LIPID REACTIONS IN FROZEN STORED COHO SALMON AND AUTOXIDIZING LINOLEATE - MYOSIN SYSTEMS

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This is to certify that the

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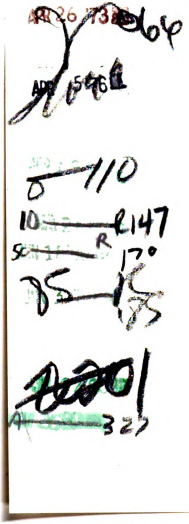

ABSTRACT

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Reactions which contribute to denaturation, destruction and quality changes of fish muscle protein and lipids were studied. Compounds with characteristic fluorescence spectra were isolated by extraction and TLC from an autoxidizing system consisting of sodium linoleate, Coho salmon myosin and buffer. Similar compounds were also present in extracts from freeze-dried salmon steaks and salmon kept frozen at -20 C for one year. TBA values and oxygen uptake of the autoxidizing system showed initial rapid increases with time followed by a significant decrease in TBA values and gradual leveling off of oxygen uptake upon prolonged oxidation. Infrared spectra before and after borohydride reduction and u.v., visible and fluorescence spectra indicated the presence of C=N functional groups in the extracts from the various

samples. These compounds were not extractable from the control myosin solutions allowed to oxidize without addition of linoleate. Amino acid analyses of the myosin from the autoxidizing system, when compared with non-oxidized myosin-linoleate and myosin systems, indicated significant decreases in the amounts of histidine, lysine and methionine following oxidation.

Fatty acids present in the neutral and phospholipids of the Lake Michigan Coho salmon were identified. Phospholipid hydrolysis was shown to occur in the frozen salmon as evidenced by losses of total phospholipid, phosphatidyls -choline and -ethanolamine and lyso-phosphatidlycholine, while the lyso-phosphatidylethanolamine fraction increased during six months storage at -20 C. A preferential hydrolysis of phosphatidylethanolamine containing $C_{16:0}$, $C_{18:1}$, and $C_{22:6}$ fatty acids was implied from fatty acid analyses which showed that the $C_{16:0}$ and $C_{22:6}$ acids were more concentrated and the $C_{18:1}$ acid less concentrated in the remaining lysophosphatidylethanolamine following six months storage at -20 C.

The results of this study show that in such complex systems as frozen fish and the simpler, yet still complex linoleate-myosin system, chemical changes detrimental to the maintenance of original characteristics are occurring.

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INTRODUCTION

Foods containing significant quantities of the polyunsaturated fatty acids (PUFA) are readily susceptible to lipid oxidations leading to deterioration of quality attributes. These oxidations result in complex chemical involvements between the various food constituents. Of significant concern are reactions between PUFA breakdown products, such as radicals, peroxides, carbonyl compounds and proteins in the food system. These reactions can result in irreversible changes to the constituents involved and generally increase during storage for many products, depending on the product and the storage conditions.

Frozen and processed seafoods and fishery products are very complex food systems which are highly susceptible to oxidative deteriorations because of the large amounts of PUFA present in their lipids, notably eicosapentaenoic ($C_{20:5}$) and docosahexaenoic ($C_{22:6}$) acids. Oxidation in both frozen and freeze-dried fish can be very extensive and may result in severe impairment of quality. In a study of nutritional changes in heated freeze-dried herring

press cake, it was postulated that even a mild degree of heating was sufficient to allow any small quantities of reducing sugars, or of carbonylic products of fat oxidation that may be present, to take their toll of available lysine or other amino acid side chains (1). A more recent study reported that decomposition products of oxidized lipid in freeze-dried salmon probably reacted with the amino groups of the protein to increase browning (2). Meanwhile, it was also suggested that protein radicals were formed as a result of interaction between proteins and radicals produced by lipid oxidation in freeze-dried gelatin-methyl linoleate systems (3) and in menhaden oil-protein solutions (4, 5).

There is evidence which suggests that production of radicals, peroxides and carbonyl compounds from autoxidizing PUFA (linoleate) is a function of different stress conditions in the reacting systems (6). In fish tissue, where reactive constituents are formed from PUFA oxidation, many reactions can take place because of the biologically active compounds capable of reacting with the oxidizing lipids (7, 8). One of these biologically active compounds, malonaldehyde, has been implicated in many instances in protein-lipid breakdown product reactions, and has been shown to participate in cross-linking, aggregation

and denaturation of the protein species (9-14). It is possible that other carbonyl compounds could also take an active part in these reactions.

Changes during frozen storage in the major phospholipids of fish, phosphatidycholine (PC) and phosphatidlyethanolamine (PE), resulting in free fatty acid (FFA) production and the formation of the lyso-derivatives (LPC and LPE) may also be a factor contributing to product quality and protein denaturation (15-17). Also, because of the complexity of the changes, it is difficult to evaluate quality differences resulting from changes in the FFA, phospholipids and proteins in the muscle. Protein extractability determinations have been devised to predict the degree of severity of some frozen storage deteriorations (13).

There is some disagreement about the use of freeze-drying as a means of processing seafood. For example, it was reported in one instance that freeze-dried salmon and tuna salads rehydrated readily and rapidly in cold water and that taste panel acceptance of the products was excellent (19); while Connell (20) emphatically stated that "No authenticated instances have been reported of the production of freeze-dried fish which have not suffered some unacceptable degree of textural denaturation."

Storage life and quality after storage of frozen fish are also important factors which have been the subject of much research, with some success reported, when one considers life expectancy of frozen seafoods. In a review, the reported life expectancy at 0 F for whole frozen salmon was 5-9 months, while 3 months was the expectancy of cellophane packed salmon steaks (21).

This research gives additional supporting evidence for some observations reported in the literature concerning phospholipid changes in frozen fish; also, new evidence is presented indicating the production of C=N compounds as a result of reactions between autoxidation products of the PUFA and amino groups present in a biological system. Simulated systems containing sodium linoleate and extracted fish muscle myosin in solution also produced C=N compounds when subjected to oxidative stress conditions. The use of linoleate in a model lipid-protein system is justified. Since linoleic acid is a constituent of so many food lipids and is readily oxidized, it is frequently used in model systems to elucidate the factors that characterize the differences in oxidative mechanisms (22).

Numerous difficulties are encountered when attempts are made to prepare and store fish and fish products under conditions which would insure high quality products after processing and storage.

Although knowledge in these areas has increased, the subtle interactions of lipids with other biologically important compounds in the fish have only been partly explored. It was the intent of this research to attempt to contribute to a better understanding of some of these interactions and changes during frozen storage of fish, and to increase the store of knowledge which will someday make possible the control of such changes.

LITERATURE REVIEW

Protein Denaturation

Research into methods of preventing deterioration in cold-stored and processed fish began extensively with the impact of better transportation and refrigeration following World War II. Certain antioxidants were shown to retard development of oxidative rancidity and resulting quality deteriorations in frozen fish (23). Moreover, it was recognized that addition of sulfur dioxide and removal of free sugars and amino acids by washing with cold water would markedly reduce browning of subsequently heated fish flesh (24).

Dyer (25) recognized that protein denaturation was occurring with increasing storage time of frozen cod and Atlantic halibut. Decreasing actomyosin solubility and taste panel preference evaluations with increase in frozen storage time led him to suggest that lipids, fatty acids, nuclectide compounds, or even carbohydrates may protect or modify the properties of actomyosin in the muscle. Frozen storage temperature was shown to affect the rate of FFA production and protein denaturation in fish muscle when it was discovered that production of FFA was much greater

at +10 F than at -10 F. In addition, the FFA which developed appeared to be related to a decrease in quality during storage as shown by actomyosin denaturation (26).

Browning reactions also result in damage to proteins and amino acids of fish muscle. In dehydrated and salt fish, browning involving ribose and amino acids resulted in a loss of ribose and amino acids; also, systems consisting of amino acids, ammonia and trimethyl amine in a phosphate buffer showed a loss of reactants upon browning (27).

Olley et al. (28) found that FFA released during cold storage of cod, lemon sole and halibut were remarkably similar, with evidence showing that in these species most of the FFA arose through hydrolysis of the phospholipids. Concurrent with the increase of FFA, a decrease in extractable protein was noted. In a later paper, it was reported that there were no immediately obvious trends either in the nature of the FFA produced or in the composition of the phospholipids or neutral lipids from which the FFA were derived. However, species with higher percentages of $C_{22:6}$ in the FFA liberated during storage did show greater rates of protein denaturation (29), indicating that the $C_{22:6}$ in the FFA might be of more than average significance in causing protein denaturation.

Fish actomyosin in solution was rendered increasingly insoluble during storage at 0 C in the presence of small concentrations of linoleic and linolenic acids (30). Also, the amount of sodium linoleate necessary to add to muscle homogenates of seven species of fish in order to reduce the extractable proteins to one-half the initial value depended on the total lipid content of the muscle, a greater amount being required for species with higher lipid contents (31). This was also observed by Hansen and Olley (83), who compared the denaturation of protein in both lean and fatty species of fish and proposed that protein is protected against FFA insolubilization by the neutral lipid within the cells.

A series of related studies were carried out in an attempt to find a basis for the proposed relationship involving FFA formation and protein insolubility in frozen fish (30, 31, 84). The addition of fatty acids to solutions of fresh cod actomyosin was shown to reduce the solubility; however, variable results were obtained using several fatty acids and different experimental conditions which did not lead to an understanding of the fundamental mechanism of insolubilization.

Lipid Hydrolysis

It was reported above that the FFA increase during frozen storage of fish muscle resulted in a decreased solubility of the extracted muscle protein. Through study of the different lipid fractions in the muscle during frozen storage, it was ascertained that the increase in FFA was primarily due to a breakdown of the phospholipids by phospholipases in the muscle and a release of FFA, which could then cause protein denaturation (32-35).

