An Hypothesis for the Mechanism of the Heme Catalyzed Lipid Oxidation in Animal Tissues[1,2,3]

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The catalytic activity of the heme compounds on lipid oxidation in muscle tissues has been reviewed. Evidence from experiments found in the literature, and unpublished studies in this laboratory on meats and fishery products, lead to the conclusion that the ferric heme compounds initiate lipid oxidation, while the ferrous heme compounds are inactive in the absence of preformed peroxides. The latter are decomposed by both types of heme compounds, but by a different mechanism.

A comprehensive mechanism of the different catalytic activities of the various heme compounds has been presented, based on both experimental evidence and theoretical considerations.

THE MECHANISM of the autoxidation of the unsaturated fatty acids was elucidated by Bolland (8) and Farmer (18,19). According to this mechanism, fat oxidation is initiated by the removal of hydrogen from a labile methylene group in the molecule of the unsaturated fatty acid. A free radical is thus formed to which oxygen is attached to form a peroxyl radical, which subsequently abstracts a hydrogen from a nearby labile site of another unsaturated fatty acid molecule to form a hydroperoxide and propagate the chain reaction. The hydroperoxides thus formed may decompose yielding free radicals which can initiate new reaction chains.

The catalysis of fat oxidation by heme compounds was first observed by Robinson (54).[a] Oxyhemoglobin, methemoglobin, and hemin were added to linseed oil and the oxygen consumption was followed manometrically. Robinson concluded that the catalytic activity should be attributed to the complexed iron and not to the oxygen released by oxyhemoglobin.

Wright, Conant, *et al.* (85) have studied the catalytic effect of potassium ferricyanide on oleic acid. Because of the production of ferrocyanide upon oxidation of the fatty acid, they concluded that the iron is reduced during the oxidation.

Barron and Lyman (7) added ferrihemichrome to linseed oil and oleic acid and they observed formation of ferrohemochrome upon oxidation of the substrates. They have postulated that the iron of the hemichrome is reduced during fat oxidation and it is reoxidized by atmospheric oxygen. They further suggested that the function of the catalyst is to facilitate the formation of initial centers for development of chain reactions.

The decomposition of organic hydroperoxides by iron or metal coordination compounds has been extensively studied by many researchers and has been reviewed by George (21), Leffler (37), Waters (78), Weiss (84), and others. The initial step of the discussed mechanism is the donation of an electron by iron II to the hydroperoxide. Iron is thus oxidized to the iron III state, to be later reduced to iron II by the decomposition products of the hydroperoxides. This agrees with Weiss' explanation of the catalytic activity of the iron-porphyrin enzyme catalase on hydrogen peroxide (82), as well as with the mechanism of the catalytic decomposition of hydrogen peroxide by different metals (83). The decomposition of peroxides may be also catalyzed by methemoglobin (17,23,24,34,35,51,52). In this case the electron donated to the peroxide is derived probably from the porphyrin ring. This mechanism will be discussed in detail later.

Maier and Tappel (43,44) have demonstrated that the decomposition of hydroperoxides from fatty acids is catalyzed by heme compounds. Tappel's postulated mechanism for heme compounds catalysis of fat oxidation is based entirely on peroxide decomposition (59–63,67,68). If this were the only mechanism involved, then heme compounds should not initiate fat oxidation in the absence of hydroperoxides. In the presence of peroxides, on the other hand, the ferrous hemes should catalyze as readily as the ferric hemes.

Younathan and Watts (76) have shown experimentally that the cooking of meats, which converts the ferrous pigments to ferrimyochromogens (66,71) sets off a rapid oxidation of tissue lipids upon storage even in the freezer. According to the same workers (88) nitric oxide myochromogen of cured meats is inactive as a catalyst of fat oxidation. Tarladgis *et al.* (69) have also shown that the ferromyochromogen of irradiated meats is much less active as a catalyst of fat oxidation than the metmyochromogen of cooked meats.

