Metal-Catalyzed Lipid Oxidation and Changes of Proteins in Fish[1]

CHARLES H. CASTELL, Fisheries Research Board of Canada, Halifax Laboratory, Halifax, Nova Scotia, Canada

Abstract

The addition of metals to pure fats and model systems has given us a picture of the part they play in lipid oxidation. But in complex substrates such as fish flesh the same metal ions catalyze reactions in other components of the muscle as well. A more complete picture of deterioration is obtained when we also examine the effect of metals on the other components of the muscle.

Although lean fish muscle contains 0.5% to 1% unsaturated lipids, during frozen storage it rarely goes rancid, as indicated by thiobarbituric acid (TBA) values or rancid odors. The lipids do oxidize, but instead of forming carbonyls and other compounds associated with rancidity, they become bound up in lipid-protein complexes, which accounts for the toughened texture of overstored or poorly stored frozen fish. It has been generally accepted that the formation of these lipid-protein polymers is brought about by a reaction between the proteins and oxidizing fatty acids. For fish of the gadoid or cod family, this is further complicated by the enzymic production of formaldehyde, which forms in the muscle during cold storage. The direct addition of formaldehyde to give concentrations of 0.001% to 0.05% caused marked reductions in the extractable protein of cod muscle. Because formaldehyde and dimethylamine (DMA) are produced in the stored muscle by the same reaction, the accumulation of the DMA can be used as a measure of this process which leads to protein insolubility. Removal of the dark lateral muscle from the fillets before freezing reduced the production of DMA and formaldehyde during storage and resulted in less loss of extractable protein.

Introduction

This study is concerned with commercial, marine fish caught in the Atlantic off the east coast of Canada. The interest is primarily directed towards the chemical changes taking place in these fish as they slowly deteriorate during frozen storage.

For those who are not familiar with fish, it should be pointed out that, in a general way, they can be divided into two groups, depending upon the location in their body where they store their lipids. In the "fatty fish" such as herring, mackerel or tuna, much of their depot fat is interspersed in the muscle. In "lean fish," such as cod or haddock, most of the lipid is stored in the liver. The muscle of these lean fish, however, does contain anywhere between 0.5% and 1.5% fat. These are functional lipids rather than storage or depot fat. Instead of being in a free state, these muscle lipids, mostly as phospholipids or lipoproteins, are incorporated into the cell structures, such as the cell membranes. Such functional lipids are of course present also in the muscle cells of "fatty" fish.

During frozen storage, the depot lipids in the muscle of fatty fish oxidize readily. The limiting factor for the storage time of the fatty fish is usually the development of rancidity.

This is not the case with many of the lean fish. For example, we have stored large numbers of cod fillets at temperatures ranging between −3 and −26 C for periods from three weeks to three years. Never once have we encountered rancidity as indicated either by the odor of the fish or the content of thiobarbituric acid (TBA) reactive substances of the muscle. These fish do deteriorate during storage, rapidly at temperatures above −10 C and more slowly as the storage temperature is decreased. Their texture becomes tougher; they first lose their characteristic flavors and odors and then slowly develop various off odors and off flavors. But there is no evidence that lipid oxidation, resulting in rancidity, is part of this spoilage pattern.

If these lipids are extracted from the muscle, they readily oxidize, developing high TBA values and the typical odors of rancid fish oils.

Our problem has been to try to find out why these highly unsaturated fats apparently do not become oxidized in the fillets in frozen storage. We believed that an answer to this question might throw light on some of the changes that ordinarily take place in the frozen fish. We felt that, in a complex substrate such as fish muscle, what is taking place as it deteriorates cannot be fully understood if the changes in the lipids are dissociate from the changes that are simultaneously taking place in the nonlipid components.

