BACTERIAL AND AUTOLYTIC CHANGES IN WATER RETENTION PROPERTIES AND DEGRADATION OF ADENOSINE NUCLEOTIDES IN COD MINCE

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John H. Muyonga

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ABSTRACT

This study was conducted to determine the role of bacteria and endogenous enzymes in changes in water retention properties and degradation of adenosine nucleotides in Atlantic cod. In mince, unlike whole fish, bacteria are distributed throughout the fish and are in contact with the internal substrates. Therefore, their potential to participate in deterioration may be better expressed.

NaOCl (150 ppm of OCl^-) was added to destroy bacteria. The same amount of deionised and distilled water was added to the normally spoiling samples. Standard plate counts were used to determine the bacterial kill achieved by the NaOCl treatment. All samples were packaged in sterile polythene bags and were stored on ice/ice salt mixtures (1 and 1.6%) in insulated chests which were held in a cold room. The concentration of trimethylamine (TMA), and the nucleotide catabolites, hypoxanthine (Hx) and inosine (HxR), the pH of tissue homogenates, the water uptake ability (WUA), and the expressible moisture (EM) were determined after 2, 5, 9, and 14 days.

NaOCl treatment reduced the bacterial load of the mince by about 99%. The level of TMA in all samples was extremely low (<0.02 TMA-N mg/100g) until day 9 when the level had increased significantly in the normally spoiling samples. The TMA levels in the NaOCl treated samples did not increase significantly throughout the 14 days of storage at -3°C but had increased significantly ($p<0.05$) by day 14 in mince at 0 and -2°C. The level of TMA was significantly ($P<0.05$) higher in normally spoiling mince than in NaOCl treated samples at all three storage temperatures. The pH for both normally spoiling mince and the treated samples increased during storage regardless of the storage temperature. The increase in pH tended to

be higher in spoiling mince stored at 0°C as compared to the NaOCl treated samples, but there was no significant difference in the pH of normally spoiling mince and NaOCl treated samples stored at -2 and -3°C. The level of Hx + HxR increased until day 9. The increase was most rapid at 0°C, less at -2°C and least at -3°C. Normally spoiling mince had significantly higher levels of Hx + HxR at days 5 and 9. At day 14, however, all samples except NaOCl treated samples stored at -3°C had lower levels of Hx + HxR than the levels in the same samples at day 9 and NaOCl treated samples had significantly higher levels than normally spoiling samples at all three storage temperatures.

WUA for normally spoiling mince increased with time at all storage temperatures while no change in WUA was observed in the NaOCl treated samples. Treated samples also had lower EM. The EM for normally spoiling samples were higher and increased with time.

A second set of experiments was conducted at 0°C with a lower level of NaOCl (25 ppm ClO^-) applied to all samples. Treatment samples were inoculated with bacteria recovered from the gills of gutted fish while the controls were stored without inoculation. The bacterial count for the controls was about 5% of the count for the inoculated mince. The Hx + HxR concentration increased during storage and the increase was higher in inoculated mince. WUA for inoculated samples increased during storage while that for controls remained approximately constant. EM for inoculated samples decreased during storage while that for the controls increased. This study showed that bacteria are capable of causing changes in chilled fish even within the first week of storage.

BIOGRAPHICAL SKETCH

John Herbert Muyonga was born in Mbale, Uganda, on June 24 1969. He graduated with a B. Sc. in Food Science and Technology from Makerere University, in Kampala, Uganda in June 1993. After graduation, he worked as a research assistant and assisted in the teaching of undergraduate courses in the Department of Food Science at Makerere.

In the fall of 1994, he accepted funding from the Uganda National Agricultural Research Organization (NARO) to pursue an M. S. at Cornell University. He completed his M. S. in Food Science, with a minor in Agricultural Economics, in August, 1996.

The author is a member of the Institute of Food Technologists.

DEDICATION

Dedicated to my parents Birah and Sam Muyonga who gave up plenty for my sake and to Faith for her friendship

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INTRODUCTION

Successful marketing of fishery products is to a large extent dependent on delivery of high quality product. This requires development of reliable quality evaluation criteria and prevention of processes that lead to quality loss. An understanding of the factors that cause perceived loss in quality is a valuable step towards prevention of deterioration and to the development of quality evaluation systems.

The deterioration of fish during chilled storage is a complex process resulting from the composite activities of autolysis, spontaneous chemical reactions, bacterial attack and leaching by ice melt water (Jones et al., 1964). While some changes, such as the production of volatile sulfides (Herbert and Shewan, 1975), can be attributed to just a single one of these processes, many including nucleotide degradation, proteolysis and changes in protein functional properties have been attributed to both endogenous enzymes and bacterial activity.

The level of various nucleotide catabolites is one of the most useful characteristics employed for measuring freshness of fish. In live animals, the energy rich compound adenosine triphosphate (ATP), which is broken down to adenosine diphosphate (ADP) to provide energy, gets regenerated through the process of rephosphorylation, during respiration. After death, however, glucose gets depleted and ATP cannot be regenerated. Instead, ADP is progressively broken down to adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine (HxR) and hypoxanthine (Hx). The levels of the nucleotides HxR and Hx as a measure of freshness in fish, usually in combination as the K-value: the ratio of Hx and HxR to total nucleotides

has been widely used. The level and rate of accumulation of Hx and HxR varies with species. It is believed that microorganisms do not play a role in the nucleotide catabolic process during the useful storage life of fish (Fletcher and Hodgson, 1988; Fletcher and Statham, 1988). Some authors, however, have reported correlations between bacterial counts and nucleotide catabolites (Handumrongkul and Silva, 1994; Toledo-Flores, 1988).

Water retention properties have also been used to assess the quality of both fresh and frozen meat. During storage at around 0°C, meat proteins are known to undergo proteolysis leading to an increase in water retention by the meat system. Water retention properties have, therefore, been used to determine the microbial quality of meat.

The aim of this study was to investigate the role of bacteria and autolytic enzymes in the production of the adenosine nucleotide catabolites Hx and HxR and in the changes in water soluble protein and water retention properties during chilled and superchilled storage. Atlantic cod, one of the most important commercial fish species, was used. NaOCl was used as an antimicrobial and samples were stored at -3, -2, and 0°C.

LITERATURE REVIEW

Atlantic Cod (*Gadus morhua*)

Atlantic cod is a ground (demersal) fish, i.e., it lives close to the bottom of the ocean. The cod family includes the most important commercial fish species world wide and is only second to the herring family in world catch volumes. In 1987 alone, 30 billion pounds of fish belonging to the cod family was landed worldwide.

In a side view, the Atlantic cod is oval with three distinct dorsal fins and two anal fins (Figure 2.1). They average 3 ft long and weigh 10-25 Lb (Flick et al., 1990).

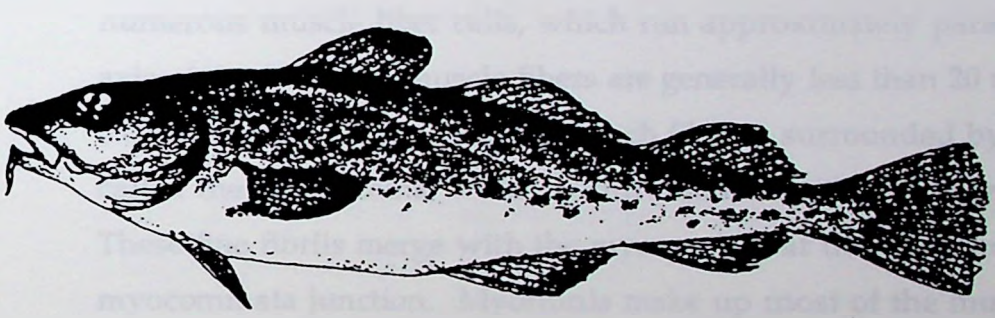


Figure 2. 1. **Side view of Atlantic cod** (*Adopted from Flick et al., 1990*).

The Muscle of Marine Fish

Fish muscles just beneath the skin contain a high level of myoglobin which gives them a dark color. The large lateral muscles, which are the major component of fish fillets, lie beneath the subcutaneous dark muscles. These muscles are whitish because they are low in myoglobin. The dark muscles contain chromoproteins and two to five times more lipid than the white muscles.

Fish muscle is divided by thin connective membranes known as the myocommata, creating segments called myotomes. The thickness of the myocommata depends on the species, age and/or length of the fish, and on the nutritional status of the fish. The number of myotomes corresponds to the number of vertebrae in the backbone. Each myotome is composed of numerous muscle fiber cells, which run approximately parallel to the long axis of the fish. The muscle fibers are generally less than 20 mm long and 0.02 mm to 1.0 mm in diameter. Each fiber is surrounded by a membrane called the sarcolemma, which is surrounded by thin collagenous fibrils. These fine fibrils merge with the mycommata at the myotome-myocommata junction. Myofibrils make up most of the muscle cell volume. Each myofibril is 5μm in diameter and runs parallel to the long axis of the fiber (Figure 2.2). The myofibrils are segmented into sarcomeres, which are composed of thin and thick myofilaments and are bordered by Z-lines. The movement of the myofibrils form the basis for muscle contraction and post mortem stiffening of the body (Sikorski et al., 1990).

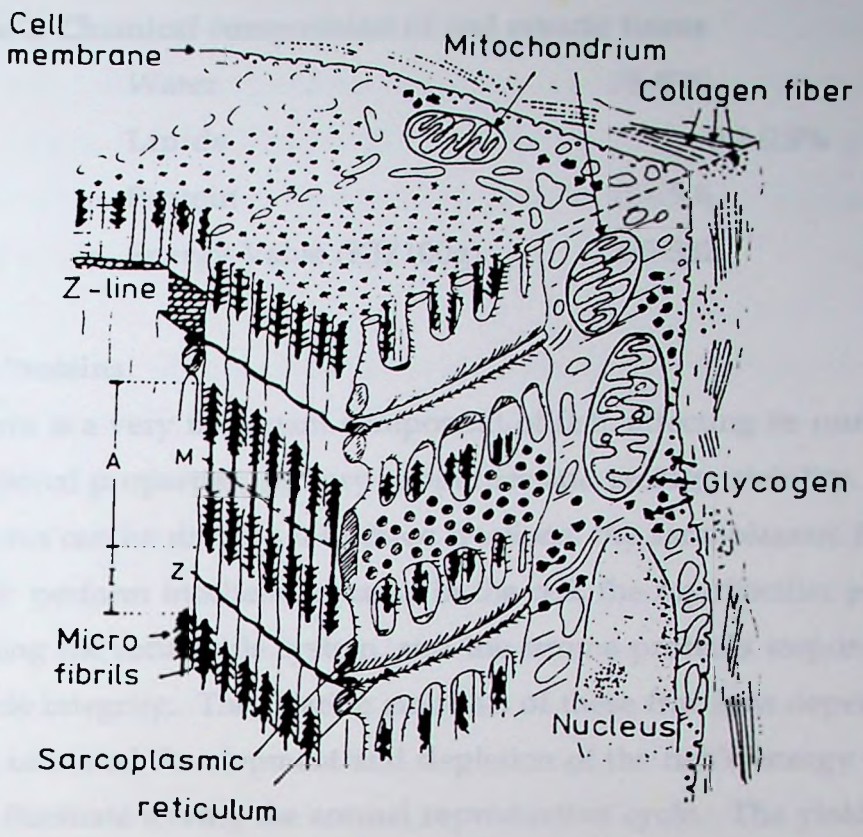


Figure 2.2. **Schematic diagram of the ultrastructure of cod muscle fiber** (*Adopted from Sikorski et al., 1990*).

Chemical Composition

The chemical composition of fish varies with species, age, sex, feed intake and location of the catch. Depending on the fat content, fish are divided into lean (<5% fat in edible portion) and fatty (>5% fat in the edible portion) species. Cod is a lean species and Table 1 gives its composition (Huss, 1988).

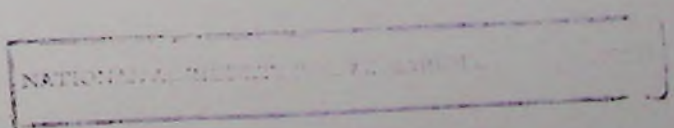
Table 1. **Chemical composition of cod muscle tissue**

Water	78-83%
Lipids	0.1-0.9%
Protein	15-19%
Energy Value (KJ/100g)	295-332

Fish Proteins

Protein is a very important component of fish, affecting its nutritive value, functional properties, sensory properties and storage stability. The muscle proteins can be divided into three fractions: the sarcoplasmic fraction which perform biochemical tasks in the cell, the myofibrillar proteins forming the contractile system, and the stroma proteins responsible for muscle integrity. The relative amounts of these fractions depend upon the level of sexual development and depletion of the fish's energy reserves and may fluctuate during the annual reproductive cycle. The yield of these fractions is affected by the conditions of extraction, mainly the grinding, mixing and centrifugation procedures, the pH, salt concentration, and dilution, as well as the degree of denaturation due to storage and processing.

Myofibrillar proteins represent 60-75% of the total muscle protein and consist of organized arrays of thick and thin filaments. They can be extracted from a comminuted fish homogenate with neutral salt solutions of ionic strength above 0.15, usually ranging from 0.3-1.0. The myofibrillar proteins participate in the post mortem stiffening of fish tissue (*rigor mortis*) and are also responsible for the water retention properties of the fish, the characteristic texture of fish products, as well as for the functional properties of fish minces and homogenates. Myosin accounts for 50-58% of the myofibrillar fraction and is the major protein in the thick filaments.