Extensive experimental evidence obtained with meat and fishery products (20) treated various ways and assayed by the peroxide and the 2-thiobarbituric acid test (70) give strong evidence that lipid oxidation

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[a] The following nomenclature for the heme compounds will be followed throughout this paper:

NAME	COMPOSITION
Heme	Iron-porphyrin coordination complex
Hemin, Hematin	Ferriheme (acidic and basic respectively)
Ferrohemochrome	Ferroheme with 2 nitrogen bases
Ferrihemichrome	Ferriheme with 2 nitrogen bases
Hemo(myo)globin	Ferroheme, native globin and 1 mol of water
Oxyhemo(myo)globin	Ferroheme, native globin and 1 mol of oxygen
Methemo(myo)globin	Ferriheme, native globin and 1 mol of water
Ferrohemo(myo)chromogen	Ferroheme, denatured globin, or one coordination bond with globin and the other with some nitrogen base
Ferrihemi(myo)chromogen	Ferriheme, denatured globin, or one coordination bond with globin and the other with some nitrogen base
Methemi(myo)chromogen	Ferriheme, denatured globin and 1 mol of water

is actually initiated by the ferric heme compounds of cooked tissues, as suggested by Younathan and Watts (87). Just after cooking of meat, both peroxide and TBA numbers are extremely low, if not nonexisting. Upon storage, however, rancidity increases very rapidly. This is better demonstrated with fishery products by the marked difference in the increase of oxidation in the cooked red muscle of fish and that of the white muscle (20).

When meat is cured by the addition of sodium nitrite, fat oxidation is not observed over a long period of time. Sodium nitrite is an oxidizing agent, oxidizing oxymyoglobin to metmyoglobin, while nitrite is reduced to nitric oxide. Yet although an oxidizing agent is added to the meat, the pigment is ultimately converted into the ferrous form upon cooking and no fat oxidation is observed (20,87). When imidazole is added to cooked meats, which converts the pigment from the ionic to the covalent form (71), again fat oxidation is not observed (81).

It becomes therefore apparent that the catalytic activity of the heme compounds on fat oxidation is closely related to the valence of iron in their coordination complexes. It is still not clear however whether the oxidized or reduced form of the iron in an iron-porphyrin coordination complex, or both, is the active catalytic form; whether either form, or both, can initiate fat oxidation or be active only in the presence of hydroperoxides and the reasons for such catalytic behavior.

Theoretical Considerations. The critical stages of the already discussed unsaturated fatty acid oxidation are the initial removal of an electron from a labile site of the molecule and the decomposition of the hydroperoxides, the products of which carry on the initiation of new reaction chains. According to the accepted mechanisms, the decomposition of the hydroperoxides requires either some form of energy, or the donation of an electron in order to take place. These are, therefore, the only two stages of the unsaturated fatty acids oxidation in which a catalyst could accelerate the chain reaction, by a reversible acceptance and donation of electrons to the fatty acid and the hydroperoxides.

The oxidation-reduction potentials of the heme compounds have been extensively studied by Barron (3–6), Conant (12,13), and others (31,39,73–75). These studies show very clearly that the coordinated iron of the various heme compounds can be reversibly oxidized and reduced. The heme compounds are, therefore, ideally suited for the role as catalysts of the unsaturated fatty acid oxidation. It still remains to be resolved whether the ferric or the ferrous form, or both, can initiate fat oxidation or accelerate the decomposition of hydroperoxides, as well as the reason of such catalytic activity.

When an electron is removed from the labile methylene group either between two double bonds of a highly unsaturated fatty acid or adjacent to the single double bond of oleic acid (47,57), a free radical is formed. The formation of a free radical involves the uncoupling of the spins of the two electrons which form the covalent bond between carbon and hydrogen. Thus a biradical is formed (38). In quantum mechanical terms, this is a transition between states of different multiplicity and it is a "forbidden" one, under ordinary circumstances (16,38). This quantum mechanical interdiction can be lifted however in the presence of a magnetic field (38,50). Hence such a field should accelerate any reaction involving change in multiplicity, in other words, the formation of a free radical (16,38).