The investigations were carried out in two separate phases. To begin with we worked with homogenized muscle, prepared by blending whole fillets with three times their weight of water. Such a system has severe limitations; blending disintegrates the tissues and incorporates large amounts of atmospheric oxygen into the homogenate. But it did have some advantages. It is possible to add a metal catalyst or some other reagent and have it evenly distributed throughout the blend. (It is difficult to do this with a whole fillet.) And the problem of taking a representative sample for analyses is much simpler with the blended muscle.

Although our ultimate aim was to study the whole frozen muscle, we did get a lot of very useful preliminary information from our experiments with blended muscle. The results of some of these tests were reported by Schmidtsdorff (1) at the 1967 symposium in Sweden, and more fully in a series of papers published in the Journal of the Fisheries Research Board of Canada (2–9).

A brief look at some of these earlier studies shows that lipid oxidation could be readily induced in the flesh of both lean fish and fat fish, as well as crustaceans and shellfish, by the addition of trace amounts of certain heavy metal ions (7). In general,

[1] One of 28 papers presented at the Symposium "Metal-Catalyzed Lipid Oxidation" presented at the ISF-AOCS, World Congress, Chicago, September 1970.

Cu^{++}, Fe^{++} and V^{++} were the most active catalysts; Cd^{++}, Co^{++} and Zn^{++} produced rancidity in fat fish but not in lean fish. The activity of individual heavy metal ions in producing lipid oxidation was not the same for all species of fish. Crustaceae, and to a lesser extent the flesh of shellfish, were extremely resistant to the Cu^{++}-induced rancidity.

In addition to producing rancidity, some of these same metal ions brought about other changes in the muscle. Cu^{++} and Cr^{++}, but not Fe^{++} or V^{++}, rapidly decreased the extractable protein in the muscle (9). Fe^{++} and V^{++} reduced trimethylamine (TMA) oxide in the muscle to TMA; Fe^{++}, but not V^{++}, reduced TMA oxide to dimethylamine (DMA) and formaldehyde (FA).

Comparing the effects of specific metal ions on changes taking place in several components of the muscle helps to clarify the part played by some of the nonlipids in the development of rancidity. For example, when added to extracted cod muscle lipids, Cu^{++}, Fe^{++} and V^{++} were all very active lipid oxidation catalysts and with all three, the development of rancidity has a very short induction period. But this was not the case when they were added to blended cod muscle (7). With Fe^{++} and V^{++} rancidity developed almost immediately, while with Cu^{++} there was an induction period of 12 to 15 hr. On the other hand, Cu^{++} reacted quickly with the extractable protein while the Fe^{++} and V^{++} had no effect. It was also observed that certain free amino acids, especially cysteine, histidine and tryptophane, protected the lipids in the muscle against copper-induced rancidity (6), as well as against copper-induced changes in the proteins (9).

As well as being active lipid oxidation catalysts, Fe^{++} and V^{++} were able to reduce TMA oxide in the muscle to TMA. However, conditions that favored the development of rancidity depressed the production of TMA, and vice versa. Increased pH, anaerobic conditions and the presence of cysteine (Table I) all favored the production of TMA and depressed the development of rancidity. These few examples as well as many other similar experiments emphasized the interrelationship between the changes taking place in the lipid and in the nonlipid components of the muscle.

Production of Dimethylamine and Formaldehyde in Fish Tissues

In 1963 Amano and his associates in Japan published the first of a series of papers (10–16) dealing with the enzymic reduction of TMA oxide in fish tissues to form DMA and formaldehyde (FA). Most of their results up to 1969 have been summarized in a lengthy review by Yamada (17). Of the more than 45 species of fish tested, DMA and FA were found in species of the cod family and in squids, but not in flesh of other fish or marine invertebrates. A crude extract of the enzyme was produced and it was shown to require a heat stable cofactor. Although present in the muscle, the enzyme was more abundant in the pyloric caeca, stomach, gall bladder and other visceral organs. The enzyme activity was not inhibited by temperatures below freezing (0 to −25 C). Tokunaga (18,19) reported that during frozen storage there was an increase in the DMA and FA of Alaska pollock at temperatures as low as −25 C.

