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THE CONTRACTING MUSCLE: A CHALLENGE FOR FREEZE-SUBSTITUTION AND LOW TEMPERATURE EMBEDDING

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Abstract

Frog sartorius and semitendinosus muscles are quick-frozen either in the resting state or during contraction by means of a LN_2 cooled falling copper block. The frozen specimens are freeze-substituted (acetone + OsO_4 + uranyl acetate) in a REICHERT JUNG CS auto and either embedded in Spurr's resin and polymerised at a high temperature (60°C) or embedded and polymerised in the Lowicryls K4M, K11M or HM23 at low temperatures (below -30°C). Excellent morphological results are obtained when freeze-substitution, embedding and polymerisation are all carried out below -50°C. Muscles in which a major portion of cellular K^+ions has been replaced by electron dense Cs^+ or Tl^+ ions are also cryofixed at rest or during contraction, freeze-substituted in pure acetone for 1 week at -80°C and polymerised in K11M at -60°C. A characteristic uneven distribution of the electron dense ions - known from earlier published control experiments - can be observed in sections of resting muscles. Electrically stimulated muscles show ion redistribution. It is concluded that freeze-substitution and low temperature embedding of quick-frozen contracting muscle may be used to investigate changes of ultrastructure, redistribution of cellular water and intracellular movements of mobile ions during muscle contraction.

Key words: Quick-freezing, freeze-substitution, low temperature embedding, muscle contraction, A-band shortening, ion localization, potassium binding, cesium, thallium, cell water.

Introduction

The main components of living cells are water, proteins and ions. Electron microscopy may be used to investigate the interactions of these different components provided that the preparation of biological material can be carried out in such a way that life-like ultrastructural details, ion distribution and water containing spaces can be evaluated. Since this goal cannot be achieved by conventional fixation- and dehydration- procedures new cryotechniques have been developed: after stabilization of the biological material by proper quick-freezing (cryofixation) several methods may be used for further processing of the specimen (for review see [23]). Among them is the freeze-substitution technique (for reviews see [10, 23 pp 461-499, 24, 28] that can nowadays be carried out in the best reproducible manner by means of commercially available freeze-substitution units (BALZERS, REICHERT-JUNG). The frozen specimens are dehydrated automatically at low temperatures under controlled conditions. Afterwards the specimens can be infiltrated with a resin at different selected temperatures and are then polymerised. The main difficulty is now to find out the optimum conditions for obtaining results closest to the above mentioned goal.

In this paper the striated muscle of the frog cryofixed either in the resting state or during contraction was used to test the freeze-substitution technique. Apart from the aim to improve structure preservation by freeze-substitution and embedding exclusively at low temperatures (for reviews of freeze-substitution and low temperature embedding see [10, 28]) it is asked whether muscles in which most of the cellular K^+ ions had been replaced by electron dense Cs^+ or Tl^+ ions can be freeze-substituted and embedded in such a way that these heavy ions are retained in the muscle preparation.

Materials and Methods

Frog sartorius and semitendinosus muscles were isolated from Northern American leopard frogs (Rana pipiens, Schreber) and used either when they contained their normal amount of K^+, or after the major portion of cell K^+ (about 80%) was reversibly replaced by Cs^+ or Tl^+ [12, 14].

Resting or contracting muscles are cryofixed by the highly polished base of a LN_2 cooled container made of copper as shown in Figs. 1a-e. As a rule, contracting muscles were cryofixed after 100ms of direct stimulation (2-10ms, 10-30V) at a rate of 50/s. These muscles were spring loaded and kept at a tension of about 1g (isotonic contraction). After cryofixation the carrier with the frozen specimen (see Fig. 1d) was transferred into LN_2. Small pieces of frozen tissue were prepared under visual control with a stereo light microscope on a metal block permanently cooled by LN_2 in a styrofoam container. Afterwards the specimens were transferred into the REICHERT JUNG CS auto cryosubstitution unit.

Freeze-substitution was carried out either in pure acetone or in acetone containing 2.5 % OsO_4 and 0.2 % uranyl acetate (dissolution of 0.2 % uranyl acetate in acetone requires time: about 2 h when shaking the solution). At the end of the freeze-substitution process the specimens (still in the CS auto) were rinsed in pure acetone and gradually infiltrated with Spurr's resin at room temperature or with Lowicryl at a low temperature. Specimens infiltrated with Spurr's resin were transferred into flat embedding moulds and polymerised at 60°C outside the CS auto. Specimens infiltrated in Lowicryl (K4M, K11M or HM23) were placed into flat embedding moulds (accessory of the CS auto) and polymerised by UV irradiation (lamp: PHILIPS TW 6W) at low temperatures inside the freeze-substitution unit [24]. Freeze-substitution and polymerisation schedules (time and temperature) are given in the figure legends of the micrographs in the next section. The polymerised preparations were sectioned with DIATOME diamond knives on a REICHERT JUNG Ultracut E. Sections were stained with uranyl acetate and lead citrate and examined in a ZEISS EM 109 if not mentioned otherwise.

Results

Three different sets of results are shown:

1. Muscles freeze-substituted in acetone containing OsO_4 and uranyl acetate and polymerised in Spurr's resin (Fig. 2).
2. Muscles freeze-substituted in acetone containing OsO_4 and uranyl acetate and polymerised in different Lowicryls at low temperature (Figs. 3 and 4).
3. Muscles freeze-substituted in acetone exclusively at -80°C and polymerised in Lowicryl K11M at -60°C (Figs. 5 and 6).

