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X-RAY MICROANALYSIS OF CALCIUM CONTAINING ORGANELLES IN RESIN EMBEDDED TISSUE

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Abstract

The localization of calcium in cell organelles at the electron microscope level is often achieved through cytochemical techniques, and verified by X-ray microanalysis. Various methods have been used to cytochemically detect calcium or calcium-binding sites : calcium loading, calcium substitution by strontium, barium, or even lead, and calcium precipitation by oxalate, phosphate, fluoride, or pyroantimonate. Their results may have heuristic value, particularly in preliminary studies of poorly known cell types. A complementary and more physiological approach is offered by quantitative measurement of the total calcium content of organelles after cryofixation.

Resin embedding is less demanding than cryomicrotomy and gives better images : it can be used after cryosubstitution in the presence of oxalic acid. This technique was tested, and applied to several cell types.

Key words : X-ray microanalysis, calcium, organelles, cytochemistry, freeze-substitution

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Introduction

The unequal distribution of calcium in various subcellular compartments is of major importance in cell biology. A very low Ca^{2+} concentration in the cytosol is achieved by Ca^{2+} transport through the plasma membrane, but also by Ca sequestration in membrane bound organelles. A variety of mechanisms allows rapid fluctuations of free Ca^{2+} in the cytosol, the organelles and the extracellular milieu acting as sources or sinks (see Campbell, 1983, Carafoli, 1987). In the last twenty years, the main interest of workers interested in the cell biology of Ca has been focused on cytosolic Ca^{2+} and its variations. However, the cytosol is excluded from X-ray microanalytical studies firstly because in most cases it is too narrow for the electron beam diameter and, mainly, because its Ca^{2+} content stays well under the sensitivity limit of the technique. This limit is of the order of 5 mM in our hands, as well as for Gupta and Hall (1978; also Hall, 1979a), somewhere between 5 and 10 mM for Ornberg and Reese (1981a), and at best 0.3 mM (see Somlyo 1985), which is still two orders of magnitude higher than the cytosol (*e.g.*, Somlyo et al., 1982). However the *total* Ca content of cells is much higher than the cytosol ionized Ca^{2+}, generally in the mM range or higher, within the limits of detection by electron probes (Gupta and Hall, 1978). Changes in total Ca occur in cell organelles during various physiological processes, and can be detected and even quantified by X-ray microanalysis (*e.g.*, Brown et al., 1975; Amos et al., 1976; Somlyo et al., 1981; Wendt-Gallitelli and Jacob, 1982; Bond et al., 1984; Yoshioka and Somlyo, 1984; Chang et al., 1986). Analysis of organelles *in situ* avoids the redistribution which is known to occur during homogenization and cell fractionation (Clemente and Meldolesi, 1975), provided that one finds a way to control the movements of Ca during the preparation.

There is a good agreement on the reliability of the cryotechniques : major results have been obtained by shock freezing the tissues and making thin cryosections, eventually examined by quantitative X-ray microanalysis (see Dorge et al., 1974; Gupta et al., 1976; Somlyo et al., 1977, among the first). However, numerous authors have used sections of resin embedded tissue as alternatives to cryosections, either after aqueous cytochemistry or after quick freezing and freeze-substitution. In the present paper on calcium localization in resin embedded tissues, we shall review first the cytochemistry of chemically fixed cells, then the use of X-ray microanalysis to measure physiological concentrations of total Ca in frozen and freeze-substituted specimens.

Autoradiography, a complementary approach to X-ray microanalysis of cryofixed tissues which demonstrates freshly exchanged ^{45}Ca (instead of total Ca) will not be reviewed, except in the evaluation of the preparative techniques.

Electron probe X-ray microanalysis

The Hall method of quantitation in X-ray microanalysis of thin sections is well established and widely used (see Hall et al., 1973; Shuman et al., 1976; Chandler, 1977; 1978; Gupta and Hall, 1978; Hall, 1979a; 1979b; Roomans, 1980; Roomans and Shelburne, 1983; Kitazawa et al., 1983; Somlyo, 1985). Ca is a particularly convenient element to analyze in an electron microprobe, stable under the electron beam and with a relatively large signal per mass of analyzed atoms. In cryosections of well frozen tissues, potassium is abundant and can impair the detection of small quantities of Ca, as the potassium Kβ peak overlaps the Ca Kα peak. As shown by X-ray spectra in the present paper, the potassium may be washed off when the freeze-substitution (resin embedding) technique is used instead of cryosections.

However, the experimental conditions are very diverse using the different instruments commercially available and few recommendations may be generalized.

Instruments. We used two very different instrumental configurations for transmission analytical electron microscopy: energy-dispersive spectrometer (EDS) associated with a 120 kV high resolution transmission electron microscope (TEM) or wavelength dispersive spectrometer (WDS) associated with a scanning electron microscope (SEM). The second configuration was a CAMEBAX®, operating at a maximum of 50 kV; it was equipped with a TEM accessory, an EDS which was mainly used to estimate the continuum, and a light microscope.

Readers interested in quantitative analysis of thin biological specimens with the CAMEBAX will find technical advice in Blaineau et al. (1987). We generally use aluminum grids with no supporting film in this instrument.

In most recent TEM-EDS configurations the commercially available programs are satisfactory and quantitation is open to users who do not know how it works. We use Formvar-coated copper grids to analyze the sections in our TEM-EDS configuration (Philips CM12 + Tracor TN 5400). The supporting film is needed by the program and we avoided aluminum to be able to switch easily to frozen dried cryosections with the same reference artifacts (an Al grid may interfere with elements of interest such as Mg).

Standards. It is often stated that the standard must have a composition which is the closest possible to the specimen. This is particularly pertinent with the continuum method, and even more so in WDS which necessitates high probe currents; it is mainly justified by the quasi-instantaneous mass loss which occurs in the specimen when the electron beam hits the section (see ref. in Hall, 1979a; 1979b; Roomans, 1980; Amsellem et al., 1983). We first tried to make a direct Ca standard by mixing a Ca-macrocyclic polyether with resin (see Spurr, 1975; Chandler, 1976) but were only able to make a homogeneous standard of higher concentration than 0.025 % one time by this method. Ca standards of concentration inferior to 0.5 % will introduce excessive statistical error in the quantitation. This difficulty of obtaining a sufficient Ca concentration is also reported by Roomans (1980) but Chandler (personal communication) made standards up to 1% of Ca with crown-ethers in resin. We occasionally used an indirect peripheral 5% sulphur standard, obtained by adding 40 % Thiokol LP3 (Ciba-Geigy) to the Epon mixture (Jessen et al., 1974; Amsellem et al., 1983). This sulphur standard was tested by coulometric titration in the Service Central d'Analyses du CNRS (Vernaison, France). We also used Chelex 100 beads loaded with Ca (given by Dr De Bruijn - see De Bruijn, 1981) and embedded in Epon, which make a 6.2 % standard. We mainly used a 1% Ca naphtenate standard in resin (see Ornberg and Reese, 1981a; Hagler et al., 1981), provided by Dr Ornberg. The standard deviations (SD) of relative peak intensities (peak minus background/continuum of the specimen minus continuum in a hole of the grid), converted to percentages vary from 17 % to 23 % for the polyether-Ca complex in Epon, containing 0.025 % of Ca, from 12 to 16 % for the Chelex beads embedded in Epon, from 4 to 18 % for a Ca naphtenate in resin, containing 0.5 % of Ca. These standard deviations were obtained with 4 to 6 series of measurements. A single experiment (10 measurements) with the polyether-Ca 1% provided by Dr Chandler gave a SD of 12%. Separate tests run by Dr Quintana on our Ca-naphtenate 1% with a different instrument allowed her to estimate the margin of error to 10 %. All these results suggest that it is important to take numerous measurements of the standard to reduce the statistical error; a large probe may average the local variations but at the risk of a different mass loss.

