BIOCHEMICAL BASIS OF FRESH MEAT COLOUR

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Abstract

This article highlights the importance of the chemistry of myoglobin and the principal biochemical mechanisms which regulate the meat colour stability rapidly after slaughter or during storage. It will be first discussed if the differences in discoloration rate are more influenced by biochemical factors such as oxygen consumption and myoglobin autoxidation rate or by the metmyoglobin reducing enzyme systems (reducing capacity). Secondly, the relationships between lipid and myoglobin oxidation, with the production of reactive oxygen species and their importance in colour regulation, will be highlighted.

Introduction

For the meat industry and the consumer, meat colour is a very important quality attribute. The colour of meat depends of many factors such as concentration of haeminic pigments, and particularly of myoglobin, the physical characteristics of the meat and the chemical state of these pigments. Reduced (or deoxy) ferrous myoglobin (Mb) is the purple pigment of deep muscle and of meat surface under vacuum. On exposure to air, myoglobin combines with oxygen to form bright red ferrous oxymyoglobin (MbO_2) which is thought to indicate freshness and considered attractive by the consumer. With time, contact of myoglobin with oxygen also leads to the formation

of the oxidized form, ferric metmyoglobin (MetMb), which is brown and unattractive. It is well established that beef muscles exhibit a wide range of colour stabilities when stored in various air containing atmospheres (Renerre and Labadie, 1993). Rapidly post-slaughter, and during storage, the rate of metmyoglobin accumulation on the surface of meat is governed by many intrinsic factors (pH, muscle metabolic type, animal, age, breed, sex, diet, etc....), extrinsic ones : temperature, oxygen availability, type of lighting, surface microbial growth, storage mode (air, modified atmospheres, vacuum) or by a combination of many factors (Renerre, 1990). The mechanisms involved have been not yet well established neither completely understood (Faustman and Cassens, 1990a,b; Renerre, 1990; McMillin, 1996).

Myoglobin oxidation-reduction mechanisms

Myoglobin, which is located in muscle, provides a reserve supply of oxygen and facilitates its movement within muscle. Myoglobin is the most important of haem proteins for meat colour. Myoglobin is a monomeric, globular haem protein with a molecular weight of approximately 17000 and is formed by 140 to 160 amino-acid residues and a heme group in a crevice of the molecule. It is reported in the literature that in a well bled carcass, the myoglobin content will account for 90 to 95% of the total iron. The iron atom of the heme has four of its coordination sites involved with bonds to porphyrin; the fifth site is linked to proximal histidine (E93) (Kagen, 1973). Some changes are at the the sixth coordination position of heme iron: in myoglobin, it is empty; in oxymyoglobin, it is occupied by O_2; in metmyoglobin, it is occupied by water. The oxidation state and the type of ligand bound to the iron atom determine the colour and the reactivity of myoglobin.

Many reports have shown that meat colour stability is muscle-dependant (Ledward, 1971, O'Keefe and Hood, 1980; 1982; Ledward, 1985; Renerre and labas, 1987a, 1987b; Faustman and Cassens, 1990a,b; Chan et al., 1996). It was shown in our laboratory, from beef carcasses of different age, sex, and breed that *Longissimus dorsi* and *Tensor fasciae latae* are the most stable muscles, *Semi-membranosus* being of intermediate stability, *Gluteus medius*, *Psoas major*, *Supra-spinatus*, *Triceps brachii caput longum* are less stable and *Diaphragma medialis* is the least stable (Renerre 1984). The percentage of MetMb for these muscles, determined by the method of Stewart et al. (1965), and confirmed by the %R630-%R580 method (Van den Oord et al., 1971), showed a variation between 25 and 50% MetMb (Renerre in press, 1999).

Oxygen consumption rate

Contrarily to oxygen uptake in living animal, oxygen uptake in meat has always received only little attention and more work is needed. The bright red colour of fresh meat depends on the depth of MbO_2 which is determined by the rate of O_2 diffusion, O_2 consumption rate (OCR) and by the partial pressure of O_2 at the meat surface (Giddings, 1974; Skibsted et al., 1994). In air and at low temperature, during the first days of storage, the depth of oxygen penetration in meat increases, due to a decrease in tissue oxygen consumption by mitochondria, and a better O_2 diffusion (Bendall and Taylor, 1972; Renerre, 1984, Lanari and Cassens, 1991); this reaction takes place when meat is packaged in an oxygen-permeable film and known as « blooming ». For Skibsted et al. (1994), for a given fall in temperature, the diffusion rate decreases less than the respiratory activity and the bright red layer of MbO_2 increases at the beginning of the storage. At a few mm below the surface, there is a region where the partial pressure of oxygen is in the optimum range for the formation of a metmyoglobin layer (Ledward, 1970). In meat, the first order rate constant for myoglobin oxidation shows a well defined maximum value for an oxygen pressure of 6 mm Hg at 0°C and 7.5 mm Hg at 7°C. After storage, MetMb becomes visible at the surface and the meat becomes brown. When meat is stored with an oxygen-permeable film, the spoilage flora in their logarithmic growth phase have a high oxygen demand and may reduce the oxygen partial pressure to increase the rate of formation of MetMb causing brown discoloration (Skibsted et al., 1994).

