Ultrastructure of the Myofibrillar Component in Cod (*Gadus morhua* L.) and Hake (*Merluccius merluccius* L.) Stored at −20 °C as a Function of Time

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Transmission electron microscopy and image analysis techniques were used to study the ultrastructure of the myofibrillar component in cod and hake muscle stored at −20 °C for varying periods of time. Cod muscle showed a deformation of the hexagonal array of thick filaments with the storage time, reflected in an increase in the eccentricity value, a parameter defined to measure changes in the ratio of maximum to minimum hexagon diameter, and an increase in the cross-linkings between the filaments. Degradation of cod thick filaments leading to detachment was also visible upon prolonged storage. In hake muscle significant changes were not found in the arrangement and morphology of thick filaments during frozen storage, suggesting a high incidence of intrafilament aggregation. The ultrastructural differences in the array of thick filaments between species were accompanied by a difference in the textural measurements.

Keywords: *Ultrastructure; frozen storage; hexagonal network; myofilament aggregation; Gadus morhua; Merluccius merluccius*

INTRODUCTION

Fish muscle undergoes chemical and physical alterations in frozen storage leading to loss of water, changes in flavor and taste, and undesirable softening, processes that are dependent on storage time and temperature (Matsumoto, 1980; Haard, 1992; Sikorski and Kolakowska, 1994). Denaturation and aggregation of myofibrillar proteins are of fundamental importance in the alterations of muscle texture during frozen storage. For this reason, a number of biochemical studies have been undertaken to determine the type of bonds and proteins involved in the aggregation (Connell, 1965; Laird et al., 1980; Matthews et al., 1980; Ohnishi and Rodger, 1980; Tsuchiya et al., 1980; Lim and Haard, 1984; Careche et al., 1998; Tejada et al., 1996). From another perspective, these studies have been complemented by ultrastructural studies on changes occurring in muscle fiber during frozen storage, although the nature of these changes has not been elucidated. Disturbances have been described in the pattern and structure of hexagonal network filaments in frozen muscle. According to Howgate (1979), disruptions to the filament pattern can be caused by the action of ice crystals on the myofibrils, reducing the space between thick filaments along the line of compression exerted by the ice. However, upon thawing, freshly frozen tissue regained its original filament spacing.

Changes in the hexagonal pattern were observed by Jarenbäck and Liljemark (1975a) in studies on cryofracture in frozen cod muscle. On comparing muscle kept frozen for a long period with unfrozen muscle, these authors found no differences in the interactions of myofilaments via cross-bridges or in dimensions within the single filaments.

On the basis of cross-bridges between thick and thin filaments in normal contraction and rigor mortis, it has been postulated that removal of water by freezing and, further, during frozen storage could reduce the distance between filaments, favoring the formation of cross-bridges between them (Jarenbäck and Liljemark, 1975a). On the basis of this idea, some studies have concentrated on measuring the distance between thick filaments. Liljemark (1969) concluded that there was no significant reduction in interfilament spacing after frozen storage, concluding that the distance between thick filaments is not implicated in toughening. Aitken and Connell (1977) found no changes in interfilament distances upon examining frozen cod muscle using X-ray diffraction. However, Jarenbäck and Liljemark (1975a) reported a significant reduction in the distance between thick filaments using cryofracture techniques. Howgate (1979) concluded that electron microscopy was perhaps not sensitive enough to detect changes in interfilament distance resulting from formation of cross-links.

Other ultrastructural studies were carried out by Jarenbäck and Liljemark (1975b). These authors indirectly observed structural changes in the thick myofilaments of frozen stored cod by the electron microscope examination of myofibrillar proteins extracted with saline solutions and residues insoluble in these solutions. To account for their observations, these authors suggested progressive cross-linking of myosin molecules within the thick filaments.