Compounds creating an intense magnetic field are the paramagnetic compounds, due to the presence of their unpaired electrons (16,22,27,37,38,50,58,78). They are therefore effective catalysts for free radical formation as it has been demonstrated (38,78,84).

The Heme Compounds. The electronic configuration of the iron in the heme compounds has been elucidated by Pauling and co-workers (14,15,48,49,72). This is as follows:

	ORBITALS			
	3d	4s	4p	4d
Oxyhemo(myo)-globin	⇅ ⇅ ⇅ ⇅ ⇅	⇅	⇅ ⇅ ⇅	— —
Hemo(myo)globin	⇅ ↑ ↑ ↑ ↑	⇅	⇅ ⇅ ⇅	⇅ ⇅
Methemo(myo)-globin	↑ ↑ ↑ ↑ ↑	⇅	⇅ ⇅ ⇅	⇅ ⇅

Iron has 26 electrons occupying the different orbitals as follows:

Fe^{o} $1s^2$ $2s^2$ $2p^6$ $3s^2$ $3p^6$ $3d^6$ $4s^2$
Fe^{+2} $1s^2$ $2s^2$ $2p^6$ $3s^2$ $3p^6$ $3d^6$
Fe^{+3} $1s^2$ $2s^2$ $2p^6$ $3s^2$ $3p^6$ $3d^5$

In the above scheme the electrons occupying the inner 1s, 2s, 2p, 3s, and 3p orbitals of the d^2sp^3 or sp^3d^2 hybrid iron-porphyrin coordination complexes are omitted for simplification. The formation of the metal coordination complexes is discussed in detail by Bailar (2), Martell and Calvin (45), Moeller (46), and others, while the orbital picture is given by Coulson (16), Gould (27), Syrkin and Dyatkina (58), and many others.

As can be seen, the coordinated iron in oxyhemoglobin is diamagnetic. Such compounds have all their orbitals filled with electrons and therefore their spins are coupled mutually, cancelling out their magnetic fields. Oxymyoglobin would not be expected therefore, to initiate free radical formation and consequently fat oxidation. It can only occur when the oxygen occupying one of the ligands in the iron porphyrin coordination complex is released at low oxygen tensions. Then oxymyoglobin is oxidized to metmyoglobin, probably by the released oxygen (31), while fat oxidation is initiated by the paramagnetic oxygen molecule.

In methemoglobin the iron is electron deficient, in addition to being paramagnetic. Its five unpaired electrons create a strong magnetic field favoring initiation of a free radical formation and consequently fat oxidation. It is further thought that it can also accept the electron from the hydrogen it removes from the unsaturated fatty acid molecule, the ferric ion thus being reduced to the ferrous state. The remaining proton forms a hydronium ion with water.

After the formation of the free radical, fat oxidation proceeds by the chain reaction mechanism with the accumulation of hydroperoxides (8,18,19). The latter decompose when an electron is received from some electron donating compound according to the mechanisms mentioned before (21,37,78,82–84). This electron may be donated by a diamagnetic heme compound as follows:

$$H\text{–}\underset{|}{\overset{|}{C}}\text{–}OOH + FeII \longrightarrow H\text{–}\underset{|}{\overset{|}{C}}\text{–}O\text{–}:^{-} + \cdot OH + FeIII \qquad (1)$$

The hydroxyl radical formed can attack another hydroperoxide:

$$\text{H–C–OOH} + \cdot\text{OH} \rightarrow \begin{cases} \text{H–C–OO} \cdot + \text{H}_2\text{O} \\ \text{H–C}\cdot\text{–OOH} + \text{H}_2\text{O} \end{cases} \quad (2)$$

or another labile methylene group:

$$\text{H–C–H} + \cdot\text{OH} \longrightarrow \text{H–C}\cdot + \text{H}_2\text{O} \quad (3)$$

