Scanning Electron Microscopy of Squid, *Loligo pealei*: Raw, Cooked, and Frozen Mantle

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Introduction

The main body portion of a squid is the cone-shaped mantle which encloses the viscera. Early studies of squid mantle structure (Pierce, 1950; Tanaka, 1958; Wilson, 1960; and Young, 1938) are scant and incomplete, but outline the basic muscular arrangement: Bands of radial and circumferential muscle fibers arranged in an orthogonal manner and sandwiched between layers of connective tissue. With the advent of better transmission electron microscopic techniques, Ward and Wainwright (1972) gave a complete description of the entire mantle structure as it relates to locomotory function, and Moon and Hulbert (1975) define the fine structure of an individual squid muscle fiber. Structural analysis of squid tissue using scanning electron microscopy (SEM) has not been reported, nor has SEM been used to describe ultrastructural changes caused by cooking of invertebrate tissue.

The major intent of this study was to illustrate, with scanning electron photomicrographs, the ultrastructure of the tissue components of the mantle from squid, *Loligo pealei*. The structural description of the raw tissues is useful for interpretation of the locomotory function of the mantle, and for understanding the unique metabolic aspects of these tissues which function such that the squid is the fastest swimming marine invertebrate. In turn, the structural description of raw tissues provided a basis for comparative analysis of structural alterations caused by cooking and freezing.

SEM has been used successfully to demonstrate cooking alterations in beef tissues (Schaller and Powrie, 1972; Alexander and Fox, 1975; Cheng and Parrish, 1976; Jones et al., 1977), and the freezing alterations in crab (Giddings and Hill, 1976). Application of this microscopic technique, in combination with tests of physical strength and sensory textural analysis, could be used to evaluate food texture of squid as affected by cooking and freezing (Otwell and Hamann, 1979a, b).

Materials and Methods

Squid

Squid used in this study were harvested by commercial vessels fishing the North Carolina coast adjacent to Cape Hatteras. Average mantle length of squid used in this study was 14 ± 4 cm (5.5 ± 1.6 inches). Squid were packed on ice, transported to Raleigh, North Carolina, and cleaned for use (skin, head, and viscera removed) within 72 hours subsequent to harvest. It was assumed that at this time the squid musculature was in the post-rigor state. No squid was used which showed evidence of pigment "staining" on the mantle, which is an indication of mishandling and/or early spoilage (Takahashi, 1965). A portion of the squid was packed in 10×20 cm (3.9×7.8 inch) Whirl-pak[1] plastic bags, then frozen in a $-29°C$ walk-in freezer. Samples frozen for 20 days were used to determine the effects of freezing. Cooked samples consisted of mantles which had been boiled for 1 minute in distilled water. Water and samples were heated from room temperature to boiling at a warming rate of $5°C/$minute, and the temperature of the internal musculature and water were the same as determined by microthermoprobes.

SEM Procedure

Fresh, cooked, and frozen squid mantles were prepared by the same methodology prior to SEM evaluation. Thin slices (approximately 1 mm or 0.04 inch) of whole mantle cooled to $4°C$ were cut with a razor blade in a specific manner according to muscle fiber direction. Cuts were made parallel and perpendicular to the circumferential fiber direction. All radial fibers were viewed in longitudinal section. Awareness of fiber orientation was essential for interpretation of photomicrographs (see Figure 1 for orientation).

ABSTRACT–Scanning electron microscopy (SEM) was used to investigate the tissue structure in raw, frozen, and cooked mantles from squid, Loligo pealei. *The mantle consists of five distinct layers of tissue: Outer lining, outer tunic, muscle fibers, inner tunic, and visceral lining. Each layer in the raw state is described. The freeze fracture techniques used to prepare samples for SEM viewing revealed tissue structural alterations caused by freezing the mantle to $-29°C$. The same technique was used to observe thermal alterations caused by cooking the mantle to $100°C$. Loss of structural differentiation in the muscle fibers was the only discernible alteration caused by freezing, but cooking caused gross distortions in all mantle tissues.*

[1]Mention of trade names, commercial products, or firms does not imply endorsement by the National Marine Fisheries Service, NOAA.

