Rickansrud, D. A. and Hendrickson, R. L., 1967; Glötze, 1969).

REVIEW OF LITERATURE

The occurrence, structure, function, and much of the general chemistry of myoglobin and hemoglobin constitute well documented "textbook" information, and are referred to in this review only as required for clarification of specific points.

Radiation Chemistry of Myoglobin and Hemoglobin

Uncertainty regarding the oxidation state and sixth coordination position ligand of the heme iron of the derivatives produced by ionizing irradiation of myoglobin and hemoglobin arose in the 1950's and has continued to persist. Laser (1955) stated that the effect of X-ray irradiation on the oxidation state of hemoglobin depends only on the initial state of the pigment and is independent of the presence or absence of oxygen. He reported, as did Barron and Johnson (1956) a year later, that oxyhemoglobin is oxidized to methemoglobin by X-irradiation in the kilorad dose range, and that methemoglobin is reduced by irradiating in the absence of oxygen followed by a further oxidation to a green compound. The latter authors found the same to be true with myoglobin. Ginger et al. (1955) and Ginger and Schweigert (1956) observed that irradiation of meat extract containing a high proportion of metmyoglobin produced a bright red compound spectrally indicative of oxymyoglobin, and similar to oxymyoglobin in its reactions with hydrosulfite, ferricyanide and cyanide, and carbon monoxide. When the extract to be irradiated contained a high proportion of oxymyoglobin, the formation of metmyoglobin and/or a green oxidized porphyrin derivative was favored. Also based

upon spectral evidence, Tappel (1956) concluded that oxymyoglobin is formed by irradiation of metmyoglobin in meat and in purified solution. Bernofsky et al. (1959) reported that, upon irradiation, oxymyoglobin in partially purified solution is first converted to metmyoglobin which is in turn converted by further irradiation to a red compound. This compound was stable to still higher doses of irradiation and appeared spectrally similar to oxymyoglobin except for a lower A_{540}/A_{560} ratio. Irradiation of recrystallized metmyoglobin produced the same red pigment. They also observed that addition of irradiated water to non-irradiated metmyoglobin solution produced a red pigment having an absorption spectrum identical to that of "peroxymetmyoglobin" and distinct from that of both oxymyoglobin and the red compound produced by irradiation of metmyoglobin solutions.

Brown and Akoyunoglou (1964) concluded that absorbance changes in the visible region suggested that irradiation of nitrogen flushed pigment solutions caused partial oxidation of oxymyoglobins and converted metmyoglobins to substances with spectra similar to that of oxymyoglobins, differing by only a few nm in the positions of the absorbance maxima. Ho (1967) similarly removed practically all oxygen in myoglobin solutions by repeated nitrogen flushing and immediate sealing of the vials before irradiating to 500 Krad. The irradiated metmyoglobin was converted to a red pigment having an absorption spectrum very similar to that of oxymyoglobin. Clarke and Richards (1971) concluded from absorbance spectral peaks at 411, 542, 582 and 620 nm that the iron of metmyoglobin, myoglobin,

or a mixture of oxy- and metmyoglobin irradiated in either oxygen, air, or nitrogen atmosphere was in the ferric (+3) oxidation state, but that the normal metmyoglobin structure was destroyed. They concluded that the 411 nm Soret peak ruled out oxymyoglobin and that the 620 nm band was "a result of the irradiation." Satterlee et al. (1971) reported that irradiation of metmyoglobin in meat and in several differing states of purity converted it to a red pigment that is similar to but not identical with oxymyoglobin because of its Soret peak at 412 nm. They speculated that the red pigment might be formed by the addition of a small molecule to the heme iron followed by reduction of the heme iron from the ferric to the ferrous state. They noted that the red pigment formation was greatest in a nitrogen atmosphere, was slightly inhibited in air, and was greatly inhibited in an oxygen atmosphere, implying, as did Clarke and Richards, that differences due to atmosphere were quantitative only. Employing reflectance spectrophotometry to study the effect of ionizing radiation on the color of intact beef samples, Ballantyne (1970) observed a direct dose rate effect on the rate of conversion of oxymyoglobin to deoxymyoglobin and ferrimyoglobin.

Related to the radiation redox chemistry of ferrimyoglobin and ferrihemoglobin is the recent demonstration of direct reduction of heme proteins by solvated electrons resulting from water radiolysis. Three of the more recent reports on this subject are by Pecht and Faraggi (1971), Land and Swallow (1971) and Wilting et al. (1971). The absence of oxygen, an effective scavenger of hydrated electrons, favors reduction reactions and generally establishes a reducing environment in irradiated aqueous media.

Reaction of Hemoproteins with H_2O_2

In a related development, a number of investigators have been studying the interaction of hemoproteins with hydrogen peroxide. George (1952) revived a long overlooked theory according to which the products of the interaction of ferricatalase, ferriperoxidase, ferrihemoglobin and ferrimyoglobin with H_2O_2 have a ferryl, (+4), oxidation state. The simple higher oxidation state theory was at variance with the then accepted hemoprotein-peroxide, enzyme-substrate complex model advanced by Chance (1951) and others. George and Irvine (1955, 1959) subsequently demonstrated the ferryl structure. King and Winfield (1963), using a combination of electron spin resonance, manometric and low temperature spectrokinetic techniques provided further evidence for the quadrivalent heme iron resulting from the metmyoglobin - H_2O_2 reaction. Additional confirmation of the ferryl structure was presented by Peisach *et al.* (1968), who described the ferrylmyoglobin iron as having four 3d valence electrons and an effective spin of 1, complexed to an oxygen atom, in agreement with the structure proposed by George and Irvine (1955). More recent support for the ferryl hemoprotein structure was presented by Mochan and Nicholls (1971), Coulson *et al.* (1971), and by Dolphin *et al.* (1971). In addition to electron spin resonance spectroscopy evidence for the tetravalent heme iron of myoglobin and other hemoproteins, following reaction of the trivalent form with H_2O_2, Mössbauer Spectroscopy has also been employed in demonstrating the ferryl state (Lang, 1970; Maeda *et al.*,

1971). Stable tetravalent iron porphyrins have also recently been prepared and studied (Felton et al., 1971). Unlike myoglobin, hemoglobin, catalase and peroxidases, cytochrome c is apparently unable to exist in a stable quadrivalent state; however ferricytochrome c has also been shown to react with H_2O_2, thereby catalyzing the peroxidatic oxidation of ferrocytochrome c (Mochan and Degn, 1969; Davison and Hulett, 1971).

Hydrogen peroxide is one of the two known molecular products of water radiolysis (H_2 being the other) and its production is enhanced by the presence of oxygen in the irradiated aqueous medium, being minimal in the absence of oxygen. This suggests that irradiation of oxygen containing metmyoglobin solutions may give rise to ferrylmyoglobin via the reaction with radiation-generated H_2O_2. However the superoxide anion, O_2^-, and its conjugate acid the hydroperoxy radical, $HO_2^{\cdot}$, are able to exist for finite lengths of time (i.e., milliseconds) in irradiated aqueous media (Rabani and Nielsen, 1969; Nilsson et al., 1969). Although both rapidly disproportionate to H_2O_2 plus O_2, it is conceivable that if formed by irradiation, both could interact with any hemoprotein or other solute present instead of undergoing self dismutation. Rabani and Stein (1962) had earlier proposed that in irradiated ferricytochrome c solution containing oxygen, hydrated electrons or hydrogen atoms produced by water radiolysis may reduce O_2 to O_2^- which could, in turn, reduce the cytochrome iron. They further postulated that the protein moiety could serve to eliminate hydroxyl ($^{\cdot}OH$) radicals, thereby preventing reoxidation of the iron.

Autooxidation of Myoglobin and Hemoglobin by Oxygen

The exact mechanism(s) of oxymyoglobin and oxyhemoglobin autooxidation, particularly the all-important initial event(s) is an open question. Keilin (1961) reported results of several experiments, each of which indicated that H_2O_2 is not generated during autooxidation of oxyhemoglobin under the influence of mild acidification. This upset a long-held conviction, first advanced by Lemberg and Legge (1949), that autooxidation is an intramolecular mechanism wherein that portion of bound O_2 not liberated during autooxidation becomes H_2O_2 by extracting one electron from the heme iron and a second electron from a site on the globin moiety. Keilin concluded by stating that "the problem of the mechanism of autooxidation of hemoglobin and myoglobin therefore still remains open for further investigation." Although the autooxidation mechanism question has received some attention during the subsequent decade, the statement remains a valid one. That this H_2O_2 aspect has not been finally resolved is evidenced by the claim of Shtamm et al. (1970) that the autooxidation of oxyhemoglobin at neutral pH is retarded by catalase addition to their system. Kikuchi et al. (1955) had earlier observed a similar catalase effect upon hemoglobin autooxidation. A primary question upon which the elucidation of a detailed mechanism hinges is that of the fate of the bound oxygen during autooxidation. That the heme iron oxidizes from the ferrous to the ferric state during the process is clear. But if that fraction of bound oxygen that is not evolved during autooxidation does not end up as H_2O_2 (assuming the results reported by Keilin are correct) then the fate of this oxygen remains a critical unanswered question.

The first detailed, systematic study of autooxidation of oxygen binding hemoproteins was that of George and Stratmann (1952a, b; 1954). The latter two papers in the series address the mechanism specifically, based principally on the measured kinetics of the process under varying conditions and on a theoretical energetics analysis. In contrast to Lemberg and Legge (1949), George and Stratmann proposed an intermolecular mechanism, but also involving a redox site on the globin moiety, however. Perhaps the most interesting aspect of their proposed mechanism is the initial event which they depict as either the dissociation of a superoxide anion from the oxyheme complex, or, the univalent reduction of oxygen by deoxyferrous heme during a bimolecular, diffusion-controlled collision. Their energetics analysis indicates that such a (reversible?) process is at least as energetically feasible as the association-dissociation of molecular oxygen from ferrous myoglobin.

The binding of O_2 to ferrous myoglobin and hemoglobin is known to be exothermic and the analysis of George and Stratmann (1954) indicates that a reversible binding of superoxide anion to the ferric hemoprotein, if it occurs, would be equally as exothermic if not more so. Additional support for the hypothetical reversible dissociation of superoxide anion from the oxyheme complex is afforded by the new model for the structure of the iron-oxygen complex of MbO_2 and HbO_2, and now oxygenated sulfmyoglobin (Berzofsky et al., 1971a), which has both a theoretical and an experimental basis. This model has the heme iron formally ferric due to partial transfer of a 3d electron to the liganded oxygen which is depicted as bound superoxide anion (Weiss, 1964; Maggiora et al., 1965; Vol'kenshtein,

1969; Williams, 1970; Wittenberg et al., 1970). Also, there is evidence to suggest that the ferric heme iron of two other hemoproteins, myeloperoxidase and tryptophan 2,3-dioxygenase, binds O_2^- (Hirata and Hayaishi, 1971; Odajima, 1971). The binding of superoxide anion to the heme of cytochrome P-450 has also been suggested (Strobel and Coon, 1971). The George and Stratmann mechanism calls for a consumption of 0.25 mole of O_2 per mole of oxidized heme iron which was at variance with their measured 2.5 moles of O_2 consumed per mole of oxidized heme iron. Some years later, however, the rigorous experiments of Brown and Mebine (1969) demonstrated that, in fact, 0.25 mole of O_2 is used and 0.75 mole of O_2 is evolved during the autooxidation of oxymyoglobins, thus bringing George and Stratmann's proposed mechanism in line with the true oxygen evolution-utilization.

Kikuchi et al. (1955) proposed a mechanism of autooxidation of oxyhemoglobin similar to George and Stratmann's for oxymyoglobin in that the bound oxygen is depicted as a dissociating superoxide anion radical leaving ferric hemoglobin. Their proposed mechanism involves a somewhat different intermolecular process in that it does not involve the protein moiety, although it also requires utilization of 0.25 mole of, and evolution of 0.75 mole of bound O_2 per oxidized iron ion. However, their manometric data indicated less than the expected O_2 evolution, and they also invoked groups on the protein moiety to explain this.