Skeletal muscle myosin has a MW of ~480,000 and is composed of two large subunits called heavy chains (MW~200,000) and four small subunits called light chains (MW 16,000-27,500). The two heavy chains form the rod portion and a large part of the myosin head. Two light chains are located in each of the myosin heads (Betchel, 1986).

C protein and M-line proteins are also part of the thick filament. C protein (MW~140,000) binds to myosin at low ionic strengths (Moos et al., 1975). The function of C proteins is not well understood (Callaway and Bechtel, 1981). The M-line (Figure 2.2) of skeletal muscle is where the enzyme creatine kinase and M-line protein are located. Creatine kinase is believed to have two roles, one as a structural component of the M-line and another as the enzyme involved in the reaction that forms ATP and creatinine from creatine phosphate and ADP. The other protein associated with the M-line has a MW of 165,000 and binds to both myosin and creatine kinase. Its function, however, is not well understood (Bechtel, 1986).

The thin filaments are composed of actin (MW 42,000) which accounts for 15-29% of the total myofibrillar proteins. Actin exists in the monomeric (G-actin) and the polymerized (F-actin) form (Sikorski et al., 1990). The polymerization process requires ATP. F-actin forms the backbone of the thin filament and also provides binding sites for troponin and tropomyosin. One end of the F-actin is bound to the Z-line. When calcium is present in muscle, F-actin comes in contact with the myosin heads of the thick filaments and there is a rapid breakdown of ATP, ultimately resulting in muscle contraction (Bechtel, 1986).

Post slaughter muscle contains actomyosin which is a complex of actin and myosin. Other components of the myofibrillar fraction include

tropomyosin, troponin, actinin, nebulin, and titin. These make up about 10% of the total myofibrillar proteins (Sikorski et al., 1990). Tropomyosin and troponin are both components of the thin filaments. Actinin is a component of the Z-line. The Z-line provides a framework for connecting the thin filaments of the myofibrils and maintains the correct spacing between these filaments. Actinin is thought to be involved in the anchoring of the thin filaments into the Z-line. Titin proteins are thought to be located throughout the sarcomere, which is the distance from Z-line to the next Z-line. Titin proteins are elastic and may be responsible for the elastic properties of skeletal muscle (Bechtel, 1986).

Sarcoplasmic proteins constitute about 30% of the total fish proteins and 90-95% of the protein in a fish muscle extract with water or solutions of neutral salts with an ionic strength below 0.15 . This fraction is made up of over 100 proteins with a range of molecular weights and isoelectric points. They include enzymes, glycoproteins, nucleoproteins, the components of lipoproteins, as well as the chromoproteins of muscle and blood. Enzymes of the glycolytic pathway and the hydrolytic enzymes of the lysozymes are known to influence the quality of fish as food. Proteinases, oligopeptidases, di- and tripeptidases, adenine dinucleotidase, adenosine triphosphatase, adenine deaminase, and trimethylamine oxide (TMAO) demethylase are examples of enzymes that have been found in fish that may affect its quality (Sikorski et al., 1990).

The stroma or connective tissue proteins fraction is insoluble in dilute solutions of HCl or NaOH. This fraction, which is composed of collagen, elastin, reticulin and denatured myofibrillar and possibly sarcoplasmic proteins, accounts for a mere 3% of the total muscle protein. The low

content of connective tissue is the reason for the tenderness of fish (Sikorski et al., 1990).

Protein Hydration

The hydration of proteins is responsible for the rheological properties and juiciness of muscle foods. The muscle of fish contain from about 50 to about 85% water. Water retention by fish depends largely on the interaction between water and proteins. The hydrophilic amino acid residues participate in hydrogen bonding with water molecules, while hydrophobic groups induce layers of highly ordered water structures around themselves (Sikorski et al., 1990).

Myofibrillar proteins are primarily responsible for the holding of water in meat tissue. About 4-5% of the total water in muscle is tightly bound to muscle proteins and is hardly affected by changes in protein structure and charge. This portion of the water is called the true hydration water. The remaining water, called free water is strongly influenced by the spatial structure of the muscle proteins. Water that can be eliminated from muscle tissue by the application of force is a part of the free water and is referred to as loose water, while the portion of free water that is not removed by the application of force is called immobilized water (Hamm, 1960).

The true hydration water participates in hydrogen bonding between polar groups of the protein and is involved in stabilizing the native structure of protein. It is bound by hydrophilic groups of the side chains of the protein, such as the carboxyl, amino, hydroxyl and sulfhydryl groups, as well as the nonionized carboxyl and imido groups of peptide chains. This water is unavailable for chemical reactions and is unfreezable at -40C (Kinsella, 1981).

About 85% of the water in muscle tissue is present within the myofibrils. Many attempts to explain the loss and gain of water by muscle tissue are based on changes in myofibrillar volume (Hamm, 1960; Offer and Trinick, 1983; Offer and Knight, 1988). Offer and Knight (1988) suggested that water holding of the muscle results from changes in the distance between neighboring filaments and not just the myofibrillar volume.

Cross-bridges between thin and thick filaments, as well as the M- and Z-lines may act as a constraint to the enlargement of the interfilament space and therefore deter increases in myofibrillar volume. Disruption of cross-bridges and M- and Z-lines leads to an increase in meat hydration by reducing constraints to the enlargement of the interfillament space. According to Betchel (personal communication) only a limited amount of water can be absorbed by a meat system without change in myofibrillar volume. However, significant changes may occur in the ability of such meat systems to retain water. Changes in the water retention properties of meat systems without changes in volume is largely due to changes in protein charges.

Measurement of Water Retention Properties

Two distinct methods have been applied to measure the water retention properties of meats: Measuring the amount of water liberated by meat tissue under specified conditions and measuring the amount of water that is absorbed under specified conditions by meat tissue placed in excess water (Hamm, 1960).

Hamm (1986) coined terms to describe water retention properties of meat tissue depending on the conditions applied to the meat. The amount of

water lost by meat tissue may be referred to as drip loss (no external force), thawing loss (thawing, without external force), cooking loss (heating, with or without application of external force), expressible juice (external force applied to thawed or unfrozen tissue, no heating). Regenstein (1984) proposed the term water binding potential (water uptake ability) as a measure of the amount of water that a system can hold in the presence of an excess of water and with application of an external force. He indicated that this property is anion dependent while expressible moisture (EM) or expressible juice is cation dependent. The water uptake ability (WUA) is expressed in mL of water per mg of protein in the pellet. It is therefore a measure of the uptake ability of the water insoluble proteins.

Factors Affecting the Hydration Capacity of Proteins

The pH of meat, the presence of neutral salts, salts of weak acids and bases, processing as well as the fat content of meat influence its water retention (Hamm, 1960).

An increase in pH increases the negative charges on the thick filaments causing repulsion between the filaments and expansion of the filament lattice. According to Offer and Knight (1988), meat retains the least water at about pH 5.0, close to the mean isoelectric pH of the major myofibrillar proteins and the further the pH is from 5.0 on either side, the more water is retained. Wismer-Perdersen (1987) reviewed the effect of constraints to myofibrillar volume increase. When meat structure is not disturbed by comminution, increases in myofibrillar volume are constrained by the connective tissue fibers and membranes which surround the muscle fibers. The effect of increasing pH on water uptake is therefore less significant in intact than in comminuted tissue.

When a neutral salt such as NaCl is added to a meat system at a pH greater than the isoelectric point, the Cl^- are preferentially adsorbed to the positive groups of myosin or actomyosin. As a result, the electrostatic repulsion between adjacent protein molecules, and consequently the interfilament space is increased and more water is retained. On the acidic side, however, the protein has excessive positive charges which repel each other. When NaCl is added, the repulsion is reduced due to the adsorption of anions to the protein. As a result, the protein structure is tightened and less water is retained (Hamm, 1960). The effect of NaCl on hydration increases with concentration up to an ionic strength of 1.0, above which hydration drops. The drop may be due to reduction in the repelling activity of carboxyl groups of myosin by the effect of cations and/or salting out (Hamm, 1986).

Salts of weak acids, such as phosphates, have been shown to increase the water retention of meats (Sherman, 1961; Hellendoorn, 1966; Chang, 1996). Hamm (1971) summarized the effects caused by phosphates that lead to improved water retention properties as: increase in pH, dissociation of actomyosin, increase in ionic strength, and sequestering of metal ions.

Hamm (1986) indicated that divalent cations decrease water retention of meats. Binding of cations such as Ca^{2+} reduces the electrostatic repulsion between the negatively charged peptide chains and leads to tightening of the protein structure. The removal of divalent cations, e.g., by cation exchange or chelating agents leads to improvement in water retention properties of meats.

Ragnarsson (1987) observed that addition of sodium acetate (NaAc), Na_2SO_4 and NaCl prior to freezing fish mince leads to a decrease in toughness and EM while NaI, NaSCN, $CaCl_2$ and $MgCl_2$ were found to increase toughness and EM. Using SDS gel electrophoresis, he showed that protein crosslinking occurred in cod and whiting during storage at -7°C. Addition of NaSCN prior to freezing was found to eliminate the crosslinking.

The effect of fat content on water retention was reported by Miller et al. (1968). They indicated that EM increased when fat content was increased. Froning and Norman (1966) compared the cooking losses of white meat and dark meat and found that the dark meat had a higher cooking loss. They attributed this to the lower ratio of protein to water in the dark meat.

Gill et al. (1979) studied changes in texture of red hake and haddock muscle stored at -5 and -17°C. At -5°C, muscle from both species was found to harden over time. Textural deterioration observed in red hake was far greater than that observed in haddock. SDS gel electrophoresis revealed that covalent crosslinking between troponin and myosin occured in hake but not in haddock. The higher level of textural deterioration in hake was attributed to production of formaldehyde by the TMAO-ase enzyme . This enzyme is lacking in haddock. The textural deterioration in haddock was thought to result from secondary bonds such as hydrogen and electrostatic.

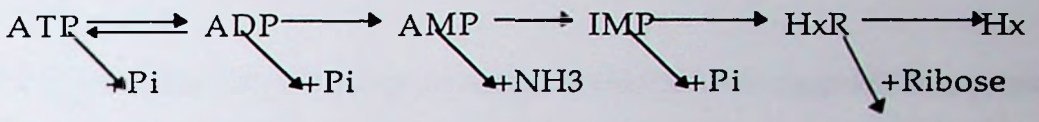
Biochemical Changes in Fresh Fish

At death, the normal metabolic control of fish breaks down and the supply of oxygen stops. Breakdown of glycogen proceeds anaerobically by glcolysis, leading to the accumulation of lactic acid and a decrease in pH. The ultimate pH (lowest pH achieved) is dependent on the level of glycogen at death and on the species. For cod, the pH decrease is usually

from about 7.0 to 6.3-6.9. This post mortem decrease in pH leads to a decrease in water binding properties of the tissue proteins.

Changes in Adenine Nucleotides

ATP is broken down by a series of dephosphorylation and deamination reactions to IMP which, in turn, is degraded to Hx and ribose.



The speed of each of the above autolytic changes varies with species (Spinelli, 1965).

Factors Affecting Nucleotide Degradation

Greene et al. (1990) reviewed the nucleotide catabolites accumulated by a number of species. Flatfish generally accumulate Hx with the exception of halibut, which like salmon and a number of tropical fish accumulate HxR, while the Japanese red and black bream, Atlantic pollock, and haddock accumulate IMP.

The maximum concentration of Hx that can be reached during storage varies with species (Jones et al., 1964). Greene et al. (1990) indicated that salmon, Pacific cod, Pacific pollock and Atlantic cod are slow to form Hx, averaging about 0.5 µmol Hx/g by day 3 of storage on ice. The same authors studied a number of flatfish species and reported that rex sole showed a gradual formation of HxR and a slow degradation of IMP, a characteristic associated with many fish popular for sashimi, such as tuna, mackerel, yellowtail, and bream. Jhaveri et al. (1982) reported that the Hx

concentration in ice stored mackerel increase until day 16 although the rate was slower after day 10. After day 16, Hx concentration was found to decline. Jacober and Rand (1982) reviewed the pattern of changes in concentration of Hx and diamine development in fish, showing that a decline in Hx concentration occurs at the time when diamines begin to accumulate. They suggested the use of these two methods together in assessing fish quality.

The potential of using accumulation of nucleotide degradation products as a measure of loss of seafood freshness has been widely studied. Breakdown of adenine nucleotides is facilitated first by endogenous enzymes and later by microorganisms (Fletcher and Statham, 1988). Controversy, however, still exists on the role of microbial degradation on the loss of quality within the useful life of seafood. Fletcher and Hodgson (1988) compared the level of Hx and HxR in sterile and naturally spoiling snapper fillets at 0°C and found no difference in the two groups for the 15 days of storage, a period sufficiently long for the fish to become unacceptable. Fletcher and Statham (1988) also found that sterile fish stored at 4°C rapidly accumulated Hx and developed unpleasant flavors and odors, becoming unacceptable after 4 - 7 days. They reported a high correlation between Hx concentration and panel scores for off-flavor (r^2=0.93).

Toledo-Flores (1988) reported that application of 5% potassium sorbate had no effect on the K-values for silk and cardinal snapper stored at 0, -2, and -3°C, but significantly increased the time for the K-value to reach 20% in queen snapper at the same temperatures. Boyle et al. (1991) compared K-values and Hx concentration in whitefish fillets stored in CO_2 modified atmospheres and those stored in air and found no difference in their K-values. Hx concentrations, however, were lower in fillets stored in the

modified atmosphere. Samples dipped in 5% potassium sorbate prior to storage had lower Hx accumulation rates than those that were not dipped for both aerobic and modified CO_2 storage. Handumronakul and Silva (1994) reported a high correlation between Hx concentration and the aerobic plate counts for striped bass. No cause effect relation, however, was established.