Sliced mantle was submerged in 5 percent glutaraldehyde (Electron Microscopy Science Company) in 1.M cacodylate-0.25M sucrose buffer for 16 hours fixation time. After the first 8 hours of fixation, submerged slices were cut into smaller lengths (4 mm or 0.16 inch) to assure fixative penetration. Glutaraldehyde was washed from the fixed tissue by two successive baths (12 hours each) of buffer containing half of the previous concentration of sucrose. Post-fixation of the tissue by exposure to osmium tetraoxide vapors was omitted from the procedure since it did not appreciably improve resolution. Fixed tissue was dehydrated by exchange in a graded ethanol series (15 minutes each in 30, 50, 70, 95, and 100 EtOH), and stored 24 hours in 100 percent ethanol.

Slight modifications of the freeze fracture procedure of Giddings and Hill (1976) were used to expose the surface for microscope viewing. Initially, the dehydrated tissue was cooled for 15 seconds in Freon 22 (chlorodifluoromethane, Dupont Company), then frozen by direct contact with liquid nitrogen. Frozen samples were fractured by a sharp blow to a single-edge razor blade positioned perpendicular to sample surface. Fracture orientation was selected with reference to fiber direction. Fractured pieces were slowly thawed in cool 100 percent ethanol, then solvent exchanged in a graded Freon 113 (trichlorotrifluorethane, Dupont Company) series. The procedure consisted of immersing 20 minutes each in 30, 50, 70, 95, and then placing in the 100 percent Freon 113. Immersion time in 100 percent Freon 113 did not exceed 12 hours. Preliminary tests indicated that prolonged exposure to Freon 113 (>12 hours) destroys fine tissue structures.

Samples packaged in Freon 113-impregnated envelopes of filter paper were dried in a Bomar SPC-50 critical point drier charged with Freon 13 (chlorotrifluoromethane, Dupont Company). Dried samples were mounted on aluminum stubs and coated with DAGS (colloidal graphite in isopropanol base; Ted Pella Company), then gold-coated for 5 minutes in a
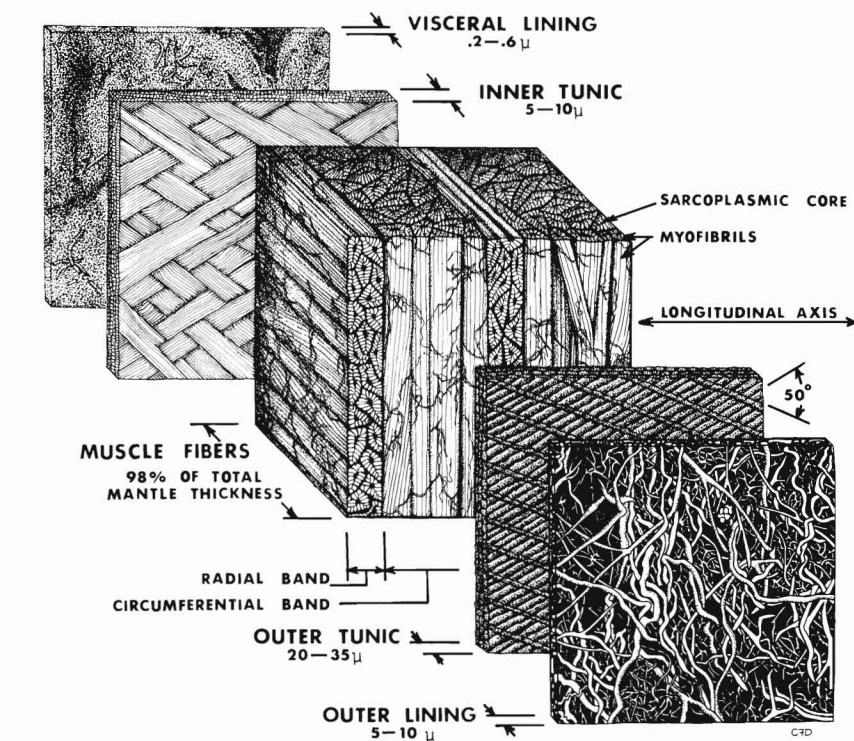

Figure 1.—Artist's rendition of the tissue composition in the mantle of squid, *Loligo pealei*. The view is an expanded cube cut from the entire thickness of the mantle. Skin has been removed from the outer lining, and the profuse sarcoplasmic tubular network typically surrounding the surface of the muscle fibers has been omitted to reveal muscle fiber structure. The longitudinal axis refers to the head to "tail" axis of the squid mantle.