The use of osmium tetroxide. In most of the cytochemical studies, the authors use osmium tetroxide post-fixation, or even primary fixation, before dehydration and embedding. Omitting OsO_4 may give a poor chemical fixation of the lipids, particularly the phospholipids which are an essential component of membranes, but once the tissues have been fixed with OsO_4 it is possible to get rid of osmium with hydrogen peroxide (André and Marinozzi, 1965) or microincineration (Thomas and Greenawalt, 1968). Osmium tetroxide is also used to vapor stain cryosections (Somlyo *et al.*, 1977) or to improve the contrast of cryosubstituted tissues (see Harvey, 1982).

OsO_4 is known to dissolve calcium in various intracellular stores. This dissolution can be prevented by adding Ca^{2+} to the fixative (Reynolds, 1965; Oschman and Wall, 1972; Skaer et al., 1974). After osmium fixation, an electron dense structure is generally considered to be osmiophilic, or calciphilic if it is only seen in the presence of Ca. Qualitative X-ray microanalysis may be needed to establish such statements, especially as the same anionic sites may bind both Ca and Os, as in the case of phospholipids ; X-ray spectrum processing will show in some cases that highly electron dense structures, presumed osmiophilic, contain very little osmium (*e.g.*, Beaulaton et al., 1986). OsO_4 has been shown to redistribute Ca from one organelle to another in at least one example, in the vitelline cells of the parasitic worm *Schistosoma* (Erasmus and Davies, 1979).

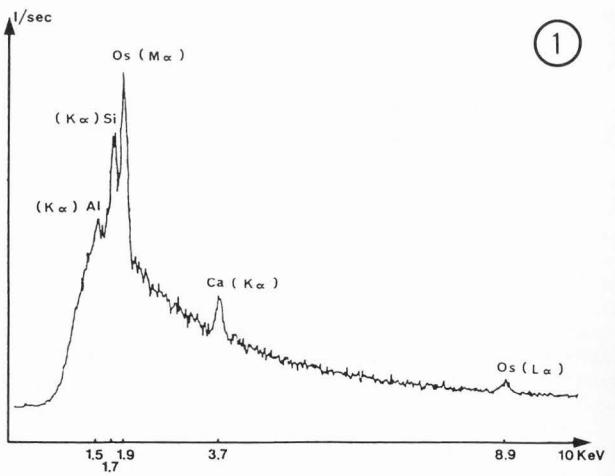


Fig. 1. Energy dispersive spectrum of a droplet of osmium tetroxide solution prepared and stored in an ordinary glass vial, then dried on a piece of graphite. A similar solution prepared in plastic would not show a Ca nor Si peak.

Although the best use of X-ray microanalysis is to study physiological elemental distributions, which are not preserved by chemical fixation, it may sometimes be relevant to quantitatively measure by this technique the concentrations of Ca in osmium-treated specimens. One may want to compare two series of specimens after aqueous chemical fixation (*e.g.*, Maunder et al., 1977 in Hall, 1979a), or two areas of diffuse precipitates if a precipitating cytochemical method is used, or even to measure the total Ca content after freezing and freeze-substitution in the presence of Os. It is often recommended to avoid Os if one intends to perform quantitative microanalysis (*e.g.*, Quintana, 1980) but in theory the microanalytical bias introduced by Os can be corrected (Hall, 1979b). In that case the main inconvenience is a lesser sensitivity due to an increased background; the continuum theory can be applied, provided that the mean atomic number of the specimen is less than 21. The mean atomic number of glutaraldehyde-fixed and OsO_4 post-fixed cardiac tissue has been estimated after destructive chemical analysis and is close to 10 (Blaineau et al., 1989). Quantitative analysis is thus feasible, provided that the local mass fraction of Os is not too high; our experience of osmium-treated tissue indicates that the Os signal is rather constant (*e.g.*, Hernandez-Nicaise et al., 1982) but may increase more than four times its minimal value on the same type of organelle (Nicaise and Bilbaut, 1975). It was found that the tissue Ca content can increase as a consequence of Os treatment, as in the sarcoplasmic bodies in distal myopathy (Edström and Wroblewski, 1981) or most organelles in heart muscle (Blaineau et al., 1989) ; if heart muscle has been submitted to OsO_4 post-fixation it can contain 10 times more Ca than if the tissue had simply been fixed by glutaraldehyde, without deliberate calcium addition to the fixatives (Blaineau et al., 1989). This would be due to an accidental Ca enrichment of OsO_4 aqueous solutions stored in glass (Fig.1); it shows that there is a widespread transfer of Ca from the OsO_4 Ca-enriched solution to the tissue, without any particular Ca-binding site visually detected (Blaineau et al., 1989).

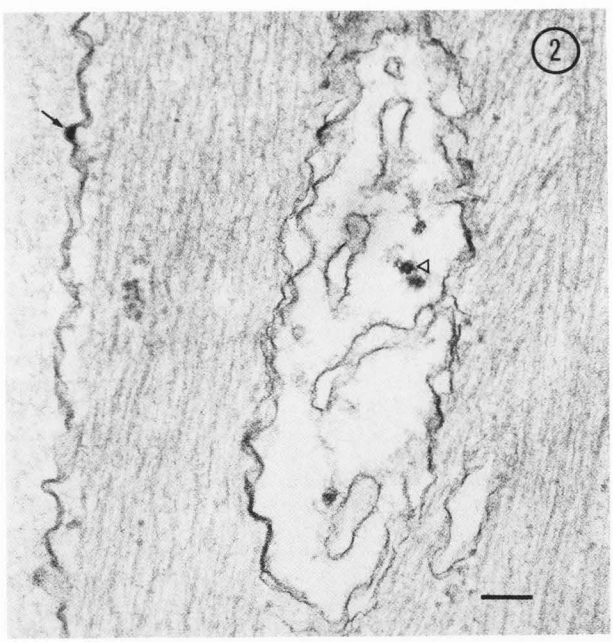


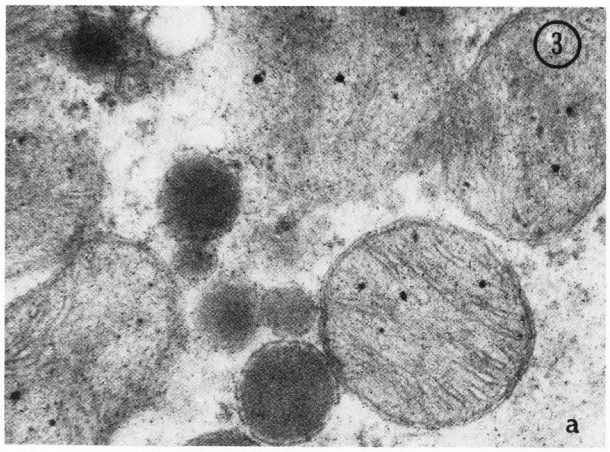
Fig. 2. Periphery of a giant smooth muscle fiber from the planctonic invertebrate *Beroe*. A piece of body wall has been fixed in Ca-containing glutaraldehyde. A Ca-binding site can be seen under the plasma membrane (arrow). The matrix intramitochondrial granules (triangle) present an increased electron opacity which is also due to the presence of Ca in the fixative. Bar = 0.1 μm.

