SEASONAL VARIATION OF ANTIFREEZE GLYCOPROTEIN AND SENSITIVITY TO BIOCHEMICAL DAMAGE BY PARTIAL FREEZING IN MUSCLE FROM ATLANTIC COD, GADUS MORHUA, L

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Seasonal variation of antifreeze glycoprotein and sensitivity to biochemical damage by partial freezing in muscle from Atlantic cod, *GADUS MORHUA, L*

By

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A thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of

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Dedicated to
All my children
Ewurabena, Baaba and Aba Mansah

Abstract

Live Atlantic cod *Gadus morhua* were acclimated at 0°C or 10°C for at least three weeks and studied as well as cod fish held at ambient water temperature to determine, the effect of habitat temperature on the keeping qualities of fish during low temperature storage. Cod fillets were stored in ice (0 ± 0.2°C) or partially frozen (-3 ± 0.1°C) for various time intervals to evaluate the effectiveness of the two methods in preserving fish freshness. The criteria employed for the evaluation of biochemical deterioration were changes in (i) extractable protein, (ii) free amino acids, (iii) "extracellular area", (iv) levels of TMAO-N, TMA-N, and DMA-N, (v) free drip and (vi) cell fragility.

Freezing point and melting point measurements of the muscle fluids or blood plasma derived from cod fish acclimated to cold temperature (0°C) or from cod fish caught in the winter months demonstrated thermal hysteresis, unlike similar samples derived from cod fish acclimated to warm temperature (10°C) or cod fish maintained at ambient water temperature caught in the summer months. This finding implies that antifreeze proteins are present in the muscle fluid of cod fish when the habitat temperature is low, but absent when the habitat temperature is high.

Partial freezing proved to be a more effective method of minimizing biochemical damage than ice storage as judged by the capacity of the partially frozen samples (i) to retain relatively higher levels of extractable protein, (ii) to exhibit less depletion of free amino acids, (iii) to form relatively lower levels of TMA and DMA, (iv) less increase in extracellular area, and (v) to maintain better consumer acceptability during sensory evaluation studies.

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List of Abbreviations

AFP = antifreeze polypeptide

AFGP = antifreeze glycoprotein

ASWF = ambient sea water fish

CFU = colony forming units

CTAF = cold temperature acclimated fish

DMA = dimethylamine

DMA-N = dimethylamine nitrogen

EP = extractable protein

FAA = free amino acids

FFA = free fatty acid

FP = freezing point

RD = relative damage

SF = summer fish

TAPC = total plate count

TH = thermal hysteresis

TPC = total plate count

TMA = trimethylamine

TMA-N = trimethylamine nitrogen

TMAO = trimethylamine oxide

WF = winter fish

WTAF = warm temperature acclimated fish

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Chapter 1

Introduction

1.1. Background

The switch from a nomadic lifestyle to a more sedentary one revolutionalized man's method of food acquisition. While nomadic man obtained his food from the wild, the formation of settlements made it essential to cultivate as well as preserve and store seasonal surpluses of food. The choice of sites for settlements appears to have been influenced by the availability of water and of salt. As such, most early settlements were located near rivers, lakes, lagoons and oceans which provide food materials such as fish, crustacea, gastropods, to mention a few. Fish provides man with food for both pleasure and nutritive sustainance. Fish consists primarily of water (66 - 84%), protein (15 -24)%, lipid (0.1 - 22%) and mineral substances (0.8 - 2%) (Matsumoto, 1979). Although fish can provide a useful addition to man's calorific, mineral and vitamin requirements, its most valuable contribution to human diet is that of high quality protein.

Early man was able to develop various methods to improve the keeping qualities of his food. Pre-Neolithic man (possibly 250,000 years ago), discovered that heat and smoke from fire could aid food preservation and also cause desirable changes in the eating quality, and the Paleolithic 'food technologist' discovered the effectiveness of sun-drying as a means of preserving food as early as 18,000 B.C. (Stewart and Amerine, 1982).

1.2. General Methods of Fish Preservation

Even though modern food technology has improved tremendously upon the simple processes which evolved with ancient man, the underlying principles of the 'new' processes have remained essentially unchanged. Most of the known methods of fish preservation i.e. salting, drying, freezing, freeze drying, chilling, gas storage, storage under vacuum (hypobaric storage), irradiation and the application of antibacterial substances especially antibiotics are aimed primarily at reducing or eliminating the effects of spoilage bacteria. Chemical, biochemical and physical processes also contribute to the deterioration in food quality. Chilling, storage at roughly $0^{o}C$, is a widely used method and this can be done in various ways. Usually the fish is stored in melting fresh water ice which can achieve a temperature as low as $0^{o}C$ or slightly below. Sea water ice which has a lower melting point has been used with little success (Merritt, 1965). Chilled sea water at $-1^{o}C$ is used in a limited number of cases notably in the halibut and salmon fisheries. Storage under mixtures of carbon dioxide and other gas is used commercially (Lee and Toledo, 1984). The use of the antibiotic tetracycline in melting ice has given some improvement in keeping time an extra 1 to 3 days for cod and haddock after 14 days storage in melting ice although its approval for use in Canada has been discontinued (Dassow,1976).

1.2.1. Superchilling or Partial freezing

Fresh fish normally has been, and continues to be, the consumer's primary choice in seafood. Fresh fish is usually stored in fresh water ice until sold. Although different species vary in their shelf-life, the limit for shelf-life of fish stored in ice is usually less than a week (Lee and Toledo, 1984). For long-term storage, fish is usually dried, fermented, cured or frozen. A frequent problem of freezing fish is that of texture and flavour deterioration during storage. Thus since quality deterioration remains an unsolved problem in the fishing industry, superchilling conditions therefore became attractive to evaluate as a possible method for holding fresh fish.

Superchilling or partial freezing is a process whereby fish are held at temperatures just below the point where the fish flesh begins to freeze. The superchilling temperatures are slightly lower than those obtainable in ice and are in the range of -4°C and -1°C.

Several decades ago, superchilling was observed to extend the shelf life of cod and haddock (Reay, 1930). As early as 1935, interest in the commercial use of superchilling was expressed in a trade journal "Fishing Gazette" (Anon. 1935). The article gave a description of a refrigeration procedure called the Bellefon-Falliot process. In this process, 110 lb of whole fish were packed in individual galvanized sealed iron boxes which could be hermatically sealed by means of a rubber gasket and metal clasps. A continuous stream of brine maintained at -2°C to -3°C was pumped over the closed boxes stacked on shelves in a vessel's hold or at a shore side installation. The article claimed that the quality of the fish remained essentially the same, after 30 to 40 days of

storage, as when the fish were first caught. The reasons given for this high product quality were exclusion of air in the sealed boxes; maintenance of a regular refrigerated temperature at -2 to -3°C, no sweating, no evaporation, no damage from excessive fish handling, no bruising from ice (since no ice was used) and no freezing of the fish flesh. Apart from this article no additional published information was found concerning the use of the Bellefon-Falliot process and it was therefore assumed that the process did not meet with universal acceptance.

The use of superchilling to preserve fish remained in the background until 1963 when Ranken (1963) briefly mentioned that a system had been developed in Portugal for holding fish aboard a fishing vessel at about -1°C (30°F). Following this, in 1964, new interest and activity were generated in the superchilling process for fish refrigeration. Scarlatti (1965) gave a detailed description of superchilling on board ship and this stimulated a wide-spread investigation of the process (Anon., 1967). However, until recently there has been relatively little application of superchill storage and this appears to be due to the problems involved, as described in the next section.

1.2.1.1. Problems of Superchilling

Superchilled fish have a shelf-life extending from two to three weeks beyond that of normally ice-stored fish (Anon., 1967; Gillespie, 1968). Before the tenth or eleventh day the superchilled fish is considered to have a poorer quality compared to ice-stored fish (Merritt et al., 1966; Waterman and Taylor, 1967; Power et al. 1969). After this period, the rate of deterioration in superchilled fish is lower from 9 to 26 or 32 days (Power et al., 1969) and also bacterial activity is greatly retarded (Power and Morton, 1965; Tomlinson et al., 1965).

According to various researchers superchill temperatures are in the range where maximal endogenous biochemical damage occurs; that is the most rapid glycolysis (Sharp, 1935), lipid hydrolysis (Lovern and Olley, 1962), protein insolubilization (Love and Elerian, 1964) and nucleotide degradation (Nowlan and Dyer, 1969). This accelerated biochemical damage is probably the reason why superchilled fish may be rated lower than ice-stored fish during the first ten or eleven days. After this storage time bacterial spoilage in ice-stored fish becomes predominant and proceeds more rapidly in the normally iced fish than in the superchilled fish (Roach et al. 1966).

The texture of superchilled fish has been the most criticised (Partmann, 1965; Power and Morton, 1965; Merritt et al., 1966). This has been described as dry and tough for haddock (Carlson, 1967), mushy, soft, and fragile for pacific cod and halibut (Roach et al., 1966). Objective testing such as protein insolubilization and free fatty acid formation have shown similar results to those of the taste panel. Merritt (1965) and Love (1966a), on the other hand found almost no change in cell fragility or sensory values in cod superchilled up to 20 days between -1° and -3°C (cited in Love, 1966b ; Merritt, 1965).

Several systems had been used to superchill fish but with all of these systems the fish flesh is indisputably partially or shallow-frozen, and this is evidenced by gaping myotomes and large ice crystals that produce considerable thaw-drip (Merrit, 1965; Roach et al., 1966; Carlson, 1967), and by biochemical proof of cellular disruption (Gould, 1970). Such flesh is indeed considered frozen although partially (Merrit, 1965; Partmann, 1965; Power and Morton, 1965; Carlson, 1967) and therefore may not be considered fresh in this respect.

Another critical factor in superchilling is the temperature control (Merritt, 1965; Carlson, 1967). According to Watermann and Taylor (1967) a change of about one degree lower than -2°C makes the fish unsuitable for filleting or smoking due to ice formation.

Some fish species are more successfully superchilled than others; large sized fish with firm texture fare better than small fish (Anon., 1967). In salmon, superchilling not only retards bacterial growth, it also delays "belly-burn".

1.2.1.2. Present Status of Superchilling

Superchilling has been done using either refrigerated sea water (RSW) (Tomlinson et al., 1965) or refrigerated ethylene glycol solution (Uchiyama and Kato, 1974; Kato et al., 1974; Nowlan et al., 1975) and it has been practised successfully in some countries for several years (Scarlatti, 1965; Anon., 1967). Presently several fish processing plants are using this process. In Japan it is extensively used as a means of preserving several types of fish (Uchiyama et al., 1978 ; Aleman et al., 1982 ; Kakuda et al., 1984 ; Kakuda and Uchiyama, 1983). In China superchilling has also been effectively used to prevent bacterial spoilage of fish and also to suppress enzymatic decomposition (Ming, 1981). In New Zealand, several processing plants have started using the method (Lee and Toledo, 1984). In Canada, the method is currently used by Fishery Products International to hold fresh fish for longer periods (personal communication with Tom Morse, Fishery Product International, St. John's).

However, further study is needed to understand the type and extent of deterioration which occurs during storage of different species of fish and to learn how

intraspecific factors influence deterioration during storage of fish muscle at partial freezing temperatures.

In summary, it appears from previous studies that superchilling low[redacted]e quality of fish more so than 0°C storage during the early stages of storage beca[redacted]lerates endogenous biochemical reactions. However, because superchilling retards bacterial action more so than 0°C storage, it appears to extend the edible storage life considerably longer than 0°C storage.

1.2.1.3. Ice Formation in Superchilled Fish Muscle

Ice formation normally begins in fish muscle at temperatures of about -1°C and increases as the temperature drops, so that at -4°C about, 75% of the water in the fish is frozen (Dyer, 1968). Freezing of water in biological tissue proceeds in two steps, namely nucleation and crystal growth, and these steps occur as a result of heat removal from the fish. During the first stage of slow freezing, the temperature falls fairly rapidly to just below 0°C, the freezing point of water. During the second stage the temperature remains essentially constant for a period of time and this stage is known as the "period of thermal arrest". Approximately 75% of the water is turned into ice during the second stage. The temperature again begins to fall during the third stage of freezing where the rest of the water freezes. As the water in the fish freezes out as pure crystals of ice, the concentration of salts and other compounds in the remaining unfrozen water increases substantially.

1.3. Factors Governing Fish Spoilage

The process of fish spoilage is very complex and is influenced by a number of factors including fish species, catching method, fishing ground, season, nature and load of bacterial contamination, as well as post-harvest physical or chemical changes. Above the freezing point of fish, bacterial action is by far the most important factor in producing the undesirable alterations in the flavour, odour, and appearance of the fish. However in frozen fish severe alterations of protein structure, usually termed denaturation, occurs and this results in a significant deterioration of the texture of the fish or product. Another important deteriorative reaction which is important in certain species of fish during iced or frozen storage is hydrolysis and / or oxidation of fat.

1.3.1. Protein Denaturation of Fish Muscle during Frozen Storage

The term "protein denaturation" has been defined by Sikorski et al. (1976), as "the complex phenomenon involving the alterations of the secondary and tertiary structures of proteins due to breakage of the bonds that contribute to the stability of the native protein conformation without rupture of the covalent linkages between carbon atoms in the polypeptide chains". Protein denaturation in frozen fish muscle alters the protein so it is no longer soluble or extractable by salt solutions under conditions in which the native protein is soluble or extractable (Cowie and Mackie, 1968).

Fish protein deterioration during frozen storage is reflected mainly by a drastic decrease in protein solubility, due to alterations in the salt soluble or myofibrillar fraction. The sarcoplasmic proteins do not undergo significant changes in solubility during frozen storage. Most of the alterations occur in myosin or actomyosin. It has been

demonstrated that isolated fish myosin aggregates upon incubation in frozen solution (Connell 1959, 1960, 1962, 1963). Aleman et al. (1982) found that the molecules of cod myosin in solution aggregate upon standing especially rapidly when frozen. Myosin from mammalian and avian muscles appears to be much less susceptible to denaturation during frozen storage (Hultin, 1985).

The factors that appear to contribute to this protein denaturation can be grouped as : factors related to changes in fish moisture; factors related to changes in fish lipids; and the enzymic formation of formaldehyde from trimethylamine oxide.

1.3.1.1. Moisture

[redacted]duction in the free water content of frozen fish appears to create an environment conducive to protein denaturation. Ice formation may lead to damage caused physically by formation and accretion of ice crystals, or by dehydration of the sarcoplasm and an increase in concentration of salt and other solute.

Tissue damage as a result of ice crystal formation is well documented. For instance, it has been shown that freezing at a slow rate causes the formation of inter- and intracellular ice crystals which lead to breakage of the cells, rupturing of membranes, and disordering of the ultrastructure of the cells and tissues. According to Love (1968), the size and location of ice crystals found in frozen fish are influenced by the physiological status of the fish, the freezing rate, the storage time, and temperature fluctuations. During frozen storage of food, the small ice crystals have a tendency to melt and aggregate to form larger ones. Kent (1975) showed that there is a gradual accretion of ice in the frozen (ice) system at the expense of the unfrozen (bound and liquid) water fraction.

Fluctuation in food storage temperature, even at low freezing temperatures, accelerates the growth in the size of the ice crystals. According to Dyer and Dingle (1961), the effect of temperature fluctuation on ice accretion is more prominent in the critical freezing zone of -0.8°C to -5°C. Also, storage at temperatures below freezing results in a continuous pressure being exerted upon the cellular ultrastructure by the ice crystals, causing a disruption in the orientation and organization of the sarcoplasmic organelles.

Proteins are amphiphilic and are built up from hydrophobic and polar amino acids. Proteins vary in their residue content and amino acid sequence. The integrity of the native structure of proteins is influenced by a variety of forces, such as hydrophobic interactions, hydrogen bonding, ionic bonding, etc. For example, the stability of the three dimensional structure of protein molecules is highly dependent on a network of hydrogen bonds, many of which are mediated through water molecules (Shenouda, 1980). Thus, dehydration of protein molecules through freezing can result in a disruption of the H-bonding system, as well as, exposure of the surface regions of the protein molecule, and consequently, leaving these surfaces available for other interactions. Hydrophobic-hydrophobic and hydrophilic-hydrophilic interactions could then take place, either within the same protein molecule, causing deconformation of the three dimensional structure, or between adjacent protein molecules, indicating protein-protein interactions and consequently aggregation (Sikorski et al., 1976).

1.3.1.2. Increase in Salt Concentration

During the slow freezing of fish muscle, part of the water becomes unavailable as a solvent, but a considerable portion may remain unfrozen, even at temperatures well below the initial freezing point. According to Kent (1975), more than 90% of the moisture freezes out at common freezing temperatures (-10°C to -20°C), and this causes about a tenfold increase in the concentration of soluble solutes in the remaining free water. An increase in salt concentration is known to affect cell permeability and protein properties. Calcium and magnesium have the ability to form ionic cross-linkages between polypeptide chains and the increase in their concentration has been shown to cause the contraction of muscle actomyosin associated with ATP splitting (Briskey and Fukazawa, 1971). Both ions are involved in the hydrolysis of the organic phosphates in the tissues, as well as, polymerization of actin and myosin. Calcium ions enhance the polymerization of G-actin to F-actin, thus favouring protein interactions. Sodium and calcium chloride may also be indirectly involved in protein changes by activating the hydrolysis of lipids. Furthermore, inorganic salts can alter the conformation of proteins by participating in the formation of lipid-protein complexes.

1.3.1.3. The Influence of Lipids and Fatty Acids

The effect of lipids on protein during frozen storage of fish varies according to the state of the lipids. Intact fats and phospholipids (unhydrolyzed, not oxidized) differ in their reactivity with protein from their hydrolyzed and/or oxidized products. Also free fatty acids (FFA) influence proteins differently from the oxidised fatty acids. Even though intact lipids can play a protective role for proteins in situ they can also have a detrimental effect on proteins by forming lipoprotein complexes which denature the protein or make them more liable to denaturants. It appears that in fatty fish the neutral

triglyceride lipid droplets dissolve the FFA and thereby neutralize their hydrophobic effect on protein or compete with them for the binding sites on protein. Love and Elerian (1965) in a study on the cryoprotective effect of glycerol on frozen cod muscles postulated that the added glycerol might indirectly protect the protein as a solvent by lowering the concentration of cell salts formed in the frozen material.

During frozen storage of whole or minced fish the pressure exerted by ice crystals on the cellular structure and the breakdown in membranes or deformation in other microorganelles can lead to disorientation or liberation of lipid and protein components from natural compartments and open the way to new forms of contact between lipids and proteins. Since these moieties are derived from different locations in the cell, their interaction can form new lipid protein complexes and this may affect the textural quality of the muscle tissue.

A correlation between decreased protein extractability and the accumulation of FFA in frozen-stored tissue has been observed. Polyunsaturated fatty acids insolubilized more fish myofibrillar proteins than did less unsaturated ones (King et al., 1962) and short chain FFA were more reactive than long chain FFA. FFA are believed to interact primarily with the myofibrillar proteins. Actomyosin was the prime target according to many researchers and it is largely unextractable in salt solution after reaction with FFA. According to Sikorski et al. (1976), the FFA attach themselves hydrophobically or hydrophilically to sites on the protein surface, consequently creating a more hydrophobic protein surface and this can result in a decrease in protein solubility. Lipid oxidation, in addition to causing rancidity, can result in free radicals and oxidised products that

interact with proteins. Therefore, lipid oxidation can cause changes in the nutritional and functional properties of fish proteins (Sikorski et al., 1976).

