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MEMBRANAL LIPID OXIDATION IN MUSCLE TISSUE - MECHANISM AND PREVENTION

By

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ABSTRACT

MEMBRANAL LIPID OXIDATION IN MUSCLE TISSUE - MECHANISM AND PREVENTION

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Oxidative reactions which cause deterioration in meats during storage are initiated at the membrane level. This study addressed the mechanism of lipid oxidation in meats with specific emphasis on cholesterol oxidation.

Several methods for determining total cholesterol were evaluated in order to choose one which was most efficient, accurate and reproducible. A direct saponification technique, followed by extraction and analysis by gas-liquid chromatography, was chosen and used to survey a variety of meats for total cholesterol content before and after cooking.

The relationship between membranal lipid stability and the overall oxidative stability of a variety of raw and cooked meats was also investigated. In both muscle and membranal lipid stability studies, cooked pork oxidized to a greater extent than beef, while dark chicken oxidized more than white chicken. The stability and composition of muscle membranes reflected the overall stability of meat lipids.

Incorporation of α-tocopherol into veal muscle membranes and the resultant oxidative stability of muscle lipids and cholesterol as a result of dietary vitamin E supplementation was investigated. Growth performance of the veal calves was not enhanced by supplementation.

Supplementation increased muscle and membranal α-tocopherol concentrations almost 6-fold over those of control animals. Oxidative stability of muscle, membranes and cholesterol was enhanced as a result.

Studies on the stability of cholesterol in different lipid environments were conducted. Cholesterol in its linoleate ester form oxidized to a greater extent than cholesteryl stearate or free cholesterol. Also, cholesterol oxidized in dispersions with both phosphatidyl choline and adipose tissue. These observations supported the results of the feeding study by demonstrating that cholesterol oxidation is affected by the surrounding lipid environment.

To my parents
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INTRODUCTION

Recently, much concern has arisen among consumers about the presence of saturated fats and cholesterol in their diets. Consumption of red meats, in particular, has declined because of saturated fat and cholesterol contents (Cross et al., 1986). Dietary fat has been shown to be a common factor in several disorders such as obesity, hypertension, diabetes, cancer and cardiovascular disease (the number one killer in the United States). High plasma lipids and cholesterol have been implicated in the occurrence of cardiovascular disease, resulting in suggestions for reduction of dietary levels. Thus, there is a trend towards processing and marketing of fresh and precooked low-fat meat products.

Also of concern is oxidative rancidity, which is a major deteriorative problem in meat during storage. Oxidative deterioration in meat has been associated with flavor and color changes, losses of protein functionality and nutritive value as a result of reaction of oxidation products with proteins, and adverse biological effects such as cardiovascular disease and cancer. It has been suggested that consumption of cholesterol oxides may have more of an angiotoxic effect than cholesterol consumption itself (Peng et al., 1979; Addis, 1986). The oxidative stability of lipids, particularly cholesterol, in low-fat meat and meat products has not been given much attention in the literature. Several researchers have studied various

aspects of the mechanism of lipid oxidation in meats, however, the findings are not conclusive (Asghar et al., 1988).

Initially, it was proposed that initiation of lipid oxidation in meat occurred in the adipose tissue fraction (Pearson et al., 1977). However, now it is believed that initiation of oxidation occurs in muscle membranes as opposed to adipose tissue (Gray and Pearson, 1987). The research presented here is an investigation of various aspects of membranal lipid oxidation.

The overall objective of this study was to investigate mechanisms of lipid oxidation in meats, with specific emphasis on cholesterol oxidation. Specific objectives of this study were to:

1. Evaluate methods for cholesterol determination in meat and use the chosen method to survey a variety of meats for cholesterol content and losses of cholesterol during cooking.

2. Investigate the relationship between membrane lipid stability and overall oxidative stability of a variety of raw and cooked meats.

3. Evaluate the effects of vitamin E supplementation on the incorporation of α-tocopherol into veal muscle membranes and to determine the resultant oxidative stability of muscle and membranal lipids.

4. Investigate the mechanistic features of cholesterol oxidation in muscle tissues, using model systems and meat systems.

LITERATURE REVIEW

Lipid Content, Cholesterol Content and Their Distribution in Meat

Lipid Composition

Animal fats are distributed intermuscularly, intramuscularly, in adipose tissue, in neural tissue and in blood (Dugan, 1987). Lipids account for 18-30% of the carcass weight of steers and 12-20% of the live weight of market hogs. Several lipid species exist, the most predominant being triacylglycerols. A small proportion of mono- and diacylglycerols are also present. Other lipid substances include phospholipids, sterols, sterol esters and free fatty acids.

Intramuscular lipids include marbling and membrane-bound lipids. Marbling mainly consists of triacylglycerol deposits in fat cells (Pearson et al., 1977). Phospholipids and lipoproteins are the principal constituents of the membrane-bound lipids (Love and Pearson, 1971). According to Dugan (1987) the phospholipid fraction is 0.5-1% of the total weight of muscle tissue. The phospholipid content is inversely related to the lipid content of meat, indicating that the phospholipid fraction is quite constant even though the fat content may be variable. There is, however, variation in phospholipid content between species (Kaucher et al., 1944) and between locations within the same species (Gray and Macfarlane, 1961; Dugan, 1987). Data were

compiled by Allen and Foegeding (1981) to demonstrate that muscle lipid content and composition are influenced by muscle function (Table 1).

Table 1. Neutral lipid and phospholipid composition of lipid from various muscles[a]

Species	Muscle or type	Content		
		Lipid (%)[a]	Neutral lipids (%)	Phospholipids (%)
Chicken[b]	White	1.0	52	48
	Dark	2.5	79	21
Turkey[c]	White	1.0	29	71
	Dark	3.5	74	26
Fish	White	1.5	76	24
(Sucker)[d]	Dark	6.2	93	7
Beef[e]	Longissimus	2.6	78	22
	dorsi	7.7	92	8
		12.7	95	5
Pork[f]	L. dorsi	4.6	79	21
	Psoas major	3.1	63	37
Lamb[g]	L. dorsi	5.7	83	10
	Semitendinosus	3.8	79	17

[a] From Allen and Foedeging (1981)

Fatty acids in meat are typically straight chained with an even number of carbons (Dugan, 1987). In triacylglycerols, the dominant fatty acids are saturated (palmitic and stearic acid) and monounsaturated (oleic acid). The principal fatty acids present in various animal fats are presented in Table 2.

Table 2. Component fatty acids in animal fats[a,b]

Acid	Lard	Beef Tallow	Mutton Tallow	Butter
Butyric				3
Caproic				1
Caprylic				1.5
Capric				3
Lauric		0-0.2		3.5
Myristic	0.7-1.1	2-8	1-4	12.0
Stearic	12-16	14-29	25-32	13.0
Palmitic	26-32	24-33	20-28	38.0
Arachidic		0.4-1.3		1
Lauroleic				0.4
Myristoleic	0-0.3	0.4-0.6		1.5
Palmitoleic	2-5	1.9-2.7		3
Oleic	41-51	39-50	36-47	28.5
Linoleic	3-14	0-5	3-5	1.0
Linolenic	0-1	0-0.5		
Arachidonic	0.4-3	0-0.5		0.5

[a] All values given in percentages
[b] From Dugan (1987)

Igene et al. (1981) demonstrated that the phospholipids in meat contain approximately 15-fold higher levels of polyunsaturated fatty acids than triacylglycerols. The degree of unsaturation in the phospholipids contributes to their chemical and physical properties (Dugan, 1987). Typically, the fatty acids with the greatest degree of unsaturation are located at the 2-position of the phospholipid, while the saturated fatty acids occupy positions 1 and 3. The various phospholipid types tend to have characteristic fatty acid patterns (Pearson et al., 1977). The unsaturated fatty acid content of phospholipids from a variety of species was summarized by Melton (1983) (Table 3).

Table 3. Unsaturated fatty acid content of phospholipids in some muscle foods[a]

Fatty acid	Content (%)				
	Lamb[a]	Beef[b]	Pork[c]	Chicken[d]	Fish[e]
C18:1	19.51	33.44	12.78	20.25	19.59
C18:2	18.49	10.52	35.08	14.20	5.88
C18:3	0.43	1.66	0.33	0.90	8.07
C20:2	0.34	0.69	—	—	0.20
C20:3	0.62	2.77	1.31	1.30	0.36
C20:4	13.20	8.51	9.51	11.60	3.75
C20:5	—	0.76	1.31	1.55	7.16
C22:4	—	0.88	0.98	2.10	0.65
C22:5	—	0.92	2.30	5.75	2.39
C22:6	—	—	2.30	5.75	2.39

[a] From Melton (1983)

Cholesterol Content and Distribution

The greatest concentrations of cholesterol are reported to be in neural tissue, liver and in certain fat depots (Dugan, 1987). As an important component of many animal cell membranes, cholesterol, found in association with phospholipids, may act in controlling the passage of substances across such membranes (Dugan, 1987).

When expressed on a mg/100 g tissue wet weight basis, it is generally agreed that adipose tissue contains a higher level of cholesterol than lean muscle (Rhee et al., 1982b; Eichhorn et al., 1986). Stromer et al. (1966) reported total cholesterol concentrations for Longissimus dorsi muscle and adipose tissue as 34-46 mg/100 g tissue and 113-150 mg/100 g lipid, respectively. Reiser (1975) reported that adipose tissue had lower levels of cholesterol than muscle, however, these values were calculated on a dry weight basis.

A question that develops from this information is whether cholesterol content is related to the degree of marbling. If meat with increased marbling is consumed would this mean a higher cholesterol intake? Rhee et al. (1982a) found no significant differences in cholesterol among eight marbling groups except for raw "Practically Devoid" steaks which had significantly less cholesterol. Berg et al. (1985) also looked at the effects of marbling on cholesterol content. Although their data were not entirely consistent with that of Rhee et al. (1982a) they could find no significant pattern in cholesterol with respect to marbling.

Hoelscher et al. (1988) sought to clarify the distribution of cholesterol between the muscle and the intramuscular fat by studying cholesterol distribution at the subcellular level. The membrane of muscle contained 60-80% of the total cholesterol while the cytosolic storage component contained 20-40%. The distribution in adipose tissue was quite different. Membrane cholesterol was 8-12% of the total and the cytosolic storage cholesterol was 88-92%. The investigators suggested this as an explanation for total cholesterol per gram in raw muscle not increasing much with increase in intramuscular fat, even though adipose tissue has approximately twice the cholesterol content of muscle. Also, in studying different grades of meat it was noted that as intramuscular fat increased the content of membranes per gram of tissue decreased. Thus, a change in cholesterol distribution occurred but total cholesterol

remained the same. The conclusion reached was that cholesterol intake would not be lowered by consuming meats which had less intramuscular fat (marbling).

Sweeten et al. (1990) separated intramuscular adipose tissue from well-marbled Longissimus dorsi and analyzed subcellular cholesterol. They found a total cholesterol concentration of 118 mg/100 g of intramuscular adipose with 54 mg/100 g in the membrane fraction and 64 mg/100 g in the cytoplasm. The distribution of cholesterol was different from that reported previously (Hoelscher et al., 1988).

Cholesterol in adipose or muscle tissue may be found in the free or esterified forms. Studies on the content and distribution of cholesterol in meat have resulted in wide ranges of reported values. Kritchevsky and Tepper (1961) determined the free and ester forms of cholesterol in a variety of foods and reported the esterified cholesterol content of meat to be predominant (i.e., 50-70% of total cholesterol). Tu et al. (1967) reported esterified cholesterol values for beef and pork muscle to be approximately 6% of the total cholesterol. The cholesterol ester content of chicken was determined by Pikul et al. (1984). They did not report total cholesterol. Cholesterol esters were expressed as a percentage of total lipid for breast (1.2%) and leg (0.9%) muscles. Very few reports of cholesterol esters in muscle tissue exist. The influence of the esterification of cholesterol on its oxidative stability in muscle tissue remains to be determined.

Variation in the reported total cholesterol values in the literature may be due to several factors such as diet, sex, age of the animal and the analytical methodology used in cholesterol determination (Kunsman, 1981). In their summary of the cholesterol content of a variety of foods, Sweeny and Weihrauch (1976) discussed the methods used in the quantitation of cholesterol and pointed out the limitations of these methods. Comparison of data from various laboratories is often difficult because adequate information about methods used is not available. Variations in concentrations reported for cholesterol in foods point to the need for development of an efficient, accurate method so that consistency in values may be achieved.

Lipid Oxidation in Meat Systems

Mechanism

Lipid oxidation is one of the major causes of deterioration in meats. In order to control oxidative deterioration, it is important to understand the mechanism of oxidation.

Lipid oxidation proceeds via a free radical mechanism involving initiation, propagation and termination (Gunstone and Norris, 1983).

Initiation: $RH \xrightarrow{-.H} R.$

Propagation: $R. \xrightarrow{+O2} ROO.$

$ROO. \xrightarrow{RH} ROOH + R^{\cdot}$

Termination: $R. + R. \longrightarrow RR$

$R. + ROO. \longrightarrow ROOR$

$ROO. + ROO. \longrightarrow ROOR + O_2$

Initiation involves abstraction of a hydrogen atom from an unsaturated fatty acid (RH) producing a free radical (R•). The initiation step is not fully understood (Gunstone, 1984). Free radical production may occur by action of heat, metals, light and the decomposition of hydroperoxides (Frankel, 1980). Propagation involves interaction of the free radical with oxygen and abstraction of another hydrogen atom by the peroxy free radical. Termination involves the interaction of free radicals to produce non-initiating and non-propagating products.

Initiation and Catalysis

As stated above, the initiation of lipid oxidation is not fully understood. The oxidation reaction has been shown to be catalyzed by both enzymatic and nonenzymatic factors (Rhee, 1988). As lipid oxidation in meat systems is an important issue with regard to flavor, color and safety, several studies investigated the role of various components as catalysts of oxidation. Tappel (1962) reported heme compounds such as hemoglobin, myoglobin and cytochromes to be the dominant catalysts of lipid oxidation in meat systems. The mechanism proposed was that hematin compounds catalyze the decomposition of lipid hydroperoxides into free radicals which could then propagate the free radical chain reaction. This of course depends on the presence of preformed hydroperoxides and thus does not explain the initiation process.

Non-heme iron has also been implicated for its ability to decompose lipid hydroperoxides and form alkoxy radicals which would also propagate lipid oxidation (Ingold, 1962). Sato and Hegarty (1971) studied warmed-over flavor (WOF) development in cooked meats. Their work with water-extracted muscle led them to conclude that catalysis of lipid oxidation was not by heme compounds but by nonheme iron. Results of this study were confirmed by Love and Pearson (1974) who studied oxidation in water-extracted tissue to which various concentrations of purified metmyoglobin (MetMb) (1-10 mg/g) and ferrous iron (Fe^{2+}) (1-4 parts per million (ppm)) were added. They reported no prooxidant or antioxidant effects of MetMb, while concentrations of Fe^{2+} as low as 1 (ppm) resulted in increased thiobarbituric acid (TBA) values. Igene et al. (1979) determined that nonheme iron was the major catalyst of oxidation in cooked meat and meat products and that heme proteins contributed by releasing iron upon heating. Schricker et al. (1982) and Schricker and Miller (1983) also showed that iron was released from the heme complex on cooking, most likely due to oxidative cleavage of the porphyrin ring. The various studies on heme and nonheme iron and their role in lipid oxidation have been extensively reviewed by Love (1983) and Asghar et al. (1988).

Ingold (1962) presented two mechanisms by which iron may act to catalyze oxidation by decomposing hydroperoxides:

$$ROOH + Fe^{2+} \longrightarrow RO\cdot + OH^{-} + Fe^{3+}$$

$$ROOH + Fe^{3+} \longrightarrow RO_2\cdot + H^{+} + Fe^{2+}$$

This together with the theory of Tappel (1962) may explain increased propagation of lipid oxidation in cooked meats (Asghar et al., 1988) but not necessarily how lipid oxidation is initiated in meat with no preformed hydroperoxides. The search for the actual initiators and their mechanisms continues.

A number of mechanisms for initiation of lipid oxidation have been proposed (Table 4).

Table 4. Proposed initiators of lipid oxidation[a]

Proposed initiator	Representative investigators/reviews
Singlet oxygen	King et al. (1975); Kellogg and Fridovich (1977); Foote (1985)
Superoxide radical	Misra and Fridovich (1972); McCord and Petrone (1982)
Hydroperoxyl radical	Uri (1961); Bielski et al. (1983)
Hydroxyl radical	Fong et al. (1976); Gutteridge et al. (1979); Gutteridge (1984); Willson (1984)
Crypto-hydroxyl radical	Youngman (1984)
Perferryl radical	Hochstein et al. (1964); Svingen et al. (1979); Ernster et al. (1982); Morehouse et al. (1983; 1984)
Ferryl radical	Koppenol and Liebman (1984)
Oxygen-bridged di-iron	Tien and Aust (1982); Minotti and Aust (1987)
Porphyrin cation radical	Harel and Kanner (1985a); Roozen (1987

[a] From Asghar et al. (1990)

Some of the more commonly proposed mechanisms will be discussed in this review. The hydroxyl radical (HO·) has received much attention as one such initiator (Asghar et al., 1988). It has been suggested that OH· is able to

abstract hydrogen from unsaturated lipids and thus promote lipid oxidation. Production of HO· occurs by the following mechanisms:

(1) $O_2^{-\cdot} + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$

(2) $2O_2^{-\cdot} + 2H^+ \longrightarrow O_2 + H_2O_2$

(3) $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + HO\cdot$

Steps (1) and (2) represent the Haber-Weiss reaction and step (3) is the Fenton reaction.

Tien and Aust (1982) described lipid oxidation initiated by NADPH-cytochrome p450 reductase and by xanthine oxidase. In either case ADP-Fe^{3+} is reduced by either the reductase or by superoxide radicals ($O_2^{-\cdot}$) to form ADP-Fe^{2+}-O_2. This complex will either react with polyunsaturated fatty acids (PUFAs) to initiate lipid oxidation, or it may be reduced to the ferryl ion (ADP-$[FeO]^{+2}$) which could initiate oxidation. A similar mechanism was also proposed by Ernster et al. (1982).

Kanner and Harel (1985a; 1985b) studied membranal lipid oxidation initiated by hydrogen peroxide (H_2O_2)-activated MetMb. The interaction of hydrogen peroxide and metmyoglobin resulted in an activated species capable of initiating lipid oxidation, whereas neither of the two by themselves was capable of such initiation. This species was

described as a porphyrin cation radical (P^{+}-Fe^{4+}=O). The proposed mechanism is as follows:

$$P^{+}\text{-}Fe^{4+}\text{=O} + RH \longrightarrow P\text{-}Fe^{4+}\text{=O} + R^{\cdot} + H^{+}$$

$$R^{\cdot} + O_2 \longrightarrow ROO^{\cdot}$$

$$P\text{-}Fe^{4+}\text{=O} + ROOH \longrightarrow P\text{-}Fe^{3+} + ROO^{\cdot} + {}^{-}OH$$

$$ROO^{\cdot} + RH \longrightarrow ROOH + R^{\cdot}$$

Since H_2O_2 and $O_2^{-\cdot}$ alone are not capable of initiating oxidation, studies of iron as a more reactive species were undertaken (Minotti and Aust, 1987). In a liposomal model system with Fe^{2+} and H_2O_2, these researchers concluded that Fe^{2+} and Fe^{3+} are required for lipid oxidation and that the rate of reaction is controlled by the Fe^{3+}:Fe^{2+} ratio. They suggested that a Fe^{3+}-dioxygen-Fe^{2+} complex was the initiator.

A number of investigators have reported enzymatic oxidation in muscle microsomes (Hochstein and Ernster, 1963; Lin and Hultin, 1976; Rhee et al., 1984; German and Kinsella, 1985; McDonald and Hultin, 1987). As expected, a number of mechanisms have been proposed.

German and Kinsella (1985) explained that fish microsomal lipid oxidation was initiated by lipoxygenase located in the skin. Lipoxygenase would initiate free radical production and thus accelerate lipid peroxidation. Lipoxygenase has also been implicated as a contributor to oxidation in chicken meat on frozen storage as the activity of the enzyme was only slightly lowered by freezing (Grossman et al., 1988).

Rat liver microsome lipid oxidation was studied by Hochstein and Ernster (1963). The mechanism involved NADPH-induced oxidation with activation by ADP and iron. This occurs coupled to the NADPH oxidase system of microsomes.

An enzymatic system for oxidation in chicken skeletal muscle was described by Lin and Hultin (1976). This mechanism was similar to that proposed by Hochstein and Ernster (1963) yet was tested in skeletal muscle. Also NADH was found to work in the reaction although at a lower rate (preferred NADPH). This enzymatic reaction was decreased by heat and by the detergent sodium deoxycholate. This system was also described in beef skeletal muscle (Rhee et al., 1984).

Site of Oxidation

Phospholipids have been implicated as the primary site of attack for initiation of lipid oxidation in meat systems (Igene and Pearson, 1979). The actual contributions of triacylglycerols to oxidative flavor development was not as significant. This led to the suggestion that lipid oxidation is initiated in muscle membranes as opposed to adipose tissue. That lipid oxidation in meats is initiated in membranes has been supported by a number of observations.

Slabyj and Hultin (1984) extracted lipids from herring muscle and prepared a lipid emulsion to study the influence of a microsomal fraction on the oxidation of the emulsion system. This was based on prior knowledge of a reported enzymic oxidation system in the microsomal membrane

(McDonald et al., 1979; Slabyj and Hultin, 1982). Oxidation of the emulsion lipids was dependent on the presence of this enzymic membrane system. This enzymic system capable of initiating oxidation in membranes such as the microsomes is not present in the adipose tissue.

Phospholipids in meat are located primarily in muscle membranes (Love and Pearson, 1971). The observations of Corliss and Dugan (1970) and Igene et al. (1981) that the concentrations of phospholipids decline significantly on oxidation lends support to the hypothesis that initiation occurs in the membranes.