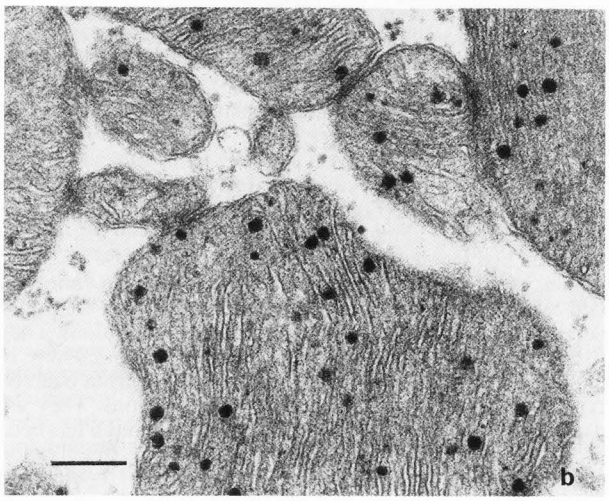
Calcium localization in chemically fixed tissues

Calcium-enriched fixatives. In the electron microscope, bone is black or, more precisely, electron opaque: beyond a certain mass per unit volume, calcium can be seen directly. This statement has however no general value : the possibility of identifying a given structure is primarily due to the difference in contrast between this structure and the surrounding matrix; it is likely that the relative electron opacity of a calcium-containing organelle will be higher in a frozen-dried than in an Epon-embedded or even Lowicryl-embedded section. However a Ca^{2+} load, prior to fixation or in the fixative, will often reveal electron-dense sites of Ca accumulation in epoxy embedded tissues. There is no necessary relationship between normal calcium metabolism and such accumulations but they may reveal otherwise unsuspected mechanisms or structures. In most cases, the authors use osmium tetroxide fixation or post-fixation, and the observations of completely unstained preparations of epoxy embedded tissues are rare.

Although it is not proved that they cannot occur *in vivo*, the **calcium binding sites of the inner side of the plasma membrane** (Fig. 2) are usually demonstrated after calcium-enriched aldehyde fixation (Sampson et al., 1970a; Clawson and Good, 1971; Oschman and Wall, 1972). These binding sites or micropapillae are found in a wide variety of cell types but their occurrence or abundance is highly dependent on physiological or experimental conditions. They form bumps on the plasma membrane, often 50 nm in diameter, but their size may vary considerably from one cell type to the next, or with experimental conditions. They are usually filled with an electron dense deposit, which has been presumed to contain Ca as it is not seen after Ca-free fixation and disappears after EDTA or EGTA treatment (Clawson and Good, 1971; Oschman and Wall, 1972; Tsuchiya, 1976; Geyer et al., 1978; De Araujo Jorge et al., 1979). The deposits have been proved to contain Ca by X-ray microanalysis (Oschman et al., 1974; Hillman and Llinas, 1974; Plattner and Fuchs, 1975; Fisher et al., 1976; Tsuchiya, 1976; Przelecka and Sobota, 1976; Goffinet, 1978; Sobota et al., 1978; De Araujo Jorge et al., 1979; Ripps et al., 1979; Stockem and Klein, 1979; Sobota and Przelecka, 1981; De Chastellier and Ryter, 1981; Nicaise et al., 1982; Dougherty, 1983). Oschman and Wall (1972) and subsequent authors (*e.g.*, De Chastellier and Ryter, 1981, but not Plattner, 1975) reported that osmium tetroxide post-fixation dissolves the electron-dense deposits unless Ca is present. Calcium chloride is generally added to the aldehyde primary fixative at the concentration of 5 mM, but this concentration can be lowered considerably. It is possible that in some experiments the calcium deposits are formed or preserved by calcium ions present in the fixative or the buffer as impurities (Oschman and Wall, 1972). They have not been reported to occur in cells which have been frozen without any preliminary fixation (*e.g.*, Skaer et al., 1974). There is at least one demonstration that these structures can be shown after Ca^{2+} loading without adding Ca^{2+} to the fixative, with oxalate-containing OsO_4 (Plattner, 1975).

The addition of Ca^{2+} to the fixative also reveals at least two other categories of calcium binding sites, in the mitochondrial matrix of various cells and in the synaptic vesicles of nerve terminals.





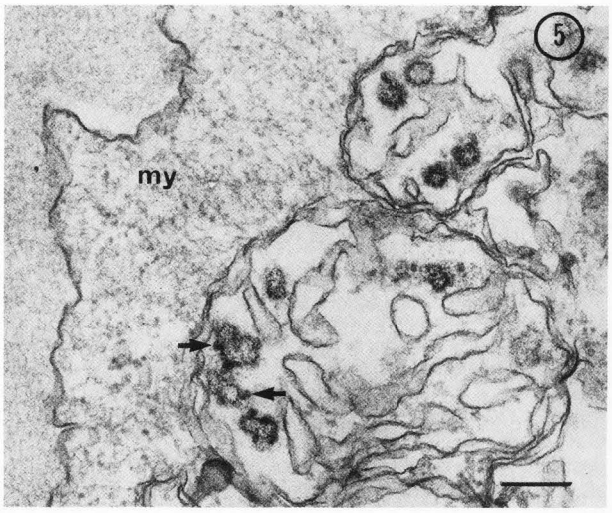
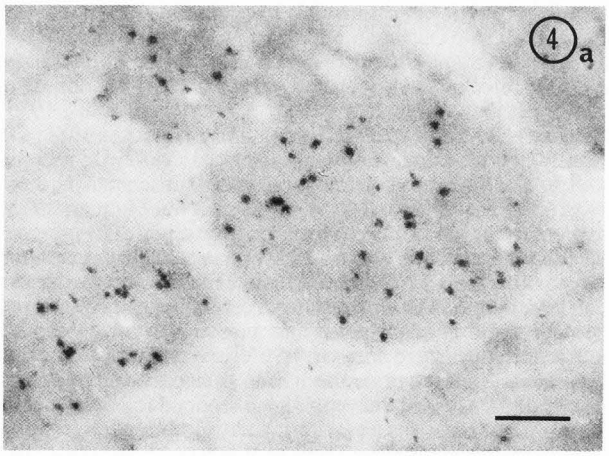


Fig. 3. Matrix intramitochondrial granules (MIGs) in muscle cells of the mouse auricle fixed with phosphate-buffered glutaraldehyde (post-fixed with OsO_4 but otherwise unstained). Bar = 0.2 µm.

3a. Control.

3b. Mitochondria from a piece of tissue which has been incubated in Ca^{2+}-rich (10 mM Ca^{2+}) physiological solution : the matrix granules are more numerous and larger.



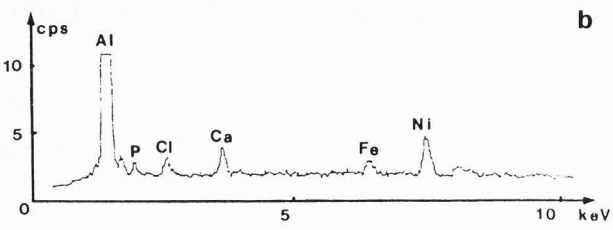


Fig. 4. X-ray microanalysis of frozen and freeze-substituted mouse auricle which has been incubated in Ca^{2+}-rich (10 mM Ca^{2+}) physiological solution.

4a. Electron micrograph of mitochondria containing electron-dense deposits of the same size as MIGs (unstained). Bar = 0.2 µm .

4b. Energy dispersive spectrum of these deposits showing a conspicuous Ca peak (the section is collected on an Al grid, the Fe and Ni signals come from the column); this peak is not seen in areas devoid of electron-dense deposits (not shown).

Fig. 5. Periphery of a giant smooth muscle fiber from the planctonic invertebrate *Beroe*. The animal has been fixed in Ca-containing glutaraldehyde after a 70 min incubation in artificial sea-water. The mitochondrial matrix contains hollow spherules ; electron-dense spots of the size of MIGs (arrows) can be seen in the periphery of the spherules. my : myofilaments. Bar = 0.2 µm.