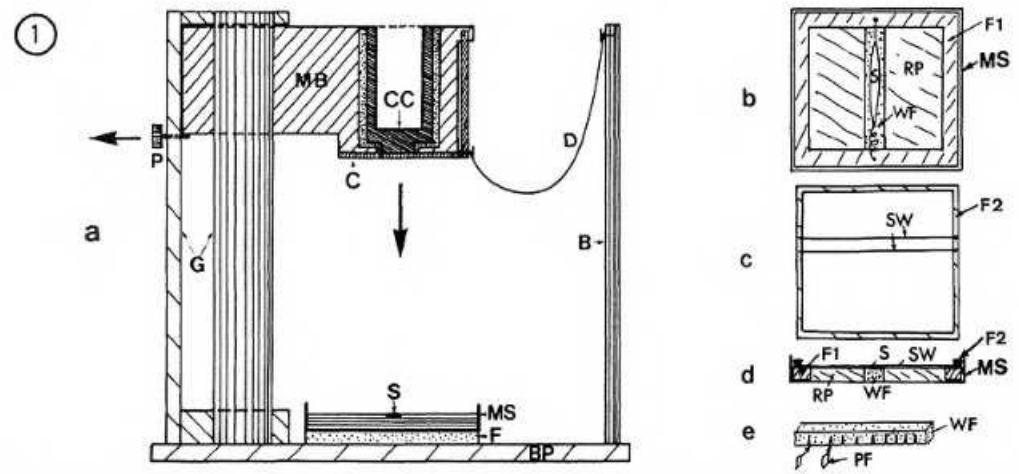

Fig.1. Procedure of cryofixation a) Freezing apparatus. A LN_2 container made of copper is mounted about 20 cm above the specimen S in such a way that removal of pin P causes falling of the mounted container along a guide G towards the specimen. During falling of MB an anticontimination cover C is pulled away by a cord D fixed to bar B from the highly polished Cu-surface (base of CC). The Cu-mirror is cooled by filling LN_2 into the container CC. IM, insulating material; MB, metal block; F, soft rubber foam; MS, metal support (see b and d); BP, base plate. b)-e) Preparation of a muscle for cryofixation. b) a muscle (S) is fixed to the plastic frame F1 (this procedure is carried out in Ringer's solution in a Petri dish) and transferred on a metal support MS in which two rubber plates RP and a wet felt WF formerly soaked in Ringer's solution are already placed. c) shows a plastic frame F2 with two stimulating wires SW which may be placed on top of the preparation shown in (b). d) cross-section of the mounted muscle preparation. e) shows the piece of felt WF which is used as a soft support for the specimen. The felt shows incisions into which small pieces of parafilm PF are placed before soaking the felt in Ringer's solution. Once the muscle is prepared completely it is covered by a humidity chamber (not shown) and the metal support MS is placed on a soft rubber foam F for cryofixation (see a). Electrical stimulation is started by a microswitch in such a way that the falling MB (see a) triggers this switch (not shown) fixed at a predetermined height of G; falling of MB lasts 200 ms, hence longer stimulation times require that the falling of MB is started at a predetermined space of time after the beginning of electrical stimulation.

