Influence of Temperature, pH, and Phospholipid Composition upon the Stability of Myoglobin and Phospholipid: A Liposome Model

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An oxymyoglobin (OxyMb)-liposome model was used to study the effects of temperature, pH, phospholipid (PL) polar head group, and fatty acid composition upon oxymyoglobin and PL oxidation (TBA). Both oxidations were enhanced with increased temperature (37 > 20 > 10 > 4 °C) and decreased pH (5.6 > 6.4 > 7.2) ($P < 0.05$). OxyMb and PL oxidation in OxyMb-liposomes prepared from commercial PLs followed the order phosphatidylserine (PS) > phosphatidylethanolamine (PE) > phosphatidylcholine (PC) ($P < 0.05$). Since both PL polar head group and fatty acid composition were believed to affect stability of OxyMb and the PL bilayer, PC and PE with defined fatty acids at Sn1 = 16:0 and Sn2 = 18:2 or 20:4 were used to construct OxyMb-liposomes. When fatty acid composition was held constant, PE-based OxyMb-liposomes showed a greater increase in both oxidations than PC-based OxyMb-liposomes ($P < 0.05$). When the PL polar head group was held constant, increased chain length and unsaturation of fatty acids resulted in greater OxyMb and PL oxidation ($P < 0.05$). The in vitro model provided evidence that OxyMb and PL oxidations are interrelated.

INTRODUCTION

The color of meat is regarded as an important parameter of meat quality (Faustman and Cassens, 1990). It is principally dependent upon the redox state of the heme iron of myoglobin (Mb) and the extent of oxygenation of the heme protein. The cherry red color that consumers prefer in fresh meat is due to oxymyoglobin (OxyMb). The discoloration of meat from red to brown during storage results from the oxidation of OxyMb to metmyoglobin (MetMb) in which heme iron is converted to the ferric state and the heme protein is no longer oxygenated.

Rancidity in meat involves the oxidation of unsaturated fatty acids, in particular polyunsaturated fatty acids (PUFA) (Kanner et al., 1988). The content of PUFAs associated with membrane phospholipids (PLs) is believed to be the major factor for the development of off-flavor in meat (Moreck and Ball, 1974; Fogerty et al., 1989). OxyMb oxidation and PL oxidation appear to be interrelated in meat (Greene, 1969; Kanner and Harel, 1985). The radical species produced during PL oxidation may act to promote pigment oxidation (Little and O'Brian, 1968). Conversely, superoxide anion released from oxidized OxyMb can dismutate to hydrogen peroxide and hydroxyl radical, each of which is a potent prooxidant of PL (Tajima and Shikama, 1987). Although the role of fatty acid composition in meat upon PL oxidation has been studied (German and Kinsella, 1985), the relationship between OxyMb and PL oxidation remains unclear.

Liposomes have been used extensively as model membranes; they present a simple and controllable system for biological membrane study (Yuasa et al., 1987; Kagan et al., 1990). Water-soluble constituents can be incorporated into the aqueous phase of liposomes, while lipid-soluble species are embedded in the membrane. Farmer and Gaber (1987) used liposomes to encapsulate hemoglobin for the production of synthetic red cells to be used as an emergency blood replacement. Hemoglobin and Mb are closely related and behave in a similar manner physiologically.

A logical approach for studying Mb and PL oxidation behavior would be to use liposomes to encapsulate OxyMb. Many factors such as temperature (Brown and Mebine, 1969; Lanier et al., 1977; Hood, 1980), pH (Shikama and Sugawara, 1978; Chow, 1991), and partial oxygen pressure (Okayama, 1987; Forrest et al., 1975) have been examined in meat to determine their roles in discoloration or PL oxidation. The effects of these parameters on OxyMb and PL oxidations have not been studied simultaneously.

In this investigation, the influence of temperature, pH, and phospholipid composition upon OxyMb and PL oxidation was studied by a liposome model system; the long-term goal of the work is to model these phenomena in meat.