Cheah and Cheah (1971) had shown that the functionnality of mitochondria was not altered for 6 days at 4°C but that the decrease of oxygen uptake was due to a loss of substrates. For Bendall and Taylor (1972), the OCR in *post-mortem* muscle decreased exponentially during 2 to 6 days. For Atkinson and Follett (1973), the OCR is higher in lamb than in beef, and for these authors, is responsible for the relative instability of colour in lamb compared to beef. In beef, muscles and breeds of lower color stability had the highest levels of oxygen consumption rate and contained a higher NAD content (Lanari and Cassens, 1991); Holstein beef discolored more rapidly than beef from crossbred animals and differences may be dependent on inherent metabolic differences in coenzymes (NAD), or in reducing agents (GSH) (Faustman and Cassens, 1991). It was shown that a high OCR decreases MbO_2 formation and favours more rapidly MetMb at the surface; consequently, beef muscles with high OCRs tend to be less colour-stable than those of low OCR (O'Keefe and Hood, 1980; Renerre, 1984; Renerre & Labas, 1987a,b; Lanari and Cassens, 1991; Renerre et al., 1992; Ledward, 1985; Madhavi and Carpenter, 1993). The consumption of oxygen by mitochondria is believed to maintain myoglobin in a reduced state and it is admitted that this deoxymyoglobin is less stable than oxymyoglobin.

For Reddy and Carpenter (1991), a sufficient reduction in O_2 tension could resulte in little or no formation of H_2O_2 resulting in greater stability of pigments. Effectively, H_2O_2 which is a prooxidant compound may be cleaved by Fe^{++} to yield highly reactive hydroxyl radicals (OH°), by the Haber-Weis and Fenton reactions (Halliwell and Gutteridge; 1989), which oxidize lipids and proteins. Feldhusen et al. (1995) have also done direct measurements of pO_2 in beef to establish the connections between the myoglobin oxidation and the colour stability. One of the difficulty to control the action of molecular oxygen is to accurately determine its concentration *in situ* because there are many intracellular gradients at various points of the muscle. It was shown (Renerre and Labadie, 1993), that the packaging environment can impact significantly partial oxygen pressure and have profound effect on the colour stability of fresh meat. It has been observed (Rousset and Renerre, 1991) how the creation of « true »anaerobic conditions (in 100% CO_2 with an oxygen scavenger) is one of the simpliest way to control reactions with molecular oxygen; in these conditions, the oxygen residual concentration was reduced rapidly to less than 0.1%. After modified atmospheres (Allen et al., 1996) or after 100% CO_2 for many weeks (Santé et al., 1994; Renerre et al., 1997) with oxygen scavengers, it was shown that retail cuts demonstrated significantly greater retail colour shelf-life than those which were not exposed to oxygen scavengers.

Myoglobin autoxidation rate

A reaction of central importance in myoglobin chemistry is the oxidation of the native ferrous form to metmyoglobin.. The mechanism involves a nonenzymic spontaneous oxidation by free oxygen, which determines the rate of discoloration (Giddings, 1974). Exposed to oxygen, myoglobin is slowly and continuously oxidised to metmyoglobin in fresh meat. It increases not only at low oxygen partial pressure, but also at high temperature and ionic strength, at low pH, in the presence of concentrated salts, oxidation-reduction mediators and catalytic amounts of heavy metals (Georges and Stratman, 1952; Antonini and Brunori, 1971). By a computer simulation Wazawa et al. (1992) provide a picture of the oxidation reaction of myoglobin as a function of oxygen pressures. Bruun-Jensen et al. (1997) studied the pressure effects on acid-catalysed autoxidation of oxymyoglobin.

The finding that autoxidation is more rapid with deoxymyoglobin (Georges and Stratman, 1952), indicate that this form is an intermediate in the reaction. Oxymyoglobin is more stable towards oxidation than deoxymyoglobin due in part to hydrogen bonding between the bound oxygen and a distal residue (histidine 64 or E7) of the apoprotein (Giddings, 1977). For Antonini and Brunori (1971), an hydrophobic environment around the heme would be responsible for the greater stability of the oxygenated derivative of myoglobin. In some abnormal adult hemoglobins, distal histidine is replaced by tyrosine and the autoxidation is abnormally rapid

(Antonini and Brunori, 1971). By comparing different species (yellowfin tuna, green sea turtle, sperm whale), it was found that the autoxidation rate was more dependent on ligand accessibility than on the dynamic stability of the myoglobin (Livingston et al., 1986). When compared with sperm whale oxymyoglobin, tuna oxymyoglobin was found to be much more suceptible to autoxidation (rate constants 10-time higher) ; Kitahara et al. (1990) found that the distal side of the heme iron in tuna was apparently less hydrophobic. If replacement of His 64 by other amino acids increases the rate of autoxidation, the increase of the polarity by substitution of Val 68 (in the distal pocket) with other amino acids such as Ser accelerates autoxidation (Brantley et al., 1993).