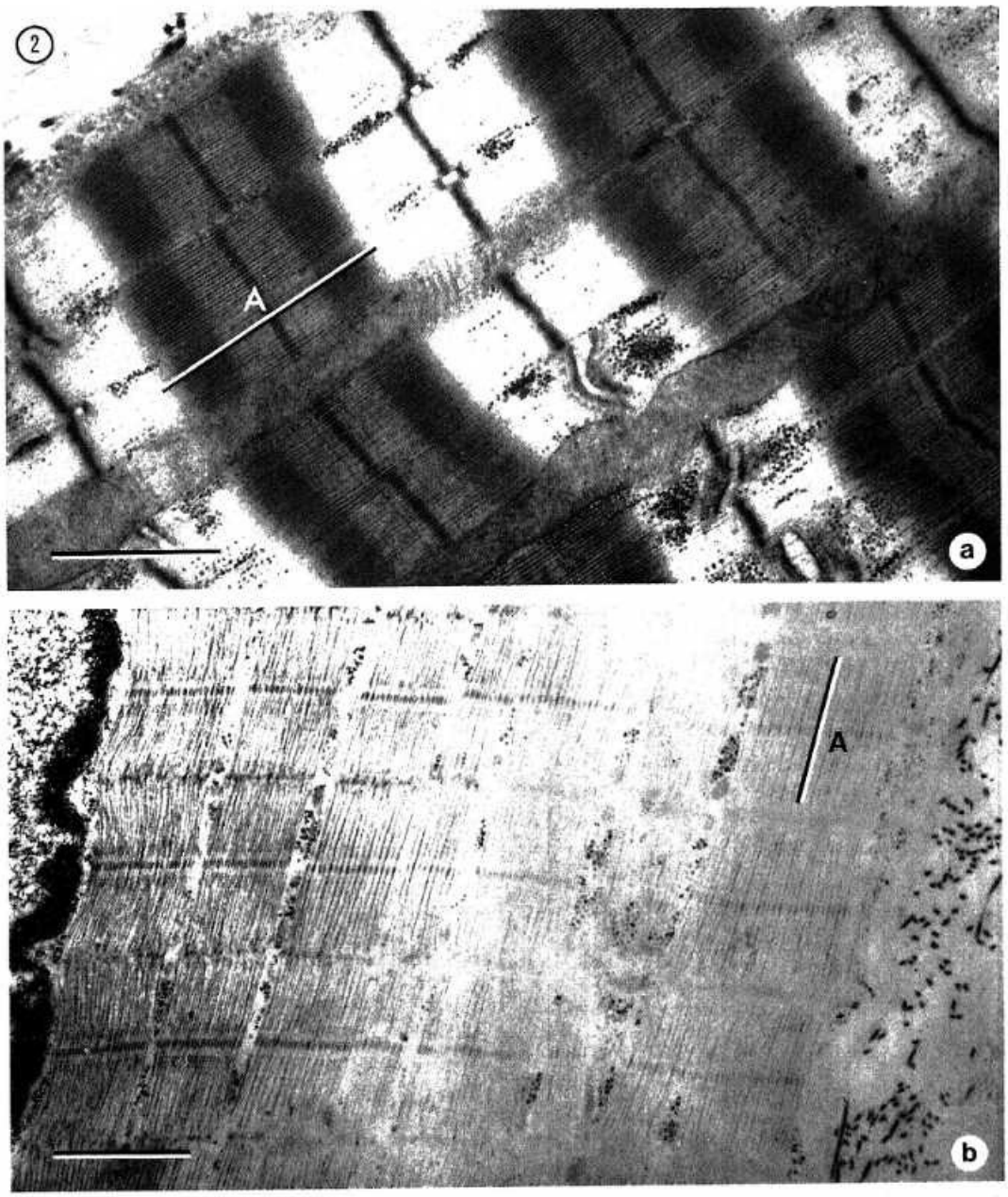

Fig.2. Freeze-substituted frog sartorius muscles (acetone, 2,5% OsO_4, 0.2% uranyl acetate). a) resting muscle, freeze-substitution: 2d -80°C, temperature increase 4°C/h up to 0°C, embedding in Spurr's resin. Bar = 1µm. Micrograph taken with a ZEISS EM 902. A-band (A): 1.5 µm. b) contracting muscle, stimulation : 100 ms, rate: 50/s; freeze-substitution: 2d -80°C, temperature increase 3°C/h up to -50°C, 8d -50°C, temperature increase 5°C/h up to room temperature embedding in Spurr's resin. Bar=1µm. A-band (A, only visible in the well preserved zone on the right side): 1.1µm.

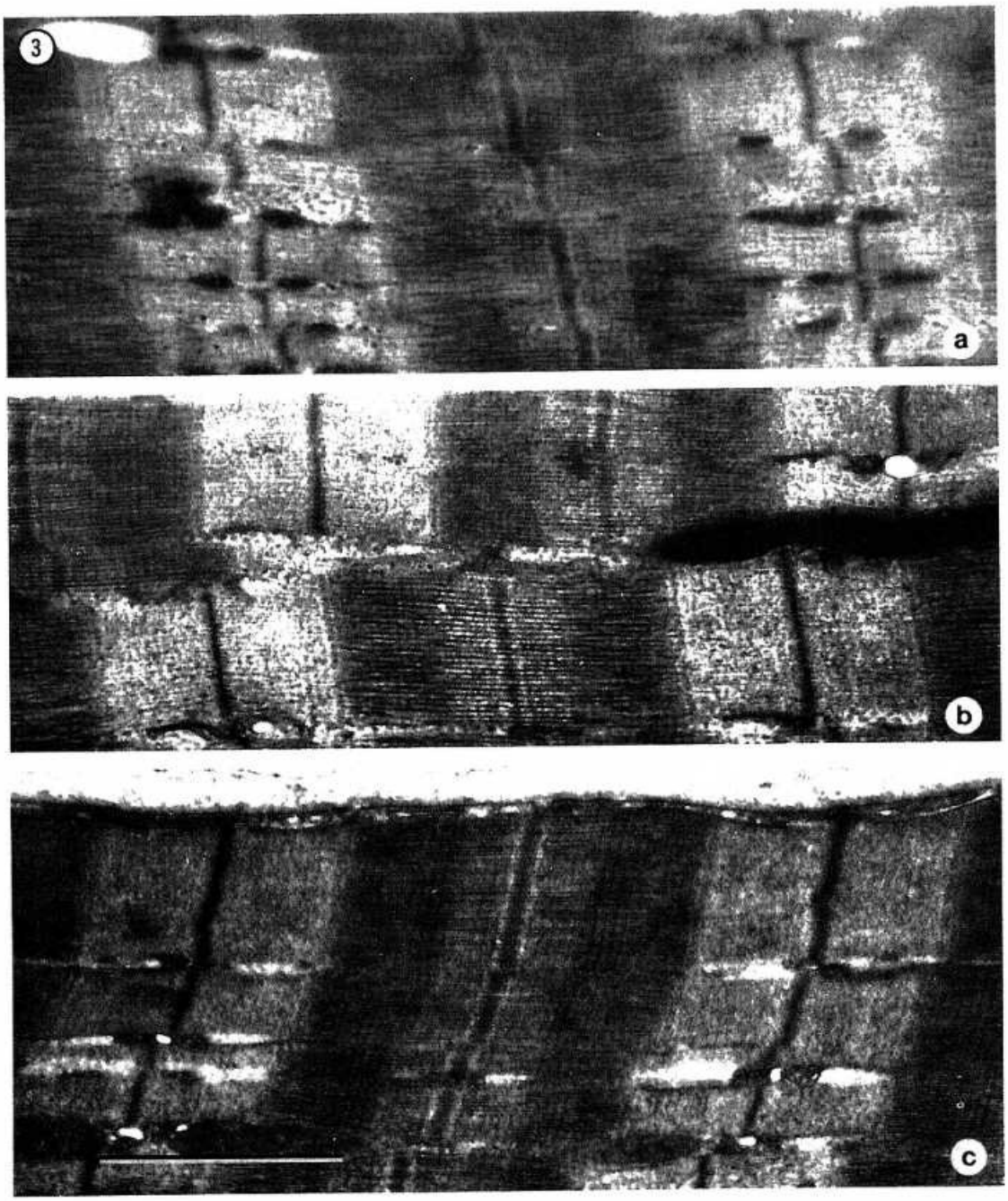

Fig.3. Freeze-substituted resting frog muscles (acetone 2.5% OsO_4, 0.2% uranyl acetate). Bar = 1µm, A-band:1.5µm. a) sartorius muscle, freeze-substitution: 2d -80°C, temperature increase 5°C/h up to -30°C, 10h -30°C, embedding in K4M, polymerised at -30°C. The embedded specimen yielding a) appeared black and was not perfectly polymerised. b) and c) semitendinosus muscle; 3d -80°C, temperature increase 3°C/h up to -50°C, 60h -50°C. b): embedding in K11M, polymerised at -50°C; c): embedding in HM23, polymerised at -50°C. The embedded specimens yielding b) and c) appeared light brownish and were well polymerised.