The susceptibility of polyunsaturated fatty acids (PUFAs) to oxidation increases with increasing sites of unsaturation (Moerck and Ball, 1974). Phospholipids typically are highly unsaturated. The phospholipid fractions studied by Igene et al. (1981) were 15.5% polyunsaturated, while no PUFAs were found in the triacylglycerol fractions of fresh beef and only 1.96% in fresh white chicken meat. Thus, it would be reasonable to expect oxidative initiation to occur more readily in membranes as opposed to adipose tissue.

Sato and Hegarty (1971) reported large increases in TBA values for raw ground beef within an hour after grinding. They proposed that disruption of muscle membranes results in exposure of lipids to not only oxygen but also to reaction catalysts.

The above studies support the view that lipid oxidation is initiated in membranes and does not initially involve adipose tissue lipids. If this is so, the susceptibility of muscle tissue to lipid oxidation should not depend on the fat content of the meat per se. Lipid oxidation in membranes will thus occur in low-fat meat and meat products. However, little work has been done on such low-fat products. The stability of cholesterol in such products also deserves attention.

Cholesterol Oxidation in Meat Systems

Mechanism

Interest in cholesterol oxidation has increased because of the many associations of cholesterol oxide consumption with various health disorders (Smith, 1981; Addis et al., 1983). Cholesterol oxidation is initiated by abstraction of hydrogen leading to hydroperoxide formation by a free radical reaction as presented previously for lipid peroxidation (Maerker, 1987). These hydroperoxides are unstable and are eventually converted to more stable products. A scheme for cholesterol oxidation is presented in Figure 1. More than 70 products of cholesterol oxidation have been reported (Smith, 1980).

Smith (1980) proposed two free radical mechanisms for cholesterol autoxidation. These represent hydrogen abstraction and formation of peroxy radicals at C-7 or in

the side chain (Equation 1) and formal alcohol dehydrogenation at C-3 with peroxy free radical formation (Equation 2).

Equation 1: $RH + O_2 \longrightarrow ROOH$

Equation 2: $RCH(OH) + O_2 \longrightarrow RC{=}O + H_2O_2$

In Equation 2 the C of RC=O represents the number 3 carbon of the A ring of cholesterol. This review by Smith (1980) details these reactions as well as several subsequent reactions. The most likely site for hydrogen abstraction is at C-7, a carbon adjacent to the double bond (Maerker, 1987). Side chain oxidation and oxidation of the A ring is less favorable. The most predominant cholesterol oxides therefore are 7-α-hydroxycholesterol, 7-β-hydroxycholesterol and 7-ketocholesterol.

Site of cholesterol oxidation

Cholesterol in lean meat tissues is principally located in the membranes (Pearson et al., 1983). When muscle is cooked, phospholipid levels have been shown to decline (Igene and Pearson, 1979). This decline, coupled with a decline in PUFAs has led to the suggestion that these substances are involved in WOF development. However, the susceptibility of cholesterol in these membranes to oxidation has not been adequately studied. Being in such close contact with other unsaturated lipid components (such as phospholipids) may have great impact on the mechanistic

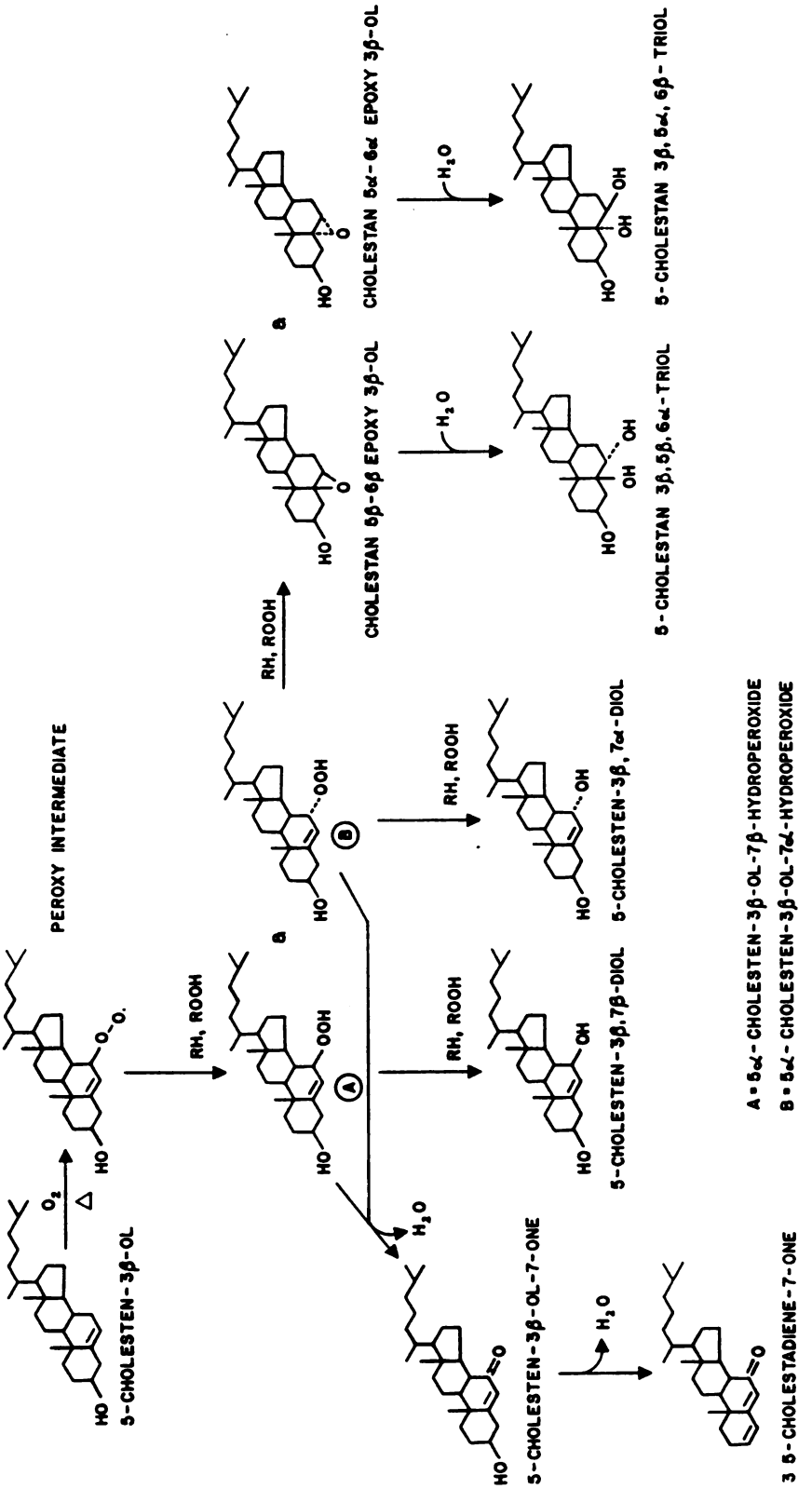


Figure 1. Scheme for cholesterol oxidation

features of cholesterol oxidation in meat systems. Factors influencing PUFA and phospholipid oxidation should be investigated for their role in cholesterol oxidation.

Inhibition of Lipid Oxidation in Meat Systems

Control of lipid oxidation in meat systems has been accomplished by a number of different substances. These include nitrite, phosphates and other metal chelators, ascorbate, various antioxidants, Maillard reaction products, smoking of meat and possibly a variety of extenders and fillers (Gray and Pearson, 1987). Packaging of oxidizable materials, such as meats, may also be used as a means of controlling oxidation and extending the shelf life of the product (Harte and Gray, 1987).

Packaging

Oxidation of lipids may be controlled by various packaging techniques such as vacuum packaging, gas flushing, shrink and skin packaging, and use of barriers to oxygen and light (Harte and Gray, 1987).

Nolan et al. (1989) demonstrated the beneficial effects of vacuum packaging in retarding lipid oxidation in cooked pork and turkey as measured by TBA and sensory analyses. These researchers also studied modified atmosphere packaging and found storage in 100% CO_2 and 100% NO_2 resulted in less oxidation than that occurring in samples stored in air, yet not as protected against oxidation as in vacuum packaging.

Hwang et al. (1990) conducted a similar study with cooked beef stored in air, vacuum packaging or modified atmosphere (80% N_2, 20% CO_2). Samples held in the freezer under modified atmosphere had similar TBA values, hexanal contents and sensory scores to frozen, vacuum packaged samples. Those samples packaged in air were much more oxidized.

Nitrites, Phosphates and Ascorbate

In cured meat products, nitrites, phosphates and ascorbate each play a role in inhibiting oxidation (Pearson et al., 1977). The protective effect of nitrites on meat lipids was demonstrated by Sato and Hegarty (1971). These investigators completely inhibited oxidation using 2 mg/g $NaNO_2$ in cooked ground beef. Several investigators have confirmed the antioxidative properties of nitrite and several mechanisms of action have been proposed (Zipser et al., 1964; Kanner et al., 1980; MacDonald et al., 1980; Gray et al., 1981; Kanner et al., 1984; Igene et al., 1985; Freybler et al., 1989). The theory that nitrite forms a complex with an iron porphyrin in heat denatured meat to form nitric oxide hemochromagen was proposed by Zipser et al. (1964). Pearson et al. (1977) suggested that nitrite stabilizes the lipid containing membranes. That nitrite may act in the quenching of free radicals was proposed by Kanner et al. (1980; 1984). Igene et al. (1985) proposed three mechanisms for antioxidant activity of nitrite. Nitrite may form a complex with heme compounds to prevent release of

iron in cooking, stabilize unsaturated lipids in membranes or interact with metal ions to prevent their catalysis of oxidation. More recently, Freybler et al. (1989) demonstrated that nitrite acts by forming a nitroso-nitrosite derivative with the unsaturated fatty acids and also by stabilization of heme to prevent the release of iron.

Phosphates act as antioxidants by chelating metal ions, such as iron, a major catalyst of lipid oxidation (Tims and Watts, 1958). Other chelators, such as citric acid, exist which are also effective against oxidation. Benedict et al. (1975) reported a slight reduction in lipid oxidation of ground beef with addition of citric acid (0.005%). Sato and Hegarty (1971) also reported slight inhibition of oxidation in cooked ground beef upon addition of sodium citrate (5 mg/g). Sato and Hegarty (1971) found greater inhibition of oxidation in the cooked ground beef samples with EDTA (2.5 mg/g). EDTA is a powerful chelator but is not approved for human consumption (Gray and Pearson, 1987).

Ascorbic acid has been shown to act by a number of mechanisms. It has been proposed to maintain reducing conditions as well as to act as an oxygen scavenger (Sato and Hegarty, 1971). At low concentrations (<100 mg/kg) ascorbate was found to act as a prooxidant (Sato and Hegarty, 1971). At higher concentrations (>1000 mg/kg), it acted as an antioxidant. This behavior was suggested to be due to the fact that at low concentrations ascorbic acid

acts to maintain a portion of iron in the ferrous state. However, at higher concentrations it may upset the balance between ferrous and ferric forms (Sato and Hegarty, 1971). Ascorbate has been shown to interact with vitamin E in prevention of oxidation by regenerating vitamin E from its radical form (Packer et al., 1979).

Maillard Reaction Products

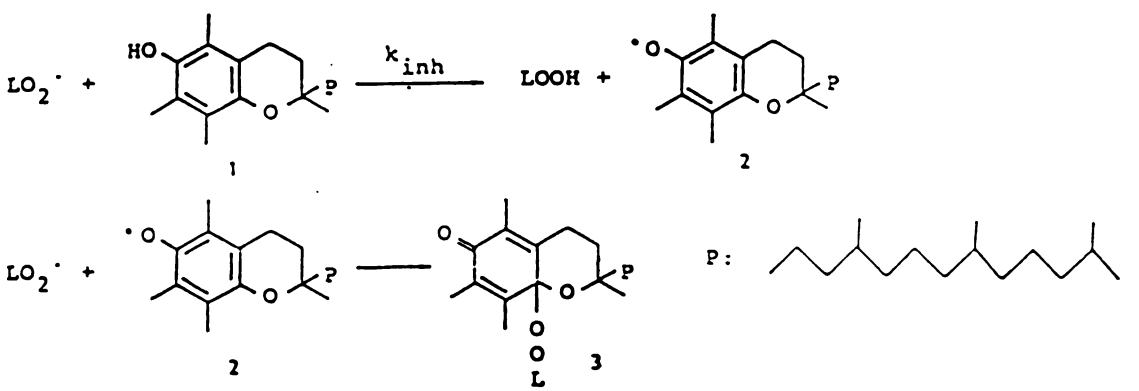
Maillard reaction products also have antioxidant properties which were reviewed by Bailey (1988). Development of such products as antioxidants is desirable because they are derived from naturally occurring substances and impart desirable flavors to meats.

Antioxidants

Antioxidants, some of which have already been discussed, may be either naturally occurring or synthetic (Pearson and Gray, 1983). Use of these substances in meat systems has resulted in varying degrees of inhibition of oxidation. Benedict et al. (1975) added solutions of various naturally occurring antioxidants (0.018% of total lipid) to ground beef and stored samples at -1.1^{o}C for 10 days. They reported slight inhibition of oxidation with most antioxidants (α-tocopherol, ascorbyl stearate and citric acid) with the exception of ascorbate. Several synthetic and natural antioxidants were added to ground pork prior to cooking to measure their ability to retard lipid oxidation as indicated by TBA numbers (Shahidi et al., 1987). Of some of the commonly encountered synthetic

antioxidants, BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), TBHQ (tert-butylhydroquinone), and PG (propyl gallate) were effective in reducing oxidation at 200 ppm. Watts (1961) demonstrated the problem of lipid soluble antioxidant usage in intact meats. Phenolic antioxidants were more effective in ground tissue than in intact tissue due to the difficulty of distributing the antioxidant to the proper location.

Vitamin E is a powerful antioxidant and functions as a lipid-soluble free radical scavenger (Linder, 1985). Several tocopherol structures exist. The form with the highest biological activity (α-tocopherol) will be considered in this discussion. A hydrogen from an -OH group of α-tocopherol is donated to a peroxy radical to stop free radical propagation, i.e.,



Some of the mechanistic features of vitamin E action as an antioxidant in solution have been clarified, however, the role of vitamin E as an antioxidant in membranes remains unclear (Niki, 1987). Several questions pertaining to its transport, incorporation, location and inhibition of oxidation in membranes need to be answered. This

information would be particularly important in controlling oxidation in muscle tissue as it is initiated in the membranes (Gray and Pearson, 1987).

Alpha-Tocopherol Supplementation in Diets of Food Animals

The fact that α-tocopherol is a naturally occurring, lipid soluble antioxidant makes it very desirable for use in retarding lipid oxidation in meat systems. Synthetic antioxidants (such as BHA and BHT) may be very powerful antioxidants yet receive much criticism due to the fact that they do not occur naturally in foods (Bailey, 1988). Today's consumers are concerned about the use of synthetic chemicals in food processing; thus, there is a move towards using natural ingredients for greater acceptance (Bailey, 1988).

In cells, α-tocopherol is located primarily in the membranes where it functions to maintain intracellular membrane integrity (Diplock, 1985). Alpha-tocopherol acts as a free radical scavenger and thus protects against lipid oxidation (Linder, 1985). One hypothesis is that α-tocopherol is situated in the membranes such that the chromanol ring is at the polar surface and the phytol side chain is in contact with the polyunsaturated fatty acids of the phospholipids in the nonpolar interior (Molenaar et al., 1980). This positioning of α-tocopherol would allow effective radical scavenging as it would be adjacent to the membrane-bound enzymes such as NADPH oxidase, which generates free radicals.

McKay and King (1980) have proposed that hydrogen peroxide, generated by superoxide interacting with hydrogen in the cell, is distributed in both the membranous and aqueous phases. Glutathione peroxidase, a selenium-containing enzyme that is a primary defense against lipid oxidation, destroys hydrogen peroxide in the aqueous phase. That remaining in the membranous phase may react with superoxide anions to produce hydroxyl free radicals, which may be potent catalysts for oxidation of membranal lipids. The α-tocopherol present may prevent lipid oxidation catalyzed by the hydroxyl radical by donating a hydrogen. This is demonstrated in Figure 2.

As an essential nutrient, α-tocopherol is not synthesized by the body and therefore must be consumed in the diet (Linder, 1985). It has been shown that α-tocopherol concentrations in animal tissues can be increased by dietary supplementation (Buckley and Connolly, 1980; Faustman et al., 1989b; Lin et al., 1989). If initiation of oxidation occurs in membranes, dietary supplementation of vitamin E to increase membrane levels should improve the stability of muscle lipids to oxidation. Incorporation of α-tocopherol into the muscle prior to slaughter by dietary supplementation with vitamin E has a more beneficial antioxidative effect than addition of α-tocopherol to meats just prior to processing. This is expected, as it is difficult to distribute lipid soluble antioxidants to the sites of lipid oxidation in intact meats (Watts, 1961;

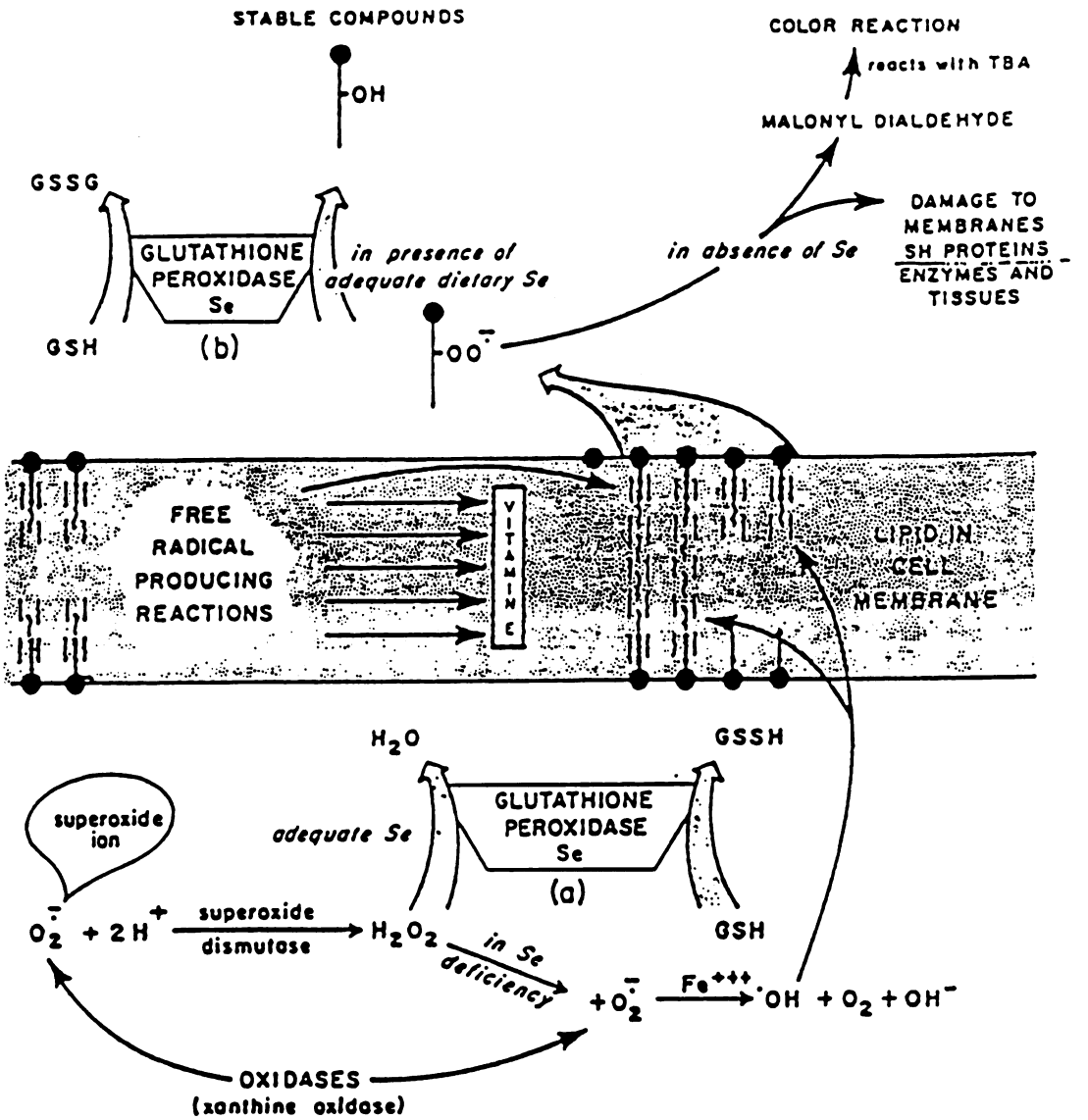


Figure 2. Protection of membranal lipids by α-tocopherol (vitamin E) (From Machlin, 1984)

Faustman et al., 1989b). Also, free radical scavengers are most effective during the initial stages of oxidation so that a build-up of hydroperoxides can be prevented.

Stabilization of lipids by dietary supplementation of vitamin E has been shown in a variety of food-producing animals, including poultry (Marusich et al., 1975; Lin et al, 1989), pigs (Buckley and Connoly, 1980), cattle (Faustman et al., 1989a; 1989b), and calves (Ellis et al., 1974; Shorland et al., 1981). Some of the more pertinent studies, particularly those in beef and veal muscle, will be discussed.

Improvement in the stability of red color in sirloin steaks as determined by Hunter colorimeter values was noted as a result of supplementation with 370 IU vitamin E/day in diets of Holstein steers (Faustman et al., 1989a). The fresh reddish color of meat has been shown to deteriorate to a brownish color due to oxidation of myoglobin to metmyoglobin (Faustman and Cassens, 1989). The exact connection with lipid oxidation is unclear (Greene et al., 1971). It has been noted that pigment oxidation occurs with lipid oxidation, but there is uncertainty whether lipid oxidation promotes this pigment oxidation or whether pigment oxidation promotes lipid oxidation. The other possibility is that they are co-oxidized. Whatever the case, deterioration of fresh meat color is often used as an indicator of lipid stability. Faustman et al. (1989b) also reported more than two-fold greater tissue levels of α-

tocopherol in supplemented animals than in controls. Greater pigment and lipid stability were noted in the supplemented animals due to absorption of α-tocopherol and incorporation into cellular membranes where it functions as an antioxidant.