MATERIALS AND METHODS

Oxymyoglobin Preparation. All preparation steps were carried out at 4 °C. Oxymyoglobin was prepared according to Brown and Mebine (1969) as follows. Commercial MetMb (Sigma Chemical Co.) was dissolved in buffers of desired pH (pH 5.6, sodium citrate buffer; pH 6.4 and 7.2, sodium phosphate buffer), chemically reduced by mixing with sodium hydrosulfite (0.1 mg/mg of MetMb), and oxygenated by bubbling air through the solution. The residual hydrosulfite was removed by column chromatography on a mixed-bed ion-exchange resin (Bio-Rad Ag 501-X8). The OxyMb concentration was determined via spectrophotometry by the absorbance at 525 nm (Krzywicki, 1982) and adjusted to 2.5 mg/mL for OxyMb-liposome preparation, a concentration representing an average of four beef muscles (longissimus dorsi, psoas major, biceps femoris, and semitendinosus) (Rickansrud and Henrickson, 1967).

Oxymyoglobin-Liposomes Preparation. All PLs [commercial phosphatidylcholine (PC); phosphatidylserine (PS); phosphatidylethanolamine (PE); and PC, PE with defined fatty acids] were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Fatty acid composition of commercial PL was analyzed by gas chromatography (LePage and Roy, 1986). Liposome preparation followed the general procedure of New (1990) for multilamellar vesicles and was as follows. Thirty milligrams of PL, 12 mg of cholesterol, and 3 mg of dicetyl phosphate were dissolved in 5 mL of chloroform/methanol (2:1 v/v) and placed in a 100-mL round-bottom flask. Solvent was removed and a lipid film was formed by rotary evaporation. OxyMb solution (10 mL) and 1 mg of glass beads (1- mm diameter) were mixed with the lipid film. OxyMb-liposomes were formed by mechanical shaking of the mixture for 30 min at 5 °C on a table top shaker, and allowing to stand 1 h.

Measurement of OxyMb-Liposomes Oxidation. The effects of four temperature (4, 10, 20, and 37 °C) and three pH conditions (5.6, 6.4, and 7.2) were studied using PC-based OxyMb-

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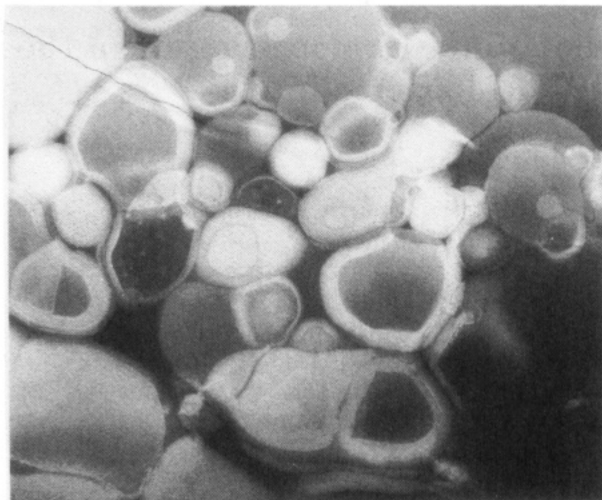

Figure 1. Electron micrograph of phosphatidylcholine (3 mg/mL) multilamellar liposomes (85000×, negative stain). (The figure is reproduced here at 70% of the original.)

liposomes. The four temperatures represent four different practical conditions of meat: refrigerated storage, retail display temperature, room temperature, and physiological temperature. The three pH values represent the pH of normal post-mortem meat (5.6), DFD (dark, firm, and dry) meat (6.4), and physiological pH (7.2). The effects of PL polar head group and fatty acid composition were studied by PC-, PE-, or PS-based OxyMb–liposomes incubated at pH 5.6 and 10 °C. Oxymyoglobin oxidation was measured spectrophotometrically from 650 to 500 nm by use of a diffuse-integrating sphere. Percent metmyoglobin was determined according to the method of Krzywicki (1982). Lipid oxidation was measured by the thiobarbituric acid assay (TBA assay) of Schmedes and Holmer (1989). One milliliter of Mb–liposomes was mixed with 0.5 mL of 20% trichloroacetic acid (TCA) and the mixture centrifuged at 1400g for 5 min at 4 °C. One milliliter of supernatant was mixed with 1 mL of 2-thiobarbituric acid (TBA) reagent (0.02 M) and the mixture stored in the dark for 20 h at 25 °C. At the end of 20 h, the absorbance at 532 nm was recorded and reported directly as TBA number.

Preliminary investigations (data not shown) revealed that for commercial PC-based OxyMb–liposomes, MetMb formation and lipid oxidation (TBA no. [A_{532}]) were linear up to values of 70% and 0.500, respectively. For the experiments reported herein, measurements of MetMb formation and lipid oxidation were made at several time points during the incubations. Initial rates of the oxidative processes were calculated from the linear portion of the reaction curve. The effect of each experimental condition was analyzed on OxyMb–liposomes from five different preparations.