TABLE I

Effect of Adding 50 ppm Fe^{++} and 0.05 M Cysteine on the Production of Trimethylamine and Rancidity in Blended Cod Muscle Held for 24 hr at 0 C

Treatment	Trimethylamine mg/100 g	Thiobarbituric acid value, mg malonaldehyde/1000 g
Blend alone	0.45	0.42
Blend + cysteine	0.57	0.69
Blend + Fe^{++}	0.68	14.64
Blend + Fe^{++} + cysteine	15.42	1.58

In a long series of experiments at our Halifax Laboratory, we also found small but consistant increases in DMA in cod fillets during frozen storage (20). We were struck by the observation that the production of DMA seemed to fit into a pattern of other changes that took place in the frozen muscle. Because of our previous interest in the relationship between the production of TMA and rancidity in the blended muscle, we decided to take a rather long look at the production of DMA in frozen fish, and to try and determine whether it was a relatively isolated reaction or whether it was an integral part of the complex changes that take place in the muscle as it deteriorates in frozen storage.

At this point we stopped working with blended muscle and began working whole frozen fillets. The procedures were quite simple. Whole fillets were individually packaged in heat-sealed plastic bags. They were frozen in a plate freezer at −40 C and then placed in storage at −5 C. This storage temperature was selected because we had previously learned that it stopped the growth of psychrophilic bacteria and at the same time permitted a rapid development of chemical changes that take place as frozen fish deteriorate in storage. Most of these experiments involved storage periods of 30 to 50 days.

First we took a look at the effect of species. We simultaneously froze fresh, high quality fillets of cod, haddock, pollock, hake, cusk, flounders, halibut, ocean perch, ocean catfish, shad, mackerel and muscle from lobster, shrimp and scallops. They were placed side by side in the same storage compartment at −5 C. Periodically samples of each species were withdrawn and a number of tests were carried out to determine some of the chemical changes that had taken place. Figure 1 shows the DMA values. The results divided

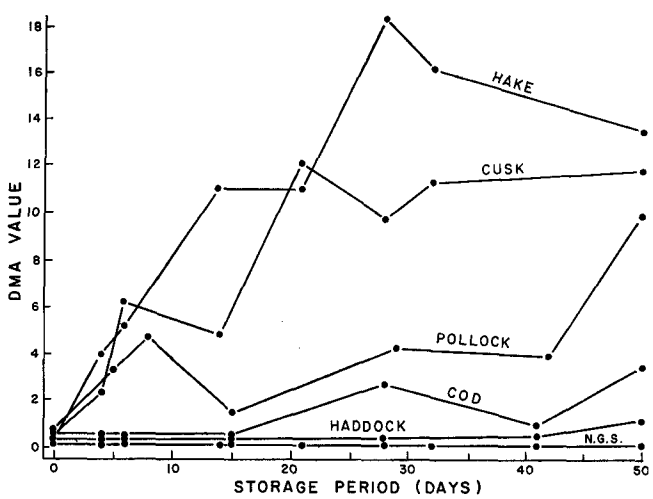

FIG. 1. Development of dimethylamine (DMA) in whole frozen fillets stored at −5 C. Six nongadoid species of fish, as well as lobster, shrimp and scallop muscle, did not produce DMA as indicated by the line marked N.G.S. (nongadoid species).

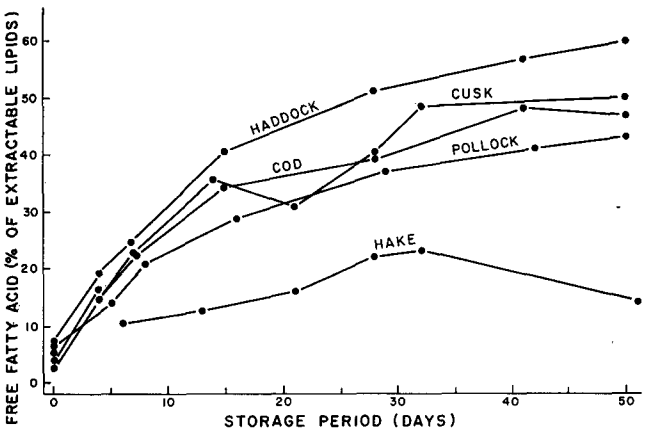

FIG. 2. Free fatty acid development in whole frozen fillets from five species of gadoid fish, held at −5 C.