Winfield (1965) proposed an elaborate intra-intermolecular chain reaction mechanism for oxymyoglobin autooxidation based upon George and Stratmann's erroneous oxygen utilization-evolution data plus detection

of protein aromatic amino acid radicals by his group. Chain initiation is described as extraction of a proton from a phenylalanine residue by the bound oxygen, with simultaneous transfer of an electron from the ferrous ion to the bound oxygen. The initial product after further redistribution of oxygen and hydrogen atoms between the iron and the amino acid is depicted as ferrimyoglobin ($MbFe^{3+}$ OH) plus a tyrosyl residue carrying an oxygen radical instead of a hydroxyl group. The latter radical gives rise to an intermolecular chain propagation mechanism, followed ultimately by chain termination. As Brown and Mebine (1969) pointed out, this complex mechanism was elaborated to explain the erroneous oxygen evolution data of George and Stratmann, and therefore its validity is questionable.

Working with beef and tuna myoglobin, Snyder and Ayres (1961) and Brown and Dolev (1962) confirmed the findings of George and Stratmann on horse myoglobin that autooxidation is first order with respect to unoxidized myoglobin over a wide range of pH, temperature and oxygen concentration conditions. Highly purified myoglobin oxidized more readily than that in crude extract, possibly due to reducing factors in the latter. Robach and Costilow (1961) demonstrated that microbial growth on the surface of prepackaged fresh red meat can accelerate pigment oxidation by either reducing in-package oxygen content to a level at or near that of the maximal autooxidation rate (ca. 1-2 torr) or by producing H_2O_2, or both.

Snyder (1963) presented evidence in support of his proposed mechanism of myoglobin autooxidation which has the heme dissociating from, and

reassociating with the globin in a dynamic equilibrium at near neutral pH. Oxidation of the iron is suggested to occur when dissociated heme interacts with 0_2, the oxidized heme rejoining the globin to form metmyoglobin. Rossi-Fanelli and Antonini (1960) had previously reported that such a dynamic equilibrium state does exist in aqueous hemoprotein systems at neutral pH, although their Keq indicates very little dissociation at any given time. Fronticelli and Bucci (1963) repeated Snyder's experiments and found that addition of dithionite to the unbuffered solutions lowered the pH to about 4.7 where heme dissociates much more readily, and autooxidation is much more rapid than at pH 6.6 (the initial pH). They were unable to detect any appreciable free heme in deoxyferrous myoglobin solutions at pH 6. Recently, Banerjee and Stetzkowski (1970) reported experimental results which they claim lend support to the mechanism proposed by Snyder. Employing peptides obtained by trypsin cleavage of, and cyanogen bromide cleavage of the globin moiety of horse myoglobin they found that peptides having a high binding affinity for ferroheme and a low affinity for ferriheme resulted in a high rate of deoxyhemoglobin oxidation, compared with that of 0_2 saturated oxyhemoglobin, when the peptides were in the presence of the two hemoglobin derivatives under the same nonsaturating oxygen pressure. The authors expressed the view that the increase in oxidation rate of deoxyhemoglobin in the presence of the ferroheme binding peptides is a consequence of the capacity of the peptides to enhance heme dissociation by binding the free ferroheme. Their proposed mechanism calls for oxidation by 0_2 of the ferroheme bound to the peptides after dissociation from globin. Following this the peptides,

having a much lower affinity for the ferriheme, release the newly oxidized heme group. The free heme, now in the ferric form, reassociates with globin to form methemoglobin. They suggest that complete saturation of hemoglobin with O_2 would block the process due to a stabilizing effect of O_2 on the heme-globin linkage. However, as noted by Kiese (1966), at saturating O_2 pressure hemoglobin and myoglobin still undergo autooxidation at 1/4 the maximal rate and 1/2 the maximal rate, respectively. Kiese further points out that accumulated evidence indicates that both hemoglobin and myoglobin have, in principal, the same autooxidation mechanism. Regarding autooxidation in the absence of an added heme acceptor, Banerjee and Stetzkowski relate their findings to Snyder's proposal that globin-free heme is oxidized by O_2 after which it reassociates with globin to form ferrihemoprotein. While this highly speculative model for hemoprotein autooxidation cannot be ruled out as a possible contributing factor in the overall autooxidation process, evidence in support of it is suggestive at best and is generated by experiments which are somewhat removed from the typical situation during autooxidation of hemoglobin and myoglobin in solution and in meat. Although it is seemingly consistent with the fact that autooxidation and heme dissociation both increase with decreasing pH, there are other equally plausible reasons for the pH affect. For example, lowering the pH causes changes in the globin conformation, undoubtedly to one less able to stabilize the iron-oxygen complex. Also, a proton may bind to the electronegative complexed oxygen thus loosening the iron-oxygen bond. The oxygen might then leave the (ferric) iron as a hydroperoxy radical. Castro (1971) develops these pH effect concepts,

as well as others in his theory of hemoprotein reactivity. Perhaps the most important argument against the dissociation-oxidation-association theory of autooxidation is that there is so little heme dissociation near neutral pH where the autooxidation rate can be quite rapid, especially in highly purified hemoprotein solution at low (but finite) 0_2 concentration. Whatever the significance or lack thereof of this interesting theory, it does not include the fate of oxygen during the crucial iron oxidation although a univalent reduction to 0_2^- seems to be implied.

Snyder and Skrdlant (1966) noted that the variable autooxidation rate of their purified oxymyoglobin preparations was due to metal ion contamination, particularly copper. They presented evidence indicating that cupric ion acts as a catalyst for autooxidation rather than as a direct oxidant. A likely explanation for the cupric ion effect, one which the authors hint at but do not develop, is that the basic globin protein has a high binding affinity for cupric ion which, in high concentration, can denature the protein (Marks et al., 1971). In lesser amounts it can likely induce a conformational change in the globin rendering it less able to stabilize a heme iron-oxygen complex. An interesting side observation of Snyder and Skrdlant was that, at equivalent degrees of purification, hemoglobin is approximately as prone to autooxidation as is myoglobin. A commonly held view is that myoglobin oxidizes more easily than hemoglobin. Whether or not this is true in-situ is unknown. As pointed out earlier, Brown and Mebine (1969) made a critically important contribution where through rigorously controlled experiments they demonstrated that about 25% of the bound 0_2 is utilized during oxymyoglobin autooxidation while

about 75% of the bound oxygen is evolved. George and Stratmann (1952a, b) had reported 2.5 moles O_2 utilized per mole of heme iron oxidized. They attributed George and Stratmann's erroneous results to utilization of oxygen by oxidation products of the dithionite employed. While this well may be the reason, it should be noted that during reduced myoglobin oxygenation by shaking a flask of myoglobin solution in air after addition of a small quantity of dithionite, excess dithionite and its oxidation products could have undergone a reaction with oxygen prior to actual experimentation. Brown and Mebine offer a simple, stoichiometrically correct mechanism of autooxidation which has O_2 first dissociating from ferrous heme, followed by oxygen plus a proton extracting an electron from the iron to become H_2O. As with previous mechanisms this one is obscure about the exact manner in which the electron is transferred from the ferrous heme iron to the oxygen-plus-proton. An interesting, possibly significant observation was recently recorded by Morell et al. (1970). They present data which suggests that a spectral shift observed during deoxygenation of hemoglobin in sealed cuvettes may involve a reversible autooxidation of hemoglobin. The finding by the authors that deoxygenation is completely inhibited by alkylation of reactive -SH groups of the hemoglobin globin suggests either that (a) heme iron oxidized during deoxygenation is reduced by the sulfhydryl groups, or (b) that such groups provide the second of the two reducing equivalents required to reduce bound O_2 to H_2O_2 during autooxidation, as indicated by Williams (1970) and Ingraham (1966). It should be noted that mammalian myoglobins do not have sulfhydryl groups. Morell et al. (1970) suggest that a reversible

autooxidation of hemoglobin might account for the depletion of oxygen which shifts the equilibrium in the sealed cuvettes to the deoxy state. Ueda and Tynma (1971) similarly observed extensive deoxygenation of dialyzed hemolysate and "stripped" (of phosphate compounds) oxyhemoglobin in closed cuvettes. They attributed this to oxygen-consuming factors in the preparations which induce deoxygenation of oxyhemoglobin by lowering the 0_2 content of the closed systems. The nature of the reducing factor(s) and oxygen consuming factor(s) was not discussed.

Another possible explanation for excess 0_2 consumption reported by George and Stratmann is presented by Possani et al. (1970). They point out that if bound 0_2 is fully reduced to H_20 during autooxidation of hemoglobin (or myoglobin) then 0.75 mole of the 0_2 should be released per mole of heme iron oxidized (barring secondary oxygen consuming reactions), in agreement with the results of Brown and Mebine (1969). Possani et al. propose that a photodynamic effect can account for 0_2 utilization in excess of 0.25 mole per mole of heme iron oxidized, and they present evidence in support of singlet (photoexcited) oxygen involvement. They found that in the presence of moderately strong incident white light 0_2 is utilized in excess of that utilized during autooxidation in the dark. They concluded that the oxidant was oxygen bound to the heme iron which became photoactivated, perhaps to the singlet state which is a high energy oxidant. Earlier, however, Sajgo (1963) reported that, during photooxidation of ferrimyoglobin, the 2 moles of 0_2 utilized per mole of hemoprotein can be accounted for by oxidation of two histidyl residues. Histidine, an especially important amino acid of hemoglobin and myoglobin is particularly sensitive

to photooxidation involving singlet oxygen (Foote, 1968). Thus, photocatalytic utilization of 0_2 during myoglobin oxidation need not necessarily involve only the heme and its liganded oxygen. Assaf et al. (1971) reported that frozen crude extracts of myoglobin from beef muscle showed a marked increase in pigment autooxidation under flourescent illumination, but that unfrozen extracts did not. Extract dialyzed prior to freezing exhibited much less photooxidation than comparable undialyzed extract. This effect was experimentally attributed to enhancement of photooxidation by dialyzable metal ions. Employing intact beef muscle, Solberg and Franke (1971) found slight increases in oxidized pigment on the surfaces of cuts exposed to light at selected wavelengths spanning the visible spectrum, as compared with face-matched cuts similarly held in the absence of light. Temperature was eliminated as a factor during the experiments. The photocatalytic mechanism they proposed is that of activation of a compound such as riboflavin which could, in turn, oxidize oxygenated hemoprotein.

Although it does not explain autooxidation in the absence of light, photoexcitation and the excited singlet state of oxygen may very well play a role in hemepigment autooxidation in the presence of electromagnetic radiation in the U.V. and visible wavelength range. In certain systems at least, such as in dimethyl sulfoxide solution, singlet molecular oxygen can be demonstrated as a 'decay' product of superoxide anion (Khan, 1970). Kearns (1971) suggests that singlet oxygen, including that arising from enzymatically generated superoxide anions, may be of significant general importance in biological systems. Among the biological sensitizers which

can cause excited singlet oxygen generation by transferring light energy to ground-state (triplet) oxygen is protoporphyrin, the porphyrin of hemoproteins (Politzer et al., 1971; Dalton and McAuliffe, 1972). Thus the porphyrin ring of heme can conceivably mediate photoexcitation of oxyhemoglobin or oxymyoglobin bound oxygen. Riboflavin, which was invoked by Solberg and Franke (1971) is another potent sensitizer in producing singlet oxygen from triplet oxygen. The first conference devoted entirely to this newly established entity, singlet molecular oxygen, has been published (Trozzolo, 1970), and recognition of its role in biology, including the photooxidation of both the heme and the globin of hemoproteins is sure to increase with time.