For many authors (Georges and Stratman, 1952; Brown and Mebine, 1969), the reaction is first order, with respect to MbO_2, with a rate maximum at low oxygen pressures, and the experimental results of these authors supported the conclusion that 0.25 mole of oxygen in oxymyoglobin was used for the oxidation and 0.75 mole was released. Now, it is proposed that the oxygenated complex MbO_2 separates into ferrimyoglobin and a free superoxide anion $O_2^{-\circ}$. The radical anion superoxide formation is due to a nucleophilic displacement of $O_2^{-\circ}$ from MbO_2 by a water molecule or a hydroxyl ion OH° that can enter the heme pocket from the surrounding solvent (Gotoh and Shikama; 1974; Sugawara and Shikama, 1980; Shikama, 1990). Tajima and Shikama (1987) proposed that, in a second etap, H_2O_2 produced from $O_2^{-\circ}$ by a spontaneous reaction, can be eliminated or decomposed mostly, if not completely, by the MetMb presumably by the formation of the ferryl species. More recently it was confirmed (Mikkelsen and Skibsted, 1991; Newman et al., 1991; Kanner, 1994) that in meat ferryl-myoglobin (MbFe IV=O) was formed by reaction of MetMb with H_2O_2 and was a primary instigator of lipid peroxidation and, consequently, of rancidity. During the first 24hours p.m., the NAD(H) in beef muscles is high (Renerre, 1984) and in these conditions the level of ferryl-myoglobin is kept at low level (Skibsted et al., 1994). For Kroger-Olsen and Skibsted (1997), in aerobic conditions, autoxidation of MbO_2 and growth of lactic acid bacteria act synergetically in the production of H_2O_2 and, consequently, in the production of ferryl-myoglobin.

The autoxidation reaction is highly temperature dependent, with a Q_{10} value of about 5 and an activation energy of 24 to 27.8 kcal mol^{-1} (Brown and Mebine. 1969). The rate of discoloration with temperature is muscle dependent and, in aerobic conditions, the rate is 2 to 5-fold higher at 10°C than at 0°C, with variations between muscles (O'Keefe and Hood, 1980-81). High temperatures favour greater scavenging of oxygen by residual respiratory enzymes leading to low oxygen tension which facilitates autoxidation of myoglobin. In parallel, it is known that lipid oxidation increases with elevated temperatures with essentially the contribution of non enzymic processes. In frozen meats, light in conjunction with oxygen increased lipid and pigment oxidation and singlet oxygen ($^1O_2$) could be implicated to initiate lipid oxidation (Mc Millin, 1996). For Berthelsen and Skibsted (1987), light was the most important factor in discoloration of frozen meat.

Many authors such as Georges and Stratman (1952), Brown and Mebine (1969), Shikama and Sugawara (1978) or Yin and Faustman (1993) also showed that the rate of autoxidation increased markedly with decreasing pH. Low pH reduces the stability constant for the haem-globin linkages (Livingston and Brown, 1981) and increases the autoxidation rate; moreover, oxymyoglobin denatures at pH values below 5. In these conditions, the protonation of bound oxygen is accelerated and the release of superoxide anion is favored. For Sugawara et al. (1995), the unfolding of the globin moiety (with 8M urea) allows much easier the attack of the water molecule or OH° on MbO_2 in the autoxidation process. Because the rate of oxymyoglobin autoxidation increases with decreasing pH, whilst the enzymic reduction is much reduced at low pH, it is not surprinsing that, in general, muscles of low pH discolour more rapidly than those of high ultimate pH (Ledward, 1985). For Skibsted et al. (1994), a decrease in pH of 0.3 by lactic acid formation double the rate of oxymyoglobin autoxidation and in stored meat, the brown layer broaden toward the surface.

If ultimate pH has a limited effect on meat colour and rate of discoloration (Hood, 1980; Renerre, 1990), the rate and extent of pH fall (influenced by biological as extrinsic factors) can play a more marked role. Heat and low pH enhance autoxidation of myoglobin and are responsible for the colour fading observed in PSE (pale, soft, exsudative) pig meat (Renerre, 1990). Tam et al.(1998) have shown an effect of halothane genotype on porcine meat quality, on metmyoglobin accumulation and on myoglobin autoxidation rate. In others species, such as turkey, the onset of rigor mortis can be extremely rapid with negative consequences on the colour of the packaged meat (Santé et al., 1994). A few researchs have been conducted to establish if the changes in temperature / pH regimes during glycolysis modify the perceived colour of the meat, possibly independently of MetMb formation during retail display (Ledward, 1985; Renerre and Bonhomme, 1991). For Ledward (1985), exposing the meat to high temperature and low pH, as occurs in electrical stimulation, may decrease the activity of the oxygen-utilizing enzymes and thus improve the colour stability of the muscle.

In beef, pre-slaughter stress may result in dark-cutting beef (DFD) which is translucent and more susceptible to bacterial spoilage particularly under vacuum where H_2S is rapidly produced. For Ledward (1983), the formation of sulfmyoglobin may involve a ferryl (FeIV) ion as an intermediate. Moreover the high pH will also accelerate respiratory activity of the tissue and the formation of purple reduced Mb. As it is known that Mb is more susceptible to oxidation than MbO_2 (Lawrie, 1985), DFD meat can autoxidize more rapidly than normal meat. If further oxidation of the porphyrin ring occurs, then a green verdohaem complex may form.