The **matrix intramitochondrial granules** (MIGs) have been studied by numerous authors, but the question of the relationship between these granules and larger mineral deposits of calcium particularly observed in cases of heavy Ca loading is still open (see next paragraph). MIGs are osmiophilic spherical inclusions of variable size, typically 25 to 35 nm in diameter, observed in the mitochondrial matrix of most cell types, sometimes associated to the mitochondrial inner membrane. Like the calcium binding sites of the plasma membrane, MIGs are very susceptible to changes in physiological conditions. For example their number can increase in some cells in case of moderate Ca^{2+} loading prior to fixation (Blaineau and Nicaise, 1976; Fig. 3). There is good evidence that they specifically contain phospholipids (Wendel and Barnard, 1974; Barnard and Ruusa, 1979) responsible for their osmiophilia, and cytochrome C oxidase (Hertsens et al., 1986). After fixation in presence of Ca^{2+}, they have been shown to contain Ca, as suggested by microincineration (*e.g.*, Sampson et al., 1970a), or more directly by X-ray microanalysis (Goffinet, 1978; Barnard and Ruusa, 1979;

Nicaise and Hernandez-Nicaise, 1980; Fig. 2). In mouse atrial tissue which has been cryofixed and freeze-substituted without Os, the mitochondria of certain muscle cells contain small electron-dense calcium deposits, as shown by X-ray microanalysis, which have the same diameter (30 nm) as the MIGs seen after chemical fixation (Blaineau, 1987; Fig. 4).

In the same mitochondria which normally presented MIGs, a heavier Ca loading (prior to fixation) will sometimes induce the disappearance of MIGs and the formation of **large calcium precipitates**, under the form of spicules or hollow spherules (60 nm in diameter or more; Fig. 5), as first demonstrated by Peachey (1964). The Ca content of these large mitochondrial deposits is often deduced from indirect evidence, but was also directly tested by X-ray microanalysis (in chemically fixed tissues : Sutfin et al., 1971; Buja et al., 1976; Parducz and Joo, 1976; Barnard and Ruusa, 1979; Hagler et al., 1979; Nicaise and Hernandez-Nicaise, 1980; Karcsu et al., 1983; in frozen tissues : Somlyo et al., 1975; Saetersdal et al., 1977; Mergner et al., 1977; Ali et al., 1978; Seveus et al., 1978; Landis and Glimcher, 1978; Somlyo et al., 1979; Hagler et al., 1979; James-Kracke et al., 1980; Wendt-Gallitelli and Wolburg, 1984; Meyran et al., 1986). Similar large calcium-rich mitochondrial inclusions are found without any particular loading in bone tissues (see ref. in Sayegh and Abousy, 1977), even after cryofixation and cryomicrotomy (Ali and Wisby, 1975; Landis et al., 1977; Ali et al., 1978) or in various calcifying tissues of invertebrates, particularly at certains stages (Mizuhira and Ueno, 1983; Meyran et al., 1986). As they are more easily seen in frozen tissues, Christensen (1971) suggested that they could be artifactually formed by segregation of ions in the process of freezing (see also Mergner et al., 1977; Seveus et al., 1978). However, in tissues which are not involved in calcification, they seem to be only found in cells which suffered or which were calcium-loaded prior to fixation (see ref. in Somlyo et al., 1979; Somlyo, 1985); it is most likely that the numerous Ca-containing mitochondrial inclusions seen in some frozen blocks result from cell damage. In case of aqueous chemical fixation, the presence of 5mM Ca in the fixative (at least in osmium tetroxide) is needed to preserve these large electron opaque deposits in liver (Reynolds, 1965); this observation was confirmed by Yates and Yates (1968) in nerve cells (see also Normann and Hall, 1978) and Martin and Matthews (1969) in chondrocytes; unfortunately this precaution was often ignored in more recent studies (*e.g.*, Blaineau and Nicaise, 1976). However, Hirokawa and Heuser (1981) mention that after chemical fixation in the presence of 50mM $CaCl_2$ the preservation of large electron-dense mitochondrial granules is much poorer than after cryofixation and cryosubstitution (in the presence of OsO_4 and oxalic acid). Workers interested in these inclusions and wanting to avoid cryotechniques may try non-aqueous room temperature fixation (Landis and Glimcher, 1978; Manston and Katchburian, 1984). A convenient distinction between MIGs and large spherules of Ca may be made, but intermediary forms also occur (*e.g.*, Nicaise and Hernandez-Nicaise, 1980), and the relationship between these two categories of mitochondrial inclusions remains unclear (Fig. 5).

Calcium binding sites have also been demonstrated in **synaptic vesicles** after the use of Ca^{2+} containing fixatives (Sampson et al., 1970b; Heuser et al., 1971; Boyne et al., 1974; Politoff et al., 1974; Pappas and Rose, 1976; Benshalom and Flock, 1977; Gautron, 1978; Goffinet, 1978; De Araujo Jorge et al., 1979; Ripps et al., 1979; Pellegrino de Iraldi and Corazza, 1981). The presence of Ca in these electron-dense deposits has been tested by X-ray microanalysis but the authors had difficulties, linked to the small size of the deposits (Goffinet, 1978; Ripps et al., 1979). Biochemical data suggest that there is a physiological Ca store in at least a subpopulation of synaptic vesicles (see Israel et al., 1980, other ref. in Phillips and Boyne, 1984; Oorschot and Jones, 1987). After rapid freezing and freeze-substitution with tetrahydrofuran (but not with acetone), Ca could be detected in synaptic vesicles by X-ray microanalysis (Ornberg, in Phillips and Boyne, 1984). However the synaptic vesicles examined in freeze-dried cryosections of the cerebellar layer have a rather low Ca content, of the order of 1 mmol/l (Andrews et al., 1987).

Other forms of electron-dense calcium-containing intracisternal inclusions or **granules** can be demonstrated without Ca addition to the fixatives, although such an addition increases their electron opacity, in blood platelets (Skaer et al., 1974; 1976; Martin et al., 1974) and glial cells (Gambetti et al., 1975).

In *Aplysia* ganglia the cytosomes (lysosome-like bodies or pigmented granules) sometimes contain local electron-dense accumulations of Ca in fresh frozen and freeze-substituted unstained specimens (unpublished). Again a Ca loading during or before fixation increases the organelle Ca content (Henkart, 1975; Brown et al., 1975).

Calcium substitutes of higher atomic number. Instead of Ca, it is possible to load living cells with other divalent cations from the group II of the periodic table; **strontium** (Sr) and **barium** (Ba) have the advantage of a higher atomic number (particularly Ba) and may help to trace the pathway of exchangeable and freshly incorporated Ca without using radioisotopes (*e.g.*, Krefting et al., 1988). Sr and/or Ba will increase the contrast of Ca-binding sites at the inner side of the plasma membrane (Oschman and Wall, 1972; Fisher et al., 1976; Arjamaa, 1982), in *Aplysia* cytosomes (Henkart, 1975), in mammalian atrial granules (Blaineau and Nicaise, 1976; Fig. 6) or in snail neurosecretory granules (Joo et al., 1977). These elements, like Ca, will form precipitates in the mitochondrial matrix either in small dots of the same size as MIGs (Fig. 7) or more often under the form of larger and sometimes hollow spherules (Peachey, 1964; Somlyo and Somlyo, 1971; Somlyo et al., 1974; Hernandez-Nicaise and Amsellem, 1980; Nicaise and Hernandez-Nicaise, 1980; Fig. 8).

If electron dense sites appear after the use of these cations, they may be due to Sr or Ba accumulation, but also to Ca displaced from another location, or to Os, if osmiophilic sites have been exposed. A control by X-ray microanalysis is therefore highly recommended. An inverse staining can also indicate a local accumulation of Sr or Ba : in Sr-loaded aldehyde-fixed and osmium-treated (otherwise unstained) mouse auricle the relative electron opacity of atrial granules is weak, but if OsO_4 post-fixation is omitted, the granules appear denser than in Ca loaded tissue (Blaineau and Nicaise, 1976). In Ba-injected snail neurones, the heterochromatin is clearer than the surrounding euchromatin in conventionally stained sections, but much denser than usual if heavy metal staining is omitted (Nicaise and Meech, unpublished; Fig. 9). This indicates that in these cases Sr and Ba were masking sites that would have been normally occupied by the heavy metals used in classical electron microscopic processing.