In recent years technical means have improved the sensitivity of microscopes by incorporating energy filters, which increase the contrast by eliminating the background produced by inelastic scattering and reduc-

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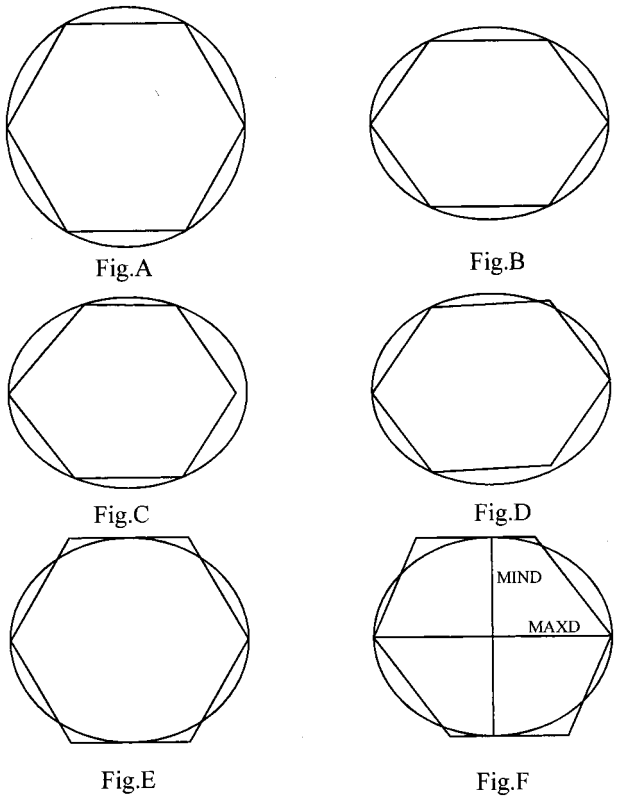

Figure 1. Scheme representing the method used to calculate the eccentricity values: MAXD, maximum diameter; MIND, minimum diameter; (A, B) hexagons drawn inside a circle and an ellipse, respectively; (C, D) examples of some ellipses that do not perfectly circumscribe the hexagons irregularly deformed during frozen storage; (E, F) eccentricity defines one ellipse around each hexagon, perfect (E) or deformed (F).

ing the effects of chromatic aberration (Peachy et al., 1986; Bauer et al., 1987). The purpose of this paper was to study ultrastructural changes of the filamentous components in cod and hake muscle stored at −20 °C. Transmission electron microscopy was used with energy filters and image analysis to detect changes in the pattern and/or structure of the myofilaments during frozen storage of the muscle.

MATERIALS AND METHODS

Fish Source. Cod (*Gadus morhua* L.) and hake (*Merluccius merluccius* L.) were supplied by Torry Research Station (TRS), Aberdeen, U.K., and Instituto Portûges de Investigacâo Maritima (IPIMAR), Lisbon, Portugal, respectively. Fillets were prepared from whole fish in post-rigor condition.

Cod fillets were blast-frozen at −40 °C in 4 h and packed in laminated vacuum pouches (30-μm nylon + 120-μm polyethylene). Frozen fillets were air-freighted with dry ice, vacuum packed in Cryovac BB-1 bags, and stored at −20 °C for 70 weeks.

Hake fillets were frozen at −40 °C on board and vacuum-packed. The fillets were air-freighted with dry ice and stored in chambers at −20 °C.

Transmission Electron Microscopy. A minimum of three individuals was used for each storage time. Frozen muscle after arrival was been considered to be the control. Rectangular pieces (3 mm × 2 mm) were removed from the fillets after they were thawed in air at room temperature. The pieces were immediately fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, postfixed in 1% osmium tetraoxide, dehydrated in a graded series of acetone, and finally embedded in Epon. Thin sections were stained with 2% uranyl acetate followed by lead citrate. Electron micrographs were obtained with a Model 902 electron microscope (Carl Zeiss, Oberkochen, Germany) with a Canstaing−Henry−Ottensmeyer energy filter.

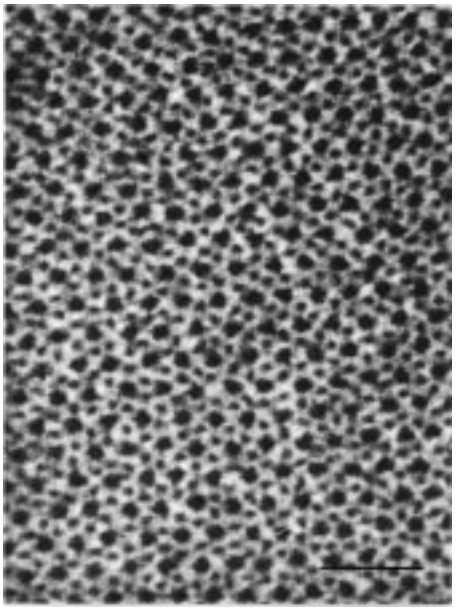

Figure 2. Cross section of cod muscle control. Note the regularity of the network's myofilaments. Bar = 0.09 μm.