Polaron #5000 diode gold coater. Coated specimens were stored at room temperature in a vacuum desiccator until examined with an Etec Autoscan microscope operating in the linear mode at 20 kev, 12-8 working distance, 2.5 contrast, and 0 darkness. Both fractured and nonfractured surfaces were examined. Photomicrographs were taken with a Polaroid camera packed with type 55 Polaroid 5 × 5 Land film.

Other Evaluations

Samples of different layers of mantle tissues were separated by sectioning with a freezing microtome. Moisture, crude protein, and hydroxyproline content was determined in three samples each from the separate layers, with at least three replications of each analysis

per sample. Moisture and protein was determined by standard methods (AOAC, 1975). Protein was converted from nitrogen with multiplication by 6.25. Hydroxyproline content, as an indirect measure of collagen content, was determined by the colorimetric method of Woessner (1961).

Results and Discussion

Raw Squid

Figure 1 is an artist's rendition of the composite analysis of squid mantle ultrastructure. The figure is drawn slightly off scale to emphasize specific features of the various tissue components. The mantle is composed of five different layers of tissue. The muscle

fiber layer fills the bulk of the mantle thickness; the remaining layers account for approximately 2 percent of the total mantle thickness. The relation of mantle length (ML) to mean mantle thickness (MT) using cm units is: $ML = (32.26\ MT) - 0.686$ (Otwell, 1978). The terminology used to describe the various tissues in squid are consistent with that expressed by Ward and Wainwright (1972) and Moon and Hulbert (1975). The figures and descriptions provided are representatives from numerous hours of viewing numerous specimens and photographs.

Outer Lining

An outer lining of randomly ordered and sized fibers of connective tissue lies immediately below the skin (Fig. 2). This layer functions to attach the skin to the mantle. No previous report on squid mantle structure has identified this layer. This lining, with a thickness varying from 5 to 10μ, is composed of primary fibrils (0.1μ diameter) which aggregate into larger fibers (0.1-4.0μ). Bacteria was present on the outer lining in some specimens viewed. Immediately below this outside lining is an outer tunic of connective tissue (Fig. 3).

Outer Tunic

The outer tunic is comprised of layers of collagenous fibers (Ward and Wainwright, 1972) arranged in a mesh which is 20-35μ thick (Fig. 4). Fibers within one layer are parallel, running at a consistent angle (approximately 50°) to fibers in lower layers (Fig. 5). The angle is bisected by the longitudinal axis of the mantle. Individual fibers are 2-7μ thick, comprised of fibrils, $0.1 \pm 0.05\mu$ diameter (Fig. 6). Lack of observable banding typical for collagen fibrils may be due to the high carbohydrate content reported for squid collagen, a mucopoly-saccaride (Hunt et al., 1970). The outer tunic appears identical to that described for squid by Ward and Wainwright (1972), but is distinctly different from the inner tunic which has been reported to have similar construction (Ward, 1970).

Inner Tunic and Visceral Lining

The inner tunic is the most difficult layer to observe because it is tightly covered by the nonfibrous visceral lining (Fig. 7). A fine point needle was used to puncture this lining to expose portions of the inner tunic. The inner tunic is connective tissue comprised of 1-5μ diameter fibrous aggregates of 0.1μ diameter fibrils. Fibrous aggregates are loosely bound and interwoven in a 5-10μ thick mesh. A few samples of the 0.2-0.6μ thick visceral lining is laden with pockets of bacteria (Fig. 8).

Although the construction of the outer and inner tunics appears distinctly different, their chemical composition is similar. Both tunics are stained in the same manner by Masson's Trichrome stain, or Verhoeffs elastic stain (stains: Manual of Histologic Staining Methods, AFIP, 1968), and contain hydroxyproline (Table 1). The tunics were shaved free of the mantle with a freezing microtome prior to hydroxyproline determinations. Higher hydroxyproline content in the outer tunic could be evidence for a difference in molecular construction. Hydroxyproline from the muscular layer substantiates the report by Ward and Wainwright (1972) of intramuscular collagenous fibers.

