Protein denaturation in frozen stored hake (*Merluccius merluccius* L.) muscle: The role of formaldehyde

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Protein solubility of whole and minced muscle of hake (*Merluccius merluccius* L.) was evaluated at different temperatures of storage: $-5°$, $-12°$ and $-20°C$. Proteins soluble in 1% NaCl did not show any change at any of the temperatures of storage, while the 5% NaCl-soluble proteins decreased significantly when whole hake was stored at $-5°$ and $-12°C$ ($p < 0.01$). A correlation was found between the decrease of solubility and the production of formaldehyde at $-5°$ and $-12°C$ ($p < 0.001$ and $p < 0.01$, respectively). Bound formaldehyde (formaldehyde not extractable) increased with storage time at $-5°$ ($p < 0.001$).

The addition of formaldehyde to the minced muscle brought about a decrease in both the 1% and the 5% NaCl-soluble proteins. The decrease was dependent upon the temperature, the amount of formaldehyde added and the time of storage. The role of formaldehyde in the denaturation of sarcoplasmic proteins was confirmed by measuring trimethylamino oxide demethylase activity during the experiment.

INTRODUCTION

Frozen storage offers a means of preserving fish; however, during frozen storage, quality is lost due to a deterioration of texture, flavour and colour, especially after long periods of storage, when poor freezing practices are employed or when the initial fish quality is low. The main problem is the change of texture which reduces consumer acceptability (Sikorski *et al.*, 1976; Shenouda, 1980). Texture changes are the result of denaturation of muscle proteins, particularly those in the myofibrillar fraction (Haard, 1992). For these reasons, different methods of measuring protein denaturation have been used to follow textural deterioration. Among them, protein solubility is one of the most often chosen, because of its simplicity and relatively good correlations with textural characteristics (Shenouda, 1980; De Koning & Mol, 1991). Solubility of muscle proteins, in particular buffers, reflects changes in their unique chemical and three-dimensional structure, changes that are usually referred to as denaturation (Nakai *et al.*, 1991).

Denaturation of muscle proteins has been attributed to different factors, fish species being one of them. Basically, fish species can be divided into two groups based on the possession or not of trimethylamine oxide demethylase (TMAOase), which degrades trimethylamine oxide (TMAO) to dimethylamine (DMA) and formaldehyde (FA) during iced and/or frozen storage. The enzyme is mostly found in the viscera and red muscle of fishes belonging to gadoid species (Mackie & Thomson, 1974; Hebard *et al.*, 1982; Haard, 1992). During frozen storage, a strong relationship between the production and accumulation of FA and the deterioration of texture in muscle of this group of fish has been found (Dingle *et al.*, 1977; Gill *et al.*, 1979; Matthews *et al.*, 1980; Parkin and Hultin, 1982*a*; Kelleher *et al.*, 1981; Jahncke *et al.*, 1992). Although FA might react with proteins during frozen storage, it is still not clear how FA accelerates protein denaturation (Ang & Hultin, 1989).

Hake, fish belonging to the Order *Gadiformes*, are gaining importance as fisheries, as cod are increasingly being depleted (Morrow, 1992). These species represent the main high-quality white-fleshed fish in some European countries, such as Spain (Whitaker, 1980), where they are very often sold in the frozen state. It is also an important source of derived frozen products. There are some data on storage properties of frozen South Atlantic hake, *Merluccius hubbsi* and *M. gayi* (Almandos *et al.*, 1984; Ciarlo *et al.*, 1985) *M. bilinearis* (Hiltz *et al.*, 1976; Licciardello *et al.*, 1980), *M. productus* (Crawford *et al.*, 1979) and *M. capensis* (De Koning & Mol, 1991). Also, it has been reported that there are changes
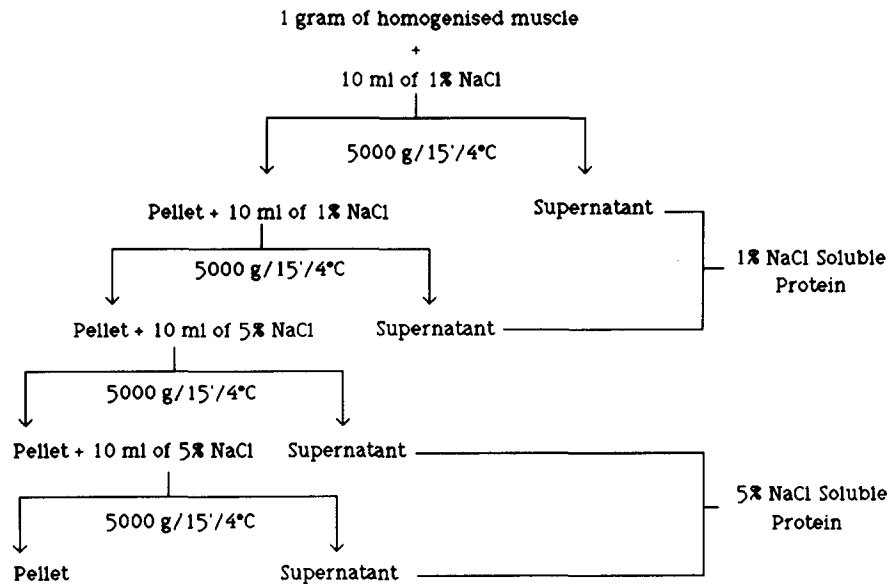
1 gram of homogenised muscle
+
10 ml of 1% NaCl