The radicals formed from reactions (1) and (2) can further reduce iron III to iron II:

$$\text{H–C–O}:^- + \text{FeIII} \longrightarrow \text{H–C–O} \cdot + \text{FeII} \quad (4)$$

or interact with a methylene group:

$$\text{H–C–OO} \cdot + \text{H–C–H} \longrightarrow \text{H–C–OOH} + \text{H–C}\cdot \quad (5)$$

or decompose to a carbonyl and another hydroxyl radical:

$$\text{H–C–OOH} \longrightarrow \text{C}{=}\text{O} + \cdot\text{OH} \quad (6)$$

Iron III can also be reduced by initiating another chain:

$$\text{H–C–H} + \text{FeIII} \longrightarrow \text{H–C}\cdot + \text{FeII} \quad (7)$$

According to this mechanism, originally proposed by Waters (78) and modified by the author, iron II is oxidized by the hydroperoxides to iron III, from which state it can be reduced again either by the decomposition products of the hydroperoxides or, in line with its paramagnetic properties, by attacking another labile site on a fatty acid and removing an electron.

Keilin and Hartree (34,35) have shown that the decomposition of hydroperoxides can be accomplished by methemoglobin also. They have noticed however that upon mixing the reactants a red color appears, which they attributed to a reduction of the iron, after which the reaction proceeds rapidly. George and Irvine (22–24) and Polonovski *et al.* (51,52) have postulated that this reduction of the iron proceeds through the formation of a complex between methemoglobin and hydrogen peroxide or hydroperoxides and an intermediate formation of iron IV, which is in turn reduced to the iron II state by a two electron process.

The formation of this higher oxidation state of iron has been achieved electrolytically only and at a very high pH and not by such mild oxidizing agents as hydrogen peroxide and hydroperoxides (36). It is extremely unstable and by a slight drop of pH oxygen is immediately liberated (36). Such an oxidation state of the iron therefore would not be expected in animal tissues, as Tappel has suggested (63).

George and Irvine favor the iron IV scheme over the possibility of the removal of an electron from the porphyrin ring, although their evidence for iron IV formation is purely qualitative (23). Recent electron paramagnetic resonance studies gave conclusive evidence that the electron is removed from the porphyrin ring rather than the iron, when hydrogen peroxide reacts with methemoglobin, porphin, and hemin (25).

The mechanism of the hydroperoxide decomposition is now better understood. The electron donated to the hydroperoxides is removed from the π electron cloud of the porphyrin ring, which can be excited more easily than an electron from iron III (33). This electron starts the decomposition of the hydroperoxides and iron is reduced to the iron II state by their decomposition products, thus explaining the red color noted by Keilin and Hartree (35) and George and Irvine (23).

Gouterman (28), Longuet-Higgins *et al.* (41), and Seely (56) have used the Linear Combination of Atomic Orbitals method to calculate the Molecular Orbitals (LCAO-MO) of several porphins and porphyrins. Their results show certain areas of the molecule with high electron density and others with low. The areas with high electron density are localized around the nitrogens of the pyrrole rings, which, therefore, are susceptible to attack from electrophyllic reagents, while the areas with the low electron density are around the carbon atoms of the methene bridges. This part of the molecule is more susceptible to attack by nucleophyllic reagents.

It can be therefore hypothesized that when hydrogen peroxide or hydroperoxides are added to methemoglobin, a π electron is removed from the molecular orbital of the porphyrin ring. This leaves an unpaired electron behind, which can be detected by electron paramagnetic resonance (24). Now the electron cloud of this molecular orbital has a positive charge and therefore repulsion forces are created between it and the iron ion of the ring, which has a partial positive charge. In the mean time, the donation of this electron has decomposed the hydroperoxide. Its decomposition products include such nucleophyllic species as $\cdot$OH, $\cdot O_2H$, etc. (29,84). These could attack the carbon atom of the methene bridge and opening at this point results (42), which facilitates the dissociation of the iron from the already unstable coordination complex. Appearance of green pigments, attributed to billirubin and billiverdin (39) is thus explained.