Statistical Analysis. Data were treated by analysis of variance (ANOVA) and computed using the SAS General Linear Model (GLM) procedure (SAS, 1985). Temperature and pH data were evaluated by two-way ANOVA. Differences among means were determined by the least significance difference test.

RESULTS AND DISCUSSION

An electron micrograph of PC liposomes (multilamellar vesicles) used in these investigations is presented in Figure 1. A spectral curve of deoxy-, oxy-, and met-Mb–liposomes was recorded and is presented in Figure 2. The spectral curves of the Mb–liposomes are consistent with those of the protein in vitro or in meat (Broumand et al., 1958; Fox, 1966).

The influence of temperature and pH upon the rate of MetMb formation and PL oxidation is shown in Table I. There was significant interaction between temperature and pH ($P < 0.05$). In this OxyMb–liposome system, higher temperature led to an increase of both OxyMb and PL oxidations. Metmyoglobin formation and PL oxidation

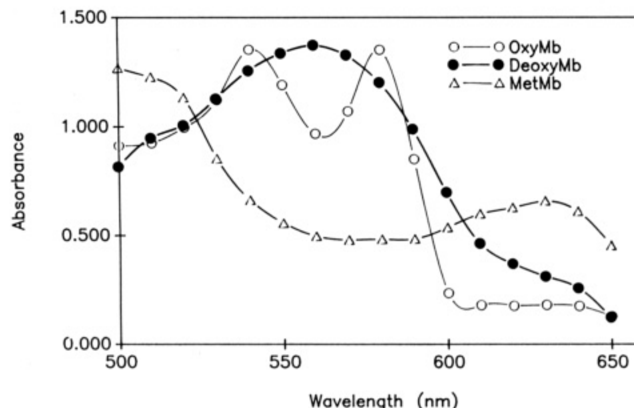

Figure 2. Absorbance spectra for oxy-, deoxy-, and metmyoglobin liposomes.

Table I. Effect of Temperature and pH on Metmyoglobin (MetMb) Formation Rate and PL Oxidation (TBA) Rate in Oxymyoglobin–Phosphatidylcholine Liposomes (n = 5)[a]

temp (°C)	pH	% MetMb/h (mean ± SD)	TBA/h (mean ± SD)
4	5.6	$1.4^{b} \pm 0.05$	$0.006^{a} \pm 0.001$
	6.4	$0.7^{a} \pm 0.01$	$0.003^{a} \pm 0.001$
	7.2	$0.2^{a} \pm 0.05$	$0.001^{a} \pm 0.001$
10	5.6	$3.0^{d} \pm 0.22$	$0.014^{b} \pm 0.001$
	6.4	$2.7^{cd} \pm 0.09$	$0.005^{a} \pm 0.001$
	7.2	$2.2^{c} \pm 0.05$	$0.003^{a} \pm 0.001$
20	5.6	$3.7^{e} \pm 0.11$	$0.022^{c} \pm 0.002$
	6.4	$2.9^{d} \pm 0.12$	$0.017^{bc} \pm 0.001$
	7.2	$2.7^{cd} \pm 0.18$	$0.018^{bc} \pm 0.001$
37	5.6	$33.5^{h} \pm 0.61$	$0.141^{f} \pm 0.006$
	6.4	$12.7^{g} \pm 0.38$	$0.046^{d} \pm 0.003$
	7.2	$11.8^{f} \pm 0.55$	$0.051^{e} \pm 0.005$

[a] SD, standard deviation. Values in columns with different superscripts are different ($P < 0.05$).

each followed the order 37 > 20 > 10 > 4 °C ($P < 0.05$). Matsuura et al. (1962) reported that the autoxidation rate constant of tuna Mb in vitro was doubled when temperature increased from 20 to 40 °C. O'Keefe and Hood (1982) also indicated that the OxyMb oxidation rate of beef increased with an increase in temperature. The enhanced oxidation of OxyMb in meat observed with increased temperature may be caused by the increased rate of various pro-oxidant reactions, decreased oxygen solubility (O'Keefe and Hood, 1982), and/or increased oxygen consumption by the tissue (Bendall and Taylor, 1972). Igene and Pearson (1979) also observed that cooking temperature (70 °C) resulted in a concomitant decrease in the stability of membrane phospholipid; this may further increase the rates of OxyMb and PL oxidation.

