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Catalytic "Free" Iron Ions in Muscle Foods

Joseph Kanner,* Bella Hazan, and Linda Doll

Catalytic "free" iron was determined in muscle foods by the bleomycin method. The results demonstrated that turkey and chicken muscle contain significant amounts of free catalytic iron. Base propenal derived from DNA during the incubation of bleomycin with muscle water extracts was inhibited by the iron chelator desferrioxamine. The amounts of free iron in turkey and chicken dark muscles were found to be 2.5 and 0.5 $\mu g/g$, and those of light muscles, 0.9 and 0.2 $\mu g/g$, respectively. The amount of free iron in turkey and chicken dark muscle correlates well with the tendency of this muscle tissue to undergo lipid peroxidation. Turkey and chicken dark and white muscle stored at 4 °C for 1 week releases free iron ions, the concentration of which increases by 2–3-fold over fresh samples. The implication of free iron in the quality of muscle foods is discussed.

Iron is an important catalyst in biological systems. Transition metals, e.g. iron and copper, with their labile d-electron system, are well suited to catalyze redox reactions. The stable oxidation states of iron in aqueous systems are exclusively the ferric (Fe^{3+}) and ferrous (Fe^{2+}) forms (Spiro and Saltman, 1969). Free iron, especially ferric ions, is insoluble in water above pH 3.0, and therefore in order to participate in aqueous-phase reactions they must be chelated in some form of soluble ligand complex, such as EDTA, ascorbate, or ADP. The redox chemistry of iron is itself directly affected by the coordinating ligands (Richter and Waddel, 1983).

The two oxidation states of iron allow it to act as an electron donor and acceptor, which give it important catalytic properties. In the presence of oxygen, ferrous ions produce the superoxide anion radical which dismutates to H_2O_2. Ferrous ions further reduce H_2O_2 to hydroxyl radicals (Cohen and Sinet, 1980). The hydroxyl radical ($HO^{\bullet}$) can attack and oxidize any bioorganic compounds present in live cells. Recently, we demonstrated that hydroxyl radicals are generated during the iron redox cycle by ascorbic acid; however, it seems that most of the membrane lipid peroxidation was stimulated by breakdown of hydroperoxides during interaction with the reduced metal (Kanner et al., 1986).

Ferrous ion can stimulate polyunsaturated fatty acid (LH) peroxidation by decomposing preformed lipid peroxides (LOOH) to the alkoxyl radical ($LO^{\bullet}$), which will attack other lipid molecules.

In muscle foods, e.g. meat, poultry and fish, iron accumulates in myoglobin, hemoglobin, ferritin, and transferrin. A small pool of non-protein-bound iron moving among transferrin, cell cytoplasma, and ferritin could provide

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"free" iron ions at micromolar concentrations. These iron ions are removable by chelators such as bleomycin, phenanthroline, or EDTA (Gutteridge, 1984).

Various workers (Igene et al., 1979; Chen et al., 1984) have presented data suggesting that non-heme iron is the major catalyst accelerating lipid peroxidation in cooked meat. Most recently we found that free metal ions seem to be the main catalyst of lipid peroxidation in turkey muscle tissues (Kanner et al., 1986). Non-heme iron content of meat was determined by several researchers using methods of acid digestion (Schrinker and Miller, 1983), chelation by EDTA at a low pH, and separation from proteins by precipitation with trichloroacetic acid (Igene et al., 1979) or acetone (Chen, 1982). This iron could be chelated in a form preventing its catalytic effect. Recently, Gutteridge et al. (1981) developed a method for detection of catalytic free iron ions in biological systems by using bleomycin-dependent degradation of DNA. Bleomycin is the name given to a mixture of structurally similar antitumor antibiotics. Bleomycin binds a number of metal ions, but only its complex with free iron ions is capable of releasing TBA-reactive compounds from DNA (Dabrowiak, 1982; Gutteridge et al., 1981). During DNA degradation, the deoxyribose sugar is cleaved to give base propenals that form a colored adduct with thiobarbituric acid under acidic conditions (Giloni et al., 1981). Once the bleomycin–iron complex has bound to the DNA, the degradation cannot be prevented by antioxidants and so the assay can be applied directly to body fluids (Gutteridge and Shute, 1981). The degradation of DNA by bleomycin is absolutely dependent on the presence of ferrous ions (Sansville et al., 1976). The assay conditions have been carefully designed to prevent any interference from iron proteins, such as hemeproteins, transferrin, or ferritin. The method has been used to determine the increase of free iron in the synovia of rheumatoid patients (Rowley et al., 1984) and in cerebrospinal fluids.

We conducted a study to determine catalytic free iron ions in muscle foods by the bleomycin method.

