Intracellular Site of Sr^{2+} and Ba^{2+} Accumulation in Frog Twitch Muscle Fibres as Determined by Electron Probe X-ray Microanalysis

B. Uhrík and D. Zacharová

Centre of Physiological Sciences, Slovak Academy of Sciences, Vlárska 5, 833 06 Bratislava, Czechoslovakia

Abstract. Strontium and barium can substitute for calcium at different levels of the excitation-contraction-relaxation cycle. The problem of sequestration of these ions in cellular microcompartments may be settled only by direct evidence obtained with analytical methods. Isolated frog twitch muscle fibres were perfused with increasing concentrations of potassium in Ca-free solution supplemented with Sr^{2+} (10 mmol/l) or Ba^{2+} (5 mmol/l). After equilibration in a Ca-free Ringer with Sr^{2+} or Ba^{2+} for 30 to 60 min the fibres were frozen in liquid propane (at 80 K) to immobilise ions. Ultrathin (150 nm) cryosections were cut at 170 K, freeze-dried, carbon-coated and analysed in an electron microscope equipped with an X-ray spectrometer. The ultrastructure of the superficial layer of the fibres was satisfactorily preserved. The terminal cisternae (t.c.) of the sarcoplasmic reticulum (SR) were dark and contained various amounts of Sr or Ba in addition to Ca. In Sr loaded fibres the longitudinal SR occasionally showed electron dense content with significant amounts of Ca; no Sr was present. The results suggest that t.c. is the common sequestering compartment for Ca, Sr and Ba. Essentially the same distribution pattern of Sr was found following precipitation of Sr with a solution containing digitonin and K-oxalate.

Key words: Frog muscle fibres — Strontium — Barium — Cryosections — X-ray microanalysis

Introduction

It is well known that alkali-earth cations, strontium and barium, can substitute for calcium at different levels of the excitation-contraction-relaxation cycle. Strontium is able to replace calcium in the activation of contraction and to be accumulated in the microsomal fraction of skeletal muscle cells (Edwards et al.

1966). Both these cations can enter the muscle fibres via the slow inward calcium channel (Potreau and Raymond 1980; Cognard and Raymond 1985) and elicit contractile response after micro-injection into the sarcoplasm (Heilbrunn and Wiercinski 1947; Caldwell and Walster 1963).

It is generally assumed that strontium is sequestered also by the sarcoplasmic reticulum (SR) of intact muscle cells. However, the only morphological evidence of Sr accumulation in vesicles of SR has so far been presented for smooth muscle cells. In contrast to Sr, Ba was absent in SR of smooth muscle cells and was accumulated only in mitochondria (Somlyo and Somlyo 1971; Somlyo et al. 1974). Similarly, the heart muscle microsomal fraction exhibited only a poor affinity for Ba (Vanderkoi and Martonosi 1971). On the other hand, progressive decline in tension in frog skeletal muscle fibres upon increasing intracellular Ba concentration was interpreted in terms of irreversible replacement of Ca by Ba in SR (Potreau and Raymond 1980).

The lack of morphological evidence of Sr and Ba sequestration in intact skeletal muscle cells and the discordant findings concerning Ba accumulation in preparations of different origin, have prompted us to study this problem with a technique allowing direct localisation of divalent cations at the subcellular level. The method of electron probe X-ray microanalysis meets this requirement and has been used in the present study. The results have already been published in abstract form (Uhrík and Zacharová 1987, 1988) and have shown that the terminal cisternae of SR are the site of accumulation of both Sr and Ba.

Materials and Methods

Muscle preparation

Isolated twitch muscle fibres from frogs (*Xenopus laevis* or *Rana temporaria*) were dissected from m. semitendinosus in Ringer's solution and then mounted in a chamber allowing quick exchange of the bathing solution (Zachar et al. 1964). One tendon was mechanically attached to the chamber, and the other was connected to a silicon tensometer (Marko et al. 1986) to record mechanical responses.

Sr or Ba loading

The fibres were perfused for 10—20 s with increasing concentrations (20, 40 and 80 mmol/l) of potassium (substituted for Na) in Ca-free Ringer's at a constant $[K]_o \times [Cl]_o$ product (propionate substituted for Cl) containing 10 mmol/l $Sr(OH)_2$ or 5 mmol/l $BaCl_2$. In the period (25—30 min) between potassium contractures and at the end of the perfusion series the fibres were equilibrated in Ca-free Ringer's containing Sr^{2+} (10 mmol/l) or Ba^{2+} (5 mmol/l). The final equilibration lasted 30 to 60 min; during this time the fibres were mounted to holders for rapid freezing.