In a study of the specificity of phospholipase A for egg lecithin, it was observed that marked changes occurred in the composition of the LPC fatty acids and in the composition of the fatty acids released from the β-position of PC during the first ten minutes of the reaction (36). The hydrolysis of phospholipids in frozen cod fillets was reported to decrease and then cease after 60-80% had been hydrolyzed (37); it was also discovered that oleic acid had a strong inhibitory effect on the phospholipase B activity of cod muscle. Frozen storage of cod muscle at -12C for nine months resulted in an increase of the FFA content from 5 to 325 mg/100 gm flesh due to the hydrolysis of PE and PC. PE hydrolysis ceased after storage for 4

months, whereas PC hydrolysis continued thereafter at a slower rate (38).

Separation of cod muscle homogenates into cell particulate matter and supernatant by centrifugation caused considerable hydrolysis and loss of phospholipids, even after only two hours at 0 C (85). These losses, which were enhanced by time and by the extraction of proteins and accompanying lipids into strong salt solutions, were thought to be largely due to oxidation during the extraction procedure. Bosund and Ganrot (86) have shown that storage of frozen herring for up to twelve weeks at -15 C resulted in an increase of the FFA content from 50 to 1000 mg/100 g flesh in dark muscle and from 17 to 280 mg/100 g flesh in white muscle. The increase was due to hydrolysis of PC, PE and to a varying extent, also of triglycerides.

Additional evidence for enzymatic phospholipid hydrolysis in frozen fish was reported when the formation of glyceryl-phosphorylcholine and choline increased in cold-stored rainbow trout muscle (39). Since FFA and the lyso-derivatives arise from phospholipid hydrolysis in the frozen flesh, Jonas (40) studied the effect of LPC in fish muscle homogentaes and reported that the addition of LPC caused increases in protein solubility.

More recent research has shown that phospholipid hydrolysis in frozen muscle during storage is not confined to fish. During storage of chicken muscle at -10 C, decreases in PC and PE and increases of LPC and FFA were observed; these results suggested that lipid hydrolysis occurred throughout frozen storage and that lipid hydrolysis and protein denaturation may be interdependent phenomena (41). In frozen bovine muscle, a slight decline in the total phospholipids was evident during the first two weeks of storage, then the phospholipid loss was high between the second and fifth weeks; a net increase in the FFA content was also observed (42).

Lipid-Protein Interactions

The previous discussions were concerned with hydrolysis of phospholipids to FFA plus derivatives and subsequent interaction of these molecules with proteins in the muscle, resulting in denaturation of the protein. This section will be devoted to lipid-protein interactions which arise primarily because of protein reactions with products of lipid oxidation.

It has been shown that the presence of amino groups was an important chemical prerequisite to cross-linking by formaldehyde, whereas proteins or polypeptides poor in free amino groups were not

readily cross-linked (43). It was also observed that yellow-brown copolymers were formed by the oxidation of unsaturated fats in the presence of proteins (44). This paper suggested that aldehydes of unsaturated fat oxidation would readily react with amino acids and proteins forming aldimines which could lead to the formation of brown nitrogenous polymers and copolymers. In another study designed to determine whether or not the active carbonyl-amine browning reaction was a dominant mechanism for the formation of brown-colored polymers in oxidized menhaden oil-egg albumin systems, carbonyl-amine browning reactions were found to play only a minor role (45).

Complexes were formed between egg albumin and oxidized linoleate, with little or no complex formation when unoxidized linoleic, oleic or lauric acids were used; however, the number of amino, SH and OH groups in the complexed and the original protein were found to be the same (46). These authors felt there was little likelihood of aldehyde-amine condensations taking place in the reactions leading to the formation of a fatty acid-protein complex. On the other hand, when low density lipoproteins were exposed to methyl linoleate hydroperoxide, extensive denaturation of the proteins occurred, but no change was observed upon exposure to fresh

methyl linoleate (47). It may be possible that the active oxygen in the hydroperoxide of methyl linoleate which was incorporated into low density lipoprotein molecules could be transferred into the unsaturated fatty acid portion of lipoproteins by a free-radical chain reaction and could thus facilitate the physical denaturation and chemical degradation of the lipoproteins (47).

Other results indicated that lipid-protein complexes were not formed from lipid and protein unless the fat or fatty acid was oxidized or polymerized; however, all lipids, saturated or unsaturated, fresh or oxidized, seemed to denature protein to some extent by occupying specific sites in the protein molecule (48). To interpret this statement, it must be reasoned that denaturation of the protein does not necessarily mean complex formation. Furthermore, keto and epoxy groups in oxidized fats seemed to have a pronounced influence on complex formation with proteins, while hydroxy and hydroperoxide groups were observed to be less reactive (49). In the case of this lipid-protein complex, it is not possible to express the absorption of the lipid on the protein merely on the basis of either polar or non-polar Van der Waals forces or the regular electrostatic forces for two reasons: 1) The lipid-protein

complexes are extremely stable; and 2) The lipid does not carry any ionic charges. However, it is possible that hydrogen bonding may exist between the carbonyl oxygen in the protein and the hydrogen of hydroxy or hydroperoxide groups in the oxidized lipid (49).

The development of rancidity in fish muscle from fatty species such as the salmon can be rapid, even at frozen storage temperatures, and is governed by complex factors common to many biological systems such as inherent metal ions, presence of natural antioxidants, kind and amount of fatty acids, age, season of harvest and storage conditions before and following processing. Reactions involving oxidation of PUFA leading to the production of reactive free radicals, peroxides, aldehydes and ketones occur during this development of rancidity. These oxidation products may be very complex in type and quantity, depending on the conditions of oxidation and the degree of involvement with other constituents in a system, and play an important role in autoxidizing food systems, especially animal tissues.

Many of these autoxidation products, in particular, carbonyl compounds such as malonaldehyde, may be probable reactants in lipid-protein interactions. In a system similar to one studied in this thesis, the

compounds, n-hexanal, 2-octenal and 2,4-decadienal, were the primary products formed in a buffered ammonium linoleate solution allowed to autoxidize at 37 C (50). The spectrum of fatty acids in Coho salmon has been shown to be very complex (87) and it would be expected that the oxidation products from these fatty acids which could be available for reaction with other constituents in the system, such as proteins, should be many and complex. Indeed, this is so, since positive identifications have been made of malonaldehyde and 15 monocarbonyl compounds from autoxidized salmon oil; these included the C_2, C_3, C_5, C_6, C_7, C_8 and C_9 alkanals, the C_3, C_4, C_5, C_6, C_8, C_9 and C_{10} 2-enals and hept-2,4-dienal, and tentative identification of butanal, 2,4-decadienal and 2-undecenal (51).

There appeared to be a high correlation between the amount of malonaldehyde and the development of rancid odors in some autoxidizing foods, particularly products of animal origin (52). As oxidation of a system proceeds, increasing the amounts of malonaldehyde-like components, the chance of reactions between these components and other components in the system increases. The salmon studied in the research presented in this thesis contained significant proportions of the fatty acids, oleic, linoleic, linolenic and arachidonic (87),

all of which have been shown to produce malonaldehyde, or malonaldehyde-like products under oxidative conditions (88). These malonaldehyde-like products, in the presence of water, have been postulated to exist mainly as novolatile enolate ions, and as such they can react with amino acids, proteins, glycogen and other food constituents to produce a bound complex hydrolyzable by acid and/or heat (53).

Buttkus (10) reacted trout myosin with malonaldehyde and showed that the rate of reaction with the ε-amino groups was greater at -20 C than at 0 C and was almost as great as that at +20 C. The increasing rate of reaction in the frozen system was explained as a concentration effect. Other reactions involving products of lipid oxidation interacting with proteins have been reported. Pokorny et al. (89) showed that oxidized lipids formed complexes with casein in the presence of water, and that practically the total amount of oxidation products were bound to the casein in a nonselective manner, probably linked to the casein molecule either by multiple H-bonds or similar physical forces. Tannenbaum et al. (90) studied a model system consisting of casein and methyl linoleate and noted losses of methionyl residues as a consequence of lipid oxidation at different relative humidities. They proposed that methionyl residues may act as

peroxide decomposers concomitant with carbonyl compound formation, which in turn could lead to non-enzymatic browning.

Two proteins, gelatin and insulin, were chemically modified in the presence of autoxidizing methyl linoleate (73). It was shown that lipid oxidation intermediates reacted with the ε-amino group of lysine, and with glycine and phenylalanine of insulin, making the protein non-reactive to a fluorodinitrobenzene reagent. Recent research has confirmed that the lipid oxidation product, malonaldehyde, can react in solution with amino acids to yield conjugated Schiff bases possessing characteristic absorptions in the infrared, u.v. and visible regions of the electromagnetic spectrum (66). Compounds of this type are very likely produced in autoxidizing biological systems (67) and will be discussed in this thesis in relation to autoxidizing fish. Other researchers have documented the possible existence of such autoxidation products in deteriorating frozen fish. The presence of unidentified dark spots was observed at or near the solvent front of thin-layer chromatograms of total lipids extracted from stored whitefish muscle (16). These researchers indicated that the new spots, increasing as muscle storage progressed, were probably autoxidation products, but did not confirm their statement.

In a model system consisting of amino acids dispersed in oxidizing menhaden oil, cysteine was oxidized completely to cystine, and cystine thiolsulfinate was a product whenever palmitic acid was included in the system (54). Using C^{14} labeled linolenate, it was shown that at low levels of oxidation, about 1 out of 80 linolenate peroxy radicals underwent an addition reaction resulting in C^{14} incorporation into cytochrome C (55). In addition, histidine, serine, proline and arginine were found to be most labile to peroxidative damage, while methionine and cystine were next in order when the cytochrome C was allowed to react with linolenate hydroperoxides. When n-heptanal-tyrosine ethyl ester mixtures were reacted at 10, 20, 30 and 40 C, colored pigments were produced, suggesting an aldehyde-amine condensation reaction, since infrared spectra showed a band at 1640 cm^{-1} for the imine linkage (56).

The natural antioxidant, tocopherol, may influence the development of rancidity and subsequent lipid-protein interactions in fishery products. Ackman (91), in a review of the influence of lipids on fish quality, reported that certain cod were particularly resistant to Cu^{++} induced oxidation during June and July, and postulated that high levels of tocopherol were responsoble for this lipid stability. However,

in some fish with a high degree of susceptibility to lipid oxidation, he proposed that the presence in the fish of materials or biological processes causing destruction of tocopherol resulted in the abnormal development of rancidity. Other workers showed that the addition of tocopherol to fish muscle extracts containing linoleic acid diminished increasing rancidity, as measured by a TBA reaction, and prevented insolubilization of actomyosin (92).