Muscle Fibers

Muscle fibers are arranged in rows of orthogonal bands (Fig. 1). Radial bands (10-15μ thick) are comprised of fibers which connect the two tunics of connective tissue (Fig. 9). Wider circumferential bands (100-200μ; $\bar{x}$ = 130μ thick) are comprised of fibers running about the entire circumference

of the mantle cone. Length of individual circumferential muscle fibers was not determined.

Fiber anatomy (Fig. 10) is the same regardless of orientation. Average fiber diameter is 3.5$\pm$2.5μ. Each fiber has a central sarcoplasmic core which houses numerous mitochondria and at least one nucleus. The core is formed by a periphery of myofibrils. Due to the small fiber size, the SEM was unable to detect myofilaments or striations, typically seen with scanning electron microscopes in the larger (50-100μ diameter) mammalian fibers, (Cheng and Parrish, 1976; Jones et al., 1977).

The tendency for fibers to fracture obliquely (Fig. 10) is consistent with the view that squid muscle is truly obliquely striated (Ward and Wainwright, 1972; Moon and Hulbert, 1975), and that the weak lines for fracture are the dense "Z-bodies," similar to "Z-line" fractures reported by Jones et al. (1977) and Schaller and Powrie (1972). All fibers are surrounded by the profuse sarcotubular network noted by Moon and Hulbert (1975).

Frozen Squid

Fracture patterns, larger interfiber spacing, and diminished fine structure of the muscle fibers were the only discernible alterations in mantle tissue caused by freezing (Fig. 11 A, B). Previously frozen fibers fractured at right angles to the fiber direction, rather than obliquely as with fresh fibers. These results are most likely due to freeze alteration of myofibrillar proteins which has been detected in other species by quantitative changes in protein extractions (Powrie, 1973), and with transmission electron microscopy

Table 1.—Percent protein and hydroxyproline in separate layers of the squid mantle. Standard deviation cited ($\pm$) was computed across three separate samples per tissue, and at least three replications of each analysis.