Williams et al. (1993) studied the effect of low dose irradiation on the K-value and Hx concentration in fillets from five species. Of the species studied, sea perch irradiated with 1 KGy and 3 KGy had lower K-values and Hx concentrations than unirradiated muscle through 9 days of storage. Fillets of mullet, sweetlip and king snapper irradiated with 1 KGy and 3 KGy were also found to have lower K-values than controls except for king snapper irradiated with 3 KGy, which had a significantly higher K-value than the control. Irradiation, however, was reported to have no significant effect on the K-value of barramundi fillets at either of the two doses. It was also noted that barramundi had lower initial K-value compared to the other species.

Vieciana-Nogues et al. (1990) reported correlation between Hx concentration and the biogenic amines histamine (r^2=0.88) and tyramine (r^2=0.86) for anchovy stored at refrigeration (4-6°C) and room temperatures (18-22°C). These biogenic amines were also found to correlate with trimethylamine (TMA) concentration, volatile base nitrogen (VBN) and pH. These characteristics were all found to correlate with length of storage ($p<0.0001$).

Jones et al. (1964) alluded to species difference in the accumulation of hypoxanthine. They reported that the Hx concentration in cod rose

relatively slowly during early chill storage but more sharply with the onset of bacterial attack. In lemon sole, they reported a rapid change in flavor score with an initial increase in Hx concentration. In both cases, Hx was found to be a good predictor of odor.

Toledo-Flores (1988) found that cardinal snapper stored at -2 and -3°C required 8 days to reach a K-value of 20%, while at -3°C, the K-value for silk snapper increased to exceed 20% within five days. He reviewed studies showing extended storage life in rainbow trout and mackerel. Storage at -3°C increased the time to reach a K-value of 20% to 10 days instead of one day in iced cultured rainbow trout (Uchiyama et al., 1978) and from 4 days to 7 days in mackerel (Aleman et al., 1982).

Vieciana-Nogues et al. (1990) found a close to linear increase (r^2>0.95) in Hx with time of storage in anchovy stored under refrigeration and at room temperature for 140 hours. Damoglou (1980) reported a linear increase of Hx in herring for up to 20 days at 0°C and up to 10 days at 5 and 10°C.

Nucleotide degradation has also been shown to affect other processes. Hultin (1992) reviewed the interaction between the nucleotide breakdown system and the initiation of lipid oxidation. Under anaerobic conditions, the enzyme xanthine dehydrogenase is converted into xanthine oxidase through a process believed to be mediated by oxidation of sulfhydryl groups or by proteolysis. The two enzymes produce different products from dehydrogenation of Hx. Xanthine dehydrogenase transfers electrons from Hx to NAD+ while xanthine oxidase transfers them to molecular oxygen.

The xanthine oxidase catalyzed reaction produces superoxide and hydrogen peroxide, which are believed to be initiators of lipid oxidation.

Organoleptic Properties of Adenosine Nucleotides

The organoleptic importance of the autolytic degradation of ATP is only partially understood. IMP and other 5'-nucleotides function as strong flavor enhancers in quite low concentrations, and together with glutamic acid, they give rise to a "meaty flavor." HxR is said to be more or less flavorless, while Hx has been reported to impart a bitter flavor to spoiling fish (Spinelli, 1965).

Changes in Proteins

The autolytic changes in the proteins are less pronounced than the changes in nucleotides. Many proteases have been isolated from muscle tissue (Reddi et al., 1972). Wojtowicz and Odense (Huss, 1988) showed that the main proteases in fish muscle, the cathepsins, have low activity and proposed that the rapid autolytic rate of many fish cannot be attributed to these enzymes.

Enzymes of the digestive tract play an important role in the autolysis of uneviscerated fish. Fish caught during heavy feeding periods are more susceptible to tissue degradation. This has been attributed to the low post mortem tissue pH, which leads to weakening of tissue (Love, 1980). According to Granroth et al. (Huss, 1988), the most important digestive proteases include tryspin-like endopeptidases, cathepsin, and other pepsin-like enzymes. These enzymes split proteins into large peptides which can be degraded further by exopeptidases.

Bacteriological Changes

The interior of healthy fish tissue before slaughter is essentially sterile and bacterial invasion occurs at a rather late stage (Shewan and Murray, 1979). However, the skin, gills, and intestines contain 10^2-10^7 cfu/cm^2, 10^3-10^9 cfu/g, and 10^3-10^9 cfu/g, respectively. Fish from clean environments tend to have lower counts (Shewan, 1962). The microbial flora on newly caught fish in temperate waters is dominated by aerobic or facultative psychtrophic, gram negative rods of the genera *Pseudomonas, Alteromonas, Moraxella, Acinetobacter, Flavobacterium, Cytophaga* and *Vibrio,* while fish from tropical waters are dominated by gram positive bacteria such as *Micrococcus, Bacillus and Coryneforms* (Shewan, 1977).

Huss (1988) reviewed the quantitative and qualitative changes that occur in the bacterial flora of fish during storage. These changes are greatly influenced by storage conditions such as temperature, gaseous environment, and presence of chemical preservatives. During storage of marine fish at low temperature, *Pseudomonas* and *Alteromonas* become the dominant genera regardless of the composition of the initial flora because the generation time for these genera at low temperature is short. The total number of bacteria reaches 10^8-10^9 cfu/g when spoilage is apparent. Under anaerobic conditions, counts of about 10^6 cfu/g are reached, and facultatives dominate. Only a small percentage of the total microflora is capable of causing spoilage. Spoilage microorganisms include *Alteromonas putrefaciens* and certain *Pseudomonas, Vibrio* and *Aeromonas spp.*

Carbohydrates, nucleotide fragments and the rest of the non-protein nitrogen fraction including TMAO are readily available substrates for bacteria. The growth of aerobic organisms results in the formation of partly

anaerobic microclimates on the surface of the fish. Under these conditions, facultatively anaerobic bacteria such as *Proteus* are favored. A number of TMAO reducing bacteria such as *Alteromonas*, although classified as obligate aerobes, obtain energy by reducing TMAO. In sterile cod muscle, TMAO is not reduced. However, the digestive system and kidney tissue seem to contain a TMAO reductase, which may play a role in minced products. The end product, TMA, has a typical unpleasant "fishy" odor. Trimethylamine (TMA) constitutes a large portion of the total volatile bases (TVB) when the TVB reaches the rejection level for cold stored fish.

Generally many odors connected with spoilage are the degradation products of amino acids. The bacterial breakdown of the sulfur containing amino acids, cysteine and methionine, results in the formation of H_2S, methylmercaptan, and dimethylsulfide. These volatiles are organoleptically detectable at levels as low as a few parts per billion.

Herbert and Shewan (1975) investigated the role of autolytic and bacterial enzymes in the production of volatile H_2S, methylmercaptan and dimethylsulfide in chilled North Sea cod and concluded that these volatile sulfides arise from microbial breakdown of the amino acids cystine, cysteine and methionine. All of the thirteen strains of *Pseudomonas* studied were found to produce H_2S and methylmercaptan. Six strains were found to produce dimethylsulfide. They found no evidence for the involvement of autolytic enzymes.

Bacterial Deterioration of Proteins

Most of the work reported on the role of microorganisms in the causing of changes in protein and water holding properties of fresh meats has been done with beef. Ockerman et al. (1969) compared changes in the protein

fractions and percent free water in aseptic beef to tissue inoculated with pure cultures of *Achromobacter* and *Pseudomonas* and with general flora stored at 3°C. The sarcoplasmic fraction was found to decrease in all samples, but the decrease was more rapid ($P<0.01$) in the aseptic than in the inoculated samples. The difference was attributed to microbial breakdown of other tissue components leading to solubilization of components that are normally insoluble. They concluded that the observed decrease in protein extractability was a result of enzymatic and/or chemical changes and not due to microbial activity.

Myofibrillar proteins on the other hand were found to increase initially for about 15 days, then remain constant for about 5 days before starting to decline. Inoculated tissue tended to have slightly higher myofibrillar proteins levels. Bacteria may have lead to an improvement in extraction of myofibrillar proteins before day 15. After day 15, however, bacterial degradation of proteins led to a drop in the levels of myofibrillar proteins. The stroma fraction increased in inoculated samples, but no change was observed in aseptic tissue.

The nonprotein nitrogen also increased during storage. There was no significant difference between inoculated and aseptic tissue until the later stages of storage when inoculated tissue had a more rapid increase.

Percentage EM was relatively constant in aseptic tissue but dropped significantly in inoculated samples. The pH of inoculated samples was found to increase from 5.4 to >6.2 within about 17 days of storage. Jay (1964) and Borton et al. (1968) indicated that in beef and pork respectively, drip (extract release volume, ERV) is not due to the presence of large numbers of bacteria per se but to bacterial action during growth.

Shelef and Jay (1969) reported a correlation between microbial counts and the level of amino sugars and suggested that amino sugars may be responsible for the increase in hydration capacity of spoiling meats.

Use of Chlorine Compounds as Antimicrobials

Antimicrobials are an essential component of the food industry. They are used to deter spoilage processes as well as to destroy pathogens.

Chlorine in different forms is widely used in the food industry as a sanitizer. Chlorine compounds are utilized as sanitizing solutions for food contact surfaces, an additive to water used for conveying raw food products and for cooling of heat sterilized cans and in the treatment of raw pork (Fandido et al. 1989), beef, poultry, and fish to reduce microbial load and extend shelf-life (Cords and Dychdala, 1993). In the US, chlorine and hypochlorites are accepted for use in food processing and for bottled water as described by the 1958 amendment to the Federal Food, Drug and Cosmetic Act (FD&C Act) of 1938. They are regarded as Generally Recognized as Safe (GRAS) compounds under the FD&C Act, the Poultry Products Inspection Act and the Meat Inspection Act. In other food processing uses, chlorine is considered an additive under the FD&C Act (Wei et al., 1985).

Types of chlorine compounds available include gaseous chlorine, hypochlorous acid, sodium and calcium hypochlorites, chloramine-T, dichlorodimethyl-hyantoin, di- and trichlorocyanuric acid. Sodium hypochlorite is the most widely used of these (Dychdala, 1983). Chlorine dioxide, which has a different legal status and follows a different chemical pathway, has been shown to increase the shelf-life of fish (Toledo-Flores, 1988).

Mechanism of Chlorine Disinfection

Wei et al. (1985) reviewed several theories that have been proposed to explain the antimicrobial action of chlorine. One of the first theories to be advanced was that chlorine kills bacteria by reacting with cell membrane proteins, resulting in the formation of N-chloro compounds that interfere with cell metabolism. A later theory proposed by Rudolph and Levine (1941) suggested that the chlorine penetrates bacterial cell and reacts with cellular protoplasm to form toxic N-chloro compounds. Based on the low chlorine requirement for bactericidal action, Green and Stumpf (1946) postulated that chlorine acts by inhibiting some key enzymatic reactions within the cell. They found that the rate of inhibition of glucose oxidation in the cell was correlated with the extent of bacterial destruction. The inhibition of enzymes was probably due to chlorine oxidation of sulfhydryl groups (Knox et al., 1948). Campers and McFeters (1979) reported that chlorine impaired the function of the bacterial membrane in transporting extracellular nutrients.

More recent studies suggest that chlorine is capable of producing lethal reactions at or near the cell membrane as well as affecting DNA. It has also been suggested that NaOCl reacts with the DNA of living cells, causing mutations by oxidation of purine and pyrimidine bases while inactivation by ClO_2 has been attributed to a disruption in protein synthesis (Cords and Dychdala, 1993).

The destruction of bacterial spores by chlorine is thought to be due to the disruption of spore coats and underlying layers, resulting in an increase in spore permeability and a concomitant increase in susceptibility to destruction (Foegeding, 1983). Chlorination may also inactivate the

germination mechanism of spores. Wyatt and Waites (1975) reported a reduced rate of germination in spores that were treated with chlorine.

Chemistry

The antimicrobial properties of chlorine compounds are influenced by the form of chlorine in aqueous solution. When elemental chlorine or hypochlorites are added to water, they undergo the following reactions:

$$Cl_2 + H_2O \longrightarrow HOCl + H^+ + Cl^-$$

$$NaOCl + H_2O \longrightarrow HOCl + NaOH$$

$$HOCl \longrightarrow H^+ + OCl^-$$

The term "free available chlorine" is usually applied to the three forms of chlorine that may be present in an aqueous solution: HOCl, OCl^- and Cl_2. At pH 4-5, most of the chlorine is present as HOCl. As the pH is decreased below 4, increasing amounts of Cl_2 are formed. Above pH 5 the proportion of OCl^- increases. HOCl is the most bactericidal of the three chlorine species (Cords and Dychdala, 1993).

Antimicrobial Activity

Chlorine is considered a broad spectrum germicide. Hypochlorites have demonstrated antimicrobial activity against viruses, non acid-fast bacteria, acid-fast bacteria, bacterial spores, fungi, algae, and protozoa (Dychdala, 1983).

Vegetative bacteria are generally more susceptible to chlorine inactivation than spores. Different organisms including the pathogens *Listeria monocytogenes, Campylobacter jejuni, Yersinia enterolitica, Salmonella typhimurium, Pseudomonads* and several gram negative bacteria are

completely destroyed by chlorine under practical use conditions of time, temperature, and concentration. *Mycobacterium fortuitum, Candida parapsilosis, Escherichia coli* and some strains of *Staphylococcus aureus* show a greater resistance to chlorine (Cords and Dychdala, 1993).