Quality changes resulting from lipid oxidation in frozen fish can be compared with changes in fish flesh and other systems processed by freeze-drying. Generally, freeze-dried fish products need improvements in initial quality, storage properties and cost, before they can be successfully exploited commercially. Such improvements will depend on a much deeper understanding of the physical and biochemical effects of drying and storage on the muscle protein systems (93). In a review, it was reported that browning of dried food systems containing oxidizable lipids and proteins may also involve complex lipid-protein interactions and reactions implicating other constituents (57). The complexity of the components contributing to quality deteriorations in a system such as freeze-dried fish is undisputable. Browning of freeze-dried fish muscle was accelerated in samples containing

added ribose and retarded in those in which a large proportion of the sugars were removed by enzymatic treatment. Storage in nitrogen retarded development of the browning but did not eliminate it, indicating that oxidation was responsible for much of the discoloration (58). The formation of the brown color during smoking of fish was found to be due to a reaction between the fish proteins and smoke components, the presence of free amino groups deemed essential to the reaction (59). Water also plays an important part during browning of dried products containing autoxidizing lipids and certain antioxidants, as has been shown recently, using freeze-dried model systems containing linoleate, manganese and histidine (60).

EXPERIMENTAL

Systems

Sodium Linoleate

Buffered solutions containing either sodium linoleate (L), Coho salmon myosin (M) or sodium linoleate-myosin (LM) were prepared for each experiment. The buffers used consisted of either 0.45M KCl-0.001M tetrasodium pyrophosphate or of 0.04M tetrasodium pyrophosphate, pH 7.5, both of which proved suitable for reaction purposes. Sodium linoleate solutions (7.5×10^{-3}M) were prepared by rapidly stirring 0.5 ml cis-linoleic acid (Sigma Chemical Co.) into 10 ml buffer pH 9.0 to form a lasting emulsion. Then 1-3 NaOH pellets were added and dissolved until a clear solution was obtained. This solution was brought to a final volume of 200 ml with pH 9.0 buffer, adjusting to the desired pH with concentrated HCl.

Myosin

Myosin (M) solutions were prepared from frozen Lake Michigan Coho salmon according to the procedure of Richards *et al.* (61) who prepared myosin from frozen yellowfin tuna. Prepared myosin solutions were shown

to be free of actomyosin by the light scattering test of Rice et al. (62) and stored at -20 C in 50% glycerol-buffer. When needed, the myosin was precipitated by the addition of nine volumes of distilled water, collected by centrifugation and dissolved in the desired buffer (63). Final concentrations of myosin solutions used in this experiment were approximately 1 mg/ml as determined by a biuret test (64). Aggregation of the myosin preparations during storage at -20 C did not affect the results of this experiment.

Linoleate-Myosin

Equal volumes of L and M (1:1) were mixed in the prescribed buffer, placed in a closed flask under oxygen atmosphere in a 50 C water bath and shaken vigorously at periodic intervals. Experiments were performed using duplicate samples consisting of L and buffer, M and buffer and LM in sufficient volume to perform several analyses.

Fish

Lake Michigan Coho salmon were obtained from the Michigan State Department of Natural Resources in September, 1968 (87) and in July, 1969. These fish were kept on ice for approximately 15 hours and then frozen and stored whole in a blast freezer at -20 C. Fish used for analyses were kept in separate

polyethylene bags and, when needed, steaks were sawed vertically from the dorsal fin region of the frozen fish (87, 94) and the skin, belly flap and dark muscle were discarded. Whenever it was necessary to compare component changes, such as fatty acids and phospholipids, before and after storage, muscle was used from the same fish. The 1968 salmon had an average weight of 7-8 lbs and a total lipid content of 22% (g fat/g dry weight flesh), while the 1969 salmon were 4-5 lbs and approximately 11% fat, respectively. The fish were not differentiated on the basis of sex.

Chemical Analyses

Oxygen Absorption

Peroxide values were determined iodometrically and were converted to moles of oxygen absorbed per gram of linoleate or myosin, assuming conversion of one mole of oxygen to one mole of peroxide. In some instances, absorbed oxygen was also measured with a Gilson respirometer. The two methods showed close agreement until about 12-15 hours, at which time the respirometer readings decreased considerably, while the peroxide values only leveled off.

TBA Reactives

TBA reactive compounds were determined in the oxidizing L, M and LM systems by periodically removing 1 ml samples, adding 1 ml 0.02M 2-thiobarbituric acid in 90% glacial acetic acid, holding in a boiling water bath for 20 minutes, cooling, adding 0.5 ml distilled acetone to relieve turbidity and measuring the absorbance at 532 nm. A ten minute centrifugation at 3,000 x g was necessary to obtain a clear solution in some cases. TBA tests were performed on frozen and freeze-dried salmon using a distillation procedure (65).

Fluorescent Compounds

Blue-fluorescing compounds containing C=N functional groups were extracted from oxidized LM solutions by shaking with chloroform or by freeze-drying and extracting with chloroform-methanol (2:1). These compounds were also extracted with chloroform-methanol (2:1) from whole frozen salmon kept for one year at -20C, freeze-dried salmon steaks stored at laboratory temperatures for six to eight months and salmon steaks maintained at -20 C under an oxygen atmosphere for three months.

Separation of three major fluorescing fractions was achieved by TLC on silica gel G. Extracts were pipetted in a thin band onto 20 cm x 20 cm glass plates

and developed in hexane:diethyl ether (60:40). After drying the plate by evaporation under a stream of nitrogen, a second development was achieved in chloroform:methanol:H_2O (65:25:4). Fluorescing bands containing C=N groups could be visualized under u.v. radiation at R_f values of approximately 0.95, 0.7 and 0.35. Additional purification of each band was accomplished by further TLC.

Spectrophotometric analyses of the fluorescing compounds were performed to characterize the C=N functional groups (12, 66, 67). Infrared analyses were performed before and after sodium borohydride reduction (12) using a Beckman IR-12 spectrophotometer. Fluorescence spectra were obtained with an Aminco-Bowman spectrophotofluorometer and u.v. spectra were obtained with a Beckman Model DK-2A spectrophotometer.

Amino Acid Analysis

Amino acid analyses of precipitates from unoxidized and oxidized duplicate samples of LM were made with a Spinco Model 120-C amino acid analyzer. Hydrolysis of samples was performed in 6N HCl at 110 C for 24 hours only, hence values were not extrapolated to zero time to correct for hydrolysis losses.

Fatty Acid Analysis

Total lipid samples from the salmon were obtained using the method of Bligh and Dyer (68) in which the tissue was homogenized with chloroform and methanol in such proportions that a miscible system was formed. Neutral lipid and phospholipid fractions were then separated by TLC on silica gel G (Pet Et_2O;Et_2O:HOAc, 70:30:2). The phospholipids remained at the origin of the plate, while the glycerides, free fatty acids and other neutral lipids migrated up the plate. The band containing the triglycerides (TG) was identified by chromatographing corn oil (Mazola), spraying with 2',7'-dichlorofluorescein and observing the plate under u.v. radiation. This band was scraped from the plate directly into 30 ml Et_2O and saved for methyl ester preparation. All TLC was performed under a CO_2 atmosphere produced by placing a piece of solid CO_2 in the TLC solvent tanks prior to development of a plate.

The phospholipid band from the above plate was eluted on a sintered glass funnel with three-15 ml portions of chloroform:methanol (4:1), concentrated under a nitrogen stream and rechromatographed in chloroform:MeOH:H_2O (65:25:4). The phospholipids were separated into phosphatidylcholine (PC),

phosphatidylethanolamine (PE) and their respective lyso derivatives (LPC and LPE). The bands were identified through the use of standards, ninhydrin and molybdate spray reagent (69) and scraped into Et_2O for preparation of methyl esters.

Methyl esters were prepared from the TG and phospholipid fractions by the low temperature method of Kook (70). GLC of the methyl esters was performed using a Beckman GC-5 gas chromatograph equipped with dual flame ionization detectors. Resolution of the methyl ester peaks was achieved at 190 C using a 6' x 1/8" stainless steel column packed with 20 per cent DEGS-chromosorb W 80/100 mesh.

To aid in the identification of the various PUFA methyl esters, TLC (Pet Et_2O:Et_2O:HOAc, 90:10:1) was performed on silica gel G plates sprayed with 20 per cent silver nitrate dissolved in ethanol:H_2O (90:10). These impregnated plates separated the methyl esters according to the total number and geometry of double bonds in the molecules; fully saturated molecules travelled faster than monoenes, which preceded dienes, etc. Each band was scraped from the plate, eluted with Et_2O and injected into the gas chromatograph.

Fatty acid methyl ester standards used for comparison in all of the TLC and GLC work were obtained from Supelco. Much use was made of Supelco PUFA mixture #7033 which contained the methyl esters $C_{16:1}$, $C_{18:1}$, $C_{20:1}$, $C_{22:1}$, $C_{20:5w3}$, $C_{22:5w3}$ and $C_{22:6w3}$.

Additional techniques which aided in identification of the fatty acids from the neutral and phospholipid fractions of the Lake Michigan Coho salmon included the following: 1) Reference to previously published research concerning identification of fatty acids from Pacific Coast Coho salmon and the Lake Michigan alewife (94-98). 2) Comparisons of the GLC retention times of known fatty acid methyl esters with unknown peaks obtained from Coho salmon fatty acid methyl esters and graphs of log retention time vs carbon chain length for series of fatty acid methyl esters (99, 100).

Phospholipid Analysis

Phospholipid bands consisting of PE, PC, LPE and LPC were scraped from the TLC plates as described above and placed into micro-Kjeldahl flasks for digestion with perchloric acid following a procedure described by Harris (71). Blanks containing silica gel G were also digested to obtain quantative values for the absorbance at 820 nm.