The photocatalytic autooxidation of the hemoprotein pigments has significance with respect to discoloration and lipid oxidation of meat, especially at the retail level, and proper packaging and display conditions should prevent its occurrence. More difficult to control perhaps is autooxidation which takes place independently of photoexcitation. In his review article, Wang (1970) presents a valuable discussion of myoglobin and hemoglobin autooxidation and he speculates about possible initial events in the process. He argues against superoxide anion dissociation from ferric heme iron on the grounds that charge separation should be hindered by the hydrophobic environment surrounding the heme. However other charged species (e.g., dithionine and cyanide ion) are known to diffuse to the site of, and react with ferric heme iron. Using elegant model systems, Wang demonstrates that the nature of the environment around the heme in the globin cleft, as elucidated with X-ray crystallography,

facilitates the binding of O_2 by the heme iron while at the same time inhibiting univalent oxidation of the iron by the ligand during reversible oxygenation-deoxygenation. Such factors tend to favor the concept of autooxidation resulting from conformational changes in the protein and/or association-dissociation of the heme from the protein. They do not appear to shed light, however, on the autooxidation rate-oxygen partial pressure relationship.

Williams (1970), Ingraham (1966) and Castro (1971) cite the difficulty of univalently reducing molecular oxygen, and the relative ease with which O_2 accepts two electrons in their arguments against superoxide anion production during autooxidation of oxyhemoproteins. However, in the following section it will be seen that univalent reduction of oxygen, even in biological systems, is not unusual and may indeed occur during autooxidation, especially if liganded oxygen is photoexcited to a higher energy state.

Globin conformational changes have been observed with NMR spectroscopy during oxygenation and deoxygenation of myoglobin by Shulman et al. (1970) as well as by other workers employing X-ray crystallography, etc. This lends further support to the widely held view that the protein conformation plays an important role in stabilizing the oxygenated complex against oxidation. This also suggests that liganded oxygen would not oxidize the heme iron unless some other factor (e.g., decrease in pH) caused protein conformational changes.

Interaction of Superoxide Anion with Hemoproteins

Like the excited singlet state of molecular oxygen, the superoxide anion radical, O_2^-, has only recently had its existence positively established (Knowles et al., 1969; Bray et al., 1970). In this case the anions were produced by univalent reduction of oxygen by the xanthine oxidase enzyme during the course of its normal reaction with substrate (xanthine). This first, and most widely studied of the systems which generate superoxide was thought capable of univalent reduction of oxygen as early as 1958 (Fridovich and Handler, 1958). Recently a number of other biological, organic and inorganic systems have been shown to reduce oxygen univalently. (Massey et al., 1969; Nilsson et al., 1969; Orme, Johnson and Beinert, 1969; Misra and Fridovich, 1971; Forman and Fridovich, 1972; Nakamura, 1970; Ballou et al., 1969; McCord and Fridovich, 1970).

The most widely employed method of detecting superoxide anion production (Beauchamp and Fridovich, 1971) is that of monitoring spectrophotometrically the reduction of ferricytochrome c by the anion in a diffusion-controlled, bimolecular redox reaction, and the competitive inhibition of the reaction by "superoxide dismutase" (a cupric ion-requiring erythrocyte protein also known as hemocuprein or erythrocuprein).

Fridovich (1962) had earlier reported that the globin of horse and pig heart ferrimyoglobin competitively inhibits the reduction of ferricytochrome c by the aerobic xanthine-xanthine oxidase system, and that the heme group is not involved. Subsequently, McCord and Fridovich (1968) attributed superoxide dismutase activity to the bovine erythrocuprein

(hemocuprein) protein, and they also expressed the view that the globin of myoglobin has inherent superoxide dismutase activity. Although globin has a high binding affinity for cupric ion, neither this nor any other feature of the globin molecule was suggested as an explanation for the claimed activity. Although the heme of ferricytochrome c was known to undergo a redox reaction with O_2^-, the authors did not suggest that perhaps the heme group of myoglobin and other hemoproteins might similarly interact with O_2^-. They later attributed the apparent superoxide dismutase activity of myoglobin-globin to erythrocuprein contamination (McCord and Fridovich, 1969), and the possibility of a myoglobin-superoxide reaction was not further investigated. Recently McCord et al. (1971) have proposed a theory of obligate anaerobiosis based upon the apparent wide distribution in nature of superoxide dismutase activity. On the other hand, Muraoka et al. (1967) had earlier expressed the view, with some experimental support, that the oxygen requiring reduction of cytochrome c may not have biological significance since a natural electron carrier may have been lost during purification of xanthine oxidase. In any event, oxygen is essential for ferricytochrome c reduction in isolated systems, in the absence of an artificial electron transfer mediator. Oxygen is also probably an important electron acceptor in the in-vivo functioning of xanthine oxidase, as well as various other oxidases, mixed-function oxidases and oxygenases. Production of superoxide anion in addition to hydroperoxide and hydroxyl radicals, at least in aerated model xanthine oxidase systems, has been established (Beauchamp and Fridovich, 1971).

Probably the only direct, unequivocal way of demonstrating superoxide anion production in a given system is that of low temperature trapping coupled with ESR spectroscopy. While this has been employed successfully with systems which generate large amounts of O_2^- at a rapid rate, it is most unlikely that autooxidation of oxyhemoglobin or oxymyoglobin can generate enough O_2^- rapidly enough, assuming O_2^- is generated at all, to allow this method of detection to be used. Therefore, in this work another accepted method of demonstrating O_2^- production, that of monitoring ferricytochrome c reduction, and inhibition of this reduction by Tiron, was employed. The ability of Tiron to mimic the activity of "superoxide dismutase" by competitively inhibiting reactions involving O_2^- has been similarly put to use at other laboratories (Fridovich and Handler, 1962; Muraoka et al., 1967; Miller, 1970; Strobel and Coon, 1971).

MATERIALS AND METHODS

BIOCHEMICALS:

Myoglobin was prepared from the semitendinosus muscle of USDA Commercial grade beef round. Partially purified oxymyoglobin (preparation A) was prepared by homogenizing x grams of ground lean beef with 3 x ml of chilled 75% saturated $(NH_4)_2\ SO_4$ solution. After centrifuging, the red supernatant was passed through several layers of cheesecloth and exhaustively dialyzed against cold deionized distilled water. After a final dialysis against 0.01M phosphate buffer, pH 7, the preparation was vacuum filtered through Whatman #5 qualitative paper.. A more highly purified oxymyoglobin (preparation B), and a highly purified ferrimyoglobin (preparation C) were prepared essentially according to the method of Hardman et al. (1966) with the following modifications: The fresh beef muscle was minced by passing through a 1/8" grinder plate before blending in 3 liters of chilled, deionized water per Kg of meat. Three high-speed blends of 3 to 5 seconds each were employed with 5 minute pauses in between (the high water/meat ratio and the brief high speed bursts aid in preventing emulsion formation). After passing the blendate through two layers of cheesecloth the solid material was hand squeezed in the cloth and discarded. After centrifuging (45 min @ 8000xg), the red supernatant was passed through several layers of cheesecloth (brown ppt was discarded) to remove floating solids, followed by suction filtration through coarse (Whatman #4) filter paper. The lead precipitation of globulins and other non-myoglobin proteins, and the zinc precipitation of oxymyoglobin (plus small amounts of other protein) from 25% ethanol solution at -15°C, and

the resolvation of the oxymyoglobin-zinc ppt were carried out as per Hardman et al. From this point on oxymyoglobin (prep B) and ferrimyoglobin (prep C) were further purified separately.

To prepare further purified oxymyoglobin (prep B), the oxymyoglobin which was brought into solution with EDTA was exhaustively dialyzed with chilled deionized water and then brought to 70% saturation with $(NH_4)_2\ SO_4$ by slowly adding the required amount of the salt with vigorous stirring. After several minutes of stirring, the material was centrifuged (45 min @ 8000xg) and the red supernatant was exhaustively dialyzed as before (ppt was discarded). The dialyzed oxymyoglobin solution was of very high purity at this stage as evidenced by an $A^{418}/A280$ greater than 4, and by disc gel electrophoresis employing both a protein and a heme stain (see "Disc-gel Electrophoresis"). The dialyzed solution was typically about $5x10^{-4}M$ in oxymyoglobin based upon an E_M^{418} of $13.3x10^4$ for oxymyoglobin, and was around pH 7. Adjustment of the pH, up or down, was done by adding a minimal amount of the appropriate dry mono- di- or trisodium phosphates with stirring. Adjustment of concentration was accomplished either by dilution or by ultrafiltration in an Amicon #402 cell with a UM-10 membrane. Further purification of this preparation was not found to be necessary, but could be done according to the DEAE cellulose (or DEAE sephadex) method of Yamazaki et al. (1964).

Preparation of highly purified ferrimyoglobin (prep C) began with the addition of an excess of potassium ferricyanide to the EDTA solubilized oxymyoglobin-zinc ppt after it was dialyzed. Following oxidation of myoglobin to the trivalent state the solution was brought to 70% $(NH_4)_2$

SO_4 as per "prep B", and was similarly centrifuged and dialyzed. Following dialysis, the ferrimyoglobin solution was vigorously stirred while a generous amount (about 1/4 lb per liter) of reactor grade MB-3 mixed-bed deionization resin (Rohm & Haas) was added. This was done to assure complete removal of any ferricyanide or ferrocyanide that might still be attached to the myoglobin, as well as to further deionize the preparation. The solution was then concentrated (500 ml down to about 20 ml) in an Amicon #402 ultrafiltration cell with a UM-10 membrane. Ten ml of concentrate was applied to a 2.5x25 cm column of DEAE Sephadex which was equilibrated with 0.01M Na-phosphate, pH 6.0. The ferrimyoglobin was retained at the top of the column, and was gradient eluted with 0.01 M Na Phosphate, pH 6.0 and 8.0. When the eluent reached about pH 7, the ferrimyoglobin moved down the column as a brown band and was collected with a fraction collector. At this stage the preparation was practically free of non-heme protein as indicated by disc gel electrophoresis, and by an $A_{409}/A280$ greater than 5. The pH of the solution was approximately 7 and this was adjusted up or down with a minimal amount of the appropriate sodium phosphate. Preparation A could be stored for several days at 0°C, and preparations B and C for several weeks. Several batches of each were prepared during the course of the work in order to avoid working with preparations that had been stored too long.

Heme-free Globin was prepared from ferrimyoglobin by the 2-butanone method of Teale (1959) as modified by Yonetani (1967). Following dialysis against 0.01 M Na-PO_4, pH 7.8, the concentration of the globin solution

employed was 2.8×10^{-7} M based upon an E_M^{280} of 1.6×10^4 for globin and an E_M^{409} of 1.60×10^4 for the parent ferrimyoglobin.

Human Ferrihemoglobin, 2x crystallized ("Pentex", Miles Laboratories, Inc.) was solubilized in 0.1M NaCl and passed through a 1.5x90 cm column of Sephadex G-100 gel with 0.1M NaCl as eluant. This treatment removed catalase contamination of the hemoglobin as well as other impurities (Aebi et al., 1964). Following gel filtration, the ferrihemoglobin fraction was exhaustively dialyzed against deionized, distilled water after adding a few crystals of K_3 Fe $(CN)_6$ to assure complete oxidation. Following dialysis the ferrihemoglobin was mixed with MB-3 deionization resin (see "Myoglobin"), suction filtered through Whatman #5 filter paper, and the pH was adjusted with sodium phosphate. Oxyhemoglobin was made from the ferrihemoglobin by first reducing the pigment with hydrosulfite and then exhaustively dialyzing the reduced pigment against deionized water. During dialysis the hemoglobin oxygenated. Following dialysis the oxyhemoglobin was mixed with MB-3 deionization resin (see "Myoglobin") and suction-filtered through Whatman #5 filter paper. The pH was adjusted finally with the appropriate sodium phosphate salt. The preparation was quite stable against autooxidation at 0-5°C.