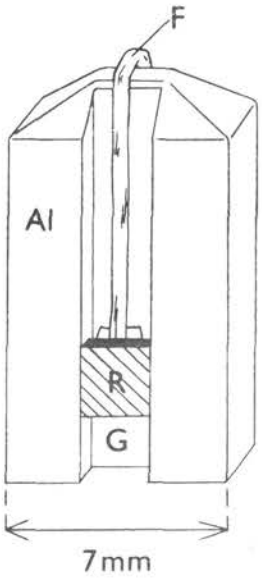

Fig. 1. Aluminium holder (Al) with a fibre mounted for rapid freezing. F — fibre protrusion at the top of the holder; R — a piece of rubber pressing upon the tendon; G — groove for accommodating the fibre.

Freezing and cryoultramicrotomy

The fibres were placed in a groove of an aluminium holder and mechanically fixed with pieces of rubber pressing upon the tendons. On the top of the holder the fibres were bent forming a protrusion (Fig. 1). The holder was picked up with a pair of forceps, blotted with filter paper and plunged with the fibre protrusion downwards into liquid propane. The propane was stirred with a propeller in an Al-container and was pre-cooled to 80 K with liquid nitrogen (LN) contained in a styrofoam box. The freezing rate was estimated by plunging into liquid propane a copper-constantan thermocouple with a wire diameter of approx. 80 μm. The freezing rates as measured with a HEXLAND EMSCOPE CRM 250 cooling rate meter were between 10,000—20,000 K/s. The freezing rates attainable with liquid propane are generally higher than with other liquid coolants (Elder et al. 1982). The holders were kept under LN until cryosectioning. The design of the holders allowed them to be mounted directly to the chuck of a Reichert Ultracut E equipped with FC 4D cryoattachment. Approx. 60 % of fibres had to be discarded because of a thick layer of frozen Ringer's upon them. Ultrathin (150 nm) cryosections from the fibre protrusion were made with glass knives at the nominal temperature of the holder around 160 K. The knife was kept at 170 K and the bottom of the cryochamber at 130 K. The cryosections were transferred with an eyelash to Formvar coated Ti or Cu grids and placed on a LN-pre-cooled specimen stage block of the freeze-etching device (JEOL EE-FED.B) and freeze dried in a JEOL JEE-4X vacuum evaporator at $1—3 \times 10^{-4}$ Pa. A LN-chill-

ed cold trap was used, the specimens were protected with a LN-pre-cooled knife stage block. After rewarming, the grids were carbon-coated and transferred within two minutes into HEXLAND analytical cooling holder with titanium or beryllium (in case of Ba analysis) inserts.

Electron microscopy and X-ray analysis

The sections were studied and analysed at a tilting angle of 45° and at the temperature of the cooling holder of 85—90 K, in the scanning transmission mode of a JEOL JEM 1200 EX electron microscope equipped with a LINK 860 X-ray spectrometer. The pressure inside the microscope column was below 1×10^{-4} Pa and an anticontamination device and a cold trap were used. Analysis life-time was 100—200 s at 80 kV with a count rate of about 1000 counts per second.

From Sr- and Ba-loaded preparations respectively, three fibres could be successfully sectioned and analysed. The terminal cisternae of SR (t. c.), longitudinal SR, I- and A-bands and mitochondria were analysed, each compartment was represented by 10—15 spectra from each fibre. The presence of Sr or Ba in a given compartment was assumed if the integral X-ray counts P (gross integral of the characteristic X-ray peak) and b (background of the same peak) fulfilled the relation $P - B > (P + b)^{1/2}$ (Chandler 1977). Relative concentrations of Ca and Sr, or Ca and Ba, in t. c. were estimated from relative efficiencies of the detecting system for Ca, Sr and Ba, using spectra from $CaSO_4$, $SrSO_4$ and $BaSO_4$ normalised to sulphur (Chandler 1977).