Tappel has postulated that the decomposition of hydroperoxides is catalyzed by the heme compounds, through the intermediate formation of a complex (59–63, 67, 68). This complex is formed between the $\diagup$–Fe–OH group of the "hematins" and the –C–OOH $\diagdown$ group of the hydroperoxide.

The only heme compounds known with an –OH group attached to the iron through the $3d_z2$ coordination bond is the alkaline methemoglobin and the hematin. The 3d orbital of the ferric ion in these compounds is occupied by 3 unpaired electrons (2,14, 15,27,45,46,48,49,50,58). It has been clearly demonstrated that hematin is not the only heme compound catalyzing the oxidation of the unsaturated fatty acids (7,20,43,44,54,59–63,67,68,71,76,85,87,88). The electronic configuration and structure of all heme compounds is equally clear, as already discussed. Catalysis of fat oxidation by them, through the postulated complex formation implies replacement of the various ligands occupying the $3d_z2$ coordination bond with the iron in the different heme compounds by the –OH group. which does not seem possible at the pH of the meat and in absence of alkali.

Formation of unstable intermediate complexes with metal chelate compounds could be achieved through the use of a higher energy orbital of the coordinated

metal. In the case of the heme compounds however this has not been demonstrated.

Acceptance of an electron by any other orbital of the iron of the heme compounds besides the 3d orbital, implies the use of a higher energy 5s or 4d orbital. The 5s orbital can not be used, being spherical, non-directional, and its approach being hindered by the other directional orbitals of the atom (16).

The use of a 4d orbital requires a large amount of energy which will not be compensated by the formation of a stronger bond (16,45). Furthermore, if an electron is accepted by either one of these two orbitals, the very stable octahedral coordination complex has to be altered into one of the following unstable configurations, in order to accommodate this extra electron (2,45,46):

a) Triagonal prism, with hybrid states d^4sp^2, d^4p^3, d^5p^2 or
b) Octahedron, with hybrid states d^5sp, d^3sp^3 or
c) Pentagonal bipyramid, with hybrid states sp^3d^3.

Existence or synthesis of the above iron-porphyrin coordination complexes has never been demonstrated (2,27,28,45,46).

Furthermore, formation of an unstable heme compound coordination complex with a coordination number of 7 has been suggested by Brdicka (9) and Clark (11), but has not been confirmed (2,27,28,32, 40,45,46,53,86).

The iron in hemoglobin is paramagnetic but since it is in the reduced form it cannot accept an electron, as explained above. Therefore it would not be expected to initiate a fast fat oxidation in absence of preformed hydroperoxides. Furthermore hemoglobin or myoglobin exists in tissues at very low oxygen tensions (1,26,79). When oxygen becomes available these compounds rearrange to diamagnetic oxy-compounds or they are oxidized to the respective ferric forms. They are expected however to donate an electron for the decomposition of hydroperoxides as above.

Denatured Heme Compounds. Barron (3–6) and others (31,39,73–75) have shown that the oxidation-reduction potential of the coupled hemo-hemi-chromogen system is higher than that of the respective oxy-methemoglobin coupled system. Therefore the denatured heme compounds are even better oxidation-reduction catalysts than the respective native hemes.

Tappel (66) has identified the heme compounds of cooked meats as a mixed denatured globin-nicotinamide hemichrome. Such a compound would be expected to have only a single unpaired electron in the 3d orbital of its coordinated iron, being analogous to the ferrihemichromogen of which the magnetic susceptibility has been measured by Pauling (48,49). Although the magnetic properties of cooked meat pigment have not been measured, spectroscopic evidence to be discussed in detail in a later publication (71) leads to the conclusion that this compound is in fact an ionic, outer, paramagnetic, ferric-porphyrin coordination complex, analogous to methemoglobin and having 4–5 unpaired electrons.