5000 g/15'/4°C

Pellet + 10 ml of 1% NaCl   Supernatant

5000 g/15'/4°C   1% NaCl Soluble
                                                            Protein

Pellet + 10 ml of 5% NaCl   Supernatant

5000 g/15'/4°C

Pellet + 10 ml of 5% NaCl   Supernatant

5000 g/15'/4°C   5% NaCl Soluble
                                                            Protein

Pellet   Supernatant

Fig. 1. Protocol for the extraction of 1% and 5% NaCl-soluble proteins in minced muscle with FA added.

in some sensory and chemical parameters in European hake (*Merluccius merluccius*) during ice and frozen storage (Pérez-Villarreal & Howgate, 1987, 1991). However, there are no data on changes in muscle protein solubility of frozen stored European hake.

Temperatures such as −18°C and −20°C are recommended for storage of frozen fish in retail cabinets, but these temperatures may rise to −12°C or even higher. This might adversely affect the quality, depending upon the period that fish are maintained under those conditions (Singh & Wang, 1977). The objective of this study was to follow changes in protein solubility and FA production of hake (*Merluccius merluccius* L.) muscle when the whole fish was stored at temperatures that might be attainable in retail outlets or home freezers, −5°C, −12°C and −20°C. Also, minced muscle was used as a model system for adding different amounts of FA and

for evaluating the role of excess of FA on protein solubility during storage at the above temperatures.

MATERIALS AND METHODS

Storage of whole hake

Hake (*Merluccius merluccius* L.), varying in weight from 0·5 to 0·8 kg, were obtained from the local fish market 24 h after catching. The fish had been eviscerated when landed and each individual was measured and weighed. Each fish was immediately blast-frozen until it reached a temperature of −40°C (2 h). Each frozen fish was packed in a polyethylene bag and randomly divided into three batches, which were stored at −5°, −12° and −20°C respectively. At definite inter-
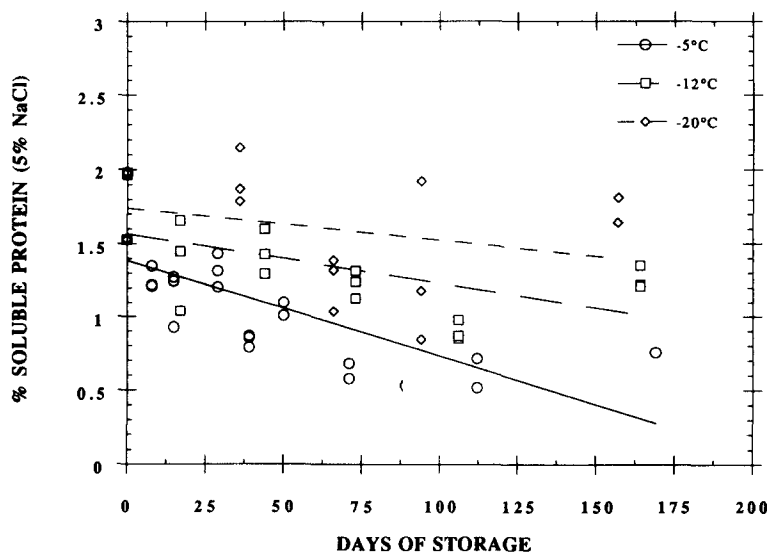

Fig. 2. Decrease in 5% NaCl-soluble protein of whole hake muscle during frozen storage. Regression lines were plotted in each case; coefficients (r^2), significance and number of individuals analysed (n) were as follows: −5°C: $r^2 = 0·615$ ($p < 0·0001$), $n = 22$; −12°C: $r^2 = 0·352$ ($p < 0·01$), $n = 18$; −20°C: $r^2 = 0·00$ (n.s.), $n = 14$.

vals, 1–3 individuals were removed from frozen storage, thawed (4°C, overnight) and prepared for analysis by deboning and mincing the flesh.