To examine the relationships between the autoxidation rate of oxymyoglobin and the discolouration rate of muscles during display, a few experiments have been done. Firstly, it is possible to rapidly purify reduced oxymyoglobin required to study the autoxidation mechanism (Renerre, 1977; Krzywicki, 1982; Gatellier et al., 1993; Trout and Gutzke, 1996) Secondly, a few experiments were done to see the links between the autoxidation rate and the colour stability. For Ledward (1983), the results found for myoglobins in solution can not be applied to meat, as in meat, there are a reducing system, a catalytic process and gradients of oxygen concentration. Trout and Gutzke (1996) found no direct relationships between the oxidation of purified myoglobin and the discolouration rate of the meat and concluded that (unknown) endogenous oxidative systems may play a significant role in determining the rate of metmyoglobin formation. It is effectively not known to-day how endogenous factors affect the autoxidation (and / or the enzymatic reduction) rate in meat. Santé et al. (1993) observed that myoglobin autoxidation rate of turkey breast and thigh muscles were identical even if differences in colour stability between these muscles were observed (Mercier et al., 1998b). By examining the autoxidation rate of myoglobin extracted at 2h *post-mortem*, we did not observe muscle-dependent effect (Renerre et al., 1992). However, after an extraction at 192 h *post-mortem*, it is noteworthy that MbO_2 from *Psoas major* muscle (PM, unstable muscle) showed a greater autoxidation rate than myoglobin purified from *Longissimus dorsi* muscle (LD, stable). By utilizing ^1H NMR, it was confirmed that oxidative processes during meat storage led to greater oxidative susceptibility of myoglobin after extraction from *Psoas major* than for *Longissimus dorsi* muscle (Foucat et al., 1994). These differences in myoglobin autoxidation rates could be partially due to the surrounding environment particularly lipidic (Faustman and Cassens, 1991; Gatellier et al., 1992; Anton et al., 1993b; Mercier et al., 1995). Nevertheless, more clarification is still required to develop a better understanding of the influence of the pro-and anti-oxidant pool of the muscle food and of the oxidation-reduction mechanisms on the properties of myoglobin *in situ*.. Lanari et al. (1994) showed that vitamin E supplementation stabilized the MbO_2 complex by enhancing the Mb oxygenation rate and by decreasing MbO_2 autoxidation rate by unknown mechanisms. Actually, as earlier (Renerre, 1990), it is always not known if bacteria plays or not a role in oxidation of oxymyoglobin (Chan et al., 1995).

Metmyoglobin reduction

In vivo, the metmyoglobin reducing enzyme systems are important in maintening myoglobin in its physiological role because metmyoglobin is unable to bind oxygen reversibly; moreover, these systems allow to maintain reduced myoglobin, or to reduce metmyoglobin if formed and consequently can extend the shelf-life of fresh meat. As reported by Faustman et al. (1988), many investigations after Giddings (1974) have provided evidence for the enzymic phenomenon but without explanation of the mechanism. (Ledward, 1971; Lanier et al., 1978; O'Keefe and Hood, 1982; Ledward, 1985; Renerre and Labas, 1987a). Possible enzymic pathways of metmyoglobin reduction have been studied and it is now accepted that the reduction process in meat is primarily enzymic in nature with NADH as coenzyme which facilitates the conversion of ferric myoglobin to its ferrous form. A loss of reducing activity in meat during storage is due to the combination of factors including fall in tissue pH, depletion of required substrates and co-factors and ultimately, complete loss of structural integrity and functional properties of the mitochondria (Giddings, 1977; Renerre, 1984; Ledward, 1983). Despite some speculations (Watts et al., 1966) the pool where the NADH comes from is not yet known and can involve mitochondria and/or submitochondrial particles with the reversal of electron transport which would allow reduction of NAD (Giddings, 1974). For Renerre (1984) and Faustman and Cassens (1990a), NAD(H) decreased more or less rapidly in muscles during storage

Stewart et al. (1965) first described the enzymatic reduction of metmyoglobin in ground beef: the metmyoglobin reducing ability (MRA) measured by reflectance; MRA was greatest at 35°C and at pH values above 5.8. But the use of potassium ferricyanide (to obtain 100% MetMb) has been criticized by many authors and, consequently, the rates of reduction using MRA must be carefully considered (Faustman and Cassens, 1990b). If for Watts et al. (1966), MRA was favored by anaerobic conditions, for Ledward (1983) the enzymic reducing system was believed to be oxygen independent. It must not be forgotten that in meat *in situ* the reduction of MetMb must be more complex to explain than in *in vitro* reduction, with the purified enzyme, with the presence and the relationships between many substrates and intermediates. Ledward (1972) described an other method to measure the pigment reducing capacity: the aerobic reducing ability (ARA). ARA uses low pO_2 (1% O_2/99%N_2), conditions to oxidize meat pigments, and avoids the use of components as ferricyanide which can influence the native reducing system of meat. Faustman and Cassens (1990a) have used this method and described the calcul mode of the reducing capacity.

Enzymic reducing systems for MetMb have been studied in fish and mammals but the physiological importance of these enzyme activities in colour stability must be always questionned because of the use of an artificial mediator, e.g. methylene blue (Livingston and Brown. 1981) with NAD(P)H as active reductants. For Livingston et al. (1986), reductases requiring methylene blue are categorized as diaphorases. MetMb reductase enzymes have been purified and characterized from blue-white dolphin (Matsui et al., 1975), blue-fine tuna (Al Shaibani and Brown., 1977) and yellow-fin tuna (Levy et al., 1985). From mammals, the first MetMb reductase which does not use artificial mediators, but an activator as ferrocyanide or cytochrome b_5, has been purified from beef heart muscle (Hagler et al.,