Results of studies with veal have followed similar trends. Oxidative stability of rendered veal fat from calves fed milk/milk starter supplemented with vitamin E was greater than that of veal and pork fat from commercially reared, unsupplemented animals (Ellis et al., 1974). Also demonstrated was a protective effect of α-tocopherol on lipid stability in fat from animals fed high levels of linoleic acid (C18:2) (approximately 14% of the lipid) as compared to the other group whose diet contained approximately 3% C18:2 of the lipid. Both groups received dietary vitamin E (level not reported). Because of the greater degree of unsaturation present in fat from animals with increased C18:2, it was expected that the fats from these animals would have shorter induction periods. In the presence of α-tocopherol this reduction in induction period did not occur.

Shorland et al. (1981) also studied dietary vitamin E supplementation in veal calves and the resultant lipid stability and composition. Concentrations of α-tocopherol were increased by supplementation of 500 IU vitamin E/day. Vitamin E supplementation affected the fatty acid composition and increased the amount of stearic acid

present. A beneficial effect of vitamin E supplementation was found with regards to lipid oxidation, however, the effect was not consistent from tissue to tissue.

Reddy et al. (1987) determined the optimal dietary requirement of Holstein heifer calves from birth to 24 weeks based on overall performance, which included weight gain, feed consumption, fecal scores, enzymatic activity such as creatine kinase, glutamic oxalacetic transaminase and lactic dehydrogenase (indicators of cell membrane damage), other serum metabolites and hematological values. Conventional diets were supplemented with 0, 125, 250 and 500 IU of vitamin E. Based on the above criteria, these researchers concluded that supplementation of 125 and 250 IU vitamin E per day increased overall performance of these calves.

The above studies on lipid stability on beef and veal did not investigate membrane lipid composition or stability, which is very important with respect to the initiation of lipid oxidation. Studies by Lin et al. (1989) and Asghar et al. (1990) have demonstrated incorporation of tocopherol in membranes and improved stability of the membranes towards oxidation upon dietary supplementation with α-tocopheryl acetate.

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CHAPTER ONE

TOTAL CHOLESTEROL DETERMINATION - EVALUATION OF ANALYTICAL METHODS AND SURVEY OF MEAT PRODUCTS

Abstract

Literature values for total cholesterol content in meat vary widely. Variation may be a result of several factors including diet, sex and age of the animals and analytical methodology. The primary objective of this study was to evaluate methods for total cholesterol determination for accuracy, efficiency and repeatability and to apply the chosen method to survey a variety of meats for cholesterol content and changes in total cholesterol with cooking.

An enzymic procedure, a colorimetric method and a number of gas-liquid chromatographic (GLC) techniques were compared. The method chosen as most desirable was a direct saponification procedure without derivatization, followed by GLC analysis with a packed column (1% SE 30 on 100/120 mesh Gas Chrom Q). Total cholesterol in beef bottom round was 50-60 mg/100 g sample. This method was then used to survey a variety of meats for total cholesterol content before and after cooking. Values agreed well with those reported in the literature. Total cholesterol values were higher for cooked than raw meats on a per gram basis. However, adjustment based on moisture content resulted in similar cholesterol values.

Introduction

Several methods for total cholesterol determination in foods have been reported in the literature. These methods, which include enzymatic (Karkalas et al., 1982), colorimetric (Schoenheimer and Sperry, 1934; Searcy and Berquist, 1960; Rhee et al., 1982a), gas chromatographic (Punwar, 1975; A.O.A.C., 1984; Adams et al., 1986) and high performance liquid chromatographic (Newkirk and Sheppard, 1981) procedures, have been applied to a variety of foods with a high degree of variation in the results reported. Sweeny and Weihrauch (1976) reviewed the earlier methods for cholesterol determination and pointed out the lack of available and reproducible analytical data.

Variation in the reported total cholesterol content of meat products may be the result of several factors such as diet, sex and age of the animals, and analytical methodology, including poor reporting of the procedures applied (Sweeny and Weihrauch, 1976). The problem of obtaining accurate and reproducible cholesterol information is intensified with increased public awareness and interest in the cholesterol content of food (Karkalas et al., 1982).

A number of practices have been promoted as a means of improving the nutritional quality of various meats, such as trimming away excess fat prior to cooking and selecting lean meats with little marbling. Although these practices would reduce the fat intake from such sources, it is of interest to determine whether cholesterol intake would also be

affected. Thus, information on cholesterol concentrations in cooked and trimmed meats as well as in meats of various fat contents is very important.

Cooking has been shown to decrease the cholesterol content of meats (Kritchevsky and Tepper, 1961). This has been partly attributed to cholesterol oxidation and to cholesterol loss in the drip (Tu et al., 1967). Several researchers (Rhee et al., 1982a; Prusa and Hughes, 1986; Hoelscher et al., 1988) found increases in cholesterol with cooking and attribute these not to an increase in cholesterol but a concentration of the cholesterol present as a result of moisture loss. There was an increase in cholesterol as a percentage of cooked weight. Kregel et al. (1986) investigated cooking effects on cholesterol concentrations using ground beef of three different fat contents. Although the cholesterol concentration was different in the raw beef according to fat content (i.e., the least fat sample had the least cholesterol), there was no difference in the cooked samples. These researchers concluded that the consumer did not decrease cholesterol consumption by choosing leaner ground beef. Berg et al. (1985) studied the effects of trimming meat and indicated that samples which were not trimmed had less moisture loss and greater cholesterol contents than trimmed cooked samples.

The primary objective of this study was to evaluate some existing methods for total cholesterol determination in order to find the most accurate, efficient and reproducible method and to apply the chosen method in surveying a variety of meats for cholesterol content and changes in total cholesterol with cooking.

Materials and Methods

Materials

Meat and seafood samples were purchased from a local supermarket on the day of analysis. Cholesterol and 5 α-cholestane standards were purchased from Steraloids Inc., Wilton, NH.

Moisture and Fat Content

Moisture content of the meat samples was determined by the A.O.A.C. official method (1984). Total lipid content was determined by the method of Folch et al. (1957).

Evaluation of Methods for Total Cholesterol Determination

Ground raw beef bottom round was used in the first part of this study in an evaluation of several methods for determining total cholesterol. Methods evaluated included an enzymatic procedure (Boehringer Corp., Indianapolis, IN), a colorimetric method (Rhee et al., 1982a) and two gas-liquid chromatographic (GLC) techniques (A.O.A.C., 1984; Adams et al., 1986). Each of these methods will be described here in detail.

Enzymatic

Enzymatic cholesterol determination was carried out using a kit obtained from Boehringer Corp., Indianapolis, IN. Cholesterol was oxidized to cholestenone by cholesterol oxidase with production of hydrogen peroxide. Catalase was added to facilitate the oxidation of methanol to formaldehyde, which was then reacted with acetylacetone to form a yellow lutidine dye in the presence of ammonium ions. Color was determined spectrophotometrically (Bausch & Lomb Spectronic 2000, Rochester, NY) at 405 nm.

Colorimetric

Colorimetric determination of cholesterol was done by the method of Rhee et al. (1982a). A Folch lipid extract (Folch et al., 1957) was saponified, followed by a hexane extraction of the unsaponifiable fraction. Cholesterol content was determined by the colorimetric procedure of Searcy and Berquist (1960), which involved the reaction of $FeSO_4$-acetic acid and concentrated H^2SO_4 to produce color to be spectrophotometrically determined at 490 nm.

Gas-Liquid Chromatographic

Two methods, a direct saponification procedure (Adams et al., 1986) and the official method (A.O.A.C., 1984), were evaluated. The A.O.A.C. official method (1984) involved lipid extraction prior to saponification. The procedure was modified slightly to use a Folch extraction (Folch et al., 1957). Following extraction, 100 ml of the lipid extract were filtered through anhydrous Na_2SO_4. The Na_2SO_4 was

rinsed with chloroform and the extract was evaporated under nitrogen in a steam bath. The residue was dissolved in petroleum ether (boiling range: 35-80^{o}C) and filtered through anhydrous Na_2SO_4, rinsed and dried again under nitrogen in a steam bath. The sample was saponified in a refluxing system containing 8 ml concentrated KOH and 40 ml reagent alcohol. This mixture was heated, with stirring for an hour. Then, 60 ml reagent alcohol were added and the sample was cooled to room temperature. Extraction of the unsaponifiables was done using 100 ml benzene which was slowly added and then vigorously stirred. Because of the hazardous nature of benzene this work was done wearing gloves and performed in a fume hood. The contents were transferred into a 500 ml separatory funnel and 200 ml 1 N KOH was added. After shaking for 10 seconds the layers were allowed to separate and the lower layer was discarded. The benzene layer was washed with 40 ml 0.5 N KOH, rotated gently for 10 seconds, and the lower layer was discarded. The benzene layer was then washed with water 4 times, each time discarding the lower layer. The benzene extract was dried over anhydrous Na_2SO_4; after which a 50 ml aliquot was removed and evaporated to dryness on a rotary evaporator (Brinkmann Instruments, Inc., Westbury, NY). Three ml of acetone were added and again rotary evaporated to dryness. The residue was finally dissolved in 3 ml dimethylformamide.

The method of Adams et al. (1986) involved direct, accurate weighing of the ground meat sample (3-5 g) into a refluxing flask to which 8 ml 50% KOH and 40 ml reagent alcohol were added. This mixture was saponified while refluxing and stirring for 1 hr. Sixty ml of reagent alcohol were added and the mixture was cooled. Toluene (100 ml) was added and the mixture was vigorously stirred for 1 min. In a 500 ml separatory funnel, the mixture was extracted with 110 ml 1 N KOH, shaking vigorously for 30 sec and discarding the lower aqueous layer upon separation. To the toluene layer was added 40 ml 0.5 N KOH and the separatory funnel was rotated gently. Again the lower layer was discarded and the extract was washed five times with water (100 ml each) with increasing degrees of agitation. The toluene extract was dried over anhydrous Na_2SO_4 as above. A 50 ml aliquot was removed and rotary evaporated, washed with acetone, dried, and redissolved in 3 ml dimethylformamide.

Extracts prepared by each method were analyzed in either derivatized (silylated) or underivatized form. Derivatization was done according to the method of Adams et al. (1986) using hexamethyldisilazane and trimethylchlorsilane. GLC analysis was carried out using capillary (polydimethylsiloxane-15 m x 0.25 mm) and packed (1% SE-30 on 100/120 Gas Chrom Q) columns. A Hewlett Packard 5890A gas chromatograph (GC) (Hewlett Packard Co., Avondale, PA) at 190^{o}C for 9.5 min and programmed at

20°C/min to 260°C for 30 min, was used for the capillary analyses. Packed column determinations were achieved with a HP 5840A GC using isothermal conditions (230°C for 20 min).

Survey of Meat Products

The method chosen to be most accurate, efficient and reproducible was then applied to a variety of meat and seafood products. Cholesterol content was determined before and after cooking. Samples of ground beef, pork loin chops, lamb shoulder blade chops and veal shoulder blade steak were broiled in a conventional electric oven to an internal temperature of 170°F (measured using a copper constantan thermocouple: Omega Engineering, Inc.). Chicken breasts and leg quarters (both with skin on) were baked at 350°F to an internal temperature of 170°F. Salmon was baked under similar conditions, while shrimp was peeled and boiled for 5 min.

Statistical Treatment of Data

Statistical analyses were carried out using MSTAT-C (Microcomputer Statistical Program, Michigan State University, East Lansing, MI). For evaluation of different methods a randomized complete block factorial design was developed for factor A (method), with factors B (packed column vs capillary column) and C (derivatized or non-derivatized) as split plots on A. Statistical significance was declared at $P<0.05$.

Results and Discussion

Evaluation of Methods

Methods for total cholesterol determination were evaluated in the first part of this study. Beef bottom round samples were analyzed by each of the following methods.

Colorimetric

Colorimetric analysis by the method of Rhee et al. (1982b) was found to be time consuming and quite difficult, with low reproducibility. Extreme care must be taken in such colorimetric procedures, especially consistency in mixing after the addition of sulfuric acid to the cholesterol extract. Inconsistent temperatures may provide variable results (Tonks, 1967). Bubble formation must also be avoided as this will interfere with spectrophotometer readings. Sweeny and Weihrauch (1976) have expressed other concerns with such colorimetric procedures including color reactions with other sterols and possibly other compounds, time dependence and moisture effects.

Enzymatic

This procedure provided reproducible data, i.e., duplicate extractions gave similar absorbance readings. The value obtained with sample alone was consistent with literature reports for beef round. This procedure involved saponification (approximately 35 min) and one hour color development followed by cooling. The procedure therefore was not very rapid and was quite expensive. Karkalas et al.

(1982) compared this procedure to GLC analysis and found the results to be very similar, especially with animal products. Karkalas et al. (1982) also noted that the enzymatic procedure will not be as accurate for foods containing vegetable oils as there may be interference from the phytosterols in the vegetable oils.

Gas-liquid Chromatographic

Based on the results of the above analyses, the main emphasis of this study was placed on GLC analysis. Upon evaluation of the results obtained for beef bottom round samples, the method chosen as most efficient and reproducible was direct saponification (Adams et al., 1986), without derivatization, followed by analysis on the packed column system. Studies comparing samples with and without spiked cholesterol indicated that the recovery was 85-95%. Results from the samples of both extraction techniques with or without derivatization, and packed versus capillary systems were quite similar (Table 1). The greatest difference observed was between samples analyzed on packed versus capillary columns ($P<0.05$). Slight differences were noted as a result of the extraction technique, where the A.O.A.C. method which involves chloroform-methanol extraction of the beef lipids gave cholesterol values which were somewhat lower than those obtained by direct saponification.

Table 1. Cholesterol concentrations (mg/100 g) of beef bottom round determined by capillary or packed GLC systems in either derivatized or non-derivatized form as extracted by the A.O.A.C. (AOAC) or the direct saponification (DS) method[a,b]

	Capillary		Packed	
	Deriv.	Non-deriv.	Deriv.	Non-deriv.
AOAC	49 ± 1.7[c]	52 ± 3.4[c]	49 ± 1.7[d]	49 ± 3.1[d]
DS	57 ± 2.9[c]	54 ± 3.0[c]	49 ± 2.1[d]	51 ± 1.5[d]

[a] Each value represents the mean of at least 3 samples ± standard error

[b] Means with different superscripts are significantly different ($P<0.05$)

Kovacs et al. (1979) discussed the benefits of direct saponification over saponification of a lipid extract as in the A.O.A.C. official method (1984). Not only is it less time consuming, there also is the advantage that the cholesterol may be more efficiently extracted since cholesterol in muscle membranes is bound amongst phospholipids and proteins and may not be completely extracted in a chloroform-methanol lipid extraction.

The silylation process is undesirable as it involves an additional reaction step and is therefore more time-consuming. Also, the chromatograms from the packed column system were less complex and easier to interpret (Figures 1 and 2). The analysis time on the packed column system was also shorter (approx. 20 min) in comparison to that on the capillary system (approx. 40 min).

Survey of Meat Products

The second part of this study involved application of the direct saponification method, without derivatization, to meat and seafood samples for analysis by packed column GLC. Total cholesterol, as well as lipid and moisture contents were determined before and after cooking. These values are presented in Table 2. Lipid and moisture values and cook loss percentage are given in order to relate cholesterol to dry weight and fat content. Each value in Table 2 represents the means of duplicate samples obtained on two different days from the supermarket. Even though there were duplicate extractions done on each and duplicate injections

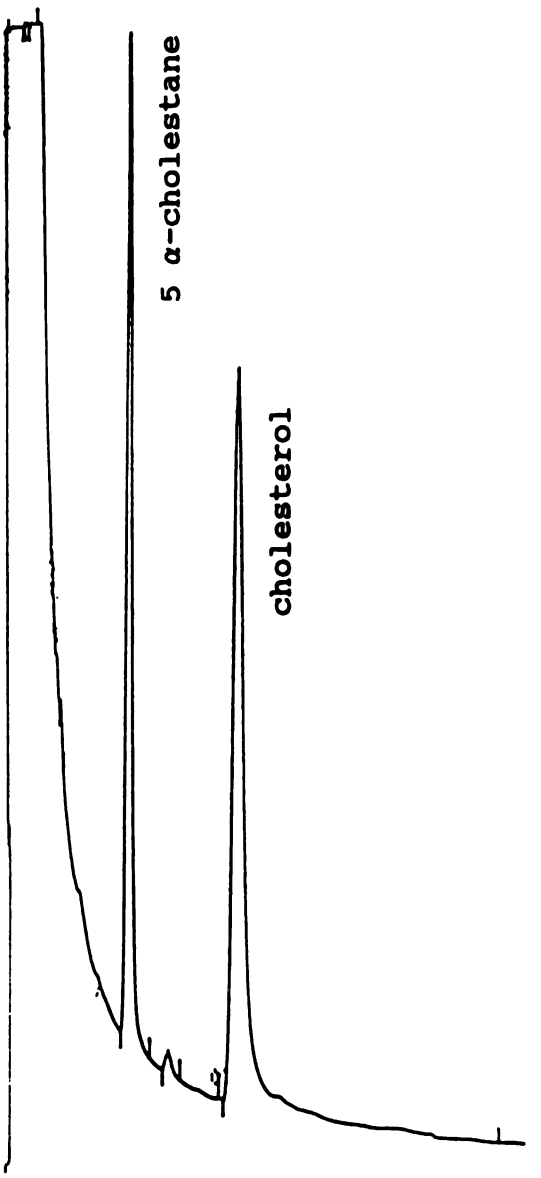

Figure 1. Packed column gas chromatogram of beef round cholesterol extract

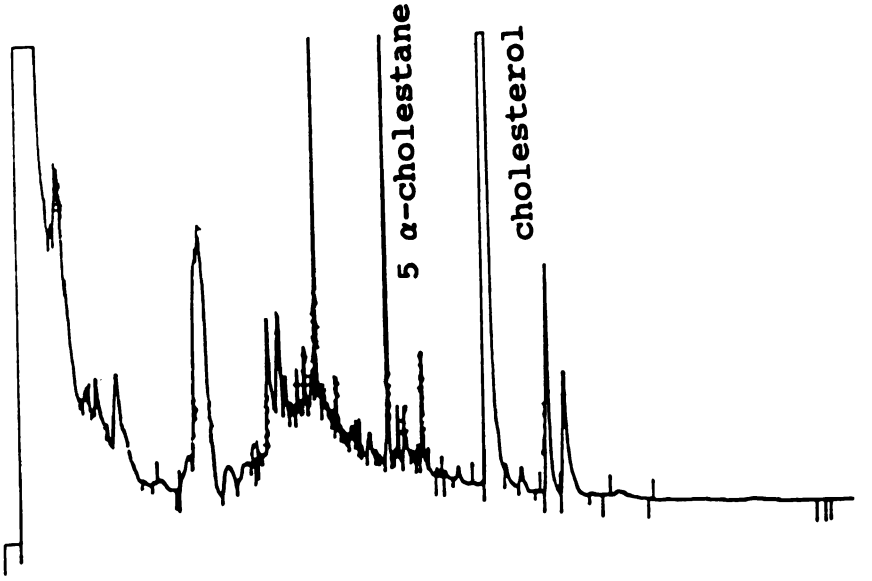

Figure 2. Capillary column gas chromatogram of beef round cholesterol extract

Table 2. Cholesterol contents of various muscle foods as determined by the direct saponification - gas chromatographic procedure[a]

Food	Moisture (%)		Cook Loss (%)	Lipid (%)		Cholesterol (mg/100g)	
	Raw	Cooked		Raw	Cooked	Raw	Cooked
Beef round	70			6		62	
Ground beef	70	57	34	9	11	59	109
Veal steaks	74	68	22	6	6	184	189
Pork chops	71	59	25	7	14	60	87
Lamb chops	62	51	25	22	28	99	139
Chicken-dark	72	65	14	12	18	123	138
Chicken-white	71	66	15	8	11	86	105
Shrimp	79	73	30	1	2	262	270
Salmon	66	65	16	7	18	100	150

[a] Mean of dupllicate samples

done on each extract, the greatest source of variability would be expected between samples collected at the supermarket on different days. It is not possible to do statistics on these two samples.

Cholesterol values obtained in this study generally agree with the values reported by a number of investigators including Kritchevsky and Tepper (1961), Tu et al. (1967), Karkalas et al. (1982), Rhee et al. (1982a; 1982b) and Prusa and Hughes (1986). These literature values were obtained by a number of methods including those investigated in the present study.

Total cholesterol in the cooked products increased in all cases. This increase was expected, as there would be concentration of cholesterol after drip loss. Only a few reports of changes in total cholesterol with cooking have been published (Kritchevsky and Tepper, 1961; Karkalas et al., 1982; Rhee et al., 1982a; 1982b; Kregel et al., 1986; Prusa and Hughes, 1986; Hoelscher et al., 1988). A report of decreased cholesterol upon cooking by Kritchevsky and Tepper (1961) was explained as due to cholesterol oxidation and loss of cholesterol in the drip (Tu et al., 1967). Most of the other reports found increases of cholesterol upon cooking (Rhee et al., 1982a; Prusa and Hughes, 1986; Hoelscher et al., 1988). They concluded that although cholesterol did not actually increase in cooked meat, there

was an apparent concentration of cholesterol as a result of cook loss. Thus, cholesterol increased as a percentage of cooked weight.