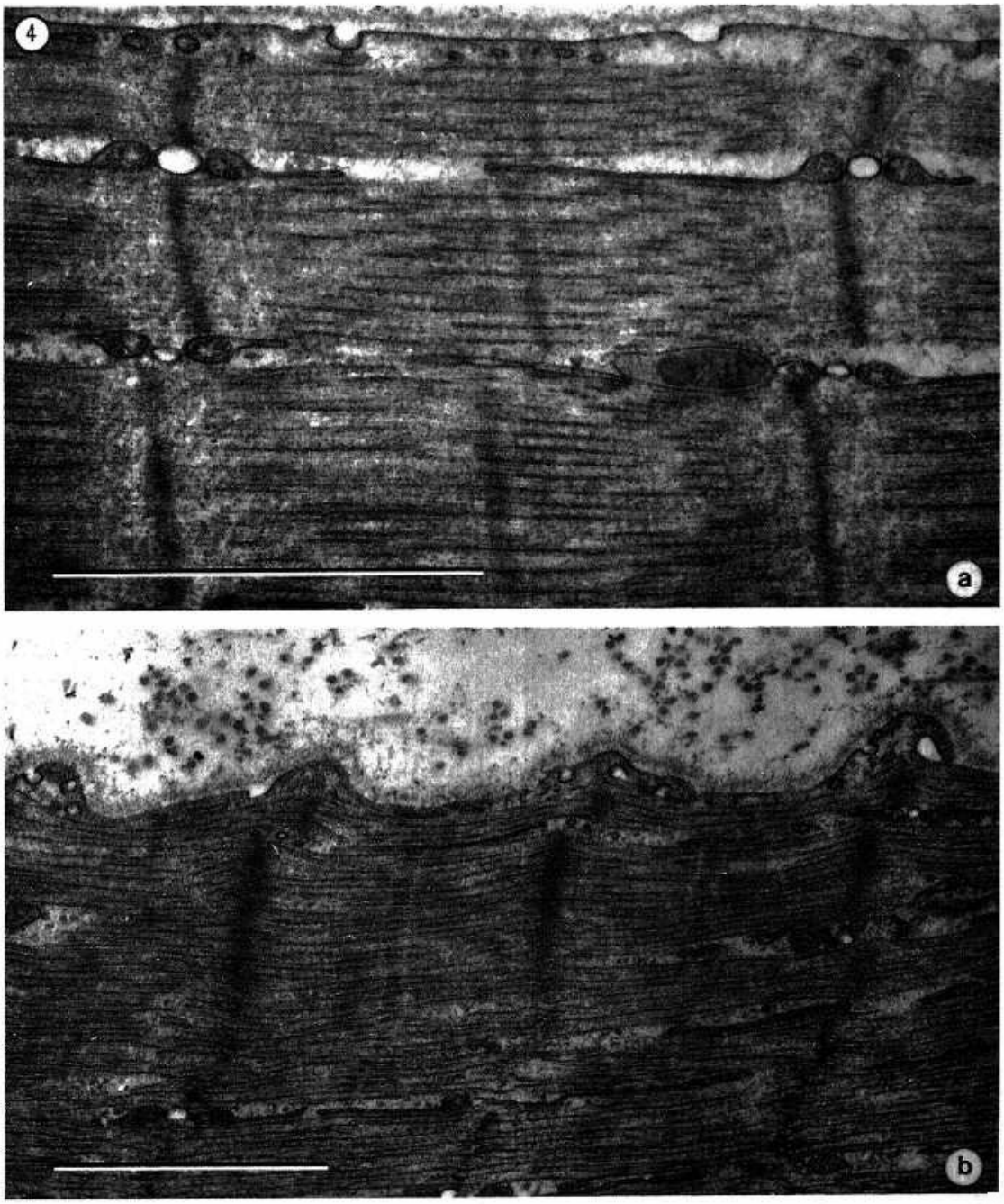

Fig.4. Freeze-substituted contracting frog sartorius muscles (acetone, 2.5% OsO_4, 0.2% uranyl acetate). Stimulation: 100 ms, rate 50/s; freeze-substitution: 2d -80°C, temperature increase 3°C/h up to -60°C, 1d -60°C, temperature increase 3°C/h, 4d -50°C; embedding in HM23, polymerised at -50°C. a) and b) show different states of contraction. Bars = 1µm; a): A-band:≤1.3µm b): sarcomere length: 1.1µm.

The results may be summarized as follows: Contracting muscles always show shorter A-bands than resting muscles prepared under the same conditions. Staining of several ultrastructural details (e.g., membranes, actin filaments) is poor or absent in preparations obtained after polymerisation in Spurr's resin (Fig. 2). Ultrastructural details are best seen in preparations obtained after freeze-substitution in OsO_4 containing acetone exclusively below -50°C and polymerisation at -50°C in the Lowicryls K11M and HM23 (Figs. 3 and 4). It is noteworthy that a freeze-substitution duration of at least 5 days (see figure legends) is required to obtain these results. Ultrathin sections obtained from muscles freeze-substituted for 1 week at -80°C and polymerised in K11M at -60°C reveal a remarkably good structure preservation (Fig. 5); dry cut sections of Cs^+ or Tl^+ loaded resting muscles show a preferential accumulation of the electron dense ions in A-bands and Z lines (Figs. 6a, c). Contracting muscles reveal different patterns of distribution of the electron dense ions: Sections of Cs^+ loaded and contracted muscles appear with a rather poor contrast (Fig. 6d) suggesting that Cs^+ ions have been liberated from A-bands and Z lines. Many Tl-precipitates can be seen in sections of Tl^+ loaded and electrically stimulated muscles (Figs. 6e, f) indicating that Tl^+ ions have been liberated and have combined probably with free anions (most likely phosphate ions).