1.3.1.4. TMAOase Activity

The formation of formaldehyde (FrHO) and dimethylamine (DMA) has been observed in fish species that are characterised by poor keeping qualities during frozen storage (Shenouda, 1980). The fate of trimethylamineoxide (TMAO), a component that occurs in marine fish and shell fish, was carefully studied and its degradation in frozen fish muscle into the secondary amine DMA and FrHO in a 1:1 molar ratio was elucidated (Amano and Yamada, 1965). A positive correlation between the formation of DMA and deterioration in fish texture, particularly in the gadoid family, has been demonstrated. TMAOase associated with muscle cells catalyzes the following reaction at freezing temperatures :

$$\underset{\text{(TMAO)}}{(CH_3)_3NO} \xrightarrow{\text{Enzyme (TMAOase)}} \underset{\text{(DMA)}}{(CH_3)_2NH} + \underset{\text{(FrHO)}}{HCHO}$$

TMAO is naturally present in most marine animals and its physiological role is believed to be that of osmoregulation. TMAO is a major part of the relatively high non protein nitrogen content of fish muscle.

The deterioration in quality and texture of frozen stored fish associated with the enzymatic breakdown of TMAO is basically attributed to the formation of FrHO rather than DMA. A proposed mechanism by which FrHO affects proteins at the molecular level is based on the ability of FrHO to bind covalently to various functional groups in

the protein and hence cause a deformation accompanied by cross-linking between the protein peptide chains via methylene bridges. Walker (1964) identified various reaction sites of FrHO in protein molecules and these included amino, amido, guanido, thiol, phenolic, imidazole, and indolyl residues.

The extent to which each of these different factors contribute to protein denaturation in frozen fish is not completely understood at this time.

1.4. Distribution of Antifreeze

Many marine fish, including the Atlantic cod *Gadus morhua* may live in an environment that is below the normal freezing point (FP) of most other organisms. Fish do not survive freezing temperatures if protective measures are not taken. One means by which marine organisms protect themselves against freezing is the formation of proteins that lower the freezing temperature of the blood. As early as 1953, some significant observations on freezing resistance were made on the blood sera of various polar fish (Scholander et al., 1953, 1957; Gordon et al., 1962). These antifreeze proteins were found to be soluble in trichloroacetic acid and were shown to lower the freezing point of water to a greater extent than equimolar concentrations of NaCl. Scholander and Maggert (1971) and Hargens (1972) also confirmed the presence of the antifreeze-like substance in the saffron cod *Eleginus gracillis*.

DeVries and Wohlschlag (1969) first reported the existence of an antifreeze glycoprotein in Antartic fish. More recent confirmation of the presence of antifreeze has been reported in the winter flounder *Pseudopleuronectes americanus*, (Hew and Yip, 1976; Fourney et al., 1984).

The antifreeze proteins can be classified into two groups based on the presence or absence of carbohydrate. Antifreeze polypeptides (AFP) with no carbohydrate moiety have been described in the winter flounder *Pseudopleuronectes americanus*, (Raymond et al., 1975); Bering Sea sculpin *Myozocephalus verrucosus*, (Duman and DeVries, 1976); shorthorn sculpin *Myozocephalus scorpius*, (Hew and Yip, 1976); sea raven *Hemitripterus americanus*, (Hew et al., 1981; Slaughter et al., 1981); and the ocean pout *Macrozoarces americanus*, (Hew et al., 1984).

Flounder AFP is the most studied of the lot described above. The molecular weights of AFP have been reported to be 6,000, 8,000, and 12,000 daltons (Raymond et al., 1975; Duman and DeVries, 1976). Hew and Yip (1976) described AFP of molecular weight 10,000 daltons which could fragment into at least seven components with molecular weights ranging from 3,300 to 4,500 daltons (Fourney et al., 1984).

Glycoprotein antifreezes (AFGP) have been described in saffron cod *Eleginus gracilis*, polar cod *Boreogadus saida*, Labrador rock cod *Gadus ogac*, the tomcod *Microgadus tomcod*, and the Atlantic cod *Gadus morhua*, (DeVries et al., 1970; Hargens, 1972; Raymond et al., 1975; Van Voorhies et al., 1978; Osuga and Feeney 1978; Hew et al. 1981; Fletcher et al., 1982a).

In contrast to the structural diversity exhibited by the AFP, all of the glycoprotein antifreezes isolated have similar, if not identical structures in which the basic repeating unit is a glycotripeptide of alanine-alanine-threonine with a dissacharide linked to the threonine residue (Fletcher et al., 1982a). These antifreeze glycoproteins have molecular weights ranging from 2,600 to 33,000 daltons.

1.4.1. Properties of the Antifreeze Glycoproteins and Polypeptides

The two classes of antifreeze appear to function in the same way. They depress the freezing point in a non-colligative manner, that is they lower the freezing point much more than what would otherwise be expected based on the osmolality of their solution and they do not affect the melting point (DeVries, 1980). That is, these antifreezes show thermal hysteresis. When the thermal hysteresis of these antifreeze glycoproteins and polypeptides are plotted against the concentration of the antifreeze a convex curve is obtained indicating a saturation effect of the thermal hysteresis at higher protein concentrations. Freezing point depression due to thermal hysteresis is additive with that due to the colligative properties of the antifreeze proteins.

The noncolligative lowering of the freezing temperature is unique to the antifreeze proteins and this has formed the basis for their identification, purification and quantitation in plasma. The mechanism(s) whereby these antifreeze proteins prevent(s) ice crystal nucleation and growth is (are) not well understood. However, it appears that the antifreeze proteins bind to ice surface and somehow prevent the addition of water molecules to the ice lattice (DeVries, 1980). Recently, it was demonstrated that mixtures of various compounds with both low and high molecular weight fractions of antifreeze glycoprotein show synergistic depression of the freezing temperature in solution (Kerr et al., 1985; Caple et al., 1984).

1.4.2. Seasonal Variation of the Antifreeze Proteins

For certain species of fish, the freezing point depression, thermal hysteresis and antifreeze protein levels in the blood plasma have been found to show annual cycling with the maxima in winter months and minima in the summer months (Fletcher, 1977; Hew et al., 1980). The maxima levels of antifreeze occur during the winter months when the water temperature is about -1°C to -2°C and the minima in the summer months with water temperatures of about 10°C to 14°C. Fletcher et al. (1982b) found this seasonal change to occur in the antifreeze, sodium and chloride ion levels in the blood plasma of Newfoundland Atlantic cod *Gadus morhua*. Sodium and chloride ions are also thought to play a role in the low temperature acclimation of some fish species (Umminger, 1976; Fletcher, 1977).

1.5. Rationale for Study

Over the past decade, investigators have established that many polar and subpolar marine fish possess protein and glycoprotein antifreezes that lower the freezing temperature of blood plasma below that expected on the basis of colligative properties (Feeney and Yeh, 1978 ; Devries, 1980). It is believed that these antifreeze proteins are a major means by which such fish are protected from freezing.

The Atlantic cod *Gadus morhua* lives in an environment whose temperature periodically falls to subzero levels (-1°C to -2°C). These fish appear to have specialized means of protecting themselves from freezing (Fletcher et al., 1982b).

According to Love (1968) the extracellular fluid of muscle has a similar composition as the blood plasma. Thus the presence of antifreeze in the blood may be indicative of

antifreeze in the muscle tissue. Varying the muscle freezing point may reflect differences in susceptibilty of muscle protein to denaturation during partial freezing. It was therefore hypothesized that *freezing point and the degree of protein denaturation and cellular damage during frozen storage at the superchilling temperature of -3°C differ with the acclimation temperature of Atlantic cod* (**Gadus morhua**).

Chapter 2

Materials and Methods

2.1. Materials

2.1.1. Atlantic Cod Gadus morhua

Atlantic cod (about 100) were caught at Portugal Cove near St. John's, Newfoundland, from May to August by local fishermen using cod traps. The fish were brought live to the Marine Sciences Research Laboratory and maintained in a 40,000 L sea water aquarium at ambient sea water temperature and photoperiod. The fish were fed capelin and the average ambient water temperature of the aquarium was recorded daily and the average computed for each month.

Some of the fish were transferred from the main aquarium to two 2,500 L aquaria, one maintained at 10°C and the other at 0°C. These cod were acclimated at 0°C (cold temperature acclimated fish, CTAF) or 10°C (warm temperature acclimated fish, WTAF) for a period of three weeks while being fed on capelin prior to sacfifice for studies aimed at deciphering whether acclimation temperature had any effect on parameters such as freezing point (FP) and thermal hysteresis (TH) of plasma and muscle fluid of the fish as well as moisture and ash composition of the fish muscle. The rest of the cod fish in the 40,000 L aquarium, referred to as ambient sea water fish (ASWF), were studied alongside CTAF and WTAF. The influence of partial freezing on

deteriorative reactions related to eating quality were also investigated. The Atlantic cod used in the study were about 55cm to 65cm long.

2.1.2. Chemicals

The chemicals listed below were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A.: sodium chloride, copper sulphate, sodium hydroxide,bovine serum albumin (BSA), trichloroacetic acid (TCA), sodium sulphate,trimethylamine (TMA) HCl, potassium hydroxide and titanium chloride.

The following chemicals were purchased from Fischer Scientific Company Ltd: xylene, ethanol, eosin Y, formaldehyde, toluene, ethylene glycol, and picric acid.

The following microbiological reagents were obtained from Difco Laboratories, Detroit, Michigan, U.S.A.: yeast extract, tryptone, peptone, dextrose and agar.

Thioglycolate was purchased from BBL Microbiol. Becton Dickinson and Co., Cockeystille, MD, U.S.A. BBL-Anaerobic system containing a holding jar, with rack, palladium catalyst and CO_2 generator envelope and indicator were purchased from Canlab Division of McGraw Supply Ltd., Canada.

Gentamycin sulphate was purchased from Schering Corporation, Kenilworth, N.J., U.S.A. Paraplast tissue embedding medium (paraffin) was purchased from Sherwood Medical Industries, St. Louis, Missouri, U.S.A.

Histoclad and Mayer's albumin fixative were purchased from Clay Adams division of Becton Dickinson and company (BD), Parsippany, New Jersy, U.S.A.

Dimethylamine (DMA) hydrochloride was purchased from J.T. Baker Chemical Co., Phillipsburg, New Jersey, U.S.A.

2.2. Methods Used

2.2.1. Treatment of the Cod

Fish were sacrificed by stunning them on the head with a blow and immediately cutting the jugular vein and tail in order to facilitate bleeding. After bleeding, they were filleted and skinned. From one fillet, sections of about 10g to 20g each were removed from the anterior end of the muscle between myotomes 9 to 20 (Appendix 1) and dipped in 0.3% gentamycin sulphate solution for 2 sec. The excess antibiotic was drained off using paper tissue and the muscle sample was then vacuum packed in Kapak heat sealable pouches (Kapak Corporation, Bloominton, Minnesota, U.S.A.) and stored at 0°C for 3, 6, 9, 12, 15, 18 or 21 days. The samples were vacuum packed in order to exclude air from the pouches. The fillet obtained from the other side of the fish was similarly sampled and dipped in 0.3% gentamycin sulphate solution (recommended by Dr. T. Patel, Biochemistry Department, Memorial University of Newfoundland) also for 2 sec, vacuum packed, and stored at -3°C $\pm$ 0.1°C in a Haake's water bath (Fisher Scientific Co. Ltd., Ottawa, Canada) for 0, 3, 6, 9, 12, 15, 18, or 21 days. The effective concentration of the gentamycin sulfate on the surface of the fillet after the dip was not determined. These vacuum packed and sealed samples will be referred to as "muscle specimen" throughout the text. After each "muscle specimen" was stored at the storage time indicated it was analysed for extractable protein (EP), pH, % drip loss, % extracellular area, physiological free amino acid, TMAO, TMA, and DMA. For this and all other experiments or analyses described below, measurements were run in duplicate on one fish and then repeated with "muscle specimen" from a second fish.

2.2.2. Measurement of Freezing and Melting Points

Freezing and melting points of blood plasma and muscle fluid were measured at monthly intervals with cod samples obtained from the main aquarium (40,000L sea water aquarium) and from cod acclimated at 10°C or 0°C for three weeks.

The blood plasma was obtained according to the method of Fletcher et al., (1982b). Approximately 2.5 mL of blood was taken from cod fish caudal blood vessel using plastic syringes equipped with 21-gauge needles. The plasma was separated from the cells by low speed centrifugation (4000xg) and was stored at -20°C. Antifreeze protein activity was determined by measuring the plasma freezing and melting points using the method of Slaughter and Hew (1981), with a freezing point osmometer (Model 3R, Advanced Instruments, Needham Heights, MA). Thermal hysteresis, a measure of antifreeze activity, was determined from the difference between the freezing and melting points of the sample (Slaughter and Hew, 1981).

About 10g of cod fillet was taken from the anterior end and homogenized for 30 sec using a Polytron homogenizer (Brinkman model PCU 1, Rexdale, Ontario, Canada) at setting number 5. The homogenate was centrifuged at 8000xg at 0°C for 30 min and the supernatant was collected and stored at -20°C. The freezing and melting points of muscle fluid were determined using the method of Slaughter and Hew (1981). About 1 mL of the muscle fluid was dialysed using a spectraphor membrane tubing, 6,000 to 8,000 mol. wt. cutoff. After dialysis, the increase in volume of the muscle fluid was diluted with the same amount of water as found after dialysis. The FP for this diluted muscle fluid was measured as well as the undiluted muscle fluid.

2.2.3. pH Determinations

The surface pH of each "muscle specimen" was measured at the respective storage times using a surface electrode (Fisher Accumet pH Meter Model 140).

2.2.4. Free Drip Measurements

Free drip was collected into a measuring cylinder from the polyethylene pouches of each "muscle specimen" after allowing the samples to thaw at about 23°C for 1 hour. The "muscle specimen", from which free drip was collected after 0, 3, 6, 9, 12, 15, 18, or 21 days, was weighed and the weight loss used to calculate percentage free drip.

2.2.5. Estimation of Extractable Protein (EP)

Total protein in the fish tissue was determined using a modified form of the procedure by Cowie and Little (1960), and it involved transferring 1 g of "muscle specimen" taken from the region of myotomes 9 to 12 quantitatively with the aid of 5 mL de-ionized water into a pyrex test tube and digested using the standard micro-Kjeldhal technique (Lang, 1958) to estimate total protein nitrogen. For estimation of water extractable protein a sample of "muscle specimen", taken from near the head of the fish in the region of myotomes 9 to 12, was freed from connective tissues (myocomata) using a scalpel and approximately 1g of this muscle was weighed and placed into a flask containing 50 mL of cold de-ionized water (~0° to 4°C). The suspension was homogenized using a Polytron (Brinkman model PCU 1) at setting 6 for 1 min. The resulting macerate was then centrifuged at 8000xg for 30 min at 4°C. The clear supernatant was analysed for protein using Snow's (1950) modification of the Biuret method. The precipitate was further macerated in 50 mL of cold 5% NaCl (~0°C to 4°C) solution adjusted to pH 7.0 with 0.02M $NaHCO_3$. The resulting macerate was

centrifuged for 30 min at 8,000xg at 0°C and the clear supernatant was analysed for salt soluble protein using the method of Snow (1950).

2.2.6. Measurement of "Extracellular Area"

"Extracellular area" of partially frozen and ice stored "muscle specimen" was measured according to the method of Love (1966c), who described the space as "extracellular ice areas" to measure freezing damage. However,since part of the study was done for iced fish, it was considered appropriate to describe this area as "extracellular area". Specimens stored at 0°C and -3°C for various times were first rapidly frozen in liquid nitrogen. A uniform (5 mm thick) slice of the frozen specimen was then cut transversely with a microtome and placed in a Speedivac Pearce tissue dryer (Edwards High Vacuum Ltd., Manor Royal, Sussex, England) and dried under vacuum at -30°C for 4 h. The dried specimen slices were then embedded in melted "tissue preparation-paraffin wax" for 60 h. This method is essentially that of Koonz and Ramsbottom (1939). Transverse sections (~10 microns thick) were cut with a rotary microtome and mounted on a slide. The tissues on the slides were then washed in a series of solutions in the following order : xylene (5 min), xylene (5 min), 100% ethanol-xylene (1:1, for 5 min), 100% ethanol (2 min), 100% ethanol (2 min), 95% ethanol (2 min), 70% ethanol (2 min), 50% ethanol (2 min), 35% ethanol (2 min), excess de-ionized water (2 min), excess tap water (2 min), haematoxylin (5 min), rinse in excess water (2 min), 35% ethanol (2 min), 50% ethanol (2 min), 70% ethanol (2 min), 95% ethanol (2 min), eosin (5min), rinse in 95% ethanol (2 min), 100% ethanol (2 min), 100% ethanol (2 min), 100% ethanol-xylene (1:1 for 5 min), xylene (2 min), xylene (2 min).

The tissue was then fixed in histoclad solution and the sections were covered with a cover slip. Each slide was then mounted on a photo microscope and pictures of the transverse sections were taken at 160X magnification. The relative damage (RD) due to "extracellular area" was calculated by cutting out the extracellular areas and comparing their weight with the weight of the total space. Percent relative damage due to extracellular area was calculated as follows:

$$RD = \frac{\text{Extracellular area}_{(\text{day } x)} - \text{Extracellular area}_{(\text{day } 0)}}{\text{Extracellular area}_{(\text{day } 0,\ \text{iced sample})}}$$

For each sample two transverse sections were prepared and 6 field photomicrographs for each section were examined and used for the calculations. * See legend to Tables 3-9 and 3-10.

2.2.7. Microbiological Analysis

Approximately 10 g of "muscle specimen" from fish held at 0°C prior to sacrifice was prepared for total aerobic plate counts (TAPC), using sterile 0.1% (w/v) peptone water containing 0.5% NaCl as the diluent. To this "muscle specimen" was added 90 mL peptone water and the mixture was homogenized for 1 min using a Waring Blendor. Serial dilutions were made for pour plates from the prepared mixture. Duplicate samples were incubated at 20°C for 4 days and a volume of 1mL was plated in duplicate for each dilution. One mL of the diluted sample was withdrawn into sterile petri dish and Difco plate count agar was used as the medium (Gilliland et al., 1976).

For total anaerobic plate counts, the same homogenate and dilutions used in the determination of total aerobic plate count was done on thioglycolate agar plates prepared by dissolving 36.5 g thioglycolate and 15 g of Bacto agar in 1 L of de-ionized water. The plates were incubated at 20°C for 4 days in an anaerobic chamber containing BBL gas packets and indicator (Lee and Toledo, 1984).

2.2.8. Cell Fragility Method for Measuring Frozen Damage

About 200 mg of "muscle specimen" was freed of connective tissue by using a double scapel and homogenized using a polytron at setting 6 for 30 sec in 20 mL cold 1.15% formaldehyde solution incubated in an ice bath. The $A_{430\ nm}$ of the homogenates were measured spectrophotometrically using a Beckman DU-8 spectrophotometer at 2 sec intervals for 20 sec at 10°C. This method was essentially that described by Love and Mackay (1962).

2.2.9. Determination of Non-nitrogenous Components in God Muscle

Moisture, ash and some mineral elements in cod muscle were determined as follows:

2.2.9.1. Moisture and Ash

Moisture and ash content of cod muscle were determined using standard procedures described in Association of Official Agricultural Chemists (A.O.A.C, 1980). For the moisture content determination, weighed "muscle specimen" was heated to constant weight (for about 24 h) in a porcelain dish at 100°C.