These cations do not necessarily behave like Ca^{2+}, for example Sr^{2+} and Ba^{2+} do not permeate all calcium channels, and they may even work as antagonists rather than substitutes; Sr may also have a different effect from Ba and not necessarily closer to that of Ca. For example isolated kidney mitochondria present minimal damage after Sr loading, more swelling after Ba, and still more important after Ca accumulation (Peachey, 1964). In smooth muscle, Sr is preferentially accumulated by the sarcoplasmic reticulum and Ba by the mitochondria (Somlyo et al., 1974; Fig. 8). Therefore the interpretation of the results obtained with these substitutes is difficult.

Anderson and Josephson (1983) demonstrated electron

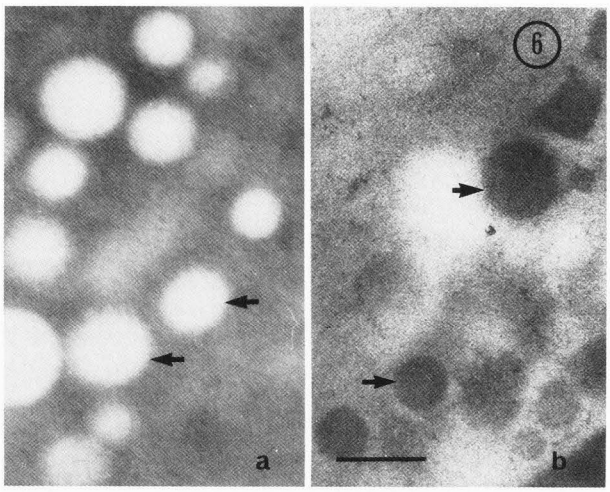


Fig. 6. Sections of unstained mouse auricles which have been fixed in glutaraldehyde, dehydrated and embedded in Epon, then observed in regular transmission electron microscopy without any other treatment (after Blaineau and Nicaise, 1976).

6a. Control; the atrial granules (arrows) are distinctly clearer than the surrounding cytoplasm .

6b. Tissue incubated in Sr-rich (10 mM) physiological saline ; the atrial granules (arrows) are sometimes more electron-opaque than the surrounding cytoplasm; a similar incubation in Ca-rich saline would induce a much poorer increase in electron opacity (not shown).
Bar = 0.2 µm.

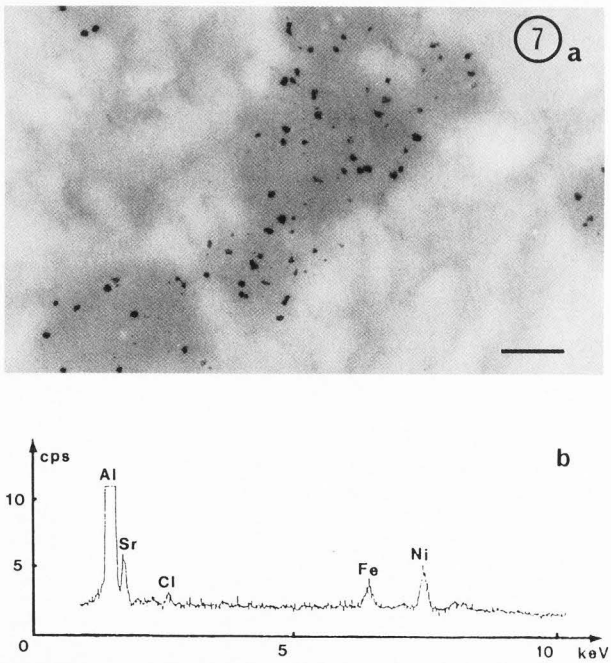


Fig. 7. X-ray microanalysis of frozen and freeze-substituted mouse auricle which has been incubated in Sr^{2+}-rich (10 mM Sr^{2+}) physiological solution.

7a. Electron micrograph of mitochondria containing electron-dense deposits of the same size as MIGs (unstained). Bar = 0.2 µm .

7b. Energy dispersive spectrum of these deposits showing a conspicuous Sr peak (the section is collected on an Al grid, the Fe and Ni signals come from the column); this peak is not seen in areas devoid of electron-dense deposits (not shown).

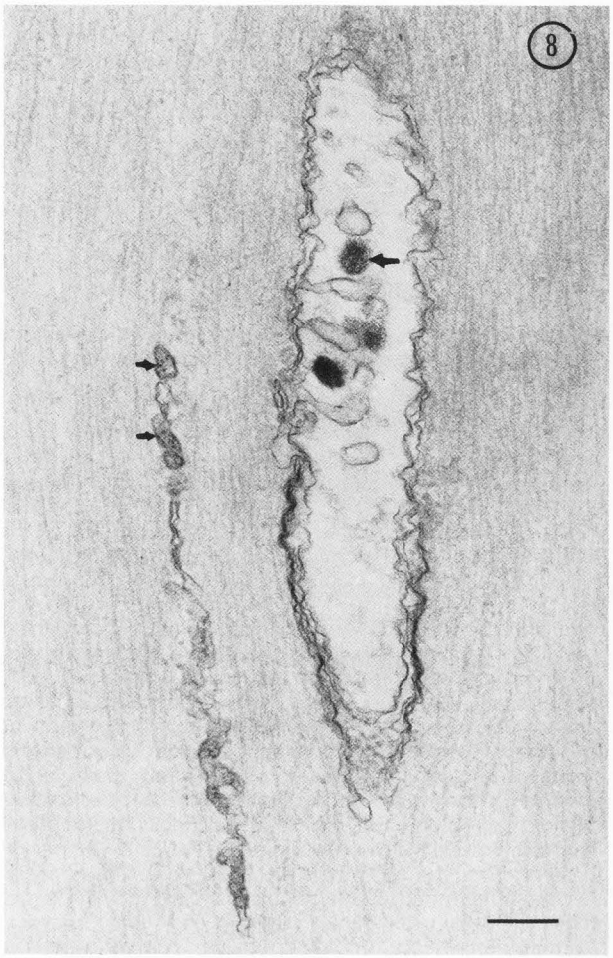
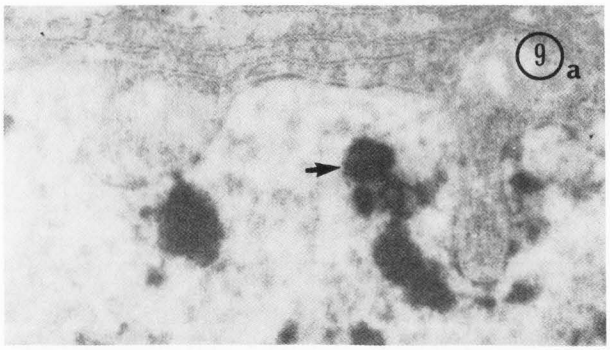
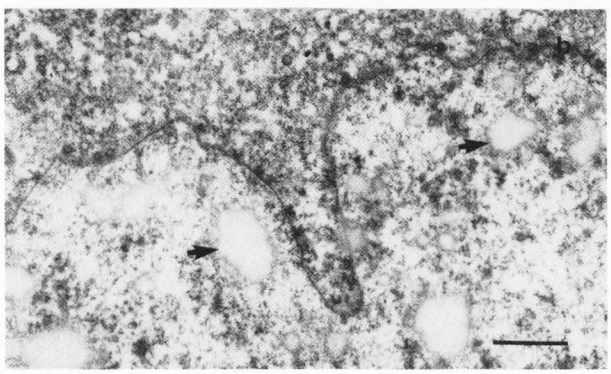