Karkalas et al. (1982) investigated changes in the cholesterol content of chicken with cooking. Based on fresh weight, they observed an increase in the cholesterol content of white meat (67 mg/100 g raw meat; 80 mg/100 g cooked meat) and a decrease in dark meat (107 mg/100 g to 92 mg/100 g). Data obtained in this study showed similar trends when corrected for cook loss (white meat 86 mg/100 g to 90 mg/100 g; dark meat 123 mg/100 g to 119 mg/100 g).

Prusa and Hughes (1986) compared cholesterol levels in pork tenderloin steaks upon cooking by different methods. The change in cholesterol content upon conventional cooking to an internal temperature of 77^{o}C was from 45 mg/100 g to 78 mg/100 g. This was comparable to the change obtained in this study (60 mg/100 g to 87 mg/100 g).

Summary and Conclusions

Several methods for determining the total cholesterol content of muscle foods were evaluated. The method chosen as most desirable was a direct saponification procedure without derivatization (silylation), followed by gas chromatographic analysis with a packed column (1% SE-30 on 100/120 mesh Gas Chrom Q). This method was direct, gave reproducible results and was simple to perform. This method was used to survey a range of meat products for total

cholesterol content before and after cooking. Total cholesterol values were higher in the cooked products. Values in general were comparable to those reported in the literature.

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CHAPTER TWO

RELATIONSHIP OF MITOCHONDRIAL AND MICROSOMAL MEMBRANE FATTY ACID COMPOSITION AND OXIDATIVE STABILITY TO OVERALL OXIDATIVE STABILITY OF MUSCLE LIPIDS

Abstract

The relationship of membranal lipid composition to the oxidative stability of beef, pork and chicken was investigated. The neutral lipids of beef and pork muscle contained more saturated fatty acids than those of chicken. Cooked pork oxidized to a greater extent than beef, while dark chicken underwent more oxidation than white chicken.

Membranal lipid fatty acid composition was similar to that of the muscle fractions. There was a higher degree of unsaturation in microsomal fatty acids than those from mitochondria. This explained the trend for greater oxidation occurring in microsomal lipids. Trends in membranal oxidation were parallel to those in muscle. Dark chicken oxidized to the greatest extent followed by pork, white chicken and beef. The stability and composition of muscle membranes tends to reflect the overall stability of meat lipids.

The trends in composition and stability lead to the suggestion that the stability of lipids in muscle tissue is affected by not only the fatty acid composition, but also by other factors such as lipid content, proportions of neutral and phospholipids and the unknown history of retail meat.

Introduction

Lipid oxidation is a major deteriorative factor in meat systems during storage. Lipid hydroperoxides and their breakdown products, have been implicated in a number of deleterious effects, including off-flavor and color development (Benedict et al., 1975; Pearson et al., 1983), possible reaction with certain food components such as amino acids and proteins with concomitant losses of nutritional value and functionality (Matsushita, 1975; Gardner, 1979), and a variety of health-related problems such as heart disease (Addis, 1986; Yagi, 1988) and cancer (Pearson et al., 1983; Addis, 1986).

Lipid peroxidation proceeds by a free radical chain mechanism (Gunstone and Norris, 1983). In meat systems, the initiation of peroxidation most likely occurs at the membrane level (Gray and Pearson, 1987). This is supported by a number of observations. Slabyj and Hultin (1984) found that an enzymatic oxidative system in the microsomal membrane of herring muscle was necessary for the oxidation of emulsified lipids. This enzyme system was not found in adipose tissue. Disruption of muscle membranes results in increased lipid oxidation due, in part, to the fact that lipids are being exposed to oxidative factors such as enzymes (Pearson et al., 1977). Highly unsaturated fatty acids, which are very susceptible to lipid oxidation, are associated with the phospholipid fraction of muscle membranes (Gray and Pearson, 1987). Also, phospholipid

content decreases with lipid oxidation (Corliss and Dugan, 1970; Igene et al., 1981) indicating oxidation at the membrane level.

The susceptibility of lipid material to oxidation is affected by its degree of unsaturation. Lipids with greater degrees of unsaturation are more susceptible to oxidation (Moerck and Ball, 1974). As a result, the oxidative stability of meats can be related to their degree of unsaturation (Wilson et al., 1976). The order of susceptibility would thus be: turkey>chicken>pork>beef>lamb. Red (dark) muscles were also more susceptible than white (light) muscles. In accordance with this, Harel and Kanner (1985) demonstrated higher rates of lipid oxidation in the microsomes isolated from turkey muscle than those from chicken muscle as measured by thiobarbituric acid (TBA) analysis and oxygen absorption. They also noted faster rates of oxidation in microsomes from dark chicken muscles than in those from light chicken muscles.

Since lipid oxidation is initiated at the membrane level and is influenced by fatty acid composition, the present study was designed to investigate the relationship of membrane lipid composition and stability to overall muscle lipid stability. It would be of interest to note whether the membrane composition and stability can predict the lipid stability of the muscle itself.

Materials and Methods

Samples

Meat samples (whole chicken fryers, beef round steak and pork loin chops) were obtained from a local supermarket on the day of analysis. Veal samples were obtained from the MSU Meat Laboratory (as explained in Chapter 3).

Membrane Isolation

On the day the samples were purchased, the mitochondria and microsomes were isolated by a procedure similar to that reported by Kanner and Harel (1985). Two hundred grams of ground muscle were blended with 4 volumes of 0.12 M KCl, 5 mM histidine buffer (pH 7.3) in the following manner. The first blending was with 200 g sample and 200 ml buffer for 30 sec in a Waring blender with an explosion proof base at moderately high speed (obtained by use of a variable powerstat). Three 100 ml portions of the buffer were added with 30 sec blending between each addition. This muscle homogenate was equally distributed in 4 polycarbonate centrifuge bottles (250 ml capacity) and centrifuged in a GSA rotor at 600 x g (2000 rpm) for 10 min (Sorvall centrifuge, Model RC-2B) at 4^{o}C. The supernatant was poured through 8 layers of cheesecloth into a 1 L beaker (on ice). Another 300 ml aliquot of buffer was used to rinse the blender jar and then was equally distributed to the centrifuge bottles containing the pellets. The contents of each bottle were stirred gently with a spatula and recentrifuged for 10 min at 600 x g. The supernatant was

combined with that from the previous extraction and centrifuged for 10 min at 1000 x g (2500 rpm) to sediment the nuclear fraction and other cell debris. The pH of the supernatant was checked and readjusted to 7.3, if necessary, and centrifuged at 10,000 x g for 15 min to precipitate the mitochondrial fraction, which was collected and weighed into a tared vial and frozen under nitrogen. Microsomes were isolated from the supernatant by centrifuging at 100,000 x g for 1 1/4 hour using a Beckman L7 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) at 4°C. Samples were centrifuged in capped polycarbonate centrifuge tubes (70 ml capacity) using a Type 35 rotor. The microsomes were further purified by resuspending in 0.6 M KCl, 5 mM histidine buffer (pH 7.3) in polycarbonate tubes (30 ml capacity) and centrifuging at 100,000 x g for one hour in the same ultracentrifuge. Microsomal pellets were collected and weighed into a tared vial and frozen under nitrogen.

Sample Preparation

Meat samples were refrigerated until the next day at which time some of each sample (described below) was cooked in a conventional oven. Chicken samples (3 split chicken breasts) were baked at 350°F to an internal temperature of 170°F (measured using a copper constantan thermocouple: Omega Engineering, Inc.). Beef (round steak, approximately one pound, 1 1/2 inches thick) and pork (3 loin chops, approximately 3/4 inch thick) samples were broiled to an internal temperature of 170°F.

Oxidative Stability of Muscle Lipids

The extent of lipid oxidation was determined immediately on raw and cooked samples (Day 0) and again after one and two days of storage at 4°C. The procedure used was a modification of the thiobarbituric acid (TBA) distillation procedure of Tarladgis et al. (1960). Meat samples were ground in a Rival meat grinder (Model 2300), (Kansas City, MO). Thirty grams of ground muscle were homogenized (Ultra-Turrax, Model # SDF 1810, Tekmar Co., Cincinnati, OH) with an equal weight of deionized water to which an antioxidant solution (butylatedhydroxytoluene, BHT) was added. The BHT solution was prepared in propylene glycol and added to a concentration of 0.02% of the fat in the sample. Twenty gram portions of the homogenate were weighed into plastic weigh boats in duplicate. These portions were transferred to distillation flasks (500 ml long-necked), containing glass beads, using 87.5 ml deionized water. To these flasks were added 2.5 ml HCl/H_2O (2:1) and four sprays of antifoam (10% silicone emulsion in water, Thomas Scientific, Swedesboro, NJ). Samples were distilled until 50 ml of distillate was collected in a 150 x 25 mm screw-capped test tube. Five ml of distillate were added to 5 ml of TBA reagent in a 150 x 18 mm screw-capped test tube. The capped tubes were placed in a boiling water bath for 35 min; after which they were transferred to a sink containing cold water until cooled to room temperature. Absorbance was determined at 532 nm using a Bausch and Lomb

Spectronic 2000 (Bausch & Lomb, Rochester, NY). Absorbances were adjusted to TBARS (TBA-reactive substances) values by multiplying by a factor of 6.2 (Crackel et al., 1988) and expressed as mg malonaldehyde (MDA)/kg tissue.

Fatty Acid Analysis

When the samples above were ground (Day 0), 5 gram portions were removed for fatty acid analysis. Total lipid extracts were prepared by a dry column method (Marmer and Maxwell, 1981). The extract was collected in a 500 ml distillation flask and rotary evaporated (Brinkmann Instrument Co., Westbury, NY) to near dryness. The sample was then transferred to a volumetric flask using methylene chloride and brought to a final volume of 25 ml. Extracts were frozen until further analysis. A one ml aliquot was removed and dried on a Nitrogen evaporator (N-Evap Model 111, Organomation, Inc., Northborough, MA). Methylene chloride/methanol (400 μl, 9:1, v:v) was added and 200 μl were streaked on a thin layer chromatography (TLC) plate, which had been poured to a thickness of 250 μm of silica gel type G, particle size 10-40 μ (Sigma Chemical Co., St. Louis, MO). The plate was developed using a petroleum ether:ethyl ether:glacial acetic acid solvent mixture (80:20:1) (modified procedure of Pikul et al., 1984). The plate was removed, dried and sprayed with rhodamine G (0.05% in 95% ethanol) prior to viewing under ultraviolet light. The bands of interest (phospholipids, R_f approx. 0.0; neutral lipids, R_f approx. 0.5) were marked, scraped from

the plate and the silica gel collected under vacuum in a disposable pipette plugged with glass wool. The neutral lipid and phospholipid fractions were eluted with methylene chloride/methanol (9:1, v:v) into tared vials and dried on the nitrogen evaporator. They were transferred to 15 ml graduated centrifuge tubes with the 9:1 solvent mixture and dried prior to derivatization.

Derivatization was conducted by the method of Maxwell and Marmer (1983), in which 1 ml hexane was added to the dried lipids and also 100 μl 2 N KOH (in methanol). After vortexing 30 sec, the samples were centrifuged at speed 4 on an IEC clinical centrifuge (Damon/IEC division, Needham, Hts, MA). The lower layer was removed with a disposable pipette and 0.5 ml saturated ammonium acetate was added; the tube was vortexed (30 sec) and centrifuged as above. The lower layer was again removed and 0.5 ml deionized water was added; contents were vortexed and centrifuged. The lower layer was removed and sodium sulfate was added. The tubes were kept at room temperature 30 min and then either frozen or analyzed by gas-liquid chromatography (GLC).

GLC analysis was conducted on a HP 5890A system (Hewlett Packard Co., Avondale, PA) with a 30 m, narrow bore 0.25 mm id DB-225 capillary column (0.25 μ film, J&W Scientific/Anspec, Ann Arbor, MI) with a flame ionization detector and a HP 3392A integrator. The GLC was programmed from 175^{o}C (10 min) to 200^{o}C at 1.5^{o}C/min and held for 40 min, with injector temperature of 275^{o}C and detector

temperature of 300°C. The GLC was run in the split mode with a split ratio of 16. Peaks were identified by comparison of retention times with standard reference mixtures of fatty acid methyl esters (Supelco, Bellefonte, PA). Analysis of areas from the integrator was done using Lotus 1.2.3. (Que Corp., Carmel, IN).

Oxidative Stability of Membranal Lipids

Membranal oxidation studies were performed by the method reported by Kanner and Harel (1985). Previous to analysis, membranes were solubilized in 0.1 M KCl, 0.005 M lactic acid buffer (15 ml, pH 5.5) sonicated and protein determinations were done as described by Lowry et al. (1951). Membrane solutions of equal protein concentrations (0.34 mg/ml) were prepared at a final volume of 14 ml in a 150 ml beaker. The beakers were placed in a shaking water bath (GCA/Precision Scientific) at 37°C. Immediately added were 1 ml metmyoglobin (30 μM) and 100 μl hydrogen peroxide (30 μM). Aliquots (1 ml) were taken immediately (time 0) and at time 8, 15, 30, 60, 90, 120, 150 and 180 min. These 1 ml aliquots were added to 2 ml TBA reagent (TBA:TCA:HCl, 1:25:6.25) in 100 x 15 mm screw-capped test tubes and capped and vortexed. At the end of the 3 hour incubation the tubes were placed in a boiling water bath for color development. The tubes were centrifuged for 10 min at speed 5 (IEC clinical centrifuge, Needham Heights, MA) prior to determination of the absorbance at 532 nm on the B&L Spectronic 2000. Using an extinction coefficient of 1.56 x

10^5 $M^{-1}cm^{-1}$ (Harel and Kanner, 1985), concentrations of malonaldehyde per gram of protein of the membrane solutions were calculated.

Results and Discussion

Fatty Acid Composition of Meats

Fatty acid compositions of neutral lipid and phospholipid fractions of each muscle sample are presented in Tables 1 and 2, respectively. Each fatty acid is expressed as a percentage of the total in that fraction. The degree of saturation in the neutral lipid fraction was greater for beef and pork muscles than for both white and dark chicken muscles. Similar trends were reported in beef and chicken by Igene and Pearson (1979). In the phospholipid fraction, however, the ratio of saturated to unsaturated fats was slightly lower in beef and pork than chicken. In most cases, the degree of saturation increased slightly with cooking. The lack of dramatic changes in fatty acid composition with cooking was in agreement with previous studies (Giam and Dugan, 1965; Campbell and Turkki, 1967; Igene and Pearson, 1979).

Muscle Lipid Stability

Raw and cooked meat samples held at 4°C for 48 hours were analyzed for oxidative stability by the TBA assay and expressed as TBA-reactive substances (TBARS), equivalent to mg malonaldehyde/kg tissue (Table 3). Initial TBARS values (day 0) for raw meats were low (0.33-0.35 mg/kg) for all

Table 1. **Percent fatty acid composition of neutral lipids from raw and cooked meats**

Fatty Acids	Beef Round		Pork Loin Chop		White Chicken		Dark Chicken	
	Raw	Cooked	Raw	Cooked	Raw	Cooked	Raw	Cooked
C14:0	4.91	5.42	1.84	3.66	0.65	0.74	1.14	1.01
C16:0	30.28	30.48	28.47	36.78	18.16	23.14	21.87	23.62
C16:1w7	3.28	3.50	5.07	4.46	11.25	9.87	8.03	9.26
C18:0	15.53	15.96	11.83	10.36	5.42	4.14	5.06	4.74
C18:1	43.02	42.03	48.37	38.29	43.31	40.27	43.35	40.62
C18:2w6	2.98	2.23	4.42	6.46	18.18	19.84	17.77	18.75
C18:3w6	--	--	--	--	2.56	0.33	0.70	0.23
C18:3w3	--	0.37	--	--	0.46	0.91	0.27	0.74
C20:0	--	--	--	--	--	--	0.69	0.46
C20:1	--	--	--	--	--	--	--	--
C20:3w6	--	--	--	--	--	0.18	0.17	--
C20:4w6	--	--	--	--	--	0.46	0.37	0.29
C20:5w3	--	--	--	--	--	0.04	0.18	--
C22:0	--	--	--	--	--	--	--	--
C22:1	--	--	--	--	--	--	--	--
C22:4	--	--	--	--	--	0.09	0.08	0.06
C22:5w3	--	--	--	--	--	--	0.08	0.08
C22:6w3	--	--	--	--	--	--	0.11	0.13
C24:0	--	--	--	--	--	--	0.13	--
%SAT.	50.72	51.86	42.14	50.80	24.23	28.01	28.89	29.83
%MONO	46.30	42.03	53.44	42.75	54.56	50.14	51.39	49.88
%DI	2.98	2.98	4.42	6.46	18.18	19.84	17.77	18.75
%POLY[a]	--	0.37	--	--	3.03	2.01	1.95	1.54
%UNSAT.	49.28	48.14	57.86	49.20	75.77	71.99	71.11	70.17

[a] POLY = Fatty acids with three or more double bonds

Table 2. Percent fatty acid composition of phospholipids from raw and cooked meats

Fatty Acids	Beef Round		Pork Loin Chop		White Chicken		Dark Chicken	
	Raw	Cooked	Raw	Cooked	Raw	Cooked	Raw	Cooked
C14:0	0.53	0.42	--	--	--	1.40	0.64	0.62
C16:0	6.67	7.22	4.43	9.59	11.42	17.30	10.67	11.87
C16:1w7	11.27	4.58	18.52	12.42	6.54	5.94	4.82	4.36
C18:0	6.89	11.67	7.20	9.75	11.74	13.01	12.90	13.93
C18:1	31.90	22.28	37.59	25.68	29.50	33.06	21.73	19.41
C18:2w6	24.96	34.88	19.62	26.75	19.14	14.59	17.67	22.32
C18:3w6	12.62	--	12.63	3.21	2.51	1.14	9.22	3.35
C18:3w3	--	3.66	--	--	4.58	2.56	2.01	2.96
C20:0	--	--	--	--	--	--	--	--
C20:1	--	--	--	--	--	--	--	--
C20:3w6	--	1.46	--	--	0.90	0.51	0.64	0.69
C20:4w6	5.16	8.76	--	12.60	11.71	7.20	11.12	12.52
C20:5w3	--	2.45	--	--	0.96	0.54	1.83	1.55
C22:0	--	--	--	--	--	--	--	--
C22:1	--	--	--	--	--	--	--	--
C22:4	--	--	--	--	1.01	0.93	--	--
C22:5w3	--	2.62	--	--	--	0.67	1.71	1.98
C22:6w3	--	--	--	--	--	1.14	3.68	4.43
C24:0	--	--	--	--	--	--	1.36	--
%SAT.	14.09	19.31	11.63	19.34	23.16	31.71	25.57	26.42
%MONO	43.17	26.86	56.11	38.10	36.04	39.00	26.55	23.77
%DI	24.96	34.88	19.62	26.75	19.14	14.59	17.67	22.32
%POLY[a]	17.78	18.95	12.63	15.81	21.67	14.70	30.21	27.49
%UNSAT.	85.91	80.69	88.37	80.66	76.84	68.29	74.43	73.58

[a] POLY = Fatty acid with 3 or more double bonds

Table 3. TBARS values (mg malonaldehyde/kg sample) for muscle samples stored at 4°C for 2 days[a]

		Beef Round	Pork Loin	White Chicken	Dark Chicken
Raw	Day 0	1.1	0.3	0.3	0.4
	Day 1	2.0	0.2	0.6	0.4
	Day 2	1.9	0.2	0.4	0.4
Cooked	Day 0	1.2	1.2	0.7	0.6
	Day 1	1.3	3.1	1.5	2.7
	Day 2	3.3	4.3	3.4	4.7

[a] Mean of duplicate determinations

samples except for beef round (1.12 mg/kg). These samples were purchased at a local supermarket and the prior history of the meat was unknown.

Other investigators have studied TBARS development in retail meats and have noted similar trends. In a survey of meats from various supermarkets, Siu and Draper (1978) reported higher initial TBARS values for raw beef than for chicken and pork samples. Similar data were obtained by Rhee and Ziprin (1987). These trends differed from that reported by Shamberger et al. (1977), who found that the TBARS values for both raw retail beef and chicken, were greater than those of raw pork. Siu and Draper (1978) also noted great variations in TBARS values of meat of the same type purchased from different supermarkets. These differences were attributed to methods of handling, storage conditions, age of animal and source of sample. Another factor which may contribute to these differences is that raw beef may be vacuum packed and held at refrigerator temperatures for 30 days or more prior to retail packaging.

Storage of cooked meats at 4^{o}C resulted in a general increase in TBARS values over time. On the other hand, raw samples did not change appreciably over time, except in beef samples which increased noticeably in 24 hours. This trend was similar to that found by Rhee and Ziprin (1987). They proposed that TBARS values increased more rapidly in beef due to a greater concentration of heme pigments in raw beef than in chicken and pork. This may mask the effect of

increasing degrees of unsaturation in pork and chicken on the oxidative stability. The fact that the beef samples oxidized more in the raw state may also be a reflection of the higher initial TBARS values.

Cooked samples had higher TBARS values than raw samples. This is consistent with literature reports (Igene et al., 1979; Pearson and Gray, 1983; Rhee and Ziprin, 1987; Rhee, 1988). Even though cooked meat is more susceptible to oxidation than raw meat, oxidation of raw meats may be critical in periods of long storage or when the meat is cut into smaller pieces (thereby exposing more lipids to oxidative catalysts and increasing surface area for exposure to oxygen), or subject to temperature abuse (Rhee et al., 1986). Initial oxidation of raw meat may also adversely affect the oxidative stability of cooked meat and meat products because of the free-radical nature of the oxidative reaction (Rhee, 1988).