The ash content of "muscle specimen" was determined by incinerating the dried

sample, from moisture analysis, in a furnace at 500°C for 24 h or until constant weight was obtained.

2.2.9.2. Determination of Na, Cl, Mg, Cu, Zn, K, and Ca

Samples of about 400 mg were weighed accurately into numbered teflon beakers. To each sample was added 5 mL of nitric acid in a beaker and it was covered with a watch glass. The beaker was heated gently for 3 h, then the watch glass was removed and the acid was boiled to near dryness. The residue was taken up in 100 mL of 0.2% nitric acid for flame atomic absorption analysis with a Varian AA-5 atomic absorption spectrophotometer to measure the cations listed above.

Chloride in the samples was separately determined as follows : samples (~400 mg) were accurately weighed into numbered teflon beakers, then 3 mL of nitric acid were added to each sample which were then covered with watch glasses. The beakers were heated gently for 3 h, then the residue was made up to 100 mL with water for analysis by Technicon Auto Analyzer II, (Environmental Protection Agency, E.P.A. 1983).

2.2.10. Determination of Nitrogenous Components in Cod Muscle

Trimethylamine (TMA), dimethylamine (DMA), and trimethylamine oxide (TMAO) and physiological free amino acids in cod muscle were separately determined as follows : approximately 10 g of the "muscle specimen" was homogenized in 40 mL of 10% TCA solution for 30 sec using a Polytron at setting 6. The extract was filtered using Whatman No. 1 filter paper. The total volume of the filtrate was noted. Aliquots of the filtrate were taken for the following analyses :

2.2.10.1. TMA Determination

TMA in the 10% TCA extracts was analysed using a modified form of the procedure by Dyer (1945), as described by Shewan et al. (1971). Approximately 1mL of the extract in a pyrex tube was diluted with 4mL of de-ionized water, 1mL of 4% formaldehyde reagent, followed by 10 mL toluene and 3 mL 45% potassium hydroxide solution. The tube was stoppered and shaken vigorously by hand about 40 times. Five mL of the toluene layer was pipetted into a small test tube containing about 0.3 g anhydrous sodium sulphate. The tube was then gently shaken a few times to dry the toluene. One mL aliquots of the dried toluene mixture was pipetted into a cuvette and 1 mL of 0.02% picric acid reagent was added and this was swirled gently to mix. The absorbance was read at 410 nm against a blank carried through the procedure. One mL aliquots of 0.682 mg% TMA hydrochloride standard solution were also carried through the above procedure.

2.2.10.2. TMAO Determination

TMAO was determined using a modified form of the procedure by Bystedt et al. (1959) as described by Yamagata et al. (1969). Approximately 2 mL of the 10% TCA extract were mixed with 1 mL of 1% titanous chloride solution in a test tube. The mixture was then heated in a water bath at 80°C for 1.5 min until the reddish-violet colour of titanous chloride solution had faded away. After the reduction was finished, the solution was quickly cooled under running water. Approximately 1 mL of this mixture was then used to determine the total TMA content using the method described above. TMAO content was given by the following equation:

$$\text{TMAO-N mg \%} = \text{Total TMA-N mg \%} - \text{TMA-N mg \%}$$

where total TMA-N mg % is the TMA-N mg % obtained by heating the sample solution with titanous chloride solution and TMA-N mg % is the TMA-N in the sample solution not reduced. TMAO (18.6% N, Regenstein et al., 1982) was calculated from TMAO-N by multiplying the latter by a factor 5.38, and TMA (23.7% N, Regenstein et al., 1982) was calculated from TMA-N by multiplying the latter by a factor 4.21.

2.2.10.3. DMA Determination

DMA was determined using the method of Dyer and Mounsey (1945). This involved diluting 1 mL aliquot of 10% TCA extract (containing 1 μg to 6 μg DMA-N) to 10 mL with de-ionized water. To this were added 1 mL of copper-ammonia reagent (20 g ammonium acetate and 0.2 g copper sulphate dissolved in 30 mL concentrated ammonium hydroxide all diluted to 100 mL) and 10 mL of 5% carbon disulphide solution in benzene. The mixture was heated in a water bath at about 45°C for 5 min. The tubes were then stoppered and shaken rapidly on a Burrel wrist action shaker (Model 75) for 5 min. Next, 1 mL of 30% acetic acid was added to the mixture which was shaken about 10 to 20 times until the solution was clear. The benzene layer was decanted, dried by shaking with about 0.4 g anhydrous sodium sulphate and the yellow colour of copper dimethyldithiocarbamate was measured in the DU-8 spectrophotometer at 440 nm against a blank made with de-ionized water instead of the sample solution. DMA (31.1% N, Regenstein et al., 1982) was calculated by multiplying DMA-N by a factor 3.19.

2.2.10.4. Physiological Free Amino Acids

Approximately 1 mL of the 10% TCA extract was deproteinized with 10% sulfosalicylic acid and analysed for physiological free amino acids using a Beckman 121 MB amino acid analyzer.

2.2.11. Sensory Evaluation of Commercial Fillets

Sensory quality of commercial fish fillets (purchased from Fish Plaice, St. John's, Newfoundland) was evaluated by a panel consisting of 20 individuals who were not trained, but were familiar with the taste of cooked cod fish. The fish fillets were vacuum packaged and stored on ice or partially frozen for various intervals of time before cooking and evaluation by the panelists. A sample of the evaluation chart is provided as Appendix 2: The panelists were asked to evaluate baked cod fish for color, texture, appearance, flavor and acceptability on a 5 point scale. About 3 cm^3 dices of white meat were individually wrapped in aluminium foil and baked on trays at 190°C for 20 min. Analysis of variance and Student's t-test were employed to analyze the data for statistical significance.

Chapter 3

Results and Discussion

3.1. Freezing Point and Thermal Hysteresis of Blood Plasma and Muscle Fluid of Ambient Sea Water Cod Fish and Cod Fish Acclimated at 0°C or 10°C

The freezing points (FP) of blood plasma from fish taken from the 40,000 L aquarium at different times of the year are presented in Fig. 3-1. The results indicate that plasma FP was minimum in February (-1.11°C), the same month in which the lowest ambient water temperature was observed. A similar pattern was observed for the the FP of the muscle fluid except that a lower value was found in March (-1.30°C) than in February (-1.08°C). The thermal hysteresis (TH) data presented in Fig 3-2 indicates that TH was very low from May up to December for both plasma and muscle fluid. The highest values for both plasma and muscle fluid were obtained in the coldest month (February). Table 3-1 summarizes the results of the FP and TH of samples derived from WTAF or CTAF muscle fluid. The freezing point of muscle fluid from CTAF (-1.02°C) was lower than that derived from WTAF (-0.90°C). Table 3-1 also indicates that the samples derived from CTAF showed more thermal hysteresis (0.40°C) than those derived from WTAF (0.06°C). Table 3-1 also shows that dialysed muscle fluid from CTAF and WTAF had similar freezing points. Dialysis increased the freezing points of muscle fluid derived from both CTAF and WTAF. This finding indicates that dialyzable components (< 6,000 daltons) contribute to the depression of FP of the muscle fluids.

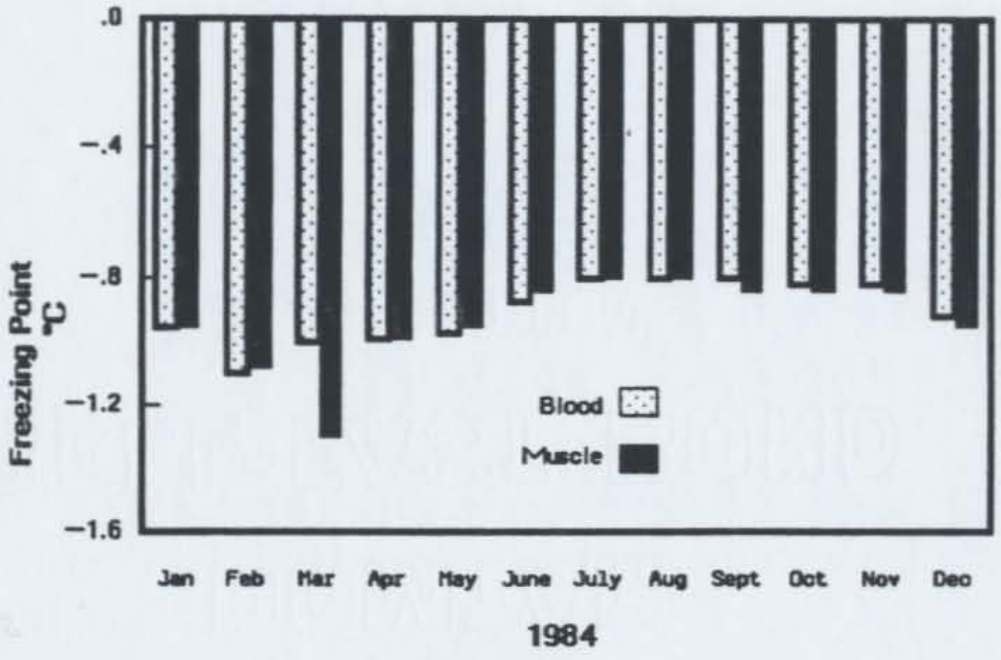

Figure 3-1: Freezing point of blood plasma and muscle sarcoplasm from cod at different times of the year

Data presented in Fig. 3-1 are averages of duplicate determinations with each of four fish.

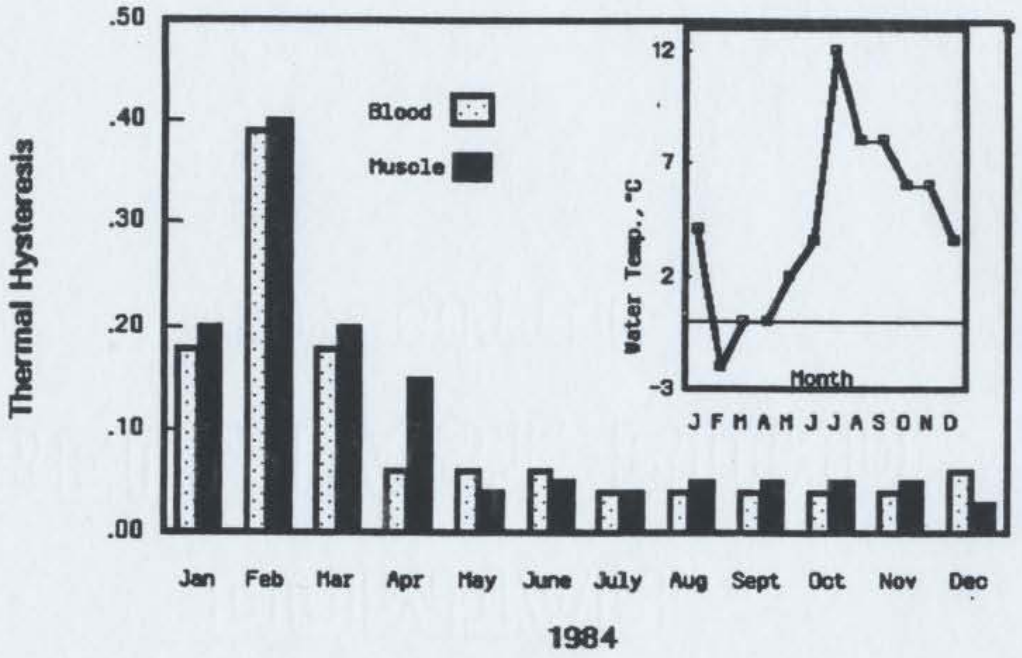

Figure 3-2: Thermal hysteresis of blood plasma and muscle sarcoplasm from cod at different times and different ambient water temperatures of the year

Data presented in Fig 3-2 are averages of duplicate determinations with each of four fish.

Table 3-1: Freezing point and thermal hysteresis of muscle fluid from fish acclimated at 0°C or 10°C (mean values with standard deviations

	Dialyzed Muscle Sarcoplasm	Muscle Sarcoplasm	
Sample	FP[1] (°C)	FP[2] (°C)	TH[2] (°C)
CTAF	-0.07 ± 0.01	-1.02 ± 0.03	0.40 ± 0.00
WTAF	-0.09 ± 0.01	-0.90 ± 0.02	0.06 ± 0.00

[1] freezing point of dialysed muscle fluid (dialysed against water using 6,000 to 8,000 mol. wt. cut off dialysis membranes) ; [2] data for raw muscle fluid (undialysed). Data presented in Table 3-1 are averages of duplicate determinations with each of four fish.

3.1.1. General Discussion : Freezing Point and Thermal Hysteresis as a Function of Season and Temperature Acclimation

The study indicates that the most thermal hysteresis occurred in the muscle fluid as well as the blood plasma of the fish during February the same month in which the lowest ambient water temperature of -2°C was observed. This time of year was about the same as that when antifreeze glycoproteins were identified in blood plasma of Atlantic cod (Fletcher et al., 1982a). According to Fletcher (1981) low water temperatures do not appear to play a role in stimulating antifreeze production in the winter flounder, it might probably be the case for the Atlantic cod. Since the CTAF exhibited TH (which is indicative of the presence of antifreeze glycoproteins), it is not surprising then to find parallelism in freezing point of muscle fluid and blood plasma since the two have been described to have similar composition (Love, 1968). Going by the findings in Fig. 3-2, it may be inferred that antifreeze glycoproteins were more or less absent in the period from July to November for both muscle fluid and blood plasma. Fletcher (1977) has also established that the antifreeze glycoproteins disappeared from cod plasma in May and June. The CTAF's antifreeze glycoprotein as determined by TH is an indication of adaptations to resist freezing. The disappearance of antifreeze glycoproteins in WTAF muscle fluid is an indication of the adaptive changes, i.e. such species (e.g. Atlantic cod) synthesizes these substances when it needs them in the colder months, then rid themselves of it as the habitat temperature warms up. Umminger (1976) suggested similar adaptations in the killifish during winter brought about by increased levels of glucose. These levels dropped to minimum in the summer. The study further indicates that these AFPs are present following the death of the animal and thus may play a role in the postmortem changes of the fish. There is no literature available for any fish muscle fluid antifreeze protein activity.

3.1.2. Non-nitrogenous Components Identified in the Muscle of Fish Acclimated at 0°C or 10°C

The moisture content measured for "muscle specimen" derived from CTAF appears to be slightly lower than that measured for the sample from WTAF (Table 3-2). By difference, the total solids of CTAF (18.97%) appears to be only slightly higher than that of WTAF (18.64%). Table 3-2 further indicates that there appears to be more ash - and hence more mineral elements - in the samples derived from CTAF (1.41%) than those derived from WTAF (1.26%). The difference in the ash content could therefore imply differences in mineral content in the fish samples and may partly be responsible for the observed FP depression of CTAF muscle fluid, since statistical analysis indicated significant differences in the ash contents derived from CTAF and WTAF at the 5% level.

Table 3-2: Non-nitrogenous components identified in the muscle of cod fish acclimated at 0°C (CTAF) or 10°C (WTAF).

Component	CTAF	WTAF
Moisture (%)	81.03 ± 0.22	81.89 ± 0.57
Total Solids[1] (%)	18.97 ± 0.23	18.11 ± 0.08
Crude Ash (%)	1.41 ± 0.03[a]	1.26 ± 0.09[a]
	Mineral Elements in mg/100 g tissue	
Calcium	12.22 ± 1.22	8.11 ± 0.24
Copper	0.19 ± 0.03	0.20 ± 0.06
Zinc	0.53 ± 0.01	0.40 ± 0.02
Potassium	327.52 ± 25.01	287.25 ± 42.43
Magnesium	25.33 ± 1.20	20.25 ± 2.80
Sodium	60.61 ± 2.70[a]	57.26 ± 1.18[a]
Chloride	192.30 ± 2.05[a]	155,59 ± 2.16[a]

[1] By difference from moisture content. Data presented in Table 3-2 are averages of duplicate determinations with each of four fish. [a] values in same row were significantly different ($p<0.05$).

3.1.2.1. Mineral Composition of Muscle from CTAF or WTAF

The result of the mineral composition of the ash derived from muscle samples from both cold and warm temperature acclimated fish samples were similar (Table 3-2). The potassium content was found to be the highest of the lot measured and this appeared to be higher for CTAF (~330 mg%) than WTAF (~290 mg%). Statistical analysis revealed no significant difference between the potassium content of the two samples ($p<0.05$). However, there was a significant difference ($p<0.05$) between the sodium and chloride levels of the two samples and these differences in sodium and chloride levels could be due to seasonal variation which would reduce the muscle fluid's FP and probably give some measure of thermal protection. Similar observations in the plasma of cod have been made by Fletcher (1977) and Hew et al. (1981) regarding the sodium and chloride levels, being highest in the coldest month and lowest in the warmer months. Fletcher (1981) and Fletcher et al. (1982b) has also indicated that temperature appears to be the major environmental factor promoting the changes in Na and Cl concentrations. Similar observations have been made in studies with North Sea cod (Harden-Jones and Scholes, 1974). Increased plasma electrolyte concentrations following acclimation to low temperature appears to be the rule in marine fish (Umminger, 1969). It is also clear from Table 3-2 that the minerals analyzed do not account for all the ash derived from the fish muscles. This is probably because not all the mineral elements that make up the ash were looked for. For instance, both sulfur and phosphorous are known to be present in significant amounts (~200 mg% each) in fish muscle. Aside from P and S, there are other minor elements such as Fe, F, Mn, I, etc. whose levels were not determined.

3.1.3. Nitrogenous Components Identified in Cod Muscle From Fish Acclimated at 0°C or 10°C

The influence of acclimating cod muscle at 0°C or at 10°C on various nitrogenous components in cod muscle is summarized in Table 3-3. Crude protein content of muscle from CTAF (18.70%) was found to be higher than the crude protein content of muscle from WTAF (16.20%). These values were found to be statistically different at the 5% level. The crude protein values were greater than the sum of water and salt soluble protein. Other sources of nitrogen in cod muscle include ~ 2.5% connective tissue protein (Ang and Haard, 1985), some contractile muscle protein and non-protein nitrogen compounds such as peptides, TMAO and free amino acids (Hultin, 1985). The free amino acid content of WTAF was found to be lower than that of CTAF (Table 3-3). These were found to be statistically different at the 5% level. The TMAO content of CTAF and WTAF was similar prior to storing the samples (Table 3-3). These data indicate that the concentration of TMAO, a major low molecular weight solute involved with osmoregulation, does not appear to vary with temperature adaption and therefore cannot account for the lower freezing point of CTAF. The crude protein and extractable protein from cod fish acclimated at ambient temperature and photoperiod in February and July were similar to that of CTAF and WTAF respectively.

Table 3-3: Nitrogenous components identified in cod muscle[1] from fish acclimated at 0°C or 10°C

Sample	Crude Protein[2] (%)	EP[3] (%)	FAA[4] (mg%)	TMAO (mg%)
CTAF	18.70 ± 0.62^{a}	12.70 ± 0.45^{c}	500 +20	415.27
WTAF	16.20 ± 0.79^{a}	9.71 ± 0.21^{c}	340 +20	415.81
WF (Feb)	18.25 ± 0.86^{b}	13.20 ± 0.58^{d}	ND	ND
SF (July)	16.88 ± 0.59^{b}	9.87 ± 0.46^{d}	ND	ND

[1] Fish muscle was less than 1 h postmortem at 0°C; [2] determined by multiplying Kjeldahl N by 6.25 (includes non-protein N, see section 2.2.5.); [3] sum of water extractable and salt extractable protein; [4] free amino acids. Data presented in Table 3-3 represent averages of duplicate determinations of four fish. TMAO values are averages of duplicate determinations with each of two fish. ND = not determined. Values with the same superscript are significantly different ($P<0.05$).