1979). Livingston et al. (1986) classified this enzyme as NADH-cytochrome b5 reductase. Faustman et al. (1988) purified a metmyoglobin reductase from bovine cardiac muscle and investigated the effects of cytochrome b5 or potassium ferrocyanide, pH and temperature on *in vitro* metmyoglobin reduction. For Faustman and Cassens (1990b), ferrocyanide is not a simple e^- carrier but binds to the haem protein in such a way as to make the heme group accessible to the metmyoglobin reductase. It was also shown, such as in our laboratory, that MetMb reductase activity present in skeletal bovine muscles was localized in microsomes and in mitochondria (Echevarne et al., 1990. Arihara et al. (1990) reported that NADH-cytochrome b5 reductase was responsible for MetMb reduction in muscle. It was also found that NADH-cytochrome b5 reductase reduced MetMb by using outer membrane cytochrome b at the mitochondrial surface and, in part, by using cytochrome b5 at the sarcoplasmic reticulum (Arihara et al., 1995).). Recently, Lynch et al. (1998) proposed that α-tocopherol maintained oxymyoglobin pigment via enhancement of cytochrome b5-mediated reduction of metmyoglobin. For Faustman and Cassens (1990a), psychrotrophic bacteria were demonstrated to be prooxidant in low populations number and proreductive in very high concentrations (ca. 10^8 CFU/g); the responsible agents were not identified. It was also observed (Arihara et al., 1995) that *Enterococci* species converted metmyoglobin to oxymyoglobin.

Nonspecific diaphorases capable of reducing both methemoglobin and metmyoglobin at the expense of NADH, but at lower rates than the more specific reductases previously cited, have been described (Livingston et al., 1986; Faustman and Cassens, 1990b).

Nevertheless, evidence for nonenzymatic reduction was also presented by Brown and Snyder (1969). For these authors, the non-enzymic reduction could occur at greater rates than the previous enzymic one but Hagler et al. (1979) failed to detect a non-enzymic reduction of bovine metmyoglobin. Nonenzymic reduction of metmyoglobin by NADH (NADPH) could also occur, at comparable rates to many of these enzyme systems (Livingston & Brown,1981 Renerre & Labas, 1987a). Recently, Mikkelsen et al (1999) demonstrated the presence of a MetMb reducing enzyme system in pork where NADH was mandatory for activity; they also showed that reduction was more facile as a non-enzymatic reaction.

If the autoxidation rate has been found to be influenced by many factors such as pH, oxygen partial pressure, temperature, light, salt, or lipid oxidation products, reduction of metmyoglobin was not so studied. Ledward (1985) claimed that with high temperatures and low pH, there was a reduction of the oxygen consumption rate and MetMb reducing ability. Mikkelsen and Skibsted (1991) studied the kinetics of enzymatic reduction of metMb in relation to oxygen activation in meat products. For Skibsted et al. (1994), the MetMb reducing activity, dependent on NADH, increases with decreasing pH , although to a lesser extent than for MbO2 autoxidation.

Moreover, Renerre and Labas (1987a,b) asserted that the enzymic ferrymyoglobin reduction, estimated by the metmyoglobin reductase activity method, in aerobic conditions, or by reflectance measurements in anaerobic conditions (MRA method), did not explain the differences observed in colour stability of beef muscles. As the most unstable muscles, from the colour point of view, showed the highest reducing activities, they concluded that it was not as important for muscle colour stability. Faustman and Cassens (1990a) also found that differences in colour stability between *longissimus*, stable (Renerre, 1984) and *gluteus medius* (unstable) muscles do not appear to be due to the aerobic metmyoglobin reducing capacity measured by the ARA method. Echevarne et al.(1990) also found no correlation between the metmyoglobin reductase activity, measured in aerobic as in anaerobic conditions, and the colour stability of different bovine muscles. Reddy and Carpenter (1991) failed to prove that MetMb reductase activity was higher in stable muscles than in unstable ones. Lanari and Cassens (1991) showed that the least stable muscles had the highest reducing activities and concluded that differences in oxygen consumption rates of muscle mitochondria may be a contributing factor for explaining the effect of muscle and breed on the rate of discoloration. All these results are opposed to the first results of Ledward (1971, 1985) who indicated that the activity of MetMb reducing systems was the most important factor in the color stability of meat. For Ledward (1985) and Cheah and Ledward (1997), the catalytic mechanism which encourage the formation of metmyoglobin declines rapidly following slaughter and not the reducing system; so in aged meat, the activity of the reducing system may be the controlling factor. Echevarne et al (1990) found a loss of reducing activity during storage of beef muscles, and, in pork, Zhee and Brewer (1998) indicated a drop of reducing activity during a 7 days storage. Madhavi and Carpenter (1993) found that aging and processing affected color, MetMb reductase and OCR of beef muscles; they found also that *Psoas* steaks had lower metmyoglobin reductase activity than *longissimus* steaks. During display, they observed that color stability declined concomitantly with, but not necessarily in response to, decreasing metmyoglobin reductase activity and NAD concentration. Zhu and Brewer (1998 found that, compared to normal and PSE pork, DFD pork had the highest MetMb reductase activity which appeared to retard MetMb accumulation. It is also not known to day if free radicals are capable of oxidizing enzymic or non enzymic metmyoglobin reduction systems found in skeletal muscles (Chan and Decker, 1994). It was previously observed in the laboratory that during meat storage, there was an increase in lipid and protein oxidation and, concomitantly, a

decrease in antioxidant enzyme activity, more particularly in red aerobic muscles (Renerre et al., 1996). Recently, Chan et al.(1998) showed that dietary vitamin E did not seem to affect MetMb reducing ability in beef (ARA method).