The fact that cooked samples oxidized faster than raw samples may be attributed to many factors. Researchers have demonstrated that cooking results in the release of nonheme iron, a potent catalyst for lipid oxidation (Igene et al., 1979; Schricker and Miller, 1983; Chen et al., 1984; Rhee and Ziprin, 1987). Harel and Kanner (1985) proposed that activation of metmyoglobin by hydrogen peroxide may occur in cooked meat to a greater extent than in raw meat. In raw meat, there is a greater abundance of catalase, which reduces hydrogen peroxide concentrations. Equally important

is the fact that heating of metmyoglobin did not prevent its activation by hydrogen peroxide (Harel and Kanner, 1985). Cooking also causes disruption of muscle cell membranes and thereby may bring lipid substrates into closer contact with oxidative catalysts (Rhee, 1988).

Cooked pork samples oxidized to a greater extent than beef, while dark chicken was more highly oxidized than white chicken. According to Wilson et al. (1976), the order of susceptibility of muscle tissue to warmed-over flavor development, as determined by TBA analysis, was turkey > chicken > pork > beef > mutton. With the exception of pork oxidizing to a greater extent than white chicken, the data from the present study follow this trend. Rhee and Ziprin (1987) also noticed higher oxidation in dark chicken than white chicken, pork and beef. However, they were unable to find significant differences between cooked white chicken, beef and pork. They suggested that meat (beef, pork and chicken) cooked immediately after purchase from retail stores may not differ much in warmed-over flavor development.

The fatty acid composition of the neutral lipids and phospholipids of dark and white chicken did not differ greatly (Tables 1 and 2). Fatty acid composition alone, however, must not be the only factor that influences the susceptibility of these muscle lipids to lipid oxidation. The fat content of these samples must also be considered since TBARS are expressed as mg malonaldehyde/kg sample.

Dark chicken muscle had a higher fat content (11.48%) than white chicken muscle (8.13%). This may explain its greater susceptibility to oxidation. Thus, even though the percentage of phospholipid expressed on a lipid basis is equivalent (Wilson et al., 1976), the phospholipid content expressed as a percentage of muscle is greater in dark tissue. The high concentration of polyunsaturation in the phospholipid fraction makes it very susceptible to oxidation (Igene et al., 1981). Thus, it has been suggested by Wilson et al. (1976) and Igene and Pearson (1979) that phospholipids are major contributors to warmed-over flavor development.

Fatty acid composition data for beef and pork were not helpful in explaining the higher degree of oxidation in pork muscle. The percentages of total lipid in these two samples were also very similar (approx. 6.6%). Perhaps the difference in degree of oxidation was due to the fact that the percentage of phospholipids is greater in pork than in beef (Wilson, 1976). Another explanation is that the previous history of the retail samples was unknown. It is possible that previous history (such as storage conditions, temperature, and age of animals) may have been responsible for the observed trends.

Fatty Acid Composition of Membranes

The fatty acid composition of the neutral lipid and phospholipid fractions of muscle membranes is presented in Tables 4 and 5, respectively. As was seen in the muscle

Table 4. **Percent fatty acid composition of neutral lipids from mitochondria and microsomes of various meats**

Fatty Acids	Beef Round		Pork Loin Chop		White Chicken		Dark Chicken	
	Mito	Micro	Mito	Micro	Mito	Micro	Mito	Micro
C14:0	3.94	3.45	1.64	1.66	0.79	0.88	0.88	0.81
C16:0	28.23	23.93	26.89	27.15	23.69	22.80	21.90	20.34
C16:1w7	3.98	5.05	3.11	3.14	0.62	7.23	7.57	7.76
C18:0	15.94	15.04	14.94	15.09	4.83	7.78	5.01	4.78
C18:1	42.43	41.24	42.35	42.76	45.60	38.05	43.30	39.89
C18:2w6	2.87	3.34	6.12	6.18	22.24	16.71	19.43	17.46
C18:3w6	0.49	2.61	1.07	1.08	0.44	2.81	0.26	1.64
C18:3w3	0.72	2.27	0.22	0.51	0.81	0.55	0.70	1.38
C20:0	--	--	1.52	1.54	--	--	--	--
C20:1	--	--	--	--	--	--	--	--
C20:3w6	--	--	--	--	--	--	0.18	--
C20:4w6	--	--	--	--	0.59	0.42	0.39	--
C20:5w3	--	--	0.90	--	--	--	0.19	--
C22:0	--	--	--	--	--	--	--	--
C22:1	--	--	--	--	--	--	--	--
C22:4	--	--	--	--	--	--	--	1.16
C22:5w3	0.52	--	--	0.91	--	2.77	0.08	0.57
C22:6w3	--	--	--	--	--	--	0.11	--
C24:0	0.88	3.06	1.24	--	0.39	--	--	4.20
%SAT.	48.99	45.48	46.23	45.43	29.70	31.46	27.79	30.13
%MONO	46.41	46.29	45.46	45.90	46.22	45.28	50.87	47.65
%DI	2.87	3.34	6.12	6.18	22.24	16.71	19.43	17.46
%POLY[a]	1.73	4.88	2.19	2.49	1.84	6.55	1.91	4.75
%UNSAT.	51.01	54.52	53.77	54.57	70.30	68.54	72.21	69.86

[a] POLY = Fatty acids with three or more double bonds

Table 5. **Percent fatty acid composition of phospholipids from mitochondria and microsomes of various meats**

Fatty Acids	Beef Round		Pork Loin Chop		White Chicken		Dark Chicken	
	Mito	Micro	Mito	Micro	Mito	Micro	Mito	Micro
C14:0	0.31	0.38	0.47	0.23	0.86	0.15	0.93	0.15
C16:0	8.26	11.74	10.78	9.58	16.29	14.23	13.80	13.19
C16:1w7	4.89	1.22	2.11	2.24	2.40	0.96	2.63	1.07
C18:0	8.85	14.03	10.06	10.37	11.75	13.83	13.93	16.48
C18:1	19.49	18.82	16.79	12.49	29.49	23.13	22.53	17.63
C18:2w6	23.86	32.73	27.93	35.29	19.46	16.09	24.93	22.72
C18:3w6	10.27	0.73	9.03	0.27	4.03	--	2.36	0.53
C18:3w3	4.38	0.73	--	1.08	--	4.90	0.31	0.33
C20:0	--	--	--	1.85	--	--	--	--
C20:1	--	--	--	--	--	--	--	--
C20:3w6	0.90	2.57	1.86	0.97	0.98	1.36	0.59	0.81
C20:4w6	5.22	11.28	11.97	15.12	9.53	15.15	11.42	14.24
C20:5w3	5.16	1.94	1.52	0.46	--	0.50	0.04	0.67
C22:0	--	--	--	--	--	--	--	--
C22:1	--	--	--	--	--	--	--	--
C22:4	1.57	--	4.01	2.82	5.20	6.25	--	3.38
C22:5w3	1.09	3.84	3.47	1.56	--	1.56	3.29	2.42
C22:6w3	--	--	--	2.59	--	1.89	3.22	5.98
C24:0	5.77	--	--	3.09	--	--	--	0.39
%SAT.	23.18	26.15	21.31	25.12	28.90	28.21	28.66	30.22
%MONO	24.38	20.04	18.90	14.73	31.89	24.09	25.17	18.70
%DI	23.86	32.73	27.93	35.29	19.46	16.09	24.93	22.72
%POLY[a]	28.58	21.08	31.86	24.86	19.75	31.61	21.24	28.36
%UNSAT.	76.82	73.85	78.69	74.88	71.10	71.79	71.34	69.78

[a] POLY = Fatty acids with three or more double bonds

fatty acid composition data, the neutral lipids of chicken were much more highly unsaturated than the neutral lipids of beef and pork. The phospholipid fractions were not obviously different. This was in contrast to reports by Melton (1983) that the phospholipid fraction of beef has a much higher C18:1 content than pork and chicken and pork has higher C18:2. The fatty acid composition data of the present study would lead to the expectation that the lipids of chicken mitochondria and microsomes would oxidize to a greater extent than those of beef and pork membranes.

Membranal Lipid Stability

Membranal lipid oxidation of mitochondria and microsomes is graphically presented in Figures 1 and 2, respectively. Mitochondrial and microsomal membranes from dark chicken oxidized to the greatest extent and those from pork underwent more oxidation than beef. Data presented by Harel and Kanner (1985) on microsomal oxidation support the view that dark chicken microsomes oxidize more rapidly than those from white chicken. Asghar et al. (1990) also reported greater oxidation in both mitochondria and microsomes from dark muscle than in those from white muscle membranes. Rhee and Ziprin (1987) conducted oxidation studies on microsomes from various muscle tissues using NADPH, ADP and $FeCl_3$ to measure enzymatic lipid oxidation for 30 min. They found dark chicken and beef microsomes were more susceptible to oxidation than those from white chicken, while pork microsomes were more oxidized than white

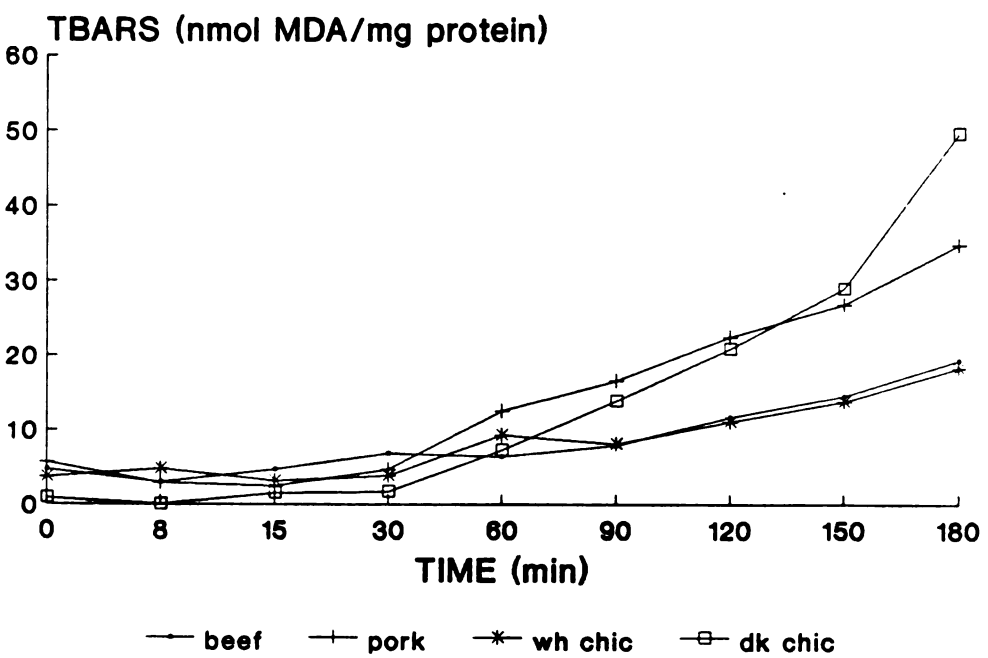

Figure 1. Lipid oxidation in mitochondria as measured by TBARS, from various meats, initiated by metmyoglobin and hydrogen peroxide (30 μM each)

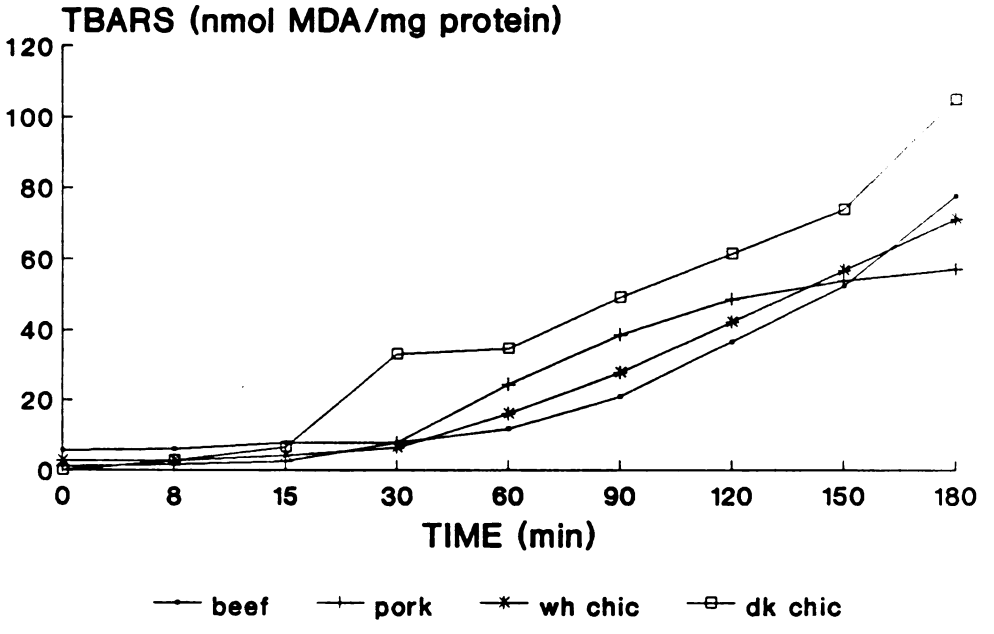

Figure 2. Lipid oxidation in microsomes as measured by TBARS, from various meats, initiated by metmyoglobin and hydrogen peroxide (30 μM each)

chicken microsomes in samples purchased at one time of the year and not significantly different from microsomes of white chicken purchased at another time of the year. In Rhee and Ziprin's study (1987) however, pork microsomes did not oxidize to a greater extent than the microsomes from two muscles of beef, in contrast to the present study where the microsomes from pork were more susceptible to oxidation than those from beef.

A comparison of mitochondrial and microsomal oxidation curves (Figures 1 and 2) indicates that microsomal lipids oxidized more rapidly than those of the mitochondria. This trend is supported by the higher degree of unsaturation in certain microsomal lipids than mitochondrial lipids (Tables 4 and 5). For example, arachidonic acid is consistently higher in the phospholipid fraction of microsomes (Table 5). This fatty acid is important as it is predominant in this fraction.

Trends in membranal lipid oxidation were similar to those for muscle TBARS development in cooked meats. Dark chicken oxidized to a greater extent than the other meats. Pork was closest to dark chicken followed by white chicken and beef. Since lipid oxidation is initiated in the muscle membranes, attempts to stabilize the membrane lipids should result in greater stability of the meat lipids. This has been demonstrated with dietary vitamin E supplementation in broilers (Lin et al., 1989; Asghar et al., 1990). Dietary vitamin E supplementation resulted in improved oxidative

stability of dark and white broiler meat and exerted a protective effect against membranal lipid oxidation.

Initiation of oxidation in meat occurs in the muscle membranes (Gray and Pearson, 1987). Even though there was variability in the initial oxidative status of muscle lipids, the trends shown in this study indicate that the stability and composition of muscle membranes tend to reflect the overall stability of meat lipids.

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CHAPTER THREE

THE EFFECT OF DIETARY VITAMIN E SUPPLEMENTATION ON THE GROWTH PERFORMANCE OF VEAL CALVES AND THE OXIDATIVE STABILITY OF LIPIDS

Abstract

The influence of dietary vitamin E supplementation on incorporation of α-tocopherol into muscle membranes and on the resultant oxidative stability of veal lipids was investigated. Daily supplementation with 500 IU vitamin E in the form of α-tocopheryl acetate for 12 weeks after birth increased muscle and membranal α-tocopherol concentrations almost 6-fold over those of control animals. Growth performance was not affected by supplementation. The oxidative stability of mitochondrial and microsomal lipids was enhanced by dietary supplementation as indicated by the results of an oxidative assay using metmyoglobin and hydrogen peroxide as initiators of oxidation. Muscle lipid stability was also improved by supplementation. Vitamin E supplementation of diets of food-producing animals will improve the freshness, quality and safety of meat and meat products.

Introduction

Lipid oxidation is a major deteriorative reaction in meats during storage. It is responsible for a wide variety of undesirable reactions such as loss of fresh meat color and flavor (Benedict et al., 1975; Pearson et al., 1983), oxidation product reactions with proteins, with concomitant losses of protein functionality and nutritional value (Matsushita, 1975; Gardner, 1979), and possible adverse biological effects such as cardiovascular disease (Addis, 1986; Yagi, 1988) and cancer (Pearson et al., 1983; Addis, 1986).

Initially, it was believed that oxidative rancidity in meats was initiated in the adipose tissue (Pearson et al., 1977). However, the current belief is that oxidation of muscle lipids is initiated in the membranes as opposed to the adipose tissue (Gray and Pearson, 1987). The membranal lipid fraction is composed of many phospholipids and this highly unsaturated environment is very susceptible to oxidation.

In the membrane portion of the cell, there exists several lines of defense against oxidation (Machlin, 1984). Glutathione peroxidase represents the first line of defense, while α-tocopherol is the secondary line of defense against oxidation in cell membranes.

Vitamin E is a naturally occurring, lipid soluble substance which functions as an antioxidant by donating a hydrogen from the chromanol ring to a lipid free-radical and

terminating the free-radical chain reaction (Tappel, 1962). Because α-tocopherol is lipid soluble, it situates itself in muscle membranes (Machlin, 1980), where it functions to maintain cellular membrane integrity.

Several studies have been conducted to observe the protective effect of vitamin E on lipid stability in various food producing animals such as veal calves (Ellis et al., 1974; Shorland et al., 1981), steer (Faustman et al., 1989a; 1989b), pigs (Asghar et al., 1989; Monahan et al., 1989) and poultry (Bartov and Bornstein, 1977; Lin et al., 1989; Asghar et al., 1990a).

It has been reported that supplementation of veal calves with vitamin E results in improved oxidative stability of rendered fat (Ellis et al., 1974), even in animals whose diets had been supplemented with high levels of linoleic acid (approximately 14%, as encapsulated safflower oil). Shorland et al. (1981) observed a protective effect of vitamin E supplementation on lipid stability in Longissimus dorsi tissues. However, this effect was not consistent from tissue to tissue. These researchers suggested that the protective effect of vitamin E was not as clearly evident in intact systems as it was in fat removed from the animals.

Faustman et al. (1989a; 1989b) theorized that the improved lipid and color stability of muscle from Holstein steers fed diets supplemented with 370 IU vitamin E/day was the result of incorporation of vitamin E into membranes,

where its physiological function is as an antioxidant. However, the α-tocopherol content of the membranes was not determined. The veal studies by Ellis et al. (1974) and Shorland et al. (1981) also did not include any membranal analyses for α-tocopherol.

Several recent studies have focused on the effects of vitamin E supplementation on the incorporation of α-tocopherol into membranes and the resultant oxidative stability of membranes and muscles in different species. Monahan et al. (1989) showed incorporation of α-tocopherol into mitochondria and microsomes as a consequence of supplementing pig diets with 200 IU vitamin E/kg feed and a resultant improvement in lipid stability. Asghar et al. (1989) reported increased concentrations of α-tocopherol in porcine tissues with increasing dietary levels of vitamin E, and a resultant improvement in muscle and membranal lipid stability. Similar trends were found in poultry (Lin et al., 1989; Asghar et al., 1990a). While no such studies have been carried out with veal, Ellis et al. (1974) pointed out that species differences in response to vitamin E supplementation do exist. The present study was designed to investigate α-tocopherol distribution in veal tissues.

Another important aspect which was not addressed in these studies was the effect of dietary vitamin E on the oxidative stability of cholesterol in meat products during storage. This will be the subject of a future paper (Chapter 4). The objective of the present paper was to

investigate the effects of vitamin E supplementation on the incorporation of α-tocopherol into veal muscle membranes and to determine the resultant oxidative stability of muscle and membranal lipids.

Materials and Methods

Samples

Eight male Holstein calves were obtained at the Michigan State University dairy barn (East Lansing, MI). They were assigned alternately at the time of birth to one of two groups. Control animals (n=4) were fed whole milk (2.82×10^{-3} mg α-tocopherol/ml) twice a day. In addition to whole milk, supplemented animals (n=4) received 500 IU of vitamin E/day in the form of α-tocopheryl acetate powder (BASF Corp., Wyandotte, MI), added directly to the milk. These feeding regimens were continued until the time of slaughter at twelve weeks. At two-week intervals, from birth to slaughter, the calves were bled and the samples collected in whole blood tubes with liquid ethylenediaminetetraacetic acid (EDTA) to prevent clotting (Becton-Dickenson, Lincoln Park, NJ). The tubes were centrifuged (speed 4, IEC clinical centrifuge - International Equipment Co., Needham Heights, MA) to collect plasma for α-tocopherol analyses. Two drops of 0.02% butylated hydroxytoluene (BHT) in ethanol were added to the

plasma samples which were then frozen for future analysis. The calves were also weighed at these intervals to record average daily gain.

At the time of slaughter, several tissues (approximately 50 g of each of liver, heart, lung, kidney and perinephric adipose tissue) were removed from each animal for tocopherol distribution studies and immediately frozen in liquid nitrogen, packaged, and then held at -80°C until removed for analysis. Leg muscle (semitendinosus, semimembranosus, adductor and biceps femoris) samples were also removed, vacuum packaged in polyethylene-laminated nylon pouches (Koch, Kansas City, MO) and frozen at -80°C for future analysis. These pouches (3.5 mil) have a water-vapor transmission rate of 0.041 $ml/m^2 \cdot day \cdot mmHg$ and an oxygen transmission rate of 0.124 $ml/m^2 \cdot day \cdot mmHg$ at 22.7°C, 50% relative humidity.

Methods

Alpha-Tocopherol Determinations

Plasma. Screw-capped test tubes (150 x 18 mm) were set up with 2 ml of 200 proof ethanol (absolute, USP) and 0.3 ml saturated ascorbic acid (3.4 g in 10 ml deionized water), to which was added the internal standard (0.1 ml of 100 μg/ml dl-α-tocopheryl acetate, Sigma Chemical Co., St. Louis, MO). Two ml of sample plasma were added, flushed with nitrogen and the tubes were capped. The tubes were vortexed on a multi-vortexer (Big Vortexer, Glas-Col Apparatus Co., Terre Haute, IN) and extracted with 6 ml of hexane containing

butylated hydroxytoluene (BHT) (0.05% w/v). Again the tubes were flushed with nitrogen, capped and vortexed for 1 min. They were centrifuged at 2500 rpm (Damon/IEC centrifuge, Model PR-6000; rotor #269; International Equipment Co., Needham Heights, MA) for 10 min at 5°C. The hexane layer (top) was transferred using a disposable pipette to another 150 x 18 mm test tube. Extraction with 3 ml BHT/hexane was repeated, and the hexane layers were combined and evaporated on a nitrogen evaporator (N-Evap Model 111, Organomation, Inc., Northborough, MA). When the tubes were dry, 200 µl of methanol were added; the tubes were quickly vortexed and the samples transferred to 1 ml microfuge tubes and subsequently frozen for future HPLC analysis.