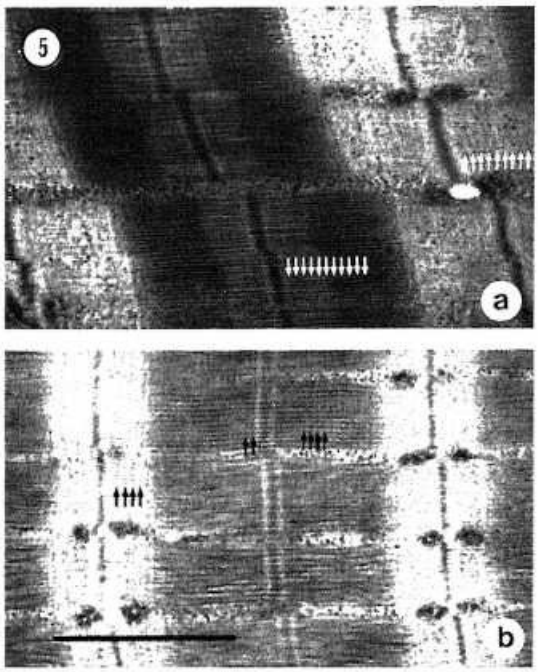

Fig.5. Freeze-substituted frog sartorius muscles. The muscles have been freeze-substituted for 1 week in pure acetone at - 80°C, embedded at - 60°C in Lowicryl K11M and polymerised at the same temperature. Bar = 1µm. a) resting muscle. b) contracting muscle. Stimulation: 100ms, rate 50/sec. The muscle shows A-band shortening. Arrows in (a) and (b) point to faintly visible axial periodicities of about 40nm. (From [8], reprinted by permission.)

Discussion

Technical problems

Cryofixation. It is well known that cryofixation of biological material is possible by a rapid contact either with a liquid or with a solid coolant (for review see [25]). Thereby either the specimen or the coolant may be moved. Moving the coolant against the specimen has the advantage that the specimen can be prepared under controlled conditions and remain situated at the very place where cryofixation occurs. Particular difficulties arise when a muscle must be prepared in such a way that renders possible electrical stimulation immediately prior to cryofixation. Although a muscle preparation with stimulating wires may be moved into a liquid coolant or against a cooled metal mirror [20, 21, 29] it appears easier to cryofix a stable muscle preparation by moving the coolant and this method has already been used by several authors [2, 9, 26, 27].

The freezing apparatus described here represents a simple automatic metal mirror freezing unit which yields reproducible results. The apparatus is particularly well suited for freezing contracting muscles because it easily permits synchronization of the electrical stimulation of the muscle and freezing. Also measurement of tension development of the muscle at the moment of cryofixation (unpublished results) poses no difficulties. In addition, even large whole intact muscles may be quick-frozen by using the described method. Of course, any other object may also be cryofixed by this new apparatus; liver, kidney and blood suspensions have already been cryofixed and the results are similar to those obtained after cryofixation with the REICHERT-JUNG KF 80 (see [25]).

The described method of freezing the specimen on a wet incised felt was developed in order to obtain preparations which are also well suited for cryosectioning: the frozen felt may be broken together with the specimen consisting of frozen felt and frozen biological material into small pieces; these pieces may be fixed in a vise type holder of a cryoultramicrotome (e.g., REICHERT JUNG, FC4) in such a way that the well frozen area of the specimen faces the knife of the microtome.