Morphometric Study. For morphometry, a Videoplan program (Kontron Bildanalyze, Germany) was used. This program is a system designed to quantify geometric parameters of images and to evaluate the measured data according to user requirements. Our studies were performed on micrographs with a final magnification of 95000×, on central myofibrils of the fibers showing well-oriented transverse sections in the A-band. Using the hexagonal array of thick filaments, 500 hexagons were measured in a total of 30 myofibrils from different fibers at each of individuals and storage periods to achieve a mean value of eccentricity. The value of this parameter is calculated by the expression ECC = $(DMAX^2 - DMIN^2/DMAX^2)^{1/2}$, which corresponds to the theoretical eccentricity of an ellipse in which the longest axis is the maximum diameter and the shortest axis is the minimum diameter of the hexagon. The justification for the method used to measure the maximum and minimum diameters of the hexagon with respect to the axes of the ellipse and hence to calculate the eccentricity value is as follows (Figure 1).

A perfect hexagon can be drawn inside a circle, in which case eccentricity is zero (Figure 1A). Likewise, when two opposite sides are brought closer together, the resulting deformed hexagon can be drawn inside an ellipse as in Figure 1B.

The hexagonal pattern in fish muscle is quasi perfect when the muscle is fresh and a section is cut normal to the fiber axis. However, during frozen storage, deformation of the hexagons is not regular. In this case we could draw any number of ellipses around the deformed hexagon and none would perfectly circumscribe it (Figure 1C,D).

To circumvent the problem, using the above expression, an ellipse has been defined so that its maximum and minimum diameters would coincide with the maximum and minimum diameters of the hexagon. Following this definition, it is only possible to draw one ellipse around each deformed hexagon, although the deformation was not geometrically perfect. Thus, in the case of a perfect hexagon, the eccentricity value of an ellipse would be 0.5 (Figure 1E). Although the hexagon is really deformed (and, moreover, irregular), the only possible ellipse is the one shown in Figure 1F.

In each species comparisons of eccentricity mean values were made between every two groups (storage time) using an ANOVA (Scheffe test).

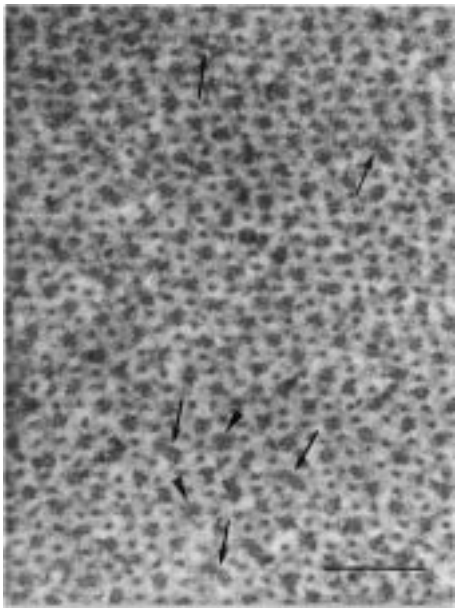

Figure 3. Micrograph from cod muscle stored at −20 °C for 49 weeks. The proximity and fusions (→) between thick filaments increase as a function of storage time. Some thick filaments show a looser appearance (▼). Bar = 0.09 μm.