Muscle and Other Tissues. Ground veal tissues (approximately 8 g) were homogenized with an equal weight of nitrogen-saturated deionized water containing 5% ascorbic acid in a 50 ml beaker. Two grams of the homogenates were weighed into 150 x 18 mm screw-capped test tubes and 1.5 ml 6 M urea and 100 µl α-tocopheryl acetate (0.2 mg/ml) were added. Tubes were flushed with nitrogen, capped and vortexed for 2 min. One ml of 0.1 M sodium dodecyl sulfate (SDS) solution was added and vortexed again for 1 min. Then 4 ml of 1% pyrogallol in ethanol was added and again vortexed 1 min. Extraction of alpha-tocopherol was accomplished by three extractions with 8 ml petroleum ether, vortexing 2 min, followed by centrifugation (speed 3, IEC clinical centrifuge). The top ether layers were collected

in another 150 x 18 screw-capped test tube and dried under nitrogen and redissolved in 200 μl ethanol.

Membranes. Mitochondria and microsomes of control and vitamin E-supplemented veal muscles were isolated by a centrifugation procedure similar to that reported by Kanner and Harel (1985) (described in Chapter 2). The membranes were assayed for α-tocopherol in a manner similar to muscle, using a buffered membrane solution (volume determined to acheive approximately 0.5 g membranes) to which 0.5 ml 0.5% ascorbic acid and 1 ml 6 M urea and 100 μl α-tocopheryl acetate (0.2 mg/ml) were added; tubes were capped and vortexed. The next step was SDS addition and the procedure was followed as for muscle.

Adipose Tissue. Perinephric adipose tissue (0.5 g) from control and vitamin E-supplemented veal calves was weighed accurately into a 150 x 18 mm screw-capped test tube and 0.5 ml 2% pyrogallol in ethanol and 100 μl 25% ascorbic acid solution (dissolved in water, then made to volume in ethanol) were added. The tubes were flushed with nitrogen, capped and placed in a water bath at 75°C for 5 minutes. Then 0.5 ml 10 N KOH was added; the tubes were capped and heated for 30 min at 75°C with periodic shaking. The tubes were cooled and 1 ml ethanol and 1 ml water were added prior to extraction with 8 ml of petroleum ether (3 times). The extracts were collected and washed with 8 ml of a dilute

sodium chloride solution in water. Then, the petroleum ether layer was evaporated under nitrogen and 200 ml of ethanol were added.

Milk. Composite milk samples were obtained from the MSU dairy barn at periodic intervals during the feeding study. One ml milk was placed in a 150 x 18 mm screw-capped test tube with 2 ml 1% pyrogallol in methanol and 0.5 ml 50% KOH solution. The samples were saponified for 30 min in a 70°C water bath. Two ml water were added and the tubes were allowed to cool to room temperature. Tocopherol was extracted with two 3 ml aliquots of hexane. The hexane extracts were combined, 100 μl α-tocopherol acetate (0.2 mg/ml) were added and they were evaporated to dryness under nitrogen. Samples were redissolved in 400 μl ethanol.

HPLC Analysis of α-Tocopherol. A Waters HPLC with a reverse phase C_{18} column (ODS, Beckman Ultrasphere, 5 μ, 4.6 mm x 15 cm) and a fixed wavelength detector (Waters, 440 absorbance detector) set at 280 nm was used. The mobile phase was 100% methanol at a flow rate of 1 ml/min. Peak areas were integrated using a HP 3380A integrator. Standard curves were prepared using dl-α-tocopherol (Sigma Chemical Co., St. Louis, MO).

Fatty Acid Analysis

Fatty acid composition of leg muscle from control and vitamin E-supplemented veal calves was determined by extracting total lipids by a dry column procedure (Marmer and Maxwell, 1981). The neutral lipids and phospholipids

were fractionated by silica gel thin-layer chromatography (modified from Pikul et al., 1984). The fatty acid composition of the lipid fractions was determined by gas-liquid chromatography (GLC) (as explained in Chapter 2).

Preparation of Samples for Oxidative Stability Studies

Muscle

Steaks (approximately 1 inch thick) from the leg muscle of each animal were cooked by broiling in a conventional oven to an internal temperature of 170^{o}F (measured using a copper constantan thermocouple: Omega Thermocouple Thermometer, Model 660, Omega Engineering, Inc.).

Membranes

Mitochondria and microsomes were isolated from muscle of each animal by the centrifugation method presented in Chapter 2.

Oxidative Stability Studies

Muscle

A modified version of the distillation method of Tarladgis et al. (1960) was used to determine thiobarbituric acid (TBA- reactive substances (TBARS)) of raw and cooked meats at 0, 2 and 4 days of storage at 4^{o}C (Chapter 2).

Membranes

The oxidative stability of mitochondrial and microsomal lipids was determined by the method of Kanner and Harel (1985), using metmyoglobin/hydrogen peroxide as the initiator (explained in detail in Chapter 2).

Statistical Treatment of Data

Statistical analyses were carried out using MSTAT-C (Microcomputer Statistical Program, Michigan State University, East Lansing, MI). Student's t-tests were done to determine significant differences between control and supplemented average daily gain, plasma tocopherol, tissue tocopherol, fatty acid composition and membranal lipid stability. Also, for TBARS values of raw and cooked meats over 4 days storage at 4°C a completely randomized factorial design was developed with factor C (raw vs cooked) being a split plot on factor A (control vs supplemented) and factor B (day of storage). Statistical significance was declared at $P<0.05$.

Results and Discussion

Effect of Vitamin E Supplementation on Average Daily Gain of Veal Calves

Supplementation with dietary vitamin E had no significant effect on the average daily gain of veal calves (Table 1). Very few reports of growth performance, as affected by vitamin E supplementation, have been published. Further work in this area may be necessary to further evaluate the relationship between vitamin E and growth performance and to determine if species differences exist. Asghar et al. (1989) reported 4 and 6% increases in daily body weight gain in the early growth stages (first 4 weeks) of pigs fed diets supplemented with 100 and 200 IU vitamin

Table 1. Average daily gain of veal calves fed control and vitamin E-supplemented diets

Week	Average Daily Gain (kg/d/calf)[a,b] Control	Supplemented
0-1	0.31 ± 0.07	0.37 ± 0.07
0-2	0.38 ± 0.02	0.37 ± 0.06
0-3	0.41 ± 0.06	0.38 ± 0.04
0-4	0.40 ± 0.04	0.37 ± 0.05
0-5	0.39 ± 0.04	0.37 ± 0.05
0-6	0.43 ± 0.02	0.43 ± 0.05
0-7	0.46 ± 0.02	0.47 ± 0.05
0-8	0.50 ± 0.02	0.48 ± 0.05
0-9	0.50 ± 0.03	0.50 ± 0.05
0-10	0.49 ± 0.04	0.46 ± 0.02
0-11	0.41 ± 0.03	0.47 ± 0.03

[a] Value represents mean of 4 animals ± standard error
[b] Control and supplemented are not significantly different at $P<0.05$

E/kg feed respectively. At later stages, the growth of the supplemented animals was parallel to that of the control animals; thus, the advantage persisted. Stuber et al. (1990) found slight increased grain-to-feed ratios in supplemented pigs (100 and 200 IU/kg feed) as compared to controls (11 IU/kg feed). Monahan et al. (1990) also supplemented pigs with 200 IU vitamin E/kg feed and like the present study found no advantages with respect to growth.

Shorland et al. (1981) evaluated various performance characteristics (live weights at slaughter, carcass weights and percent carcass yields) of veal calves fed either corn oil or coconut oil as milk fat replacer, with or without vitamin E (500 IU/day), and found no significant effects related to vitamin E supplementation. They had some difficulties with death of animals, which was attributed to the highly unsaturated diets. No death or even visible signs of stress were observed in the present study. The level of vitamin E fed to veal calves in the present study and in the study of Shorland et al. (1981) was 500 IU/day. According to Asghar et al. (1990b) high levels such as these may prevent growth enhancement. Ingestion of large amounts of vitamin E may inhibit absorption of vitamin A, which is an important vitamin for growth (Bieri et al., 1985). If further research shows a beneficial effect of vitamin E supplementation on growth performance, the optimal conditions of dosage, timing and duration need to be addressed.

Effect of Vitamin E Supplementation on Alpha-Tocopherol Concentrations in Plasma and Other Tissues

Plasma

Calves were bled every two weeks in order to determine the α-tocopherol concentrations in blood plasma. Because the calves were born on different days but bled on the same days, mean plasma concentrations for each two week period are presented (Table 2). Plasma concentrations of the supplemented animals were significantly higher ($P<0.05$) than those from control animals during the entire period, with exception of the last collection. Slaughering of the eight calves occurred on two different days, approximately 2 1/2 weeks apart. The last collection of plasma thus represents only 5 animals because they were in the second group to be slaughtered. Therefore, the great variability in a smaller group led to a lack of significance. These results agree with those of Asghar et al. (1990b), who found that supplementation of swine with vitamin E increased plasma α-tocopherol concentrations relative to those of control pigs for the entire period. Monahan et al. (1989) also measured plasma tocopherol concentrations in pigs at the time of slaughter and also observed significantly higher concentrations ($P<0.01$) in plasma from the supplemented animals. Plasma α-tocopherol concentrations were not reported in previous veal studies. Veal plasma tocopherol concentrations found in the present study were higher at 85-98 days (control = 4.13; supplemented = 8.32 μg/ml) than

Table 2. Plasma alpha-tocopherol concentrations (μg/ml) of veal calves fed control and vitamin E-supplemented diets[a]

Age (days)	Control	Supplemented
0-14[b]	1 ± 0.4	14 ± 1.9
15-28[c]	3 ± 0.6	13 ± 1.2
29-42[d]	3 ± 1.2	11 ± 1.5
43-56[e]	2 ± 0.4	10 ± 1.9
57-70[f]	1 ± 0.4	11 ± 2.4
71-84[g]	3 ± 0.8	14 ± 3.4
85-98[h]	4 ± 2.0	8 ± 2.2

[a] Value represents mean of samples from 4 animals ± standard error
[b] Control different from supplemented (P<0.01)
[c] Control different from supplemented (P<0.001)
[d] Control different from supplemented (P<0.01)
[e] Control different from supplemented (P<0.01)
[f] Control different from supplemented (P<0.01)
[g] Control different from supplemented (P<0.05)
[h] Control not significantly different from supplemented at P<0.05

those of either Asghar et al. (1989) or Monahan et al. (1989). These researchers found levels, in the plasma of pigs fed diets supplemented with 200 mg vitamin E/kg feed, of 4.07 and 5.5 μg/ml, respectively.

Tissue

Concentrations of α-tocopherol in various tissues of the veal calves are presented in Table 3. Tissues from supplemented animals had higher α-tocopherol concentrations than those from control animals ($p<0.05$) with the exception of adipose tissue, which was determined in fewer samples (control n=2; supplemented n=3) and had greater variability ($p<0.1$). Concentrations of α-tocopherol for organs from the control animals decreased in the following order: liver>kidney>lung>heart. With supplementation, the same order of tocopherol concentrations was maintained. However, the concentrations of α-tocopherol in each tissue increased to different degrees (heart>lung>kidney>liver). Adipose tissue of the control animals was intermediate between liver and kidney, but was at the highest level in the supplemented animals.

The fact that α-tocopherol increased in tissues with supplementation is consistent with other reports (Ellis et al., 1974; Shorland et al., 1981; Asghar et al., 1989; Monahan et al., 1989). However, the tissue contents and degrees of increases differed. Shorland et al. (1981) collected omental and perinephric tissues from control and supplemented (500 IU vitamin E/day) veal calves fed corn or

Table 3. Mean alpha-tocopherol concentrations (μg/g tissue, wet basis) in tissues from control and vitamin E-supplemented veal calves[a]

Tissue	Control	Supplemented
Liver[b]	6 ± 1.7	19 ± 3.64
Heart[c]	0 ± 0.0	5 ± 0.5
Lung[d]	1 ± 0.1	11 ± 0.8
Kidney[e]	2 ± 0.4	16 ± 0.9
Adipose[f] (perinephric)	3 ± 0.9	44 ± 12.8

[a] Value represents mean of samples from 4 animals ± standard error (except adipose, where n = 3)
[b] Control different from supplemented ($P<0.05$)
[c] Control different from supplemented ($P<0.01$)
[d] Control different from supplemented ($P<0.01$)
[e] Control different from supplemented ($P<0.01$)
[f] Control not significantly different from supplemented at $P<0.05$

coconut oil as milk fat replacers. Perinephric adipose tissue levels were increased upon supplementation in the group receiving coconut oil but not in those receiving corn oil as a milk fat replacer. If the α-tocopherol was expressed on a per gram lipid instead of tissue basis, supplementation increased α-tocopherol concentrations in both cases. Perinephric adipose tissue α-tocopherol concentrations in the present study were 3.0 and 44.5 μg/g tissue for control and supplemented animals, respectively. A dramatic increase in perinephric adipose tissue concentrations with dietary vitamin E supplementation was also found by Shorland et al. (1981) (19.1 and 43.7 μg/g). Ellis et al. (1974) reported approximately 3-fold higher α-tocopherol concentrations in perinephric adipose tissue from animals supplemented with vitamin E as opposed to commercial veal. The only tissues analyzed in these veal studies were adipose and muscle.

Asghar et al. (1989) reported tissue tocopherol levels in swine in the following order: liver>heart>lung>kidney. Monahan et al. (1989) found the same trend in tissue samples (liver>heart>lung>kidney) of pigs supplemented with 200 mg vitamin E/kg feed. Tissue distribution studies presented here demonstrate significant increases in α-tocopherol of various tissues; however, the percentage increase of each tissue varied from study to study.

Muscle and Membranes

Alpha-tocopherol concentrations in muscle, mitochondria and microsomes of supplemented animals were approximately 5-8 fold greater than in comparable fractions of control animals (Table 4). Shorland et al. (1981) noted increased α-tocopherol upon vitamin E supplementation in Longissimus dorsi muscle of veal calves receiving corn oil as a milk fat replacer, but not in those muscles from calves receiving coconut oil. Levels of α-tocopherol in the semitendinosus muscle of the present study are lower in the control animals than those of Shorland et al. (1981) in Longissimus dorsi (1.0 vs 3.4 μg/g, respectively). However, muscle α-tocopherol concentrations in supplemented animals were similar (6.1 vs 5.2 μg/g). The increase in muscular α-tocopherol concentration in the present study was slightly more dramatic, perhaps due to a longer feeding time of 12 weeks as opposed to 8 weeks in Shorland's study (Shorland et al., 1981). Shorland et al. (1981) did not investigate membranal α-tocopherol levels. In the other veal study presented by Ellis et al. (1974), muscle and membranal α-tocopherol levels were not investigated.

Studies of beef have investigated vitamin E supplementation and the resultant color and lipid stability (Faustman et al., 1989a; 1989b). Faustman et al. (1989b) proposed that protection of vitamin E occurs at the membrane

Table 4. Mean alpha-tocopherol concentrations in muscles and membranes of control and vitamin E-supplemented veal calves[a]

	Control	Supplemented
Muscle[b] (μg/g)	1 ± 0.2	6.1 ± 0.8
Mitochondria[c] (μg/g protein)	73 ± 38	584 ± 103
Microsomes[d] (μg/g protein)	139 ± 86	707 ± 162

[a] Value represents mean of 4 samples ± standard error
[b] Control different from supplemented ($p<0.01$)
[c] Control different from supplemented ($p<0.05$)
[d] Control different from supplemented ($p<0.01$)

level; however, none of the previous researchers in the veal or beef studies looked at membranal α-tocopherol deposition to support this proposal.

Asghar et al. (1989) have shown deposition of α-tocopherol in Longissimus dorsi muscle and subcellular membranes upon supplementation of pigs with three levels of vitamin E (10, 100 and 200 IU/kg feed). This deposition was greater as levels of dietary α-tocopherol increased. Muscle from pigs supplemented with 200 IU vitamin E/kg feed had an α-tocopherol concentration of 4.72 μg/g tissue. That from control pigs had 0.54 μg/g tissue. These values are similar to those reported for veal calves. Monahan et al. (1989) reported Longissimus dorsi α-tocopherol concentrations in pigs supplemented with 200 IU vitamin E/kg feed to be 20.6 μg/g protein (control concentration was 6.9 μg/g protein). These values expressed on a tissue weight basis would also be similar to those of the present study.

Membranal α-tocopherol concentrations were much higher in the present study than for those presented for pig membranes by Asghar et al. (1989) and Monahan et al. (1989). In the present study, microsomal concentrations of α-tocopherol were higher than mitochondrial concentrations as was reported by Monahan et al. (1989). Asghar et al. (1989) reported opposite trends.

Supplementation of poultry with vitamin E also increased α-tocopherol concentrations in muscle and subcellular membranes, particularly in dark chicken meat

(Lin et al., 1989; Asghar et al., 1990a). Dark muscle α-tocopherol concentrations were greater than those in white muscle of supplemented broilers. The values were comparable to those presented above. In dark chicken, microsomal α-tocopherol was higher than that of mitochondria. Again, the values reported for membranal α-tocopherol were lower than those of the present study. Differences in membranal α-tocopherol distribution have thus been found between species, even within species between different studies, as well as between anatomical locations. The actual impact of this differential distribution of α-tocopherol in membranes on muscle and membranal lipid stability in relationship to other factors such as fatty acid composition remains to be determined.

Fatty Acid Compositon of Veal Muscle

Mean fatty acid composition data for control and supplemented veal samples are presented for neutral lipids (NL) (Table 5) and phospholipids (PL) (Table 6). For the most part, vitamin E supplementation had no significant effect on fatty acid composition. The NL fraction (Table 5) of muscles from the supplemented animals had less 20:3w6 and 20:4w6 than those from the control animals. In the phospholipid fraction, muscles from the supplemented animals had lower 14:0, 16:0 and 18:0 contents. These changes in fatty acid composition are different than those reported by Shorland et al. (1981). These researchers reported higher C18:0 in the neutral lipid fraction with reduction in C12:0,

Table 5. Percent fatty acid composition of the neutral lipid fraction isolated from the semitendinosus muscle of control and vitamin E-supplemented veal calves

Fatty Acids	Control	Supplemented
C14:0[a]	7.26	5.48
C16:0[a]	35.36	29.94
C16:1	2.75	3.60
C18:0[a]	14.01	11.69
C18:1	37.46	38.70
C18:2w6	2.65	3.17
C18:3w6	0.13	1.99
C18:3w3	0.33	1.43
C20:0	--	--
C20:1	--	--
C20:3w6	--	--
C20:4w6	--	--
C20:5w3	--	--
C22:0	--	--
C22:1	--	--
C22:4	--	0.65
C22:5w3	0.04	0.85
C22:6w3	--	--
C24:0	0.01	2.49
%SAT.	56.63	49.60
%MONO	40.21	42.30
%DI	2.65	3.17
%POLY[b]	0.50	4.93
%UNSAT.	43.37	50.40

[a] Control different from supplemented ($P<0.02$)
[b] POLY = fatty acid with 3 or more double bonds

Table 6. Percent fatty acid composition of the phospholipid fraction isolated from the semitendinosus muscle of control and vitamin E-supplemented veal calves

Fatty Acids	Control	Supplemented
C14:0	0.73	0.46
C16:0	13.74	11.12
C16:1	3.45	1.21
C18:0	9.04	11.12
C18:1	26.45	19.36
C18:2w6	20.65	23.64
C18:3w6	5.07	0.67
C18:3w3	1.34	1.09
C20:0	--	--
C20:1	--	--
C20:3w6[a]	1.54	3.09
C20:4w6[a]	9.59	19.82
C20:5w3	1.21	2.23
C22:0	--	--
C22:1	--	--
C22:4	1.07	1.28
C22:5w3	3.73	3.94
C22:6w3	1.15	0.84
C24:0	1.25	0.14
%SAT.	24.76	22.83
%MONO	29.90	20.57
%DI	20.65	23.64
%POLY[b]	24.69	32.96
%UNSAT.	75.24	77.17

[a] Control different from supplemented ($P<0.05$)
[b] POLY = fatty acid with 3 or more double bonds

C14:0 and C16:1w7. They also found a higher level of C18:0 in the phospholipid fraction, which is opposite of the trend in the present study. Shorland et al. (1981) found reduced levels of C12:0, C14:0 and C16:0 upon vitamin E supplementation of veal calves and attributed this to the chain elongating properties influenced by vitamin E. If this is true, one would expect the trends seen in the phospholipid fraction of the present study, with the exception that C18:0 should be increased instead of decreased. It was not expected that dietary supplementation with vitamin E would affect the fatty acid composition significantly. Other researchers have demonstrated no effect of vitamin E supplementation on fatty acid composition of pigs (Monahan et al., personal communication) or broilers (Lin et al., 1989). Thus, it would appear that fatty acid composition would not be a significant factor in determining the oxidative stability of muscle lipids from the control animals versus those supplemented with vitamin E.