RESULTS AND DISCUSSION

Oxidizing Systems

Evidence is strong that deterioration of muscle protein from frozen fish and other meats is linked to the production during storage of carbonyl compounds from lipid autoxidation. To facilitate study of this concept, model systems were employed which contained L, M and LM under oxidizing conditions. The systems were permitted to oxidize at 50 C under an oxygen atmosphere in order to increase rates of reaction.

Significant amounts of myosin were rendered insoluble by increasing the concentration of linoleate in LM mixtures. The loss of soluble proteins in this system became apparent for very dilute linoleate concentrations ($<0.1 \times 10^{-3}$M). This may be compared with other research (72) showing that when sodium linoleate was added to fish muscle homogenates, no loss in soluble protein was observed until a critical linoleate concentration was reached. To determine workable proportions of linoleate and myosin for the study, myosin and linoleate were mixed and the myosin remaining in solution following centrifugation at 4,000 x g after storage at 2 C for fifteen hours was

shown to decrease as the concentration of the linoleate in the solution was increased (Figure 1). Concentrations of linoleate which, after mixing (1:1 v/v) with 1 mg/ml myosin solutions, would precipitate almost all of the myosin were found to be greater than 1.5 x 10^{-3}M. The same proportions of linoleate and myosin at 50 C caused insolubilization of all the myosin in less than one hour, while solutions containing only myosin retained some soluble protein up to seven hours at this temperature. Chloroform extractions of supernatants from unoxidized LM systems with greater than 1.5 x 10^{-3}M linoleate showed an excess of linoleate remaining in solution following denaturation of the protein. It should be mentioned that preparation of sodium linoleate solutions of greater than 7.5 x 10^{-3}M concentration was not possible in the pH 7.5 buffers used for this study.

TBA reactive components were produced during oxidation of L, M and LM mixtures. The amounts produced during an oxidation time of six days for L and M were much less than produced in LM systems, which began tc show appreciable increases after three hours (Figure 2). The amount of oxygen absorbed per gram of either L or M was clearly greater for the LM system (Figure 3). The rate of oxygen absorption began to increase rapidly between six and twelve hours,

followed by a general rate decrease. It was during the six to twelve hour interval that fluorescence of the LM system under u.v. radiation began to increase notably.

An examination of Figures 2 and 3 supports the assumption that as oxidation of the LM system proceeds, carbonyl compounds and TBA reactives increase and become involved in cross linking between ε-amino groups of the protein (12, 14, 53, 73). A continuing increase with oxidation time of TBA reactives was noted, even though they are involved in reactions with the protein. This would be possible if the extent of production of the TBA reactants by the oxidizing systems was much greater than their participation in cross-linking and other reactions with the protein. Also, reaction with the TBA reagent itself may not be specific for only free, unbound malonaldehyde (53).

Denaturation of the protein by linoleate and oxidation products in the LM systems may increase the efficiency of oxidation of the LM mixture (74). This is supported by the fact that serum albumin bound by linoleate hydroperoxide showed increased efficiency of thiol oxidation, probably due to steric changes resulting from the binding (75). On the other hand, binding of the fatty acid salts or oxidation

Figure 1.--The effect of varying concentrations of sodium linoleate on myosin solubility. Soluble myosin was measured following incubation with linoleate solutions for fifteen hours at 2 C.

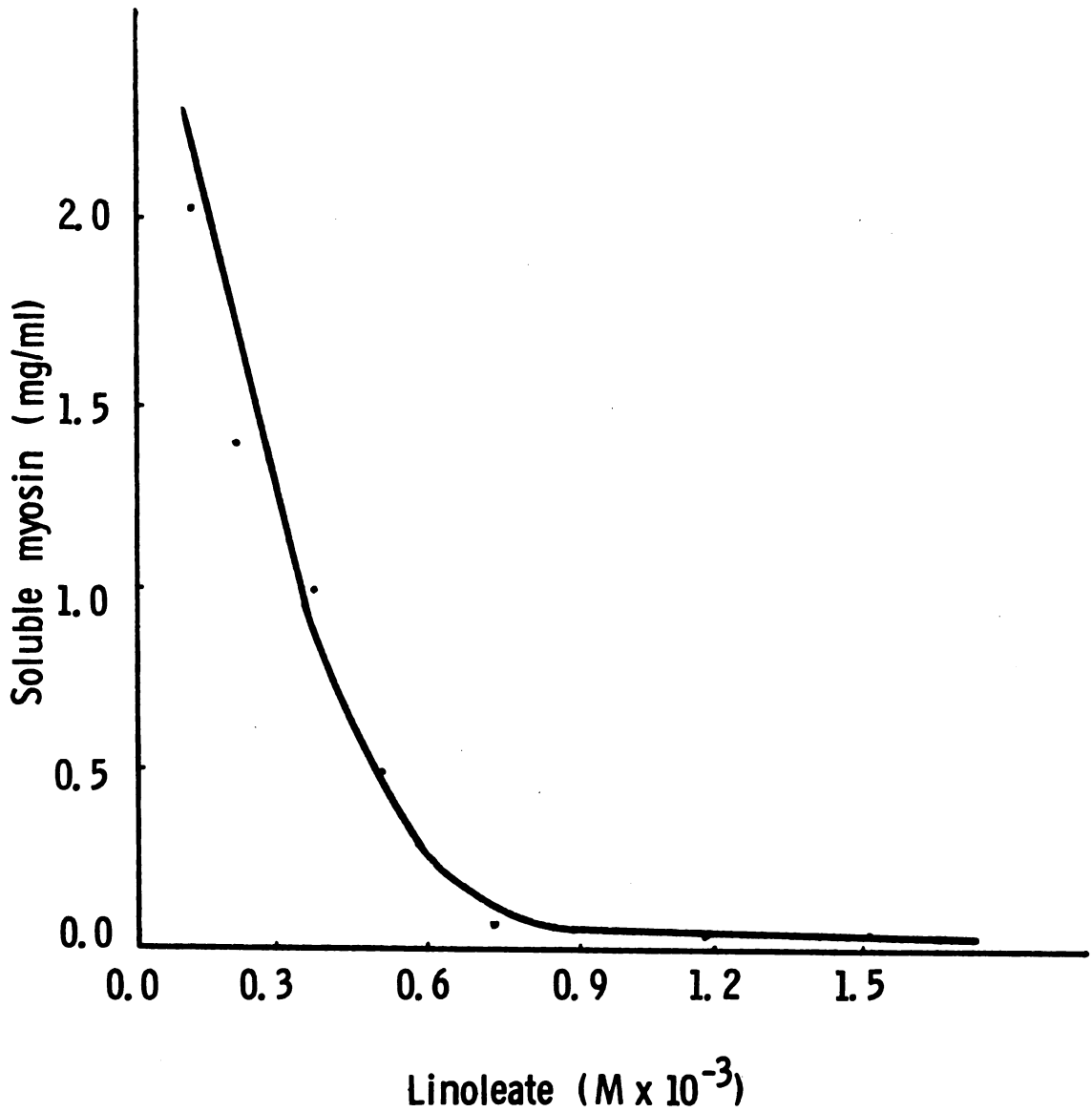

Soluble myosin (mg/ml)
2.0
1.5
1.0
0.5
0.0
0.0
0.3
0.6
0.9
1.2
1.5
Linoleate (M x 10^{-3})

Figure 2.--Amount of TBA reactive compounds produced in oxidizing linoleate, myosin and linoleate-myosin systems at 50 C. Relative concentration of linoleate = 2.5 x 10^{-3}M, myosin = 1.0 mg/ml, linoleate and myosin mixed 1:1 (V/V) with each other or with buffer.