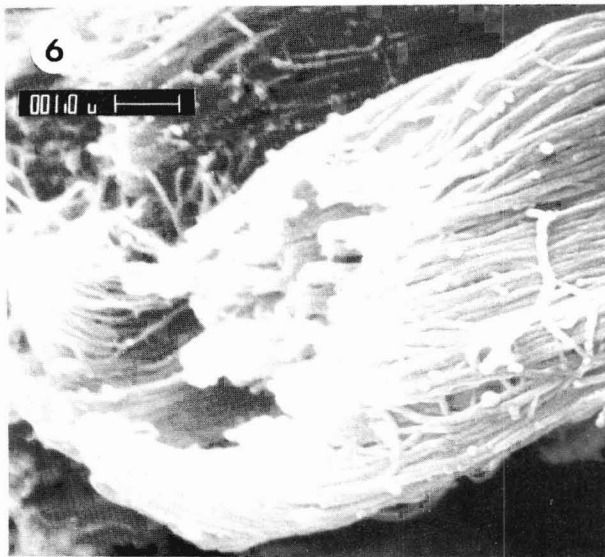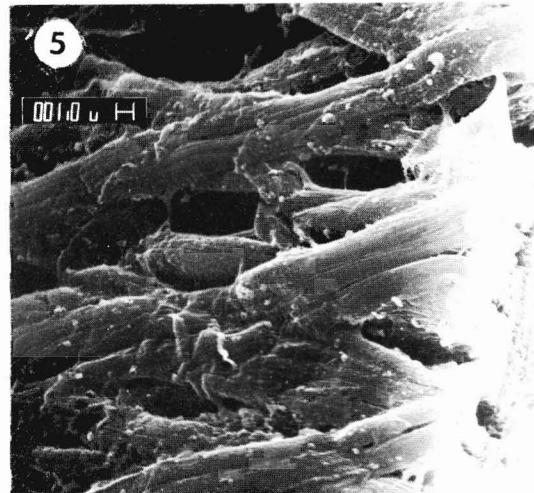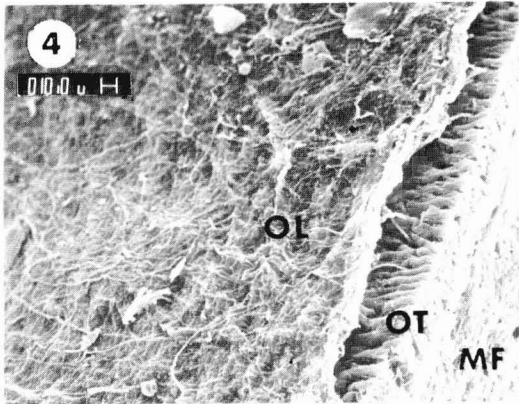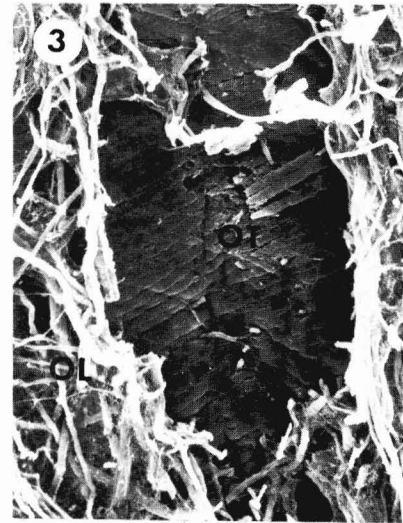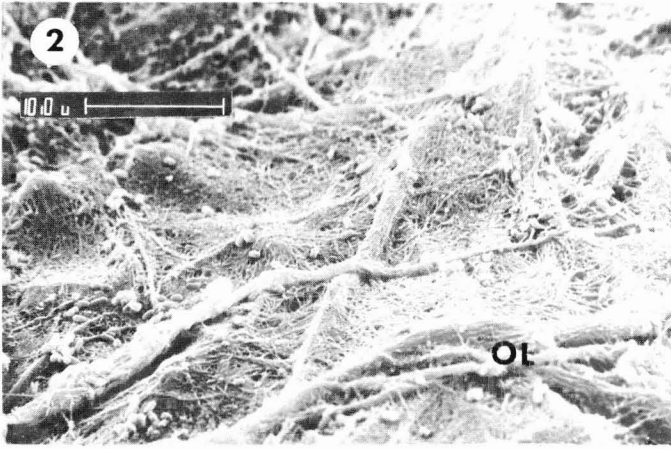| Tissue	Percent protein	Percent hydroxyproline	Percent of total mantle thickness
Outer lining and outer tunic	17.06 $\pm$ 0.30	0.270 $\pm$ 0.06	0.5-1.5
Muscle fibers	16.88 $\pm$ 0.30	0.082 $\pm$ 0.01	
Inner tunic and visceral lining	16.04 $\pm$ 0.48	0.107 $\pm$ 0.05	0.1-0.5