3.2. Biochemical Indices of Cellular Damage During Partial Freezing

3.2.1. Extractable Protein (EP)

Details of the changes in EP in the course of the storage period are presented in Appendix 3 and Fig. 3-3. Partial freezing of the fish samples slowed down the process of protein damage much more than ice storage, based on the water and salt extractable protein values for both the samples derived from CTAF and WTAF. For instance, 21 days storage caused a loss in EP in the samples as follows : 15.32% in partial frozen samples derived from CTAF ; 16.72% in partial frozen samples derived from WTAF ; 20.47% in the iced samples derived from CTAF ; and 30.18% in the iced samples derived from WTAF. Values in Appendix 3 further reveal that for water soluble protein, the iced samples showed lesser overall denaturation compared to their partially frozen counterparts. However, the salt extractable protein values indicate that for CTAF fish, the iced samples suffered *less* damage up to, and including 6 days of storage after which the situation reversed with the partially frozen samples showing lesser damage for the rest of the study. The salt extractable protein values for WTAF also indicate that the iced samples suffered *less* damage up to, and including, 3 days of storage after which the damage in the iced samples exceeded that of the partially frozen samples. Previous studies have indicated that partially frozen samples undergo *greater* damage than iced samples (Gould and Peters, 1971), similar to the situation observed with the water extractable protein in this study.

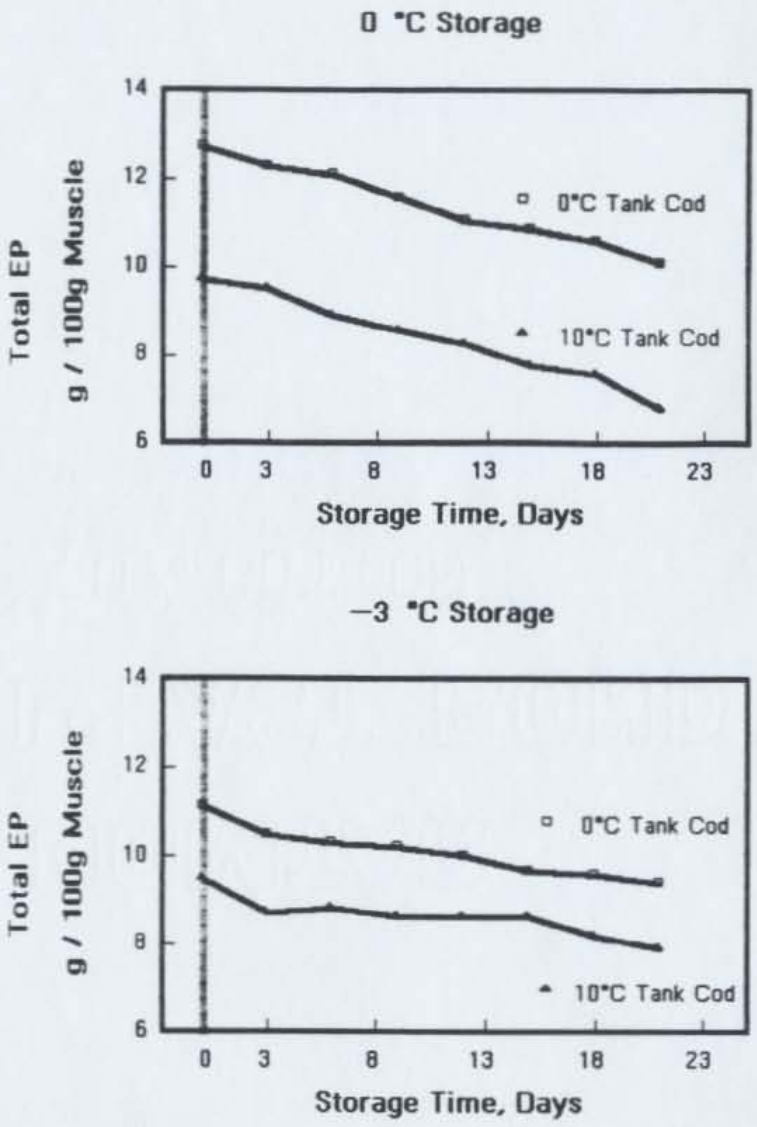

Figure 3-3: Changes in total extractable protein as a function of storage time at 0°C or at -3°C

Data presented in Fig. 3-3 are averages of two experiments analyzed in quadruplicate. Day 0 samples were analyzed after approximately 3 h storage at the appropriate temperature.

Power et al. (1969) reported that the EP of cod fillets held at -3°C dropped rapidly during storage to a value of 25% of the total EPN after 37 days storage while the samples stored on ice showed essentially no decrease in EPN over the storage period. A plausible explanation may be the good temperature control (-3 $\pm$ 0.1°C) of partial freezing together with the application of antibiotic in the present study resulted in less biochemical damage leading to loss of EP. Loss of extractable salt soluble protein is normally attributed to denaturation and aggregation of these proteins. In gadoid fish, loss of EP is associated with demethylation of TMAO to DMA and formaldehyde; the latter appears to cross-link proteins and cause decrease in extractability. In Atlantic cod, rates of glycolysis and ATP dephosphorylation and deamination, processes which increase as pre-rigor fish muscle begins to freeze, have maximum velocities near -3°C (Nowlan and Dyer, 1974). These reactions also lead to a decrease in protein solubility. It must also be pointed out that work carried out by researchers like Bramstedt (1962) and Siebert and Schmitt (1961), have implicated endogenous fish muscle enzymes as causative agents of spoilage during storage. These changes in the EP values indicate that protein aggregation, and hence protein insolubilization occurred in all the samples. On account of the foregoing, the severity of the damage is presented in the following order : partial frozen samples from CTAF < partial frozen samples from WTAF < iced samples from CTAF < iced samples from WTAF. Based on the studies carried out by previous investigators such as Dyer (1968), Dyer and Dingle (1961), Love (1966a,b), and Cowie and Mackie (1968) the decreases in EP in the samples are indicative of protein denaturation.

3.2.1.1. Extractable Protein for Muscle Specimen with Different Pre-treatment

The results of the study where the cod fillets were subjected to different pretreatment are presented in Table 3-4. The study summarized in Table 3-4 indicates that : (i) the samples from summer (July) fish had less total EP than those from winter (Feb) fish, similar to the findings reported in Appendix 3. These findings are also similar to previous observations made by Bramstedt (1962), (ii) The samples from summer fish suffered more damage than those from winter fish in all the different treatments applied, (iii) Rapid freezing with liquid nitrogen prior to storage at -3°C caused more damage in the "muscle specimen" than partial freezing or iced storage. An important observation revealed here is the fact that rapidly cooling the fish muscle to a very low temperature (that of liquid nitrogen) and then bringing the temperature up to -3°C and maintaining samples at this temperature (-3°C) results in a drastic loss in extractable protein, an indication of severe cellular damage. Ice storage appeared to cause more denaturation of "muscle specimen" protein during the ten day storage period than partial freezing. The results for iced and partially frozen samples are in agreement with those previously observed (Appendix 3).

Table 3-4: Extractable protein from "muscle specimen" from cod subjected to different pretreatment (g/100g tissue)

Sample	Month[1]	Time (day)	Water EP	Control (%)	Salt EP	Control (%)
Iced	Feb	0	3.42 ± 0.12	100.00	9.78 ± 0.46	100.00
		5	3.15 ± 0.14	92.11	9.53 ± 0.48	97.44
		10	3.13 ± 0.07	91.52	9.02 ± 0.55	92.23
PF[2]	Feb	0	3.43 ± 0.39	100.00	9.82 ± 0.53	100.00
		5	3.14 ± 0.19	91.55	9.19 ± 0.44	93.58
		10	3.11 ± 0.28	90.67	9.15 ± 0.22	93.18
PF-LN[3]	Feb	0	2.83 ± 0.22	100.00	8.82 ± 0.43	100.00
		5	2.75 ± 0.14	97.17	8.11 ± 0.35	91.15
		10	2.61 ± 0.27	92.23	7.41 ± 0.21	84.01
Iced	July	0	3.23 ± 0.23	100.00	6.64 ± 0.36	100.00
		5	2.63 ± 0.14	81.42	6.52 ± 0.23	98.19
		10	2.15 ± 0.06	66.56	6.20 ± 0.21	93.37
PF[2]	July	0	3.34 ± 0.05	100.00	6.37 ± 0.24	100.00
		5	2.88 ± 0.21	86.23	5.88 ± 0.14	92.31
		10	2.73 ± 0.21	81.74	5.49 ± 0.37	86.19
PF-LN[3]	July	0	2.67 ± 0.06	100.00	6.08 ± 0.26	100.00
		5	2.29 ± 0.20	85.77	4.38 ± 0.23	72.04
		10	1.99 ± 0.19	74.53	3.48 ± 0.30	57.24

[1]Month ASWF was removed from tank for analysis;
[2]partially frozen samples;
[3]samples were first rapidly frozen in liquid nitrogen, then stored at -3°C.
Data presented in Table 3-5 are averages of duplicate determinations with each of four fish and are representative of several other determinations.

These findings go to support the earlier assertion (see 3.2.1.) that ice storage appears to cause more destruction of fish quality during storage when compared to partial freezing. The latter process therefore proves to be the method of choice for longer term storage of fish. This deduction is in keeping with the conclusions made by Merritt et al. (1966) in their studies with fish like bream and perch. According to Love (1968), pre-freezing cod muscle in liquid nitrogen prior to holding at -3°C results in greater protein denaturation than slow freezing to -3°C because pre-freezing, unlike slow freezing, causes formation of intracellular ice with attendant solute concentration.

3.2.2. Cell Fragility

The results of a cell fragility study are presented graphically in Figs. 3-4 & 3-5. The cell fragility method was used to evaluate differences in the toughening of the cells of the cod muscles at different harvest months. The cell fragility method is based on the observation that the cells of cod *Gadus callaris L.* muscle after freezing become progressively more difficult to rupture with a homogeniser as storage time increases. A relationship between absorbance at 430_{nm} and time during the measurement in the spectrophotometer was found to be logarithmic and therefore initial cell fragility (CF_i) was calculated using the equation:

$$LnCF_i = LnCF + Bt$$

where B is a constant and t is time. By definition CF was calculated as CF_i.

The highest CF_i value was observed for fish obtained in February and the lowest value was obtained for July fish (Figs. 3-4 & 3-5). On storage of the samples for 10 days on ice or -3°C the cell fragility values decreased (Figs. 3-4 & 3-5). The results of this

analysis were quite variable and therefore the technique was not used in later studies with tank held cod.

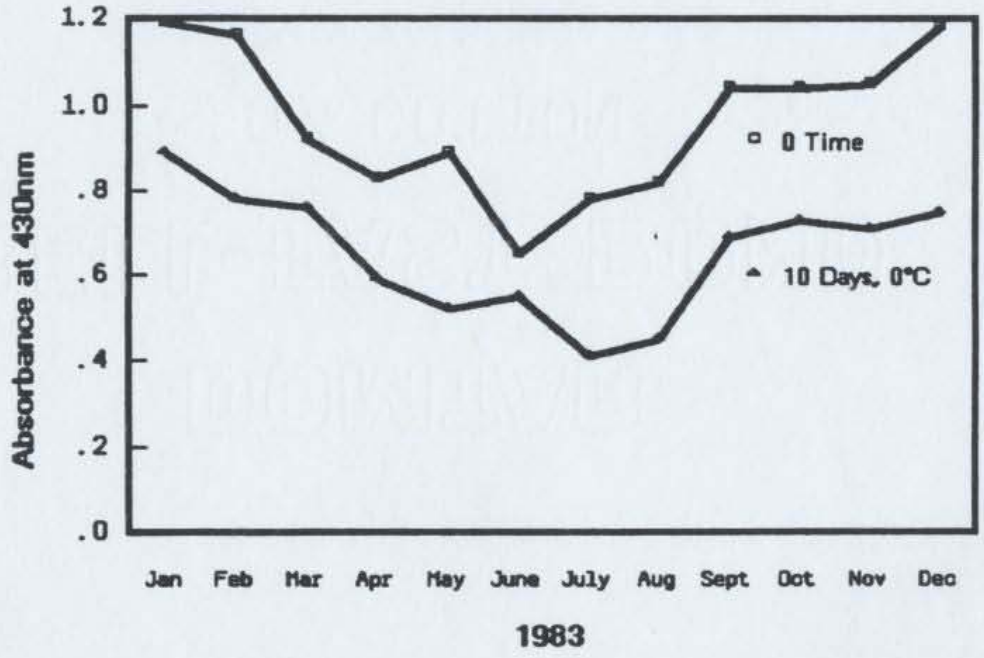

Figure 3-4: Cell fragility of cod muscle stored on ice as a function of time

Results are averages of duplicate measurements with each of two fish.

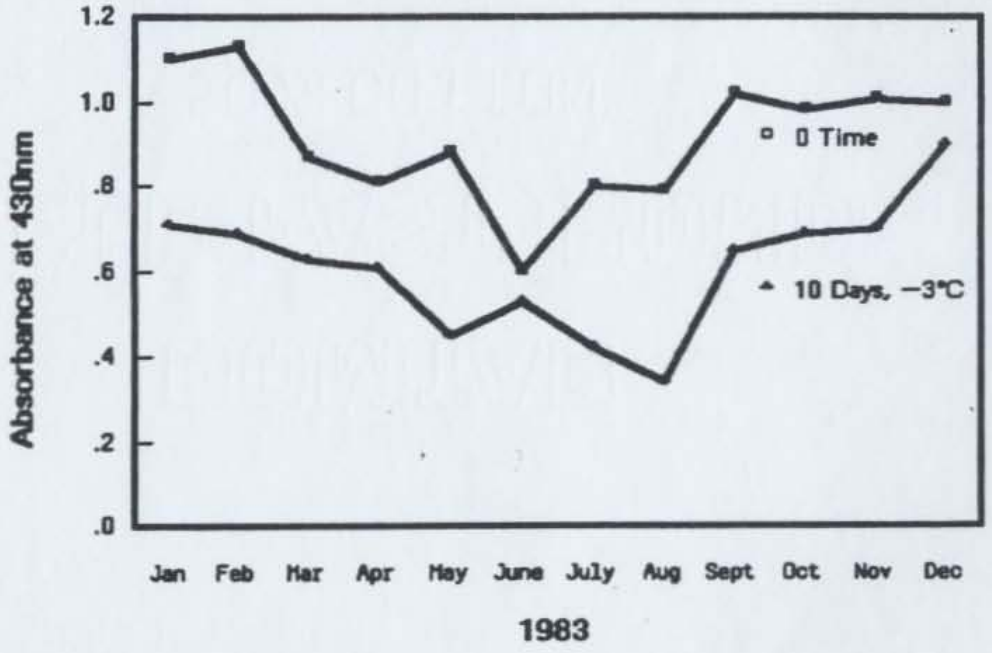

Figure 3-5: Cell fragility of cod muscle stored at -3°C $\pm$ 0.1 as a function of time

Results are averages of duplicate measurements with each of two fish.

The muscle specimens employed in these studies were not pre-treated with antibiotic and thereby differ from muscle specimens used to study changes in other biochemical indices.

3.2.3. TMA-N and DMA-N

The catabolism of TMAO to TMA in iced cod occurs in parallel with bacterial proliferation. The finding that relatively little TMAO was catabolized (Table 3-5) and relatively little TMA was formed, even after 20 days storage at 0°C or -3°C, probably reflects the minor intervention of microbial reactions in "muscle specimen" which were aseptically excised and treated with antibiotic (Figs. 3-4 and 3-15). Results of TMAO-N, TMA-N and DMA-N are presented in Appendix 4. Haard et al. (1979) reported that the TMA-N content of muscle from dressed Atlantic cod held on ice increased from ~0 mg% to ~65 mg% after 8 days at 2°C to 4 °C. Amano and Yamada (1965) have reported an initial TMAO-N level of ~90 mg% that decreased gradually to ~80 mg% after 4 days storage at 0°C to 4°C, after which there was a more rapid decrease to ~30 mg% after 14 days. These workers further reported that the levels of TMAO-N fluctuated with the location of the sample. For instance, they found that deep muscle from cod fish had more TMAO-N than the surface muscle. They also reported that DMA-N increases gradually from ~1 mg% to ~10 mg% after 14 days, while TMA-N increased gradually from ~2 mg% at day 0 to ~5 mg% after 7 days, then increased rapidly to ~80 mg% after 10 days. However, it is not apparent from Amano and Yamada's (1965) report that precautions were taken to minimize bacterial intervention, as was done in the present study. So the more enhanced catabolism of TMAO-N as well as the more pronounced formation of TMA-N or DMA-N reported in their study, compared to the findings of the present study, were probably due in part to bacterial action.

Table 3-5: Content of DMA, TMA and TMAO in muscle of CTAF or WTAF

Sample	Storage Temp.(°C)	Time (days)	DMA (mg%)	TMA (mg%)	TMAO (mg%)
CTAF	0	0[1]	1.12	2.19	415.27
		5	1.05	2.19	ND
		10	1.05	2.32	ND
		15	1.05	2.44	418.08
		20	1.25	3.33	383.86[a]
WTAF	0	0[1]	1.41	2.32	415.61
		5	1.45	2.70	ND
		10	1.35	3.08	ND
		15	1.45	2.95	418.12
		20	1.96	3.83	402.91[a]
CTAF	-3	0[1]	1.25	2.32	414.48
		5	1.35	2.32	ND
		10	1.35	2.44	ND
		15	1.54	2.32	396.56
		20	1.64	2.32	349.70[b]
WTAF	-3	0[1]	1.35	2.44	381.50
		5	1.54	2.44	ND
		10	1.54	2.57	ND
		15	1.67	2.82	388.49
		20	1.67	2.57	312.36[b]

[1] Approximately 1 h after storage at appropriate temperature. The data presented in Table 3-6 are averages of duplicate determinations for each of two fish. ND = not determined. Values with the same superscript are significantly different ($P<0.05$).

Various investigators have reported that TMAO is catabolized to DMA and formaldehyde in frozen cod flesh by endogenous (non-bacterial) enzyme systems. Some workers have reported that DMA formation occurs at a faster rate at partial freezing temperatures than at higher or lower temperatures and argued that this is the result of increased biochemical reactions due to ice damage (Gould and Peters 1971, Castell et al. 1973, and Love and Elerian 1964). The present study indicates negligible levels of DMA formed in iced and partial frozen cod when steps are taken, i.e. aseptic sampling, antibiotic treatment and vacuum packaging, to minimize microbial intervention during storage. However, it should be noted that the TMAO concentration appeared to decline a little more in samples held at -3°C. It is not clear why the significant amount of TMAO lost in -3°C stored fish was not recovered as TMA and / or DMA. It is known that the recovery of DMA by TCA extraction can be poor under certain conditions (Ito et al., 1971), therefore low recovery of DMA may explain the lack of stoichiometry of TMAO catabolism and TMA and DMA formation. The formation of DMA is accompanied by equimolar concentrations of formaldehyde causing more myofibrillar protein denaturation in samples held at -3°C than samples held at 0°C (Table 3-5 and Appendix 4).

3.2.4. Surface pH for 0°C and 10°C Fish

The surface pH for the muscle samples derived from WTAF or CTAF are presented in Table 3-7. It is apparent from the table that the surface pH of the fish fillets used in the experiment decreased only slightly or remained constant during storage. It is apparent from this that storing fish on ice or at -3°C (partial freezing) had little effect on the surface pH values.