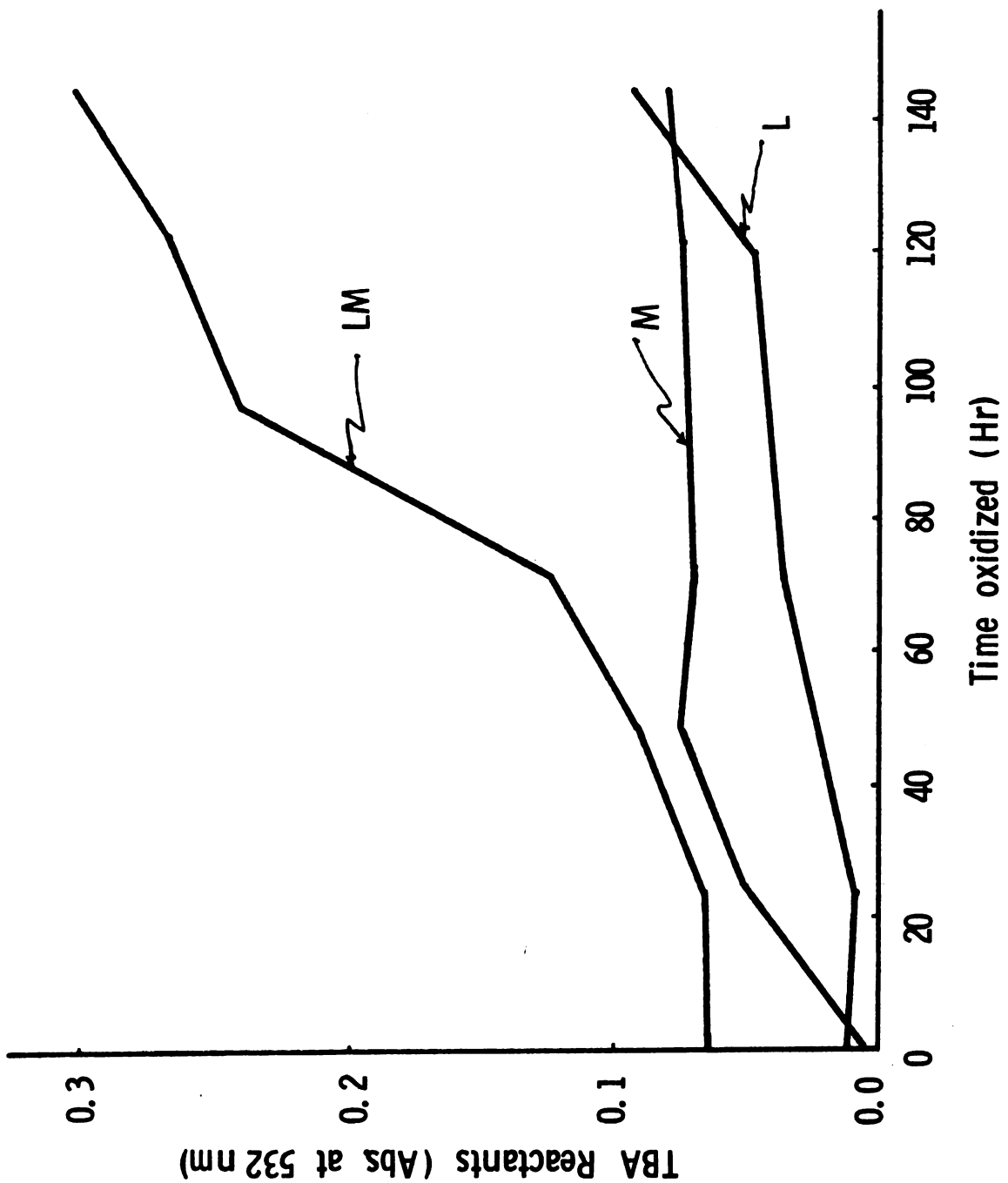
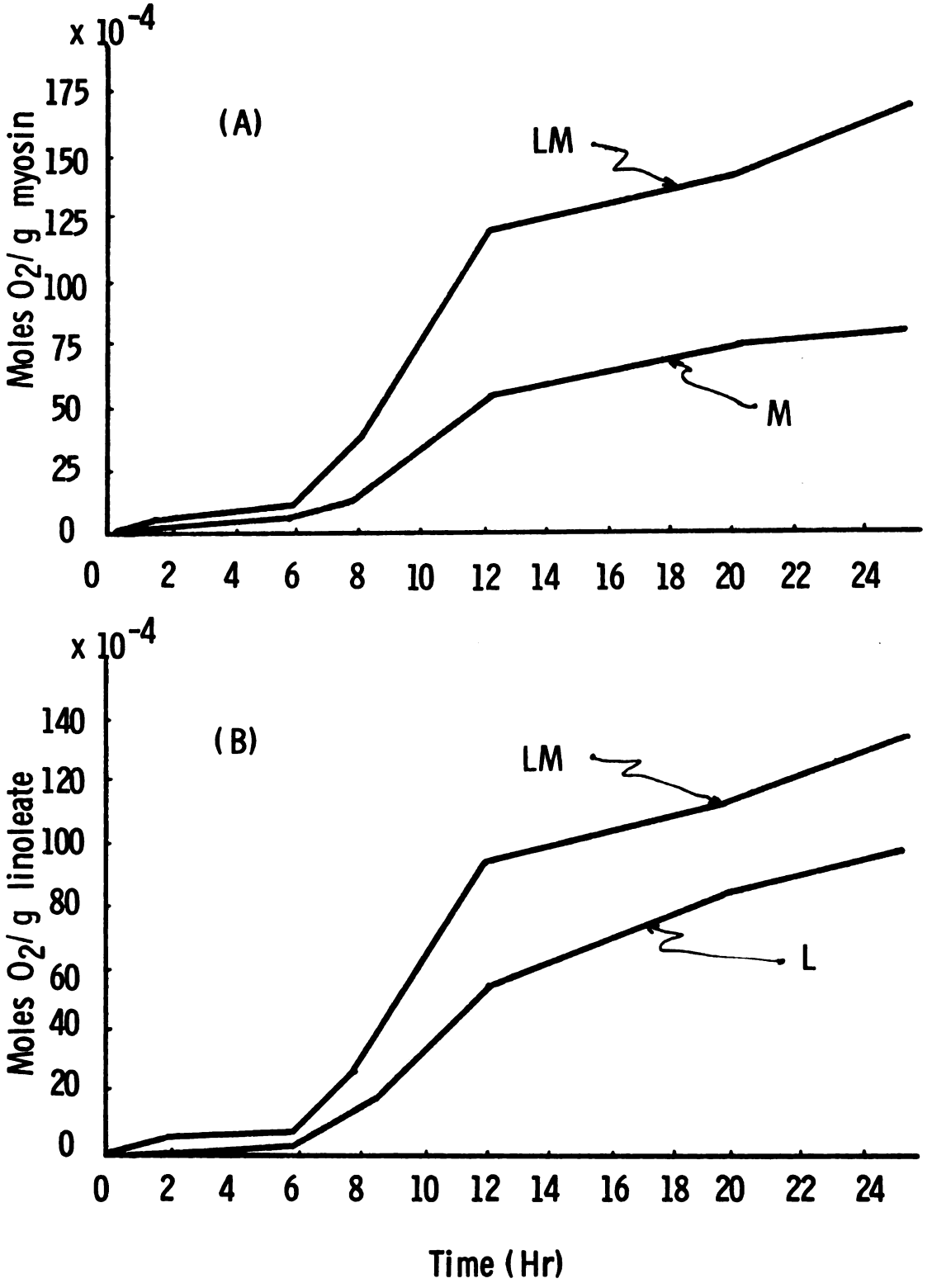

TBA Reactants (Abs at 532 nm)
0.0
0.1
0.2
0.3
0
20
40
60
80
100
120
140
Time oxidized (Hr)
LM
M
L

Figure 3.--Effect of sodium linoleate on the amount of oxygen absorbed by linoleate, myosin and linoleate-myosin systems at 50 C. Relative concentration of linoleate = 2.5 x 10^{-3}M, myosin = 1.0 mg/ml. linoleate and myosin mixed 1:1 (V/V) with each other or with buffer. A.) Moles of oxygen absorbed per gram myosin. B.) Moles of oxygen absorbed per gram linoleate.


x 10⁻⁴
Moles O2/ g myosin
175
150
125
100
75
50
25
0
(A)
LM
M
0 2 4 6 8 10 12 14 16 18 20 22 24
x 10⁻⁴
Moles O2/ g linoleate
140
120
100
80
60
40
20
0
(B)
LM
L
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (Hr)

products to the proteins in the LM systems may cause these fatty acids and products to become oriented around the protein in such a manner that would make them susceptible to oxidation, still having the effect of increasing the oxidation of the LM system.

Prolonged oxidation of LM for six days at 50 C resulted in breakdown of the complex and increasing fluorescence in the reaction mixture. A consequence of this oxidation was a loss of certain amino acids from the protein in the LM system, as presented in Table 1. Some slight changes can be observed when comparing amino acids from unoxidized and oxidized LM precipitates for such amino acids as threonine, serine, proline, alanine, leucine and phenylalanine (Table 1). These changes are not within the allowable $\pm 3\%$ error inherent to the analysis procedure, but are close enough to consider that the differences may not be significant. An unknown peak, which was in approximately equal quantities in both the oxidized and unoxidized LM samples, was observed following arginine on the recorder chart. This unknown, which was present in all the samples hydrolyzed for 24 hours, disappeared when a 72 hour hydrolysis time was used, indicating an unhydrolyzed peptide was probably responsible for the peak. The most significant changes were in losses of methionine (58%), histidine (43%) and

lysine (37%). These losses were obtained when amino acid analysis of 4,000 x g precipitates of oxidized LM systems were compared with unoxidized LM precipitates. Losses of these amino acids compared favorably with the observations of Buttkus (10, 11), who reacted malonaldehyde with trout myosin and amino acids. It was shown that lysine, histidine, arginine, tyrosine and methionine participated preferentially in the reaction, depending on the reaction temperature. He postulated that histidine residues were more accessible for reaction when the molecule unfolded during denaturation, and noted that in frozen malonaldehyde-myosin systems some lysine residues formed a product stable to acid hydrolysis.

C=N Oxidation Products

When it was apparent that rapid and extensive oxidation of LM systems led to production of TBA reactive components, losses of labile amino acids and fluorescence under u.v. radiation, attempts were made to extract and purify fluorescing components. Extraction of the LM system with chloroform after a three day oxidation period yielded a fluorescing chloroform layer and an aqueous layer which contained a fluorescing particulate residue from the protein.

TABLE 1.--Amino acid analysis of precipitates from linoleate-myosin systems oxidized for six days at 50 C and unoxidized linoleate-myosin systems.

Amino acid	g residue wt/100 g sample	
	Unoxidized LM	Oxidized LM
lysine	2.69	1.71
histidine	0.69	0.39
arginine	1.98	2.00
unknown peak	0.45	0.44
aspartic acid	2.81	2.82
threonine	1.30	1.57
serine	1.03	1.19
glutamic acid	3.88	3.93
proline	0.84	0.93
glycine	0.99	1.08
alanine	1.78	1.63
half cystine	tr	tr
valine	1.51	1.57
methionine	0.43	0.18
isoleucine	1.64	1.68
leucine	2.28	2.38
tyrosine	1.06	1.06
phenylalanine	1.06	1.31

This residue could be dehydrated by filtration and washing with ethanol, followed by extraction with

chloroform-methanol (2:1) to remove more of the fluorescing substances; however, even extensive extraction with this solvent did not result in quenching the fluorescence of the particles when resuspended in water. Intra- and intermolecular cross-linking is probably the cause of this fluorescence in the oxidized LM mixture which has become insoluble as a result of the oxidative reactions taking place (12).