the fish into two groups: Only those belonging to the gadoid or cod family produced DMA. The bottom line in the figure, labelled "NGS," shows the zero values from all the nongadoid species, including flounder, halibut, catfish, ocean perch, mackerel, lobster, shrimp and scallops.

Figure 1 also shows the considerable difference in the amounts of DMA produced by different species of gadoids. Haddock produced the least; increasingly larger amounts were found in cod, pollock, cusk and hake. The free fatty acid (FFA) content of this same gadoid group shows the same but inverted order of species (Fig. 2). Those which produced the most DMA accumulated the least amounts of FFA.

There was a similar species order for the decrease in extractable protein that took place in the muscle (Fig. 3). In this case the species producing the most DMA had the greatest and most rapid decrease in extractable protein.

Are these relationships in the production of DMA, FFA and the decrease in extractable protein mere coincidence? This is scarcely the case. The picture becomes a little clearer when the DMA values for each individual fillet are plotted against their corresponding EPN and FFA values (Fig. 4 and 5). There is almost a linear relationship between increase in DMA and decrease in EPN, but only to the point where 70% to 80% of the extractable protein has been eliminated. (This corresponds roughly to the

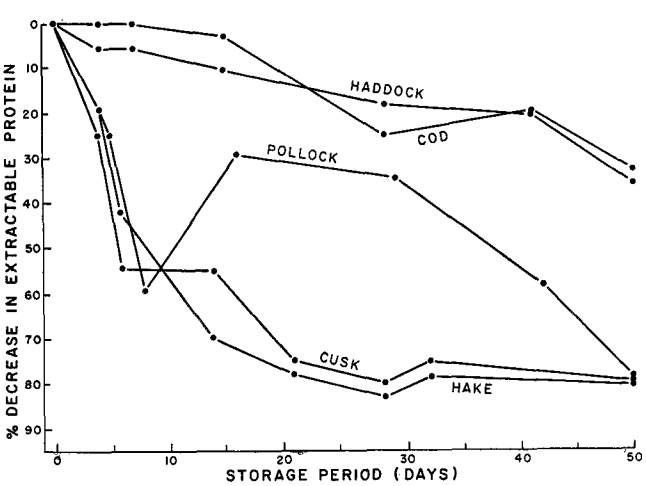

FIG. 3. Decreases in extractable protein in whole fillets stored at −5 C. These values are from the same samples used to get the dimethylamine and free fatty acid values shown in Figures 1 and 2.

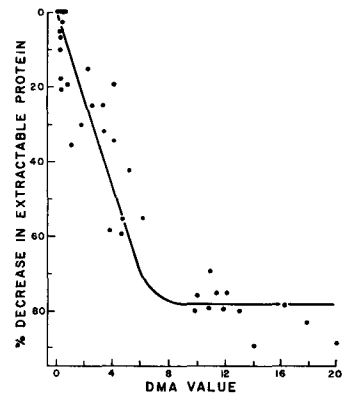

FIG. 4. Extractable protein values plotted against the corresponding dimethylamine values obtained from the storage experiments with the five gadoid species.

amount of actomyosin in the muscle.) This same relationship between increased DMA and decreased EPN occurred, regardless of species (within the gadoid group) and regardless of storage temperature and other factors that could be varied.

There was no apparent correlation between DMA and FFA where the values from all five species were bunched together (Fig. 5A). It becomes a little more meaningful when all the points from each individual species are identified. This has been done by arbitrarily joining the points for each species (Fig. 5B). It shows again that those which produce the least DMA either produce or accumulate the most FFA.

