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Effect of Total Lipids and Phospholipids on Warmed-Over Flavor in Red and White Muscle from Several Species as Measured by Thiobarbituric Acid Analysis

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TBA (2-thiobarbituric acid) analysis demonstrated that turkey meat is most susceptible to WOF (warmed-over flavor) development, followed closely by chicken, then by pork, beef, and mutton in that order. Although freshly cooked muscle from all species except mutton had higher TBA numbers than fresh raw samples, the most dramatic change occurred during storage of cooked meat at refrigerated temperature (48 hr at 4°C). Red muscles had consistently higher TBA values than white muscles under these storage conditions, indicating that red muscles were more susceptible to oxidative deterioration. Correlation coefficients between TBA numbers and total lipid levels and between TBA values and phospholipids suggest that phospholipids play a major role in development of WOF in all cooked meats, except for pork, where total lipid levels seem to be the major contributor to WOF.

Warmed-over flavor (WOF) was first noted by Timms and Watts (1958), who coined the term to describe the rapid onset of rancidity in cooked meat during short-term refrigerated storage. Although WOF was assumed to be due to metmyoglobin-catalyzed lipid oxidation (Hirano and Olcott, 1971; Kendrick and Watts, 1969; Younathan and Watts, 1960), it has since been shown that nonheme iron is the major prooxidant in cooked meat (Love and Pearson, 1974; Sato and Hegarty, 1971). Even though earlier studies have utilized beef, pork, poultry, and fish, a comparison of the various species from the standpoint of susceptibility has not been previously made. It is well known that the lipid content of red fibers is appreciably higher than that of white fibers (Cassens and Cooper, 1971; George and

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Table I. Finishing Rations Utilized by Chickens, Turkeys, Hogs, Mutton, and Beef in Present Study (Pounds/Ton)

Ingredients	Chickens	Turkey	Pigs	Mutton	Beef
Corn, ground	1228	1205	1490	---	3 lb daily
Soybean oil meal, 49%[a]	325	540	---	---	---
Soybean oil meal, 44%[a]	---	---	440	---	---
Alfalfa meal, 17%[a]	50	50	---	---	---
Meat and bone meal, 50%[a]	60	50	---	---	---
Fish meal, 60%[a]	40	50	---	---	---
Oats	250	---	---	---	---
Stabilized fat	---	50	---	---	---
Alfalfa hay, good	---	---	---	Free choice	---
Haylage	---	---	---	---	Free choice
Mixed clover-grass pasture	---	---	---	---	Free choice
Protein supplement, 32%	---	---	---	---	2 lb daily
Minerals, vitamins, etc.	47	55	70	Free choice	---
Total	2000	2000	2000	---	---
Percent fat	3.86	4.66	3.19	1.8[b]	---[c]

[a] The percentage refers to protein content. [b] Taken from fat analyses of alfalfa hay (good). [c] Since the beef steers were fed a daily ration of corn and protein supplement with pasture and haylage free choice, fat levels cannot be calculated.

Jyoti, 1955), yet virtually nothing is known about the relative susceptibility of red and white fibers to lipid oxidation.

The present study was undertaken to determine the effects of total lipids and phospholipids upon development of WOF in red and white muscle from several species of animals. WOF was followed by changes in TBA numbers of muscle analyzed immediately after cutting, immediately following cooking, and after cooking and storage for 48 hr at 4°C.

MATERIALS AND METHODS

Sources of Muscle. The chicken muscle used in this study was obtained from 8-week-old broilers fed a basic corn–oats–soybean oil meal diet (Table I) containing 3.86% fat. Turkey muscle was obtained from 14-week-old Beltsville White turkeys finished on a corn–soybean oil meal ration (Table I) containing 4.66% fat. Thigh and breast muscle were removed from both the chicken and turkey carcasses at 24-hr postmortem and utilized as the sources of red and white muscle, respectively. The pork muscle was obtained from the carcasses of market weight pigs (200–230 lb live weight) at approximately 4–5 months of age after rearing on a basic corn–soybean oil meal diet containing 3.19% fat (Table I). At 48-hr postmortem, the pig carcasses were cut and the semitendinosus muscles were divided into the red and white portions for subsequent studies.

The beef used in this study came from the carcasses of steers about 18 months of age after being fed corn–protein supplement–haylage on mixed pasture (Table I). The mutton came from aged ewes given free choice alfalfa hay (good) and minerals. The longissimus muscles were removed at 24-hr postmortem from the mutton carcasses and at 72 hr from the beef carcasses and utilized as red muscle.