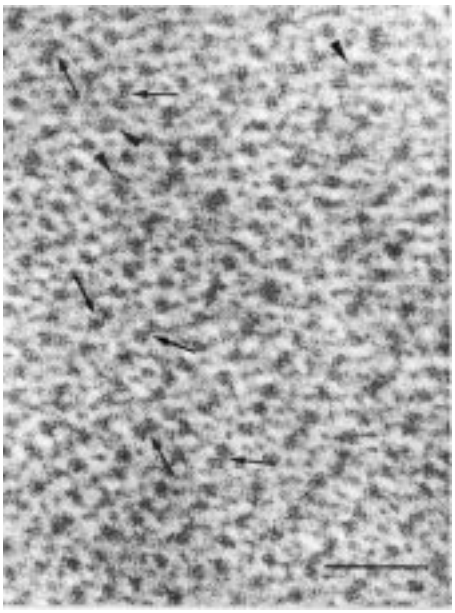

Figure 4. Transverse section of cod muscle stored at −20 °C for 70 weeks. Note the large number of cross-linkings between thick filaments in the 10−10 plane (→) and the augmentation of some thick filaments in cross section (▼). Bar = 0.09 μm.

RESULTS

Examination of the transverse sections of cod muscle revealed the loss of definition of points in the hexagonal network, a development largely attributed to the tendency of the myofilaments to fuse when muscle is kept in frozen storage. Three types of fusions occur: between actin filaments, between actin and myosin filaments, and between myosin filaments, but the predominant type of cross-linking occurs between thick filaments (Figures 2−4). In most myofibrils, these fusions lay in the same direction, so thick filaments appear as lines of dots in the 10−10 crystallographic direction (Figures 3 and 4). However, in some areas of the myofibrils, fusions can be found in different directions.

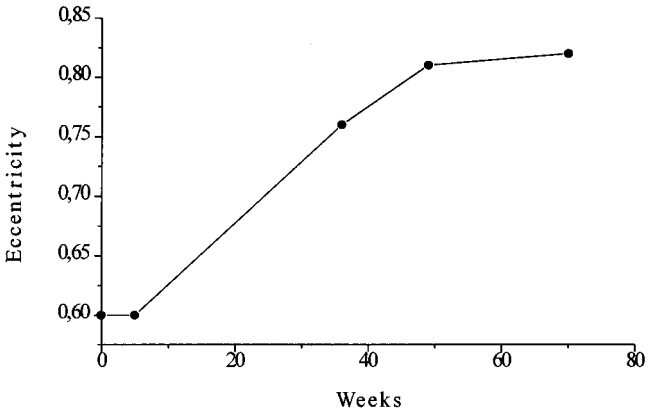

Figure 5. Graph showing mean values of eccentricity from cod muscle as a function of time.

Table 1. Morphometric Study in Cod Muscle Stored at −20 °C

weeks	max diameter, nm	min diameter, nm	eccentricity
0	66.4 ± 4.9	53.0 ± 4.5	0.60 ± 0.07[a]
5	67.5 ± 3.5	53.3 ± 2.8	0.60 ± 0.07
36	89.3 ± 6.2	57.5 ± 4.2	0.76 ± 0.06
49	88.5 ± 5.9	50.3 ± 5.7	0.81 ± 0.07
70	86.7 ± 5.0	48.9 ± 4.8	0.82 ± 0.05

[a] Standard deviation values. Eccentricity values shown in the Tables 1 and 2 represent the mean value of the individual eccentricities of each hexagon measured; they do not represent the result of deriving the eccentricity from the mean values of the maximum and minimum diameter.

Morphometric study was carried out on transverse sections of cod muscle stored at −20 °C for 0, 5, 36, 49, and 70 weeks. Figure 5 shows the mean values of eccentricity. It can be seen how the values of eccentricity rise with time, reaching a peak within 49 weeks of storage. These data indicate an increase in the ratio of maximum to minimum hexagon diameter as a function of storage time (see Table 1, which gives the mean values of the parameters measured). Significant differences were found between the eccentricity values of pairs of compared groups with different storage time ($P < 0.05$) except between the control and the muscle stored for 5 weeks and between the muscle stored for 49 and 70 weeks.

Changes in the morphology of thick filaments during frozen storage of cod muscle are also visible. These filaments appear to lose cohesiveness between their constituent polymer chains, giving them a looser appearance; some thick filaments show their diameters increased (Figures 3 and 4). Examination of longitudinal sections confirms the degradation of thick filaments when muscle is stored for 70 weeks (Figure 6).