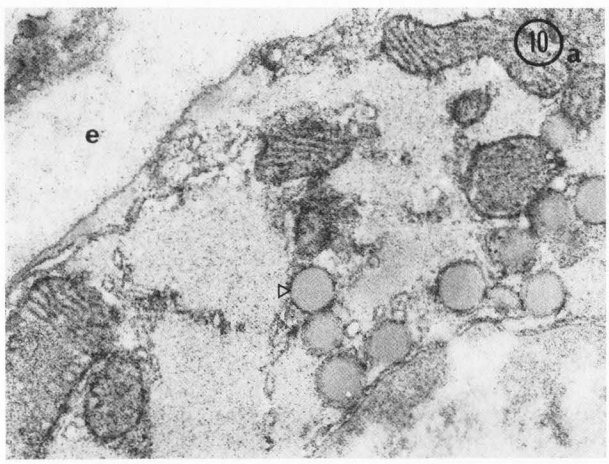
Fig. 8. Periphery of a giant smooth muscle fiber from the planctonic invertebrate *Beroe*. The animal has been fixed in glutaraldehyde and conventionally processed after one hour of incubation in K^+-rich (100 mM), Sr^{2+}-rich (70 mM) artificial sea-water. Electron opaque deposits can be seen in the mitochondrial matrix (thick arrow) and in the smooth endoplasmic reticulum (thin arrows). Bar = 0.2 µm.

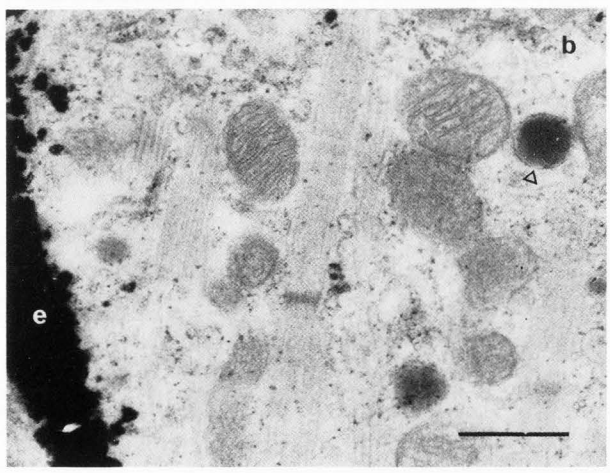
opaque deposits in the sarcoplasmic reticulum of frog striated muscle after incubation of live tissue in saline containing 50mM **cobalt** and subsequent silver intensification. As Co^{2+} ions are potent blockers of the calcium channels, it is not clear how the staining was achieved.

Calcium substitutes of higher atomic number can also be used *after* fixation, as in the **lead** method, first used in electron microscopy by Carasso and Favard (1966). The rationale of the technique is that lead will substitute for Ca in phosphates and carbonates because it forms more insoluble salts with these anions. A control can be made by rinsing the tissue in citrate buffer (Carasso and Favard, 1966; Meyran et al., 1984; Fig. 10) or EGTA (Heumann, 1976; Rubanyi et al., 1980) before incubation in lead acetate. The calcium phosphates may be formed in the tissue by the action of phosphate buffer in the fixative. A lead nitrate version of this method, giving finer deposits, has been recommended by Berridge et al. (1975), but their interpretation of a Ca store in the mitochondria of fly salivary glands has not been confirmed by later work (Gupta and Hall, 1978). It has been demonstrated for both lead techniques that Ca can also be









present in the electron opaque deposits (Berridge et al., 1975, Magloire and Nicaise, 1977, Magloire and Joffre, 1979; Meyran et al., 1984), which means that the mechanism of staining is probably more complex than a simple substitution. As for the pyroantimonate method (next paragraph), the biological meaning of the results depends on the comparison between different physiological states; if a difference in location or intensity of the staining is observed, it is suggestive of a Ca movement and represents a good start for further investigations with other techniques (*e.g.*, Blaineau et al., 1980; Nicaise and Amsellem, 1983; Meyran et al., 1984, 1986).

Cobalt nitrate has also been used, with the formation of finer deposits than lead acetate (see Fauré-Frémiet et al., 1968; Meyran et al., 1984).

Calcium precipitation. One obvious cytochemical approach to Ca localization is to take advantage of the fact that several Ca salts are insoluble in water (Figs. 11-16). Only free Ca^{2+} ions will be precipitated, either physiologically free at the time of fixation or freed by the treatment.

The use of **oxalate** on sarcoplasmic reticulum isolated vesicles (Hasselbach, 1964) and on peeled muscle (Costantin et al., 1965; Pease et al., 1965) has led to a series of papers establishing the role of the smooth endoplasmic reticulum in intracellular Ca^{2+} regulation. The oxalate anion penetrates the reticulum membrane and in the presence of ATP, the Ca transport will be demonstrated by the formation of insoluble Ca oxalate crystals inside the reticulum cisternae. Special care such as deposition of a carbon film or sectioning thicker sections (to keep the oxalate crystals embedded) must be taken to avoid losing the precipitates in the boat of the knife or later under the electron beam. The presence of Ca in the intracisternal precipitates has often been verified by X-ray microanalysis (Podolsky et al., 1970; Diculescu et al., 1971; Braatz and Komnick, 1973; Popescu and Diculescu, 1975; Heumann, 1976; Flood, 1977; Dux et al., 1978; Henkart et al., 1978; McGraw et al., 1980) and sometimes by electron diffraction (Diculescu et al., 1971). One of the difficulties of the technique for studies of organelles *in situ* is that oxalate does not permeate plasma membranes; this difficulty has been solved by mechanical peeling (Costantin et al., 1965), tissue mincing (Flood, 1977), intracellular injection (Henkart et al., 1978), plasma membrane depolarisation by hyperpotassic

Fig. 9. Sections of glutaraldehyde-fixed and directly dehydrated and embedded snail neurones which have been loaded with Ba^{2+} by incubation in a sodium-free saline where the Ca^{2+} ions were replaced by Ba^{2+}. Bar = 0.5 µm.

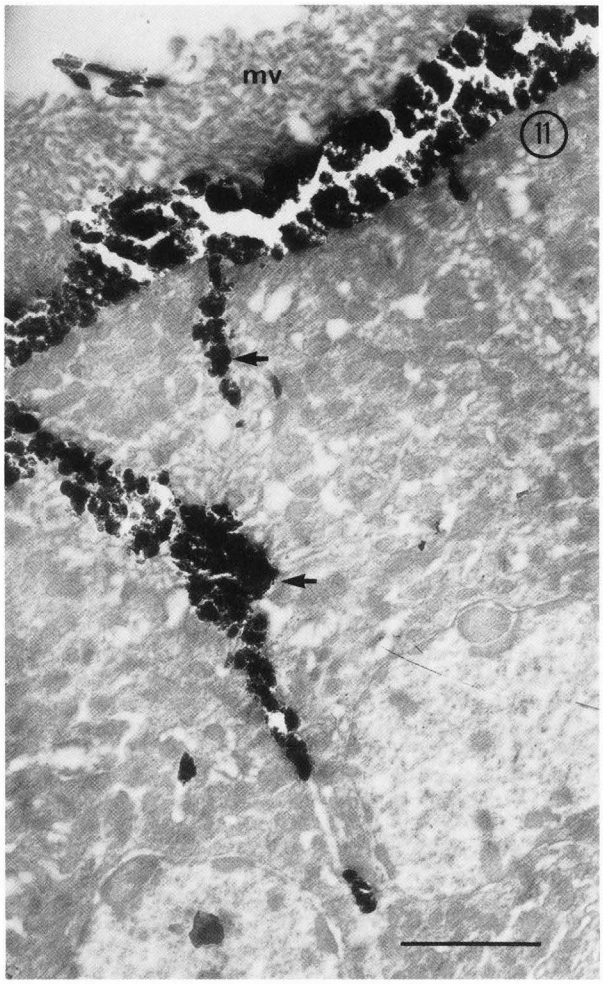
9a. In an unstained section the heterochromatin (arrow) is denser than the surrounding cell components.