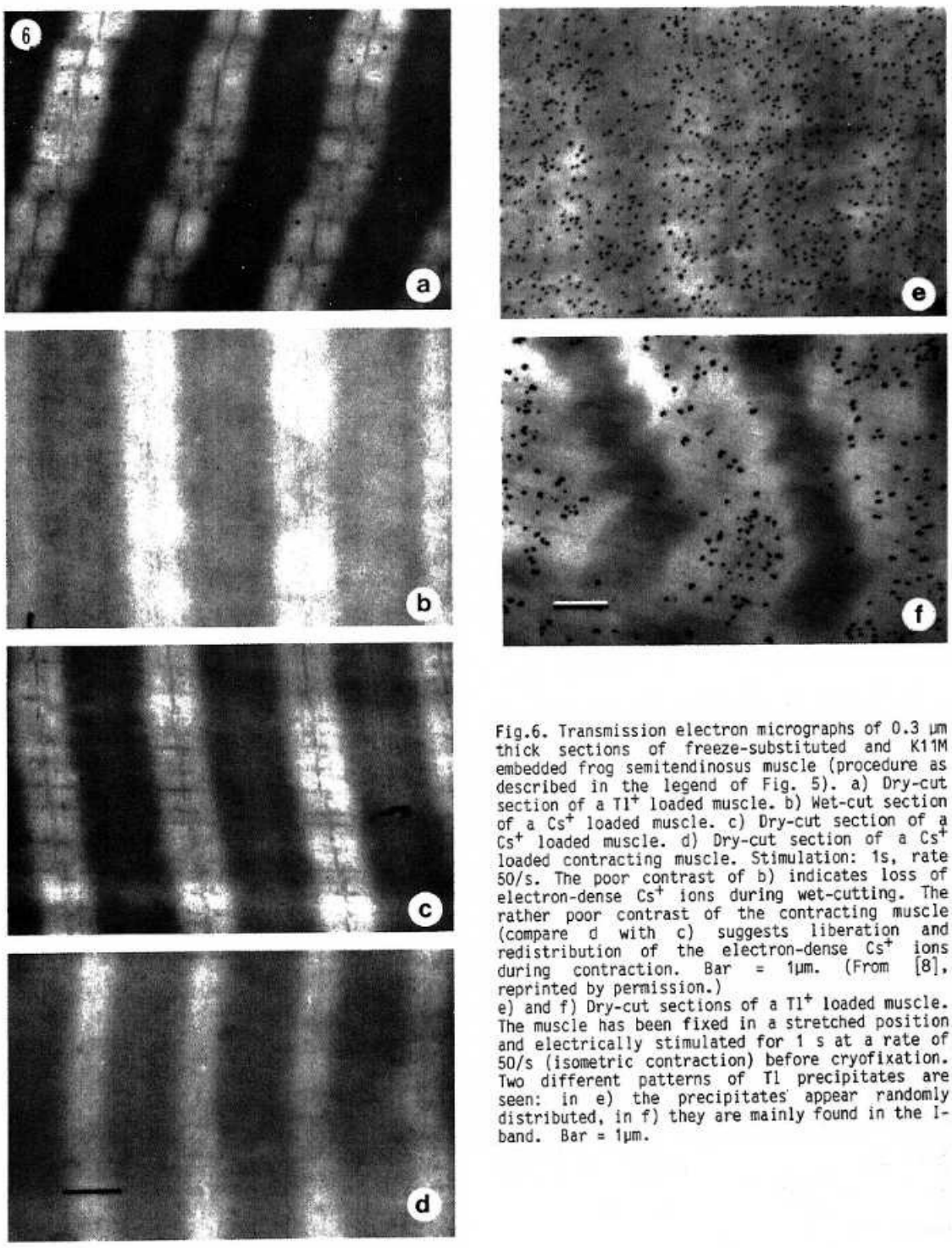

Fig.6. Transmission electron micrographs of 0.3 µm thick sections of freeze-substituted and K11M embedded frog semitendinosus muscle (procedure as described in the legend of Fig. 5). a) Dry-cut section of a Tl^+ loaded muscle. b) Wet-cut section of a Cs^+ loaded muscle. c) Dry-cut section of a Cs^+ loaded muscle. d) Dry-cut section of a Cs^+ loaded contracting muscle. Stimulation: 1s, rate 50/s. The poor contrast of b) indicates loss of electron-dense Cs^+ ions during wet-cutting. The rather poor contrast of the contracting muscle (compare d with c) suggests liberation and redistribution of the electron-dense Cs^+ ions during contraction. Bar = 1µm. (From [8], reprinted by permission.)
e) and f) Dry-cut sections of a Tl^+ loaded muscle. The muscle has been fixed in a stretched position and electrically stimulated for 1 s at a rate of 50/s (isometric contraction) before cryofixation. Two different patterns of Tl precipitates are seen: in e) the precipitates appear randomly distributed, in f) they are mainly found in the I-band. Bar = 1µm.

Freeze-substitution and low temperature embedding. Freeze-substitution, low temperature embedding and polymerisation were carried out in an automatic apparatus without problems. However, some general remarks concerning polymerisation of Lowicryls are necessary. The producer of the Lowicryls recommends polymerisation at low temperatures (e.g., K4M: -30°C, 1 day) followed by curing for 2 - 3 days at room temperature. We obtained reproducibly well hardened blocks when polymerisation was carried out solely at low temperatures (K4M:-30°C, 12h; K11M: -60°C, 24h; HM23: -50°C, 48h). These results were achieved by polymerising the resins in flat embedding moulds with a content of less than 0.1 ml. These moulds were covered tightly by a plastic UV-translucent material as shown schematically in Fig. 7 (Flat embedding moulds and UV-translucent covers are accessories of the CS auto.). The covering of the moulds seems to be necessary in order to prevent evaporation of a component of Lowicryl during polymerisation. Such an evaporation is indicated by the fact that without covering the moulds the top of the Lowicryl blocks (though irradiated directly with UV light) is usually not well polymerised and remains wet when polymerising exclusively at low temperatures. This result cannot be explained by a contamination of the Lowicryl with oxygen or water because the polymerisation is carried out in a dry nitrogen gas environment inside the CS auto. An advantage of polymerising small amounts of Lowicryl exclusively at low temperatures is that the temperature increase during polymerisation can easily be kept small (large surface to volume ratio of the resin) and conceivable deleterious effects of high polymerisation temperatures are avoided (measured temperature increase during polymerisation: less than 2°C). In addition, the used flat embedding moulds made of DELRIN appear particularly well suited for polymerising Lowicryls because UV light is back scattered by this plastic material with the result that the small amounts of resin are irradiated from all sides. The Lowicryl blocks are as a rule evenly polymerised and perfectly suited for sectioning.

It is well known that UV light induced polymerisation of Lowicryl embedded and OsO_4 containing specimens may be unsatisfying if too much Os impedes the penetration of UV light into the center of the specimen. It has been shown that OsO_4 fixation is compatible with Lowicryl embedding and UV polymerisation if the specimen appears only light brownish after OsO_4 treatment (see e.g., [10]: freeze-substitution in methanol containing 1% OsO_4, 0,5% uranyl acetate, 3% glutaraldehyde, embedding in Lowicryl K4M or HM 20 at -30°C). The method described here (UV polymerisation of Lowicryl embedded tissue substituted in acetone containing 2,5% OsO_4) also posed no difficulties. This may be due to the small size of the samples (size length less than 0.5mm) and to the fact that specimens freeze-substituted solely at temperatures below -50°C apparently do not take up much of OsO_4.

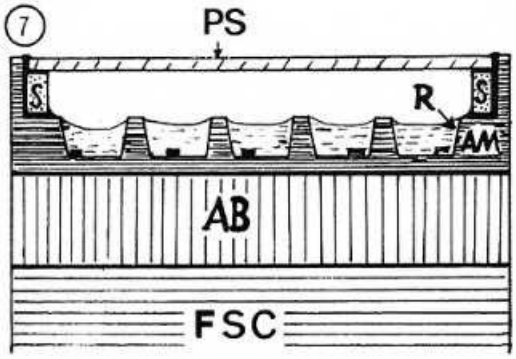

Fig.7. Schematical cross-section of flat embedding moulds for UV light polymerisation of low-temperature resins in the CS auto according to Sitte et al. [24]. The moulds are milled in an antiadhesive material AM (DELRIN) which is tightened to an aluminium base AB. This base is in thermal contact with the temperature-controlled freeze-substitution chamber FSC of the CS auto. The moulds are covered by a 1 mm thick polyethylene sheet PS. Due to capillary action the spacer S (cable fastening ribbon) between AM and PS causes the liquid resin R to ascend up to the cover PS so that a tight sealing of the space above the moulds results.

Biological significance

The results presented here may be of great significance for the direction of future scientific efforts.