Storage of minced muscle after FA addition

Another batch of eviscerated hake was purchased at the local fish market. The fish were skinned, deboned and minced, then flesh of different individuals was pooled into one batch. The homogenised flesh was distributed in plastic containers and FA (diluted with distilled water from a 37% solution, Baker) was added and evenly distributed by using an Ultra-Turrax tissue macerator (9500 rpm for 1 min). FA concentrations attained in the samples were 80, 160, 320 and 640 ppm.

The FA concentration of the stock solution was measured by iodine titration (Treadwell, 1921).

All samples were frozen at −30°C (24 hours) and after this period they were split into three batches, each of them maintained at the following temperatures: −5°, −12° and −20°C up to a maximum period of six months.

Protein solubility determination

In the case of frozen storage of whole hake, the method employed was similar to that described by Laird *et al.* (1980). One gram of minced muscle was homogenised with cold 1% NaCl, 20 mM NaHCO₃ pH 7·0, by using an Ultra-Turrax (1 min at 9500 rpm). The homogenate was then centrifuged at 13 000 × g for 20 min; the supernatant was made up to 100 ml. This fraction was considered as the 1% NaCl-soluble protein. The pellet was resuspended in cold 5% NaCl, 20 mM NaHCO₃ pH 7·0 and then centrifuged at 13 000 × g for 20 min. The supernatant was made up to 100 ml and considered as the 5% NaCl-soluble protein.

The 1% NaCl- and 5% NaCl-soluble proteins of

FA-added minced muscle were extracted as shown in Fig. 1.

The protein in all fractions was determined by the method of Lowry *et al.* (1951).

FA and DMA determination

FA was determined by the spectrophotometric method of Nash (1953) in an aqueous extract of the muscle. This extract was obtained by steam distillation of the muscle in the presence of 85% phosphoric acid (Rehbein, 1987).

DMA was determined by the spectrophotometric method described by Mackie & Thomson (1974).

Statistical analysis

Linear regression analysis of changes of soluble protein, free and bound FA and DMA *versus* time of storage were made with the data obtained from 1–3 individuals per period and temperature in the case of whole hake storage. Correlation analysis were carried out when comparing two measured variables.

RESULTS

Frozen storage of whole hake

The 1% NaCl-soluble proteins of whole hake stored at −5°, −12° and −20°C did not show any significant change (data not shown). Regression analysis of 5% NaCl-soluble protein values belonging to different individuals showed that the solubility of the hakes stored at −5° and −12°C decreased significantly ($p < 0.01$), as can be seen in Fig. 2. It also can be observed that solubility is lesser with higher temperature of storage.
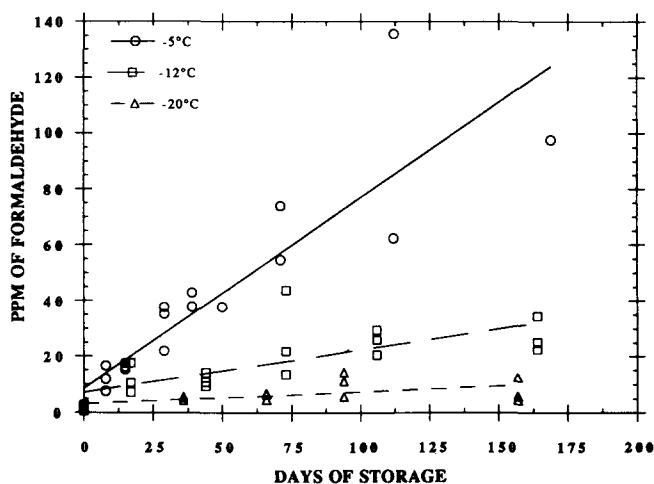