The pigments of cured meats are generally considered to be ferrohemochromogens (64,80). Recent spectroscopic studies identify the pigment of cured meats as an inner, covalent, diamagnetic, ferrous-porphyrin coordination complex (71). Irradiation also brings about at least partial reduction of the ferric pigment of the cooked meats to ferrohemochromogens (65). The pigments of the cured and irradiated meats are not expected therefore to initiate fat oxidation in the absence of preformed peroxides.

In cooked meats the catalytic process may be assumed to take place through a reversible exchange of electrons between the iron of the heme compounds and the lipids or hydroperoxides. Donation or acceptance of electrons does not take place through abnormal intermediates (9,11,23,24,51,52,61,63), but through oriented water molecule bridges (32,40,53) or diffusion through the coordination ligands (86). Eventual ring destruction (30) and dissociation of the iron from the coordination complex follows electrophyllic and nucleophyllic attacks on the π electron cloud of the porphyrin ring system, at the places of higher and lower electron density, respectively (28,41, 56). Evolution of carbon monoxide derived from opening at the methene bridge carbon, upon oxidation of hemochromogens, has been demonstrated (42).

As shown by experimental evidence, lipid oxidation is considerably delayed in cured meats (79,80,87). When lipid oxidation eventually does begin, it is accompanied by pigment fading and destruction (30). Pigment changes may be attributed to oxidation of iron II of the nitric oxide myochrome to the iron III state and by the donation of an electron for the decomposition of preformed peroxides, while nitric oxide dissociates from the complex. As decomposition products of the hydroperoxides accumulate, attacks on the ring result in the removal of a π electron and opening at the methene bridge, with subsequent appearance of green or gray colors.

Pigment fading of cured meats may also precede lipid oxidation. Walsh and Rose (77) have shown that fading of nitric oxide hemoglobin does not go through hemoglobin, but directly to methemoglobin. As a result to this effect, lipid oxidation is then initiated as previously described by the metmyochromogen to which nitric oxide ferromyochrome is converted by the absorption of light (71).

It is therefore to be expected that treatment of meats with antioxidants, in order to inhibit lipid oxidation, will affect their color indirectly. On the other hand, color changes induced by light, curing, cooking, and radiation will directly affect lipid oxidation.

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The Determination of Residual Alcohol in Defatted Alcohol Washed Soybean Flakes

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A method is described for determination of residual alcohol in soybean flakes washed with aqueous alcohol and desolventized to prepare a high-protein food supplement. The amount of alcohol removed from the flakes by extraction with acetone is determined quantitatively by esterification with phthalic anhydride. An excess of reagent is required since water present in the sample causes a secondary reaction which consumes phthalic anhydride. This analysis has been applied to flakes containing from 0.2 to 10% of methanol, ethanol, or isopropanol. Accuracy of the method was established by adding known amounts of the three alcohols to soybean flakes. A relative error of less than 1% was obtained with a standard derivation of 0.05%.

DESOLVENTIZING of hexane-extracted soybean flakes washed with aqueous alcohols is under study for the production of a high-protein food supplement (1).

An analytical method was developed to determine the residual alcohol content of these flakes after desolventization.

A method of determining the alcohol content indirectly has been used at this laboratory with alcohol-washed soybean flakes containing large amounts of both alcohol and water. In this procedure total volatiles are determined by drying the sample to constant weight, then obtaining the water content by the Karl Fischer method (2), and substracting the two values to find the alcoholic content of the flakes. The difference of the results indicates the alcohol content of the flakes. The method proved to be inaccurate for flakes containing 2% alcohol or less, but can be used for estimating the alcohol content.

The alcohol determination as used by the distilling industry was also explored. By this method the alcohol is distilled from the fermented mashes as the water azeotrope. From the specific gravity of the distillate the alcohol content is calculated. This method also

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