Ferricytochrome c. Sigma horse heart Type III and beef heart Type V, were exhaustively dialyzed against deionized distilled water after adding a few crystals of K_3 Fe$(CN)_6$ to oxidize any reduced form present. After mixing with MB-3 deionization resin (see "Myoglobin") and suction filtering, the pH of the ferricytochrome c solution was adjusted with sodium

phosphate. As expected from their great similarity, both equine and bovine cytochrome c yielded the same results.

Xanthine Oxidase (E.C. 1.2.3.2, Nutritional Biochemicals Co), obtained as a suspension in 60% $(NH_4)_2 SO_4$ was dialyzed against several changes of 0.01M Na-PO_4, pH 7.8. The solution employed was $3x10^{-6}$M Xanthine oxidase based on an E_m^{450} of 70,000. The enzyme was kept at 0-5°C in the phosphate buffered solution and aliquots were removed for the reactions. The enzyme showed no significant loss of activity during the several days it was employed as evidenced by the rate of uric acid production from Xanthine.

All biochemicals were prepared and stored at no higher than 5°C and were periodically checked at appropriate times for indications of deterioration.

OTHER CHEMICALS:

Lead Subacetate (basic lead acetate) used to remove globulins and other non-myoglobin proteins during myoglobin preparation was prepared as follows: 250 gm of lead subacetate was mixed into 500 ml H_2O and refluxed for one hour in a round bottom flask attached to a water cooled condensor. After cooling, the chalky white suspension was centrifuged 30 min @ 15,000 xg and the supernatant was suction filtered through Whatman #5 filter paper. The small amount of remaining suspended particles gradually settled out and presented no problem. One ml of lead subacetate solution added per each 100 ml of ethanol-water solution of myoglobin proved to be an excess, which was desirable, and caused precipitation of considerable non-heme protein.

Zinc Acetate, which precipitates myoglobin, serum albumins and other proteins having a high histidine content was prepared as follows: Zinc acetate was made 0.5 M in aqueous solution by simply dissolving the required amount in water. Addition of 1 ml of 0.5M Zn acetate per each 50 ml of myoglobin containing supernatant, after lead acetate treatment and pH adjustment, consistently resulted in precipitation of all oxymyoglobin, leaving a nearly colorless supernatant after centrifugation.

EDTA solutions for dissolving the oxymyoglobin - Zn precipitate were prepared with chilled deionized water. Complete dissolution of crystals to make 0.4M EDTA required continually maintaining the solution pH at about 7.8 with 6M NaOH. When completely dissolved, the 0.02M solution was made from the 0.4M solution. EDTA for the Xanthine Oxidase experiments was 3×10^{-4}M in water adjusted to pH 7.8 with disodium phosphate.

Ferricyanide and Ferrocyanide, "Baker analyzed" reagent grade potassium salts, were added to solutions both as dry crystals and in aqueous solution form. Since each method of addition gave the same result, addition of crystals was usually employed.

Sodium Hydrosulfite (dithionite), Mallinckrodt reagent grade, was added to solutions as the dry powder.

Hydrogen Peroxide, 30% "Baker analyzed" reagent grade, was used either full strength or diluted to 1% solution. Droplets were added to solutions with stirring.

O_2, CO and N_2 employed were high purity cylinder gases. The nitrogen gas was of 99.996+% minimum purity and no additional oxygen removal step was necessary.

Tiron (4,5 - Dihydroxy - m - benzene disulfonic acid, disodium salt) obtained from Eastman Organic Chemicals was added to solutions as the dry powder.

Xanthine (2,6 dihydroxypurine, Sigma Chemical Co) was dissolved in 0.1M NaOH and adjusted to 1.5×10^{-3}M Xanthine with distilled-deionized water.

Sodium Borohydride (Sigma Chemical Co) which has been used for the reduction of hemoproteins instead of hydrosulfite (Asakura et al., 1964) was similarly used in this work in the same manner as, and for the same purpose as hydrosulfite.

INSTRUMENTS & SPECIAL EQUIPMENT

Spectrophotometry. A Bausch & Lomb Spectronic 505 recording spectrophotometer was employed to obtain all recorded spectra, and also for reaction rates in the Xanthine oxidase work. Single wavelength quantitative measurements (e.g., for reagent concentrations) were made with a Beckman DU spectrophotometer. All figures are photoreduced original recorder tracings. Soret peaks (400-440 nm) were recorded after dilution of the samples used to obtain the 450-650 nm tracings. One cm matched quartz cuvettes, regular cells, and anaerobic cells with 5 ml side-arm, were used. A distilled water blank was used in all cases.

Concentration of hemoprotein solutions was accomplished by using an Amicon Corp model 402 ultrafiltration cell and UM-10 membrane under 30 psi nitrogen pressure.

Centrifugation in the preparation of myoglobin was with a Sorvall RC 2-B "superspeed" refrigerated centrifuge, SGA (4 1/4") head and 250 ml polybottles.

Irradiation was done with a 30,000 curie Cobalt-60 research gamma irradiator at a dose rate of approximately 1 Mrad/hr.

Polyacrylamide ("disc") gel electrophoresis was performed essentially according to the Davis (1964) procedure for anodic proteins with the following qualifications: Stock solution F contained ammonium persulfate (0.14 gm per 100 ml) in place of sucrose; the sample gel was omitted, and a 6.5% running gel was employed. The coomassie brilliant blue staining procedure of Chrambach et al. (1967) was used for protein band detection and the dianisidine method of Owen et al. (1958) was used for hemoprotein detection. This technique, employing the differential staining procedure, proved extremely useful in monitoring qualitatively and roughly quantitatively the increase in purity of myoglobin preparations at various stages. of the purification procedure.

EXPERIMENTAL PROCEDURE:

Irradiation Experiments: Buffered aqueous solutions of myoglobin (preparations A, B and C), ferrihemoglobin and ferricytochrome c in either

25 or 50 ml beakers or evacuated, sealed flasks were placed in the isotropic center of the cylindrical gamma irradiator and were exposed to the specified doses of irradiation. Spectra were recorded both before and immediately after irradiation. Small amounts of ferricyanide, ferrocyanide or hydrosulfite were added to solutions after irradiation to establish the oxidation state of the heme iron, and carbon monoxide gas was employed to establish the nature of the "sixth-position" ligand when oxymyoglobin was suspected. Small amounts of H_2O_2 solution were applied to ferrimyoglobin and ferrihemoglobin solutions to generate ferryl myoglobin in order to compare its spectra with that of irradiated red pigment. Anoxic solutions were prepared by alternately evacuating and nitrogen flushing the hemoprotein solution in sealed 125 ml filtration flasks several times prior to irradiation under vacuum. Oxygenated solutions had oxygen bubbled into them for several minutes prior to irradiation. These yielded the same results as solutions in equilibrium with the atmosphere prior to irradiation.

Xanthine Oxidase (Superoxide anion) Experiments: The Xanthine oxidase preparation was periodically checked during the course of ten days of these experiments by measuring the rate at which it oxidized Xanthine to uric acid (A_{290}). Activity did not vary appreciably during this period. Enzyme and other solutions were brought to room temperature (25°C) prior to use. All solutions were in equilibrium with the atmosphere at 25°C during experiments, except when deoxygenation is indicated. Reaction rates were recorded at 550 nm for cytochrome c, 582 nm for myoglobin. The 3 ml reaction mixtures consisted of 2.6 or 2.7 ml of the

hemoprotein solution under study, the balance being 0.1 ml each of EDTA, xanthine oxidase, xanthine, and either inhibitor (myoglobin or hemoglobin) or buffer solutions. Where indicated, high purity cylinder carbon monoxide or nitrogen was gently bubbled into the cuvettes. Nitrogen was applied to establish virtually anoxic conditions prior to starting reactions with deoxygenated xanthine solution. Carbon monoxide was employed after the reactions, as were ferricyanide and ferrocyanide, to aid in establishing the oxidation state of the hemoprotein heme iron. Reactions were usually initiated by adding 0.1 ml of xanthine to cuvettes via a teflon plunger. Identical results were obtained when xanthine oxidase was added last to the reaction cuvettes. For the kinetics experiments recording of absorbance increases was initiated 5 seconds after initial contact of the plunger with the reaction solution. Reaction rates were taken directly from the linear portions of the tracings. Since hemoprotein interaction with superoxide anion and its dismutation product, H_2O_2, is known to be a bimolecular, diffusion-limited process, only simple reaction rates ($\Delta A/\Delta t$) at one reactant concentration were measured. Also, the spectrum of the reaction solutions was recorded both before and after the xanthine - xanthine oxidase reaction to determine the identity of reaction products.

Autooxidation Experiments: As indicated in the "Review of Literature" the reduction of ferricytochrome c by the superoxide anion and the competitive inhibition of this reaction by Tiron presented a potentially useful method of determining directly whether or not oxymyoglobin and oxyhemoglobin can univalently reduce liganded oxygen during the 'normal' (i.e.,

not induced by any special treatment) course of their autooxidation. Based upon what is known concerning other superoxide generating systems, if during the course of oxymyoglobin and oxyhemoglobin autooxidation liganded oxygen is univalently reduced, and the product superoxide anion diffuses from the site of the heme iron into the bulk solution, then it should cause some measurable ferricytochrome c reduction. Further, if such a reduction occurs and O_2^- is the reactive agent, then the presence of Tiron would be expected to competitively inhibit the cytochrome c reduction. Based upon these premises, the following experiments were conducted in an attempt to shed light on the fate of the oxygen during autooxidation of the oxyhemoproteins.

Ferrimyoglobin and ferrihemoglobin were reduced with dithionite followed by exhaustive dialysis against chilled deionized-distilled water during which the chemically reduced hemoproteins became oxygenated. To assure complete removal of excess dithionite and its oxidation products the dialyzed oxyhemoproteins were mixed with MB-3 mixed-bed deionization resin (see "Myoglobin") followed by suction-filtration through Whatman #5 filter paper. After pH adjustment, the oxyhemoprotein solutions were mixed with equal volumes of ferricytochrome c solution in anaerobic cuvettes and zero-time spectra were recorded. The cuvettes were then evacuated to accelerate oxidation of the oxyhemoprotein, and spectra were recorded periodically over a period of several hours. Matched anaerobic cuvettes were employed in each case, one with and one without 1 mM Tiron. The hemoproteins were present at concentrations in the range of 10^{-5}M, buffered at either pH 6.5 or 8.5. Since Tiron tends to lower the pH of

the solution, the required amount to achieve a final concentration of 1 mM was divided between the oxyhemoprotein and cytochrome solutions and pH was adjusted when necessary with the appropriate $Na\text{-}PO_4$ before mixing the two solutions in the cuvette. Similar experiments were conducted with myoglobin prepared as the oxygenated derivative (preparation B) and these yielded results similar to those obtained with chemically reduced, oxygenated myoglobin.