MATERIALS AND METHODS

Bleomycin sulfate, DNA (type 1), magnesium chloride, ferrous ammonium sulfate, and 2-thiobarbituric acid (TBA) were purchased from Sigma Chemical Co. (St. Louis, MO); EDTA was from BDH Chemicals Ltd. (Poole, Dorset, U.K.); ascorbic acid, hydrochloric acid, and sodium hydroxide were from Merck (Darmstadt, West Germany); pyrogen-free water was from Travenol (Ashdod, Israel); and Chelex-100 was from Bio-Rad Laboratories (St. Albans, Herts, U.K.). Desferrioxamine methanesulfonate (desferal) was obtained from CIBA-Geigy A.G. (Basel, Switzerland). All other reagents were of the highest quality available.

Free iron was determined by the bleomycin method using pyrogen-free water treated with Chelex-100 to remove as much contaminating iron as possible (Gutteridge et al., 1981). Ascorbic acid, bleomycin, $MgCl_2$, and DNA were treated also with Chelex-100 before being used for iron detection.

The reaction mixture (1 mL) incubated in new plastic metal ion free tubes contained the following reagents: 0.5 mL of DNA (1 mg/mL), 0.05 mL of bleomycin sulfate (1 mg/mL), 0.1 mL of $MgCl_2$ (50 mM), 0.1 mL of sample, 0.1 mL of pyrogen-free water, 0.1 mL of ascorbic acid (8.8 mM), and HCl (10 mM) to pH 7.4.

Controls were set up with pyrogen-free water instead of the sample. The tubes were mixed before and after ascorbate addition and incubated at 37 °C for 2 h with shaking. The reaction was stopped by 0.1 mL of EDTA (0.1 M), and the contents were transferred to glass tubes and then mixed with 1 mL of 1% (w/v) thiobarbituric acid (in 50 mM NaOH) and 1 mL of HCl (25% v/v). Solutions were heated in boiling water for 15 min and cooled, and the chromogen was measured by its absorbance at 532 nm. A standard curve was prepared against $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ dissolved in Chelex-treated pyrogen-free water. The optimum pH for the reaction is 7.3. Samples were prepared with muscle strips chopped with a plastic knife and incubated in pyrogen iron free water.

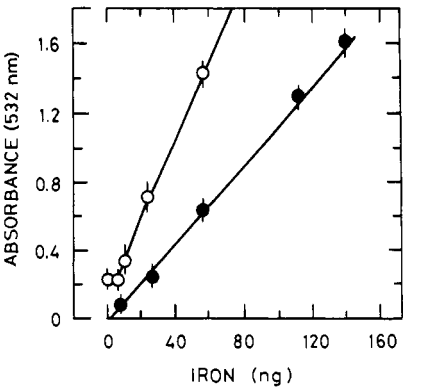

Figure 1. Standard curve for catalytic free iron detected by the bleomycin method: ●, pyrogen-free Chelex-100-treated water; ○, pyrogen-free Chelex-100 water extracts from turkey muscle.

Fresh (immediately after slaughtering) dark and white turkey and chicken muscles were stored in open polyethylene bags at 4 °C. Lipid peroxidation was determined periodically by the TBA-RS method developed by Witte et al. (1970) and calculated as malonodialdehyde (MDA) with an extinction coefficient of $\epsilon_{532} = 1.55 \times 10^5\ M^{-1}\ cm^{-1}$ (Buege and Aust, 1978).

Results are the means of triplicates; each error bar (I) in the figures denotes the standard deviation.

RESULTS AND DISCUSSION

Catalytic free iron was determined in muscle foods by the bleomycin method (Gutteridge et al., 1981). The standard curve for iron ions in pyrogen-free water treated with Chelex-100 shows data similar to those of Gutteridge et al. (1981). Recently, it was shown (Burger et al., 1985) that phosphate derivatives enhance the extent of DNA degradation by iron(II) bleomycin. As muscle food contains phosphate compounds, we made the standard curve for iron ions in muscle extracts with free iron treated water (Figure 1).

Several researchers (Sato and Hegarty, 1971; Love and Pearson, 1974; Igene et al., 1979) studied the role of heme and non-heme iron in the catalysis of muscle lipid peroxidation using a model system containing water-extracted muscle residue and cutback dialysate or diffusate fractions. Both researchers speculated that metal ions play a significant role in the catalysis of muscle lipid peroxidation; however, no one has shown by a direct and specific method the catalytic effect of the iron compounds extracted from muscle tissues. Bleomycin binds free iron, and only this complex is capable of degrading DNA to give base propenals (Dabrowiak, 1982; Giloni et al., 1981). This method is now recommended to determine catalytic free iron in biological fluids (Gutteridge and Halliwell, 1985).