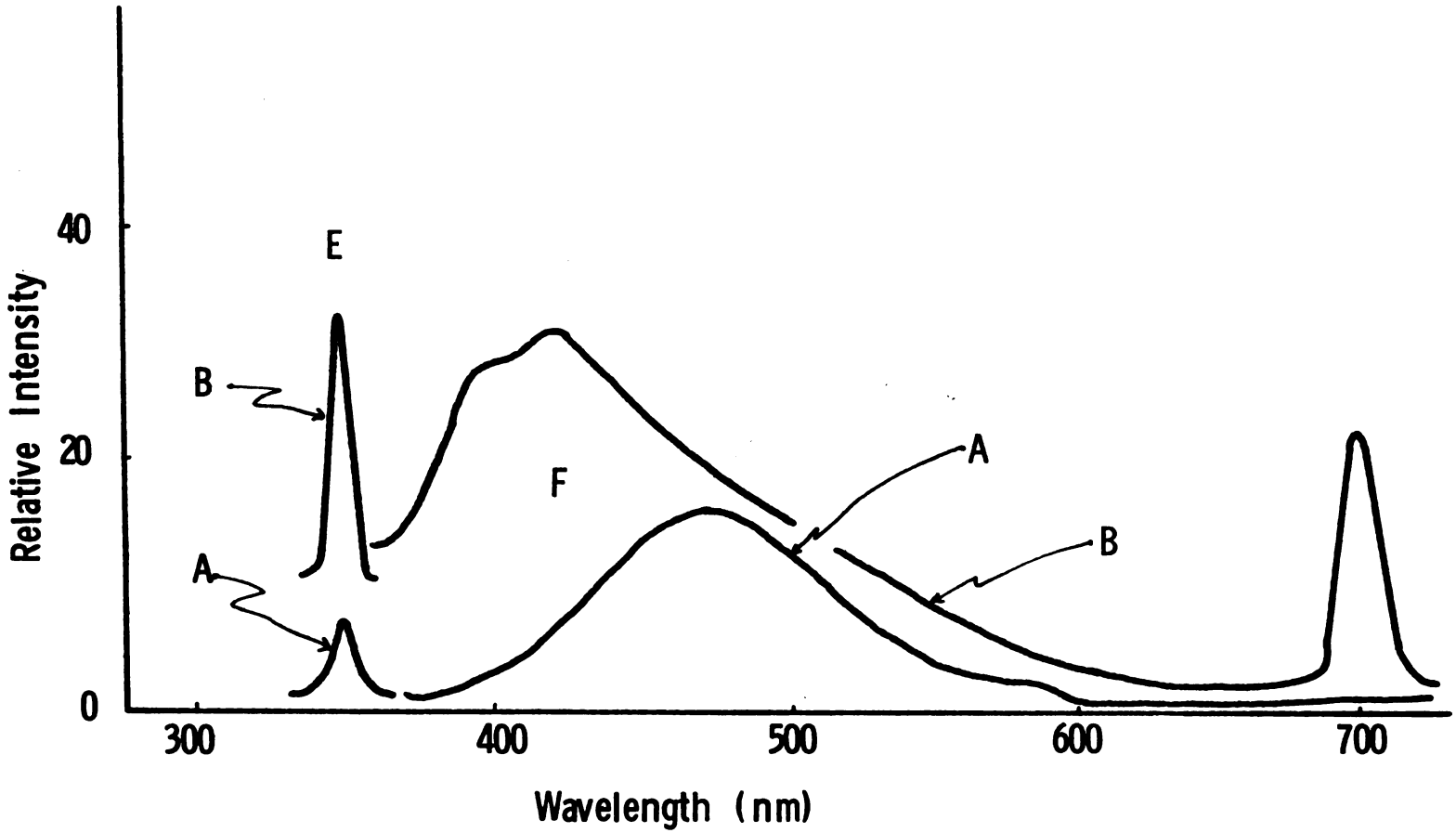
TLC in petroleum ether:diethyl ether:acetic acid (70:30:1) of the fluorescing chloroform extract resulted in separation of several bands probably containing unsaturation, as evidenced by reaction with iodine vapors for less than ten minutes. While it is known that iodine reacts with most organic compounds, reactions with compounds containing unsaturation is quite rapid and produces a dark brown color (76). One major band of interest moved slightly below the solvent front, contained carbonyl functional groups (2, 4-DNP positive), was iodine reactive and fluoresced under u.v. radiation. A much better separation of the LM chloroform extract was achieved when plates were first developed in hexane:Et_2O (60:40) causing a separation at the solvent front of the 2,4-DNP positive constituents, leaving all of the fluorescing substances at the origin. Development of this plate in chloroform:methanol:H_2O (65:25:4)

effected the separation of three fluorescing bands at R_f = 0.95, 0.70 and 0.35, respectively. Components of all three bands were reactive with iodine vapors. The band at R_f = 0.95 was very much more concentrated than the others. Purification and concentration of these components for further study was achieved by rechromatography of each band several times in the chloroform:methanol:H_2O solvent.

It should be mentioned that when the LM system was freeze-dried and then extracted with chloroform: methanol (2:1), separation of increasing numbers of fluorescing bands could be achieved. This study was confined primarily to an examination of the major fluorescing band (R_f = 0.95).

An examination of the u.v.-visible spectra of the fluorescing band (R_f = 0.95) revealed absorbance maxima at 240, 280, 375 and 431 nm (ref. solvent-methanol). Excitation and emission spectra (Figure 4) indicated an excitation peak at 350 nm and an emission in the region from 450-475 nm. An unusual emission at 700 nm was observed for the fluorescing bands found at R_f=0.70 and 0.35. These particular spectrophotometric properties (with the exception of the emission peak at 700nm) have been shown to occur in Schiff base-type compounds classified as 1-amino-3-imino propenes which were prepared by reacting amino acids

Figure 4.--Excitation (E) and emission (F) spectra of fluorescing compounds from oxidized linoleate-myosin systems. A = band from TLC (R_f = 0.95). B = bands from TLC (R_f = 0.7,0.35). Solvent = carbon tetrachloride.

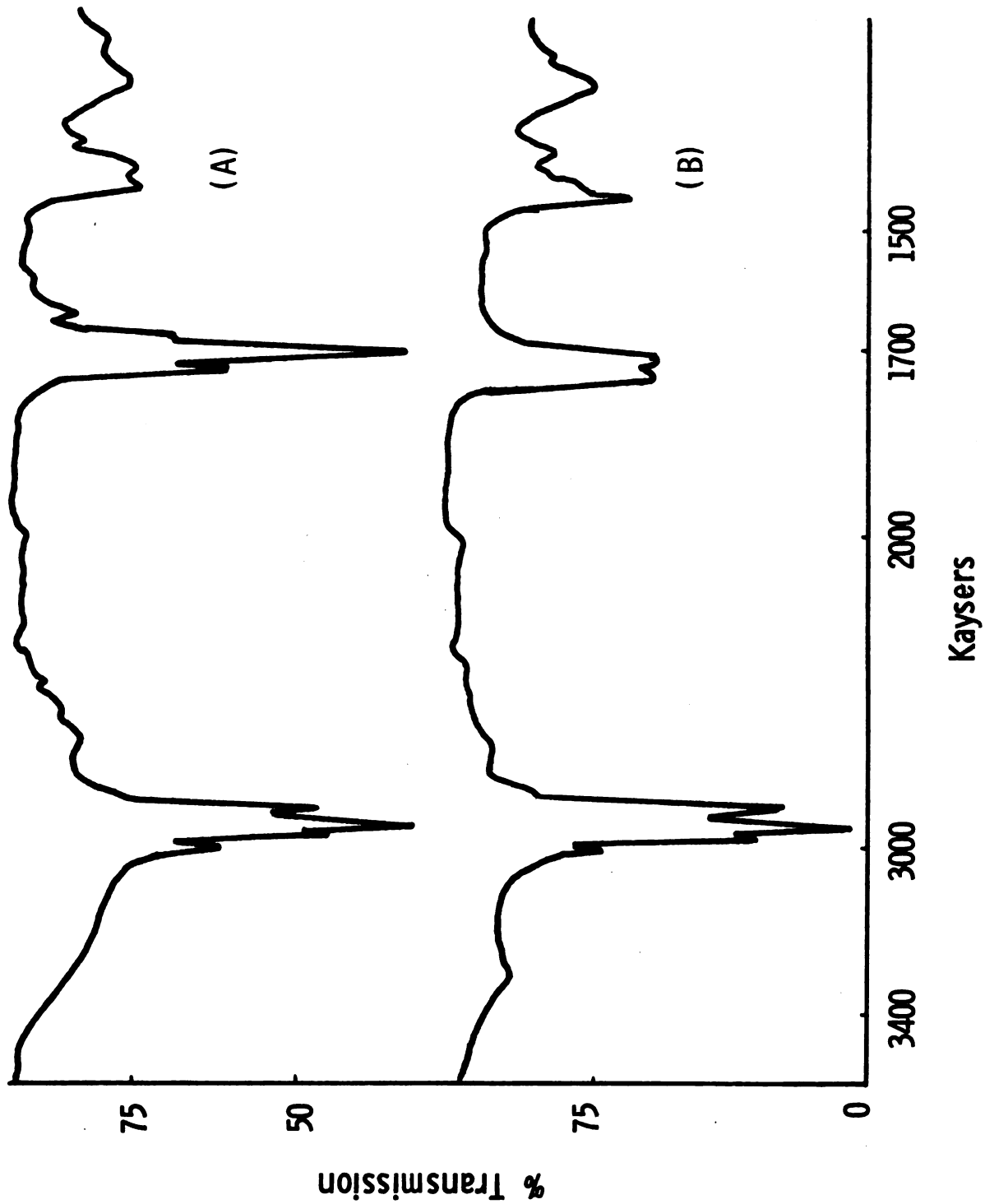

Relative Intensity
40
20
0
E
B
A
F
A
B
300
400
500
600
700
Wavelength (nm)

with malonaldehyde (66). Earlier research reported chemical and spectral evidence indicating that malonaldehyde and glycine reacted to form the enamine, N-prop-2-enalaminoacetic acid (13); however, these researchers precluded the presence of an imine linkage in the molecule.

Further elucidation of the chemical properties of the fluorescent products is possible through examination of the infrared spectra (Figure 5). Changes in the infrared spectra of the TLC isolate (R_f = 0.95) were noticeable following reduction with sodium borohydride. Bands at 1710 and 1665 cm^{-1} were greatly diminished as exhibited by the spectrum of Figure 5B. The 1710 cm^{-1} peak represents a band of the C=N bond, while the 1665 cm^{-1} peak is a band of the C=C bond. Recently, it has been shown that sodium borohydride is effective in bringing about complete reduction of the double bonds in compounds of the structure, R-NH-CH=CHCH=N-R (66). The strong band at 1750 cm^{-1} due to carbonyl absorbance is resolved more completely following reduction of the C=N bond.