Fig. 3. Formaldehyde production in the muscle of whole hake during frozen storage. Regression lines were plotted; coefficients (r^2), significance and number of individuals analysed (n) were as follows: −5°C: $r^2 = 0.508$ ($p < 0.01$), $n = 22$; −12°C: $r^2 = 0.352$ ($p < 0.01$), $n = 18$; −20°C: $r^2 = 0.056$ (n.s.), $n = 15$.
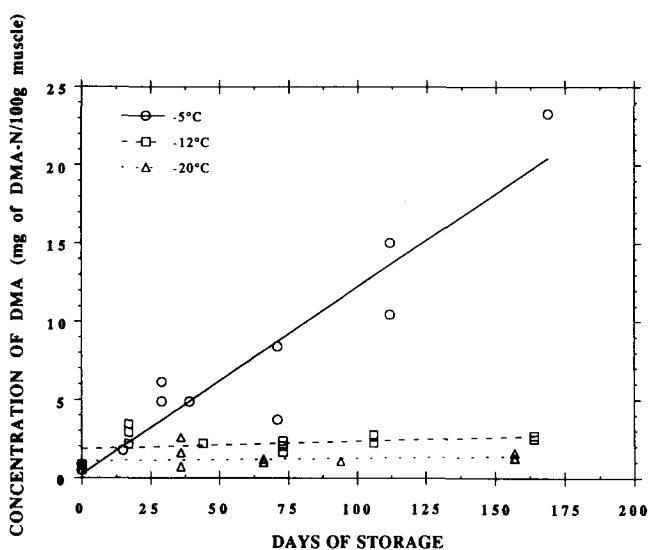

Fig. 4. Dimethylamine (DMA-N) production in the muscle of whole hake during frozen storage. Regression lines were plotted in each case; coefficients (r^2), significance and number of individuals analysed (n) were as follows: −5°C: $r^2 = 0.876$ ($p < 0.0001$), $n = 13$; −12°C: $r^2 = 0.091$ (n.s.), $n = 16$; −20°C: $r^2 = 0.042$ (n.s.), $n = 15$.
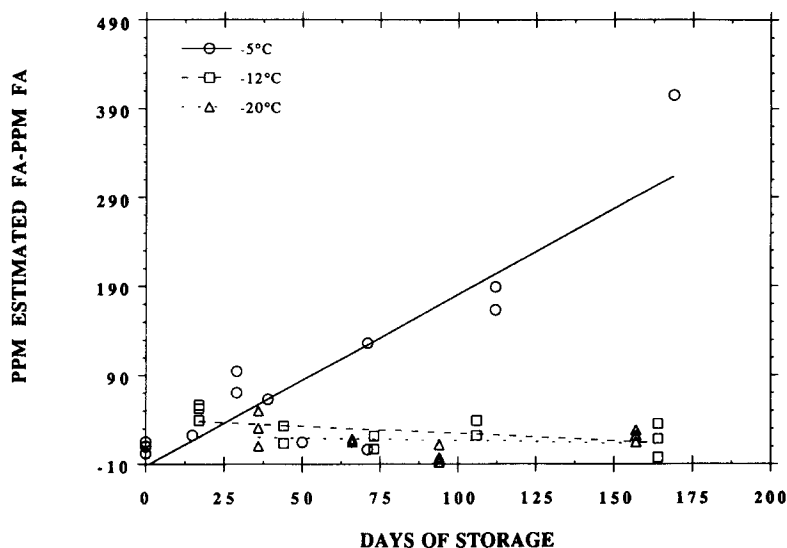
Fig. 5. Bound FA in the muscle of whole hake during frozen storage. Regression lines were plotted in each case; coefficients (r^2), significance and number of individuals analysed (n) were as follows: $-5°C$: $r^2 = 0.780$ ($p < 0.001$), $n = 13$; $-12°C$: $r^2 = 0.023$ (n.s.), $n = 16$; $-20°C$: $r^2 = 0.002$ (n.s.), $n = 15$.

FA was produced during the frozen storage of whole hake muscle. The increase in FA was very dependent upon the temperature of storage, being greatest at $-5°C$ ($p < 0.01$) and less at $-12°C$ ($p < 0.01$), while at $-20°C$ no significant changes were observed (Fig. 3).