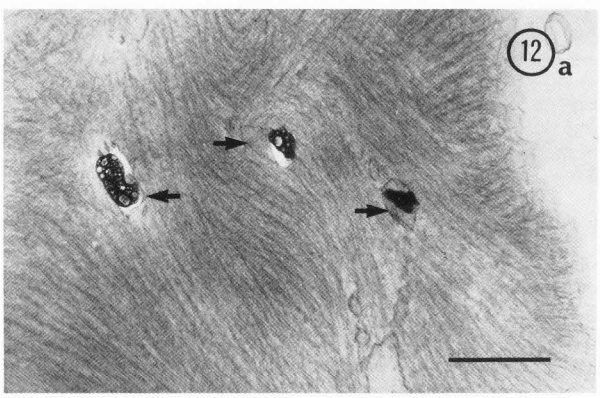
9b.After conventional staining, the contrast is inverted, suggesting that Ba already occupies the electronegative sites which would otherwise have been stained by uranyl and lead (arrow : heterochromatin).

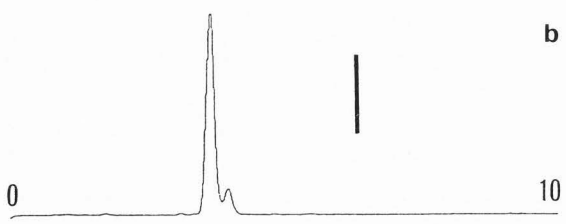
Fig. 10. Sections of mouse auricle treated by the lead method of Carasso and Favard (1966). After phosphate-buffered glutaraldehyde fixation and before OsO_4 post-fixation, the pieces of tissue are soaked in lead acetate, which is presumed to replace Ca in phosphates or carbonates. Conventional staining is omitted. Bar = 0.5 µm. (from Baux, 1973).

10a. The precipitate is observed on most intracellular membranes; the core of the atrial granules (triangle), normally rich in calcium, is unstained. The extracellular spaces (e) are not particularly rich in precipitate.

10b. Control which has been soaked in citrate buffer (after glutaraldehyde fixation and prior to incubation in the lead acetate solution), to dissolve calcium; the atrial granules (triangle) are electron-dense, possibly because osmiophilic sites have been unmasked by the citrate. Most of the precipitate is located in the extracellular spaces (e).







saline (Diculescu et al., 1971; Popescu and Diculescu, 1975), simultaneous use of OsO_4 and oxalate (Braatz and Komnick, 1973; Dux et al., 1978), and glycerin (Zebe and Hasselbach, 1966; Meyer et al., 1982), EDTA washing (Bayerdörffer at al., 1984) or saponin (McGraw et al., 1980) permeabilisation. The precipitates are not often seen in extracellular spaces; in the case of the extracellular Ca-transport pathway illustrated in Fig. 11, the spaces are narrow and the diffusion may be restricted. All the treaments based on membrane damage will cause an influx of extracellular Ca^{2+}, unless its concentration is similar to that of the cytosol. Some authors used the technique and concluded that the reticulum (or other organelles like mitochondria) actually contained Ca and therefore were potential regulators of intracellular Ca^{2+}. It is more appropriate to demonstrate that these organelles are able to accumulate Ca^{2+} at very low (cytosolic) concentrations, preferably under the control of a Ca buffer (Sorenson et al., 1980; McGraw et al., 1980; Walz, 1979, 1982; Hartter et al., 1987; Fig. 12). It is pertinent to the distinction between the respective roles of mitochondria and reticulum in Ca^{2+} regulation that oxalate seems to permeate both the reticulum and the mitochondrial membranes (see Heumann, 1976; McGraw et al., 1980); if at a cytosolic concentration of Ca^{2+} the Ca oxalate crystals are only seen in the reticulum, it can be concluded that the reticulum is the main organelle regulating cytosolic concentration in the examined cell.

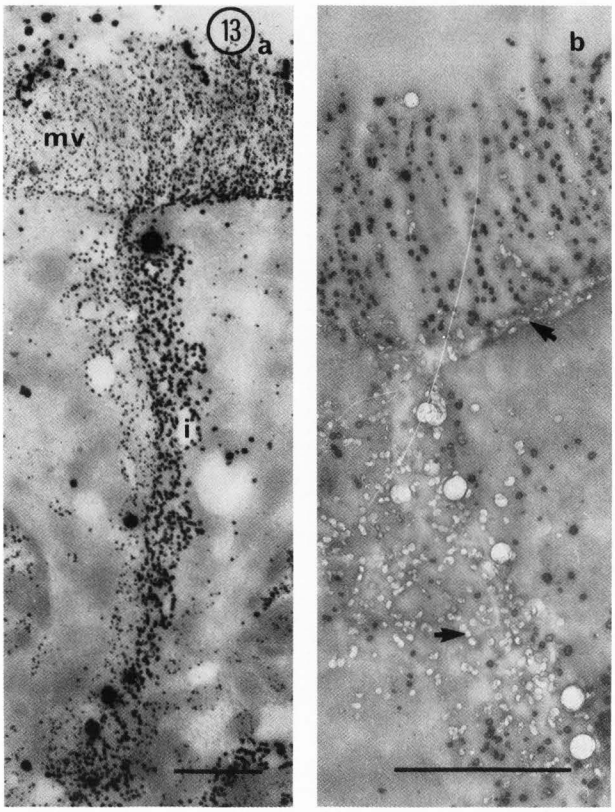
The studies on isolated sarcoplasmic reticulum vesicles show that **phosphate** can be used in place of oxalate for the formation of insoluble intravesicular precipitates, and that calcium phosphate deposits are more resistant to the electron beam (De Meis et al., 1974). A $CaCl_2$-containing gelatin gel fixed with OsO_4 retains all its Ca if the fixation is done in presence of pyrophosphate, but only 50 % in the presence of pyro-antimonate (Spicer and Swanson, 1972). Phosphate-buffered OsO_4 fixation is reported by Hagler et al. (1981) as "the only technique of aqueous fixation which resulted in ultrastructurally demonstrable early granular inclusions (in mitochondrial matrix), with small calcium peaks". However, significant losses of tissular Ca were reported after aldehyde fixation in phosphate buffer (Morgan, 1979), perhaps because of the relatively low pH used by the authors. Ca phosphate precipitates have a relatively low electron opacity, and Probst (1986) used potassium bichromate in the OsO_4 post-fixation to enhance the contrast of the Ca deposits after glutaraldehyde-phosphate fixation; other authors used pyroantimonate (next paragraph).

Fig. 11. Section of the calcium-transporting epithelium of the crustacean *Orchestia* posterior ceca fixed in cacodylate-buffered glutaraldehyde containing 50 mmol/l of oxalic acid. The calcium oxalate precipitates, which tend to bubble under the beam, are only seen in the intercellular spaces (arrows) and at the base of the microvilli (mv). (from Meyran et al., 1984). Bar = 0.5 μm.

Fig. 12. Evaluation of the Ca-pumping ability of the sarcoplasmic reticulum in a giant smooth muscle fiber of *Beroe* which has been saponin skinned and incubated before fixation in an "intracellular" medium containing ATP (5 mM), oxalate (10 mM) Mg^{2+}(5 mM) and Ca^{2+} (0.5 μM). The concentration of free Ca^{2+} was controlled with a Ca-EGTA buffer and calculated with a program written by Dr Tourneur (Univ. Lyon 1), using the constants of Fabiato and Fabiato (1979).