Table 3-6: Surface pH of fish muscle excised from fish acclimated at 0°C or 10°C tank

Sample	Tank Temp. (°C)	Storage Time (Days)	Surface pH
Iced	0	0	6.9 ± 0.2
PF		0	7.0 ± 0.0
Iced		3	6.8 ± 0.2
PF		3	6.9 ± 0.1
Iced		6	6.8 ± 0.2
PF		6	6.9 ± 0.0
Iced		9	6.8 ± 0.1
PF		9	6.9 ± 0.1
Iced		12	6.7 ± 0.1
PF		12	6.8 ± 0.1
Iced		15	6.7 ± 0.2
PF		15	6.8 ± 0.1
Iced		18	6.7 ± 0.1
PF		18	6.8 ± 0.1
Iced		21	6.7 ± 0.1
PF		21	6.8 ± 0.1
Iced	10	0	7.2 ± 0.0
PF		0	7.1 ± 0.0
Iced		3	7.1 ± 0.3
PF		3	7.1 ± 0.2
Iced		6	7.0 ± 0.2
PF		6	7.0 ± 0.2
Iced		9	7.0 ± 0.2
PF		9	6.9 ± 0.1
Iced		12	7.0 ± 0.1
PF		12	6.9 ± 0.1
Iced		15	6.9 ± 0.1
PF		15	6.9 ± 0.2
Iced		18	6.9 ± 0.2
PF		18	6.9 ± 0.1
Iced		21	6.9 ± 0.1
PF		21	6.9 ± 0.1

Data presented in Table 3-6 are averages of duplicate determinations with each of four fish and are representatives of several other determinations.

3.2.4.1. General Discussion : Surface pH for 0°C and 10°C Fish

The texture of cooked cod muscle is related to the pH (Kelly and Little, 1966; Cowie and Little, 1966). According to Kelly (1969a,b) a low ultimate pH (i.e. < 6.2) implies a tough texture. Since cold storage causes further toughening, fish of low pH would be expected to become even more unacceptable if kept frozen. It is therefore considered desirable for cod to have a pH of not less than 6.6 or 6.7 (Kelly, 1969a,b). Cooked fish with a pH (over 7.0) can have an objectionably sloppy texture and may actually be improved by cold storage.

Table 3-6 indicates that the pH of the muscle samples decreased slightly during the 21 days storage period. The increase in pH usually observed with fish samples during cold storage has been attributed to bacterial spoilage (Nowlan et al., 1975). The pre-treatment of the "muscle specimens" with the bactericidal agent, gentamycin sulfate, thus suppressed spoilage by certain bacteria and thereby prevented the pH from rising. The slight drop in the pH of the samples derived from both WTAF and CTAF indicates that there was some conversion of residual muscle glycogen into lactate during the initial storage period, as suggested by MacCallum et al., 1967. Other investigators such as Cowie and Little (1966) and Ang and Haard (1985) have demonstrated similar postmortem drops in pH of cod muscle. Storage of cod fillets for 21 days in vacuum packs at -3°C, without antibiotic treatment, results in an increase of pH to 7.3 and 7.6 respectively (Haard and Warren, 1985).

3.2.5. Microscopic Study - Extracellular Area

The partially frozen and iced samples from both WTAF and CTAF showed similar increases in "extracellular area" during the 21 day storage period, as can be seen from Fig. 3-6 and Appendices 5, 6. For the iced samples derived from CTAF, the percent extracellular area ranged from 12.0% to 20.1% with a corresponding relative damage (RD) of 0% to 67.28%, while samples from the WTAF stored on ice had extracellular area ranging from 12.8% to 22.2%, corresponding to a relative damage ranging from 0% to 72.34%. Love (1966c) reported that fresh unfrozen cod had an extracellular area of 12.5% and prerigor cod slowly frozen to -29°C had an extracellular ice area of about 55%. A higher relative damage was observed in the WTAF samples that were partially frozen at -3°C for 21 days (12.82% to 63.4%) compared to their counterparts derived from CTAF (5.66% to 58%, Fig. 3-6 and Appendices 5,6).A typical photomicrograph of cod muscle prior to storage is illustrated in Fig. 3-7. The extracellular area of such fresh fish is 12.01 $\pm$ 1.01% for CTAF and 12.87 $\pm$ 0.85% for WTAF. Partial freezing to -3°C caused an immediate (3 h).increase in extracellular area, + 0.68% for CTAF and 1.65% for WTAF (Appendices 5,6). Partial freezing caused a dramatic change in the appearance of muscle, the cell structure appeared more uniform after partial freezing (Fig. 3-8). The influence of holding cod for 10 days at 0°C or at -3°C on extracellular area is shown in Appendix 5 and Appendix 6 and in Figs 3-9 and 3-10. A similar study was done with fish from the ambient seawater tank in July or February (Table 3-7). The results obtained were similar to those observed for fish taken from temperature controlled tanks (Appendices 5,6) in that fish from cold water showed less fiber shrinkage than fish from warm water. However, muscle from ASWF formed more extracellular area during storage at -3°C than at 0°C and in this respect differed from muscle from temperature acclimated fish.

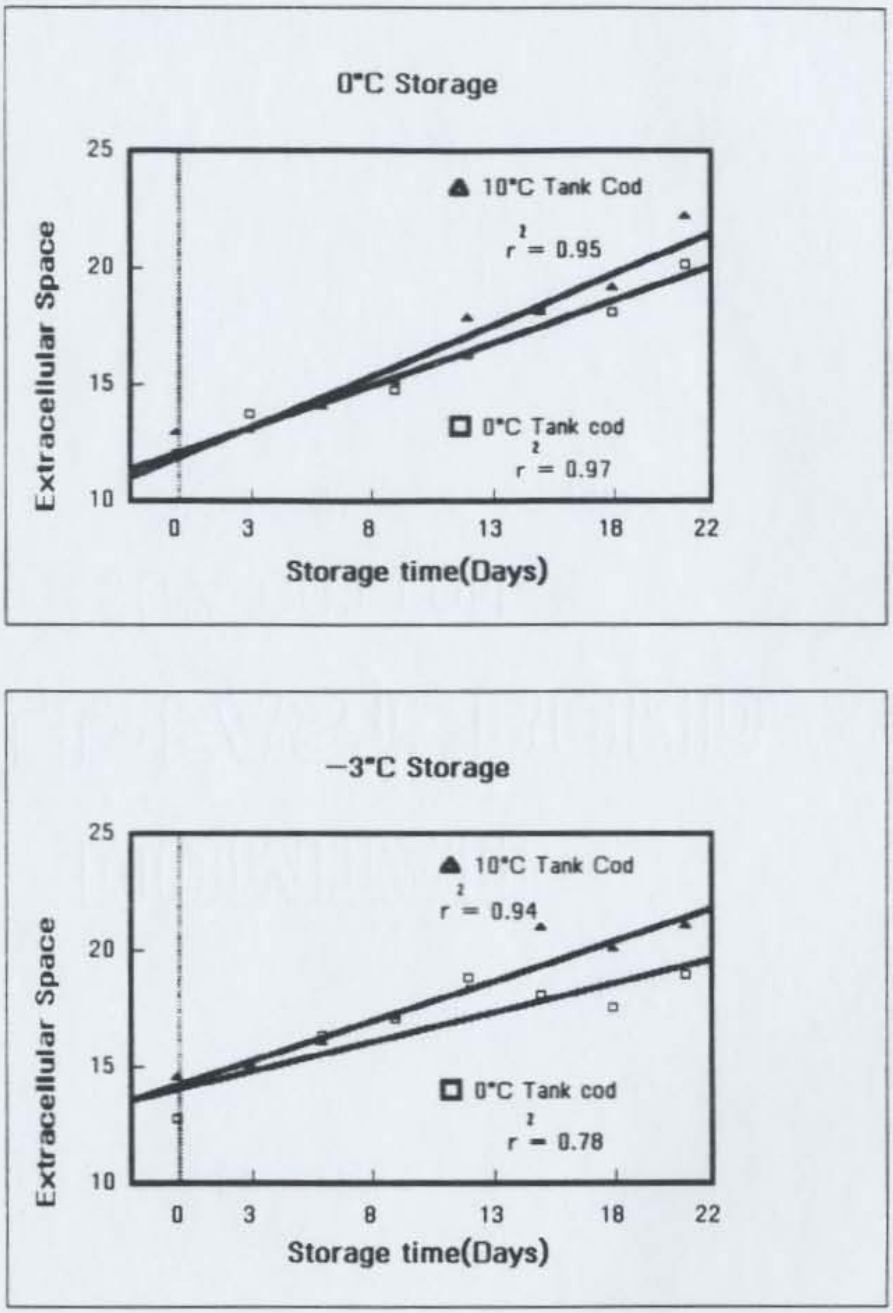

Figure 3-6: Change in extracellular area as a function of storage time at 0°C or -3°C

Data presented in Fig. 3-6 are averages of duplicate determinations with each of four fish.

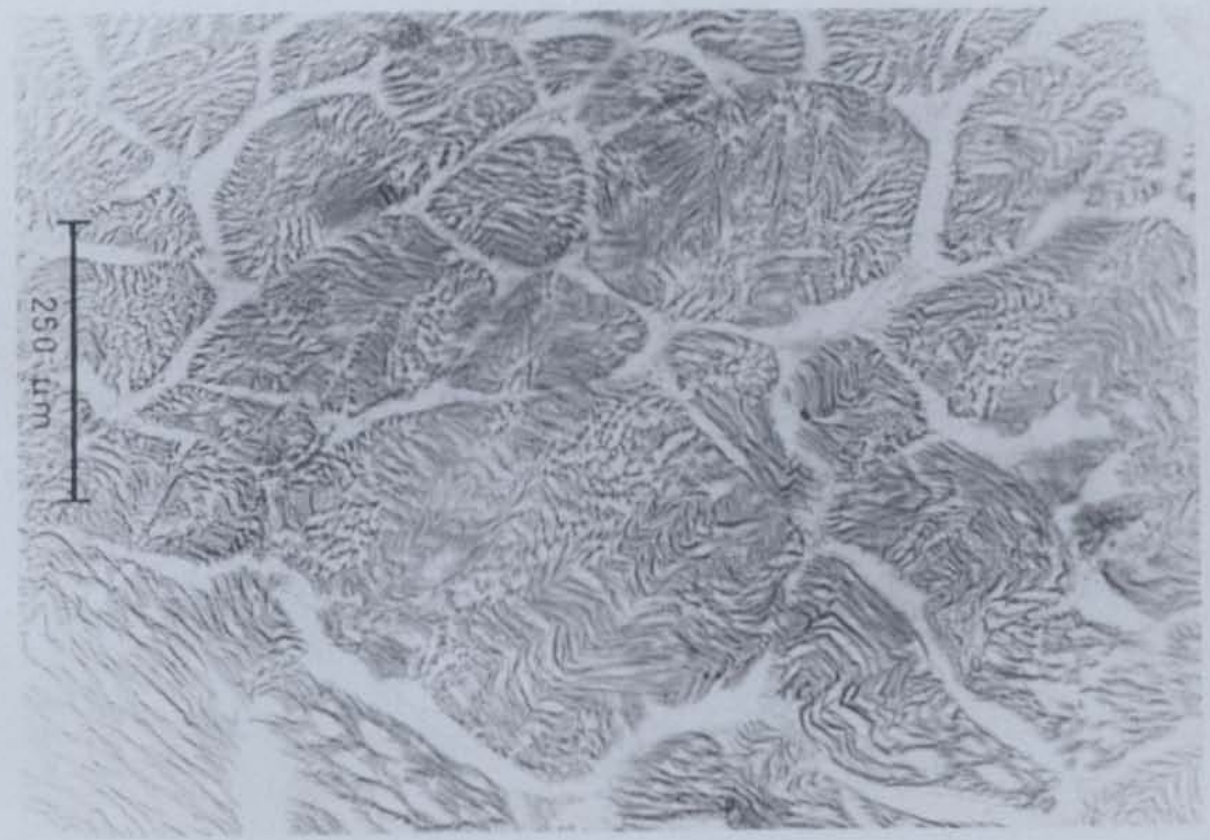

Figure 3-7: Photomicrograph of transverse section of fresh unfrozen cod muscle

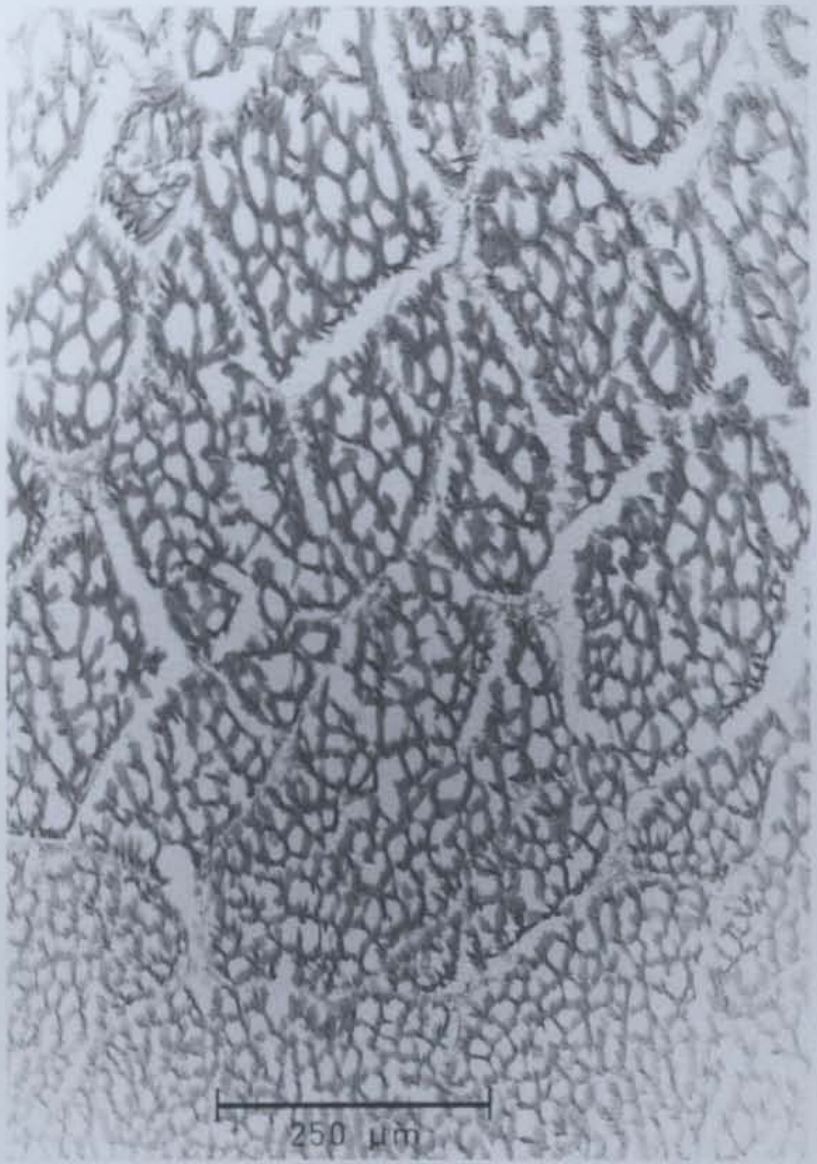

Figure 3-8: Photomicrograph of transverse section of cod muscle held at -3°C for 3 h

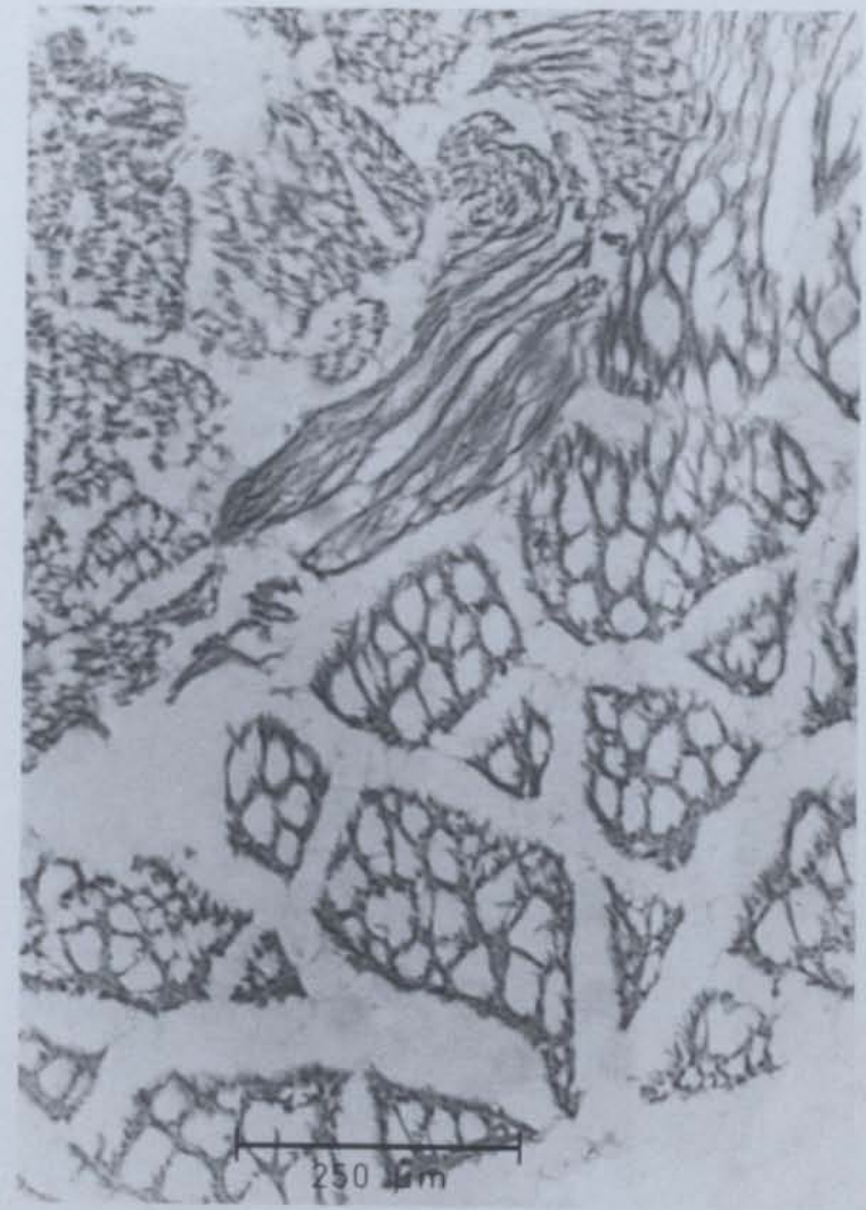

Figure 3-9: Photomicrograph of transverse section from cod muscle held at 0°C for 10 days