In the OxyMb–liposome system, a lower pH environment resulted in faster OxyMb and PL oxidation; both oxidation rates followed the order pH 5.6 > 6.4 > 7.2 ($P < 0.05$). Chow et al. (1987) reported that the autoxidation rate constant of tuna myoglobin in vitro increased with decreased pH over the range 5.5–7.1. Shikama and Matsuoka (1986) also indicated that the stability of OxyMb decreased with a decrease in pH in the acidic region (pH 4–6.5). They suggested that environmental pH affects the configuration of the globin tertiary structure, especially of the region around the heme. At low pH, the globin tertiary structure opens and the heme protein is exposed to the external environment. This results in decreased stability of the heme protein and enhanced oxidation. A lower pH environment accelerates the protonation of bound oxygen and favors the release of superoxide anion (Livingston and Brown, 1981), a pro-oxidant species.

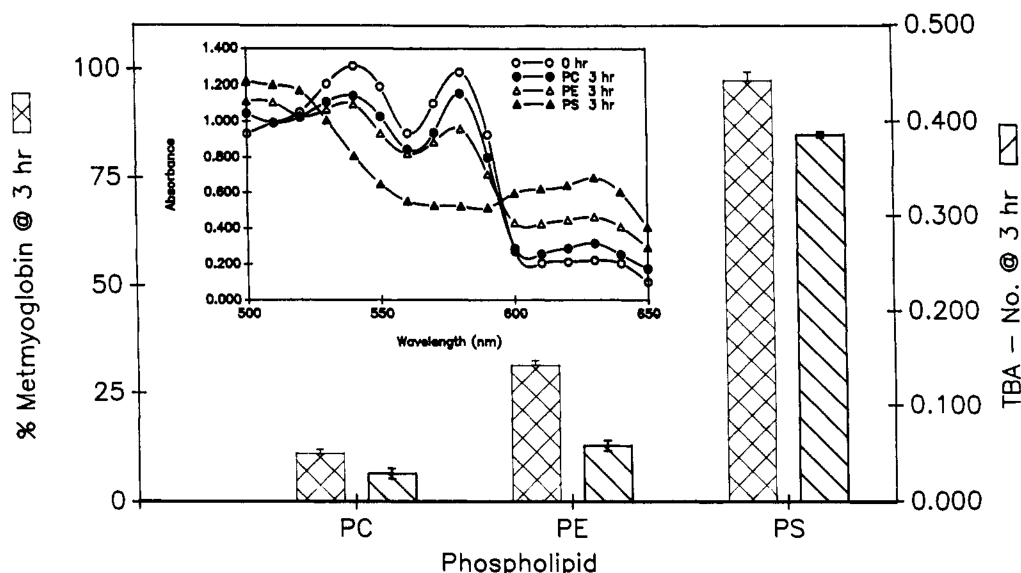

Figure 3. Metmyoglobin formation and lipid oxidation (TBA number) in phosphatidylcholine (PC), phosphatidylethanolamine (PE), or phosphatidylserine (PS) OxyMb–liposomes following 3 h of incubation at pH 5.6 and 10 °C (n = 5). Time 0 = MetMb and is the same for all phospholipid types.

Table II. Fatty Acid Composition (Percent) of Commercial Phospholipids Used To Construct Oxymyoglobin–Liposomes[a]

fatty acid	phospholipid PC	PE	PS
16:0	32.23	3.97	44.55
16:1	1.55	5.51	34.91
18:0	12.79	31.29	0.68
18:1	28.65	9.68	3.69
18:2	16.70	11.89	5.37
18:3	0.23	1.11	1.12
20:3		3.43	
20:4	4.08	15.00	1.06
20:5		10.22	
24:1	1.51	0.67	1.68
22:5	0.10	4.29	1.23
22:6	0.78	1.60	6.23
SAT.	45.02	35.26	45.33
MONO	31.71	15.86	40.28
POLY	23.04	47.54	14.27

[a] SAT., total saturated fatty acids; MONO, total monounsaturated fatty acids; POLY, total polyunsaturated fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