Figure 2.—Angled view of the outer lining: Note bacteria. Figure 3.—Punctured outer lining revealing the ordered mesh construction of the outer tunic of connective tissue. Figure 4.—Outer tunic covered by the outer lining. Figure 5.—Close view of the interwoven fibers in the outer tunic. Figure 6.—Fibril arrangement in an individual collagen fiber from the outer tunic.
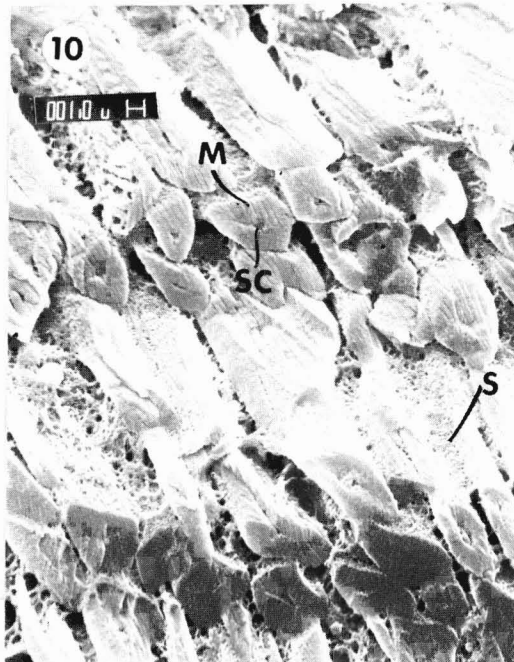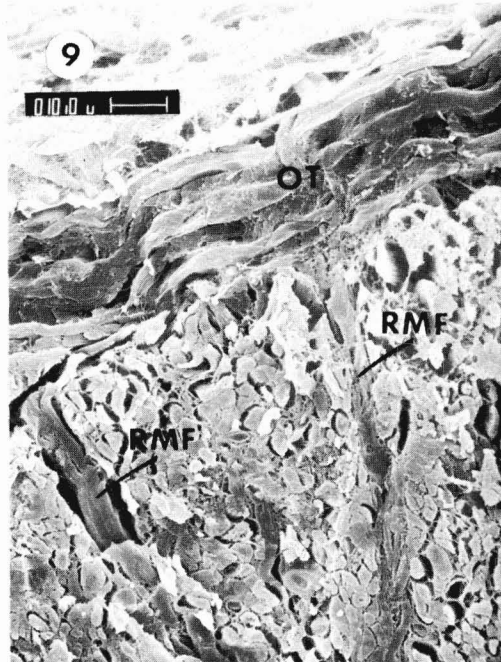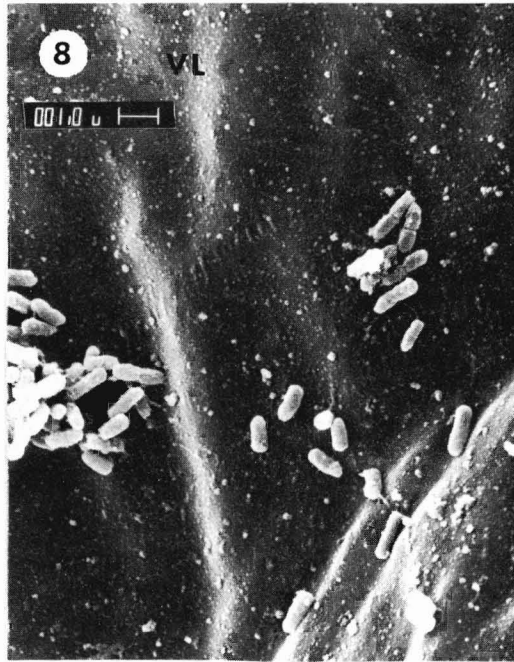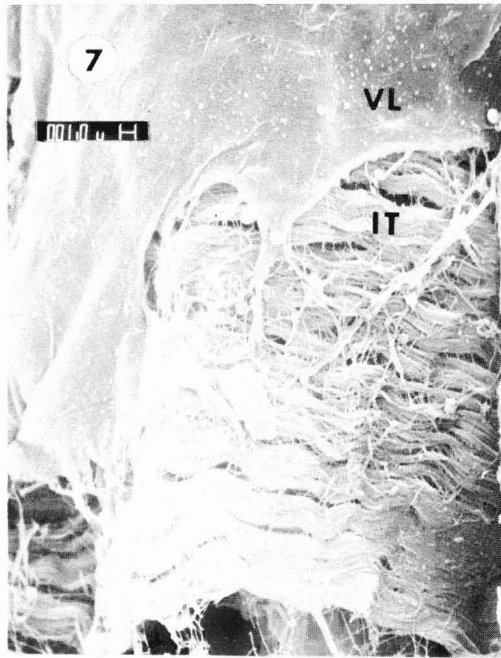
Figure 7.—Punctured visceral lining (VL) reveals the arrangement of fibers in the inner tunic (IT).

Figure 8.—Bacterial growth on the surface of the visceral lining.

Figure 9.—Intersection of radial muscle fibers (RMF) with the multilayered outer tunic (OT). The RMF form a Y-shaped intersection.