In a separate set of experiments pertaining to the initial electron-transfer events of the autooxidation mechanism, ferricytochrome c was similarly incubated over a period of hours under vacuum in the anaerobic cuvettes either in the presence of no additional hemoprotein, or, in the presence of equimolar amounts of ferrimyoglobin or ferrihemoglobin, or, in the presence of equimolar amounts of deoxyferrous myoglobin or deoxyferrous hemoglobin. The deoxyferrous derivatives were prepared as follows: Three ml of ferrimyoglobin or ferrihemoglobin was placed in the cuvette part of an anaerobic cell. A minute amount of dithionite powder (a fraction of a milligram) barely sufficient to reduce the ferrihemoprotein as determined by 'trial and error' was placed in the sidearm which was lubricated with high vacuum grease and attached to the cuvette portion. The cell was attached to an aspirator hose and suction was applied for a few minutes following which the stopcock was closed off and the dithionite was mixed into the solution. After a brief mixing suction was again applied for several minutes to remove volatile reaction products. Sodium borohydride was similarly employed in duplicate experiments. The sealed cells were allowed to stand for several hours, usually overnight, after

which they were again evacuated for several minutes. Then the cell was connected to a cylinder of high purity nitrogen and as pressure was applied the sidearm was removed. The stream of N_2 gas entering the cell above the solution level and going out the top kept air from coming in contact with the reduced myoglobin or hemoglobin so that no oxygenation occurred during the following additions. Then 0.5 ml of either ferrihemoglobin or ferrimyoglobin (previously deoxygenated by bubbling N_2 into the ferrihemoprotein solution contained in a volumetric flask with a long, narrow neck) was pipetted into the deoxyferrous hemoprotein solution to react with and use up any residual dithionite that might have still been present. Following this another sidearm containing 3.5 ml of deoxygenated (by similarly bubbling in N_2 for 30 min) ferricytochrome c solution was attached to the cuvette and the N_2 stream was stopped. Immediately the cell was evacuated for several minutes to assure complete absence of oxygen. Then the spectrum of the deoxyferrous myoglobin or hemoglobin was recorded following which the contents of the cuvette and sidearm were admixed. Immediately upon mixing a zero-time spectrum was recorded which was typically a composite of that of the deoxyferrous myoglobin or hemoglobin (plus some slight ferrimyoglobin or ferrihemoglobin absorption) and ferricytochrome c. Spectra were again recorded periodically for several hours in order to monitor spectral changes. The gradualness of spectral changes and the respective redox reactions in these oxidation mechanism experiments made it impractical to obtain quantitative reaction rates for purposes of comparison, and as a result only equilibrium, and no kinetic data were obtained. However, absorbance changes

at particular portions of the 350-650 nm spectra with time, for a given reaction mixture, yielded considerable insights into the nature of the processes going on within the cuvettes.

RESULTS AND DISCUSSION

Irradiation of Myoglobin and Hemoglobin

Figure 1 compares visible and Soret spectra of (A) untreated, oxygen containing metmyoglobin solution with that which had been treated with (B) H_2O_2, (C) 40 Krad of radiation, (D) irradiated water which stood at room temperature for one hour post-irradiation before adding 5 ml of it to 25 ml of metmyoglobin. The Soret peaks at 420 to 423 nm are indicative of ferrylmyoglobin as are the absorption bands in the 550 and 580-590 nm regions. H_2O_2, and possibly some molecular hydrogen should be the only radiolysis products in the irradiated water at the time of addition to the metmyoglobin. The slightly different pattern of that spectrum no doubt reflects fine electronic detail. Clearly, all three treatments resulted in ferrylmyoglobin production via the metmyoglobin-H_2O_2 reaction, as is further demonstrated in the next figure.

Figure 2 shows the absorption spectra of (A) oxygenated metmyoglobin solution exposed to 40 Krad, (B) "A" after ferricyanide addition, (C) "A" after ferrocyanide addition and (D) "A" after hydrosulfite addition. The same results were obtained with metmyoglobin solution that was merely equilibrated with the atmosphere. The ferrimyoglobin spectrum due to irradiation of oxygenated metmyoglobin is virtually unchanged by post-irradiation addition of ferricyanide, whereas ferrocyanide addition caused an instantaneous reduction back to metmyoglobin. The ferrocyanide/ferricyanide redox couple is ideal for this type of study in that ferrocyanide instantaneously reduces quadrivalent myoglobin to the trivalent (met) state

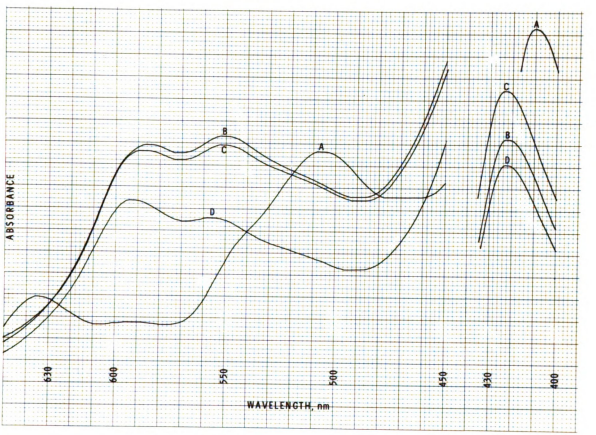


Figure 1. Absorbance spectra of oxygen containing ferrimyoglobin solution which was: untreated, Curve A; treated with H_2O_2, Curve B; treated with 40 Krad, Curve C; treated with irradiated water, Curve D.

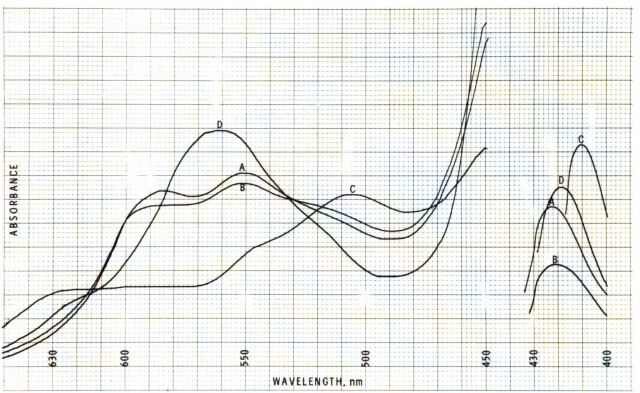


Figure 2. Absorbance spectra of oxygenated, 40 Krad irradiated ferrimyoglobin solution which was: untreated, Curve A; treated with ferricyanide, Curve B; treated with ferrocyanide, Curve C; treated with hydrosulfite, Curve D.

but not a lower one, and ferricyanide instantaneously oxidizes ferrous myoglobin to the trivalent (met) state but not a higher one, thus enabling one to distinguish the ferryl red pigment from oxymyoglobin and unequivocally establish the valency of the heme iron. The red myoglobin derivative from irradiation of oxygen containing metmyoglobin has a quadrivalent iron. The powerful, general purpose reducing agent, hydrosulfite, reduces ferrylmyoglobin to purple deoxyferrous myoglobin via a two equivalent reduction of the heme iron.

Figure 3 shows the absorption spectra of (A) oxymyoglobin (B) deoxygenated metmyoglobin exposed to 40 Krad, (C) "B" treated with ferrocyanide and (D) "B" treated with ferricyanide. When metmyoglobin solution was depleted of oxygen and subsequently exposed to 40 Krad of gamma radiation the absorption spectrum of oxymyoglobin was obtained. In addition to the spectral evidence the nature of the radiation-generated product, the following two reactions confirm the ferrous state of the heme after irradiation: ferricyanide instantaneously oxidized the radiation product to metmyoglobin as shown by the absorption spectra, whereas ferrocyanide caused essentially no change in the spectrum of the radiation product. Further, the fact that carbon monoxide gas displaced the "sixth position ligand" of the radiation-generated red pigment as evidenced by a shift in Soret and visible spectrum from that of oxymyoglobin to that of carbon monoxide myoglobin (not shown) is additional strong evidence that the radiation product is indeed oxymyoglobin. Carbon monoxide is known to readily displace liganded oxygen on the oxyhemoproteins.

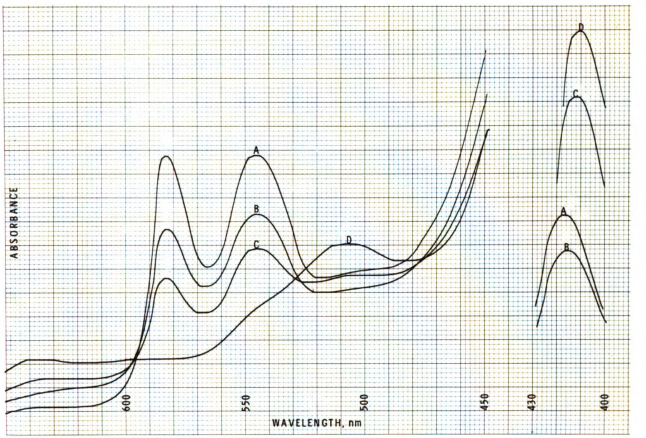


Figure 3. Absorbance spectra of: untreated oxymyoglobin, Curve A; deoxygenated ferrimyoglobin exposed to 40 Krad, Curve B; "B" treated with ferrocyanide, Curve C; "B" treated with ferricyanide, Curve D.

There are at least two possible mechanisms for the formation of oxymyoglobin under the conditions used in this experiment. The most probable one is the reduction of the heme iron by a reducing product of water radiolysis, most likely the solvated electron. This reduction would be followed by oxygenation with either residual oxygen remaining in the solution prior to irradiation, or with oxygen generated during irradiation. Some reactions leading to the generation of oxygen by the radiation are:

$$\cdot OH + HO_2\cdot \text{ ----------> } O_2 + H_2O$$

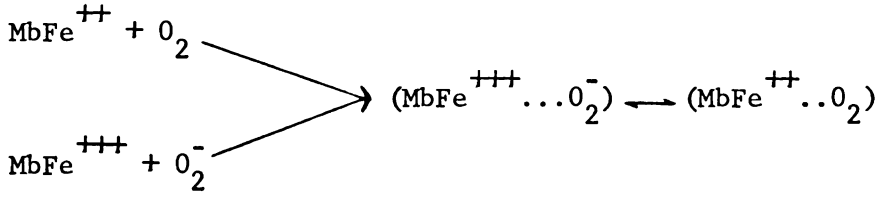
$$HO_2\cdot + Fe^{+3} \text{ ---------> } H^+ + O_2 + Fe^{+2}, \text{ and}$$

$$2HO_2\cdot \text{ ----------> } H_2O_2 + O_2$$

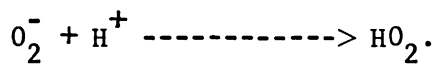
Another possibility is suggested by the work of Wittenberg *et al.* (1970) and others confirming the theory first proposed by Weiss (1964) that when ferrous heme pigments bind oxygen the heme iron becomes formally ferric by partial transfer of one 3d electron to the ligand oxygen which then becomes a becomes a bound superoxide anion, $O_2^{\bullet-}$. The solvated electron readily reacts with oxygen to generate the superoxide anion:

$$\bar{e}\ aq. + O_2 \text{ ----------> } O_2^-$$

It may be possible for the superoxide anion so generated to bind to the ferric ion of metmyoglobin to give the same oxymyoglobin structure as is normally formed by binding of molecular oxygen to ferrous myoglobin. In both cases one would end up with a formally ferric iron bound to a superoxide anion, probably in equilibrium with the oxyferrous form:



Such a reaction would be favored by an alkaline pH where the reaction



is less likely to interfere, since its pK is at about pH 4.9 (Behar et al., 1970). Although the formation of oxymyoglobin by binding of superoxide anion to the ferric ion of metmyoglobin under reducing conditions is purely speculative, such a hypothetical binding reaction has been indicated to be at least as exothermic as the binding of molecular oxygen to ferrous myoglobin (George and Stratmann, 1954). In any event, oxymyoglobin is produced under the conditions employed indicating that some sort of reductive oxygenation of the pigment takes place.

Figure 4 depicts the spectra of (A) oxymyoglobin (preparation A), (B) "A" exposed to 40 Krad of radiation, (C) "B" exposed immediately to another 40 Krad of radiation, and (D) "B" exposed to another 40 Krad of radiation after first dialyzing the solution and then oxygenating it. The oxymyoglobin preparation was oxidized completely to metmyoglobin (curve B) by the initial 40 Krad and was immediately reduced back to oxymyoglobin by the additional 40 Krad (curve C). When the radiation generated metmyoglobin solution was oxygenated prior to the second 40 Krad of radiation the ferrylmyoglobin spectrum (curve D) was obtained. Confirmation for the oxidation states of the radiation generated oxymyoglobin and ferrylmyoglobin was provided by the ferrocyanide - ferricyanide reactions.