Immobilisation of Ca and Sr by oxalate precipitation

It is known (Popescu and Diculescu 1975; Popescu et al. 1980) that high concentrations of potassium oxalate can be used for calcium immobilisation in both smooth and skeletal muscle cells. We applied this alternative procedure to 11 single fibres loaded with Sr. Oxalate solution was added immediately after 30 min-perfusion with the last potassium saline. In addition to potassium oxalate (40 mmol·l) and sucrose (100 mmol·l), the precipitating solution contained digitonin (1 mg/30 ml) and was saturated with strontium oxalate and calcium oxalate (the solubility of Sr-oxalate at 18 °C, 6 mg/100 ml, is about ten times higher than that of Ca-oxalate). Digitonin binds to cell membrane cholesterol (Elias et al. 1978) and it increases the permeability of the cell membrane to oxalate anion; this allowed to shorten the application of the precipitating solution to 10—15 s. Fixative solutions (2 % glutaraldehyde in 100 mmol·l Na-cacodylate and 1 % OsO_4, each applied for 30 min) and 50 % and 70 % ethanol contained 40 mmol·l K-oxalate and were saturated with Sr- and Ca-oxalate. After dehydration the fibres were cleared in propylene oxide and embedded in Durcupan. Sections of 0.5 μm were cut with dry glass knives, transferred to the surface of a glycerol drop, stretched with heat and picked up on copper grids.

Results

Oxalate precipitation

Fibres loaded with strontium and treated with K-oxalate and digitonin were distinguished by dark precipitates flanking the Z-lines, at the location normally occupied by terminal cisternae of SR (Fig. 2). No other compartments in the fibres contained visible precipitates.

Fig. 3*A* shows a typical X-ray spectrum generated by a precipitate in a t.c. of SR. Copper peaks are instrumental, generated by stray and scattered electrons, osmium peaks are due to fixation of the fibre with OsO_4. Significant

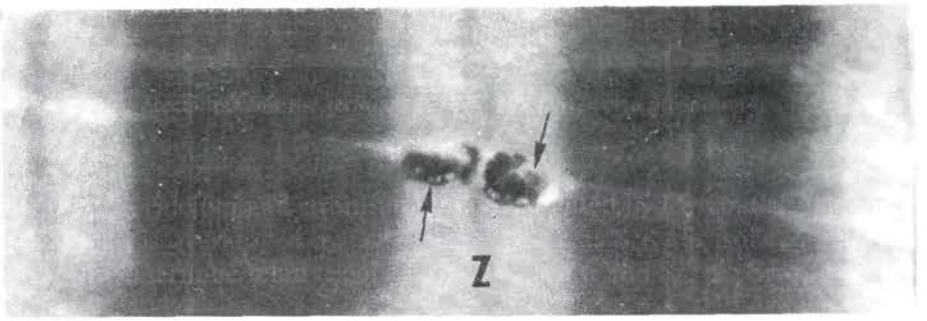

Fig. 2. Oxalate precipitates in the terminal cisternae (arrows) of a strontium-loaded fibre. Z — Z-line. × 32,200

quantities of calcium and strontium are documented by CaKα, SrLα nad SrKα peaks. SrLα is overlapped by OsMα peak, whereas SrKα is isolated and could be used to determine relative concentrations of Ca and Sr. No Sr was detected

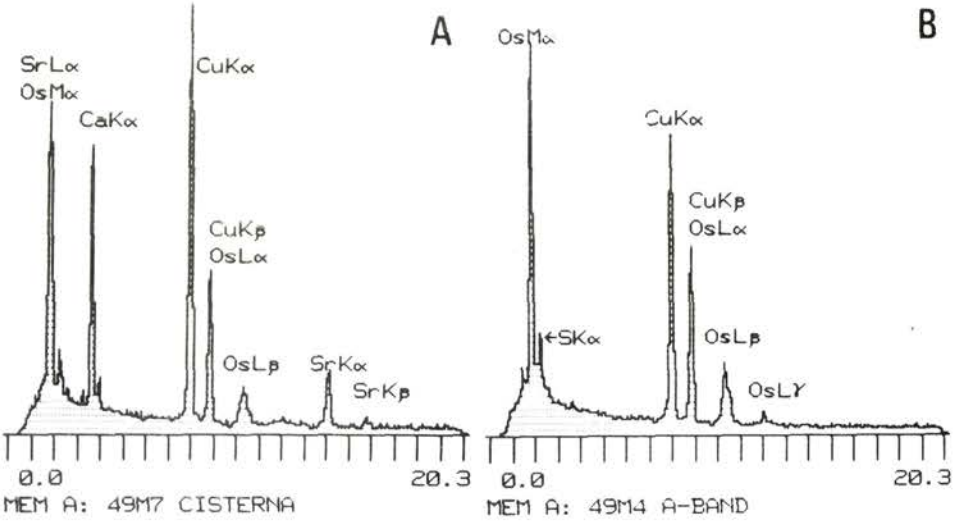

Fig. 3. X-ray spectra from a strontium-loaded fibre after immobilisation of Ca^{2+} and Sr^{2+} by oxalate. *A* — spectrum from a terminal cisterna of sarcoplasmic reticulum. *B* — spectrum from the A-band.