Figure 10.—Fractured surface of circumferential muscle fibers (CMF). Note the profuse sarcoplasmic network (S), myofibrils (M), and sarcoplasmic core (SC).
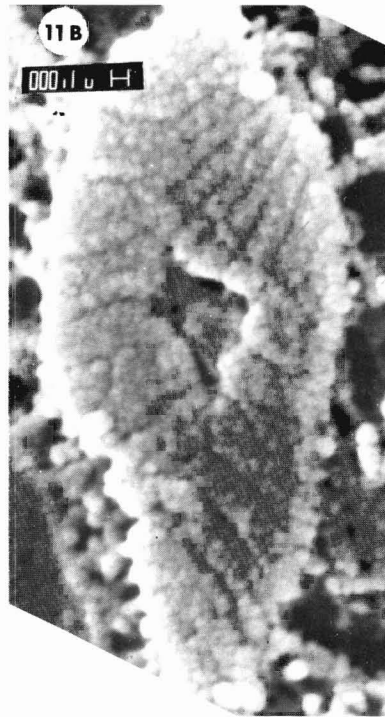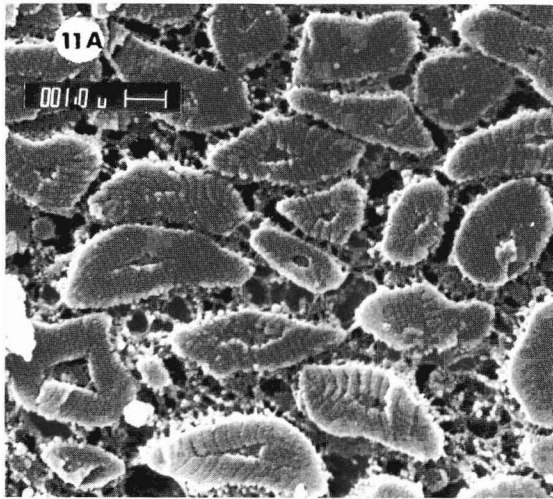
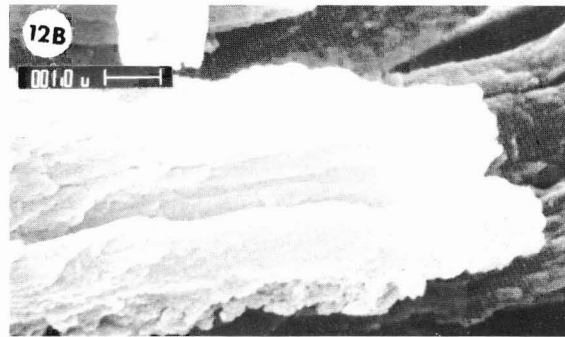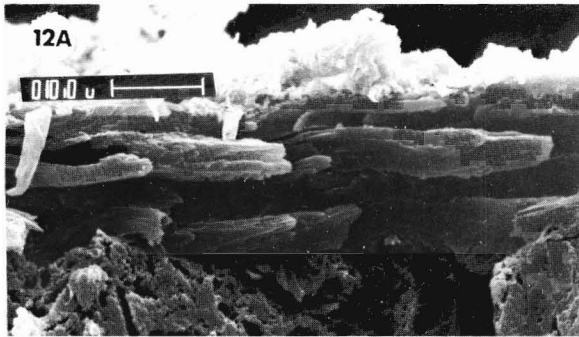Figure 11A, B.—Muscle fibers frozen for 20 days at $-29\,°C$.