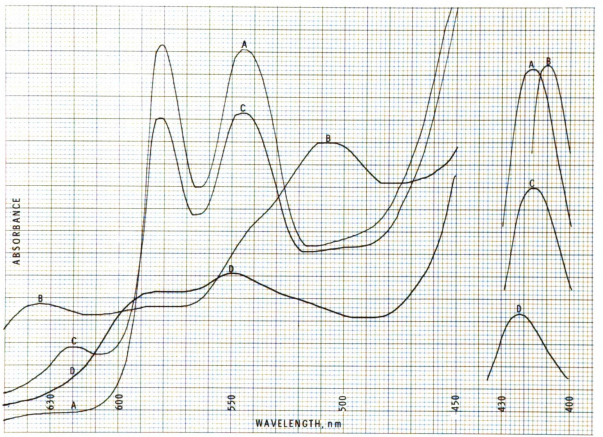


Figure 4. Absorbance spectra of: untreated oxymyoglobin, Curve A; "A" exposed to 40 Krad, Curve B; "B" immediately exposed to an additional 40 Krad, Curve C; "B" exposed to an additional 40 Krad after dialyzing and oxygenating the solution, Curve D.

The regeneration of the oxymyoglobin after oxidation to metmyoglobin, also reported by Bernofsky et al. (1959), can be explained by the depletion of oxygen in the solution during the initial 40 Krad irradiation, thus favoring a reduction of the iron during the second phase of irradiation. The 620 nm absorption band of the radiation generated oxymyoglobin indicates disruption of the normal porphyrin resonant double bond structure as a result of either oxidation of, or addition of a small molecule such as H_2S (Berzofsky et al., 1971b) to a site on the ring. The dialysis step in the production of ferrylmyoglobin from radiation generated metmyoglobin was found to be necessary to obtain complete oxidation to the ferryl state. Allowing the irradiated solution to stand for more than a day prior to oxygenation and further irradiation did not eliminate the need for dialysis. Also, pH was found not to be the factor inhibiting complete further oxidation. An interfering solute or solvent radiolysis product is therefore suggested.

When human ferrihemoglobin was treated in the same manner as ferrimyoglobin, exactly the same results were obtained. That is, ferrylhemoglobin was produced by irradiation of ferrihemoglobin in the presence of oxygen, and oxyhemoglobin was produced during irradiation of ferrihemoglobin in the absence of oxygen. The radiation results are consistent with unpublished observations made by the author when irradiating fresh red meat (Giddings, 1969). When beefsteak having a brown, metmyoglobin surface color was vacuum packaged and irradiated the brown color became purple during irradiation indicating a reduction of the surface pigment by irradiation in-anoxia. The surface pigment would then oxygenate upon exposure

of the meat to the atmosphere. Irradiation of similar meat in the presence of oxygen did not change the brown metmyoglobin to ferrylmyoglobin, as was observed with pigment solutions. This is undoubtedly due to catalase activity in the meat tissue which would rapidly break down any H_2O_2 produced during the irradiation before any significant interaction of H_2O_2 with ferrimyoglobin could take place.

Ferrihemoprotein Interaction with Superoxide Anion

Figure 5 compares the visible and Soret spectra of: (A) ferrimyoglobin and (B) ferrylmyoglobin, and (C) ferrylhemoglobin and (D) ferrihemoglobin. In both cases O_2^- generated by the aerobic xanthine oxidase system oxidized the ferric (+3) iron of the pigments to the ferryl (+4) oxidation state. In addition to the typical ferryl spectra, the +4 oxidation state of the hemoprotein iron was established by the fact that addition of ferrocyanide instantaneously reduced the pigments back to the ferric state, whereas ferricyanide or carbon monoxide produced no immediate change, but merely hastened somewhat the slow autoreduction of the ferrylheme pigments to the ferric state. Thus superoxide anions, in this case generated by reduced xanthine oxidase, further oxidized ferrimyoglobin and ferrihemoglobin to the quadrivalent state whereas they are known to reduce ferricytochrome c. Further evidence in support of this conclusion follows.

Figure 6 shows the absorption spectra of: (A) untreated ferricytochrome c; (B) ferricytochrome c in the presence of 2.5×10^{-7}M ferrimyoglobin at the completion of xanthine-xanthine oxidase reaction; (C) deoxygenated cytochrome c solution (obtained by alternately bubbling

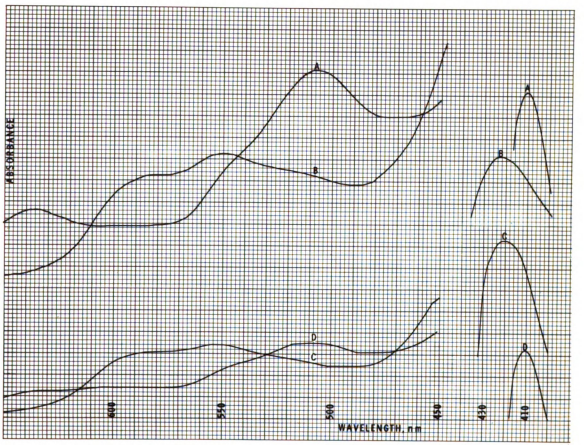


Figure 5. Oxidation of ferrimyoglobin and ferrihemoglobin to the ferryl state by the aerobic xanthine oxidase system. Conditions: 10^{-5}M ferrimyoglobin or ferrihemoglobin; 10^{-5}M EDTA; 10^{-7}M xanthine oxidase. Reaction solutions were in equilibrium with the atmosphere at 25°C and pH 7.8. Reactions were initiated by addition of xanthine. Spectra: (A) ferrimyoglobin solution before xanthine addition. (B) "A" after completion of xanthine oxidase reaction. (D) ferrihemoglobin solution before xanthine addition. (C) "D" after completion of xanthine oxidase reaction.

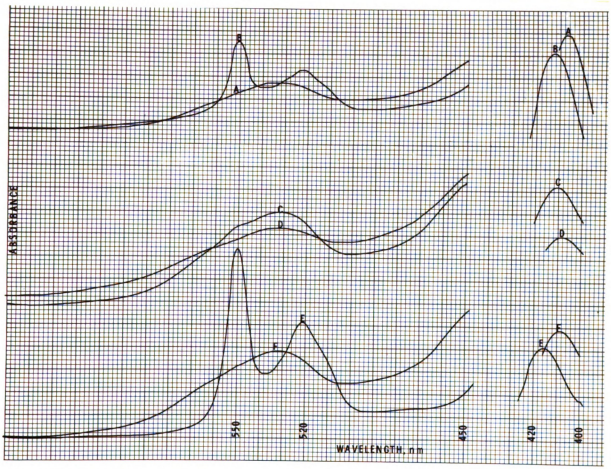


Figure 6. Effect of various treatments on cytochrome c oxidation state. Conditions: 10^{-5}M ferricytochrome c, pH 7.8 and 25°C for the six treatments shown. Spectra: (A) untreated ferricytochrome c. (B) ferricytochrome c in the presence of 2.5×10^{-7}M ferrimyoglobin inhibitor at the completion of xanthine oxidase reaction. (C) deoxygenated ferricytochrome c solution at the completion of xanthine oxidase reaction. (D) ferricytochrome c after exposure to 100 Krad of gamma irradiation. (E) ferricytochrome c, no inhibitor present, after completion of xanthine oxidase reaction. (F) ferricytochrome c after addition of 0.05 ml of 30% H_2O_2 per 3 ml reaction mixture. Reaction solutions for A, B, C, and E contained 5×10^{-5}M xanthine, 10^{-5}M EDTA, and 10^{-7}M xanthine oxidase in the total 3 ml.

nitrogen into the cuvette solution and evacuating) after completion of the xanthine-xanthine oxidase reaction; (D) aerobic ferricytochrome c solution after 100 Krad of gamma irradiation; (E) aerobic ferricytochrome c solution, no inhibitor, after completion of the xanthine-xanthine oxidase reaction, and (F) aerobic ferricytochrome c solution after addition of H_2O_2.

Clearly the xanthine oxidase reaction caused complete reduction of ferricytochrome c in the presence of ample oxygen and in the absence of inhibitor (E), whereas ferrimyoglobin greatly diminished the rate and extent of ferricytochrome c reduction (B), and deoxygenating the ferricytochrome c solution prior to initiating the xanthine oxidase reaction (C) almost completely eliminated this reduction (the small amount of reduction in this instance could be due either to residual oxygen or to a non-oxygen requiring secondary reduction mechanism). The results show that the oxygen requiring mechanism for rapid quantitative reduction of ferricytochrome c was operative and that the heme moiety of ferrimyoglobin is an effective competitive inhibitor since (1) superoxide anion oxidizes the ferrimyoglobin iron (Figure 5), and (2) heme free globin at 10 times the highest ferrimyoglobin concentration used is ineffective as an inhibitor (Table 1). Figure 6 also illustrates that neither aerobic irradiation nor H_2O_2 addition, under the conditions employed, change the oxidation state of the ferricytochrome c heme iron. This constitutes additional evidence in support of the view that H_2O_2, and not O_2^-, is the dominant reactive entity generated by aerobic irradiation of aqueous systems, whereas it is O_2^- in the case of the aerobic xanthine-xanthine oxidase reaction, at least with regard to heme iron one-electron redox reactions

of hemoproteins. (During irradiation in-anoxia the hydrated electron is generally regarded as being the dominant reactive reducing entity produced in aqueous systems).

Table 1 illustrates the inhibitory capacity of ferrimyoglobin and the virtual lack of same in the case of heme free globin. Further kinetic study was not undertaken since it is known that the reactions are diffusion controlled, bimolecular, and the inhibition is competitive for the O_2^-.

Table 1. Effect of ferrimyoglobin and heme-free globin on the xanthine-cytochrome c reductase activity of milk xanthine oxidase.[a]

Amount of globin or ferrimyoglobin	Ferricytochrome c reduction Δ A550/min
None	0.26
2.8×10^{-7}M (4.8 μg/ml) globin	0.26
2.8×10^{-6}M (48 μg/ml) globin	0.24
2.2×10^{-8}M (0.4 μg/ml) ferrimyoglobin	0.20
5.6×10^{-8}M (1 μg/ml) ferrimyoglobin	0.16
1.1×10^{-7}M (2 μg/ml) ferrimyoglobin	0.13
2.5×10^{-7}M (4.5 μg/ml) ferrimyoglobin	0.08

[a]Conditions: 10^{-5}M ferricytochrome c; 5×10^{-5}M xanthine; 10^{-5}M EDTA; 10^{-7}M xanthine oxidase; indicated amounts of globin and ferrimyoglobin.