Factors Affecting Activity

Cords and Dychdala (1993) reviewed the effects of environmental factors on the antimicrobial activity of chlorine. Lowering of the pH from about 8.4 to about 5.2 dramatically increases the efficacy of hypochlorite against *Streptococcus lactis, Pediococcus cerevisiae,* and *Saccharomyces cerevesiae.* The effect of pH on the sporicidal effect of hypochlorite was demonstrated for *Bacillus metiens.* At pH 10, 121 minutes of exposure were required to obtain the same degree of kill as 2.5 minutes at pH 6 when 25 ppm available chlorine from $Ca(OCl)_2$ was applied at 20°C.

The efficacy of chorine compounds is also affected by temperature. Generally, the lethality of these compounds increases with temperature. The effect of temperature is more pronounced at low chlorine concentrations. This is particularly true with vegetative cells. Hypochlorite is less sensitive to temperature effects than most sanitizers. The rate of kill of hypochlorite against *Micrococcus pyogenes var. aureus, E. coli* and *Pseudomonas aeroginosa* at approximately 10 ppm and temperatures of 5, 20, and 45°C were found to be similar.

The type and amount of organic material present influence the efficacy of chlorine. Addition of skim milk has been demonstrated to depress the antimicrobial activity of chlorine against *E. coli* and *Staphylococcus aureus* and the effect increases as the amount of skim milk added is increased.

Toxicity of Hypochlorite

NaOCl has been shown to be mutagenic to *Salmonella typhimurium*. It was shown to cause base substitution mutations in DNA which were presumed to involve the oxidation of purine and pyrimidine moieties by aqueous chlorine. No toxicity was found at levels up to 400 ppm when NaOCl was fed to rats and guinea pigs in milk or water.

At low levels (80-200 ppm), hypochlorite has been found to stimulate the growth rates of animals, probably due to the broad spectrum antibiotic activity of the compound in the digestive tract. High levels (2,000 ppm) have been shown to cause undesirable effects such as increase in kidney weight (Wei et al., 1985).

About 0.5-3% of the chlorine dosage applied to water treatment forms chloroganic compounds. Of the compound formed, chloroform, bromodichloromethane, chlorodibromomethane, trichloethylene, tetrachloethylene, chlorinated acetonitriles, and bis (2-chloroethyl)-ether are either suspected or confirmed carcinogens. The pyrimidine 5-chlorouracil, also found in chlorinated water, is highly mutagenic in *E. coli* and has been shown to incorporate into liver and testicular DNA of mice. It is suspected that 5-chlorouracil mutagenicity is due to a substitution for another pyramidine in DNA (Wei et al., 1985).

Biochemical Reactions in Fish Muscle During Frozen Storage

During frozen storage of fish, deterioration in quality due to microorganisms and biochemical processes is decreased. Good quality lean fish which has been properly frozen and packaged can normally be held at -20 to -30°C for more than 1 year without appreciable loss in consumer acceptability (Dyer 1968). However, measurements of

organoleptic, chemical and physical change have clearly demonstrated that the quality of fish deteriorates during frozen storage. The palatabilty of frozen fish stored for extended periods of time is normally limited by losses in either flavor and/or texture. Also, the utility of fish as a raw material for fabricated products may be limited by loss of protein functionality (Colomenero and Borderias, 1983). Gadoid fish may become progressively tough and fibrous during frozen storage giving rise to sensory attributes variously described as sponginess, stringness, dryness, rubbery texture, lack of succulence, loss of juiciness, and loss of water-holding properties. Denaturation and aggregation of fish muscle proteins, particularly those of the myofibrillar fraction, are normally associated with the texture deterioration of frozen fish. Aggregation of fish muscle proteins is associated with the formation of FA. The pronounced protein denaturation in muscle of gadoid species is attributed to their high content of both TMAO and the enzyme TMAO demethylase.

The deterioration in flavor of frozen fatty fish is due primarily to lipid oxidation, and is probably influenced by other biochemical reactions such as lipid hydrolysis, proteolysis and nucleotide catabolism.

Factors Affecting Deterioration During Frozen Storage of Fish

Rate of freezing

Freezing rate may influence quality deterioration during subsequent storage. Lee (1982) observed that freezing in liquid nitrogen resulted in less drip and muscle toughening than conventional freezing after storage at -20°C for 2 months. Reddy and Srikar (1991) suggested that the temperature range -4 to -8°C is most conducive for denaturation and that minimal loss in protein solubility can be ensured by getting below this temperature range

in no longer than 8 hours. Reid et al. (1986) studied biochemical/chemical change in fast versus slow frozen Pacific rockfish stored at -5, -12, and -20°C. Storage temperature was found to have a much larger influence on deterioration processes than freezing rate, although an influence of freezing rate on quality change was observed at the -5 and -12°C storage temperature. Accordingly, rapid freezing may be particularly important when the product is subsequently stored in less than ideal frozen storage.

Temperature and time of storage

Decreasing cold storage temperature slows the rate of reactions which contribute to quality loss. Excellent storage life is observed at very low temperatures, even for species like red hake which are particularly sensitive to frozen storage (Licciardello et al., 1982). Recent studies have shown that the temperature dependence of different deteriorative processes in frozen fish are not the same. The Arrhenius activation energy (Ea) for toughness increase is greater than for cohesiveness decrease in frozen cod, i.e., the loss of cohesiveness of frozen stored fish is less temperature dependent than toughening (Kim and Heldman, 1985). Chemical indices were found to have lower Ea than physical indices of deterioration in frozen cod fillets at -12 to -30°C for 90 days (LeBlanc et al., 1988). According to Suwetja (Haard, 1992) the Ea for lowering of K-value was shown to vary with the type of seafood, i.e., for mackerel and prawn held at -10 and -20°C the Ea are 26.2 and 33.0 kcal/mol, respectively. The author unfortunately does not indicate whether this difference is statistically significant.

Fish species

Lean species tend to be more prone to toughening during frozen storage. Wessels et al. (Haard, 1992) reported that addition of 1% sunflower oil to

hake mince gave good protection against flavor and texture deterioration during storage at -7 and -18°C. Addition of lipid to hake mince results in inhibition of dimethylamine (DMA) formation and prevention of protein functionality loss during frozen storage (Careche and Tejada, 1990). Lipids, especially oxidized lipids, may affect the hydrogen bonds and hydrophobic attractions in the proteins of frozen fish. The carbonyl groups of oxidized lipids may participate in covalent bonding, leading to the formation of stable protein-lipid aggregates (Sikorski and Kolakowska, 1994).

It has also been recognized that gadoid species, e.g., hake and cod tend to be more susceptible to protein denaturation during frozen storage. Ragnarsson (1987) compared mince from gadoid and non-gadoid species and found that crosslinking occurred in proteins of the gadoid cod and whiting but not in the non-gadoid ocean perch and founder.

While most research on changes in frozen fish has been done with northern temperate species, it appears tropical species and Antarctic are less vulnerable to texture deterioration during frozen storage species (Haard, 1992).

Intraspecies factors

Frozen stability of a given species may vary with season of harvest, nutritional status, sexual maturity, migratory status, etc. (Love, 1988). For example, spot harvested in the spring, rather than at other times of the year have a greater tendency to develop rancidity during frozen storage, while weakfish harvested at different times of the year show little variation in frozen storage stability. Seasonal differences in protein solubility have

been observed during frozen storage at -18°C for hake, but not for sardines, harvested in February, July and October (Haard, 1992).

Love (1988) showed that variation in docosahexaenoic acid (C22:6) due to season or nutritional status is directly related to the tendency for off-flavor development during frozen storage in cod.

Water temperature prior to harvest may also influence the rate of chemical reactions during frozen storage. Simpson and Haard (1987) observed that live Atlantic cod acclimated in 0 or 10°C sea water prior to sacrifice differed in the freezing point of their muscle due to formation of endogenous antifreeze proteins at 0°C. The rate of biochemical reactions and loss of sensory quality during partial freezing storage (-3°C) were lower in the muscles of cold acclimated fish.

Post-harvest history of fish prior to frozen storage

Quality deterioration of milkfish, Baltic herring , chub mackerel and gemfish is increased if the fish are held on ice prior to freezing (Haard, 1992). Jiang et al. (1987), working with milkfish, reported that adenosine nucleotides (ADP, AMP, IMP) known to occur in fresh fish retard the denaturation of actomyosin during frozen storage at -20°C, whereas nucleotides which accumulate in aged fish (Hx and HxR) accelerate this reaction.

On the other hand, species like yellow tail, rockfish (Kramer and Peters, 1981), red hake (Kelleher et al., 1982), Atlantic cod (Ragnarrsson and Regenstein, 1989) and ocean perch (Ragnarsson and Regenstein, 1989) show a slower rate of texture deterioration when they are aged on ice for several days prior to frozen storage. The finding that aged fish show less

toughening during frozen storage may be related to depletion of TMAO, and the availability of co-factors for the TMAO demethylase reaction (Lundstrom et al., 1981; Ragnararsson and Regenstein, 1989) and to the formation of TMA (Parkin and Hultin, 1982).

Partial Freezing

Some authors have suggested that superchilling (cooling below freezing temperatures) of fish give rise to mediocre quality product (degradation of texture and appearance, excessive drip loss) due to cellular damage and accompanying acceleration of biochemical reactions (Haard, 1992). More recent studies have shown that storage of fish at -3°C, where about 70% of the water is in the ice state, can retard biochemical reactions and effectively extend grade A quality (Haard, 1992; Toledo-Flores, 1988). Minimal temperature variation is critical for partial freezing temperatures to be effective in maintaining grade A quality.

Biochemical Reactions Involved in Quality Deterioration

Trimethylamine N-oxide demethylase

The products of TMAO demethylation: DMA and FA, have been identified in more than 30 species of fish, belonging to 10 families (Sikorski and Kostuch, 1982). Fish of the order Gadiformes tend to produce larger quantities of DMA and FA than other groups of fish (Rehbein, 1988). The blood and kidney tissue of gadoids contain extensive TMAO demethylase activity which accelerates the toughening of gadoid minces. For gadoids, there exists a positive relationship between texture toughening and DMA formation during frozen storage.

Fish muscle contains relatively small amounts of TMAO demethylase. Dark muscles appear to contain more enzyme than white muscle (Sikorski and Kostuch, 1982). Although there are reports of a soluble TMAO demethylase from muscle tissue (Sikorski and Kostuch, 1982), most research has shown the enzyme from muscle to be particulate (Parkin and Hultin, 1981; Lundstrom et al., 1982) and associated with microsomes (Haard, 1992). Two different co-factors systems were identified for demethylation of TMAO by red hake microsomes. The more active systems require nicotinamide adenine dinucleotide phosphate (NADP) and flavin mononucleotide (FMN) and is active under anaerobic, but not under aerobic conditions (Parker and Hultin, 1986). TMAO demethylation by microsomes at optimum pH (pH 7), is 3-4 times faster than the non-enzymatic reaction and, of the amines tested, appears to be specific for TMAO. Heat lability, sensitivity of microsomes to protease treatment, as well as, the reaction kinetics provide further evidence for an enzymatic reaction.

Evidence that microsomal enzymes produce DMA and FA was provided indirectly by Reece (1983). Removal of NADH from cod mince reduces the rate of FA formation during frozen storage at -10°C. Moreover, both in vivo and in vitro studies show that either addition of KCN or oxygen prevents demethylation of TMAO while addition of NADH stimulates DMA and FA formation (Reece, 1983).

Role of bacteria

Methyltrophic bacteria, such as *Bacillus PM6* and *Pseudomonas aminovorans*, are organisms which synthesize all their cell material from "C_1 unit" derived substrates, e.g., TMA as the sole source of carbon and energy. Methyltrophs produce DMA and FA from TMA through one of

two enzymatic pathways or by non-enzymatic oxidative N-demethylation (Colby and Zatman, 1973). Bacterial TMAO demethylase does not appear to be membrane bound and has been purified to apparent homogeneity (Meyers and Zatman, 1971). Two microorganisms, *Citrobacter freundii* and *Aeromyces hydrophilia*, from squid extracts have been shown to convert TMAO to TMA and DMA. The reaction was potentiated by ferrous salt and ascorbate (Haard, 1992).

Non-enzymatic demethylation of TMAO

The non-enzymatic reduction of TMAO to TMA, DMA and FA in the presence of cysteine with either ferrous ion or hemoglobin as a catalyst was first reported by Vaisey (1956). Non-enzymatic formation of DMA appears to occur in dried fish products (Spinelli and Koury, 1979), preheated, frozen fish (Spinelli and Koury, 1981) and heated squid (Nitisewojo and Hultin, 1986). Cysteine, cysteinesulphinic acid, hypotaurine, and taurine were shown to catalyze this reaction (Spinelli and Koury, 1981). Tozawo and Amano (Haard, 1992) suggested that the non-enzymatic formation of DMA from TMA involves free radicals. According to Bligh (1992), this reaction also involves ascorbate and hydrogen peroxide.

Protein aggregation in frozen storage

The mechanism of freeze denaturation of protein in fish is not completely understood. However, the problem has been extensively studied and various theories have been proposed (Bligh, 1992).

Theories of protein denaturation in frozen fish emphasize the contribution of:

- The immobilization of water and the associated concentration of solutes in the tissue.

- Lipid hydrolysis and/or oxidation and interaction of hydrolysis products with protein.
- The formation of FA by TMAO demethylase.

Although the most attention has been given to insolubilization of myofibrillar proteins, several recent reports also indicate that aggregation and crosslinking of collagen may also occur in frozen fish (Haard, 1992).