A correlation test was carried out between FA produced by the muscle and the 5% NaCl-soluble proteins. Correlation coefficients show that there are significant correlations between the decrease in 5% NaCl-soluble protein at $-5°C$ and $-12°C$ and the increase in FA at those temperatures ($-5°C$: $r^2 = -0.767$, $p < 0.001$; $-12°C$: $r^2 = -0.703$, $p < 0.01$; $-20°C$: $r^2 = -0.347$, $p > 0.05$, n.s.).

DMA was also determined. As expected, the data were consistent with FA changes, since they are both products of TMAOase (Parkin & Hultin, 1982b) (Fig. 4).

It has been shown that DMA and FA values may differ. Although both compounds, DMA and FA, are produced equimolecularly, the recovery of all FA produced might be reduced because of the high reactivity of FA with different compounds (free amino acids, nucleotides and proteins). In Fig. 5, FA has been subtracted, calculated from measured DMA values, and FA determined by distillation. This FA has been called bound FA. It can be observed that bound FA increases with storage time at $-5°C$ ($p < 0.001$) but does not change with storage time at $-12°C$ and $-20°C$.

Frozen storage of minced muscle and FA addition

The highest level of FA found in the experiment of the whole hake was 134 ppm. The chosen values of added FA are those expected of gadoid fish (80 and 160 ppm) or sometimes in excess values (320 and 640 ppm) (Castell et al., 1973).

Adding FA to minced hake muscle and frozen-stored resulted in a rubbery texture depending upon the amount of FA added and the temperature of frozen storage. Both 1% and 5% NaCl-soluble proteins caused changes related to the amount of FA added and the

temperature of storage (Figs 6 and 7). However, the loss of solubility was more marked in the case of 5% NaCl-soluble proteins, as was expected. When no FA was added to the muscle, 1% NaCl-soluble proteins did not cause any change at any temperature studied. When FA is added, a decrease in 1% NaCl-soluble proteins was noticed with time of storage at $-12°C$ and $-20°C$. Surprisingly, at $-5°C$ no changes could be detected with storage time, but differences were observed between each of the different levels of added FA. The same could be observed in 5% NaCl-soluble proteins.

The role of FA in denaturation of sarcoplasmic proteins was confirmed by measuring DMA produced in the minced muscle after adding FA. Figure 8 shows DMA produced at different temperatures of storage with different amounts of FA added. FA promoted the inhibition of the TMAOase (DMA and FA producing enzyme). The inhibition was greater at $-5°C$ and dependent upon the amount of FA added: the higher the amount of added FA the less DMA was produced. The same occurred at $-12°C$ although the production was less than at $-5°C$, and at $-20°C$ it was even less than at $-12°$ and $-5°C$. Although it can be said that FA inhibited the enzyme because it is a product of the catalysis of TMAOase, the solubility data might suggest a mechanism of denaturation of the enzyme. Again, it can be observed that low temperature of storage inhibits both the production of FA and denaturation of the enzyme.