Table 2 compares the relative reaction rates of the three hemoproteins as ΔA per minute at the wavelengths selected per total absorbance change. While ferricytochrome c is reduced at a rate only slightly less than that for uric acid production (and presumably O_2^- generation), the rates of ferrimyoglobin and ferrihemoglobin oxidation to the ferryl state are about

1/3 as rapid. It should be noted that the uric acid production rate was measured in the absence of hemoprotein and possibly could have been lower in their presence. However, either the xanthine oxidase molecules are generating O_2^- for at least two minutes after all xanthine is used up (i.e., after uric acid buildup ceases), which is unlikely, or another explanation

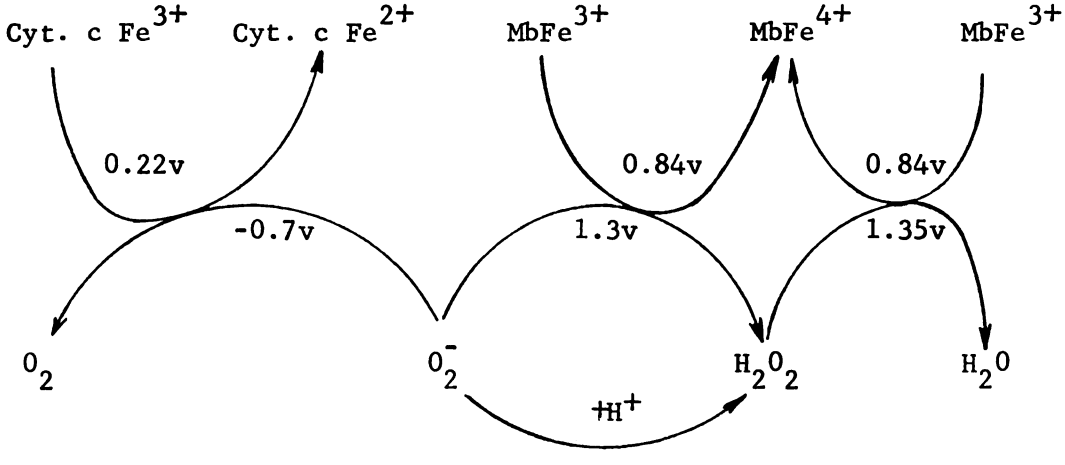
Table 2. Comparative reaction rates of ferricytochrome c, ferrimyoglobin, and ferrihemoglobin with the xanthine oxidase generated superoxide anion, and rate of production of uric acid from xanthine.[a]

Product monitored	ΔA/min.	Total ΔA	ΔA/min / total ΔA	Approx. time to complete reaction
Ferrocytochrome c	0.26 @ 550nm	0.26	1.0	1 min
Ferrylmyoglobin	0.10 @ 582nm	0.25	0.4	2.5-3 min
Ferrylhemoglobin	0.05 @ 578nm	0.13	0.38	2.5-3 min
Uric acid[b]	0.36 @ 290nm	0.32	1.13	1 min

[a]Conditions: 10^{-5}M ferrihemoprotein; 5 x 10^{-5}M xanthine; 10^{-5}M EDTA; 10^{-7}M xanthine oxidase.
[b]Based upon 1 unit of activity = 1 μ mole uric acid produced per min @ pH 7.8 and 25°C, this is equivalent to 0.2 units of activity per cuvette for all reactions, and a specific activity of approx 2.2 units per mg of xanthine oxidase.
Uric acid production was measured with no hemoprotein present.

for the lengths of time that the myoglobin and hemoglobin oxidations extend is needed, since the lifetime of O_2^- is in the millisecond range (Bray, 1970). The most likely explanation for the continuing oxidation of ferrimyoglobin and ferrihemoglobin is their redox reaction with H_2O_2. Under these conditions, the H_2O_2 is produced both by spontaneous dismutation of some of the O_2^- produced (Fridovich, 1970) and by reduction of O_2^- by that fraction of the hemoproteins present that interacts with O_2^- during approximately the first minute.

Perhaps the most important question raised by the experimental results is why ferrimyoglobin and ferrihemoglobin are further oxidized by the same system which reduces ferricytochrome c. The further oxidation results are in agreement with the finding reported by Kaplan-Bresler (1965) that, during the oxidation of reduced flavoprotein of pigeon heart mitochondria, ferrimyoglobin was oxidized to ferrylmyoglobin. She attributed this to H_2O_2 production since catalase inhibited the oxidation. This is supported by the more recent observations of Loschen et al. (1971). Muraoka et al. (1967) reported catalase inhibition of ferricytochrome c reduction by xanthine oxidase; however, McCord and Fridovich (1968) reported the absence of catalase inhibition of ferricytochrome c reduction, and they cited evidence against H_2O_2 involvement. Based upon the fact that ferricytochrome c, ferrimyoglobin and ferrihemoglobin all react with O_2^- it is reasonable to assume, however, that this is a general property of hemoproteins, and that catalase reacts with O_2^- as well. Since O_2^- can act as either a reductant, itself becoming oxidized to O_2, or an oxidant, itself becoming reduced to H_2O_2 (McCord and Fridovich, 1969), the following scheme is proposed to explain the results:



The spontaneous disproportionation of O_2^- is probably mainly of the form: $HO_2 + HO_2 \text{ -----> } H_2O_2 + O_2$ below pH 9 (Behar et al., 1970). The reduction potential (E_o') of 0.84v for the $MbFe^{3+} \longleftrightarrow MbFe^{4+}$ couple is according to George and Irvine (1955). Midpoint reduction potentials for the other reactions are taken from Wang (1970) and are in agreement with values elsewhere in the literature. Although O_2^- oxidized ferrimyoglobin and ferrihemoglobin under the conditions employed in this study, the O_2/O_2^- couple, with a midpoint reduction potential (E_o') of ca, - 0.7v, is potentially capable of also reducing these two hemoproteins, as it does ferricytochrome c. The question raised herein, whether or not the superoxide anion can under certain conditions (e.g., under anoxic irradiation) cause a reductive oxygenation of ferrimyoglobin and ferrihemoglobin remains to be answered, although extreme experimental difficulty may preclude an unequivocal answer.

If the iron of ferrimyoglobin inhibits ferricytochrome c reduction by itself engaging in a simple, bimolecular, one-equivalent redox reaction with O_2^-, as appears to be the case, then bound cupric ion such as that of hemocuprein ("superoxide dismutase") may do likewise rather than, or in addition to engaging in a disproportionation of O_2^-. It has been reported that the rate of reaction of free cupric ion with O_2^- ($Cu^{++} + O_2^- \text{ ---> } Cu^+ + O_2$) is about 25 times greater than that for ferric ion (George and Stratmann, 1952b; Barb et al., 1951). Further, the binding of cupric ion to certain proteins increases the electropositivity of the cupric ion, e.g.:

	Cu^{++}/Cu^+, $E_o' = 0.16$
hemocyanin	Cu^{++}/Cu^+, $E_o' = 0.54$

making the protein bound cupric ion potentially more reactive towards one-electron reductants. The rapid, direct reduction of the cupric ion of the Pseudomonas copper electron transport protein "azurin" by hydrated electrons in a deoxygenated system was recently reported (Faraggi and Pecht, 1971), as was the reduction of horse heart ferricytochrome c by cuprous ion of this same azurin (Greenwood et al., 1971). Therefore cupric ion of blue copper proteins can undergo one equivalent reduction by strong reducing agents such as the hydrated electron and perhaps O_2^-.

Thus the cupric ion of "superoxide dismutase" may undergo the same one-equivalent redox reaction with O_2^- as free cupric ion, but at an accelerated rate. In the case of superoxide dismutase, however, this mechanism would not account for the approximately equal rate and extent of H_2O_2 production both in the presence and absence of a catalytic amount of superoxide dismutase (McCord and Fridovich, 1969) unless the spontaneous dismutation rate was predominant in both cases (Fridovich, 1970) and the copper protein merely oxidized a small fraction of O_2^- produced which did not undergo spontaneous dismutation. Removal of O_2^- from a system by either spontaneous or catalyzed disproportionation to H_2O_2, and/or by a one-electron oxidation to O_2 all would account for competitive inhibition of ferricytochrome c reduction. It would seem that whereas cupric ion (Cu^{++}) is an absolute requirement for reaction of "superoxide dismutase" with O_2^- and that the bound cupric ion is undoubtedly the "active site", the bound cupric ion would more likely simply oxidize O_2^- to O_2, than additionally further reduce a second O_2^- to O_2^{2-}, unless the cuprous ion produced rids itself of the electron by reacting with a second O_2^- rather than by intramolecular electron transfer.

Autooxidation Mechanism Studies

Ferricytochrome c plus Oxymyoglobin and Oxyhemoglobin. When ferricytochrome c was incubated in anaerobic cuvettes either at 3°C or at 25°C with either "natural" oxymyoglobin (preparation B), or with chemically reduced oxymyoglobin or oxyhemoglobin the visible (450-650 nm) absorbance spectra in each case indicated a definite cytochrome c reduction roughly proportional with oxyhemoprotein autooxidation over time. Although the spectra of the two hemoproteins overlap somewhat, it is possible to monitor the increase in 522 nm and 550 nm peaks of reduced cytochrome c, and the increase in 635 nm absorption of ferrimyoglobin (and ferrihemoglobin) and decrease in the alpha peak (near 580 nm) of the oxyhemoprotein without difficulty. Whatever the mechanism of oxyhemoprotein autooxidation to the ferric state, and concomitant ferricytochrome c reduction to the ferrous state, it was clear from the spectra that both processes occurred simultaneously in the sealed cuvettes. That the two events were interconnected is indicated by the following observations. No reduction of ferricytochrome c occurred when it was similarly incubated in the absence of oxyhemoprotein although autoreduction of ferricytochrome c has been reported (Brady and Flatmark, 1971). Oxyhemoglobin and oxymyoglobin preparations that underwent autooxidation in the presence of ferricytochrome c, as indicated by the composite spectra, gave spectral indication of greater resistance to autooxidation when similarly incubated in the absence of ferricytochrome c. The results therefore strongly suggest that the oxidation of the oxyhemoproteins and the reduction of ferricytochrome c, when they were incubated together in the same anaerobic cuvette, were interconnected.

Regarding the mechanism of the apparent direct hemoprotein oxidation-reduction interaction, the fact that an excess of Tiron did not inhibit the process in the slightest strongly suggests that superoxide anion did not mediate the reduction of cytochrome c. It is worth noting that when Tiron was added to either oxyhemoprotein or deoxyferrous hemoprotein or ferricytochrome c solutions individually, the before and after spectra indicated no binding of Tiron to the heme iron. Tiron is a chelator of free ferric ion (forming a dark blue chromophore) and it can be assumed that if Tiron had complexed to the heme iron of the hemoproteins their spectra would have been modified as a result. Such was not the case; however, there were indications that the presence of Tiron enhanced somewhat the reduction of ferricytochrome c in the presence of oxyhemoprotein, more so at pH 8.5 than at pH 6.5. A slight but significant reduction of ferricytochrome c by Tiron at pH 8.5 was also observed by Miller (1970); however, Tiron was definitely found by him and others to be a potent inhibitor of ferricytochrome c reduction by the superoxide anion. If oxygen was in fact univalently reduced during oxyhemoprotein autooxidation under the conditions employed, it apparently was not the electron carrier for ferricytochrome c reduction, as it is in the case of the xanthine oxidase reaction, as well as in other superoxide-generating processes. On the contrary, the results point to a direct intermolecular electron transfer between the oxyhemoproteins and ferricytochrome c. If so, this may occur either before or after oxygen dissociates from the heme of oxymyoglobin and oxyhemoglobin. However, since the oxyhemoprotein preparations did not deoxygenate and oxidize to the same extent under similar conditions

in the absence of ferricytochrome c, the question arises why should they be any more prone to deoxygenation in the presence of ferricytochrome c, unless the latter somehow induces intermolecular electron transfer leaving the oxyhemoproteins no longer able to bind oxygen. That is, deoxygenation in the presence of ferricytochrome c may occur as a result of an induced intermolecular electron transfer by the latter. Further insight is contributed by the following results.

Ferricytochrome c plus Deoxyferrous Myoglobin and Hemoglobin. When a deoxygenated solution of ferricytochrome c was brought in contact with chemically reduced deoxyferrous myoglobin or hemoglobin in the anaerobic cuvettes, the spectra indicated an immediate partial reduction of cytochrome c, with partial oxidation of the deoxyhemoprotein to the ferric state occurring simultaneously. This was followed by an apparent gradual further reduction of cytochrome c with further oxidation of the deoxyferrous hemoprotein to the ferric state. Although one cannot completely rule out residual reducing agent (hydrosulfite or borohydride, or products therefrom) as the cause of cytochrome c reduction, the following observations suggest an intermolecular electron transfer between the deoxyferrous hemoprotein and ferricytochrome c. Neither deoxymyoglobin nor deoxyhemoglobin underwent oxidation to the ferric state when similarly incubated in the anaerobic cuvettes in the absence of ferricytochrome c; nor did the latter undergo reduction when similarly incubated in the absence of deoxyferrous hemoprotein. Further, as expected, ferricytochrome c did not undergo reduction when similarly incubated in the presence of either ferrimyoglobin or ferrihemoglobin, indicating that only the ferrous or reduced heme form

of myoglobin and hemoglobin can bring about ferricytochrome c reduction under the conditions employed. In this connection, it is of interest to note that ferrous ion, added as $FeSO_4$ to ferricytochrome c solution, reduces the cytochrome heme to the ferrous state (Taborsky, 1972). Metal ions, including ferrous ion, would not be expected to have been present in the rigorously deionized solutions employed in the studies discussed herein, however.