Oxidative Stability of Veal

Muscle

Veal steaks from the vitamin E-supplemented animals were more oxidatively stable than those from the control calves (Table 7). Dietary supplementation resulted in significantly lower ($p<0.05$) TBARS in both raw and cooked steaks held at 4^{o}C for 4 days. Initial TBARS values (day 0) for muscles from the control animals were much higher than

Table 7. TBARS values (mg malonaldehyde/kg meat) for raw and cooked veal steak, from control and vitamin E-supplemented animals, held at 4°C for 4 days[a,b]

	Raw		Cooked	
	Control	Supplemented	Control	Supplemented
Day 0	3.8 ± 1.0	0.3 ± 0.0	6.2 ± 0.9	0.4 ± 0.1
Day 2	6.3 ± 0.6	0.4 ± 0.0	9.7 ± 0.7	1.9 ± 0.5
Day 4	7.6 ± 0.7	0.4 ± 0.1	12.4 ± 1.0	5.0 ± 0.5

[a] Value represents mean of 4 samples ± standard error
[b] Control different from supplemented (P<0.001)
[c] Time*Treatment interaction not significant at P<0.05

expected. This can be attributed, in part, to the fact that these samples were held in frozen storage for approximately 6 months prior to analysis. TBARS values of muscles from control animals increased over time of refrigerated storage, while those of muscles from the vitamin E supplemented group did not change much over time. Vitamin E supplementation had a protective effect on the oxidative stability of muscle lipids upon frozen and refrigerated storage. This is in agreement with the findings of Lin et al. (1989), who improved the stability of both white and dark meat of broilers fed vitamin E (100 IU/kg feed), and those of Monahan et al. (1989), who improved the stability of raw and cooked pork by supplementing with 200 IU vitamin E/kg feed. Asghar et al. (1989) also found that TBARS values of raw pork supplemented with 100 and 200 IU vitamin E/kg feed were lower than control samples. Those muscles from the 200 IU supplemented group were more stable than those from the 100 IU group.

Cooked muscle TBARS values were greater than those of raw muscle. This is expected as cooking disrupts the muscle membranes, thus exposing lipid substrates to oxidative catalysts (Rhee, 1988). Cooking also results in the release of nonheme iron, a catalyst of lipid oxidation (Igene et al. 1979; Schricker and Miller, 1983; Chen et al., 1984; Rhee and Ziprin, 1981).

Membranes

Membranal lipid oxidation is graphically presented for mitochondria and microsomes in Figures 1 and 2, respectively. Mitochondrial and microsomal membranes of the control animals oxidized to a greater extent than those from calves fed the vitamin E supplement. However, these differences were not significant at the $P<0.05$ level due to great variation among membranes from different animals and the small number (n=4) of animals in each group. The protective effect of vitamin E on membranal lipid oxidation in this study was also found in pork membranes (Asghar et al., 1989; Monahan et al., 1989) and in poultry dark muscle microsomes (Asghar et al., 1990a). In the present study, oxidation was greater in control mitochondrial membranes than microsomal membranes. This was also reported by Asghar et al. (1990c) for pork membranal lipid oxidation, using the same initiators as the present study. However, Monahan et al., (1989) reported similar rates of oxidation for pork mitochondrial and microsomal lipids in iron-induced oxidation studies. The fact that Monahan et al. (1989) reported higher α-tocopherol concentrations in microsomes than mitochondria and Asghar et al. (1990b) reported higher α-tocopherol in mitochondria than microsomes implies that other factors, besides α-tocopherol concentrations played a role in the observed rates of membranal lipid oxidation.

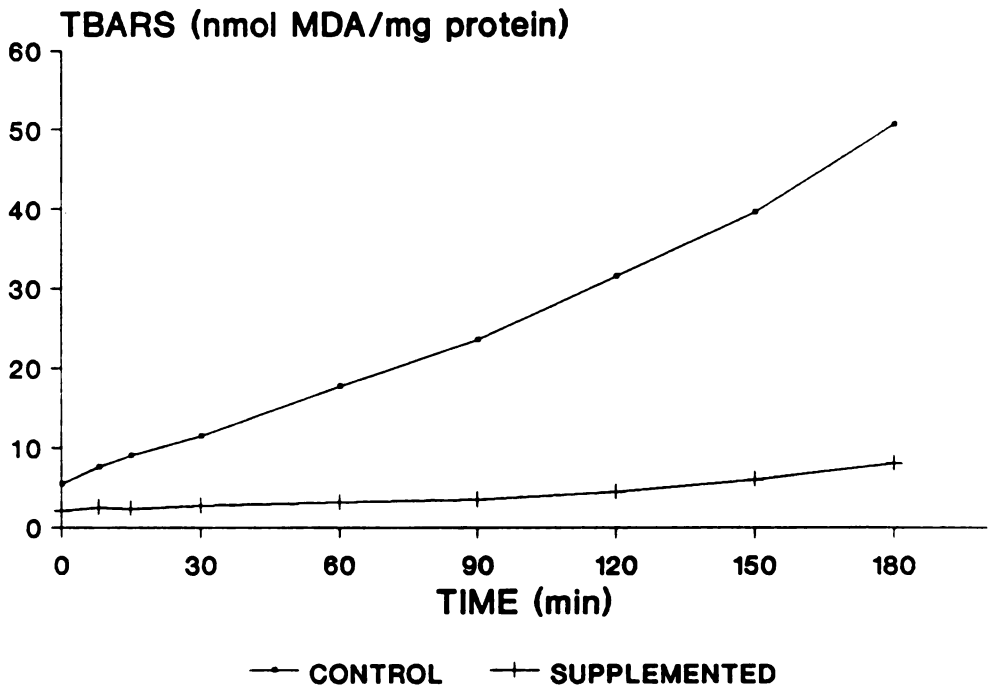

Figure 1. Lipid oxidation as measured by TBARS in mitochondria, from control and vitamin E-supplemented veal calves, initiated by metmyoglobin and hydrogen peroxide (30 μM each)

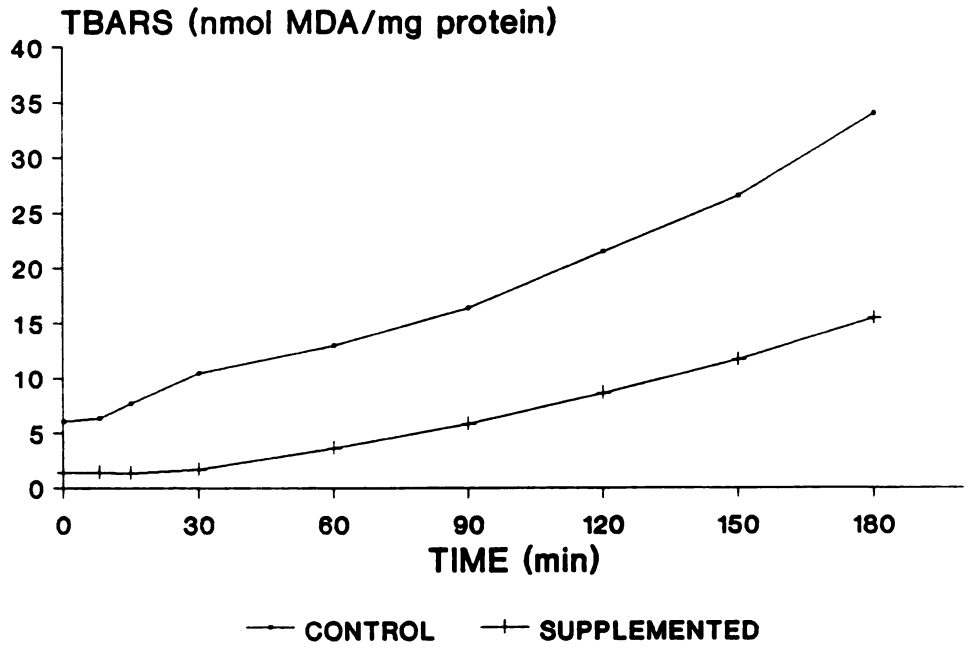

Figure 2. Lipid oxidation as measured by TBARS in microsomes, from control and vitamin E-supplemented veal calves, initiated by metmyoglobin and hydrogen peroxide (30 μM each)

Values for malonaldehyde production obtained in this study were much greater than those reported in other studies. For instance, Harel and Kanner (1985) reported malonaldehyde production after 120 min of incubation under similar reaction conditions in dark chicken microsomes to be approximately 17 nmol/mg protein. Incubation of veal muscle microsomes in this study for 120 min resulted in a concentration of approximately 22 nmol/mg protein (mitochondria >30 nmol/mg protein). Asghar et al. (1989) reported malonaldehyde concentrations at 180 min in pork mitochondria to be slightly less than 35 nmol/mg protein. This would be reasonable in comparison to those presented in this study for microsomes. Mitochondrial TBARS development was much higher due to two animals whose membranes were tested in duplicate at different times. The TBARS values of these two animals were not thrown out as outliers because they fell within 2 standard deviations of the mean.

Vitamin E supplementation of veal calves (500 IU, from birth to 12 weeks) resulted in increased incorporation of α-tocopherol into muscle membranes, where lipid oxidation is initiated in meats, and improved oxidative lipid stability of the membranes and muscle. This indicates the potential for a supplementation program in food-producing animals in order to produce higher quality meat and meat products and to prevent the consumer from the deleterious biological effects of lipid oxidation products.

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CHAPTER FOUR

MECHANISM OF CHOLESTEROL OXIDATION IN MUSCLE TISSUE

Abstract

Three model systems were designed to monitor the oxidative stability of cholesterol in different lipid environments. The cholesterol moiety of cholesteryl linoleate oxidized to a greater extent than that of cholesteryl stearate and free cholesterol. Cholesterol oxidized when dispersed with either phosphatidyl choline or adipose tissue, further demonstrating that cholesterol oxidation is affected by the surrounding lipid environment.

Oxidation of cholesterol in muscle tissue was also affected by the membrane environment. Vitamin E supplementation of veal calves improved the oxidative stability of cholesterol in veal. The oxidative stability of veal lipids, including cholesterol, was compared to that of retail beef. The results supported the hypothesis that if initiation of lipid oxidation occurs in the muscle membranes, the initial fat content of the meat should not influence the susceptibility of the lipids to oxidation.

Introduction

Cholesterol oxidation has been the subject of numerous studies in recent years (Smith, 1981; Addis and Park, 1989). Many of the oxidation products have been reported to produce a variety of adverse biological effects, such as inhibition of cholesterol biosynthesis (Kandutsch and Chen, 1973), atherogenesis (Taylor et al., 1979; Imai et al., 1980; Cox et al., 1988), cytotoxicity (Peng et al., 1979; Sevanian and Peterson, 1986), mutagenesis (Ansari et al., 1982; Sevanian and Peterson, 1986) and carcinogenesis (Bischoff, 1957; Black and Lo, 1971).

Until recently, knowledge of the occurrence of cholesterol oxidation products in foods was somewhat scarce because of limitations in methodology, the trace levels at which they occur in foods and changes in their concentrations upon isolation and quantitation (Finocchiaro and Richardson, 1983; Pearson et al., 1983; Park and Addis, 1987). As improvements in the methodology for the isolation and quantitation of cholesterol oxides were made, the significance of cholesterol oxidation in foods was realized. Cholesterol oxides have been found in a variety of food products such as eggs and egg powders (Chicoye et al. 1968; Tsai and Hudson, 1984; Missler et al. 1985; Nourooz-Zadeh and Appelqvist, 1987), dairy products (Sander et al., 1989; Nourooz-Zadeh and Appelqvist, 1988), heated fats (Ryan et al., 1981; Park and Addis, 1986a; 1986b) and meat products (Higley et al., 1986; Park and Addis, 1987). Commonly

reported cholesterol oxides in foods include 5-cholesten-3β-ol-7-one (7-ketocholesterol), 5-cholesten-3β,7α-diol (7-α-hydroxycholesterol), 5-cholesten-3β,7β-diol (7-β-hydroxycholesterol), 5-cholestan-3β,5α,6β-triol (cholestane triol), cholestan-5α,6α-epoxy-3β-ol (α-epoxide), cholestan-5β,6β-epoxy-3β-ol (β-epoxide) and 5-cholesten-3β,25α-diol (25-hydroxycholesterol).

The mechanism of cholesterol oxidation is similar to that of unsaturated fatty acid oxidation in that hydrogen is abstracted, resulting in the formation of a free radical, which then reacts with oxygen to form a peroxy radical (Maerker, 1987). This peroxy radical can abstract another hydrogen from another unsaturated molecule to propagate the reaction. The most likely site of hydrogen abstraction is at a carbon adjacent to a double bond. In cholesterol this would occur at the 7 carbon, resulting in the formation of 7 α- and β-hydroperoxides (major primary products) which then form 7 α- and β-hydroxycholesterol and 7-ketocholesterol (the most predominant products of cholesterol oxidation). Side chain oxidation also occurs at the tertiary 25 carbon, resulting in 25-hydroxycholesterol, and occasionally A ring oxidation may occur at the 3 carbon. Oxidation at these alternate sites is less common, thus there is a predominance of α- and β- isomers of 7-hydroxycholesterol (Maerker, 1987).

The present study is focused primarily on the oxidation of cholesterol in muscle tissues. There are only limited reports regarding cholesterol oxide formation in meats and meat products (Higley et al., 1986; Park and Addis, 1985; 1987). Higley et al. (1986) analyzed several samples of retail processed meats and found detectable cholesterol oxides (as determined by high performance liquid chromatography) in only a few samples, such as cooked bratwurst, cooked lean bacon, and raw and cooked hamburger. Park and Addis (1987) found cholesterol oxides in ground beef and ground turkey, which had been placed in cooking bags and cooked in a water bath at 80°C for 45 min and stored at 4°C for 0, 3 and 8 days. They also found cholesterol oxides in broiled beef steaks, but none in precooked beef which had been smoked in a vacuum cooking bag, sliced (1.5 cm thick), wrapped in polyvinylchloride film and stored at 4°C for 12 days. The extent of cholesterol oxidation in turkey was greater than that in beef, suggesting that those meats that are more prone to lipid oxidation in general, also may experience greater degrees of cholesterol oxidation. However, it is still unclear whether cholesterol oxidation occurs to any appreciable extent.

In lean muscle, cholesterol is primarily located in the membranal fraction (Dugan, 1987; Hoelscher et al., 1988). The phospholipid fraction of membranes is highly

unsaturated, thereby placing cholesterol in a lipid environment that is very susceptible to oxidation.

The objective of this study was to determine the significance of cholesterol oxidation and its relationship to fatty acid oxidation in meats. Because lipid oxidation in meats is initiated in the muscle membranes, the role of the membranal lipid environment on cholesterol oxidation was also investigated to determine whether the presence of polyunsaturated fatty acids enhances or inhibits cholesterol oxidation and whether stabilization of the membranal lipids with vitamin E (Chapter 3) will also prevent the oxidation of cholesterol.

Materials and Methods

Materials

Cholesterol, cholesteryl stearate, cholesteryl oleate, cholesteryl linoleate, phosphatidyl choline (from bovine liver) and tristearin standards were purchased from Sigma Chemical Co. (St. Louis, MO). Cholesterol oxide standards (α- and β-epoxide, 7-α-hydroxycholesterol, 7-β-hydroxycholesterol, cholestane triol, 7-ketocholesterol, and 25-hydroxy cholesterol) were purchased from Steraloids, Inc. (Wilton, NH). Bovine adipose tissue was obtained from the MSU Meat Laboratory. Veal samples (control and vitamin E-supplemented) were obtained from the feeding study as described in Chapter 3. Commercial beef round steak was purchased at a local supermarket.

Methods

Model System Studies

Dispersions. Three model system studies were undertaken for this experiment. Cholesterol compounds were dissolved in 1 ml ethanol in 150 x 25 mm screw-capped test tubes and vortexed for approximately 1 min (Vortex Genie, Fisher Scientific). Fifty ml of 10 mM phosphate buffer (pH 7) and approximately 50 mg of Triton-X-100 were added, and the mixture was vortexed for 1 minute (Vortex Genie, Fisher Scientific). In the first study, the dispersed compounds were 16 mg of cholesterol, cholesteryl stearate, cholesteryl oleate and cholesteryl linoleate. In the second study, 16 mg of cholesteryl stearate was dispersed for comparison to an equimolar concentration of cholesterol plus tristearin. The third study consisted of 16 mg of cholesterol with 16 mg of either bovine adipose tissue or phosphatidyl choline. The concentration of cholesterol in the adipose tissue was 0.7 mg/g (determined by a direct saponification method (Adams et al., 1986), modified as described in Chapter 1). Thus the contribution of cholesterol already present in the adipose tissue was considered negligible.

Oxidative Stability. Metmyoglobin and hydrogen peroxide were added to the dispersions in 125 ml Erlenmeyer flasks so that their final concentrations were 30 μM. The flasks were plugged with cheesecloth and incubated for 6 days in a shaking water bath (GCA/Precision Scientific) at 37^{o}C. Immediately after the initiators were added, and

every 48 hours thereafter, 5 ml of the dispersion were removed for cholesterol oxide analysis and 1 ml (in duplicate) for thiobarbituric acid analysis.

Cholesterol Oxide Determination. Samples (5 ml) were pipetted into 150 x 18 mm screw-capped test tubes containing 10 ml ethyl acetate and internal standard (6-ketocholestane, 0.1 mg). Tubes were vortexed for 2 min (Big Vortexer, Glass-Col Apparatus Co., Terre Haute, IN) and allowed to stand briefly for phase separation. The top ethyl acetate layer was transferred to another 150 x 18 mm screw-capped test tube using a disposable pipette. Extraction was repeated two more times using 10 ml and 5 ml portions of ethyl acetate. The ethyl acetate extracts were combined, dried over anhydrous sodium sulfate and frozen until saponification. Saponification was accomplished by a procedure described by Allen (1989). The ethyl acetate was removed under nitrogen. Potassium hydroxide in methanol (2 ml, 2 N) was added and the sample was placed in the dark for 20 hr at room temperature. Extraction of the nonsaponifiable fraction was achieved by placing the saponified solution in a glass centrifuge tube to which 1 ml distilled water and 2 ml isopropyl ether were added. The tubes were vortexed (30 sec) and centrifuged (IEC clinical centrifuge, speed 4, 1 min). This extraction was repeated two more times with 2 ml portions of isopropyl ether. The combined extracts were washed with 1 ml water and vortexed and centrifuged. The isopropyl ether layer was removed and

dried with anhydrous sodium sulfate. The extract was then placed in a 4 ml screw-capped vial and evaporated under nitrogen. When the solvent was dried, chloroform (approximately 4 ml) was added and the sample was frozen until gas-liquid chromatographic (GLC) analysis.

Samples were derivatized by drying the chloroform under nitrogen and adding 100 µl pyridine and 50 µl N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) silylating reagent (Pierce Chemical Co., Rockford, IL). The sample was wrapped in foil and held at room temperature for 30 min.

GLC analysis of the silylated compounds was carried out on a Hewlett Packard HP 5890A gas chromatograph (Hewlett Packard Co., Avondale, PA) using a 15 M x 0.25 mm id polydimethylsiloxane column (Supelco, Bellefonte, PA), with helium as a carrier gas (1-2 ml/min) and a flame ionization detector (300^{o}C). The oven temperature was programmed from 180^{o}C to 230^{o}C at 10^{o}C/min and immediately to 242^{o}C at 0.2^{o}C/min. This final temperature was held for 30 min. A split ratio of 16 was used. Areas were obtained using a HP 3392A integrator.

Total cholesterol concentrations in raw and cooked veal and beef samples were determined using a modified procedure of Adams et al. (1986), as described in Chapter 1. This allowed expression of cholesterol oxidation products as a percentage of the original cholesterol.

TBA Analysis. Sample (1 ml) was added to a 13 x 100 mm screw-capped test tube containing 2 ml thiobarbituric acid (TBA) reagent (200 ml of this reagent consisted of 0.8 g TBA, 20 g trichloracetic acid and 5 ml HCl). The tubes were lightly vortexed and capped prior to heating for 15 min in a boiling water bath. The tubes were cooled to room temperature, centrifuged (IEC Clinical Centrifuge, speed 5, 10 min) and the color intensity quantitated using a Bausch and Lomb Spectronic 2000 spectrophotometer (Rochester, NY) at 532 nm.

Meat Studies

TBA-reactive substances (TBARS) were analyzed for raw and cooked veal and beef samples stored for 4 days at 4°C. Veal steaks (approximately 1 inch thick) and beef round steaks (approximately 3/4 inch thick) were broiled in a conventional oven to an internal temperature of 170°F (using a copper constantan thermocouple: Omega Thermocouple Thermometer, Model 660, Omega Engineering, Inc.). TBARS development was monitored at 0, 2 and 4 days. Cholesterol oxide determinations were conducted at 0 and 4 days.

TBA Analysis. Muscle TBARS values were determined by a modified method of Tarladgis et al. (1960) as described in Chapter 2.

Cholesterol Oxide Determination. Total lipid was extracted from ground muscle samples (5 g) by the dry column procedure of Marmer and Maxwell (1981). The lipid extract was dried on a rotary evaporator (Brinkmann Instruments,

Inc., Westbury, NY) and redissolved in 4 ml hexane:ethyl acetate (9:1). The extract was applied to a silica gel column prepared as described by Park and Addis (1985). Four grams of silica gel 60H (E. Merck, Darmstadt, Germany) were made into a slurry with approximately 50 ml of hexane:ethyl acetate (9:1) and packed into a glass column (45 cm x 16 mm id) which was plugged with glass wool. Anhydrous sodium sulfate was added to the top of the column prior to application of the sample. Internal standard (6-ketocholestane, 1 ml of 0.1 mg/ml) was also added with the sample. Elution of cholesterol oxides was accomplished by eluting first with 26 ml hexane:ethyl acetate (9:1) and then 20 ml hexane:ethyl acetate (8:2), followed by the addition of 45 ml acetone. The acetone fraction was collected as the cholesterol oxides elute in this fraction. Acetate was removed by rotary evaporation and the cholesterol oxides were transferred to a 4 ml screw-capped vial using ethyl acetate. The ethyl acetate was evaporated under nitrogen and the sample redissolved in 4 ml ethyl acetate and frozen before derivatization. Derivatization and GLC analysis were performed as described above.