Our study demonstrated that turkey and chicken muscle contain free iron that could act as a catalyst in oxidative reactions (Table I). The degradation of DNA to base proposals was higher in water extracts of dark turkey muscles than of white muscles. DNA decomposition by bleomycin–water muscle extracts was inhibited by des-

Table I. Catalytic "Free" Iron in Muscle Food

species	iron, μg/g tissue ± SD	
	dark muscle	light muscle
chicken	0.55 ± 0.07	0.24 ± 0.09
turkey	2.50 ± 0.14	0.92 ± 0.16

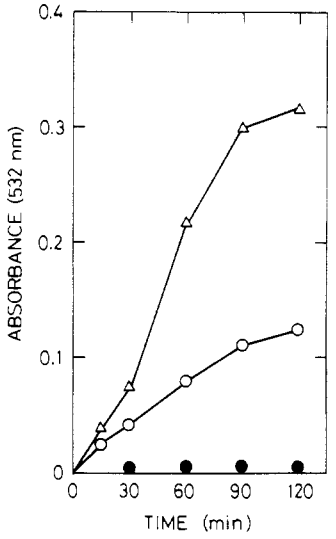

Figure 2. Base propenals derived during the incubation of DNA with bleomycin in muscle tissue extracts: ○, water extracts from light turkey muscle; △, water extracts from dark turkey muscle; ●, water extracts from dark turkey muscles without DNA or bleomycin or in the presence of desferrioxamine added before other reagents.

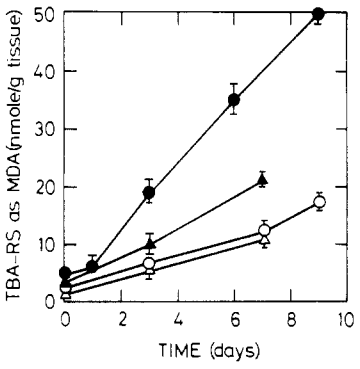

Figure 3. Lipid peroxidation in situ of muscle tissues stored at 4 °C: △, chicken light muscle; ○, turkey light muscle; ▲, chicken dark muscle; ●, turkey dark muscle.

ferrioxamine, a known specific chelator of iron ions (Figure 2). The amount of free iron in dark muscles was higher than that in white muscles (Table I) and correlates well with the greater instability of dark turkey and chicken muscle toward lipid peroxidation (Figure 3).

The results show also that there is more free iron in turkey muscles than in chicken muscles (Table I). This finding fits well with the relatively higher stability of chicken muscles to lipid peroxidation (Figure 3) (Wilson et al., 1976). Compared with the non-heme iron found in beef, the amount of free iron in dark turkey muscle is higher than that found by Igene et al. (1979) or Schrinker and Miller (1983) and lower than that reported by Sklan et al. (1983) for turkey muscles.

During storage of turkey dark muscle at 4 °C, the amount of free iron ions increases (Figure 4). Similar increases in free iron ions were found with chicken dark and light muscle stored at 4 °C (results not shown). The increase in free iron ions during storage is attributed to biochemical changes that develop in muscle postmortem.

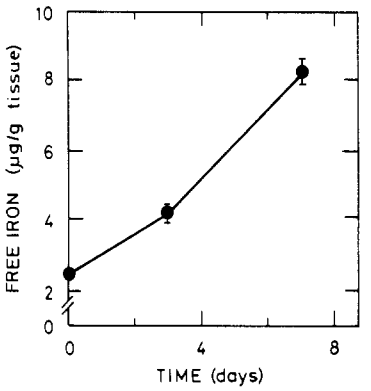

Figure 4. Catalytic free iron changes during storage of turkey dark muscle at 4 °C.

Perhaps the most significant event that occurs with the death of the animal is cessation of blood circulation. This cuts off the supply of oxygen to the muscle, which becomes anaerobic. The anaerobic condition in the muscle affects the metabolism of lactic acid, which accumulates and decreases the pH (Hultin, 1977). More recently, it was found that in cultured hepatocytes perturbation of the cell pH and hypoxia affect the release of iron from lysosomal ferritin due to conjugate action of an acidic pH and activation of hydrolytic enzymes (Starke et al., 1985). As hepatocytes and muscle cells (results not shown) contain ferritin inserted in lysosomes, these data could explain, in part, our findings concerning the release of iron during postmortem storage. It may be possible also that iron is released from heme proteins in the presence of H_2O_2 (Gutteridge, 1986), which was found to be generated during postmortem muscle storage (Harel and Kanner, 1985).