DISCUSSION

Frozen storage of whole hake

The 5% NaCl-soluble proteins were affected while the 1% NaCl-soluble proteins did not show any changes in solubility with storage time at $-5°$, $-12°$ and $-20°C$. This might reflect a higher sensitivity of the 5%
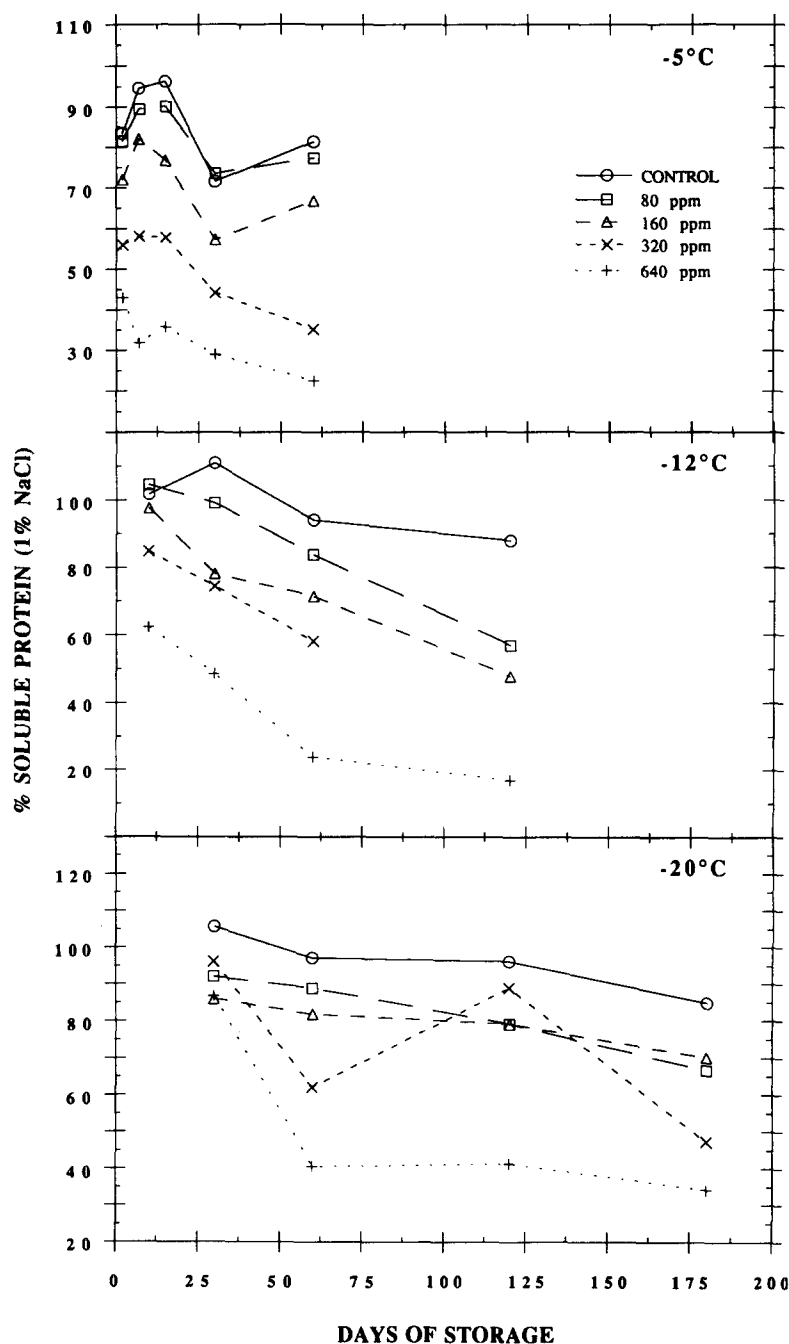
Fig. 6. Decrease of 1% NaCl-soluble protein in the minced muscle containing added FA (unfrozen samples taken as 100% solubility).

NaCl-soluble proteins to denaturation on freezing and frozen storage at different temperatures compared to the 1% NaCl-soluble protein. This is understandable as 1% NaCl-soluble proteins are mainly globular sarcoplasmic proteins, whereas 5% NaCl-soluble proteins are mainly the myofibrillar fraction of the muscle proteins (Shenouda, 1980). The same results have been found by other authors for different species of gadoids (Tejada *et al.*, 1984). However, other techniques, such as electrophoresis or liquid chromatography, have shown that the sarcoplasmic fraction might also be affected, although to a lesser extent than the myofibrillar fraction (Rehbein, 1985; Onishi & Rodger, 1980; LeBlanc & LeBlanc, 1992; Owusu-Ansah & Hultin, 1992).

It has been suggested that FA is the main causative factor of denaturation of muscle proteins in frozen stored gadoid fish (Matthews *et al.*, 1980; Parkin & Hultin, 1982*b*). As would be expected, a correlation was found between FA production and decrease in protein solubility. In high FA-producing fish, such as red hake (*Urophycis chuss*), low-ionic-strength soluble proteins were denatured (LeBlanc & LeBlanc, 1992; Owusu-Ansah & Hultin, 1992). In our study, a decrease in the 1% NaCl-soluble proteins was found when FA was added to minced muscle, indicating that the amount of FA is possibly of some importance in denaturing sarcoplasmic proteins and also in the state of tissue disruption (Parkin & Hultin, 1982*a*).