Importance of covalent crosslinking

Loss of protein extractability during frozen storage of fish can mostly be accounted for by non-covalent, hydrophobic interactions as shown by studies with Greenland halibut (Lim and Haard, 1984), cod (Mathews et al., 1980), red hake, carp, saithe and haddock (Bligh, 1992). Evidence that disulfide bonds contribute to aggregation was provided by Lim and Haard (1984) who showed that the addition of disulfide reducing agents to mince reduces protein insolubilization during freezing and by direct measurement of free cysteine in the aggregated protein.

A small percentage of muscle protein from frozen fish forms high molecular weight aggregates (Bligh, 1992). FA and diamines like cadaverine, spermine and spermidine, accelerate formation of high molecular weight polymers (>200KD) from isolated myosin, although some crosslinking of myosin occurs in the absence of FA during freezing of pure myosin (Ang and Hultin, 1989). It was suggested that FA may interact with proteins side chains and accelerate aggregation without directly causing crosslinks (Ang and Hultin, 1989).

Cryoprotective Compounds

Cryoprotective Compounds

Cryoprotective agents which lessen denaturation of proteins during frozen storage have been found to include, among others polyphoshates, sugars, polyalcohols, amino acids, and dicarboxylic acids (Haard, 1992).
Many hypotheses have been proposed to explain the mechanism of cryoprotectant action. Matsumoto (1979) proposed that cryoprotectants like sodium glutamate bind to the proteins and increase their hydration.

Osmotic cryoprotectants may function by modifying water structure and thus altering the formation and growth of ice crystals or by stabilizing protein-water interactions (Matsumoto, 1980). Methylamines containing quaternary ammonium groups like TMAO, are especially strong protein structure stabilizers, even in the presence of denaturants like urea (Haard, 1992).

High molecular weight polymers like maltodextrins (Park and Lanier, 1987; Park et al., 1988), soy protein, alginates, xanthan and carrageenan and carboxymethycellulose also stabilize proteins. The major forces responsible for interaction of these polymers with proteins are electrostatic in nature. Other interactions such as hydrogen bonding, hydrophobic or covalent bonds may also contribute to their stabilizing role (Haard, 1992).

Lim (Haard, 1992) proposed that diffusion-controlled processes in frozen systems like surimi can be influenced by the presence of the glassy state. The glassy state is a hard, noncrystalline solid state formed below the freezing point in systems with a low solute concentration, but occurs above the freezing point when the solute concentration is high (MacDonald and Lanier, 1991). Lim (Haard, 1992) observed significant retardation of protein

aggregation below the glass transition temperature (Tg') in freeze-concentrated systems. The cryoprotective effect of polymers like carboxymethylcellulose, maltodextrins and gums has been attributed to their ability to increase the Tg' (Haard, 1992). Low MW carbohydrates like sucrose prevent protein aggregation below Tg' but do not provide cryoprotection above their Tg'. It is thought that cryoprotection by these substances is achieved through solute exclusion. The added sugar molecules are excluded from the protein surface and thereby cause preferential hydration of the proteins (Arakawa and Timasheff, 1982).

Hydrocolloids have the advantage of being effective at low concentrations (0.5% w/w), producing negligible increase in caloric content and sweetness, and reducing fat absorption during frying (Ponte et al., 1987).

Although hydrocolloids improve the texture and water holding capacity of minced fish muscle, they do not appreciably influence loss of myosin solubility (Ponte et al., 1985). Krueger and Fennema (1989) observed that sodium citrate and sodium pyruvate significantly slowed the rate of pollock fillets toughening at -10°C without slowing the normal decline in protein extractability. On the other hand, the cryprotective effect of maltodextrose or polydextrose on texture appears to be associated with preventing loss of protein solubility (Park et al., 1988).

OBJECTIVES

Catabolism of adenosine nucleotides and changes in water retention properties are important quality deterioration phenomena in fish. Although extensive work has been done on the mechanisms by which these changes occur, information on the role of bacteria and endogenous enzymes in the causation of these changes in fish is limited.

The goal of this study was therefore to investigate the role of these two factors in changes in both water retention properties and catabolism of adenosine nucleotides of cod mince. Specifically, the study sought to:

1. Determine the effect of microbial inactivation on changes in water retention properties (WUA and EM) and in the accumulation of the adenosine nucleotide catabolites Hx and HxR.
2. Investigate the relationship between the changes in water retention properties and the changes in pH and water soluble proteins.
3. Determine the effect of superchilling on the above changes.

NaOCl was used as an antimicrobial because it has a wide bactericidal spectrum and unlike most other antimicrobials is effective at the near neutral pH characteristic of fish tissue.

MATERIALS AND METHODS

Fish

Fresh, adult, gutted cod fish were purchased from Steve Connolly Seafood Company (Boston, MA) during the winter season. The fish were transported to Ithaca on ice in wax-coated boxes. Upon arrival, the fish were held in a cold room (0°C) for about one hour before filleting.

Preparation of Microbial Inocula

Cotton swabs (Chesebrough Ponds, Greenwich, CT) were used to swab the gills of three fish. All the swabs used were rinsed in 200 mL of a diluent (solution containing 1.93 g of KH_2PO_4 and 5 g of NaCl per liter of distilled deionized (dd) water) in a beaker. Swabing was done until no slime was visible on the gills of the fish, to develop some level of consistency. About five swabs were used per fish.

Preparation of Minced Fish

The gutted fish were washed under running water, headed and filleted using a clean, sharp, stainless steel knife. The fillets were minced in a cold room (4°C) using a Kitchen-Aid Food Processor (KitchenAid Div., Hobart, Troy, OH) with a meat grinder attachment using a 1 mm plate.

Treatments

I. Effect of NaOCl treatment

To investigate the effect of NaOCl, 30 mL of NaOCl solution, 5% available chlorine (Sigma Chemical Co., St. Louis, MO) were added per kg of mince. An equal volume of dd water was added to the control. After the treatments, the mince was packaged into sterile Whirl pak bags (Nasco), about 30 g in each, and were stored on ice in a styroform box placed in an

insulated cooler chest (IGLO) held in a cold room (~0°C). Storage time was determined from the time the mince was placed in the storage chests. NaCl (Fisher Scientific) was added to the ice to achieve the desired below freezing temperatures (-2 and -3°C). To achieve -2°C, 1% NaCl (wt/wt) was used and 1.6% was used to achieve -3°C. The salt/ice mixtures were prepared in the cold room by manually mixing 1 kg of ice flakes at a time with the corresponding amount of NaCl.

II. Impact of bacteria

To investigate the impact of bacteria, 5 mL of NaOCl were added to 25 mL of the bacterial inocula solution. After being held in the cold room for one hour, 30 mL of the mixture were added per kg of mince to give the control. To the other portion of the mince, 5 mL of NaOCl were added per kg of mince and the mince was held in the cold room overnight to allow for the degradation of the NaOCl before addition of the bacterial inocula, in the same quantity as used in the controls. Packaging was done as in treatment I, but only plain ice (without salt) was used in the styrofoam boxes. The controls were stored under the same conditions. The time at which the samples were placed in the styrofoam boxes was taken as time zero.

Analytical Procedures

Microbiological evaluation

A portion of mince (11 g) was placed in a sterile Whirl pak bag and mixed with 99 mL sterilized diluent using a Stomacher (Cooke Laboratory Products, Alexandria, VA) for 2 minutes. Dilutions of 10^0 to 10^{-8} were prepared with diluent (same as used in preparing inocula). Portions (1 mL) of each of the dilutions were transferred in duplicate to sterile petri dishes containing sterile plate count agar (Difco Laboratories, Detroit, MI)

with 0.5% (w/v) NaCl. After four days of incubation at 25°C in a PS incubator (Precision Scientific Co. Chicago, IL), plates were removed and visible colonies counted using a colony counter (Fisher Scientific Company, Pittsburgh, PA).

Water uptake ability and protein solubility

Samples of mince (1.70 g) were weighed into 50 mL polycarbonate centrifuge tubes (Nalgene). The tubes were placed in ice and 30 mL of chilled (2°C) dd water were added to the mince. The samples were homogenized using an Omni-mixer (Sorvall, Waterbury, CT) at setting 5 for 2 minutes. The Omni-mixer blends were rinsed using 4 mL of chilled dd water. The homogenate pH was measured using a pH meter (Jenco Electrics, Taiwan). The tubes were centrifuged in a Sorvall centrifuge (Waterbury, CT) at 14,000 rpm at 2° C for 15 minutes.

An aliquot (0.1 mL) of the supernatant was placed in a 20 by 150 mm test tube for determination of protein concentration. The supernatant was decanted into a 50 mL measuring cylinder and the volume was recorded (adding the amount previously withdrawn).

The pellet was transferred using a spatula to an aluminum pan (57 mm diameter, Fisher Scientific). The weight of the pellet and the aluminum pan (W1) was recorded. The sample was dried for 24 hours in an oven (Fisher Scientific) at 106°C. The weight of the dry sample and the aluminum pan (W2) was taken and recorded.

The concentration of the protein in the supernant was determined in duplicate by the Lowry method (Lowry et al., 1951). The protein content of the pellet was calculated by difference from the total protein and the

supernant protein. The saved supernatant (0.1 mL) was diluted to 1 mL using dd water. A buffer solution (5 mL) prepared from 100 volumes of 2% Na_2CO_3 in 0.1 N NaOH, 1 volume of 1% $CuSO_4.5H_2O$ and 1 volume of 2% sodium tartrate was added to the diluted sample. The mixture was vortexed at speed 5 (Vortex-Genie Model, Scientific Industries, Inc., Bohemia, NY) for 30 seconds and then allowed to stand for 10 minutes. Folin-Ciocatleu phenol (1 N) (Sigma Chemical Co., St. Louis, MO), 0.5 mL, was added and the mixture vortexed immediately, then allowed to stand at room temperature for 30 minutes. Absorbency was read at 750 nm in a Hitachi Model 100-60 spectrophotometer (Hitachi, Ltd., Tokyo, Japan) using plastic disposable cuvets (Fisher Scientific). Protein concentration of the samples were determined from a standard curve derived using bovine serum albumin (BSA, crystallized and lyophilized and essentially globulin free, Sigma Chemical Co.), with an adjustment for impurities. Apparent protein purity of the BSA was determined by determining the absorbency of 1 mg of BSA/mL in dd water at 320 and 280 nm using quartz cuvets (Fisher Scientific) and dd water as a blank. An $E^{1\%}_{280}$ (A_{280}-A_{320}) of BSA of 6.6 (Sober, 1970) was used in calculating the apparent protein purity.

$$\text{Apparent protein purity of BSA} = \frac{(A280 - A320) \times 100}{6.6}$$

Determination of total protein concentration

Mince (0.34 g) was homogenized in 30 mL dd water and 4 mL was used for rinsing the blends. The pH of the homogenate was adjusted to 12 with 1 N NaOH. The final volume was recorded and the protein concentration was measured by the Lowry method as described above.

Calculation of WUA

$$\text{WUA (mg } H_2O\text{/mg protein)} = \frac{\text{mg of water in the pellet}}{\text{mg of protein in the pellet}} = \frac{W1 - W2}{\text{mg of protein in the pellet}}$$

The total water soluble protein was calculated using the following formula:

Total water soluble protein=Supernant volume (mL) X protein content of the supernant X the dilution factor (10).

Determination of expressible moisture

Three pieces of Whatman #3 filter paper (5.5 cm diameter, Whatman, Maidstone, England) and one piece of Whatman #50 were folded in a funnel shape, with the Whatman #50 paper innermost. The weight of the filter papers was taken using an electric balance (Mettler-Toledo, Inc., Hightstown, NJ) and recorded as M1. Approximately 1.5 g of mince samples was weighed on a 7.0 cm^2 polyester mesh (PE. 105, type 502, 690 mesh per cm^2, Henry Simon Limited, Stockport, Cheshire, England). The weight of the fish was recorded as M2 The sample, enclosed in the mesh, was placed in the filter paper and folded into a thimble shape and placed at the bottom of a 50 mL polycarbonate centrifuge tube (Nalgene) and centrifuged in a Sorvall centrifuge at 14,000 rpm for 15 minutes at 2°C. Immediately after centrifugation, the sample and mesh were discarded and the weight of the wet filter papers was taken and recorded as M3. The expressible moisture (EM) was derived from the formula below.

$$EM(\%) = \frac{M3-M1}{M2} \times 100$$

Preparation of trichloroacetic acid (TCA) extract

Mince (5 g) was homogenized with 30 mL of chilled (~0°C) 5% (w/v) TCA using an Omni-mixer at speed 5 for 2 minute. The homogenate was filtered through Whatman #1 filter paper. The extract pH was immediately adjusted to 6.5-7.0 using 15M NaOH. The extract was used for determination of TMA, Hx, and HxR.

Trimethylamine analysis

Dyer's (1945) method with some modifications was used. Unless otherwise stated, all chemicals used in this analysis were obtained from Fisher Scientific. Portions (1 mL) of the TCA extract were taken in duplicate and placed in 20 by 150 mm screw capped test tubes and diluted to 4 mL with dd water. One mL of 20% formaldehyde, 10 mL of toluene and 3 mL of 45% KOH in dd water were added. The tubes were tightly capped and then vortexed at speed 10 for 2 minutes. After standing for a period of 10 minutes, 5 mL of the toluene layer was transferred into a dry clean tube. Picric acid, 5 mL, (0.02% in toluene) was added and the tubes were gently swirled. Absorbency of this mixture was determined at 410 nm using glass cuvets (Fisher Scientific). TMA concentration was determined using a regression equation derived from measurements of solutions containing 0 to 2 mg TMA-N prepared from TMA-HCl (Sigma Chemical Co.) per mL of water.

Determination of Hx and HxR

The method of Jones et al. (1964) was used with a few modifications. An aliquot (0.5 mL) of the TCA extract was mixed with 2 mL of dd water.