Assuming that there is a direct intermolecular electron transfer between oxyferrous hemoglobin or deoxyferrous hemoglobin, or the corresponding myoglobins, and ferricytochrome c as the foregoing observations suggest, this raises the question of the mechanism. The mechanism of electron transfer within and between hemoproteins, especially those of the mitochondrial electron transport chain, is not very well understood; the subject has drawn, and continues to draw considerable attention. Merely on the basis of midpoint reduction potentials, (E_o'), the data in Table 3 suggest that both hemoglobin and myoglobin should be able to reduce ferricytochrome c. Cytochrome b, which reduces cytochrome c_1, has about the same potential as myoglobin; and cytochrome c_1, which reduces cytochrome c, has a more positive potential than both myoglobin and hemoglobin. In this connection it is of interest that an apparent direct reduction of ferrihemoglobin by human erythrocyte cytochrome b_5 ($E_o' = 0.02$) has been reported (Hultquist and Passon, 1971). However, favorable redox potentials do not guarantee that a redox reaction will proceed at a significant rate.

Table 3. Midpoint reduction potentials of selected hemoproteins.

Hemoprotein couple	E_o' (volts)	Source of E_o' value
[a] Mb^{3+}/Mb^{2+}	0.046	Mahler & Cordes (1966), P.207
[b] $Cyt.b^{3+}/Cyt.b^{2+}$	0.05	Mahler & Cordes (1966), P.207
	0.038	Dutton et al. (1970)
[c] Hb^{3+}/Hb^{2+}	0.17	Mahler & Cordes (1966), P.207
[d] $Cyt.C_1^{3+}/Cyt.C_1^{2+}$	0.22	Mahler & Cordes (1966), P.207
	0.22	Dutton et al. (1970)
$Cyt.C^{3+}/Cyt.C^{2+}$	0.25	Mahler & Cordes (1966), P.594
	0.25-0.3	Dutton et al. (1970)

[a] Mb = Myoglobin
[b] Cyt.b reduces $Cyt.C_1$ in mammalian mitochondrial electron transport chain.
[c] Hb = Hemoglobin
[d] $Cyt.C_1$ reduces Cyt.C in mammalian mitochondrial electron transport chain.

In regard to the mechanism of electron transfer between hemoproteins, attention is drawn to the following since such a transfer appears to have taken place in the case under discussion. Proteins including hemoproteins, and more specifically hydrated proteins, fall in the class of biological semiconductors in that they are capable of intramolecular charge conduction and intra- intermolecular electron transfer. According to Winfield (1965b) it is generally agreed that the passage of electrons along the mitochondrial electron transport chain cannot take place by direct interaction between the cytochrome heme groups. Based upon his proposed mechanism of myoglobin autooxidation (Winfield, 1965a), he advances a theory of hemoprotein intra- and intermolecular electron transfer with free radical sites on

aromatic amino acid residues playing a prominent role in "electron hole" transfer to and from the outer periphery of one hemoprotein molecule, and intermolecular transfer to an adjacent molecule. Chance et al. (1968) place much greater emphasis on the heme group in their proposed mechanism of intermolecular electron transfer between hemoproteins; regarding soluble (not membrane bound) hemoproteins they suggest that the relative lack of structural constraints enables vibration and rotation of the molecules to provide intermolecular contact points so that electron transfer between hemoprotein molecules occurs on contact of loci immediately adjacent to the heme groups. This is supported by the view of Dickerson et al. that globular proteins such as cytochrome c are quite "elastic" and are easily deformed from minimum energy conformation by mild perturbation. They described cytochrome c as a classical "oil drop" type molecule with the heme enveloped in closely packed hydrophobic side chains and the outer surface of the molecule covered with charged groups to facilitate intermolecular interaction and electron transfer. This description would seem to agree with Winfield's proposed electron transfer mechanism, although it does not rule out the view of Chance et al. that contact is made in the vicinity of heme groups. In his theory of hemoprotein reactivity, Castro (1971) concludes that electron transfer within and between hemoproteins involves an "outer sphere" (of the heme ring) process. He expresses the view that strong π bonding axial ligands (low spin complexes) such as O_2, CO and CN^- render the heme less susceptible to oxidation by loss of an electron. Regarding the univalent reduction of oxygen to a free superoxide anion by the ferrous heme iron of oxymyoglobin and oxyhemoglobin

Castro presents a theoretical argument against the possibility. He states that electron transfer from a low valent transition metal (Fe^{2+}) to a high field or π bonding ligand does not occur. He argues that electron transfer to oxygen only ensues upon approach of a second metal ion or a proton, the latter enabling dissociation of the oxygen as a hydroperoxy radical. This would be consistent with the known sharp increase in autooxidation on lowering the pH of the oxyhemoprotein environment, although Castro does not rule out a pH induced conformational change of the globin as being a factor. The first part of his argument supports the view that an additional electron donor is required for two-electron reduction of liganded oxygen in order for autooxidation to take place unaided by either protonation of the oxygen or a conformational change. Regarding the cytochromes, and cytochrome c in particular, since its three dimensional structure is known in some detail, Castro proposes that the conformation of these hemoproteins, with a portion of the porphyrin ring exposed, allows for easy peripheral electron transfer from the heme group of one to that of another. He even suggests the possibility of direct π overlap of heme groups of adjacent cytochromes. Conformational constraints, he feels makes myoglobin and hemoglobin, especially when liganded to a "high field" ligand such as oxygen, much less able to participate in electron transfer via the periphery of the heme porphyrin ring.

To summarize this last section having to do with the interaction of oxy- and deoxymyoglobin and hemoglobin with ferricytochrome c, the experimental results strongly suggest a direct intermolecular electron transfer leading to oxidation of the oxy- and deoxy-hemoproteins as cytochrome c

is reduced. Some recent literature having to do with this possibility is included in the discussion of results. Although the results with Tiron appear to indicate that a freely diffusable superoxide anion is not one of the products of oxy-hemoprotein oxidation, the system employed to detect such an event may have influenced the results in this respect. That is, if ferricytochrome acted to induce autooxidation by engaging in direct intermolecular electron transfer, then the fate of the liganded oxygen would perhaps be different than if autooxidation proceeded in the absence of such an governing factor. The experimental results and the literature seem to indicate that autooxidation of oxyhemoproteins is a complex process, especially in a matrix as complex as meat, and that there is likely to be more than one "mechanism" depending upon what influencing environmental factors are present. Literature is cited which suggests that a simple one electron transfer to oxygen followed by dissociation of a superoxide anion from a now ferric heme iron probably does not occur. Rather, the liganded oxygen, with the aid of the globin moiety serves to protect the iron from oxidation, until such factors as protonation of the oxygen, globin conformational change, or donation of a second electron by an electron donor site or molecule upset the delicate balance and give rise to autooxidation. However, McCord et al. (1971) express the opposite view, that autooxidation of hemoglobin and myoglobin by oxygen belong to a group of processes all of which produce O_2^- via one-electron transfer to O_2. That oxyhemoprotein autooxidation can and does occur even in-vivo is evidenced by the facts that the mammalian erythrocyte contains an enzyme system which functions to maintain hemoglobin in

the oxygen binding ferrous state, and that absence of this enzyme gives rise to a serious clinical disorder called "methemoglobinemia" which is manifested by a build-up of ferrihemoglobin in the red cells (Hsieh and Jaffé, 1971; Niethammer and Huennekens, 1971). There is every likelihood that a similar system functions in the red muscle fibers to maintain myoglobin in the reduced state.

SUMMARY AND CONCLUSIONS

The following aspects of myoglobin and hemoglobin redox chemistry were studied: (1) The effect of ionizing radiation upon the oxidation state and the coordinated ligand of the heme iron of the oxy- and ferri-hemoproteins when they are gamma irradiated in the presence and absence of oxygen, (2) the interaction of the ferric hemoproteins with superoxide anions generated during the aerobic xanthine-xanthine oxidase reactions, and the competitive inhibition of ferricytochrome c reduction by this interaction, and (3) the immediate fate of oxygen during the autooxidation of myoglobin and hemoglobin by oxygen; more specifically, the questions of whether or not oxygen is univalently reduced to superoxide anion by the ferrous heme iron during autooxidation. Purified aqueous solutions of the hemoproteins were employed in the studies, and changes in oxidation state and in the coordinated ligand of the hemoproteins resulting from the application of irradiation and of superoxide anions (parts 1 and 2, respectively) were evaluated by absorption spectrophotometry. Spectral evidence was supplemented with the aid of chemical oxidizing and reducing agents, and carbon monoxide gas. To test for superoxide anion generation in part 3, the reduction of ferricytochrome c by the anion, and the inhibition of this reaction by Tiron were employed. Ferricytochrome c reduction and Tiron inhibition were monitored spectrophotometrically.

The following conclusions are drawn from the results of the studies:

Irradiation of ferrimyoglobin and ferrihemoglobin in the presence of oxygen produces the ferryl (+4) oxidation state of the hemoproteins via

reaction with radiolytically generated H_2O_2. That H_2O_2 and not O_2^- is the reacting species is demonstrated by the fact that irradiation of ferricytochrome c in the presence of oxygen produced no change in oxidation state. Superoxide anion would have reduced ferricytochrome c, whereas H_2O_2 does not change its oxidation state. Irradiation of ferrimyoglobin and ferrihemoglobin in the absence of oxygen results in a reductive oxygenation, thus producing the oxyhemoproteins. This is probably due to reduction by hydrated electrons followed by oxygenation with oxygen generated during free radical recombination reactions.

Superoxide anions generated by xanthine oxidase reduction of oxygen further oxidize the heme iron of ferrimyoglobin and ferrihemoglobin to the ferryl (+4) state. This reaction competitively inhibits ferricytochrome c reduction by O_2^-. Contrary to previous reports, the globin moiety of myoglobin causes no competitive inhibition of ferricytochrome c reduction by O_2^-. The heme group of myoglobin must be present for myoglobin to act as a competitive inhibitor. That O_2^- oxidizes ferrimyoglobin and ferrihemoglobin although it reduces ferricytochrome c can be explained by the fact that the superoxide anion can act as either an oxidant or a reductant, itself becoming reduced to H_2O_2 in the first case and oxidized to O_2 in the second.

When together in the same solution, ferricytochrome c underwent reduction concurrently with oxymyoglobin or oxyhemoglobin oxidation. Absence of Tiron inhibition of this ferricytochrome c reduction indicates that superoxide anion was not the immediate reductant. However, this does not prove that superoxide anion (or its conjugate acid the hydroperoxy radical)

is not generated during oxyhemoprotein autooxidation under favorable conditions because the spectral results in the present case were strongly indicative of a direct, intermolecular redox reaction (electron transfer) between ferricytochrome c and myoglobin or hemoglobin. Not only did ferricytochrome c undergo reduction as the oxyhemoproteins oxidized; it was also reduced in the presence of deoxyferrous myoglobin or deoxyferrous hemoglobin. Furthermore, the deoxyferrous hemoproteins oxidized concurrently with ferricytochrome c reduction when the latter was present in the same solution; however the deoxyferrous hemoproteins did not oxidize under the same conditions in the absence of ferricytochrome c. In addition, ferricytochrome c did not undergo reduction under the conditions employed unless one of the two ferrous forms (oxy- or deoxy-) of myoglobin or hemoglobin was present. Again, a direct intermolecular electron transfer is strongly indicated. Aside from its own intrinsic interest, ferricytochrome c - induced oxidation of the oxyhemoproteins would probably render cytochrome c reduction unsuitable as a system for superoxide anion detection.

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