Preparation of Samples. All subcutaneous and intermuscular fat was removed from the muscles. The samples were then ground twice with an electric meat grinder equipped with a plate having 1/8 in. holes in order to achieve homogeneity. Samples were divided into 10-g aliquots, which were utilized for determination of TBA numbers and for analyzing for total lipids and phospholipids.

TBA Analysis. The TBA test for all samples was carried out by the method of Tarladgis et al. (1960), which expresses TBA numbers in terms of milligrams of malonaldehyde per 1000 g of meat. TEP (1,1,3,3-tetraethoxypropane) was used to construct a standard curve and its recovery was found to be 78%, which compares to a value of 68% reported by Tarladgis et al. (1960).

TBA numbers were determined for all raw muscle samples immediately following removal from the carcasses. Aliquot samples were placed in a boiling water bath until the internal temperature reached 70°C. The samples were then removed from the water bath and cooled for 20–30 min, at which time one sample was used immediately for TBA values. A second cooked sample was placed in a refrigerator at 4°C and held for 48 hr, at which time it was also subjected to TBA analysis.

Analysis for Total Lipids and Phospholipids. Total lipids were determined by the technique of Folch et al. (1957), which makes use of a chloroform–methanol mixture for extraction. Phospholipids were separated from the total lipids using the method of Choudhury et al. (1960), which involves separation on activated silicic acid by preferential removal of the neutral lipids on washing with chloroform followed by solubilization of the phospholipids with methanol.

Statistical Analysis. Correlation coefficients were calculated between TBA numbers and percentage of total lipids, and with phospholipids as percentage of tissue and as percentage of total lipid. The calculations were made by using the Michigan State University computer package program PLOTXY utilizing a Control Data Corporation 6500 computer. Significance of the correlation coefficients was determined using Fisher's "t" table (Mason, 1970).

RESULTS

TBA values, which are indicative of WOF development in meats (Sato and Hegarty, 1971; Timms and Watts, 1958), are shown in Table II for fresh raw, fresh cooked, and cooked–refrigerated (48 hr at 4°C) red and white muscles from the various species. Results indicate that turkey was the most susceptible to WOF development, followed closely by chicken, then by pork, beef, and mutton in that order. Although there was some evidence of increased oxidation in fresh cooked muscle for all species, with the exception of mutton, the most dramatic change in TBA numbers occurred in the cooked–refrigerated samples. The relatively high TBA values for the fresh raw and fresh cooked chicken and turkey do not indicate that these samples were initially suffering from WOF, although some oxidation could have occurred during the 30-min period that elapsed during preparation of these samples for analysis. Table II also shows that TBA numbers for red and white muscles were of about the same magnitude in both fresh raw and fresh cooked samples, with the exception of turkey where red muscles consistently had considerably higher TBA values than white muscles. After cooking and refrigerated storage, however, the TBA

Table II. Mean TBA Values and Standard Deviations for Raw, Cooked, and Cooked-Refrigerated Red and White Muscles from Mutton, Beef, Pork, Chicken, and Turkey[a]

Species	Raw[b]		Cooked-0 day[c]		Cooked-stored 48 hr at 4°C[d]	
	Red muscle[e]	White muscle[f]	Red muscle[e]	White muscle[f]	Red muscle[e]	White muscle[f]
Mutton	0.14 ± 0.07		0.15 ± 0.08		2.96 ± 0.18	
Beef	0.95 ± 0.25		1.92 ± 1.11		3.71 ± 0.06	
Pork	0.24 ± 0.16	0.69 ± 1.00	1.07 ± 0.74	1.55 ± 0.77	6.03 ± 0.94	5.83 ± 0.57
Chicken	1.26 ± 0.79	1.61 ± 1.44	3.96 ± 1.03	3.13 ± 1.76	9.20 ± 0.69	8.60 ± 0.72
Turkey	5.58 ± 1.28	2.42 ± 1.16	6.00 ± 1.09	4.13 ± 1.29	11.47 ± 1.06	8.63 ± 0.69

[a] Means are average values from three different animals from each species. [b] Raw samples were analyzed at once after the carcasses were cut, which was at 24-hr postmortem for mutton, chicken, and turkey, at 48-hr postmortem for pork, and 72 hr for beef. [c] Cooked samples at 0 day were analyzed immediately after cooking. [d] Cooked samples stored at 4°C were analyzed for TBA numbers after 48-hr storage. [e] Red muscles were as follows for each species: mutton and beef, longissimus; pork, red portion of semitendinosus; chicken and turkey, thigh. [f] White muscles were as follows for each species; mutton and beef, none; pork, white portion of semitendinosus; chicken and turkey, breast.