Phosphate buffer, 2 mL, (0.25 M, made from a mixture of 30.5 volumes of 0.25 M monobasic sodium phosphate and 19.5 volumes of 0.25 M dibasic sodium phosphate and pH adjusted to 7.6 using molar HCl and NaOH) was added. An aliquot (1 mL) of a freshly diluted aqueous solution containing 0.64 International Units (IU) of xanthine oxidase (XO) and 3.2 IU of nucleoside phosphorylase (NP) were added. The xanthine oxidase used had the following manufacturer's specifications; Grade IV from milk: suspension in 2.3 M $(NH_4)_2SO_4$ and containing 1 mM sodium salicylate. For xanthine oxidase, 1 IU converts 1µmole of Hx to uric acid per minute at pH 7.5 at 25°C. The nucleoside phosphorylase had the following specifications: lyophilized powder. For nucleoside phosphorylase, 1 IU leads to the phosphorolysis of 1 µmole HxR to Hx and ribose 1-phosphate per minute at pH 7.5 at 25°C. The mixture was incubated at 37°C for 30 minutes to allow conversion of Hx to uric acid. Absorbency at 290 nm was determined against a blank, without the enzyme. Hx + HxR concentrations were determined using a regression equation derived from solutions containing 0-100 µg of Hx (6-hydroxypurine). All chemicals used in determination of HxR and Hx were purchased from Sigma Chemical Co.

Statistical analysis

Data for all parameters were analyzed by one-way (including Tukey's comparisons) and two-way analysis of variance (ANOVA) using the Minitab IBM version 10 software package.

RESULTS AND DISCUSSION

The Effect of NaOCl on Bacterial Counts

The cod mince treated with NaOCl had counts in the range of 5.6×10^2 to 1.2×10^3 cfu/g at time zero compared to 2.0×10^5 to 6.2×10^5 cfu/g for untreated samples. NaOCl therefore caused a well over 99% the reduction in bacterial load of the mince. For treatment II, inoculated samples had counts ranging from 6.9×10^4 to 3.5×10^5 cfu/g while the controls had between 2.8×10^3 and 2.9×10^4 cfu/g.

Effect on TMA Production

In both fresh and two day old mince, no TMA was detected regardless of treatment and storage temperature (Fig 4.1). At day five, levels of about 0.1-0.2 mg TMA-N/100g of mince had accumulated in all samples. By day nine, the levels exceeded 2 mg TMA-N/100g mince for normally spoiling samples while the levels remained below 0.2 mg TMA-N/100g in samples treated with NaOCl.

These observations are consistent with the idea that TMA is almost exclusively a result of bacterial activity and only starts accumulating when bacterial counts exceed a certain threshold (approx. 10^6) and when spoilage is underway. This level seems to have been reached by day nine in naturally spoiling samples. In NaOCl treated samples, however, TMA levels began to significantly increase only after day 14.

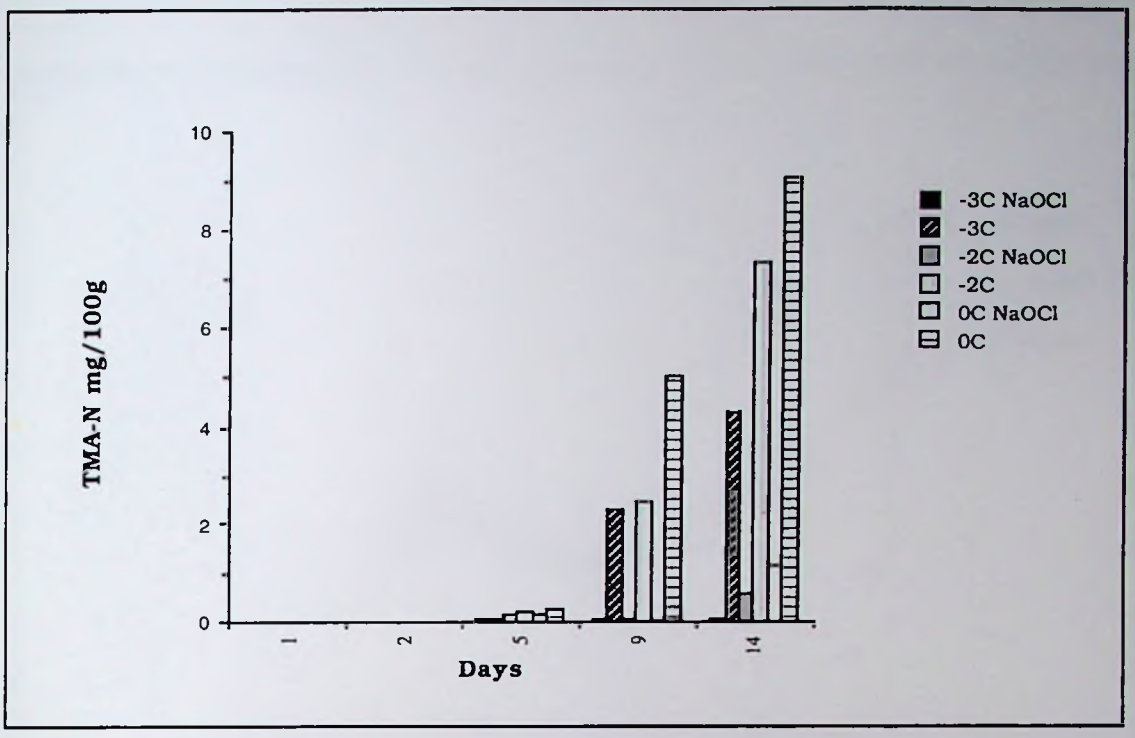


Figure 4.1. **Accumulation of TMA in chilled and superchilled cod mince with and without NaOCl**

The eventual increase in TMA in NaOCl treated samples indicates that bacteria that survive after NaOCl treatment resume growing and eventually participate in fish spoilage. This observation is suggests that the the bacteriostatic effect of NaOCl lost during storage .

Effect on pH

Fig 4.2 shows changes in pH over 14 days of storage of samples at -3, -2 and 0°C, with and without NaOCl. At -2 and 0°C, normally spoiling samples tended to have a more rapid increase in pH than samples treated with NaOCl. Samples stored at -3°C had approximately the same pH (7.1) at the end of the storage period regardless of whether they had been treated with NaOCl or not.

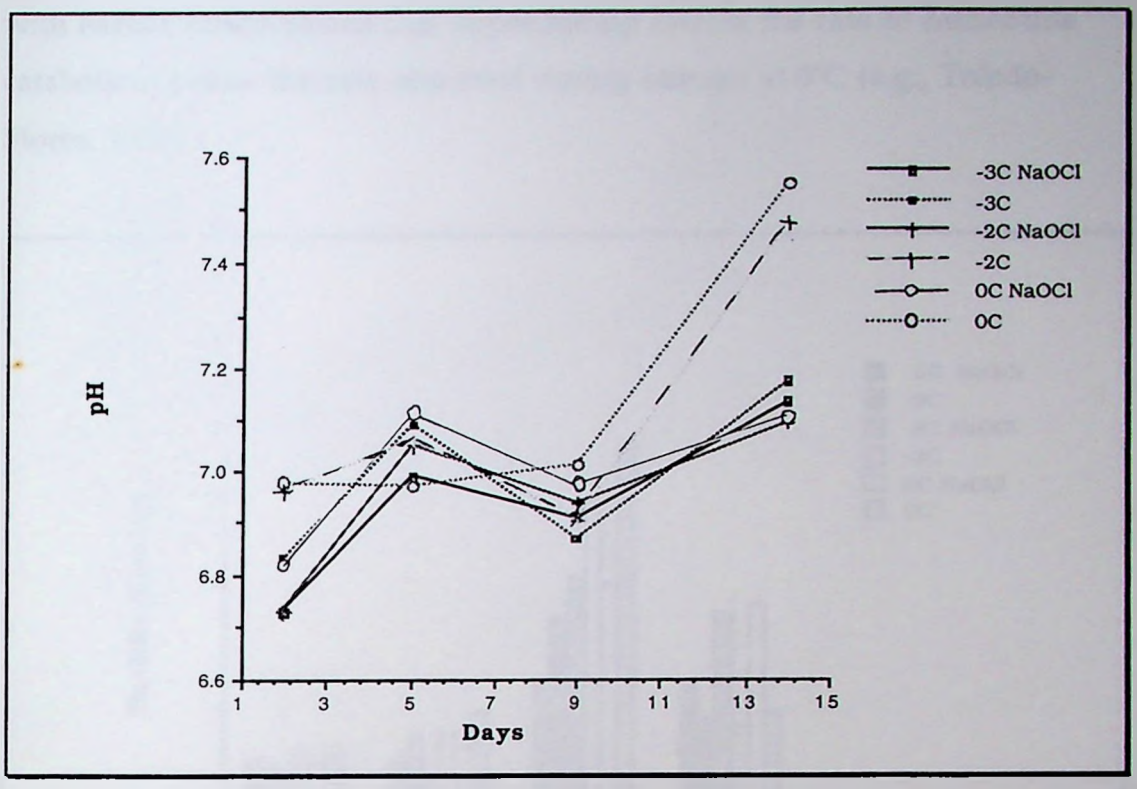


Figure 4.2. **Effect of NaOCl on pH changes in chilled and superchilled cod mince**

These results are consistent with the fact that bacteria break down nitrogenous compounds, releasing amines, ammonia and other bases leading to an increase in pH. By inactivating bacteria, NaOCl reduces bacterial production of these bases. At lower temperatures bacterial activity is low and therefore the rate of pH increase is also reduced. The increase in pH in fish seems to mainly be dependent on bacterial rather than autolytic activity.

Effect on Adenine Nucleotides

The level of the catabolites Hx and HxR increased rapidly in all samples up to day nine (Fig 4.3). The increase, however, was more rapid in samples

stored at 0°C, and was slowest in samples stored at -3°C. This is consistent with earlier observations that superchilling lowers the rate of nucleotide catabolism below the rate observed during storage at 0°C (e.g., Toledo-Flores, 1988).

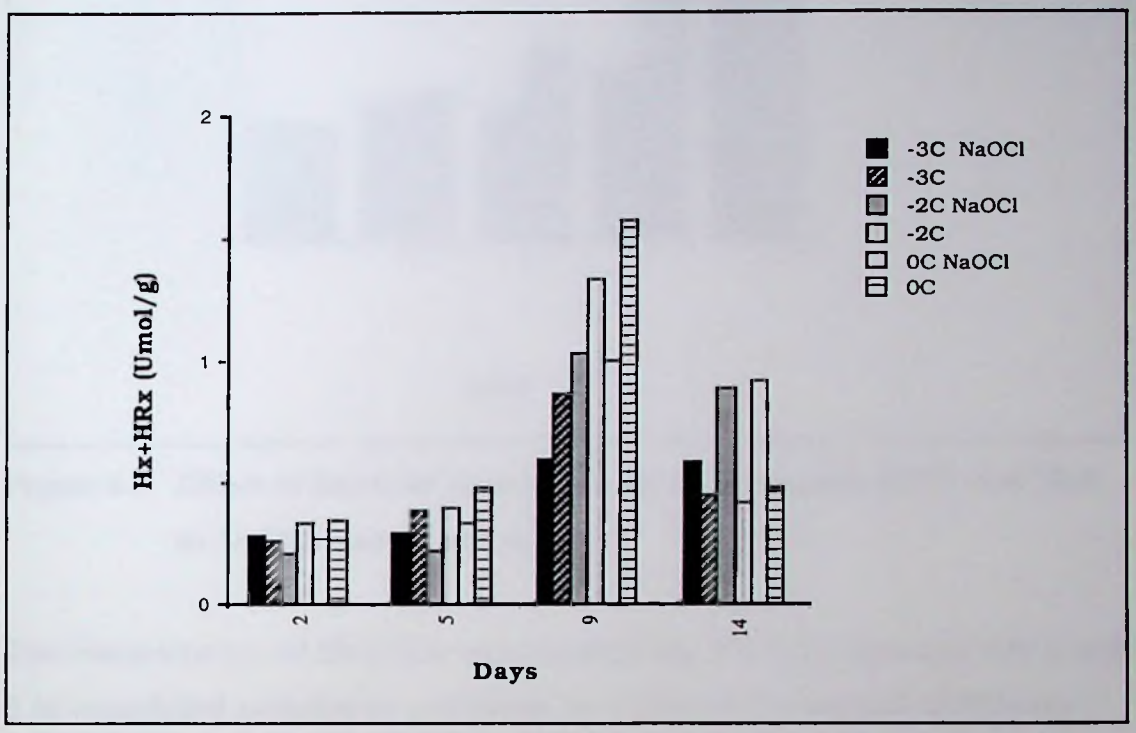


Figure 4.3. **Accumulation of Hx and HxR in chilled and superchilled cod mince with and without NaOCl**

Samples treated with NaOCl tended to have lower levels of these catabolites than untreated samples stored at the same temperature. This observation is potentially in conflict with the idea that the breakdown of adenine nucleotides during early storage of fish is entirely autolytic and that bacteria play no significant role during the useful storage life.