Long-term storage of hake muscle does not cause a change in the regularity of the hexagonal lattice of thick filaments or in the morphology of these filaments compared to the control (Figures 7−9). Although fusions between thick filaments are barely identifiable during frozen storage, cross-linkings of the thin−thin filament or the thin−thick filament type are abundant.

In the morphometric study (Table 2), significant differences were not found in the eccentricity values from 36 weeks on (Figure 10). However significant differences ($P < 0.05$) were found in hake muscle between the rest of pairs of compared groups with different storage time.

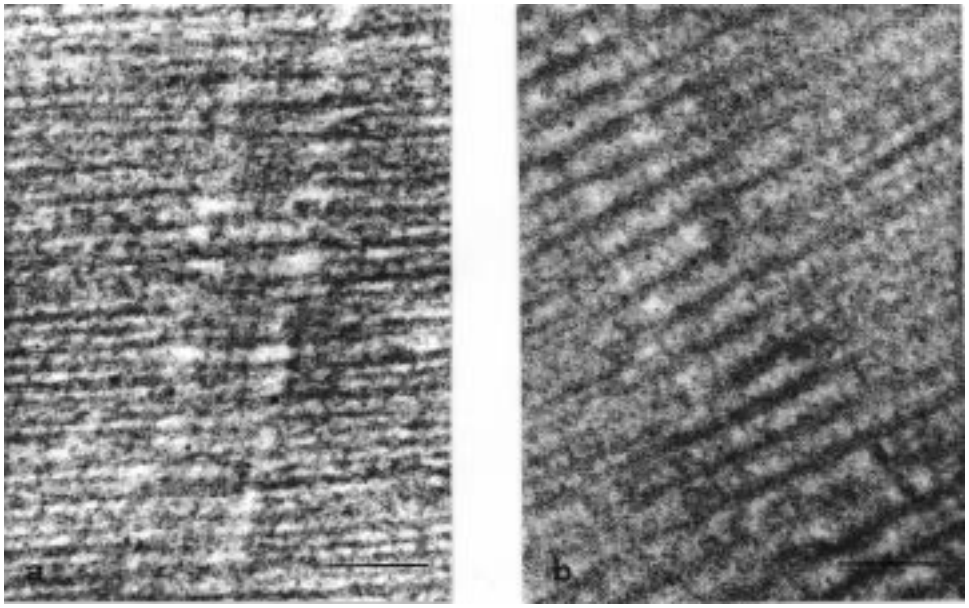

Figure 6. (a) Longitudinal section of cod muscle stored at −20 °C for 5 weeks and (b) longitudinal section of cod muscle stored at −20 °C for 70 weeks. Note the difference in the size of thick filaments. Bar = 0.09 μm.

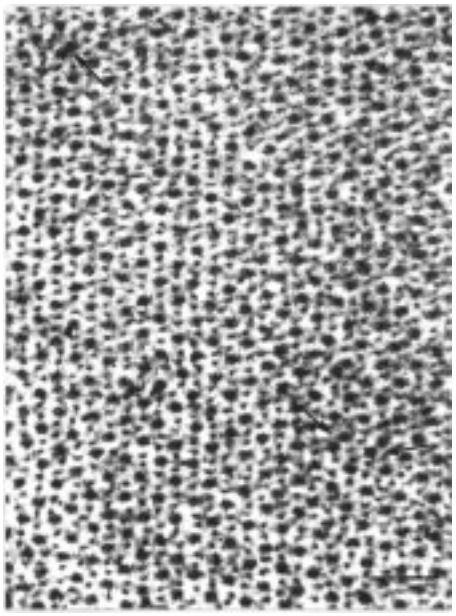

Figure 7. Transverse section through A-band region from hake muscle control showing the conventional array of filaments. Note the proximity of thick filaments in some areas of the myofibril (→). Bar = 75 nm.