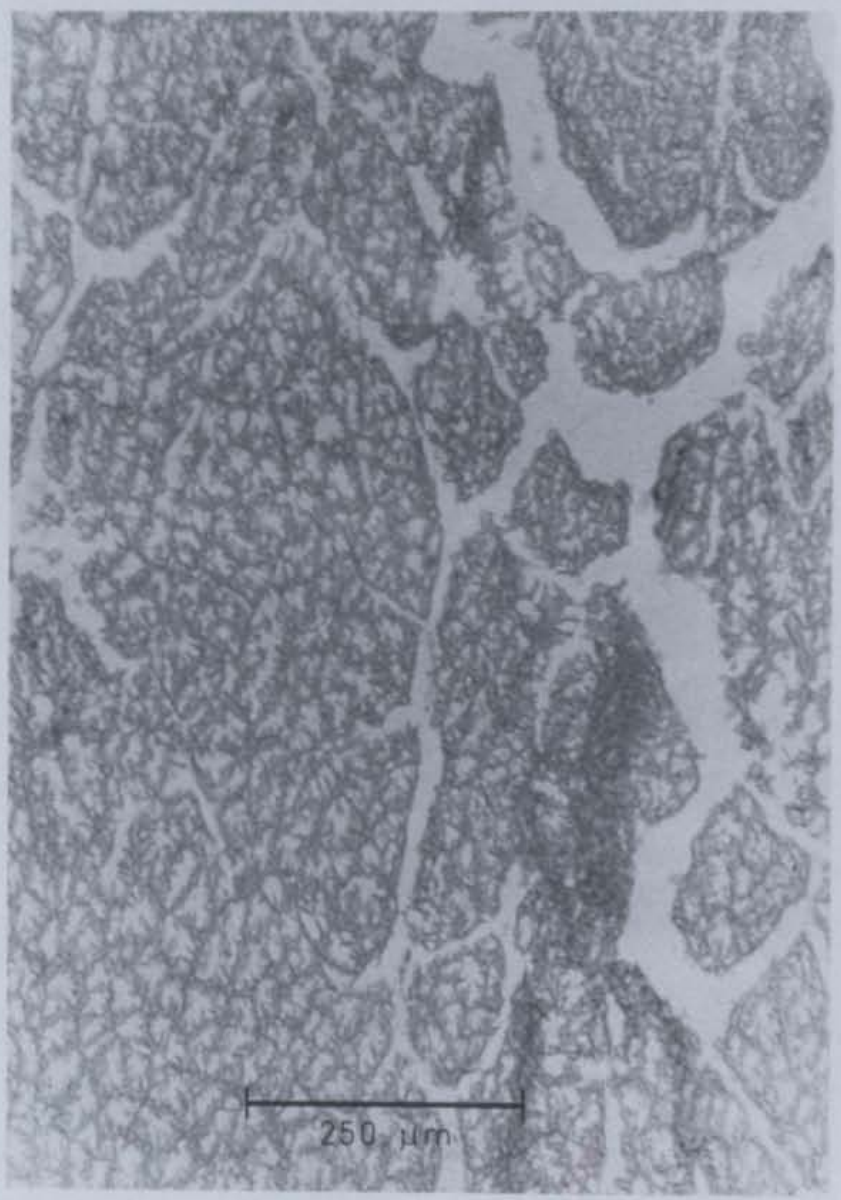

Figure 3-10: Photomicrograph of transverse section from cod muscle held at -3°C for 10 days

Table 3-7: "Extracellular area" of fish harvested in February and July and the influence of storage at 0°C or -3°C

Winter Fish

Sample	Day of Analysis	% Extracellular Area
Iced	0	10.70 ± 0.16
	5	14.13 ± 0.42
	10	14.32 ± 0.10
PF[1]	0	11.43 ± 0.05
	5	15.24 ± 0.05
	10	15.84 ± 0.28

Summer Fish

Sample	Day of Analysis	% Extracellular Area
Iced	0	11.94 ± 0.05
	5	14.26 ± 0.07
	10	17.96 ± 0.21
PF[1]	0	13.65 ± 0.38
	5	16.60 ± 0.04
	10	19.14 ± 0.22

[1] PF ≙ partially frozen samples ; data presented in Table 3-7 are averages of four determinations each run in duplicate. Values could not be computed for rapidly frozen samples as explained in the text (3.2.5).

Muscle from cod held in ambient seawater tank was collected in July and February and frozen in liquid nitrogen prior to storage at -3°C. Pretreatment of cod muscle by freezing in liquid nitrogen prior to warming to -3°C and subsequent storage at -3°C appeared to destroy the integrity of the muscle fiber sarcolemma as well as the membranes surrounding bundles of fibers (Figs. 3-11, 3-12). Because the integrity of fibers and bundles was lost by this pretreatment, it was not possible to calculate the influence of storage on ice area or extracellular area. It is generally found that rapid freezing (e.g. liquid nitrogen) gives rise to a uniform distribution of small ice crystals, both intracellular and extracellular, and results in less cell damage during freezing and frozen storage at low temperature (Fennema, 1975 ; Love 1966c). Formation of intracellular ice, by rapidly freezing cod followed by storage at -3°C was also found to be undesirable by Love (1968).

In this study, the liquid nitrogen frozen samples were warmed to -3°C and this appears to have caused massive disruption of cell integrity. The apparent disruption of sarcoplasmic membrane may relate to lower salt EP values of CTAF or WTAF. It was concluded that the rapidly frozen samples suffered most damage and therefore the procedure was discontinued.

3.2.5.1. General Discussion : Cellular Damage

Iced samples (Fig. 3-6) from both the CTAF and WTAF suffered more cellular damage than the partially frozen muscle samples. In theory, at least, the reverse situation would be expected to prevail as the lower partial freezing temperatures would be more conducive to extracellular ice formation and subsequent cellular dehydration than storage on ice. The study indicates that the 'damage' increased with time of storage

for all samples. It appears that shrinkage of muscle fibers or enlargement of extracellular area is not strictly dependent on cell dehydration due to extracellular ice formation.

A significant finding of the ice storage study is that, ***it is not accurate to impute all the spaces that form in fish muscle during frozen storage exclusively to ice formation.*** The literature does not indicate that any such study has been done in the past. However, it is well recognized that the muscle fibers tend to shrink during post-rigor storage above the freezing point. The study also indicates that cod fish derived from colder habitat temperatures would probably suffer less cellular damage during cold storage than their counterparts derived from warmer habitat temperatures.

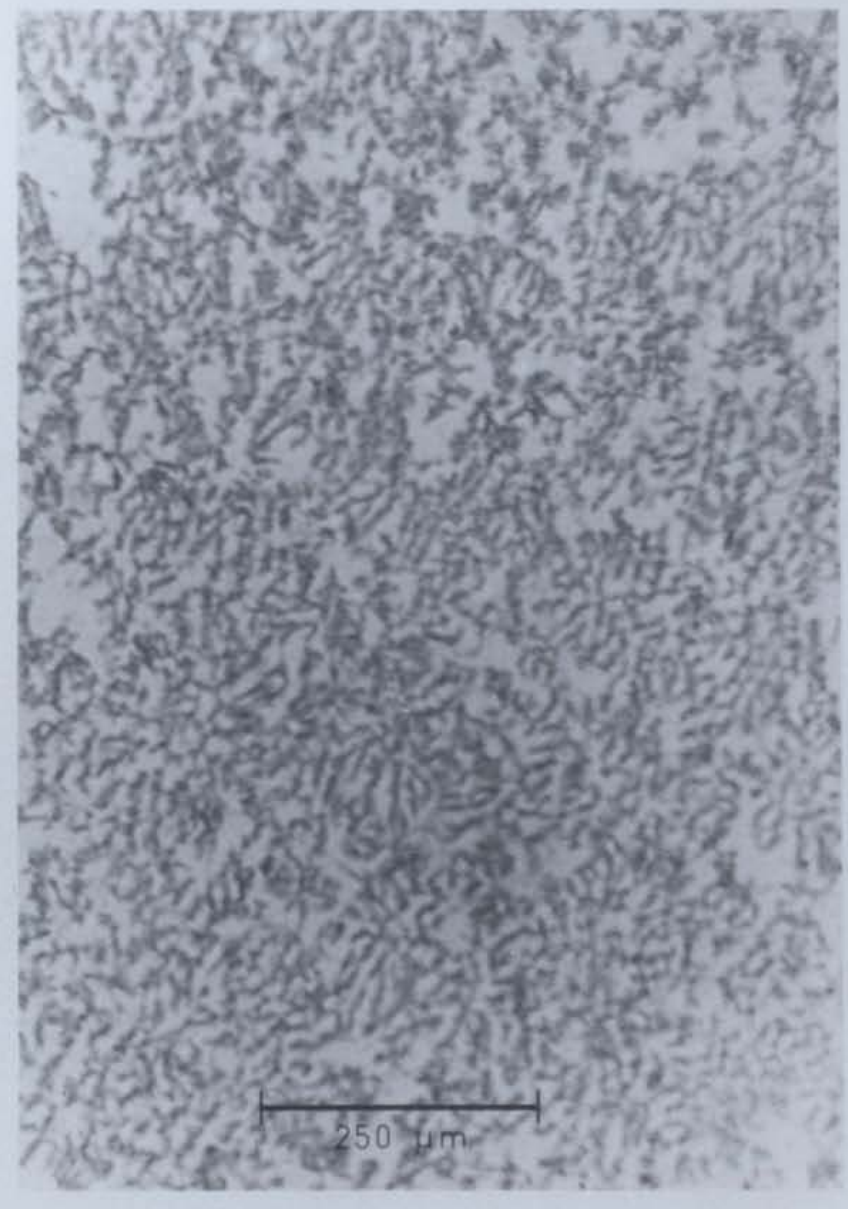

Figure 3-11: Photomicrograph of transverse section from cod muscle rapidly frozen in liquid nitrogen then held at -3°C for 3 h

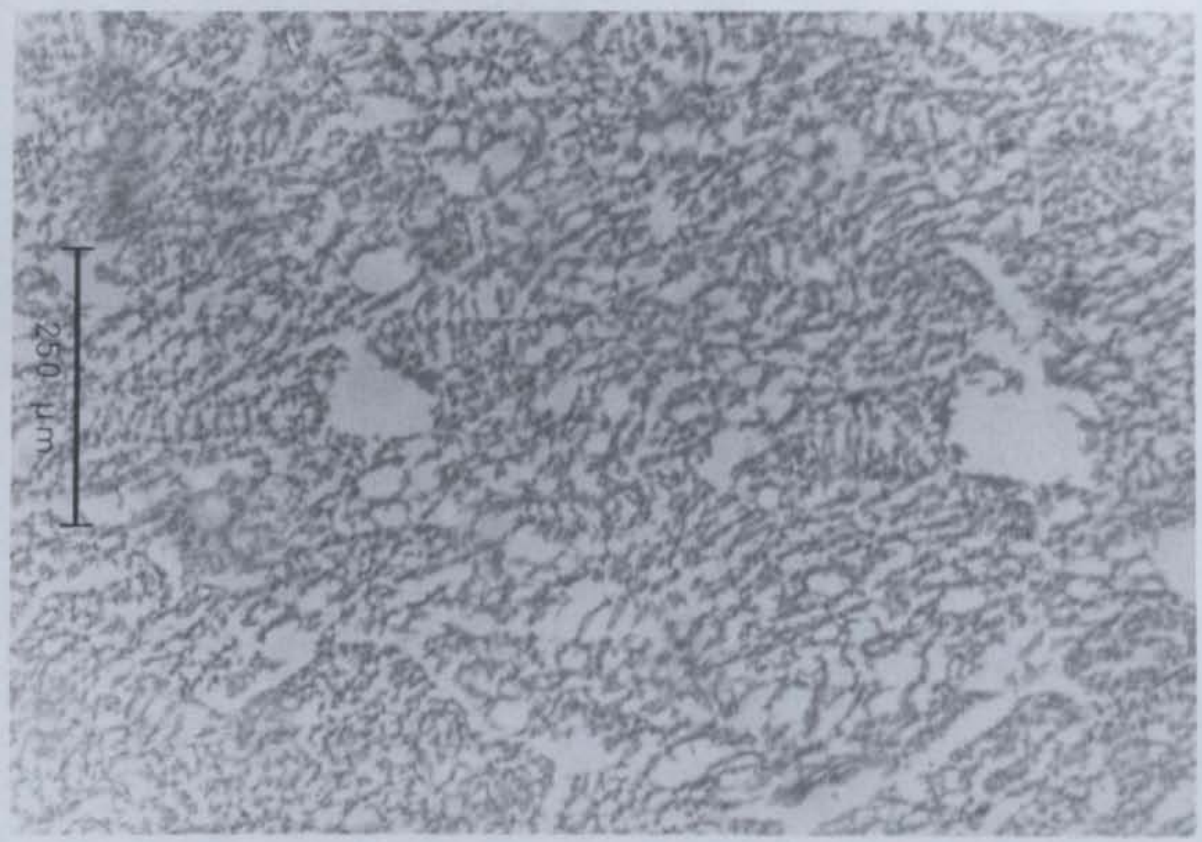

Figure 3-12: Photomicrograph of transverse section from cod muscle held rapidly frozen in liquid nitrogen, then held at -3°C for 10 days

3.2.6. Free Drip

The results of the free drip measurements on the muscle samples derived from the CTAF or WTAF fish are presented in Appendices 7,8 and Fig. 3-13. Partially frozen samples from CTAF exuded more 'drip' (9.96% to 13.77%) during the 21 days storage period than the corresponding iced control samples (6.22% to 12.22%). A similar observation was made in the case of the samples derived from WTAF, 10.08% to 18% free drip for the partially frozen samples versus 9.92% to 14.77 % for the corresponding iced control samples. Although both WTAF and CTAF showed similar trends in their percentage free drip, after the 3 day storage period, the iced samples derived from WTAF exhibited more drip (9.92%) than their counterparts derived from CTAF (6.22%). (Appendices 7,8). Again, after 21 days storage, iced samples derived from WTAF exhibited 14.77% free drip while their counterparts derived from CTAF exuded only 12.22% free drip. For the partially frozen samples the ones derived from CTAF exuded 9.96% free drip after 3 days storage compared to 10.87% for those derived from WTAF over the same period. However, after 21 days storage, the samples derived from CTAF exuded only 13.77% free drip in contrast to the 18% free drip from those derived from WTAF. At first glance, one might be inclined to deduce from the foregoing that the results from the free drip study are contradictory to the findings of the microscopic evaluation of extracellular area. However, if one takes into account the probability that the spaces found in the iced samples were not formed by (or occupied by) ice crystals, then it can be suggested that the excess drip measured in the partially frozen samples was derived in part from the melting of 'authentic ice crystals' that formed during partial freezing. Free drip may also be affected by change in tissue pH and other factors which influence binding of water to protein.

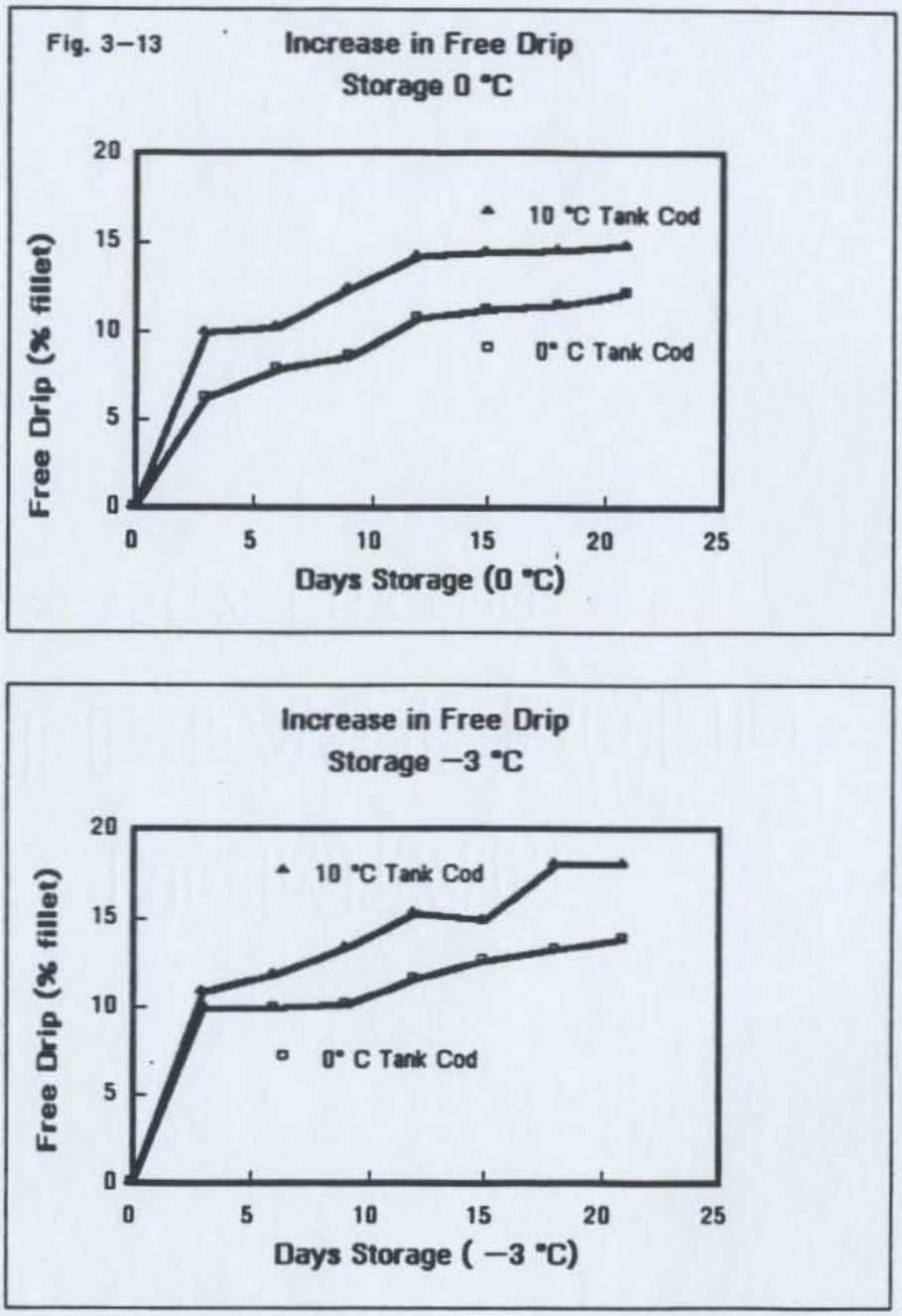

Figure 3-13: Influence of storage of cod muscle at 0°C or 10°C on free drip

Data are averages of duplicate determinations for each of four fish.

3.2.6.1. General Discussion : Free Drip

The percentage drip losses observed with samples derived from WTAF were higher compared to that of the samples derived from CTAF. The range of drip loss found in the study is similar to findings made by previous investigators. For instance, in gutted cod *Gadus morhua*, iced on a vessel for a week or so, Ellison (1934) noted losses up to 8% while Holston (1966) recorded losses as high as 18%. Based on Stroud's (1968) report that the higher the temperature at which a fish goes into rigor the greater will be the drip loss on thawing, it would be expected that the pre-rigor samples stored on ice should exude more drip than those stored at the relatively lower partial freezing temperature used in the study. However, factors other than the passage of the samples through rigor probably contributed to the drip losses. Because the muscles were removed from the frames, it is to be expected that acute 'thaw-rigor' could also contribute to drip loss in the partially frozen muscle samples. According to Stroud (1968), when frozen pre-rigor fish fillets are thawed, the muscles are capable of rapidly shrinking as soon as the ice formed within the flesh during storage has melted. As such, the fillets or pieces become shrunken and corrugated and lose a larger amount of drip.

3.2.7. Physiological Free Amino Acids from Fish Acclimated at Different Temperatures

Table 3-8 summarizes the free amino acid content of "muscle specimens" derived from WTAF or CTAF. WTAF contained less free amino acids than CTAF and exhibited a greater rate of decline in free amino acids during storage at 0°C or at -3°C (Appendices 9 to 12). The amino acid residue differing most between CTAF and WTAF samples was alanine which was present at approximately twice the concentration in the former species.