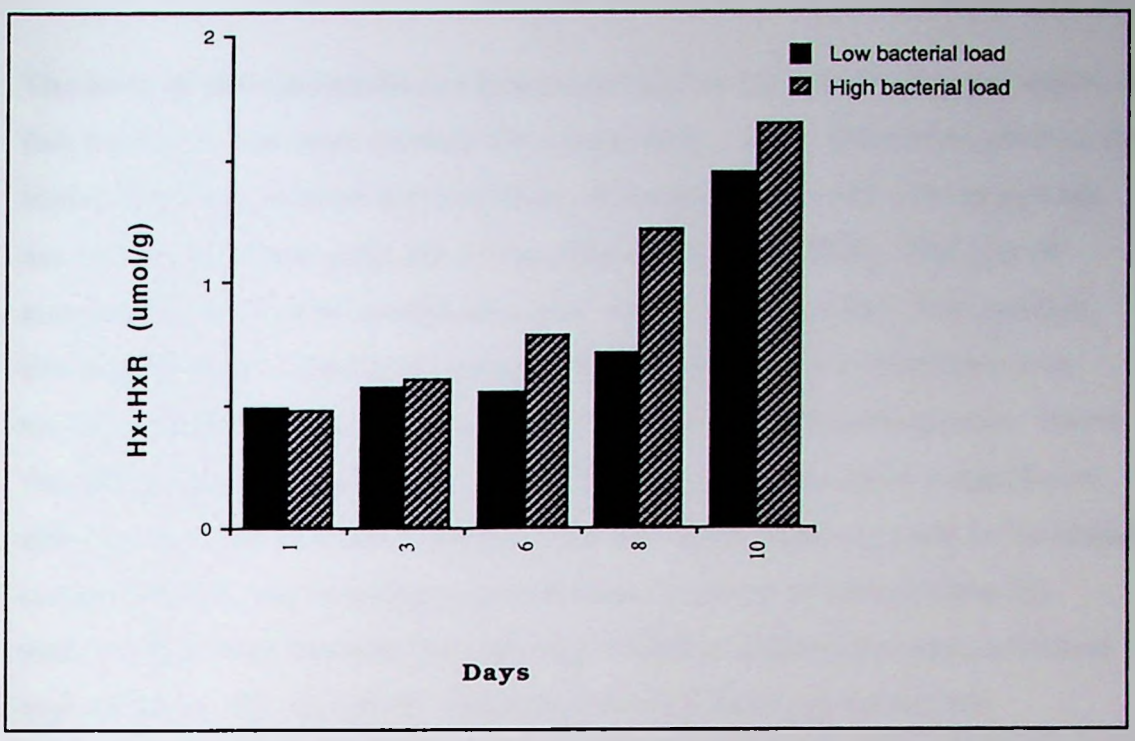


Figure 4.4. **Effect of bacterial inoculation on accumulation of Hx and HxR in NaOCl treated cod mince**

The concentration of Hx+HxR was significantly ($P<0.05$) higher at day 6 and 8 in inoculated samples as compared to controls for treatment II (Figure 4.4). This suggests that the observed differences are due to differences in bacterial content rather than merely to the presence of NaOCl.

This difference could be attributed to the fact that reports on the autolytic nature of the catabolism of adenosine nucleotides have been exclusively done with whole fish or fillets, in which the interior is essentially devoid of bacteria and bacterial enzymes. In this case, bacterial enzymes are only involved in these reactions later in storage when the fish starts to lose its integrity and the interior is invaded by bacteria. Mincing, however, brings bacterial enzymes in proximity with the substrates (ATP, ADP and AMP) causing bacteria to a play a role during early storage.

The level of the adenosine nucleotide catabolites Hx and HxR as an index of fish freshness has been studied for a long time. Wide differences exist in the initial level and rate of accumulation of these compounds. Some species are known to accumulate Hx while other accumulate HxR. The rate of accumulation of these metabolites also varies with species. This renders this method less valuable in comparison of fish of different species and makes it difficult to establish a single standard for different species. From the observations made in this study, handling may also have a significant effect on the rate of accumulation of Hx and HxR. Fish exposed to bacterial contamination and bruising/comminution is likely to accumulate Hx and/or HxR both because the mincing/bruising process releases enzymes and increases the rate of the biochemical breakdown of adenosine nucleotides and because bacteria elevates the rate of nucleotides breakdown. The level of Hx and HxR may therefore also be of little value in comparing freshness of fish that has been handled in different ways, e.g., comparison of fillets to whole fish. Because presence of bacteria affects the rate of accumulation of Hx and HxR, these metabolites should be avoided in comparison of the freshness of fish given antimicrobial treatments to those that have not received such treatment.

Fig 4.3 also shows a drop in the nucleotides Hx and HxR after day nine. The decline in these compounds may be a result of degradation to uric acid. The drop was more rapid in normally spoiling samples suggests that bacteria increase the rate of degradation.

Effect on Water Soluble Protein

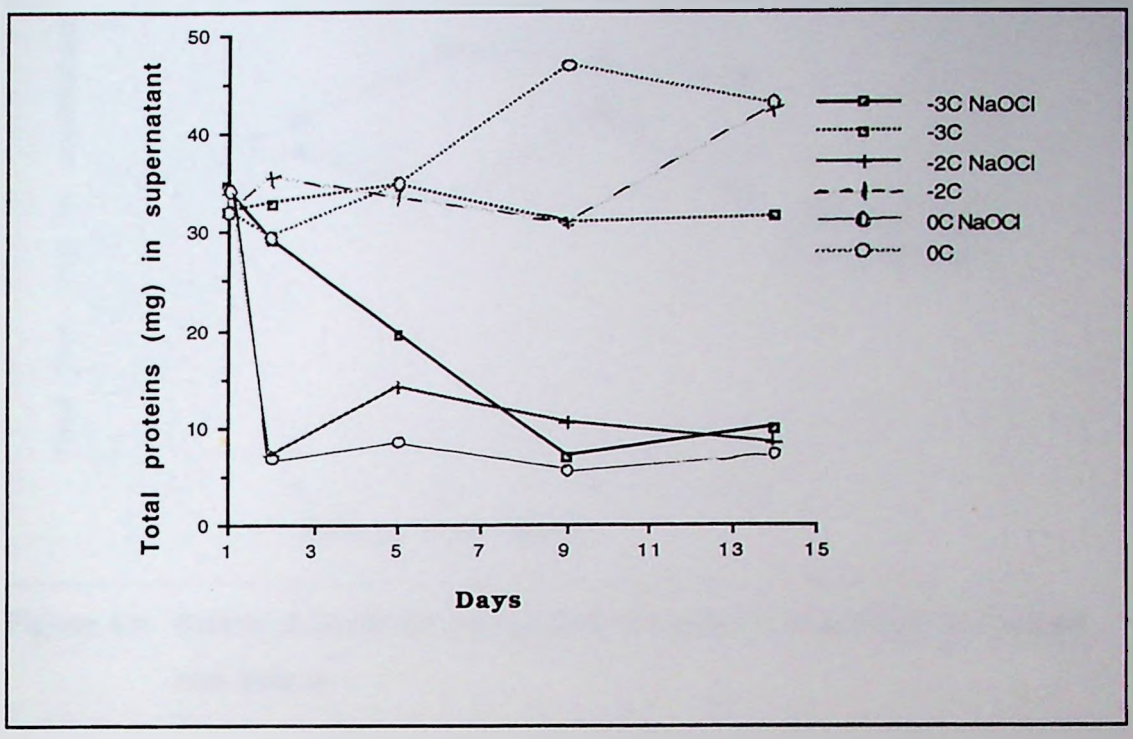


Figure 4.5. **Effect of NaOCl on water soluble proteins chilled and superchilled cod mince**

In samples treated with NaOCl, the water soluble protein fraction (sarcoplasmic proteins) decreased during storage (Fig 4.5). The decrease was most rapid in samples stored at 0°C, slower at -2°C and slowest at -3°C. Normally spoiling samples had higher levels of water soluble proteins. The level was approximately constant in samples stored at -3°C but rose after five and nine days in samples stored at 0 and -2°C respectively.

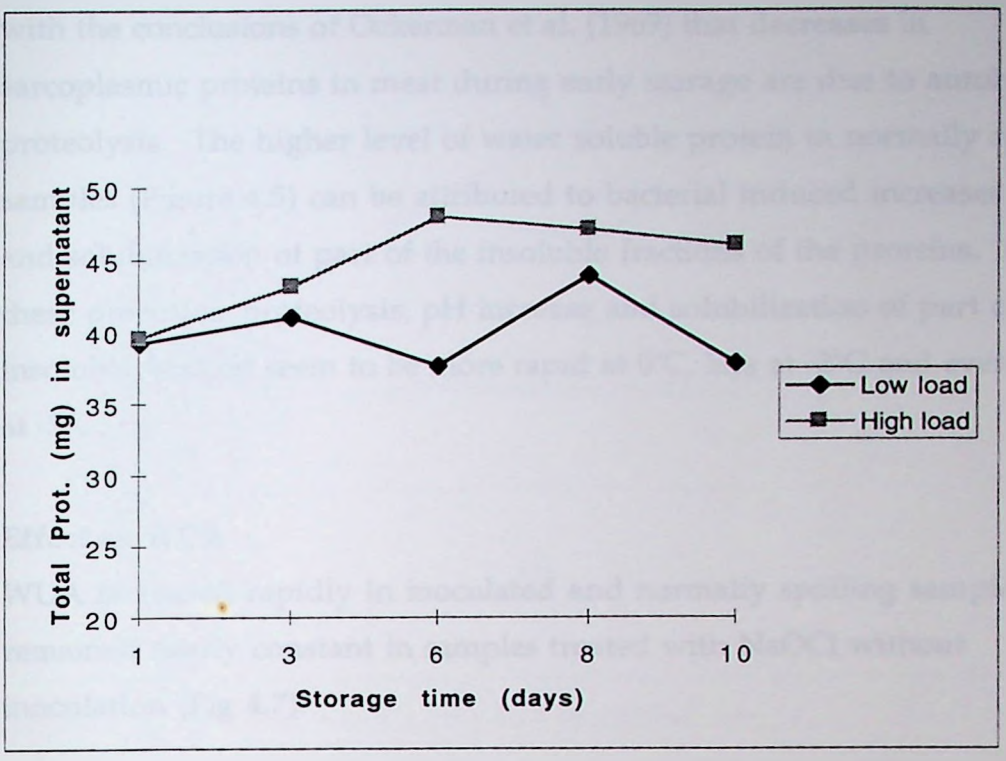


Figure 4.6. **Effect of bacterial inoculation on protein solubility in chilled cod mince**

Changes in water soluble proteins were less dramatic in samples treated with the lower level (25 ppm) of NaOCl (treatment II) as shown in Fig 4.6. It is possible that the NaOCl leads to a reduction in protein solubility. There was a difference in water soluble protein between the inoculated and uninoculated samples. This difference, however, was only significant at day 6. A possible explanation for this observation may be that microorganisms solubilize parts of the non-water soluble fractions, leading to an increase in the total water soluble protein. By day 8, bacterial growth and activity seem to have resumed in the control samples, lessening the difference in the level of soluble protein between the inoculated samples and the controls. The observation that the reduction in water soluble proteins was not increased by an increase in bacterial load is consistent

with the conclusions of Ockerman et al. (1969) that decreases in sarcoplasmic proteins in meat during early storage are due to autolytic proteolysis. The higher level of water soluble protein in normally spoiling samples (Figure 4.5) can be attributed to bacterial induced increases in pH and solubilization of part of the insoluble fractions of the proteins. All these processes: proteolysis, pH increase and solubilization of part of the insoluble fraction seem to be more rapid at 0°C, less at -2°C and even lower at -3°C.

Effect on WUA

WUA increased rapidly in inoculated and normally spoiling samples but remained nearly constant in samples treated with NaOCl without inoculation (Fig 4.7).

Increase in WUA in normally spoiling mince can be attributed to increase in pH, which increases protein hydration. Bacteria may also cause the destruction of the M and Z-lines thereby allowing expansion in the protein lattice and lowering the constraints to water uptake by the system.
From Figure 4.8, it can be asserted that an elevated load of bacteria ($\sim 10^5$) leads to an increase in the WUA of mince during storage at 0°C, since inoculated samples experienced a significant ($P<0.05$) increase in WUA while the controls did not.