The influence of commercial PC, PE, and PS upon MetMb formation and PL oxidation in OxyMb–liposomes is shown in Figure 3. Following 3 h of incubation, greater levels of OxyMb and PL oxidation were observed in PS-based OxyMb–liposomes than in PE- and PC-based OxyMb–liposomes ($P < 0.05$). The fatty acid composition of the commercial PLs is shown in Table II. The concentration of PUFA was highest in PE followed by PC and PS. The susceptibility of PLs to oxidation in muscle foods is attributed to their high content of PUFA (Younathan, 1985). The oxidation rate of fatty acids increases significantly with an increase in the number of double bonds. In muscle tissue, PLs represent only 0.5–1% of the tissue content. They contain not only C18:2 and C18:3 in significant amounts but also high concentrations of C20:4, C22:4, C22:5, and C22:6 (Igene et al., 1981; Sinclair et al., 1982). In milk PLs, the fractions with ethanolamine and serine derivatives were reported to oxidize faster than other fractions (Mattson and Swartling, 1963). Phosphatidylethanolamine was considered to be a major contributor to the development of warmed-over flavor (WOF) in chicken meat (Igene and Pearson, 1979). Corliss and Dugan (1970) also indicated that the ethanolamine fraction of PE not only increased the PE oxidation but also accelerated oxidation of other PLs. They suggested that the structure or charge of ethanolamine may reduce the stability of lipid bilayers, which favors PL oxidation. The concentration of PUFA within the PLs cannot explain the present results as PL oxidation rate followed the order PS > PE > PC ($P < 0.05$); OxyMb oxidation rate was highest in PS-based OxyMb–liposomes followed by PC- and PE-based OxyMb–liposomes ($P < 0.05$). Szebeni et al. (1988) reported that negatively charged PLs in the membrane of liposomes tend to destabilize encapsulated hemoglobin. At pH 5.6, PC and PE are dipolar zwitterions (net charge is 0), but the net charge of PS is −1. This one negative charge may contribute to the faster oxidation observed in PS-based OxyMb–liposomes.

Since both PL polar head group and PL fatty acid composition could have affected the stability of PL and OxyMb, both variables (PL polar head group and PL fatty acid composition) were examined separately. PC was chosen as the PL source because it has been the PL of choice in many previous investigations of oxidation. The influence of fatty acid chain length and degree of fatty acid unsaturation upon oxidation in PC-based OxyMb–liposomes was examined, and the results are shown in Table III. In PC-based OxyMb–liposomes, OxyMb and PL oxidation rates increased with an increase in the degree of fatty acid unsaturation ($P < 0.05$) and length of the fatty acid chain ($P < 0.05$). Several researchers have reported that there is a positive relationship between the degree of fatty acid unsaturation and PL oxidation (Kanner et al., 1988; German and Kinsella, 1985). However, the effect of fatty acid unsaturation on OxyMb stability has not been adequately addressed. Our results showed that OxyMb oxidation rate increased with an increase in the degree of fatty acid unsaturation and fatty acid chain length ($P < 0.05$). The enhanced OxyMb oxidation may result from increased free radicals produced by PL oxidation.

When the fatty acid composition of PC and PE was held constant, the influence of PL polar head group was examined and is shown in Figure 4. PE-based OxyMb–

Table III. Effect of Fatty Acid Chain Length and Degree of Unsaturation on Metmyoglobin (MetMb) Formation Rate and PL Oxidation (TBA) Rate in Oxymyoglobin-Phosphatidylcholine Liposomes at pH 5.6 and 10 °C ($n = 5$)[a]

PC fatty acid distribution	% MetMb/h (mean ± SD)	TBA/h (mean ± SD)
chain length		
di-18:1 trans	2.6[a] ± 0.12	0.009[a] ± 0.001
di-18:1 cis	3.0[a] ± 0.09	0.008[a] ± 0.001
di-20:1 cis	4.6[b] ± 0.22	0.012[ab] ± 0.001
di-22:1 bis	6.7[c] ± 0.12	0.017[b] ± 0.001
unsaturation		
di-18:1 cis	3.0[a] ± 0.09	0.008[a] ± 0.001
di-18:2 cis	14.7[d] ± 0.41	0.015[b] ± 0.002
di-18:3 cis	25.8[e] ± 0.31	0.115[c] ± 0.006
mixed		
16:0/18:0	1.6[a] ± 0.04	0.003[a] ± 0.001
16:0/18:1	1.9[a] ± 0.35	0.004[a] ± 0.001
16:0/18:2	3.1[b] ± 0.07	0.010[b] ± 0.001
16:0/20:4	3.8[c] ± 0.11	0.013[c] ± 0.001

[a] SD, standard deviation. Values in columns within chain length, unsaturation, or mixed categories with different superscripts are different ($P < 0.05$).