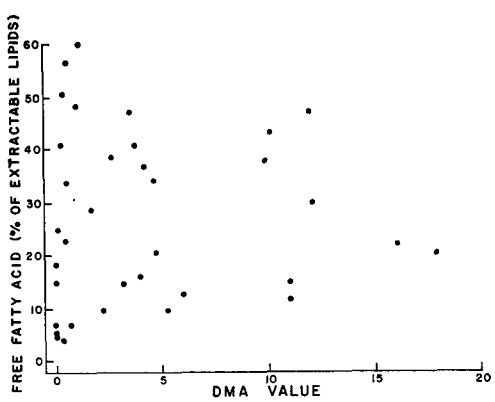

FIG. 5A. Showing the absence of any apparent close correlation between free fatty acid values and dimethylamine values when the data from all five gadoid species are taken together.

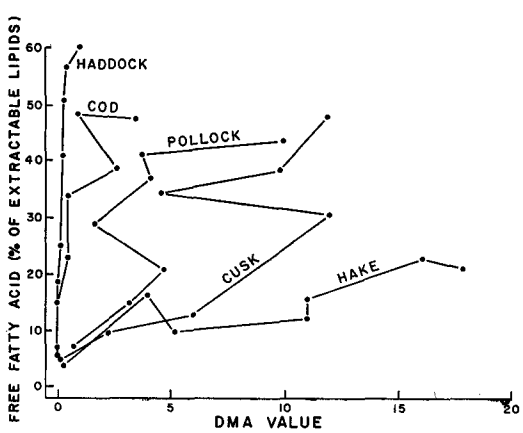

FIG. 5B. The same data shown in Figure 5A, but the points for each individual species have arbitrarily connected to facilitate seeing the results.

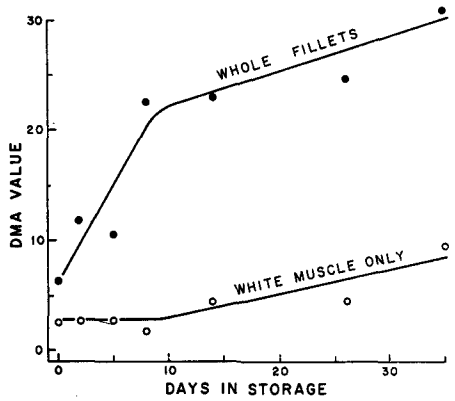

FIG. 6. Effect on the dimethylamine values of removing the dark lateral muscle from hake fillets before freezing and storage.

(It also demonstrates rather clearly that FFA, per se, are not directly responsible for reduced EPN.)

Significance of the Dark Lateral Muscle on Chemical Changes in Fillets During Storage

During these experiments it was observed that hake fillets, which produced the most DMA, had the largest proportion of dark muscle; and that for the gadoids as a group, there appeared to be a close correlation between the amounts of DMA formed and the amounts of dark muscle in the fillets. When this dark muscle was cut away from the fillets before freezing there was a marked reduction in DMA formation during subsequent storage (Fig. 6). But of more interest were the effects of excising the dark muscle on the changes in FFA and EPN. In the stored fillets freed from dark muscle, the decrease in extractable protein was either completely inhibited or greatly retarded (Fig. 7), and the FFA continued to increase to values much beyond those of the corresponding whole fillets. Some of the activity lost by removing the dark muscle was restored by addition of ferrous ion.

Formaldehyde as a Cause of Decreased EPN

In spite of the close correlation between the increase in DMA and the decrease in extractable protein, there is no reason to believe that it is a causative relationship. The direct addition of DMA to muscle does not cause a reduction in EPN, and the fact that DMA continues to increase in the fish while the extractable protein is decreasing further suggests that there is no direct reaction between these compounds. However, the formation of DMA is accompanied by an equivalent amount of FA. Because of its highly reactive nature, it is difficult to measure exact amounts

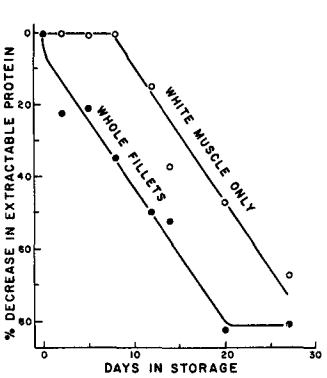

FIG. 7. Extractable protein values for stored fillets with and without the dark lateral muscle.