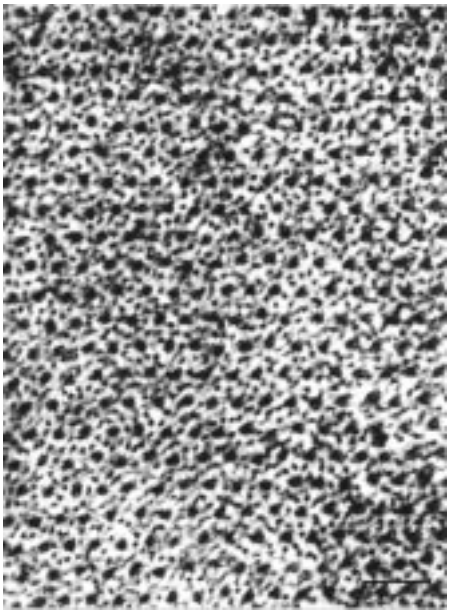

Figure 8. Micrograph from hake muscle stored at −20 °C for 36 weeks. Note the regularity in the arrangement of thick filaments in the A-band. Bar = 75 nm.

DISCUSSION

In this study electron microscopy shows a disruption in the hexagonal network during frozen storage of cod muscle. We found fusions between thick filaments, which were scarce at 1 month but which intensify from 49 weeks on. This finding had not been reported before.

The aim of the morphometric study was to determine the general tendency of deformation undergone by the hexagonal array of thick filaments during frozen storage. It is known from X-ray studies on living muscle that the sarcomere volume remains constant during contraction and relaxation and that interfilament spacing varies inversely with the square root of the sarcomere length. In post-rigor cod muscle, where sarcomere lengths vary widely, a similarly variable interfilament space would be expected (Howgate, 1979). For this reason the deformation to the network caused by frozen storage cannot be estimated by measuring a parameter from the hexagonal network, for example, the hexagon's longest diagonal or interfilament distance, because the initial value of such a parameter will vary with the degree of post-rigor contraction. To avoid this problem, deformation of the hexagonal network has been determined as a function of eccentricity.

Changes in the eccentricity value show that the hexagons in the cod muscle network become deformed, while maximum diameter increases with respect to minimum diameter as storage progresses. Obliquity of sections is one of the aspects that can affect measurements, as it distorts the hexagons. The consistency of the results confirms that the cross sections are properly orientated. If a section is oblique, the variation in the maximum diameter and the minimum diameter will be

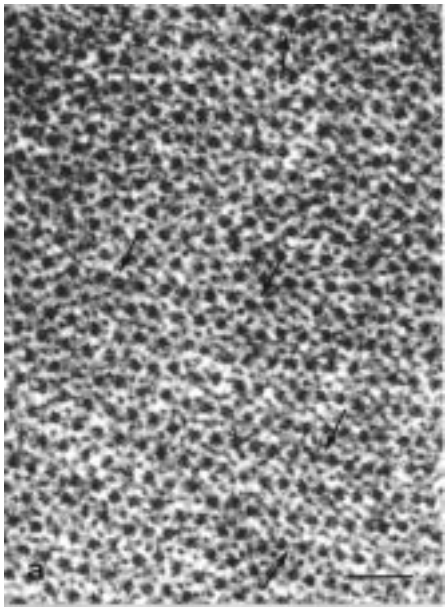

Figure 9. Micrograph from hake muscle stored at −20 °C for 63 weeks. The array and morphology of thick filaments do not change significantly after long storage. Compression exerted by frozen storage in some areas reduces the spacing between thick filaments (→), but fusions between these filaments are scarce. Bar = 75 nm.

Table 2. Morphometric Study in Hake Muscle Stored at −20 °C

weeks	max diameter, nm	min diameter, nm	eccentricity
0	77.0 ± 5.4	58.9 ± 4.5	0.65 ± 0.09[a]
5	72.9 ± 5.4	47.6 ± 3.2	0.75 ± 0.06
36	75.4 ± 6.8	53.0 ± 4.4	0.69 ± 0.08
63	78.5 ± 6.6	54.8 ± 5.5	0.70 ± 0.09

[a] Standard deviation values.