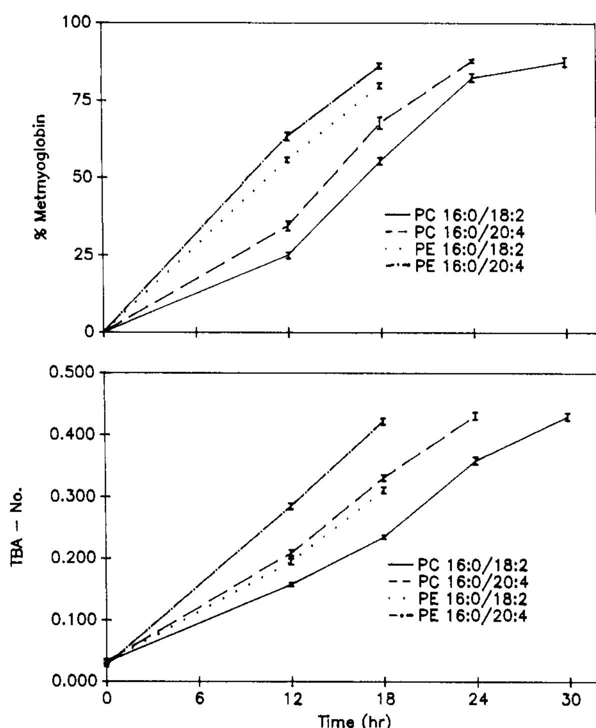

Figure 4. Metmyoglobin formation and PL oxidation (TBA number) in phosphatidylcholine (PC) or phosphatidylethanolamine (PE) OxyMb-liposomes of defined fatty acid composition (pH 5.6, 10 °C). Standard error bars are indicated ($n = 5$).

liposomes showed greater OxyMb and PL oxidation than PC-based OxyMb-liposomes ($P < 0.05$). Tsai and Smith (1972) reported that the amino group H_3^+N of the primary amine, (i.e., PE at pH 7.9) accelerated the autoxidation of methyl linoleate emulsions but that the quaternary amino group $(CH_3)_3^+N$, (i.e., PC at pH 7.9) did not affect autoxidation. These authors indicated that the presence of an N-H bond may be necessary for pro-oxidant activity of the amine. In the present study, ethanolamine of PE would be in the NH_2 state at pH 5.6 and would be expected to show amine-based pro-oxidant activity; PC, as a tertiary amine, would be expected to show a slower oxidation rate than PE, and this was observed in our study. Stewart (1990) reported that free long-chain fatty acids enhanced the OxyMb oxidation rate because they inhibited the peroxidase activity of MetMb. It was proposed that enhanced OxyMb oxidation may result from the binding of longer chain fatty acids to hydrophobic cavities of MetMb which have the ability to behave as peroxidases. Therefore, in the presence of longer chain fatty acid, the ability of MetMb to behave as a peroxidase is inhibited. The increased concentration of hydrogen peroxide produced by OxyMb oxidation may in turn increase the oxidation rate of neighboring OxyMb molecules. Stewart (1990) proposed that free radicals produced by OxyMb oxidation may in turn enhance PL oxidation. Dawson and Gartner (1983) reported that ferric heme pigments such as MetMb may also act as active catalysts which can initiate PL oxidation. In the present study, a model liposome system was used. As such, fatty acids that might influence OxyMb stability were present but as part of membrane phospholipid. This was done in an attempt to more closely mimic the condition in post-mortem muscle. Liposomes comprised of PLs containing long-chain fatty acids also resulted in higher OxyMb and PL oxidation rates ($P < 0.05$).

This investigation showed that temperature, pH, PL polar head group, and PL fatty acid composition affected both OxyMb and PL oxidation. Results supported a potential interrelationship between OxyMb and PL oxidation. A recent study has suggested that the lipid composition of meat products be altered so as to increase the relative content of unsaturated fatty acids (National Research Council, 1988). While this may have potential nutritional benefits, our results indicate that increased PUFA in meat will enhance OxyMb oxidation as well as rancidity development. Investigations are underway to determine a means by which OxyMb can be stabilized in the presence of unsaturated lipid.

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