The evidence presented proves that compounds containing C=N groups can be present as a consequence of lipid oxidation in systems containing oxidizable fat and protein. Soluble C=N compounds were present in these systems, even though the protein-lipid complex itself was insoluble. These soluble Schiff bases may

Figure 5.--Infrared spectra of the fluorescing band from TLC (R_f = 0.95) of extracts from oxidized linoleate-myosin systems. A.) Before reduction with sodium borohydride. B.) After reduction with sodium borohydride. Run as liquid, neat.


(A)
(B)
75
50
75
0
% Transmission
3400
3000
2000
1700
1500
Kaysers

be produced when the oxidative reactions of the system cause bond cleavage and formation of new compounds. For example, the losses of lysine, histidine and methionine, shown in Table 1, may reflect a loss through oxidative cleavage of some functional group from the molecules which has combined with lipid autoxidation products to form a soluble compound. The fact that no similar components were found in M-only solutions oxidized in like manner gives support to the evidence that malonalydehyde-like compounds or other carbonyls produced through lipid oxidation can react with amino nitrogen of proteins and amino acids to produce Schiff bases of various types. These compounds can then serve as intermediates leading to destructive reactions such as the non-enzymatic browning of foodstuffs. This was illustrated when LM solutions, allowed to react for many days, turned noticeably brown.

Frozen and Freeze-dried Coho Salmon

Oxidative Changes

Reactions of the type mentioned above have strong significance in consideration of quality of food products. Because of the susceptibility of frozen and freeze-dried fish to lipid-related deteriorations (16, 77), an investigation was conducted which revealed

the presence of compounds containing C=N in solvent extracts from these two products.

Extracts from frozen whole fish stored at -20 C for over a year, frozen salmon steaks kept under an oxygen atmosphere at -20 C for two months and freeze-dried salmon steaks exposed to air at room temperature for six months were separated into neutral and polar lipid fractions by TLC as described above. The more polar fraction contained fluorescing bands which exhibited C=N and the same spectral characteristics previously described for the extracts from the LM system.

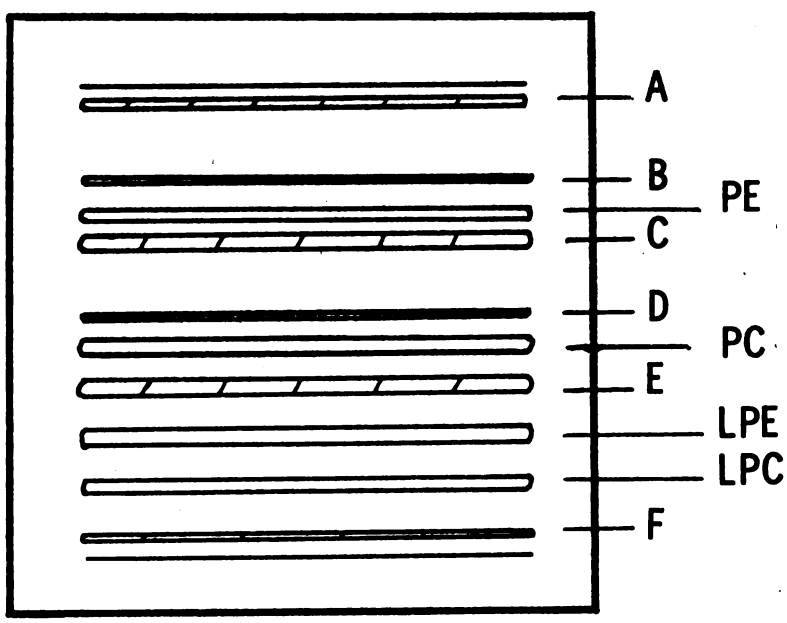
Three major fluorescing bands (A, C and E, Figure 6) with R_f values similar to the bands from the oxidized LM system were obtained from the frozen stored fish. There were also several other minor bands (B, D and F) which fluoresced similarly under u.v. radiation, but no studies were performed on these. One of the major bands (E, R_f = 0.3) showed a positive test for phosphorus by a molybdate spray and also exhibited C=N bonding in the infrared spectrum. This band moved just ahead of a band identified as LPE and may be a reaction product between a carbonyl compound and the PE amino group to give a Schiff base more polar than the PE fraction, resulting in migration between the PE and the LPE. In addition, since LPE is a

component of the lipids present in the frozen stored fish, reactions involving carbonyl compounds and the amino group of this compound may also lead to the production of Schiff bases with mobilities in the TLC solvent systems used in this experiment. Considering the probable complex nature of the autoxidation products which could react with the amino groups of PE and LPE in the frozen fish, it would be no simple task to establish the identity of these reaction products. Extracts from fresh fish treated in a similar manner did not contain these fluorescing products.

The stored frozen fish contained large amounts of TBA reactives as evidenced by a TBA test (65) which resulted in values as high as 35 mg malonaldehyde/Kg flesh; whereas, the oxidized freeze-dried fish, which had a very rancid odor, only gave TBA values in the range of from 0.5-3.5, based on the wet weight of the flesh. In an attempt to correlate TBA values with quality of fish flesh, Sinnhuber and Yu (78) reported that products with TBA values of 2 were considered acceptable, while those with values of from 4-27 were rejected.

The task of clarifying reactions involving lipid oxidations is formidable when the complexity of the lipids and proteins contained in frozen fish or of the

Figure 6.--Silica gel G TLC of the fluorescing polar lipid fraction from frozen Coho salmon stored for one year at -20 C. Solvent system, $CHCl_3$:MeOH:H_2O (65:25:4). Bands A, B, C, D, E and F showed fluorescence under u.v. light.


A
B
PE
C
D
PC
E
LPE
LPC
F

LM system is considered. The extraction of compounds containing C=N from autoxidizing food systems consisting of lipids and proteins is proof that significant reactions can occur which change the chemical nature of the original constituents. The fact that addition to and oxidation of linoleate in myosin solutions caused insolubilization of the protein is evidence for the occurrence of physical damage to the protein in such systems. These reactions and interactions could be very important to the final quality of stored food products and formulations for certain foodstuffs.

This study contains results which will aid in evaluation of some of the complex changes in food systems and organizes much of the recent research which implicates malonaldehyde-like compounds as playing a leading role in lipid deterioration reactions. The fact that malonaldehyde and other carbonyl compounds will interact with food constituents during autoxidation may account for some of the discrepancies associated with the use of TBA tests for quality evaluation of many lipid containing foods. Also, it has not been firmly established that compounds resulting from carbonyl-amine reactions do not give positive TBA reactions. It has been shown, however, that other fatty acid oxidation products, besides malonaldehyde, reacted slowly with TBA reagent to

give compounds abosrbing at 450 nm, in addition to the malonaldehyde-TBA complex abosrbing at 538 nm (88).

Lipid Hydrolysis

The complexity of the fatty acids in the neutral and phospholipids of the Coho salmon samples used in this study can be seen by examination of Table 2 and a recent publication (87). The same spectrum of fatty acids was found in both the triglycerides and the phospholipids analyzed, only quantities of specific fatty acids showed differences. For instance, when comparing the percentage composition of the fatty acids from the triglycerides and total phospholipids of fresh frozen Coho salmon in Table 2 it is apparent that the same fatty acids were present in both lipid fractions. There is a slightly greater percentage of the 16:0, 18:0 and 18:1 and less 16:1, 20:1, 18:2, 20:5 and 22:5 fatty acids in the phospholipids than in the triglycerides. After storage for one year at -20 C, the differences between the triglyceride and phospholipid fatty acids is much greater, reflected by considerably lesser amounts of 14:0, 18:1, 18:2 and 18:3w3 and more concentration of 18:0, 20:3, 20:4, 20:5 and 22:6 in the remaining phospholipid fraction. The contribution of lipid hydrolysis and FFA production

TABLE 2. Percentage composition of the fatty acids in the triglycerides and phospholipids of fresh frozen and frozen stored Lake Michigan Coho salmon.

Fatty acid	Percent (GLC peak area)			
	Triglycerides		Total phospholipids	
	Fresh	Frozen 1 yr	Fresh	Frozen 1 yr
14:0	3.2	5.4	3.0	1.8
15:0	0.4	1.1	0.5	0.4
16:0	10.3	7.9	15.6	6.0
17:0	0.8	1.8	0.9	0.6
18:0	3.5	3.7	4.7	5.5
20:0	0.2	0.4	0.1	0.1
22:0	0.5	0.7	0.5	0.4
14:1w6	0.3	1.4	0.4	0.5
15:1w6	0.2	0.8	0.2	0.6
16:1w7	10.7	8.8	8.9	7.0
18:1w9	21.2	19.4	25.3	14.1
20:1w9	3.4	3.6	2.3	1.2
22:1w9	0.5	0.6	0.6	1.5
24:1w9	0.8	tr	0.5	tr
16:2w4	1.1	1.6	1.1	0.6
18:2w6	5.6	5.5	3.6	1.9
20:2w6	1.3	1.3	0.6	0.7
22:2w6	0.1	tr	0.3	0.7
16:3w4	2.0	2.2	1.9	1.3
18:3w6	0.5	0.4	0.5	0.6
18:3w3	2.9	3.2	2.6	1.8
20:3w6	2.5	2.5	2.7	4.4
18:4w3	0.8	1.0	0.7	0.6
20:4w6	1.6	1.9	1.3	1.0
20:4w3	0.7	0.7	0.5	3.1
22:4w6	1.6	1.6	0.9	0.8
22:4w3	1.5	1.2	1.4	2.4
20:5w3	4.8	4.7	3.7	8.4
22:5w6	1.8	1.4	0.6	2.0
22:5w3	4.4	5.2	4.1	6.0
22:6w3	10.6	9.9	9.9	24.0

in frozen fish to protein denaturation and quality losses has been mentioned previously. It has generally been agreed that increases in the FFA during frozen

storage come from the phospholipids in the flesh; however, recent evidence indicates that FFA production during frozen storage may also take place in some species through the hydrolysis of triglycerides (16, 86). In this study, there were only minor changes in the FFA composition of the triglycerides during a storage period of over a year at -20C (Table 2).