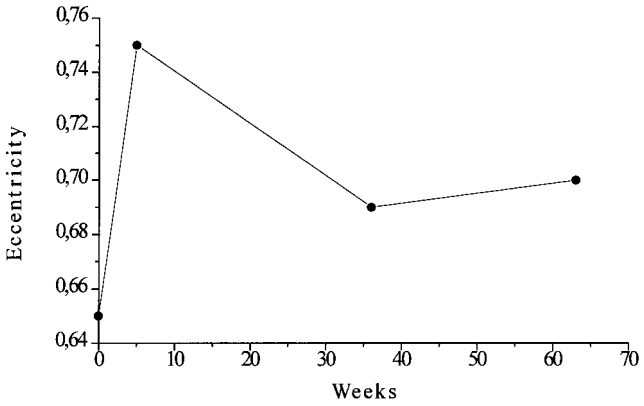

Figure 10. Graph showing mean values of eccentricity from hake muscle stored at −20 °C.

in a proportion of 1/cos α, where α is the angle of inclination of the section. If the angle of inclination is ∼20°, the diameters will increase by 6%. If α is 30°, the lengths will increase by 15%. In the present case the increase in the values of eccentricity as a function of time are >15%. Hence, the data from the morphometric study may be attributed basically to the alteration occurring in the hexagonal network during frozen storage.

A schematic diagram illustrating the evolution of the hexagonal array of thick filaments during frozen storage of cod muscle is shown in Figure 11. Figure 11a represents a transverse section through the A-band

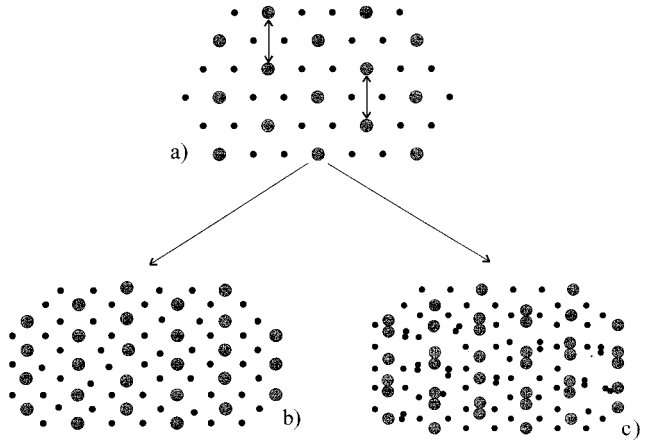

Figure 11. Diagram representing the distortion of the regular hexagonal pattern of thick filaments during frozen storage of cod muscle (a) conventional array of filaments in skeletal muscle (arrows show the 10−10 plane in which the spacing between thick filaments is reduced); (b, c) deformations observed upon prolonged storage; (b) hexagons in the network of thick filaments appear more eccentric; (c) some myofibrils show a large amount of fusions between thick filaments.

lattice, following the model proposed by Huxley and Brown (1967). As a result of the formation of myosin−myosin cross-links between the filaments in the 10−10 plane during frozen storage, the hexagons could be deformed and hence more eccentric (Figure 11b). If this model were extended, it would mean general deformation of the network with a preferential orientation. The maximum deformation degree corresponds to those myofibrils in which there are a large number of fusions between thick filaments as Figure 11c shows.

Our results are similar to those reported by Connell (1968). Sections obtained by fixing cod muscle at 0 °C directly from the frozen state showed a distortion of the regular hexagonal array of the filaments in the frozen state into a lengthened hexagonal arrangement as a result of the shrinkage produced by the migration of water to the nearest ice crystal face. As a result, the filaments appeared in transverse sections as continuous lines in the 10−10 plane, instead of a regular hexagonal pattern. However, upon thawing, muscle did not show this deformation. In our study fusions between thick filaments remain after thawing, suggesting the formation of cross-links between protein molecules in the thick filaments which prevents the tissue from regaining its original filament spacing.

In previous ultrastructural studies, Jarenbäck and Liljemark (1975a) found a significant reduction of interfilament spacing using the shortest mean distance between thick filaments upon prolonged storage of cod. On the contrary, Liljemark (1969) and Aitken and Connell (1977) did not find a significantly smaller spacing in frozen stored cod muscle. The variability of the results could be justified by the parameter used in the studies, because spacing of the filaments of muscle depends on some factors such as sarcomere length, pH, and ionic strength (Offer and Knight, 1988).