Lipid and myoglobin oxidation

In muscle, lipids consist of cytosolic droplets of triacylglycerol and, in membranes, of phospholipids and cholesterol. Phospholipid fraction is mainly composed of phosphatidyl -choline (PC) and - ethanolamine (PE); it contains also in small amount phosphatidyl-inositol and-serine, shingomyelin and cardiolipin with differences related to metabolic type of muscles. Oxidative muscles contain more phospholipids and a higher proportion of cardiolipin and PE than glycolytic ones because oxidative muscles contain more oxidative fibres which contain more mitochondria (Renerre, 1984). Fatty acids composition of phopholipids is characterised by a high proprtion of polyunsaturated fatty acids (PUFA). These PUFA are linoleic acid, long chain PUFA such as arachidonic acid and 22C fatty acids such as 22:4 n-6, 22:5 n-3 and 22:6 n-3 (Gandemer,1998; Gandemer et al., 1997). For these authors, the porportion of total PUFA in muscle phospholipids show small variation in relation with animal species, breed, dietary fat composition,, sex, ...In monogastric animals, the ratio n-3 / n-6 depends on dietary supply of linoleic and linolenic fatty acids. Lipolysis, which is one of the main (enzymatic) cause of lipid degradation in meat, and which conduce to the formation of free fatty acids, affects essentially phospholipids in fresh meat and in in dry-cured ham (Gandemer; 1998). Lipolysis is also considered as a factor promoting lipid oxidation.

Lipid oxidation (often measured by the thiobarbituric acid-reactive substances test , TBA-RS) is one of the main cause of the loss of quality in meat during storage and processing conducing to rancidity; the primary quality deterioration during lipid oxidation, and particularly after cooking, is the production of warmed-off-flavors (WOF) and off-odours. Oxidation of fatty acids also affects adversely the colour (Faustman and Cassens, 1990b; Gatellier et al., 1992) and, also, the protein oxidation / texture (Liu and Xiong, 1996; Martinaud et al; 1997), the nutritional quality and safety of meat (Gray et al., 1996).

It is also generally accepted that it is the polyunsaturated fatty acids from polar phospholipids, present in the subcellular membranes (microsomes, mitochondria), which are in contact with catalysts of lipid oxidation located in the aqueous phase rather than triglycerides, which are responsible for the initial development of WOF (Gray et al., 1996). As reported by Gray and Pearson (1987), many factors affect lipid oxidation in animal tissues such as species, anatomical location, diet, temperature, sex, age, phospholipid composition and content. For example, it is well known that feeding different dietary unsaturated fats to monogastric animals affects the composition of triglycerides and, to a lesser extent, that of phospholipids; increasing the degree of unsaturation of animal tissues as in turkeys (Mercier et al. ; 1998a,b,c) accelerates oxidative deterioration . Moreover, rapidly *post-mortem*, balance between prooxidative factors and antioxidative capacity favours oxidation (Morrissey et al., 1998). Processing of raw meat such as the method of deboning of carcasses, mechanical separation, restructuration, grinding, etc... can also increase the lipid oxidation. This is due to the disruption of the muscle membrane which alters the comparmentalized cellular systems and facilitates the formation of free radicals by interaction of iron with molecular oxygen (Buckley and Morissey, 1992). During processing, increase in temperature, UV lighting, presence of metal ions (particularly iron) and ionizing radiation can also influence greatly the rate of lipid oxidation (Kanner, 1994).

Lipid oxidation is a chain reaction, with an initiation, propagation and termination steps (Kanner, 1994). Initiation involves the loss of an hydrogen from a methylen carbon to form an allyl radical L°; during propagation, there is the formation of a peroxyl radical LOO° and lipid hydroperoxides LOOH. These hydroperoxides are both products of oxidation and substrates for further reaction with Fe^{++} and Cu^{+} to yield to peroxyl (LOO°) and alkoxyl (LO°) radicals (Morrissey et al., 1998). The LO° can also degrade to alkyl radicals and a range of aldehydes. The termination step involves reactions between two radicals to form non radical polymers.

Many potential initiators and propagators of lipid oxidation in muscle food, known as reactive oxygen species (ROS), have been described. The ROS are superoxide anion radical (O_2-°), perhydroxyl radical ($HO^{\circ}2$), hydrogen peroxide (H_2O_2) hydroxyl radical (HO°) and ferryl myoglobin radical (P-Fe(IV)=O), all of which may participate directly or indirectely in oxidative processes in meat and meat products. In food muscle tissue, the exact nature of the catalysts initiating lipid oxidation has always to be fully elucidated (Kanner et al; 1987; Baron et al., 1997) even if the hydroxyl radical OH° is the most potent oxidant encountered in biological systems (Morrissey et al., 1998). These radicals can also oxidize proteins, sugars, nucleic acids. In food muscle tissue, it is possible that O_2-° is generated from membrane e- transfer systems, autoxidation of oxymyoglobin to metmyoglobin, activation of leucocytes or oxidation of reducing compounds by free iron by direct or indirect initiation (Kanner, 1994). For Kanner et al. (1987), the kinetic barrier restricts the ability of O_2-° to oxidize directly lipids. A direct initiation involves Fe^{3+} or Fe^{2+}. Ferrous ion in aerobic aqueous solution produce ferric ion plus anion superoxide radical, hydrogen peroxide and hydroxyl radical OH°. This reaction is cycled by O_2-° and called Haber-Weiss reaction. Ferrous ion in presence of H_2O_2 can stimulate lipid peroxidation by generating OH° by the Fenton reaction (Halliwell and Guteridge, 1989). Hydrogen peroxide is present as a metabolite in muscle tissues and is produced by non-enzymatic dismutation or by SOD catalysed dismutation (Kanner et al., 1987). When fully provided with their substrates, membranes, peroxysomes and cytosolic enzymes are H_2O_2 generators. H_2O_2 is not a strong oxidant and does not react directly with PUFA. It was shown by many authors