Table III. Mean Lipid Levels and Standard Deviations for Red and White Muscles from Mutton, Beef, Pork, Chicken, and Turkey

Species	Total lipid (% tissue)[b]		Phospholipid (% lipid)[c]		Phospholipid (% tissue)[d]	
	Red muscle[e]	White muscle[f]	Red muscle[e]	White muscle[f]	Red muscle[e]	White muscle[f]
Mutton	5.58 ± 0.49		17.25 ± 1.81		0.80 ± 0.14	
Beef	14.79 ± 0.39		3.56 ± 0.16		0.50 ± 0.02	
Pork	5.47 ± 0.62	8.88 ± 0.67	16.73 ± 3.35	11.97 ± 2.77	0.83 ± 0.01	1.00 ± 0.19
Chicken	4.74 ± 0.37	1.52 ± 0.15	42.25 ± 7.60	42.40 ± 4.70	1.60 ± 0.05	0.50 ± 0.03
Turkey	1.86 ± 0.15	0.79 ± 0.05	35.43 ± 3.11	64.42 ± 3.67	0.63 ± 0.02	0.55 ± 0.01

[a] Mean values are from three different animals of the same species. [b] Total lipid as percentage of tissue weight. [c] Phospholipid as percentage of total lipid. [d] Phospholipid as percentage of tissue weight. [e] See footnote *e* to Table I. [f] See footnote *f* to Table I.

numbers for red muscles were higher than for white muscles. The rapid increase in TBA numbers during refrigerated storage of the cooked samples confirms the observation of Timms and Watts (1958) and of Sato and Hegarty (1971) that development of WOF occurs much faster than normal lipid peroxidation.

Table III presents total lipids as percentage of tissue and phospholipids as percentage of tissue and as percentage of total lipid for both red and white muscles. Total lipid levels in red muscles from mutton, pork, and chicken were quite similar (4.74 to 5.58%), while beef was unusually high (14.79%) and turkey very low (1.86%). White muscle from turkey contained less than half as much total lipid as red muscle, while white muscle from chicken was only one-third that of red muscle. The lower total lipid levels of white in contrast to red muscles from turkey are in agreement with the results of Marion and Forsythe (1964). On the other hand, white muscle from pork averaged 8.88% total lipid, which was 1.5 times higher than that for red muscle (Table III). The high total lipid content of beef was apparently the result of a high level of marbling, while the low values for turkey red and white muscles were probably the result of the early slaughter age of 14–16 weeks. Table III also shows that the level of phospholipid is inversely related to the level of total lipid, which is in agreement with the premise of Dugan (1971) that percentage of phospholipid to total lipid increases as percent total lipid decreases. Mean phospholipid levels as percentage of muscle varied from a low of 0.50% for beef red muscle to a high of 1.60% for chicken red muscle (Table III). These values are in good agreement with the range of 0.5–1.0% phospholipid as a percentage of muscle as reported by Dugan (1971).

Correlation coefficients between TBA numbers and the different lipid fractions are given in Table IV for each species for both red and white muscles. The association between TBA numbers and total lipid levels in both red and white muscles from pork was positive and significant ($P < 0.10$). This suggests that as lipid levels increase in pork muscle there is a corresponding increase in the amount of oxidation. Although none of the other correlation coefficients between TBA values and total lipid levels were significant, the relationships for turkey red muscle and chicken white muscle approached significance, but were negative. This indicates that susceptibility to oxidation may decrease as the lipid level increases.

Table IV. Correlation Coefficients of TBA Values with Total Lipid Levels, Phospholipid as Percentage of Tissue and as Percentage of Total Lipid

Species and muscle	No. of samples	Correlation coefficients for TBA value with: Total lipid as % of tissue	Phospholipid as % of total lipid	Phospholipid as % of tissue
Mutton, red muscle	3	0.39	0.39	0.50
Beef, red muscle	3	0.03	0.54	0.61
Pork, red muscle	3	0.88[a]	−0.99[b]	−0.97[b]
Chicken, red muscle	3	−0.39	0.59	0.74
Turkey, red muscle	3	−0.71	0.66	0.15
Pork, white muscle	3	0.84[a]	−0.89[a]	−0.89[a]
Chicken, white muscle	3	−0.64	0.40	−0.78
Turkey, white muscle	3	0.35	−0.18	0.44

[a] Significant at $P < 0.10$. [b] Significant at $P < 0.05$.