in other compartments of the fibres. Fig. 3*B* shows a spectrum from the A-band with Os, Cu and S peaks but without Ca and Sr peaks.

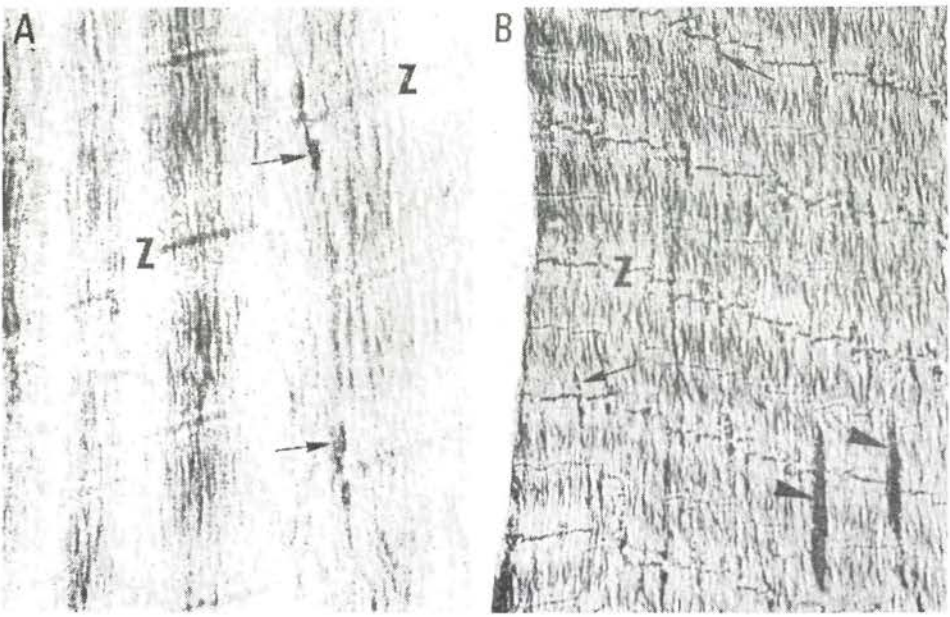

Fig. 4. Longitudinal cryosections of frog muscle fibres. *A* — a strontium-loaded fibre. Arrows point to terminal cisternae of SR. Conventional transmission image. × 23,400. *B* — a barium-loaded fibre. Arrows — terminal cisternae of SR, arrowheads — mitochondria. Scanning transmission image. × 14,000. Z — Z-line.

Analysis of cryosections

Despite the absence of any staining or contrasting substances the frozen-dried cryosections exhibited all the principal ultrastructural details. The identification of triads was facilitated by their location at the Z-lines and by a dark content of t.c. Fig. 4*A* shows a strontium-loaded fibre, two triads may be seen at neighbouring Z-lines with conspicuously contrasted t.c.

Fig. 4*B* is an overall view of a longitudinally sectioned barium-loaded fibre. A- and I-bands and Z- and M-lines are clearly visible, the mitochondria are prominent, with their long axes parallel to the long axis of the fibre, numerous t.c. can be distinguished. Frequently, mitochondrial cristae were discernible. Ice crystal damage increases from the periphery towards the fibre interior, manifested as a distortion of the myosin filaments.