(Kanner and Harel, 1985a; Rhee et al. 1987; Newman et al., 1991; Mikkelsen and Skibsted, 1991), that H_2O_2 interacts with metmyoglobin in muscle tissue to form activated metmyoglobin which promote (or initiate) lipid peroxidation. There is the generation of a short lived protein radical, perferrylmyoglobin ($^{\bullet}$MbFe(IV)=O) which is rapidly reduced to ferrylmyoglobin more stable (MbFe(IV)O) with differences between muscles (Gatellier et al., 1995). For Rhee et al. (1987), activated MetMb is more important than non- heme iron to facilitate lipid peroxidation in raw tissue. Ostdal et al. (1997) studied the formation of long-lived secondary protein radicals in the reaction between H_2O_2-activated MetMb and other target proteins.

The importance of free iron in lipid oxidation is particularly important in turkey muscles, compared to beef (Kanner, 1994). The relative contribution of different forms of iron, whether free or protein bound, haem or non-haem, oxidized or reduced, in catalysing lipid oxidation in meats have not been assigned definitively (Gray et al., 1996). Iron *in situ* is in transit between storage proteins (ferritin, hemosiderin), transport protein (transferrin) and functional proteins (myoglobin, hemoglobin, cytochromes, enzymes). If a small pool of non-protein non-hem iron provides « free »iron at micromolar concentrations in tissues, implicated in lipid oxidation, the main source of free iron in cells seems to be ferritin which is the main protein that store iron in muscle cells, but release it during meat storage (Kanner, 1994). Transit pool of iron seems also to be chelated to small molecules such as ATP, citrate, membrane lipids. It was also shown (Kanner, 1994) that *in vivo* turkey muscle cytosol contained reducing compounds such as ascorbic acid (but also NADP(H), glutathione and cysteine) which inhibited lipid peroxidation. In raw muscle, lipid peroxidation may be also stimulated by (but not initiated by) lipoxygenase or cyclooxygenase but small amounts of hydroperoxides are required for their activation (Kanner et al., 1987).

Many authors have shown that lipid and pigment oxidation were closely coupled in beef (Renerre and Labadie, 1993) and shown that lipid oxidation was a promoter of myoglobin oxidation but it was not always possible to deduce whether or not the pigment oxidation caused the lipid oxidation. As in veal (Faustman et al., 1992), in different bovine muscles, strong relationships were found between lipid oxidation, measured by TBA-RS, and myoglobin oxidation measured by MetMb % (Krzywicki, 1982) at the meat surface during an aerobic storage (Faustman and Cassens, 1989; Faustman and Cassens, 1991; Gatellier et al., 1992; Mercier et al., 1995).

Consequently, in some species, colour stability was enhanced by the addition of one of the most used antioxidants to meat, as chain-breaking vitamin E (assimilated to α-tocopherol). These results are particularly observed in beef, rich in myoglobin, compared to other meats whiter (Faustman and Cassens (1989); Arnold et al. (1993); Chan et al. (1996); Legrand et al. (1997); Liu et al., (1996); Mitsumoto et al. (1998)). The results of Hoving-Bolink et al.(1998) and Houben et al. (1998) on pork, of Monahan et al (1992) on swine, of Santé et al. (1992) on turkey, showed that the addition of vitamin E to diets improves lipid stability and, more or less successfully, pigment stability of meat. Moreover, in turkey (Mercier et al. 1998b), or in pork (Jensen et al., 1997), vitamin E supplementation had no positive effect on colour stability of meat; in white meats, such as poultry, it is difficult to appreciate oxymyoglobin formation (Millar et al., 1994; Mercier et al., 1998). In the DIETOX project, Skibsted (1998) confirmed that the effect of vitamin E supplementation on colour of pork and poultry was of minor practical importance.

The basis of the relationships between dietary α-tocopherol and lipid and myoglobin oxidation in meat is not fully understood. Arnold et al (1993) and Liu et al (1996) showed that vitamin E supplementation lowered lipid oxidation but the ranking of muscle vitamin E concentration was not the same to slow MetMb formation; and the extension of display life with vitamin E was related to metabolic type of the muscle. For Liu et al. (1995), more and smaller mitochondria in red fibers may provide more volume for potential retention of vitamin E; differences in the distribution of capillaries and residual blood could be also implicated. These authors also indicated the minimum dose of vitamin E that must contain the muscle to increase its color stability (500IU / steer daily for 126 days), with higher values when meat was frozen due to the decompartementation of many cell components. For Lanari et al. (1994), vitamin E increased the resistance of MbO_2 to oxidation but also enhanced MbO_2 stability against deoxygenation. Chan et al (1996) observed that dietary vitamin E supplementation delayed MbO_2 oxidation in beef and increased the colour shelf-life without affecting total microbial load; for Hoving-Bolink et al. (1998), in pork, the effect of vitamin E on colour was muscle-dependent and was absent on microbial counts. Zerby et al; (1998) found no effect of vitamin E on microbiological counts. We observed (Legrand et al., 1997) that the beneficial effect of vitamin E on beef color stability was influenced by its retail mode. Moreover, Monahan et al; (1994) by measurement of fluorescence anisotropy showed that α-tocopherol by its antioxidant action had a positive effect on membrane integrity. Mitsumoto et al. (1995) showed in beef that vitamin E enhanced the ability of meat to hold sarcoplasmic components implicated in oxidative processes.