A significant negative relationship between TBA values and phospholipid as a percentage of total lipid for both red and white muscles from pork indicates that high levels of phospholipids are not determinants of WOF in pork (Table IV). This is further borne out by the negative relationship between TBA values and phospholipids as a percentage of tissue (Table IV). These results suggest that phospholipids are unimportant in development of WOF in pork, but that total lipid levels play a major role in WOF development in pork.

Table V presents overall correlation coefficients between TBA numbers and the three lipid measurements. The associations between TBA values and total lipids were significantly ($P < 0.05$) but negatively related. This suggests that TBA values decreased directly with lipid

Table V. Overall Correlation Coefficients between TBA Numbers and Percent Total Lipid, Phospholipid as Percent of Tissue, and Phospholipid as Percentage of Total Lipid

Sample	Correlation coefficients of TBA numbers with: Total lipid %	Phospholipid as % of total tissue	Phospholipid as % of total lipid
All red muscles	−0.58[a]	0.24	0.66[a]
All white muscles	−0.63[a]	−0.73[a]	0.56[a]
All muscles combined	−0.59[a]	0.02	0.62[a]

[a] Significant at $P < 0.05$.

levels in all species except for pork. The reduction in TBA numbers as total lipids decrease may be a result of dilution of the off-flavors and odors as the concentration of neutral lipids becomes larger, or it could merely be a reduction in the TBA reactive material as the proportion of neutral lipids increases in the tissues, or conversely, it could be a combination of both.

Although the relationship between TBA levels and phospholipids as a percentage of muscle tissue for all muscles combined was essentially zero, there was a significant negative correlation for all white muscles, indicating that phospholipid content is not related to WOF development in white muscles (Table V). The relationship for all red muscles was positive, but not significant, which in combination with the negative relationship for all white muscles accounted for the zero overall value.

The data in Table V showed the overall correlation coefficients between TBA numbers and phospholipid as percentages of total lipid were positive and statistically significant ($P < 0.05$) for all red and all white muscles and for the combination of all red and white muscles. These results indicate that WOF becomes more serious as the proportion of phospholipids to total lipids increases. Results of the current study are in agreement with a report by Acosta et al. (1966) for turkey red muscle, in which it was found that increasing the percentage of phospholipids to total lipids resulted in greater susceptibility to rancidity. Thus, phospholipids appear to play a major role in development of WOF in cooked meats, with the exception of pork where total lipid levels seem to be the major determinant in development of WOF.

DISCUSSION

Although the samples in this study were not analyzed for individual fatty acids, the fatty acid composition of the triglycerides of muscle tissues from different species has been carefully characterized by Hilditch and Williams (1964). These analyses show that the polyunsaturated fatty acids (PUFAS) in meat triglycerides are almost always confined to linoleic acid, except for that from ruminants, which also contain linolenic acid in lesser amounts. The PUFAS of meat triglycerides from ruminants are fairly constant at 3–4% of the total fatty acid content (Hilditch and Williams, 1964), and are influenced but little by the diet (Thomas et al., 1935; Shorland, 1952) unless the trans acids are taken into account (Shorland, 1955). On the other hand, the PUFAS (almost exclusively linoleic acid) of the meat triglycerides from nonruminants vary directly with the level in the diet. Thus, typical U.S. diets involving the substantial use of corn containing about 3.5% fat with 60% linoleic acid (Hilditch and Williams, 1964) make it possible to predict approximately the composition of the triglycerides from nonruminant animals. Consequently, the triglycerides from pork fed on a corn-based diet would contain some 15% of linoleic acid in agreement with data published by Koch et al. (1968). Likewise, chicken triglycerides would contain about 25% linoleic acid, which is in agreement with the values given by Katz et al. (1966). Turkey triglycerides would also be expected to contain about 25% linoleic acid on a basic corn diet, since turkeys and chickens respond to dietary fat in a similar manner (Hilditch and Williams, 1964).

On oxidation of egg phosphatidylethanolamine dispersions at 38°C for 48 hr, Love (1972) found that the 22:6, 20:4, and 22:3 PUFAS had been completely oxidized, whereas some 18:2 PUFAS still remained. This agrees with Holman and Elmer (1947) who concluded that each additional double bond in a fatty acid increases the rate of oxidation by a factor of two. Thus, linolenic (18:3) acid oxidizes twice as fast as linoleic (18:2), and arachidonic acid (20:4) twice as fast as linolenic. This indicates that the triglycerides are much less susceptible to oxidation than the phospholipids, which in the case of phosphatidylethanolamine from lamb tissues collectively contained up to 20% of the total fatty acids as tetra-, penta-, and hexaenoic acids (Body and Shorland, 1974). Thus, the importance of triglycerides in development of WOF would appear to have a minor influence as compared to that of the phospholipids, which is in agreement with the results of the present investigation.