Figure 12A, B.—Heat denaturation of the outer lining and outer tunic. In A, tunic fibers still remain intact; in B, fibrils have "melted" into a solid mass.
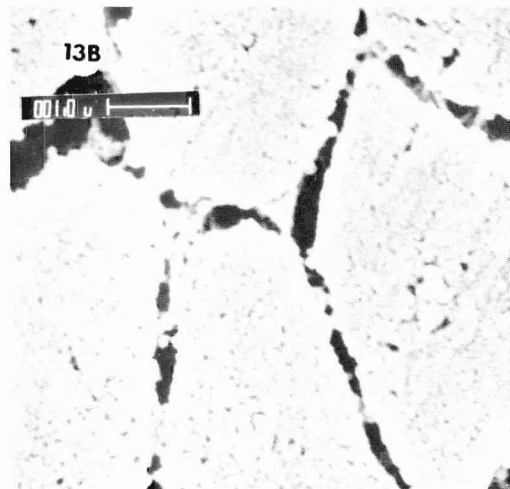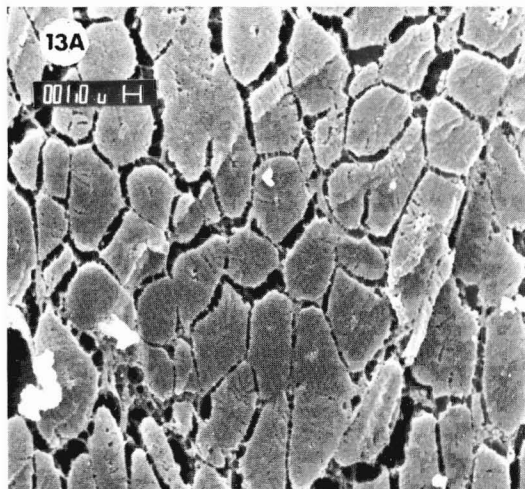
Figure 13A, B.—Heat denatured (100°C) muscle fibers. Sarcoplasmic core is shrunken and filled with heat-globularized proteins, and myofibril distinction is poor.

(Tanaka, 1965; Jarenbäck and Liljemark, 1975a, b).

It is generally thought that both slow freezing and duration of frozen storage results in fiber dehydration and compression due to growth of extracellular ice crystals. Consequently, this mode of frozen storage causes partial dehydration of intramyofibrillar spaces allowing the myofilaments to become more compact, hardened, and possibly crosslinked. Homogeneous hardening of the myofibrils caused by freezing would tend to decrease the probability of fracture along dense ''Z-bodies,'' and myofibrillar crosslinking could decrease fiber resolution.

Cooked Squid

Photomicrographs of squid mantle cooked in boiling water for 1 minute showed gross distortions of all mantle tissues. The visceral lining, the most heat labile tissue completely disintegrated. Tunics of connective tissue showed signs of ''melting'' and ''gelatinization.'' The outer tunic fibrils had congealed, and the fibers were beginning to ''melt'' into a solid gelatinous mass (compare Figures 4, 5, and 6 with 12A and 12B, respectively). The outer lining was only partially disintegrated.

Cooked muscle fibers remained intact but appear hardened and dehydrated (Fig. 13A, B). The central sarcoplasmic cores were shrunken and filled with globular structures identified as heat coagulated proteins. Only slight outlines of myofibril units remained. The muscle bands were more densely packed and the mantle had lost 37 percent of the original wet weight (Table 2).

Conclusions

Squid mantle consists of five distinct layers of tissue. The dominant layer consists of orthogonal bands of muscle fibers, which are sandwiched between two tunics of connective tissue. Ultrastructure of each layer as revealed by SEM agrees with previous TEM descriptions of squid mantles. SEM revealed structural alterations in squid mantle which had been frozen or

Table 2.—Protein and moisture content of fresh, frozen, and cooked squid mantle.

Mantle	Percent protein	Percent moisture	Cook weight loss
Fresh, raw	16.59 ± 0.21	81.26 ± 0.14	
Frozen (−29°C) 29 days	16.08 ± 0.32	81.48 ± 0.06	
Cooked (100°C) one minute	19.93 ± 0.30	77.51 ± 0.08	37%

cooked. Freezing caused discernible structural alterations in muscle fibers which suggest dehydration and homogenous hardening of the fibers. Cooking caused gross distortions of all mantle tissue. Heat coagulation of proteins and gelatination of connective tissues were obvious. Diminished structural definition and size of muscle fibers suggested fiber hardening and dehydration. Thus, it appears feasible that scanning electron microscopy can be used to subjectively evaluate changes caused by heating and freezing squid mantle. The changes observed suggest rapid freezing to minimize ice crystal formation and subsequent moisture loss during thaw is a better method of freezing squid. Cooking methods for squid should avoid excessive dehydration of the tissues.

Acknowledgments

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