It also was found that bound FA was dependent upon the temperature and time of storage, in agree-
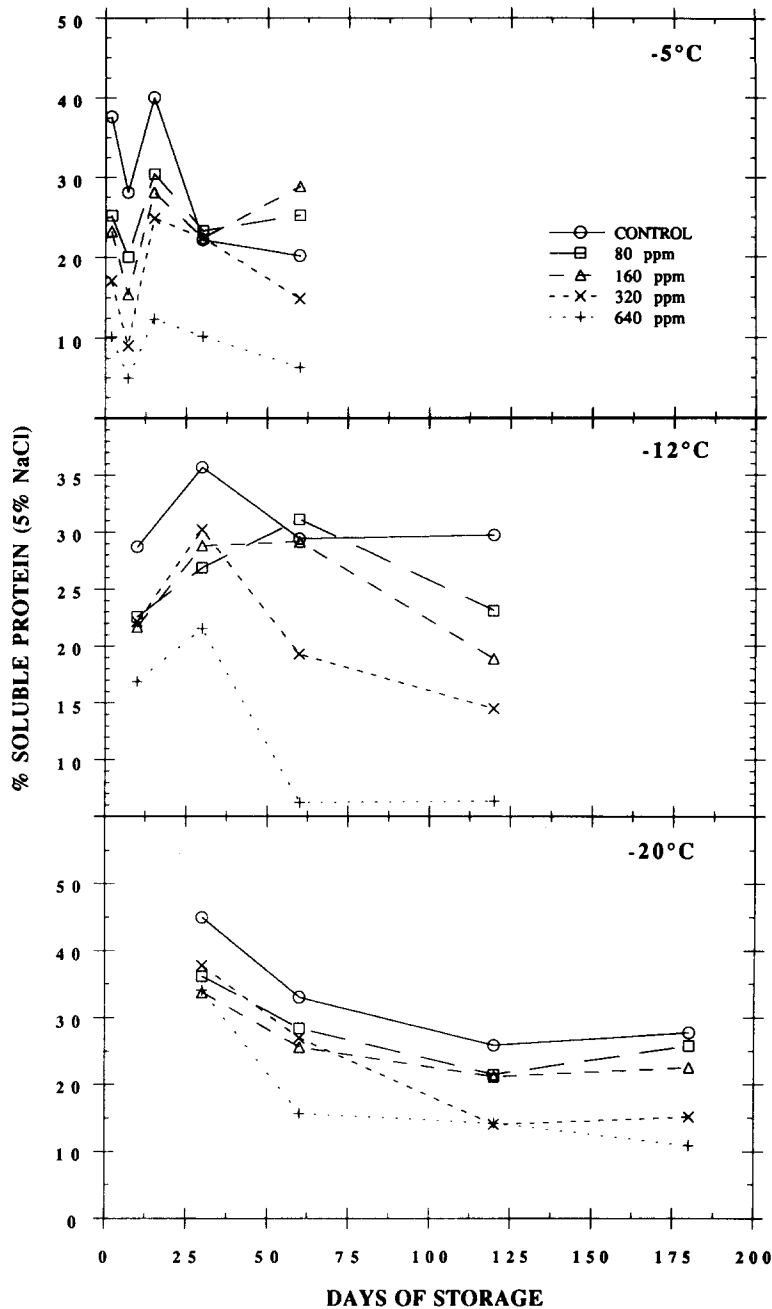
Fig. 7. Decrease of 5% NaCl-soluble protein in the minced muscle containing added FA (unfrozen samples taken as 100% solubility).

ment with the findings of Owusu-Ansah and Hultin (1986) for red hake at −7°C. The high reactivity of FA with amino acids, nucleotides and proteins (Poulter & Lawrie, 1979) might explain the existence of bound FA, and also the differences in the sensitivity of the FA and DMA tests (Kelleher et al., 1981).

If FA reacts with proteins, the highest differences between the DMA and FA concentrations would be found at the early stages of storage when the amount of FA is low and all protein target sites are, in theory, free. On the other hand, we found that bound FA increased with storage time. This suggests that changes are necessary in the target sites for the reaction to take place, or the way in which FA is produced would determine its reactivity and the time needed for the reaction to take place.