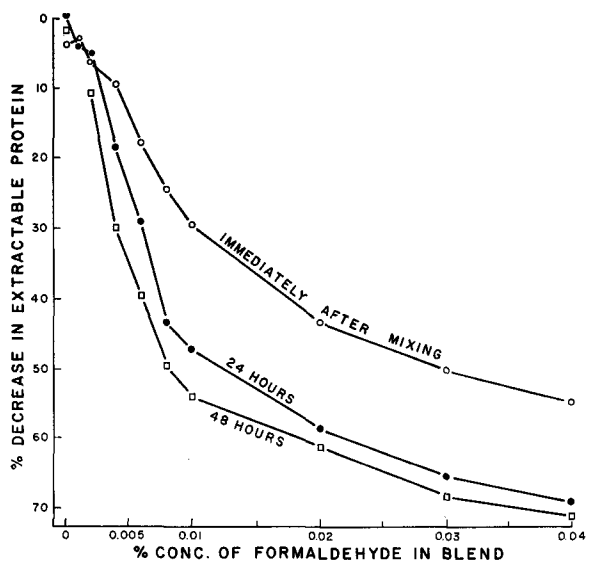

FIG. 8. Effect on the extractable protein content of blended cod muscle by adding formaldehyde to give a range of 0.001% to 0.04% in the blend. Determinations were made immediately after adding the formaldehyde and again after 24 and 48 hr storage at 0 C.

of formaldehyde when it is formed in complex organic materials, such as muscle tissue. In measurements made by Amano and Yamada (12) on changes occurring in cod muscle held at 1 to 4 C, it was found that while the DMA continued to increase with storage, the formaldehyde, after an initial increase, dropped again to relatively low values.

In our storage experigents with the gadoids the DMA values ranged between 1 and 60 mg/100 g, or 0.001% to 0.06%. Calculated on the basis of a mole of FA being formed for each mole of DMA, this gives a range of FA concentrations between 0.00068% to 0.048% in the muscle. When FA was added directly to blended cod muscle to produce concentrations within this range, very appreciable decreases in EPN were obtained (Fig. 8). When unsaturated fatty acid was added along with the formaldehyde the decrease in EPN was still greater.

Various suggestions have been proposed to explain the mechanism which brings about the insolubilization of fish proteins during frozen storage. These usually include some type of reaction between proteins and FFA's (21–23), or oxidizing fatty acids (24), or free radicals produced from oxidizing fatty acids (25), with the production of highly insoluble lipid-protein polymers. It would appear from our results, that at least with the gadoid species, consideration must also be given to the added effect of FA, and particularly formaldehyde in conjunction with unsaturated fatty acids.

Considering the complexity of fish muscle, and the variations in the chemical composition of different species of fish, it is probable that more than one mechanism may be involved in the insolubilization of fish muscle proteins; or that the mechanism may be triggered by a number of different factors. It should be pointed out that reduction in extractable protein takes place in frozen muscle of many non-gadoid species, which do not produce DMA and FA. But the relative amounts of protein change are much less with these species.

This discussion of lipid oxidation in fish muscle was started by asking why the highly unsaturated lipids in lean fish muscle apparently do not oxidize

during frozen storage. We must conclude by saying that these lipids are oxidized, but instead of forming the carboxyls and other compounds that we associate with rancidity, they are bound up in insoluble lipid-protein complexes. From the standpoint of monitoring these changes in the gadoid fish during frozen storage, the incidental production of DMA possesses some very interesting possibilities.

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