Firstly, they have demonstrated the feasibility of freeze-substituting, embedding and polymerising quick-frozen tissue exclusively at temperatures below -50°C with excellent structure preservation as a result. The problem of staining sections of freeze-substituted preparations embedded and polymerised at high temperatures remains to be investigated. The fact that the muscle shown in Fig. 2b shows an extremely poor staining may be due to the extended freeze-substitution period (see figure legend). It is conceivable that a specimen completely dehydrated in acetone and embedded in Spurr's resin cannot be stained and that small amounts of water bound to proteins are required to make staining possible.

Secondly, the visualization of the unevenly distributed electron dense Cs^+ and Tl^+ ions in resting muscle shows that mobile ions can be retained in properly freeze-substituted (acetone exclusively at -80°C) cells to or near to the same sites (mainly A-bands and Z lines) which they occupy in frozen hydrated preparations [5, 6, 8].

In the third place, the different pattern of accumulation of Cs^+ and Tl^+ in contracting muscle supports the view that the main cellular cation is redistributed during contraction of a living muscle.

Questions concerning the nature of the artifacts which are introduced by the freeze-substitution and low temperature embedding technique used here arise and one may ask whether other techniques are available yielding results with less artifacts. Ultimately these questions can only be answered by comparing the electron microscopic results with results from frozen hydrated preparations. For instance, McDowall et al. have detected structural periodicities in frozen hydrated cryosections of glycerol extracted and cryoprotected muscle which were known from diffraction measurements of unfrozen preparations and which could not be seen in muscles conventionally prepared for electron microscopy [18]. In addition, a well preserved ultrastructure of muscle has been seen in frozen hydrated cryosections of quick-frozen Tl^+-loaded muscle (Fig. 8) [8]. Electron micrographs of such preparations may be analyzed with optical diffraction methods and compared with results obtained from freeze-substituted and embedded preparations. The similarity of Tl^+ distribution in frozen hydrated cryosections and freeze-substituted embedded preparations indicates that a most difficult goal namely retention of mobile ions at subcellular sites and maintenance of ultrastructure may be reached by freeze-substitution and low temperature embedding.

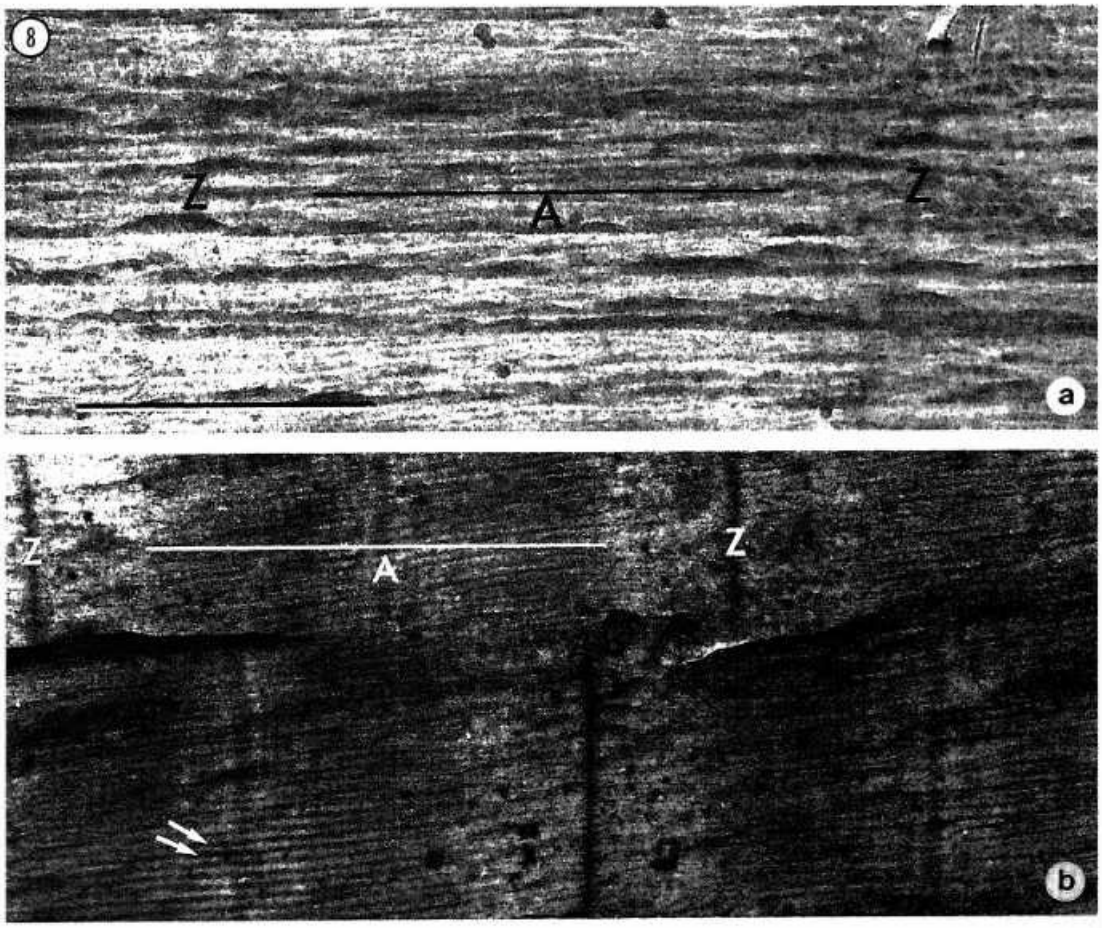

Fig.8. Frozen-hydrated cryosections of frog sartorius muscle. a) Normal K^+ containing muscle. Only very faint ultrastructural details can be seen. A, A band; Z, Z line. b) Tl^+ containing muscle. Dark myosin filaments (arrows) in the A bands (A) and dark Z lines (Z) indicate sites of preferential Tl^+ accumulation in the living cell. Bar = 1 µm. (From [8], reprinted by permission.)