Comparison of the percentage composition of the fatty acids in the fresh frozen and frozen stored salmon phospholipids presented in Table 2 reveals that the fatty acids, 20:5, 22:5 and 22:6 are more concentrated in the phospholipids remaining after frozen storage for one year at -20 C, while the fatty acids, 16:0 and 18:1 are less concentrated. These changes reflect losses of specific fatty acids from the phospholipid fractions, either through enzymatic hydrolysis or oxidative reactions, or both, and will be discussed later, when dealing with fatty acid changes in particular phospholipids in the fish (see Table 4).

Although early research reported only trace amounts of the lyso-derivatives in fish muscle phospholipids and no increase during cold storage (32), more recently, losses of phospholipids and increases of the lyso-compounds during frozen storage

have been reported for fish and chicken muscle (17, 41). In this study, a decrease in the total phospholipid content with increasing storage time of frozen salmon was apparent. Data is presented in Table 3 showing that significant decreases in PC, PE and LPC occurred after six months frozen storage. Measurements were made using samples from the same fish. There were increases in the LPE fraction and an unidentified phosphorus-containing fraction denoted as an oxidation product. If one computes the ratios of PC/LPC and PE/LPE, which are measures of the degree of hydrolysis, it can be seen that a ratio of approximately 3 is a characteristic of all but the PE/LPE for the stored fish, which has a ratio of only 1.4. Consideration of these ratios and the contents of the individual phospholipids listed in Table 3 implies that the hydrolysis rate of PE to LPE during six months frozen storage is greater than that of PC. This is in disagreement with Bosund and Ganrot (86), who showed that in frozen stored Baltic herring, the hydrolysis rate of PC was faster than that of PE in both white and dark muscle. Also, in light of previous discussions concerning oxidative changes in frozen salmon and LM systems, reactions of

TABLE 3. Phospholipid content of fresh frozen Coho salmon and after six months storage at -20 C.

Phospholipid	Phosphorus content (mg P/gm flesh) Fresh	Stored
Total P. L.	18.5	16.0
PC	9.9	6.4
PE	3.4	2.7
LPC	2.9	1.9
LPE	1.1	1.9
Ox. Product	1.2	3.1

the PE amino group with carbonyl constituents known to be present in the flesh may account for additional losses of PE and LPE.

Another explanation for the increased amount of LPE shown in Table 3 is possible. It is suggested that the release of specific fatty acids during hydrolysis may inhibit enzymatic activity resulting in incomplete hydrolysis and a buildup of the LPE. A candidate for a suitable fatty acid to participate in a reaction of this sort would be oleic acid, which has been shown to be in unusually high quantities in the Coho salmon used in these studies (87, Table 2), and which has been extensively hydrolyzed from the PE, as indicated by its decrease in the remaining

LPE shown in Table 4. Support for this statement has been presented in a research paper which has shown that oleic acid had a strong inhibiting effect on the lyso-lecithinase of cod muscle (37).

Additional data concerning lipid hydrolysis in stored, frozen salmon is presented in Table 4, which lists the proportions of certain fatty acids for different phospholipids. Changes in the fatty acids of PC and LPC fractions were different from changes in the PE and LPE fractions. Most notable were the changes in the PE-LPE, which showed concentrations of the 16:0 and 22:6 acids and a major loss of the 18:1 acid in the remaining LPE. Smaller decreases were also observed in the amounts of the 16:1, 18:0, 18:2, 20:5 and 22:2 acids present in the LPE. Phospholipid composition analyses performed on fresh frozen Coho showed very little difference in the fatty acids of PC and PE when compared before and after six months frozen storage.

The data in Table 4 implies a preferential hydrolysis of PE containing 16:0, 18:1 and 22:6 acids. A recent paper by Olley et al. (17) implies a preferential hydrolysis of phospholipids containing 16:0, 18:1 and 20:5 acids. They reported that 16:0, 18:1 and 20:5 acids became a greater proportion of the FFA and the 18:0 and 22:6 acids were concentrated in the remaining phospholipid. The fact that the 16:0

TABLE 4. Percentage composition of the fatty acid methyl esters from the phospholipids of Coho salmon frozen at -20 C for six months.

Fatty acid[a]	Percent (GLC peak area)				
	PC	PE	LPC	LPE	LPE[b]
16:0	13.4	7.6	11.0	24.7	47.2
16:1w7	3.7	8.6	4.8	5.3	7.6
18:0	4.7	4.8	5.5	1.9	5.1
18:1w9	10.7	21.7	8.6	7.4	12.8
18:2w6	5.1	5.1	3.2	1.7	3.7
18:3w3	1.8	2.2	1.3	1.0	1.8
20:4w6	2.6	1.3	2.9	2.9	1.4
20:5w3	22.8	26.5	20.5	22.2	4.3
22:2w6	1.7	5.7	4.3	2.0	0.6
22:4w6	2.7	1.9	5.9	1.6	0.3
22:5w6	2.0	0.5	1.1	2.2	0.9
22:5w3	3.8	2.4	5.6	2.7	0.8
22:6w3	24.9	11.7	16.3	24.3	13.5

[a]The number after the w denotes the position of the ultimate double bond relative to the terminal methyl group.

[b]The values in this column were obtained following treatment of Coho salmon PE with pancreatic lipase, in which the phospholipase A activity had been suppressed.

and 22:6 acids are greatly concentrated and the 18:1 acid is much less concentrated in the remaining LPE is evidence for a slower rate of hydrolysis of PE molecules containing 16:0. The situation is not the same for the PC-LPC phospholipids, both of which show similar proportions of fatty acids, implying a generally non-preferential hydrolysis.

The position of the 16:0 in the PE molecule may be important to the hydrolysis. For this reason, pure PE from Coho salmon was allowed to react for 20 minutes with pancreatic lipase at pH 9.5 in a 10% sucrose buffer. These conditions were found to repress the phospholipase activity in the pancreatic lipase preparation. The result of this hydrolysis was to hydrolyze the fatty acid from the α'position of the PE, leaving the β-acyl-LPE. Recently, Slotboom et al. (79) proved that lipase preparations from hog pancreas hydrolyze exclusively the fatty acid ester bound at the 1-position of all common types of phosphoglycerides, regardless of the nature and distribution of the fatty acid constituents. Since the 22:6 acid is concentrated and the 16:0 acid is very much more concentrated in the remaining LPE (Table 4), these acids must predominate at the β-position in the PE, and the hydrolysis in the frozen fish must largely take place at the α'-position of the PE. This is not necessarily true for the PC, however, which may

reflect hydrolysis at both positions, a characteristic of fish muscle phospholipases (17). Time of storage may be an important factor also, since it has been shown that the proportion of 16:0 and 18:0 in the fatty acids liberated by phospholipase A from the β-position of lecithin was high in the early stages of the reaction, and as hydrolysis time increased, the concentration of 18:1, 18:2 and 20:4 increased (36).

It should be recognized that pH and other conditions can regulate both the extent and rate of enzymatic hydrolysis of lipids in the muscle, and these may be different for PE and PC. It has been shown that rat intestinal phospholipase A (101) and liver phospholipase B (102) both have a pH optimum of 6.5, with markedly reduced activity below pH 6.1 and above pH 6.9 for the former and below pH 5.8 and above pH 7.2 for the latter. Such conditions, which may occur in the tissue during frozen storage of the fish, may be the regulating factor governing the hydrolysis.

Thus, apparently the 16:0 and 22:6 fatty acids at the β-position of the PE from Lake Michigan Coho salmon play a role in the preferential hydrolysis by phospholipases of this phospholipid during frozen storage. Evidence does exist that for some fish

triglycerides and phospholipids, the α'-position contains saturates and monoenes and the β-position has the long chain PUFA and palmitic acid (80). Also, the hydrolytic enzymes could be affected by many factors, such as the buildup of substrates, oxidative reactions resulting in inhibition and concentration of solutes during freezing and frozen storage. These factors may affect the mode of attack or the orientation of substrates, which, in turn, affect the selectivity and rate of hydrolysis. Such considerations would also be important to implementation of the use of FFA formation in frozen fish as a measure of muscle degradation as proposed by Jonas (81).

The results of this study show that in such complex systems as frozen fish and the simpler, yet still complex LM system, chemical changes detrimental to the maintenance of original characteristics are occurring. Changes which involve lipid oxidation products in reactions with proteins and other constituents certainly play an important role in food quality, but may also be of importance in living biological systems, since recent research has implicated lipids in changes in subcellular organelles and membrane systems (67, 82).

Further research into the identity and <u>in vitro</u> properties of the C=N compounds isolated in this

study is needed to establish the extent and importance of these reactions and compounds to food quality where autoxidation is involved. Additionally, these reactions may involve changes in the nutritional quality of foodstuffs and it should be established to what degree these changes occur in relation to such things as losses of essential amino and fatty acids.

Concurrent with the autoxidative changes in the frozen fish were enzymatic changes resulting in the production of free fatty acids from the phospholipid fractions in the flesh. A thorough study of these phospholipid enzymes in the muscle is needed. A study similar to that by Slotboom et al. (79) could be initiated, one designed to elucidate reaction parameters, preference for specific fatty acids or position of the molecule and enzyme inhibition by released fatty acids. The effect of autoxidative reactions on the enzymatic hydrolysis as well as means to control both reactions and hydrolysis might also be studied.

Other research in our laboratory has proved the direct involvement of phospholipids in production of C=N compounds in autoxidizing model systems held at different relative humidities and allowed to undergo nonenzymatic browning. These reactions in model systems

may very likely occur in natural systems such as frozen or processed fish, and serve to show that during autoxidation of the lipid constituents, that the PUFA, phospholipids, proteins and other components making up a food system are all interrelated, with each interaction contributing to product quality and stability.

Suggestions for Additional Research

1. A thorough study is needed of the lipase and phospholipase enzymes and conditions affecting their activity in the frozen fish muscle, and is essential to a complete understanding of frozen storage changes in the fish lipids.

2. More complete separation, isolation and identification of specific C=N compounds formed during storage and a study of conditions affecting their formation is needed.

3. Information obtained from 1 and 2, above, will be useful in studying ways to control product quality changes, but will be more useful if organoleptic measurements or evaluations relating these parameters to product quality are conducted.

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