Free iron and other transition-metal ions could affect the quality of muscle foods by changing the stability of the lipid fraction by three main reactions: (a) The free metals interact with reducing agents, such as cysteine, glutathione, ascorbate, and α-tocopherol, oxidize them, and thereby reduce the antioxidative tone of the tissue. (b) During the interaction with reducing agents, the metals activate oxygen to active-oxygen species such as the hydroxyl radical. (c) Metals catalyze the breakdown of hydroperoxides to free radicals, which could initiate a chain reaction of lipid peroxidation.

More work needs to be done to identify the endogenous source of the free iron ion in muscle foods.

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Antioxidant Activity of Ceruloplasmin in Muscle Membrane and in Situ Lipid Peroxidation

Joseph Kanner,* Frida Sofer, Stella Harel, and Linda Doll

Ceruloplasmin acts as ferroxidase, catalyzing the oxidation of ferrous ions to the ferric state and reducing oxygen to water. Membranal lipid peroxidation initiated by an enzymic ADP–iron or by nonenzymic iron redox cycle systems was inhibited by ceruloplasmin. However, membranal lipid peroxidation initiated by H_2O_2-activated MetMb is not inhibited by ceruloplasmin. In the presence of iron ascorbate, ceruloplasmin inhibited lipid peroxidation initiated by H_2O_2-activated MetMb at a concentration one-tenth of that which inhibits iron ascorbate membrane lipid peroxidation. This synergistic effect seems to be derived from the possible hydroperoxidase activity of MetMb in the presence of ascorbic acid and ceruloplasmin. In situ minced turkey muscle lipid peroxidation was inhibited by ceruloplasmin, which indicates that most of this peroxidation is catalyzed by free iron ions.

Ceruloplasmin (EC 1.16.3.1) is the major copper-containing protein of extracellular fluids. It has a molecular weight of approximately 134 000 with six or seven copper ions per molecule. Three biological functions are known for ceruloplasmin: (i) copper storage and supply within cells for incorporation into other copper proteins such as superoxide dismutase (SOD) or cytochrome oxidase (Marceau and Aspin, 1973a,b); (ii) maintenance of copper homeostasis in the tissues; (iii) in vivo ferroxidase enzyme, first proposed by Osaki et al. (1966). The enzyme catalyzed the oxidation of ferrous ions in the presence of oxygen to ferric ions and water by eq 1 and 2.

$$E\text{–}Cu^{2+} + 4Fe^{2+} \rightarrow E\text{–}Cu^{+} + 4Fe^{3+} \quad (1)$$

$$E\text{–}Cu^{+} + O_2 \xrightarrow{+4H^+} E\text{–}Cu^{2+} + 2H_2O \quad (2)$$

Human blood serum has been known for several years to be a powerful lipid inhibitor (Barber, 1961; Vidlakova et al., 1972). This was shown later to be dependent on ceruloplasmin activity.

The aim of this study was to identify the antioxidant effect of ceruloplasmin in three different radical-generating systems and in situ minced muscles of turkey.

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MATERIALS AND METHODS

Materials. Hydrogen peroxide (30% for synthesis), ascorbic acid, and trichloroacetic acid were purchased from Merck (Darmstadt, FGR). Myoglobin type I from equine skeletal muscle, potassium chloride, L-histidine-free base, thiobarbituric acid (TBA), bovine serum albumin (BSA), and β-nicotinamide adenine dinucleotide disodium salt reduced from type X (NADPH), and ceruloplasmin type VII, bovine (CP), were obtained from Sigma Chemical Co. (St. Louis, MO). Ferric chloride was purchased from BDH Chemicals Ltd. (Poole, England), and adenosine 5′-diphosphate salt (ADP), from Boehringer-Mannheim GmbH (Mannheim, FGR).

Methods. Isolation of the microsomal fraction from fresh turkey muscle tissue was done by a procedure described previously (Apgar and Hultin, 1982; Kanner and Harel, 1985). The enzymatic microsomal model system contained KCl (0.12 M), NADPH (0.4 mM), ADP (0.4 mM), $FeCl_3$ (0.01 mM), histidine (5 mM), and microsomes (0.5 mg of protein/mL at pH 7.3). Microsomal nonenzymic lipid peroxidation initiated by the iron redox cycle system contained ascorbic acid (0.2 mM), $FeCl_3$ (0.01 mM), acetate buffer (50 mM at pH 7.0), and microsomes (1.0 mg/mL of protein). Microsomal nonenzymic lipid peroxidation initiated by activated metmyoglobin contained MetMb (0.03 mM) and H_2O_2 (0.03 mM), preincubated for 3 min, microsomes (1 mg of protein/mL), and acetate buffer (50

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