Electron probe X-ray microanalysis of cryosections has shown that the only site of significant concentration of strontium or barium were the terminal

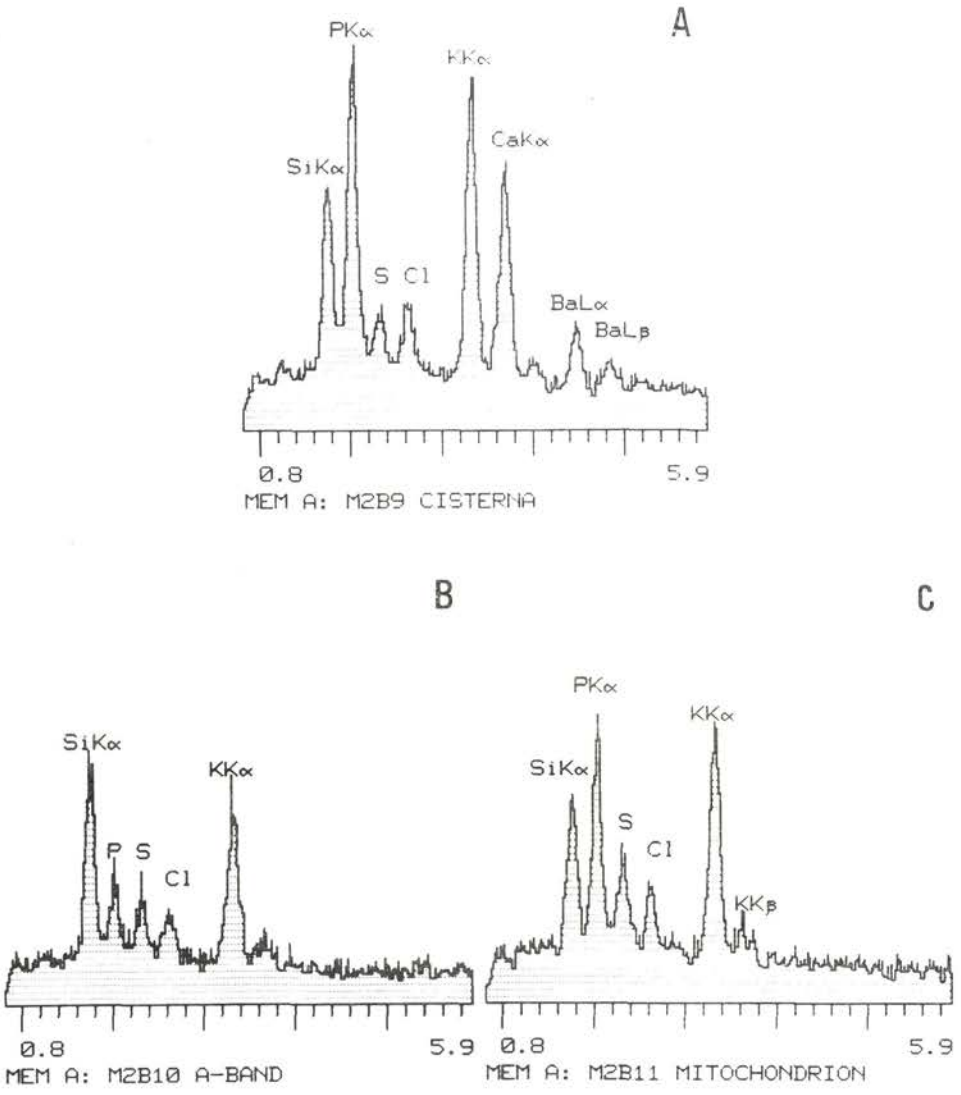

Fig. 5. X-ray spectra form cryosections of a barium-loaded fibre. *A* — spectrum from a terminal cisterna of SR. *B* — spectrum from the A-band. *C* — spectrum from a mitochondrion.