Many of the earlier analyses failed to extract and characterize the phospholipids separately, which resulted in erroneous values for phospholipids. Hornstein et al. (1961) only identified arachidonic acid in the phospholipid fraction of beef and pork; however, in a later study by the same group (Hornstein et al., 1967) they reported some three or four PUFAS in the phospholipid fraction of beef muscle. This is in contrast to the analyses reported recently for sheep muscle by Body and Shorland (1974), where they observed a wide range of C_{20} and C_{22} PUFAS and some 7% of 2,3-methylene fatty acids that had escaped the attention of other investigators. Hornstein et al. (1961) also noted that the phospholipids and the total lipids developed rancid odors much more quickly than the neutral lipids, which adds further support to our contention that phospholipids play a major role in development of WOF.

The exact role of the various PUFAS of the phospholipids upon development of WOF needs to be carefully investigated in greater detail. A study of this nature should shed light upon the exact mechanism of the reaction responsible for WOF.

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Electron Paramagnetic Resonance Studies of Actin-Lipid Interaction in Aqueous Media

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The interaction between fish lipid and fish actin in aqueous media was investigated by the use of spin-label techniques. Three types of spin-labels were used in the study. (a) 12-Doxyl stearic acid was incorporated into lipid–liposomes by sonication. The incorporation of the spin-label took place at a lipid/water ratio of 3 mg of lipid/1 ml of H_2O. Addition of actin to the labeled liposomes showed that both neutral fish lipids and polar fish lipids interact with actin. The neutral lipids showed stronger interaction than polar lipids. Results of (b) maleimide and (c) iodoacetamide spin-labels suggested that all the sulfhydryl groups and the majority of the amino groups, which bind the labels, were on the surface of the actin molecules and exhibited considerable mobility. The results also showed that these sites did not experience any conformational changes, which would restrict the spin-label mobility, during actin–lipid interaction. At the isoelectric precipitation region of action, the mobility at these sites was considerably restricted. Raising the pH to the alkaline side, i.e. pH 10.0, caused expansion of the actin molecules, accompanied by exposure of the hidden amino group sites to the surface of the molecule.

There is an increasing interest in understanding the nature of lipid–protein interaction in biological and nonbiological systems. Lipid–protein interaction in cell membranes, various enzyme reactions, the blood coagulation process, various lipase reactions, and serum lipoproteins were considered by Chapman (1969) as the areas attracting the most interest of researchers at the present time. Much work has been published covering the importance of lipid–protein interactions in other systems such as lipovitellin (Evans et al., 1968a, b), wheat flour and bread-making (Chiu and Pomeranz, 1966; Pomeranz et al., 1968), nutritional quality of foods (Varma, 1967; Rao and Rao, 1972), post-mortem stability of fish myosin (Braddock and Dugan, 1973), interaction of antigen with mylin lipids (Palmer and Dawson, 1969), and binding of botulinum toxin to membrane lipids (Simpson and Rapport, 1971).

The study of lipid–protein interaction has, as well, a valuable interest to the fish technologist. One of the methods used in extracting and concentrating fish proteins for industrial purposes is a direct application from biochemistry for extracting and purifying proteins, i.e. using aqueous buffers. The problem arose in industry of high lipid contents in the extracted proteins; this also could be an unseen problem to the biochemist, since there are usually no tests performed for the contaminating lipids in purified protein preparations. It was found (Shenouda, 1974) that the purified myosin, after Sephadex G-200 column purification, contained a significant amount of contaminating polar and neutral lipids. Taguchi and Ikeda (1968) reported the important role of lecithin in fish actomyosin ATPase activity suggesting the existence of lipid–protein complexes in the living myofibrillar tissues. The interaction of fish myosin and actin with fish lipid in aqueous media was investigated by Shenouda and Pigott (1974, 1975b) using sucrose gradient centrifugational and gel electrophoretical procedures.

The use of synthetic free radicals known as spin-labels has a wide use in probing the structure, motion, and chemical reactions of biological macromolecules. This technique proved to be sensitive to protein conformational changes. Griffith and Waggoner (1969) reviewed the advantages of using spin-labeling in studying biomolecules, in comparison to other existing biochemical–biophysical techniques. Roubal (1972) recommended the use of nitroxide spin-labels for studying lipid–protein interactions. Jost et al. (1971) have grouped spin-labels into three major arbitrary classes: (a) covalently bound spin-labels; (b) noncovalently bound, nonbiological spin-labels; and (c)

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