To prevent or delay oxidation reactions, of enzymic or non-enzymic origin, some authors such as Chan and Decker (1994) have shown the role and mechanism of endogenous antioxidant systems in meat. These endogenous antioxidants include not only chain-breaking α-tocopherol (by dietary means) but also free-radical scavengers histidine-containing dipeptides (carnosine, anserine) and antioxidant

enzymes such as superoxide dismutase, catalase, the GSH redox cycling enzymes ((Se) glutathion peroxidase, glutathion reductase and glutathion transferase), carotenoids and ubiquinone. For Chan and Decker (1994), the higher concentration of anserine and carnosine in muscle high in white fibers could help explain why these muscles exhibit greater stability to both lipid and colour oxidation. To increase the oxidative stability of muscle, it is necessary to better know the antioxidative potential present in meat which is depending of many intrinsic factors such as the metabolic fiber types. It has been shown in beef (Mercier et al., 1995; Renerre et al., 1996) that antioxidant activities were higher in oxidative muscles compared to glycolytic ones, but it did not appear to lead to increased protection against free radicals and to a better colour stability. These results obtained on beef were confirmed with turkey meat (DIETOX-project) and it was also recently indicated that vitamin E supplementation did not affect the AOE activity (Renerre et al., 1999). Lee et al. (1996) also observed that catalase and glutathion-peroxidase activities were higher in thigh than in breast muscle.

As meat is a complex middle, different model systems were developped to gain a fundamental understanding of oxidation processes and their interactions. *In vitro* approaches have utilized emulsion, liposome and microsome systems (Chan et al., 1997). In model systems, transition metals such as iron, have been used to initiate lipid oxidation via generation of hydroxyl radicals by the Fenton reaction (Fe^{++} / -ascorbic acid, / -H2O2, / -EDTA). Enzymic systems, such as cytochrome P450/P450 reductase / Fe^{+++} /NADPH, Xanthine / Xanthine oxidase / Fe^{+++}, and ferrylmyoglobin formed from the reaction of Metmyoglobin and H_2O_2, have also been used to generate different radicals in model systems. These oxidative systems can also be used to oxidize proteins *in vitro* (Mercier et al., 1998b).

Baron et al. (1997) tested the prooxidative activity of different myoglobin species in linoleic acid emulsions. Liposomes permit study of a variety of factors including fatty acid chain length and unsaturation and type of polar head group but many components such as membrane-bound proteins are absent in these compounds. Yin et al. (1993) and Yin and Faustman (1993) developed an oxyMb-liposome model and observed that vitamin E *in vitro*, and ascorbate-vitamin E, retarded lipid oxidation and MbO_2 oxidation indirectly. Genot et al. (1991) used *in vitro* systems with muscle phospholipids, extracted from different beef muscles, to relate lipid oxidation with meat colour stability. Yin and Cheng (1997) found that in phosphatidyl choline liposomes, oxymyoglobin and lipid oxidation were retarded by α-tocopherol (and β-carotene).

To study the relationships between lipid and myoglobin oxidation, microsomes were also extensively used; microsomes which are small heterogenous membranous vesicles of endoplasmic and cytoplasmic membranes contain high concentrations of PUFA and proteins (Chan et al., 1997). By combining microsomes with oxymyoglobin and cofactors (NADPH, ADP, $FeCl_3$), it was observed that enzymic microsomal lipid oxidation has led to increased oxymyoglobin oxidation (Lin and Hultin, 1977; Anton et al., 1991). By comparing different species (Yin et al., 1993) or muscles (Anton et al., 1993a), it was observed that the source of microsomes will affect lipid oxidation rates which in turn may affect oxymyoglobin oxidation.

In salted ground pork (Decker et al.,1995) as in liposomes and beef homogenates (Lee and Hendricks, 1997), it was observed that addition of different antioxidants as carnosine could inhibit myoglobin oxidation via its antioxidant activity versus lipid oxidation instead of through direct interactions with myoglobin.. Lee et al. (1998) showed the ability of carnosine and phytic acid to inhibit lipid peroxidation and formation of MetMb in raw as in cooked beef. It is consequently well admitted that more research is needed to understand the role of muscle food antioxidants in the regulation of these oxidative processes and their use in industry. This will allow more effective utilization of nutritional regimes that help increase the oxidative stability of muscles. It was also shown that after a chemical induction on meat homogenates (3000G supernatant) (Gatellier et al;, 1998) and / or on microsomal membranes from turkeys (Mercier et al., 1998a,c), a vitamin E dietary supplementation lowers lipid oxidation, and, to a less extent, protein oxidation.

Conclusion

If the oxidative and reducing mechanisms in fresh meat are described, their respective importance in colour stability is always not completely elucidated. Nevertheless, it is admitted that colour deterioration is essentially depending on the rates of myoglobin autoxidation in relation with lipid oxidation. To obtain more oxidatively stable muscle foods, and to propose healthy meat products to the consumers, more research is needed to better know the pro- and anti- oxidant potential of fresh meat and the radical oxidative processes which affect lipids and proteins during maturation.

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