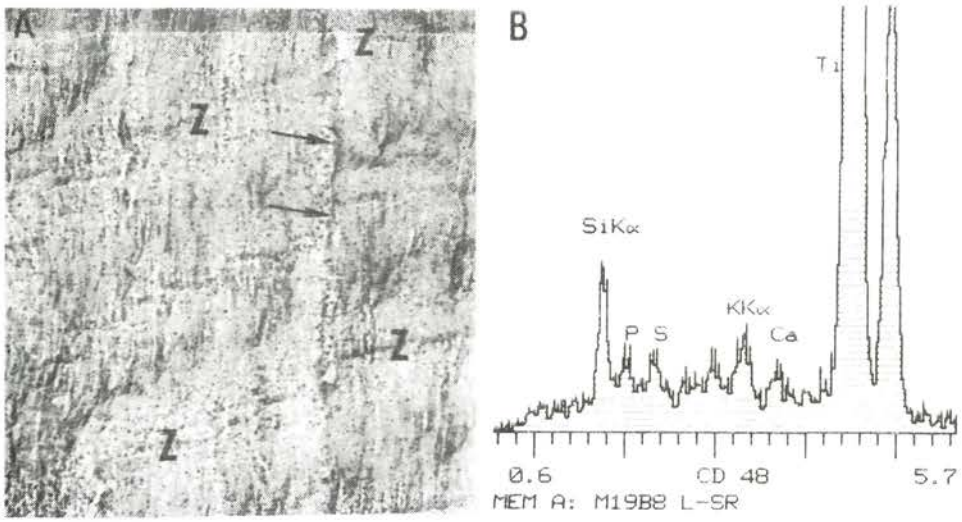

Fig. 6. Strontium-loaded fibre. *A* — conventional transmission image of a cryosection with a dark content (arrows) in longitudinal SR. ×25,800. Z — Z-lines. *B* — X-ray spectrum from longitudinal SR in *A*.

cisternae of SR. The amounts of Sr or Ba varied, numerous t.c. contained only calcium. According to the criteria for peak identification (see Materials and Methods) about one third of t.c. contained Sr or Ba, respectively.

Fig. 5*A* shows the low-energy band of a spectrum from a t.c. of a Ba-loaded fibre. Apart from expected peaks of P, S, Cl, K and Ca, BaLα and BaLβ peaks are marked. Silicon could be detected in sections staying in electron microscope over several hours and has no biological significance. Figs. 5*B* and 5*C* are from the same section and demonstrate, for the sake of comparison, the spectra from the A-band and a mitochondrion, respectively.

According to estimation of the relative molar concentrations, the amounts of strontium or barium in t.c. were maximally one fourth of those of calcium present.

Occasionally, a dark content in longitudinal SR of strontium-loaded fibres could be seen in cryosections (Fig. 6*A*). An analysis of these areas showed the presence of significant amounts of calcium (Fig. 6*B*), with no strontium present.

Discussion

The preferential site for both strontium and barium sequestration in frog twitch muscle fibres, under the conditions of our study, are the terminal cisternae of the sarcoplasmic reticulum.

As compared with data on Sr and Ba accumulation in vascular smooth muscle cells, both cations being accumulated in the mitochondria and only Sr in the sarcoplasmic reticulum (Somlyo and Somlyo 1971; Somlyo et al. 1974), the most striking difference concerns barium. However, the binding of Ba to t.c. has already been suggested by Potreau and Raymond (1980) to explain the irreversible decline in tension in frog skeletal muscle fibres upon increasing intracellular Ba concentration. Our results are consistent with their interpretation.

The terminal cisternae of SR in frog muscle cells are the principal site for Ca-storage and Ca-release as shown by Somlyo et al. (1981) in an electron probe study. Electron opaque material filling the lumen of t.c. and considered to be a Ca accumulating protein, calsequestrin, imparts sufficient contrast to t.c. even in cryosections, and facilitates the elemental analysis in this type of muscle.

The occasional finding of electron dense material in longitudinal SR (LSR) in strontium-loaded fibres, containing significant amounts of calcium (Fig. 6*A*, *B*), may indicate that apart from t.c., LSR can participate in Ca accumulation as well, at least under some unusual conditions. Ca translocation to LSR with the formation of granules containing Ca, Mg and P was observed under the influence of hypertonic solution (Somlyo et al. 1977). Dense granular content has also been found in LSR both after conventional fixation (Sommer 1982) and using the technique of freeze-fracturing, deep etching and rotary shadowing (Franzini-Armstrong et al. 1987). It may be assumed that calcium accumulated in LSR is not readily available for excitation-contraction coupling, since calcium-release channels (receptors for ryanodine), recently identified with junctional feet (Hymel et al. 1988; Lai et al. 1988), are located on the junctional membrane of t.c.

The use of the K-oxalate precipitation method for calcium (Popescu and Diculescu 1975) and strontium immobilisation yielded a precipitate pattern compatible with the results of cryosection analysis. The addition of digitonin not only reduced the time necessary for perfusion with precipitating solution but also gave more consistent results.

However, to immobilise all diffusible substances in the shortest possible time, rapid freezing combined with cryosectioning is essential.

An interesting observation on cryosections was the great variability in strontium or barium contents even in the terminal cisternae from neighbouring areas. Whether these differences correspond to different binding capacity of individual t.c. or whether they arise from insufficient loading of fibres with strontium or barium, remains to be cleared by further studies.

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