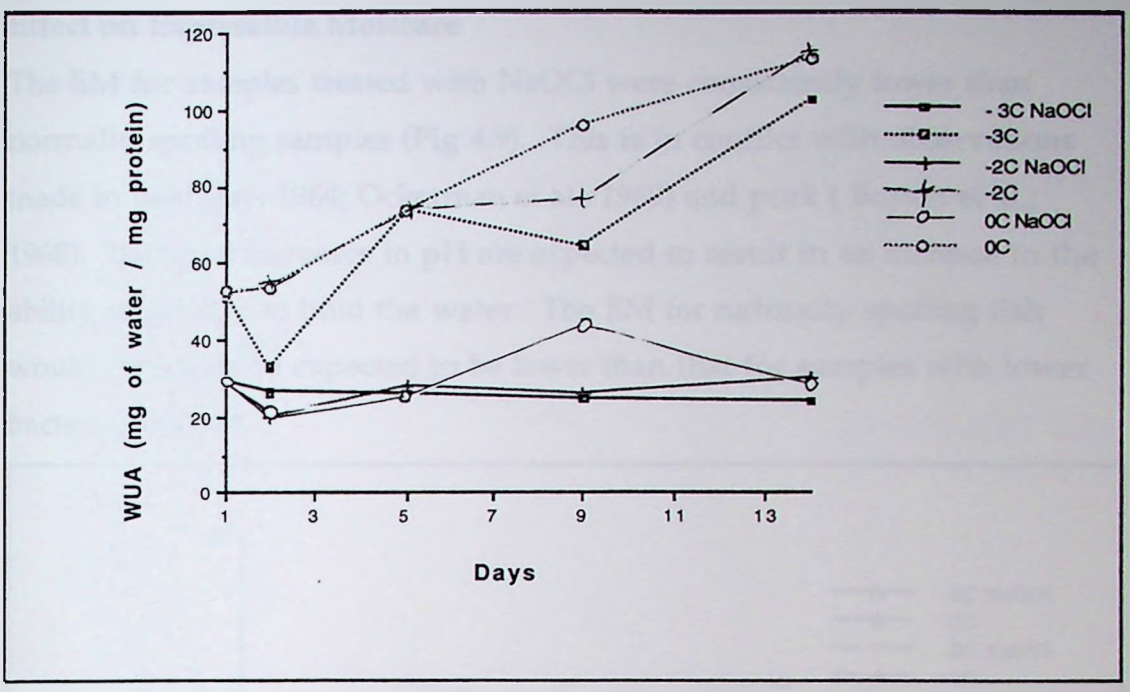


Figure 4.7. **Effect of NaOCl on WUA of chilled and superchilled cod mince**

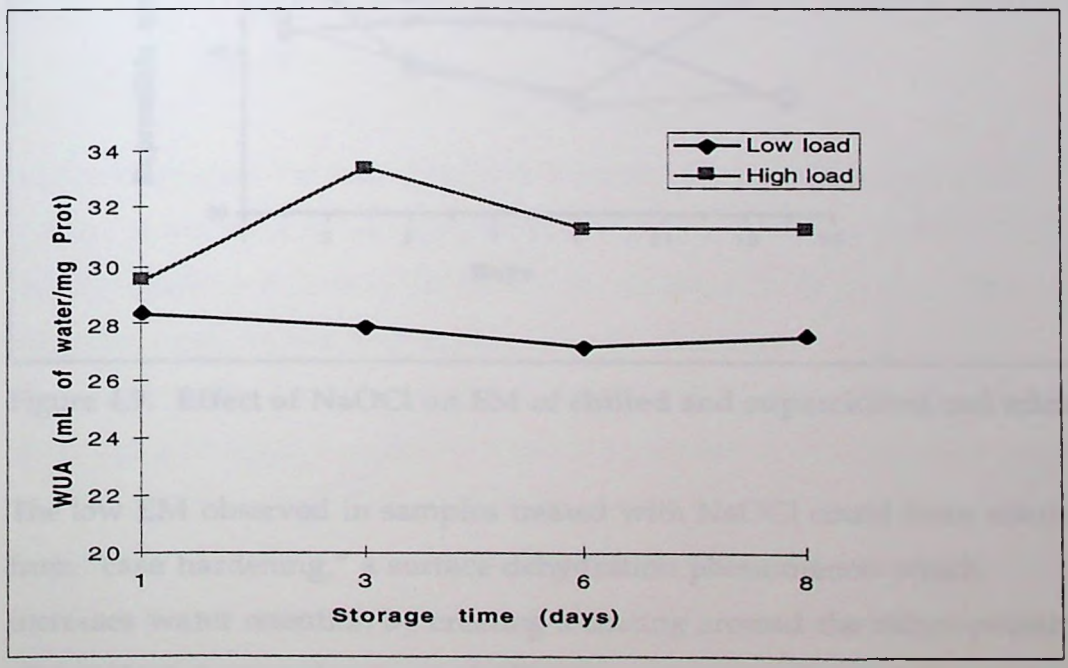


Figure 4.8. **Effect of bacterial inoculation on WUA of chilled cod mince**

Effect on Expressible Moisture

The EM for samples treated with NaOCl were consistently lower than normally spoiling samples (Fig 4.9). This is in conflict with observations made in beef (Jay, 1964; Ockerman et al., 1969) and pork (Borton et al., 1968). Bacterial increases in pH are expected to result in an increase in the ability of protein to hold the water. The EM for naturally spoiling fish would therefore be expected to be lower than that for samples with lower bacterial counts.

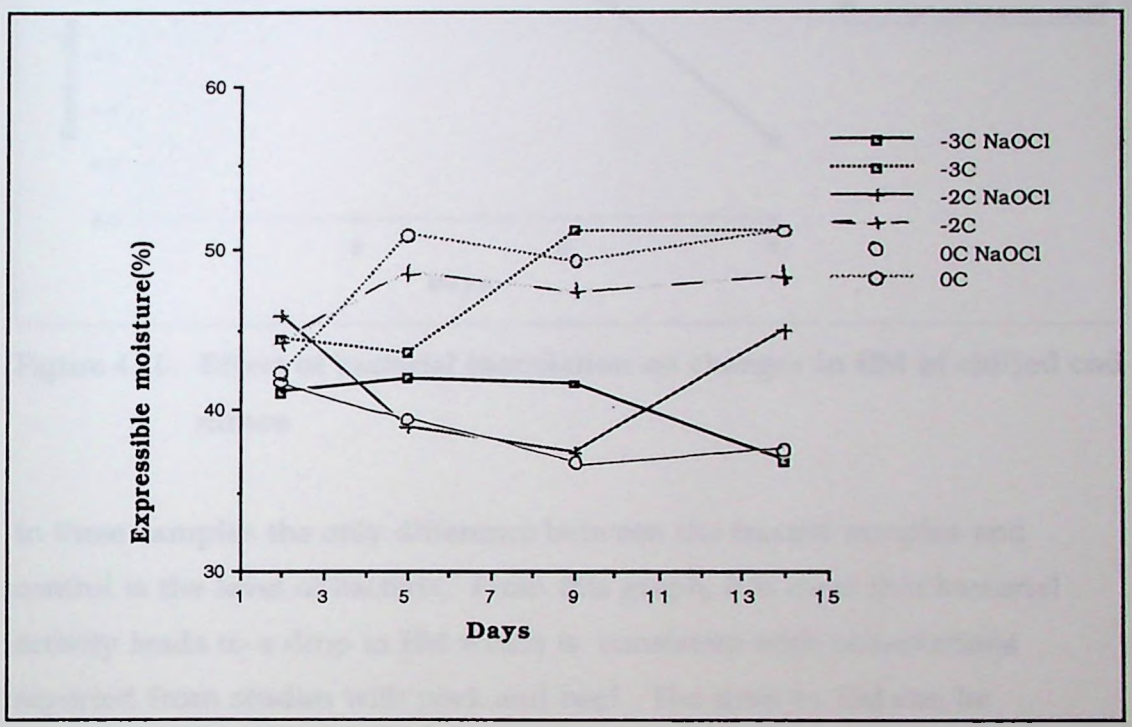


Figure 4.9. **Effect of NaOCl on EM of chilled and superchilled cod mince**

The low EM observed in samples treated with NaOCl could have resulted from "case hardening," a surface dehydration phenomenon which increases water retention by creating a coating around the mince proteins (Regenstein, personal communication).

Fig 4.10 shows changes for EM in samples where the effect of NaOCl is controlled for.

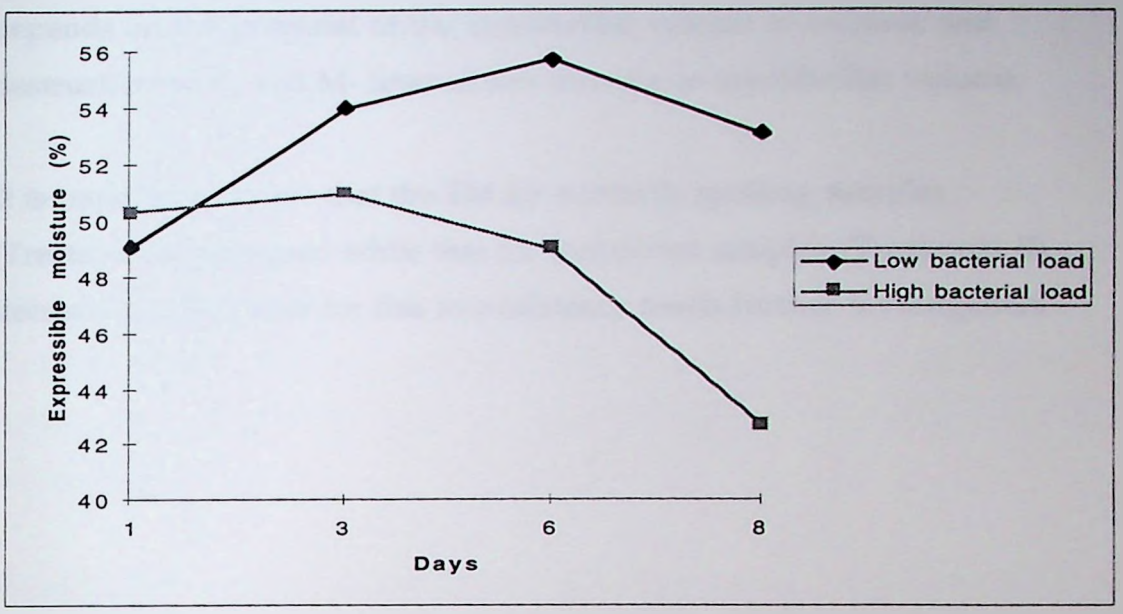


Figure 4.10. **Effect of bacterial inoculation on changes in EM of chilled cod mince**

In these samples the only difference between the treated samples and control is the level of bacteria. From this graph, it is clear that bacterial activity leads to a drop in EM which is consistent with observations reported from studies with pork and beef. The drop in EM can be attributed to bacterial destruction of the Z- and M-lines. The Z- and M-lines put a constraint on the increase in myofibrillar volume and when they are intact, only a very limited amount of water can be retained by the myofibrils. Once they are destroyed, however, the myofibrillar volume easily expands, giving the tissue a higher water retention ability. The bacterial induced increase in pH may also be a contributing factor to the decrease in EM.

Destruction of Z- and M-lines and increases in pH probably have a synergistic effect on water retention properties since the effect of pH depends on the potential of the myofibrillar volume to increase and destruction of Z- and M- lines allows increase in myofibrillar volume.

It is important to note that the EM for normally spoiling samples (Treatment I) increased while that for inoculated samples (Treatment II) decreased. The cause for this inconsistency needs further investigation.

The correlation between the changes in water retention properties, especially EM, which was found to be affected by microbial activity, and the organoleptic characteristics of fish also need further investigation. It may be worth while to consider using whole fish and fillets as well as mince, since information on the role of microorganisms in causing changes in the water retention properties of fillets and whole fish is also lacking. It may also be interesting to investigate the effect of microbial activity on other functional properties of fish such as emulsifying capacity. Such studies have been conducted in other meats with contradicting results (Borton et al., 1968).

From this study, it is apparent that the level of tissue integrity may be a contributing factor not only in accelerating the various fish deterioration processes but also in determining the causative factors for these processes. Contamination of fish with bacteria should be minimized since it may elevate the level of the nucleotide catabolites Hx and HxR and lead to the classification of such fish as less fresh than they really are. Bruising or comminution of fish may have a double effect, since it decompartmentalizes the tissue, bringing substrates and endogenous enzymes in contact and it exposes the interior of the fish to bacteria which is normally almost limited to the exterior. This work also re-ephasizes the need for greater care when handling minced fish.

The findings of this study are of greater application to fish obtained from warmer, and less clean waters, i.e., those known to have higher initial levels of bacteria. In some developing countries, fishing is mainly done by individual fishermen on a very small scale. These fishermen may transport their catch in small wooden chests. The small size of the shipping containers give a high area of contact between the container and the fish. If

the interior of these containers is not smooth, then the fish surface may get damaged, exposing the interior to bacteria from the fish surface and the container surface. This may greatly reduce the storage life of the fish.

So far most efforts to improve fish distribution and marketing in developing countries have addressed the need to use ice. While this may go a long way towards improving the storage life of fish, it should be combined with careful handling to obtain even longer storage life which is particularly important in situations where the distribution infrastructure is not well developed. Use of salt/ice mixtures would prolong the storage life further. This may also help to stabilize the fish supply available to consumers. The economic and technical feasibility of using salt/ice mixtures, however, needs to be determined. The cost and availability of clean salt and less abrasive containers needs to be determined.

By providing insights on ways to improve the storage life of fish, this study may lead to improved marketing of fish especially in countries where proper handling and storage practices have not been developed. In Uganda, for example, purchasing of fish is normally done in the morning to early afternoon to guarantee that the fish is still of high quality. For working consumers, this presents a problem, since the appropriate purchase time coincides with their working time. Improving the stability of the fish, even for one day, is likely to attract these potential customers, who represent a big part of the population and an even bigger portion of the total disposable income. The profitability of the fish industry could be enhanced by addressing the needs of this market segment. In addition, fish is only available on days when the weather is conducive to fishing. This is another hurdle for fishermen interested in providing a reliable supply to their customers.

In addition to clarifying the usefulness of proper handling and storage conditions for fish, this study validates inexpensive and fast ways to evaluate fish quality. Methods such as bacterial counts are expensive and take several days to complete. Apparently, they are also of limited use in judging the level of deterioration in fish quality, since the level of bacterial penetration and not just the bacterial load contributes to the quality loss. The water retention properties of fish and the level of Hx and HxR have been shown to have a bearing on the bacterial quality of the fish and could be utilized by firms purchasing fish to monitor fish quality before purchasing. There exist fast and inexpensive methods for determining EM by applying a fixed force on tissue placed between filter papers and weighing the amount of moisture absorbed by the filter paper. A closely related test is the measurement of extract release volume which just requires a blender, filter papers, a funnel and a scale. Both of these tests can be completed in about 15 minutes. The physical integrity of the fish should also be assessed since this may affect the keeping quality of the fish.

By adopting affordable and yet effective quality evaluation systems, firms with limited capital may be able to improve the image of their products and to compete better in local and global markets. The benefits of efforts by industrial processors to improve quality are likely to force the primary suppliers, the fishermen, to improve the quality of their product. This would ultimately improve the quality of the entire fish supply, reduce post harvest losses, facilitate improved distribution, and increase the profitability of the fish industry.

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