The differences in the hexagonal array pattern between species seen in this study could be attributed to factors that affect removal of water from the muscle during frozen storage. Tanaka (1965) observed that the pattern of ice crystal formation differed between Alaska pollack and yellowtail and suggested that it might influence the stability. Alaska pollack showed extracel-

lular freezing (~60% ice) and the myofilaments became fibrous after 2 months, as shown by the decreases in diameter and in distance between them. In yellowtail, which showed intracellular freezing, there were not such microstructural changes. The rate of actomyosin denaturation estimated by the solubility method was faster in Alaska pollack than in yellowtail, demonstrating that movement of intracellular water to the extracellular field promotes aggregation of myosin or actomyosin within the myofilaments.

In this connection, the microstructural differences between cod and hake found in our work do not seem to be based on the dehydration of muscle in relation to the distribution of water after freezing and frozen storage. Extractability studies carried out by Careche et al. (1998) and del Mazo (1997) on the samples analyzed in this study by electron microscopy showed the loss of extractability by 0.6 M NaCl during frozen storage, which indicates an effective aggregation between proteins in both species.

Moreover, these differences could be attributed to the selective nature of electron microscope studies, so the myofibrils chosen for detailed study had not effectively been compressed by ice crystals. Bello et al. (1982) observed that myofibrils situated in the periphery of the large spaces occupied by ice crystals are especially dehydrated and compressed. The analysis of the micrographs in hake muscle shows areas of the myofibrils where there is effective compression exerted by frozen storage. The spacing between thick filaments is reduced along the 10−10 plane and in some areas is reduced sufficiently for them to touch, but fusions between thick filaments are barely identified.

It is probable that the ultrastructural differences are associated with the denaturation−aggregation mechanism of myosin molecules, although the exact nature of these changes cannot be established in this study. It is known from extractability, organoleptic, and physical studies that the susceptibility of the proteins to frozen storage varies with species, biological conditions, and prefreezing history (Connell, 1968). It has been demonstrated that denaturation of myosin can contribute to myofibrillar shrinkage by a change in the shape of the cross-bridges, for example, by shortening of the subfragment-2 region of the myosin tail or by the reduction in the length of the myosin heads (Offer and Knight, 1988). Moreover, the type of aggregation between myosin molecules depends on the mechanism of denaturation. According to Sharp and Offer (1992), the mechanism of head−head aggregation between myosin molecules is different, depending on which light chain is lost. When the LC2 light chains are removed, aggregation occurs through the neck regions of the heads. When the LC1 or LC3 light chains are lost, more extensive contacts of the head were made, and the hydrophobic patch exposed with regard to the curvature of the head allows intra- and intermolecular head associations.

Frozen storage also affects the structure of cod muscle thick filaments, which become less compact, but changes are not observed in the morphology of hake filaments after a prolonged storage. These results suggest high incidences of intrafilament aggregation in hake muscle, corroborated by the absence of interfilament cross-linkings and the biochemical studies of extractability carried out by Careche et al. (1998) and Del Mazo (1997) on these samples, which showed a decrease in the amount of the actomyosin and heavy myosin chain extracted from hake muscle during frozen storage.

The results obtained after the ultrastructural study are closely related to the complementary textural studies carried out by Careche et al. (1998) and del Mazo (1997). These authors found that cod muscle stored at −20 °C showed an increase in shear resistance from week 8 (15 N/g) up to week 36 (28 N/g) followed by a decline until the end of storage. This phenomenon was attributed to loss of cohesiveness, because such samples passed through the slits easily in the Kramer shear compression cell. In hake muscle stored at −20 °C, shear resistance displayed no clear tendency to increase over storage. It seems that shear resistance is significantly higher with muscle fiber containing a large amount of fusions between thick filaments, whereas fibers with no changes in the thick filament arrangement did not show changes in toughness. It is known that factors influencing interfilament spacing in post-rigor fish such as pH are determinants of toughness. Raising the pH from 5, by giving the filaments an increasing net negative charge, results in an increase of the repulsive force, causing the lattice to expand. This should tend to loosen the structure and soften the texture (Howgate, 1977). Nevertheless, a relationship between texture, aggregation, and ultrastructural changes will be explored in future studies.

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