Whether other embedding techniques yield better results remains to be determined. For instance, it has been claimed [15, 16] that optimum results can be obtained when a vitrified biological specimen is dehydrated below the devitrification temperature by "molecular distillation" and embedded in a resin ("Molecular distillation" is used for the sublimation of the water molecules from a frozen aqueous material and hence nothing but freeze-drying. In the author's opinion "molecular distillation" is a misnomer: distillation is derived from the Latin word "destillatio" which means drip; distillation is normally used for the evaporation of a liquid and condensation of the vapour to droplets which drip into a separate container). So far however, it has neither been proven that the rather complicated "molecular distillation" apparatus [15, 16] yields better results than those obtainable with much simpler freeze-drying units (e.g., [3, 4, 7]) nor has it been shown that freeze-drying of a vitrified biological specimen is possible in such a way that the freeze-dried structure is the same as the former ice embedded structure. Even the experimental demonstration of sublimation of solid amorphous water of a cryofixed biological specimen is still missing (according to Dubochet et al. [1, 17] the transition from solid amorphous water of a biological specimen to cubic ice occurs at about -135°C. Linner et al. [15, 16] showed that in an ultrahigh vacuum the sublimation process of cryofixed biological material starts at about -128°C).There remains the question whether preservation of ultrastructure and retention of ions is better when using freeze-drying and embedding compared to freeze-substitution and embedding. Experimental results obtained from frog sartorius muscles have shown that a rather well preserved ultrastructure and subcellular retention of mobile ions can be obtained with the freeze-drying and embedding technique [7]. So far however, the author has not seen an undisturbed ultrastructure of muscle after freeze-drying and embedding. It appears that shrinking and condensation artifacts can be kept at a lower level in freeze-substituted and low temperature embedded tissue than in freeze-dried and embedded tissue. Any technique which is supposed to yield optimum results should be tested with the striated muscle whose periodical arrangements of proteins are best suited for comparative studies.

The following last comments concern implications derived from the new results described here: The findings show that many problems of muscle contraction are still unsolved. It appears that a better understanding requires not only the correct detection of protein-protein interactions but also the detection of protein-water and protein-ion interactions.

For instance, the occurrence of A-band shortening has been claimed very often in the literature (for review see [22]) but the experimental evidence was seemingly not strong enough to convince supporters of the generally accepted sliding filament theory according to which A-band shortening does not occur. It is expected that the A-band shortening shown here will lead to a more intense investigation of this controversial issue.

The finding concerning the uneven cellular cation distribution in muscle points to another more fundamental controversial issue in cell biology which has been discussed in several publications [6, 8]: Briefly, an uneven distribution of the main cellular cation cannot be explained by the widely held membrane pump theory which is based on the assumption that most of the cellular cations are freely dissolved in free cellular water. To the contrary, it confirms predictions of the association-induction hypothesis of Ling according to which the living cell represents a metastable cooperative protein-ion-water complex [11, 13]. From the point of view of this theory muscle contraction can only be understood if all the main cellular components namely proteins, ions and water are taken into consideration. In an extended chapter on muscle contraction Ling pointed to the unsolved problem of water redistribution during shortening of a contracting muscle ([13, p. 566]). He developed a tentative model in which local liberation of bound K^+ and local differences in water activities may be the cause of water-shifts within sarcomeres of the living contracting muscle. The results reported here are in accordance with this view and may stimulate others to investigate ion and water movements during muscle contraction and other fast dynamic cell processes [19].

Acknowledgements

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Discussion with Reviewers

G.H. Pollack: The observation that thick filaments shorten during contraction is intriguing. As the author mentions, numerous earlier reports of thick filament shortening fill the literature, but because of uncertainties of the methods, these reports have not gained wide attention. The author's result was observed using several embedding methods; a consistent difference between relaxed and contracted specimens was apparently seen with each method. Thus, the likelihod of artifact is diminished. The result seems genuine. One question of interest is whether the amount of thick filament shortening varied with the amount of sarcomere shortening. Was any attempt made to quantitative the magnitude of thick filament shortening? Did it vary with load, for example?

R.L. Ornberg: Might not one explanation for the A-band shortening which you see in freeze-substituted stimulated muscle be that it is caused by water removal (and shrinkage) during substitution and that stimulation and contraction initiate ion redistribution near the A-band which make it more susceptible to this shrinkage? Also what is the amount of shortening that you see and is it related to stimulation parameters or ion content?
Author: The amount of A-band shortening observed under the described conditions is about 15 - 20%. A quantitative study relating varying stimulation parameters, ion content and load of the muscles to the A-band shortening has not yet been made. The question whether local shrinkage within muscle sarcomeres may occur during freeze-substitution remains to be determined by the comparative examination of frozen hydrated cryosections and freeze-substituted specimens obtained from the same cryofixed muscles. The facts however that the observed A-band width of unstimulated muscles corresponds fairly well with generally accepted values of living muscles and that the axial periodicities seen in resting and contracting muscle are similar (see Fig. 5) speak against the idea that the observed A-band shortening could be a freeze-substitution artifact.

M. Müller: The results of MacKenzie (MacKenzie AP. Freezing, freeze-drying and freeze-substitution. Scanning Electron Microsc. 1972: 273-279) suggest that the amount of water that can be removed from the specimen is solely a function of the temperature and the polarity of the dehydrating agent (vacuum, organic solvents). Could the superior preservation of freeze-substituted, low temperature embedded samples be related to the more homogeneous distribution of the temperature within a specimen that is immersed in a liquid than within a partially freeze-dried specimen?
Author: If an ultrathin cryosection is freeze-dried very slowly in the vacuum of a cryo-electron microscope - starting at the lowest possible temperatures and going up very slowly to -80°C - the temperature gradients within the specimen should be very small during freeze-drying. I carried out such experiments with muscle sections and could not yet observe a structure preservation as good as that seen in muscles freeze-substituted exclusively at -80°C in acetone. My conclusion is that not the more homogeneous distribution of temperature but the physicochemical properties of acetone are responsible for the superior preservation. It appears that less attachment of very labile structures at neighbouring more stable structures occurs in acetone than in the vacuum. This could be due to the different dielectric constants of acetone and of the vacuum.