Results and Discussion

Cholesterol Oxidation in Model Systems

Cholesterol vs Cholesteryl Esters

Aqueous dispersions of cholesterol, cholesteryl stearate and cholesteryl linoleate that were incubated 6

days at 37°C with metmyoglobin and hydrogen peroxide as initiators of oxidation did not develop appreciable quantities of TBARS over time (Table 1). TBARS values increased from day 0 to day 2 and decreased over the remaining course of the study. This decrease is often found in TBARS analyses and is one of the limitations of this method. In muscle tissue, it has been suggested that the reason for a decline in TBARS values over time may be due to an interaction of oxidation products with proteins (Gardner, 1979). However, no such free amine groups were present in this system. Reasons for decreasing TBARS values over time need to be further clarified. Minimal TBARS development is consistent with the fact that the predominant TBARS, malonaldehyde, is produced primarily from oxidation of fatty acids with 3 or more double bonds (e.g., linolenate) (Dahle et al., 1962). Because of the nature of malonaldehyde as a breakdown product from lipid oxidation (Gutteridge and Quinlan, 1983) TBARS development is not expected to be an indicator of cholesterol oxide development.

The cholesterol moiety of cholesteryl linoleate oxidized to a greater extent than that of cholesteryl stearate and free cholesterol (Table 2). Since susceptibility to oxidation increases with increasing unsaturation (Labuza, 1971), greater oxidation in linoleate as opposed to stearate may result in increased free radical formation in close proximity to cholesterol and thus

Table 1. TBARS values (nmol malonaldehyde/mg sample) for cholesterol and cholesteryl esters in aqueous dispersions at 37°C for 6 days[a]

	C	CS	CL[b]
Day 0	0.0	0.1	0.1
Day 2	0.6	0.5	1.6
Day 4	0.0	0.0	0.1
Day 6	0.0	0.0	0.0

[a] Mean of duplicate samples
[b] C=Cholesterol; CS=Cholesteryl Stearate; CL=Cholesteryl Linoleate

Table 2. Cholesterol oxide concentrations (μg/ml dispersion) in model systems containing cholesterol, cholesteryl stearate and cholesteryl linoleate and exposed to metmyoglobin and hydrogen peroxide as initiators[a,b,c]

Oxide	C		CS		CL[d]	
	Day 0	Day 6	Day 0	Day 6	Day 0	Day 6
β-epoxide	ND	ND	ND	ND	1.5	12.4
α-epoxide	ND	ND	ND	ND	tr	2.7
7-β-OH	ND	ND	ND	ND	1.4	6.2
triol	ND	ND	ND	ND	ND	ND
7-keto	ND	ND	ND	ND	3.6	16.4
25-OH	ND	ND	ND	ND	ND	ND

[a] Mean of duplicate samples
[b] ND=Not detected (detection limit: 1 ng)
[c] tr=trace amount
[d] C=Cholesterol; CS=Cholesteryl Stearate; CL=Cholesteryl Linoleate

increase the susceptibility of cholesterol to oxidation when esterified to an unsaturated fatty acid such as linoleate.

These results agree with those of Norcia and Janusz (1965) who found cholesteryl linoleate oxidized to a greater extent than free cholesterol, which in turn oxidized to a greater extent than cholesteryl oleate. These investigators suggested a free-radical mechanism. They also suggested that the molecule would undergo a reorientation after attack by oxygen and this would render cholesterol more susceptible to oxygen attack. Stearic hindrance was the explanation offered to explain the fact that cholesterol oxidized to a greater degree than cholesteryl oleate. The fatty acid would fold over cholesterol and protect against attack at the 7-carbon. Since neither cholesterol or cholesteryl stearate was detectably oxidized (detection limit: 1 ng) in the present study this theory cannot be investigated.

Differences between the results of the present study and other literature reports could be expected due to the use of different dispersion systems. Korahani et al. (1982) found different rates of cholesterol oxidation depending on whether the cholesterol compounds were in solid state or aqueous dispersion. In the solid state, the cholesterol moiety of stearate and oleate esters was oxidized more rapidly than the polyunsaturated fatty acid esters and these more rapidly than free cholesterol. In aqueous dispersion, free cholesterol oxidized more than its stearate and oleate esters.

Using another dispersion system, Zulak and Maerker (1989) reported that cholesterol oxidized to a greater extent in a dispersion prepared with sodium linoleate as opposed to sodium stearate. However, there was a longer period initially of no cholesterol oxidation (induction period) in the dispersion with sodium linoleate, even though the rate of oxidation eventually increased to a point where it exceeded that of the cholesterol in the dispersion with sodium stearate. These investigators could not explain why the induction period was extended in the case of sodium linoleate. One suggestion was that the polyunsaturated fatty acids may protect the cholesterol by being preferentially oxidized. This theory is the subject of another experiment in the present study, to be discussed below. Zulak and Maerker (1989) also suggested that more work be done related to micellar structure and cholesterol oxidation.

Another question which arises from the present study is whether cholesterol is more susceptible to oxidation in its ester form or when dispersed as cholesterol in an environment of the same fatty acid composing the cholesteryl ester. An answer to this question might lead to speculation about influences of micellar structure and possible stearic hindrances.

Cholesteryl Stearate vs Cholesterol plus Tristearin

Again, TBARS development was minimal in these model systems. Oxidation of the cholesterol moiety in the

cholesteryl stearate dispersion or in the dispersion with tristearin was not observed under these conditions. Thus, it would be difficult to speculate whether cholesterol oxidizes more rapidly in ester form or in free form in a similar lipid environment. Other investigators have compared the oxidation of cholesterol and various cholesterol esters (Norcia and Janusz, 1965; Korahani et al., 1982). However, none have investigated the effects of keeping the fatty acid composition of the environment constant.

Cholesterol plus Adipose Tissue vs Cholesterol plus Phosphatidyl Choline

One concern addressed previously is whether the presence of polyunsaturated fatty acids, such as in the muscle membranes, actually enhances or inhibits cholesterol oxidation. A comparison of the oxidative stability of equal amounts of cholesterol in two environments: phosphatidyl choline (PC) (representing a lipid environment similar to a membranal system) and bovine adipose tissue (AT)) was done.

TBA development again was minimal over the entire incubation period (data not shown) in both environments. This may be explained by the fact that the major precursors for malonaldehyde production, i.e., polyunsaturated fatty acids with 3 or more double bonds, were not present in great quantities.

Cholesterol oxides were detected in extracts from both the PC and AT (Table 3). Minimal oxide development occurred

Table 3. Cholesterol oxide concentrations (μg/ml dispersion) in model systems containing cholesterol (C) in either adipose tissue (AT) or phosphatidyl choline (PC) with metmyoglobin and hydrogen peroxide as initiators[a,b,c]

Oxide	C + AT		C + PC	
	Day 0	Day 6	Day 0	Day 6
β-epoxide	0.6	1.5	3.1	1.4
α-epoxide	ND	0.4	ND	tr
7-β-OH	ND	0.9	ND	tr
triol	ND	ND	ND	ND
7-keto	ND	1.8	1.0	0.5
25-OH	ND	ND	ND	ND

[a] Mean of duplicate samples
[b] ND=Not detected (detection limit: 1 ng)
[c] tr=trace amount

at day 0, except for β-epoxide and 7-ketocholesterol in the PC samples. These high values were the result of correction for low recovery on one sample and may not have been so large if the sample size was larger. When analyzing trace amounts of cholesterol oxides, there may be great sample to sample variation. In the AT incubation, the oxides became more prominent after 6 days storage. It appears that cholesterol AT dispersion oxidized to a slightly greater extent than in the dispersion with PC after 6 days incubation. Without further study, one cannot conclude from this work whether polyunsaturation, as in the muscle membranes, actually enhances or inhibits the oxidation of nearby cholesterol. Several factors such as degree and mode of dispersion, micellar structure, and possible antioxidant components need to be considered in future studies.

Several changes in this study should be made for future research. One problem encountered was the dispersion of cholesterol and its esters into aqueous solution. It was necessary to use detergent, and Triton-X-100 was the detergent of choice (Maerker and Bunick, 1986). However, it was found that the presence of detergent caused many extra peaks on the chromatograms. Although these did not interfere with peaks of interest initially, they did pose problems with resolution and integration. Also, it would be interesting to note oxidative changes at more frequent

intervals. According to Zulak and Maerker (1989), changes in cholesterol oxidation among samples were noted over time within a 24-hour period.

This study has demonstrated that the environment surrounding cholesterol in muscle tissues may have an important role in determining the susceptibility of cholesterol to oxidation. It has been demonstrated that cholesterol oxidizes to a greater degree as cholesteryl linoleate than as free cholesterol or cholesteryl stearate. Also that the oxidation of nearby unsaturated lipids may result in the formation of free-radicals which may attack cholesterol. Although it was not possible to conclude whether cholesterol was more susceptible to oxidative attack in the dispersions with AT or PC, one can determine from the study that the oxidation of unsaturated lipids makes the cholesterol prone to oxidation itself.

Model system studies such as these allow the deciphering of details in mechanistic features of cholesterol oxidation. The new information may be applied to or be helpful in learning more about cholesterol oxidation in meat systems.

Cholesterol Oxidation in Meat

Previously reported results (Chapter 3) indicate that vitamin E supplementation in the diets of veal calves increased α-tocopherol levels in membranes and thus resulted in improved oxidative stability of membranal and muscle lipids.

Cholesterol oxide development in these veal muscles (raw and cooked) was determined after 0 and 4 days of storage at 4°C (Tables 4 and 5). Vitamin E supplementation was effective in controlling the development of cholesterol oxides in both raw and cooked muscles at 4 days storage. Day 0 values were not clearly reduced by supplementation. As stated in Chapter 3, these samples were frozen for several months prior to analysis. Thus, day 0 values for cholesterol oxides were much greater than expected. The problem with cholesterol oxide values from the supplemented group is that the muscles from one animal in the group had levels of cholesterol oxides much greater than the others. Because of the small sample size (n=4), the group means were high. This points to the need for larger sample sizes. Control of cholesterol oxidation by vitamin E supplementation, however, was obvious upon storage for 4 days of both raw and cooked samples. As found with other indices of oxidation, the oxide development was greater in cooked samples. This is consistent with previous findings and explained by the harsh conditions of cooking which leads to the disruption of the membranes and subsequent exposure of lipid substrates to oxidative catalysts (Rhee, 1988). One would expect this to apply to cholesterol as well, since most of the cholesterol in muscle is located in the membranes (Dugan, 1987; Hoeschler et al., 1988).

Table 4. Cholesterol oxide concentrations (μg/g sample) in raw veal held at 4°C for 4 days[a,b,c]

Oxide	Day 0		Day 4	
	Control	Vit. E[d]	Control	Vit. E
β-epoxide	0.2	0.8	3.6	0.2
α-epoxide	tr	0.1	0.4	tr
7-β-OH	1.5	1.8	3.6	2.0
triol	ND	ND	ND	ND
7-keto	0.3	1.1	6.5	0.3
25-OH	tr	ND	tr	ND
Total	2.0	3.8	14.1	2.5

[a] Mean of 4 samples
[b] ND=Not detected (detection limit: 1 ng)
[c] tr=trace amount
[d] Vit. E=Supplemented veal

Table 5. Cholesterol oxide concentrations (μg/g sample) in cooked veal held at 4°C for 4 days[a,b,c]

Oxide	Day 0		Day 4	
	Control	Vit. E[d]	Control	Vit. E
β-epoxide	1.1	6.4	4.4	1.1
α-epoxide	0.1	0.5	0.3	0.2
7-β-OH	2.7	1.5	4.0	1.8
triol	ND	ND	ND	ND
7-keto	5.5	0.2	7.2	2.7
25-OH	ND	ND	0.9	ND
Total	9.4	8.6	16.8	5.8

[a] Mean of 4 samples
[b] ND=Not detected (detection limit: 1 ng)
[c] tr=trace amount
[d] Vit. E=Supplemented veal

Previously reported (Chapter 3) TBARS values for raw and cooked veal are presented in Table 6 and show similar trends as noted for cholesterol oxidation.

Cholesterol oxidation also occurred in raw and cooked beef samples held at 4°C for 4 days (Table 7). These beef samples were analyzed immediately after purchase from a local supermarket. Thus, there was little cholesterol oxide formation on day 0. Cooked samples showed greater oxidation than raw as explained above. Cholesterol oxidation followed similar trends as noted in TBARS development (Table 8).

Park and Addis (1987) also noted greater cholesterol oxide development with increasing degrees of rancidity (as determined by TBARS values) in meats. A comparison of their results with those of the present study is presented in Table 9, where the total cholesterol oxidation products are expressed as a percentage of the cholesterol concentration in a variety of cooked meats. Although samples were not analyzed on the same days, trends in the data were observed. The sample in which cholesterol oxidation was the least was supplemented veal. This was expected as the membranal lipids would be stabilized by α-tocopherol, thus protecting cholesterol from oxidation. Beef samples in the present study oxidized to a slightly greater degree than those of Park and Addis (1987). This difference can be attributed to the fact that in both studies retail beef samples were used, the history of which was unknown. Also, it is possible that detection limits were different, thus accounting for

Table 6. TBARS values (mg malonaldehyde/kg meat) for raw and cooked veal steak, from control and vitamin E-supplemented animals, held at 4°C for 4 days[a,b]

	Raw		Cooked	
	Control	Supplemented	Control	Supplemented
Day 0	3.8 ± 1.0	0.3 ± 0.0	6.2 ± 0.9	0.4 ± 0.1
Day 2	6.3 ± 0.6	0.4 ± 0.0	9.7 ± 0.7	1.9 ± 0.5
Day 4	7.6 ± 0.7	0.4 ± 0.1	12.4 ± 1.0	5.0 ± 0.5

[a] Value represents mean of 4 samples ± standard error
[b] Control different from supplemented ($P<0.001$)
[c] Time*Treatment interaction not significant at $P<0.05$

Table 7. Cholesterol oxide development (μg/g sample) in raw and cooked beef round steak held at 4°C for 4 days[a,b,c]

Oxide	Raw		Cooked	
	Day 0	Day 4	Day 0	Day 4
β-epoxide	tr	5.2	tr	7.7
α-epoxide	tr	tr	tr	tr
7-β-OH	1.4	0.7	3.1	4.8
triol	ND	ND	ND	ND
7-keto	tr	tr	tr	4.8
25-OH	ND	ND	ND	ND
Total	1.4	5.9	3.1	17.3

[a] Mean of duplicate samples
[b] ND=Not detected (detection limit: 1 ng)
[c] tr=trace amount

Table 8. TBARS values (mg malonaldehyde/kg meat) for beef round steak held at 4°C for 4 days[a]

	RAW	COOKED
Day 0	0.6	0.5
Day 2	1.2	2.2
Day 4	0.7	3.2

[a] Mean of duplicate samples

Table 9. Total cholesterol oxidation products (expressed as a percentage of total cholesterol) in various cooked meat samples stored at 4°C[a,b]

Days	Veal[c]	Beef[d]	Beef[e]	Turkey[f]	Veal-Vit. E[g]
0	0.85	0.30	ND	ND	0.78
3	---	---	0.45	1.20	---
4	1.53	1.69	---	---	0.53
8	---	---	1.74	2.90	---

[a] ND=Not detected (detection limit: 1 ng)
[b] --- = Not determined on these days
[c] Control veal from feeding study (Chapter 3)
[d] Retail beef round (Chapter 4)
[e] Comminuted beef (Park and Addis, 1987)
[f] Comminuted turkey (Park and Addis, 1987)
[g] Supplemented veal from feeding study (Chapter 3)

different total cholesterol oxidation products to be used in calculating the percentages. One would expect to observe higher percentages in turkey samples due to the higher degree of unsaturation in the phospholipid fraction of turkey (Jantawat and Dawson, 1980). This appears to be the trend. Even though the cholesterol content of turkey is lower than that of other red meats, total cholesterol concentration, expressed as a percentage of the original cholesterol concentration, was greater than beef (8 days, 2.90%). This reflects the higher degree of unsaturation in the fatty acids of turkey as opposed to beef. Thus, it is again shown that greater unsaturation in the muscle membranes may result in greater susceptibility of cholesterol to oxidation. Protection of membranal lipids against oxidation of unsaturated lipids will aid in the stabilization of cholesterol.

The predominant cholesterol oxides found in veal and beef samples were 7-ketocholesterol, 7-β-hydroxycholesterol and β-epoxide. A high porportion of 7-ketocholesterol and 7-β-hydroxycholesterol was expected as these are the major products of cholesterol oxidation resulting from attack at the 7 carbon (Maerker, 1987). Park and Addis (1987) reported finding similar cholesterol oxides in various meats, with 7-ketocholesterol being most predominant. These researchers were also able to detect cholestane triol, a very atherogenic cholesterol oxide, for the first time in meat samples. However, it was detected in freeze-dried pork

which had been kept at room temperature for 3 years. No triol was detected in the present study. It appears that this oxide may be of importance in meat held under extended storage conditions.

This study provides more evidence to support the premise that initiation of lipid oxidation in meats occurs in the muscle membrane as opposed to the extracellular adipose tissue (Gray and Pearson, 1987). If this is true, lean muscle, such as veal, should be just as susceptible to oxidative attack as muscle with normal fat levels (represented by the retail beef in this study). Although the veal was held frozen for several months prior to analysis, it is apparent that lipids in general, including cholesterol, are as susceptible to oxidative attack in veal as in beef.

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SUMMARY AND CONCLUSIONS

A series of studies was designed to investigate the mechanisms of lipid oxidation in meats, with specific emphasis on cholesterol oxidation. Membranal lipid stability was the focus as it is believed that initiation of oxidation in meats occurs in muscle membranes.

Methods for quantitating total cholesterol in meats were evaluated for accuracy, efficiency and reproducibility. The method selected was a direct saponification procedure followed by extraction and gas-liquid chromatographic analysis. This method was then applied to survey a variety of meats for total cholesterol content and changes in the cholesterol content upon cooking. These values agreed with reported literature values.

Also studied was the relationship between membrane lipid stability and overall oxidative stability of raw and cooked beef, pork and chicken. Cooked pork samples oxidized to a greater extent than beef during storage, while dark chicken was more highly oxidized than white chicken. Trends in composition and stability lead to the suggestion that the stability of lipids in muscle tissue is affected by not only the fatty acid composition, but also by other factors such as lipid content, proportions of neutral and phospholipids and the unknown history of retail meat. Trends in membranal

oxidation were similar to those in muscle. The stability and composition of membranal lipids reflected the overall stability of meat lipids.

A third study was designed to investigate the influence of dietary vitamin E in the diet of veal calves on the incorporation of α-tocopherol into muscle membranes and on the resultant oxidative stability of veal lipids. Daily supplementation with 500 IU vitamin E resulted in nearly 6-fold increases in muscle and membranal α-tocopherol concentrations and enhanced oxidative stability of muscle and membrane lipids and cholesterol. Although growth performance was not improved by supplementation with vitamin E, a supplementation program of food producing animals would improve the freshness, quality and safety of meat and meat products to be presented to the consumer.

Model system studies were also conducted to further investigate the mechanism of cholesterol oxidation in muscle tissues. The cholesterol moiety of cholesteryl linoleate oxidized to a greater extent than that of cholesteryl stearate and free cholesterol. Also, cholesterol oxidized when dispersed with phosphatidyl choline or with adipose tissue. These studies demonstrated that the environment surrounding cholesterol in muscle tissues may have an important role in determining the susceptibility of cholesterol to oxidation. Also that the oxidation of nearby unsaturated lipids may result in the formation of free-radicals which may attack cholesterol.

Cholesterol oxidation occured in beef and veal steaks stored for 4 days at 4°C. Oxidation was detectable in raw meats but was greater in cooked meats as a result of membrane disruption. The lipids of veal, including cholesterol, were as susceptible to oxidation as those of beef. This would be expected since initiation of lipid oxidation occurs in the muscle membrane. Membranal composition greatly affects the stability of cholesterol in muscle tissue.

FUTURE RESEARCH

In this study the mechanism of lipid oxidation was addressed, with specific emphasis on cholesterol oxidation. During the course of this study, several areas for future research were revealed. It was demonstrated that oxidation of cholesterol in meats during storage may be a significant concern and that such oxidation is influenced by the lipid composition surrounding the cholesterol in muscle membranes. Development of a reliable method for determining the content and composition of cholesteryl esters in meat may be helpful in determining their susceptibility to cholesterol oxidation. Studies relating membranal composition to lipid stability of muscle (Chapter 2) should also then be extended to include cholesterol oxidation.

Protection of membranal oxidation of unsaturated fatty acids in veal calves by dietary vitamin E supplementation also reduced cholesterol oxidation. Further studies on dietary vitamin E supplementation of food-producing animals may be necessary to determine optimal duration and dosage to achieve the desired lipid stability. Closer examination of various growth characteristics may also be necessary as a means of developing greater incentive for producers to adopt such a supplementation program.

Further studies on the mechanistic features of cholesterol oxidation are also necessary. One difficulty associated with such research in model systems is that cholesterol and cholesteryl ester standards may already

contain some oxidized material. This problem has plagued many lipid oxidation studies in the past (Gutteridge and Halliwell, 1990). It is difficult to study initiation when oxidation has already occurred. Methods for obtaining pure cholesterol and cholesteryl ester standards for model system work need to be developed. Techniques such as high performance liquid chromatography may be used to eliminate peroxy compounds. Dispersion systems for model studies are also critical to the study; thus, better methods for preparing dispersions should be developed. Some alternatives include the use of bilayer liposomes and monolayer films of polyunsaturated fatty acids on silica gel. Improvements in model systems such as these, together with improvements in methodology for cholesterol oxide detection and quantitation would allow further investigation into the various factors which promote the oxidation of cholesterol. Also, study of various mechanistic features of cholesterol oxidation would be facilitated.

With the knowledge obtained from such work one may be able to control cholesterol oxidation (and overall lipid oxidation) in meat products and present consumers with meats which are more stable and fresh.

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