Frozen storage of minced muscle and FA addition

When FA was added to the minced muscle it led to a decrease in solubility that was very dependent upon the FA concentration, but was not so dependent upon the time of storage at −5°C. This means that FA reacts very quickly with proteins and nonprotein nitrogen (measured as a decrease in solubility in this study). Bearing in mind that mincing (tissue disruption) destroys the natural structures and compartments that normally exist in the tissue, it is possible that reaction limitation could in fact be a matter of availability of reactants (FA, protein targets). Is FA available to its targets, in our case proteins, during frozen storage? TMAOase has been found to be an enzymic complex, a constituent of lysosomal membranes (Gill & Paulson,
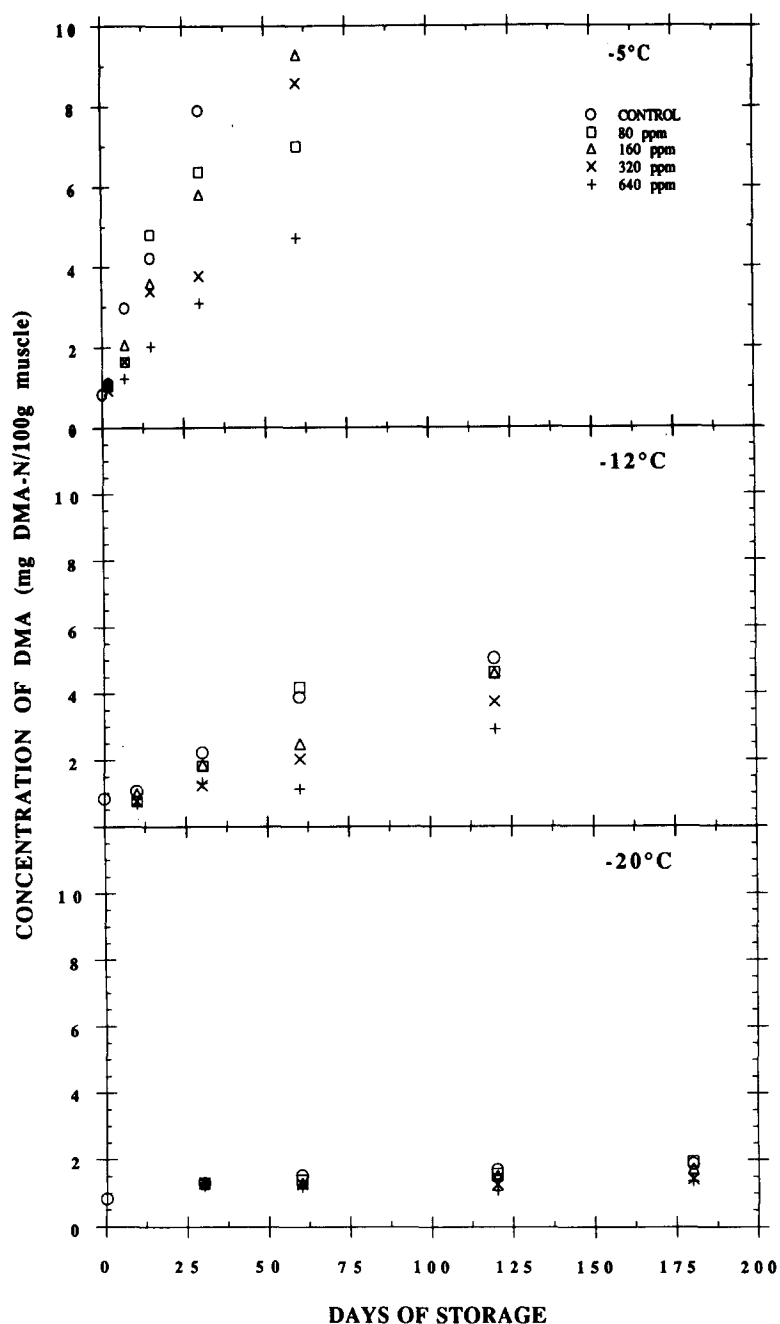
Fig. 8. Formation of dimethylamine (DMA-N) in minced muscle containing added FA.

1982; Parkin & Hultin, 1986; Joly *et al.*, 1992). Recently, it has been shown that storage of cod muscle at −12°C revealed extensive condensation of sarcoplasmic reticulum membrane (Ablett & Gould, 1992). Changes in the integrity of membranes as storage progresses might also reflect different stages in the release of FA and DMA, if both compounds were produced inside subcellullar organules (e.g. lysosomes). Raising the temperature of storage even for short periods of time, regardless of subsequent reduction of temperature, promotes diffusion and redistribution of reactants and metabolites, facilitating the occurrence of deteriorative reactions (LeBlanc & LeBlanc, 1988). This could explain the fact that insolubility increases gradually during frozen storage and bound FA also increases gradually. Also, it is known that FA reacts more easily with already-denatured proteins (Ang & Hultin, 1989;

Sotelo & Mackie, 1993). So, as frozen storage progresses, the proteins become more susceptible to reaction with FA, according to the hypothesis of Ang and Hultin (1989) in which the role of FA is suggested to be that of an accelerator of protein denaturation.

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