Table 3-8: Total physiological free amino acids from fish acclimated at 0°C and 10°C (mM)

Storage Time	0°C Storage		PF Storage	
	CTAF	WTAF	CTAF	WTAF
0	50.87 ± 3.81	32.15 ± 3.76	48.25 ± 5.47	36.66 ± 4.36
5	40.37 ± 4.36	36.56 ± 4.86	41.84 ± 5.75	34.13 ± 3.79
10	46.00 ± 4.13	31.34 ± 4.00	45.68 ± 5.09	29.55 ± 2.49
15	42.44 ± 3.83	30.99 ± 3.74	41.79 ± 3.65	32.20 ± 2.78
20	39.84 ± 3.78	23.84 ± 1.52	43.97 ± 3.65	29.75 ± 2.75

Data presented in Table 3-8 are averages of duplicate determinations each of four fish.

3.2.7.1. General Discussion : Physiological Free Amino Acids

On the whole the levels of free amino acids decreased with storage, similar to earlier observations made by Bramstedt (1962) and Shewan and Jones (1957). The following amino acids were the most predominant : glycine, alanine, taurine, threonine, lysine and proline.

Amino acids are also responsible for the taste of fish flesh, and Bramstedt (1962) has demonstrated that glycine is especially important for the individual tastes of different fish species. Some of these amino acids have been described as taste active (Lee and Sung, 1977), and it is significant that the levels of these taste active amino acids were higher in the CTAF than the WTAF. All other things being equal, one would predict that fish from colder habitat temperatures would taste better than their counterparts from warmer habitats. Comparison of Appendices 9 and 11 reveals that the content of free amino acids decreases more in ice storage (25.85%) than in partial freezing storage (18.85%) in the WTAF samples. This effect is demonstrated even more drastically with the free amino acid level from muscles derived from CTAF (Appendices 10 vs. 12) where decrease by ice storage was 21.68% versus 10% for partial freezing storage. Uchiyama and Kato (1974) also observed that free amino acids in certain fish decline at a greater rate in fish held at 0°C than those held at -3°C. The authors interpreted this observation to mean that less biochemical damage occurs in partial frozen fish than in iced fish.

The higher level of free amino acids of CTAF may be responsible for the cryoprotective effect as suggested by Suzuki (1981). According to Fletcher et al. (1982a)

glycoprotein antifreezes have as the basic repeating unit a glycotripeptide of alanine-alanine-threonine with a disaccharide linked to the threonine. Thus, the higher level of alanine observed for CTAF samples may have been derived from antifreeze proteins and may have been partly responsible for the higher TH observed. Since some of the free amino acids participate in osmoregulation (Bramstedt, 1962), it is to be expected that quantitative differences will exist in fish of the same species acclimated to different temperatures.

3.3. Other Factors

3.3.1. Microbiological Studies

Microbiological studies indicate that the aseptic technique and antibiotic used (gentamycin sulfate), was quite effective in minimizing microbial growth (Figs. 3-14, 3-15). There was a significant increase of total colony forming units in the untreated samples stored on ice during a 20 day storage period, with an aerobic total plate count (TPC) ranging from 2.5 X 10^2 CFU/g tissue at Day 0 to about 1.9 X 10^4 organisms/g tissue at Day 20. The observed changes in TPC are lower than would be expected for commercial fish held on ice, based on findings made by Haard et al. (1979). The relatively low TPC of untreated muscle specimen after 20 days at 0°C is related to care in minimizing contamination of the sample from surface and digestive tract material and to the effectiveness of vacuum packaging. Samples from the same fish that were treated with antibiotic prior to their storage on ice showed less bacterial counts ranging from 1.5 X 10^2 CFU/g tissue (Day 0) to about 5 X 10^3 CFU/g tissue (Day 20). Partial freezing was effective in further minimizing bacterial proliferation.

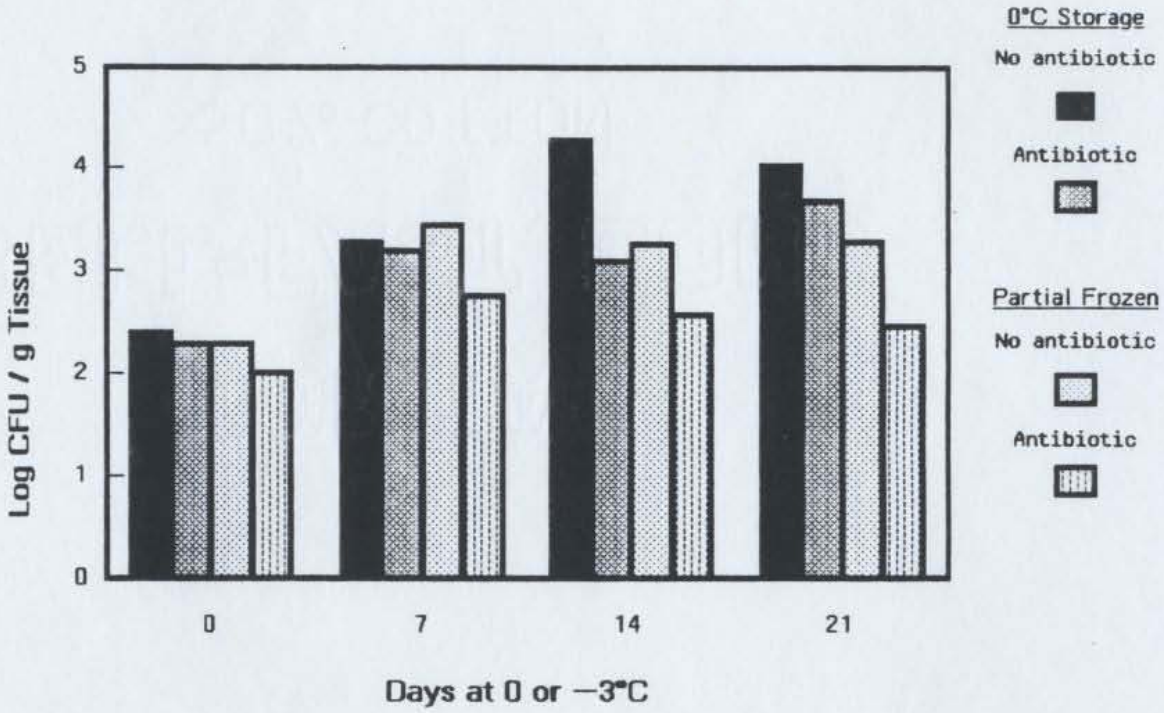

Figure 3-14: Total aerobic plate counts of cod muscle (CTAF) held at $0^{o}C$

Data presented in Fig 3-14 are average values of duplicate determinations each of four fish.

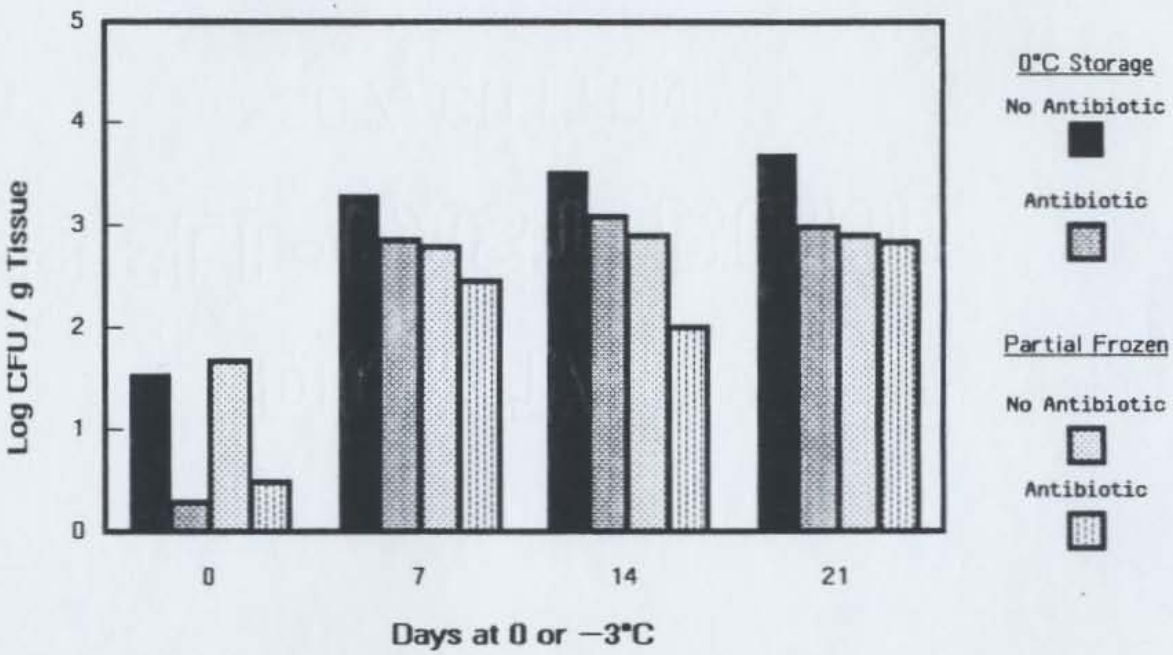

Figure 3-15: Total anaerobic plate counts of cod muscle (CTAF) treated with antibiotic and stored at 0°C

Data presented in Fig. 3-15 are average values of duplicate determinations each of four fish.

The samples that were treated with antibiotic ptior to partial freezing did not exhibit a significant increase in TPC even after 20 days storage. The purpose of treating "muscle specimen" with antibiotic in this study was to separate the contribution of microbiological metabolism and proliferation from that of endogenous "biochemical" deteriorative reactions. Although bacterial proliferation was not completely arrested in the gentamycin treated and vacuum packaged samples held on ice, the above data support the conclusion that microbial intervention was minimal. The anaerobic TPC showed similar trend to the aerobic ones. The values ranged from as low as 2 (virtually no growth) for day 0 to 5 X 10^3 CFU/g tissue after 20 days storage.

3.3.1.1. General Discussion : Microbiological Studies

The low bacterial count obtained for partial frozen cod not treated with antibiotic for both aerobic and anaerobic TPC suggests that bacterial growth was effectively retarded at -3 $\pm$ 0.1 °C. However the partially frozen samples with antibiotics exhibited considerable low counts. The vacuum packaging together with the use of the antibiotic and aseptic handling, significantly reduced growth of both aerobic and anaerobic organisms. Similar observations were made by Lee and Toledo (1984) for fish vacuum packed but untreated with antibiotics and stored at -2°C. Power et al. (1969) also made similar observation on cod stored at subzero temperatures. With round fish, partial freezing at -3°C and -4°C has been used on board trawlers and found to extend the keeping time for as long as 4 or 5 weeks although bacterial spoilage resulted in undesirable autolytic changes as well as deterioration of the appearance and texture of the fish flesh (Merritt, 1965; Partmann, 1965; Power et al., 1969; and Carlson et al., 1969). Spoiled fish , e.g. cod fish fillets held in ice for 10 to 15 days normally will contain 10^6 to 10^8 CFU/g. The highest TPC observed in this study were 10^4 CFU/g and these levels are comparable to TAPC for freshly harvested commercial fish.

3.3.2. Sensory Evaluation

The results of the sensory evaluation of fish bought from a retail store are presented in Table 3-17. Vacuum packaging and partial freezing were more effective than vacuum packaging and storage in ice in preserving the keeping quality, and hence consumer acceptability. The cod fillet samples used in this study differed from those employed for biochemical studies in that they were obtained from a retail store and they were not treated with antibiotic gentamycin sulfate prior to storage at 0°C or -3°C.

Table 3-9: Sensory evaluation of iced and partial frozen cod fish fillets[2]

Sample	Time	Odor	Appearance	Texture	Flavor	Acceptability
Iced	Day 1[1]	4.62	4.62	4.23	4.42	4.64[a]
PF	Day 1[1]	4.46	4.08	3.69	4.00	4.29[a]
Iced	Day 5	4.05	4.35	3.90	3.85	4.10[a]
PF	Day 5	4.25	4.60	3.60	4.05	4.25[a]
Iced	Day 8	2.65	3.65	2.85	3.00	2.65[b]
PF	Day 8	4.40	4.55	4.55	4.37	4.40[a]
Iced	Day 10	1.00	1.00	1.00	1.00	1.00[d]
PF	Day 10	4.33	4.38	4.52	4.38	4.57[a]

[1]24 h after catch; numbers in the same column followed by the same superscript were not significantly different ($P < 0.05$).

Chapter 4

Conclusions

4.1. Conclusions from Study

1. The freezing point of cod muscle from fish harvested in February / March is lower than that of muscle from fish harvested at other times of year. Fish held in tanks at 0°C or 10°C for a 3 week acclimation time also exhibited similar differences in freezing point as summer and winter fish. The lower freezing point of cold acclimated cod can at least partly be explained on the basis of "antifreeze" components in muscle of cold adapted fish because the muscle fluid of samples having depressed freezing point exhibited thermal hysteresis. The somewhat lower moisture content and higher free amino acid and ash content of muscle from cold adapted fish indicate that a higher solute concentration may also contribute to freezing point depression by simple colligative effects.

2. Cod muscle held at -3°C, under conditions where microbial intervention is minimal, exhibit less biochemical damage than cod muscle held at 0°C. This conclusion is supported by microscopic examination, low DMA formation, less depletion of free amino acids and retention of extractable protein. Also muscle from cod acclimated to 0°C exhibits less biochemical damage during storage at 0°C or -3°C than muscle from cod acclimated to 10°C. The results indicate adaptive changes associated with environmental temperature prior to harvest can contribute to postharvest keeping quality.

3. Cod fillet, carefully excised from cold adapted cod and vacuum packaged, but not treated with antibiotic, retain better eating quality during storage at -3°C than at 0°C.

4.2. Recommendations for Further Study

1. The identification of component(s) in the muscle fluid of Atlantic cod which contribute to thermal hysteresis should be investigated. These studies should include examination of the possible antifreeze activity of free amino acids like alanine since the alanine content was substantially higher in muscle fluid of cold adapted fish. Alanine is also a component of antifreeze glycoproteins found in blood serum of cod. The interactive effect of amino acids and antifreeze proteins should also be investigated.

2. Further practical oriented studies of partial freezing of filleted and round cod for extending the market value of this species should be investigated with a determination of the role of intraspecific factors such as season of harvest, etc.

3. Further studies to determine the minimum storage time after which the partially frozen samples cease to be acceptable to the consumer.

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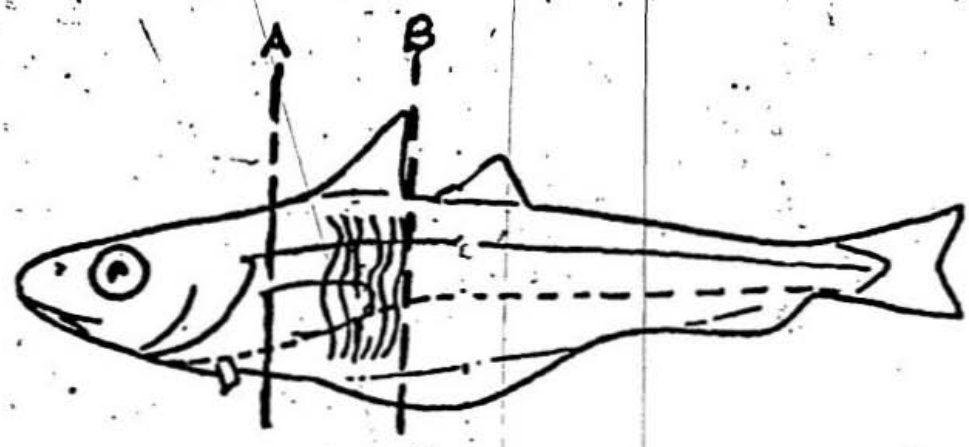
Appendix A

A.1. Appendix 1 : Diagram of cod showing region of myotomes 9 to 12



A.2. Appendix 2 : Sensory Evaluation Score Chart

Questionnaire for Hedonic Scale

Name_____ Date_____

Evaluate the sample of cooked Atlantic cod for odor, appearance, texture, flavor and acceptability by ticking the most appropriate description.

Product Number_____

Factor	Excellent	Good	Satisfactory	Borderline	Unacceptable
Odor	Typical cod odor, pleasant, aromatic.	Slight loss of fresh odor but not unpleasant.	Slight off odor	Slightly fishy unpleasant odor.	Strong fishy offensive odor.
Appearance	Nice, clear units fresh looking	Slight loss of color	Slightly dry, slightly discolored.	Dry or mushy, discolored.	Very dry or very mushy, totally discolored.
Texture	Flaky, juicy & firm, layered.	Moderately firm, slightly flaky, slightly tender.	Tacky, stringy, chewy.	Slightly soft, slightly mushy.	Very mushy, natural texture gone.
Flavor	Fresh taste, slightly sweet, juicy.	Slight loss of freshness, mod. juicy, mod. bland.	Bland.	Slight after-taste.	Strong after-taste cardboardy strong taste.
Acceptability	Good.	Fair.	Neither like nor dislike.	Slightly undesirable.	Totally undesirable

Comments:

A.3. Appendix 3

Data presented in Appendix 3 are averages of duplicate determinations each with two fish. Day 0 samples were analyzed after approxiamtely 3 h storage at the appropriate temperature.

A.4. Appendix 3: Extractable protein from muscle of fish acclimated at 0°C or 10°C, (in g/100 g tissue)

Sample	Tank Temp.°C	Time (day)	Water soluble	Control (%)	Salt soluble	Control (%)
Iced	0	0	3.50 ± 0.30	100.00	9.20 ± 0.15	100.00
x	x	3	3.50 ± 0.06	100.00	8.80 ± 0.07	95.65
x	x	6	3.19 ± 0.15	91.14	8.89 ± 0.48	96.63
x	x	9	3.19 ± 0.13	91.19	8.37 ± 0.32	90.98
x	x	12	3.12 ± 0.06	89.14	7.93 ± 0.03	86.20
x	x	15	3.06 ± 0.01	87.43	7.80 ± 0.06	84.78
x	x	18	3.15 ± 0.21	90.00	7.43 ± 0.24	80.76
x	x	21	3.07 ± 0.14	87.71	7.03 ± 0.22	76.41
Iced	10	0	3.15 ± 0.07	100.00	6.56 ± 0.14	100.00
x	x	3	3.13 ± 0.08	99.37	6.41 ± 0.10	97.71
x	x	6	3.34 ± 0.10	106.03	5.59 ± 0.01	85.21
x	x	9	2.49 ± 0.42	79.05	6.10 ± 0.27	92.99
x	x	12	3.19 ± 0.42	101.27	5.05 ± 0.25	76.98
x	x	15	2.63 ± 0.07	83.49	5.15 ± 0.29	78.51
x	x	18	2.77 ± 0.08	87.94	4.83 ± 0.25	73.63
x	x	21	2.82 ± 0.08	89.52	3.96 ± 0.35	60.37
PF[1]	0	0	3.15 ± 0.13	100.00	7.95 ± 0.40	100.00
x	x	3	2.93 ± 0.07	93.02	7.57 ± 0.03	95.22
x	x	6	2.88 ± 0.06	91.43	7.42 ± 0.44	93.33
x	x	9	2.81 ± 0.07	89.21	7.39 ± 0.43	92.96
x	x	12	2.85 ± 0.07	90.48	7.15 ± 0.29	89.94
x	x	15	2.70 ± 0.05	85.71	7.00 ± 0.11	88.05
x	x	18	2.78 ± 0.21	88.25	6.82 ± 0.29	85.79
x	x	21	2.63 ± 0.40	83.49	6.77 ± 0.21	85.16
PF[1]	10	0	3.17 ± 0.05	100.00	6.34 ± 0.21	100.00
x	x	3	2.99 ± 0.14	94.32	5.72 ± 0.13	90.22
x	x	6	3.08 ± 0.25	97.16	5.72 ± 0.07	90.22
x	x	9	2.90 ± 0.10	91.48	5.72 ± 0.17	90.22
x	x	12	2.90 ± 0.12	91.48	5.72 ± 0.13	90.22
x	x	15	3.08 ± 0.25	97.16	5.54 ± 0.17	87.38
x	x	18	2.82 ± 0.12	88.96	5.37 ± 0.34	84.70
x	x	21	2.73 ± 0.08	86.12	5.19 ± 0.08	81.86

A.5. Appendix 4: Content of DMA-N, TMA-N and TMAO-N in muscle of CTAF or WTAF

Sample	Storage Temp.(°C)	Time (days)	DMA-N (mg%)	TMA-N (mg%)	TMAO-N (mg%)
CTAF	0	0[1]	0.35	0.52	77.71
		5	0.33	0.52	ND
		10	0.33	0.55	ND
		15	0.33	0.58	77.24
		20	0.39	0.79	71.35
WTAF	0	0[1]	0.44	0.55	77.81
		5	0.45	6.4	ND
		10	0.42	0.73	ND
		15	0.45	0.70	77.25
		20	0.61	0.91	74.89
CTAF	-3	0[1]	0.39	0.55	77.24
		5	0.42	0.55	ND
		10	0.42	0.58	ND
		15	0.48	0.55	73.75
		20	0.51	0.55	65.00
WTAF	-3	0[1]	0.42	0.58	77.04
		5	0.48	0.58	ND
		10	0.48	0.61	ND
		15	0.52	0.67	72.21
		20	0.52	0.61	58.07

[1] Approximately 1 h after storage at appropriate temperature. The data presented in Appendix 4 are averages of duplicate determinations each of four fish. ND = not determined.

A.6. Appendix 5: Extracellular Area present in iced and partially frozen (-3°C) muscle from cod acclimated at 0°C

Sample	Day of Analysis	% Extracellular Area	% Rel. Damage
Iced	0	12.01 ± 1.01	-
	3	13.70 ± 0.20	14.07 ± 1.67
	6	14.35 ± 0.15	19.48 ± 1.25
	9	14.78 ± 0.20	23.06 ± 1.68
	12	16.27 ± 1.72	35.47 ± 14.32
	15	18.27 ± 1.36	52.12 ± 11.32
	18	18.07 ± 0.94	50.46 ± 7.83
	21	20.09 ± 0.68	67.28 ± 5.66
PF	0	12.69 ± 0.23	5.66 ± 1.92
	3	14.95 ± 0.05	24.48 ± 0.42
	6	16.27 ± 0.30	35.47 ± 2.50
	9	16.95 ± 0.07	41.13 ± 0.58
	12	18.78 ± 0.76	56.37 ± 6.33
	15	18.02 ± 1.03	50.04 ± 8.58
	18	17.50 ± 0.50	45.71 ± 4.16
	21	18.98 ± 0.13	58.03 ± 1.08

The calculations of %RD were made relative to the extracellular area in the iced

samples at day 0. Data presented in Appendix 5 are averages of duplicate determinations with each of four fish.

A.7. Appendix 6: Extracellular Area present in iced and partially frozen (-3°C) muscle from cod acclimated at 10°C

Sample	Day of Analysis	% Extracellular Area	% Rel. Damage
Iced	0	12.87 ± 0.85	-
	3	12.97 ± 0.20	0.70 ± 1.55
	6	14.01 ± 0.02	8.86 ± 0.16
	9	15.11 ± 0.53	17.40 ± 4.12
	12	17.75 ± 0.75	37.92 ± 5.83
	15	18.05 ± 1.06	40.25 ± 8.24
	18	19.11 ± 1.90	48.48 ± 14.76
	21	22.18 ± 0.68	72.34 ± 5.28
PF	0	14.52 ± 0.52	12.82 ± 4.04
	3	15.01 ± 0.04	16.63 ± 0.31
	6	15.99 ± 1.00	24.24 ± 7.77
	9	17.03 ± 0.26	32.32 ± 2.02
	12	18.32 ± 0.41	42.35 ± 3.19
	15	20.89 ± 0.52	62.32 ± 4.04
	18	19.97 ± 0.98	55.17 ± 7.61
	21	21.03 ± 0.26	63.40 ± 2.02

The calculations of %RD were made relative to the extracellular area in the iced

samples at day 0. Data presentaed in Appendix 6 are averages of duplicate determinations with each of four fish.

A.8. Appendix 7: Free drip for muscles derived from fish acclimated at 0°C

Sample	Time (day)	% Drip[1]
Iced	0	0
PF		Negligible
Iced	3	6.22 ± 1.53
PF		9.96 ± 1.82
Iced	6	7.84 ± 3.15
PF		9.96 ± 0.83
Iced	9	8.64 ± 0.54
PF		10.13 ± 0.73
Iced	12	10.78 ± 2.68
PF		11.59 ± 2.19
Iced	15	11.21 ± 0.51
PF		12.58 ± 0.65
Iced	18	11.50 ± 2.72
PF		13.23 ± 2.95
Iced	21	12.22 ± 1.01[a]
PF		13.77 ± 1.95[a]

[1]% Drip was calculated as follows :

$$\% \text{ Drip} = \frac{\text{Wt. of Drip} \times 100}{\text{Initial wt. of tissue}}$$

Data presented in Appendix 7 are average values of duplicate determinations with each of four fish. Values in the same row followed by same superscript were not significantly different ($P < 0.05$).

A.9. Appendix 8: Free drip for muscles derived from fish acclimated at 10°C

Sample	Time (day)	% Drip[1]
Iced	0	- 0
PF		Negligible
Iced	3	9.92 ± 2.82
PF		10.87 ± 0.47
Iced	6	10.29 ± 3.00
PF		11.77 ± 1.37
Iced	9	12.37 ± 3.33
PF		13.39 ± 1.61
Iced	12	14.22 ± 2.44
PF		15.20 ± 1.37
Iced	15	14.40 ± 0.20
PF		14.89 ± 1.68
Iced	18	14.51 ± 0.59
PF		17.93 ± 0.12
Iced	21	14.77 ± 1.80
PF		18.00 ± 0.98

Data presented in Appendix 8 are duplicates determinations with each of four fish. Values in the same row followed by same superscript were significantly different ($P < 0.05$).

A.10. Appendix 9: Total physiological free amino acids from iced samples derived from fish acclimated at 10°C, in mM

Amino Acid	Day 0	5	10	15	20
Ala	7.55 ± 0.34	8.43 ± 0.28	7.28 ± 0.34	7.61 ± 0.10	3.95 ± 0.22
Arg	0.25 ± 0.17	0.20 ± 0.09	0.19 ± 0.09	0.17 ± 0.15	0.00 ± 0.00
Asp	0.14 ± 0.01	0.14 ± 0.01	0.17 ± 0.02	0.13 ± 0.02	0.16 ± 0.02
1/2Cys	0.02 ± 0.01	0.03 ± 0.01	0.04 ± 0.00	0.05 ± 0.01	0.02 ± 0.00
Cys	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.02	0.03 ± 0.00	0.02 ± 0.00
Glu	0.38 ± 0.05	0.44 ± 0.00	0.51 ± 0.11	0.63 ± 0.03	0.42 ± 0.01
Gly	11.45 ± 0.18	10.57 ± 0.96	10.25 ± 0.12	10.39 ± 0.76	5.74 ± 0.33
His	0.41 ± 0.11	0.61 ± 0.39	0.40 ± 0.09	0.57 ± 0.23	0.19 ± 0.00
HO-Lys	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
HO-Pro	0.56 ± 0.17	0.61 ± 0.27	0.45 ± 0.18	0.67 ± 0.18	0.52 ± 0.17
Ileu	0.09 ± 0.01	0.09 ± 0.06	0.10 ± 0.04	0.12 ± 0.10	0.13 ± 0.03
Leu	0.19 ± 0.11	0.20 ± 0.08	0.20 ± 0.06	0.22 ± 0.14	0.64 ± 0.08
Lys	2.15 ± 0.94	2.59 ± 0.53	2.17 ± 0.78	2.27 ± 0.78	1.48 ± 0.11
Met	0.18 ± 0.08	0.15 ± 0.05	0.14 ± 0.04	0.14 ± 0.08	0.16 ± 0.03
Phe	0.12 ± 0.04	0.11 ± 0.05	0.12 ± 0.03	0.13 ± 0.06	0.20 ± 0.01
Pro	0.68 ± 0.28	2.20 ± 0.03	1.57 ± 0.18	1.67 ± 0.41	0.93 ± 0.00
Ser	1.04 ± 0.36	1.20 ± 0.06	0.91 ± 0.23	1.10 ± 0.19	0.03 ± 0.03
Tau	4.36 ± 0.64	5.91 ± 0.59	4.48 ± 0.63	2.27 ± 0.27	6.83 ± 0.33
Thr	2.12 ± 0.08	2.62 ± 0.14	2.17 ± 0.81	2.40 ± 0.02	1.96 ± 0.18
Try	0.05 ± 0.01	0.05 ± 0.02	0.04 ± 0.01	0.02 ± 0.02	0.03 ± 0.00
Try	0.14 ± 0.06	0.15 ± 0.04	0.15 ± 0.04	0.16 ± 0.06	0.17 ± 0.01
Val	0.22 ± 0.08	0.21 ± 0.06	0.15 ± 0.06	0.24 ± 0.13	0.26 ± 0.02
Total	32.15 ± 3.76	36.56 ± 4.86	31.34 ± 4.00	30.99 ± 3.74	23.84 ± 1.52

Data presented in Appendix 9 are average values for duplicate determinations for each of four fish.

A.11. Appendix 10: Total physiological free amino acids from iced samples derived from fish acclimated at 0°C, in mM

Amino Acid	Day 0	5	10	15	20
Ala	14.91 ± 0.86	12.29 ± 0.57	13.52 ± 0.99	12.2 ± 0.76	11.93 ± 0.79
Arg	0.04 ± 0.02	0.19 ± 0.01	0.15 ± 0.00	0.15 ± 0.04	0.11 ± 0.06
Asp	0.16 ± 0.05	0.16 ± 0.00	0.15 ± 0.01	0.14 ± 0.01	0.12 ± 0.00
1/2Cys	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.00
Cys	0.04 ± 0.01	0.05 ± 0.00	0.05 ± 0.01	0.04 ± 0.01	0.01 ± 0.01
Glu	0.58 ± 0.18	0.63 ± 0.16	0.60 ± 0.21	0.64 ± 0.08	0.63 ± 0.15
Gly	13.51 ± 0.22	11.52 ± 0.97	12.82 ± 0.90	11.53 ± 0.14	11.12 ± 0.39
His	1.45 ± 0.22	0.60 ± 0.04	1.35 ± 0.17	0.72 ± 0.07	0.60 ± 0.80
HO-Lys	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
HO-Pro	0.51 ± 0.28	0.42 ± 0.25	0.47 ± 0.30	0.50 ± 0.27	0.47 ± 0.37
Ileu	0.23 ± 0.01	0.23 ± 0.04	0.19 ± 0.05	0.27 ± 0.04	0.26 ± 0.01
Leu	0.48 ± 0.10	0.49 ± 0.16	0.46 ± 0.12	0.51 ± 0.17	0.52 ± 0.09
Lys	1.08 ± 0.28	1.80 ± 0.04	1.45 ± 0.03	1.87 ± 0.86	1.74 ± 0.37
Met	0.34 ± 0.00	0.31 ± 0.01	0.32 ± 0.01	0.32 ± 0.01	0.32 ± 0.02
Phe	0.80 ± 0.64	0.86 ± 0.74	0.84 ± 0.05	0.85 ± 0.03	0.78 ± 0.59
Pro	1.87 ± 0.41	1.32 ± 0.18	1.52 ± 0.23	1.15 ± 0.13	1.15 ± 0.11
Ser	2.51 ± 0.08	1.41 ± 0.69	1.96 ± 0.28	1.72 ± 0.53	1.72 ± 0.49
Tau	8.40 ± 0.26	5.10 ± 0.22	6.82 ± 0.59	6.96 ± 0.39	5.52 ± 0.01
Thr	2.76 ± 0.07	2.32 ± 0.71	2.66 ± 0.07	2.23 ± 0.04	2.13 ± 0.02
Try	0.06 ± 0.00	0.04 ± 0.01	0.05 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
Tyr	0.11 ± 0.03	0.15 ± 0.02	0.17 ± 0.02	0.17 ± 0.03	0.20 ± 0.04
Val	0.48 ± 0.09	0.46 ± 0.13	0.43 ± 0.08	0.41 ± 0.21	0.45 ± 0.08
Total	50.87 ± 3.81	40.37 ± 4.36	46.00 ± 4.13	42.44 ± 3.83	39.84 ± 3.78

Data presented in Appendix 10 are averages of duplicate determinations for each of four fish.

A.12. Appendix 11: Total physiological free amino acids from partially frozen samples derived from fish acclimated at 10°C, in mM

Amino Acid	Day 0	5	10	15	20
Ala	8.48 ± 0.97	7.32 ± 0.36	6.46 ± 0.34	7.28 ± 0.90	6.30 ± 0.30
Arg	0.22 ± 0.11	0.16 ± 0.06	0.24 ± 0.09	0.21 ± 0.06	0.18 ± 0.08
Asp	0.13 ± 0.01	0.08 ± 0.01	0.16 ± 0.01	0.14 ± 0.02	0.14 ± 0.01
1/2Cys	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
Cys	0.05 ± 0.01	0.04 ± 0.00	0.02 ± 0.00	0.06 ± 0.02	0.00 ± 0.00
Glu	0.44 ± 0.01	0.59 ± 0.11	0.52 ± 0.02	0.56 ± 0.04	0.51 ± 0.00
Gly	11.01 ± 0.57	9.92 ± 0.85	9.96 ± 0.67	9.89 ± 0.94	9.02 ± 0.34
His	0.86 ± 0.05	0.56 ± 0.17	0.21 ± 0.03	0.46 ± 0.08	0.36 ± 0.03
HO-Lys	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
HO-Pro	0.6 ± 0.21	0.51 ± 0.19	0.45 ± 0.17	0.52 ± 0.26	0.44 ± 0.27
Ileu	0.10 ± 0.06	0.08 ± 0.06	0.07 ± 0.05	0.06 ± 0.04	0.12 ± 0.03
Leu	0.19 ± 0.10	0.32 ± 0.24	0.17 ± 0.07	0.13 ± 0.04	0.18 ± 0.09
Lys	2.10 ± 0.73	1.98 ± 0.41	2.22 ± 0.01	2.32 ± 0.03	2.22 ± 0.08
Met	0.18 ± 0.09	0.14 ± 0.06	0.13 ± 0.06	0.16 ± 0.04	0.12 ± 0.03
Phe	0.10 ± 0.04	0.09 ± 0.04	0.11 ± 0.02	0.10 ± 0.03	0.11 ± 0.00
Pro	2.00 ± 0.38	1.85 ± 0.13	1.17 ± 0.01	0.96 ± 0.26	0.84 ± 0.01
Ser	1.15 ± 0.24	1.21 ± 0.06	0.88 ± 0.30	1.03 ± 0.15	1.20 ± 0.28
Tau	5.61 ± 0.51	6.40 ± 0.17	4.32 ± 0.02	5.74 ± 0.13	5.20 ± 0.59
Thr	2.64 ± 0.08	2.21 ± 0.73	1.82 ± 0.50	1.92 ± 0.54	1.74 ± 0.59
Trp	0.43 ± 0.01	0.33 ± 0.01	0.33 ± 0.00	0.29 ± 0.01	0.22 ± 0.01
Tyr	0.13 ± 0.05	0.14 ± 0.04	0.14 ± 0.05	0.14 ± 0.04	0.13 ± 0.05
Val	0.22 ± 0.08	0.18 ± 0.08	0.14 ± 0.06	0.20 ± 0.04	0.18 ± 0.05
Total	36.66 ± 4.36	34.13 ± 3.79	29.55 ± 2.49	32.20 ± 2.78	29.75 ± 2.75

Data presented in Appendix 11 are averages of duplicate determinations for each of four fish.

A.13. Appendix 12: Total physiological free amino acids from partially frozen samples derived from fish acclimated at 0°C, in mM

Amino Acid	Day 0	5	10	15	20
Ala	14.48 ± 0.39	13.18 ± 0.99	13.32 ± 0.08	12.48 ± 0.14	13.01 ± 0.28
Arg	0.17 ± 0.08	0.16 ± 0.00	0.10 ± 0.05	0.09 ± 0.09	0.06 ± 0.06
Asp	0.15 ± 0.03	0.16 ± 0.01	0.12 ± 0.02	0.11 ± 0.00	0.16 ± 0.02
1/2Cys	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Cys	0.04 ± 0.00	0.02 ± 0.00	0.06 ± 0.00	0.06 ± 0.02	0.01 ± 0.01
Glu	0.61 ± 0.19	0.61 ± 0.20	0.57 ± 0.14	0.59 ± 0.25	0.57 ± 0.30
Gly	13.07 ± 0.58	12.18 ± 0.76	12.49 ± 0.76	12.07 ± 0.34	12.14 ± 0.19
His	1.31 ± 0.74	0.73 ± 0.12	1.28 ± 0.17	0.68 ± 0.08	1.08 ± 0.00
HO-Lys	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00
HO-Pro	0.50 ± 0.36	0.45 ± 0.29	0.62 ± 0.47	0.60 ± 0.31	0.84 ± 0.54
Ileu	0.26 ± 0.10	0.24 ± 0.03	0.25 ± 0.01	0.18 ± 0.07	0.18 ± 0.04
Leu	0.50 ± 0.21	0.50 ± 0.14	0.47 ± 0.10	0.45 ± 0.04	0.42 ± 0.06
Lys	1.35 ± 0.54	1.61 ± 0.76	1.21 ± 0.48	1.74 ± 0.67	1.31 ± 0.68
Met	0.38 ± 0.04	0.31 ± 0.00	0.34 ± 0.01	0.31 ± 0.02	0.27 ± 0.05
Phe	0.90 ± 0.73	0.84 ± 0.68	0.78 ± 0.62	0.84 ± 0.66	0.77 ± 0.62
Pro	1.48 ± 0.80	1.42 ± 0.11	1.68 ± 0.24	1.24 ± 0.06	1.91 ± 0.42
Ser	2.33 ± 0.19	1.56 ± 0.66	1.83 ± 0.36	1.17 ± 0.14	0.67 ± 0.67
Tau	6.99 ± 0.03	4.69 ± 0.14	7.38 ± 0.67	6.63 ± 0.24	7.70 ± 0.55
Thr	3.00 ± 0.28	2.47 ± 0.69	2.54 ± 0.82	2.08 ± 0.48	2.22 ± 0.70
Trp	0.04 ± 0.01	0.07 ± 0.00	0.04 ± 0.01	0.03 ± 0.01	0.01 ± 0.01
Tyr	0.20 ± 0.00	0.16 ± 0.02	0.16 ± 0.01	0.16 ± 0.00	0.14 ± 0.00
Val	0.47 ± 0.17	0.46 ± 0.15	0.42 ± 0.07	0.43 ± 0.05	0.36 ± 0.04
Total	48.25 ± 5.47	41.84 ± 5.75	45.68 ± 5.09	41.97 ± 3.65	43.85 ± 5.24

Data presented in Appendix 12 are averages of duplicate determinations for each of four fish.