12a. Electron micrograph showing Ca oxalate crystals in the smooth endoplasmic reticulum cisternae (arrows). Bar = 0.5 μm.

12b. Energy dispersive spectrum from 0 to 10 keV of an intracisternal opaque deposit, after computer processing, showing only the Kα and Kβ lines of Ca. The spectrum of a nearby precipitate-free cytoplasmic area has been subtracted from that of the precipitate-containing sarcoplasmic reticulum area. Bar = 10 counts per second.



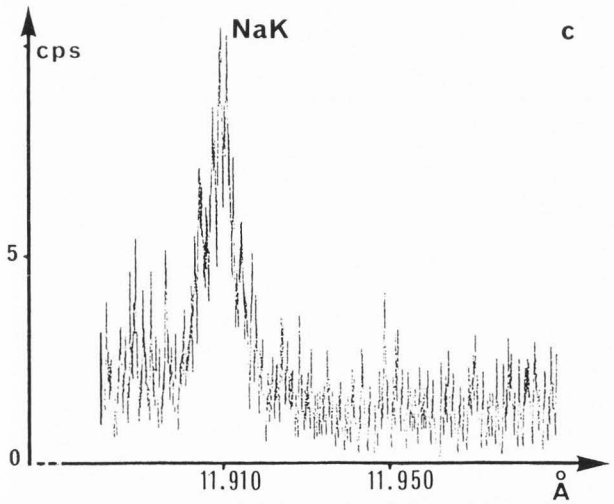


Fig. 13. Section of the calcium-transporting epithelium of the crustacean *Orchestia* posterior ceca treated with pyroantimonate (adapted from Meyran et al., 1984). Bars = 2 µm .

13a. The precipitates are particularly abundant in the lateral intercellular spaces (i) and the microvilli (mv).

13b. Section treated with EGTA to extract Ca pyroantimonate, the precipitates disappear in the lateral intercellular spaces and at the base of the microvilli (arrows); the holes left by the extracted precipitates have the same distribution as the calcium oxalate deposits shown in Fig.12.

13c. Wavelength-dispersive spectrum of the pyroantimonate precipitate remaining after EGTA treatment (as shown in 13b) showing the Na K line. On an EDS spectrum (unprocessed) this line would be masked by the Cu L line (the section is mounted on a Cu grid). The analysis also reveals that some Ca remains in the precipitates (not shown).

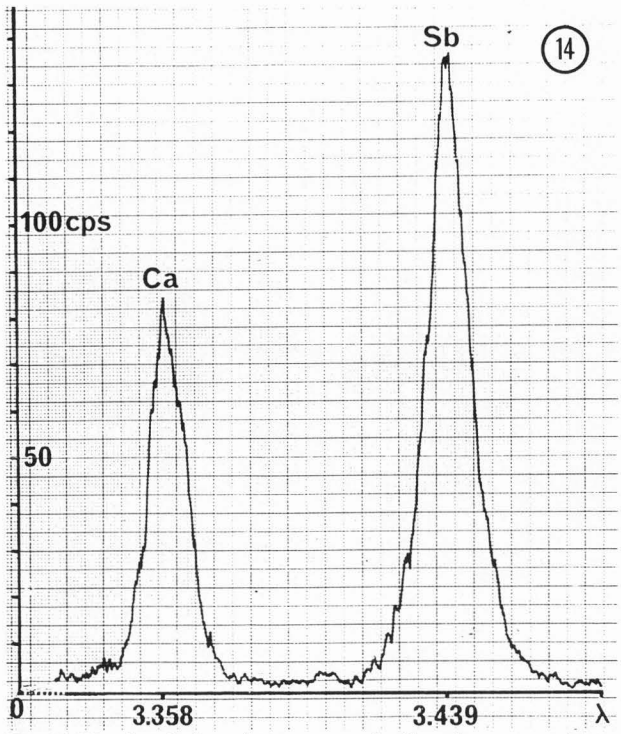


Fig. 14. Wavelength dispersive spectrum of a Ca-antimonate precipitate showing distinct CaKα and Sb Lα1 peaks. λ = wavelength in Angströms (from Meyran et al., 1984).

The **potassium pyroantimonate** method has originally been proposed by Komnick (1962) to precipitate sodium but clearly started its career as a Ca technique with the study of Legato and Langer (1969) on heart muscle. It is indeed more sensitive to Ca^{2+} (10^{-6}M) than to Na^{+} (10^{-2}M - Klein et al., 1972), but these are also near the usual cytosolic concentrations (Mentré and Halpern, 1988). EGTA has often been used to selectively dissolve the Ca pyroantimonate precipitates (*e.g.,* Saetersdal et al., 1974; Cramer et al., 1978; Davis et al., 1979; Appleton and Morris, 1979; Sampson et al., 1982; Fig. 13). The literature published with the pyroantimonate technique is very abundant and there are many methodological variants which are not equivalent (see Simson and Spicer, 1975; Wick and Hepler, 1982; Borgers et al., 1983, 1984; Mentré and Escaig, 1988; Mentré and Halpern, 1988). The main advantage of pyroantimonate over oxalate is the electron opacity of the precipitates which is certainly partly due to the relatively high atomic number of antimony. But this advantage is also an inconvenience as pyroantimonate tends to induce coarser precipitates by displacement of weakly bound Ca. These heavier precipitates, particularly seen when using OsO_4 as a primary fixative, have been attributed to extracellular calcium influx at the time of fixation (Yarom et al., 1974a, 1974b); however, it is not likely that the calcium contained in the extracellular fluids penetrates into the cells at the time of fixation, it would rather massively diffuse with a very sharp gradient through the fixative, when this fixative is devoid of calcium. A possible explanation of the increased Ca content observed by Yarom and co-workers is given by the observation of Blaineau et al. (1989) of an accidental Ca enrichment of cardiac tissue by OsO_4 solutions stored in glass. It has been shown by Garfield et al. (1972) on smooth muscle that the retention of calcium after fixation is decreased if pyroantimonate is used in the fixative, particularly in the case of OsO_4 primary fixation (see also Van Iren et al., 1979 on plant material).

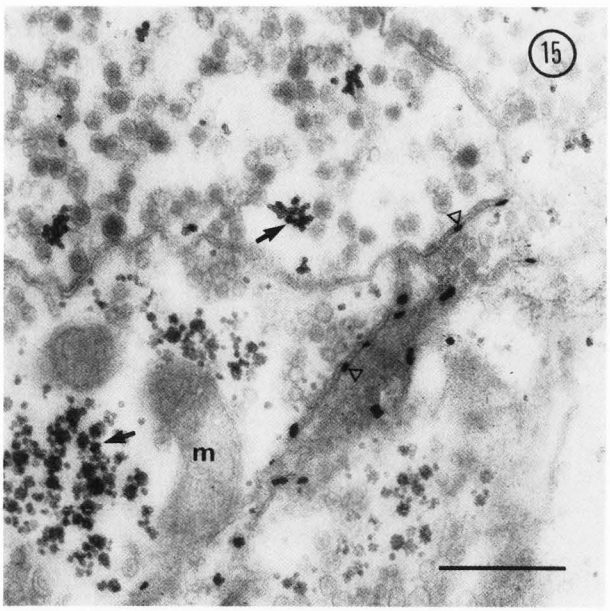


Fig. 15. Unstained section of the ganglionic neuropile of the marine slug *Glossodoris*. The ganglion was fixed in phosphate-buffered (pH 7.9) pyroantimonate-containing (4 %) glutaraldehyde and post-fixed in osmium tetroxide. The precipitates are mainly extracellular (triangles); inside the cells, they are mainly found on glycogen particles (arrows), which is probably indicative of a state of glycogenolysis at the time of fixation. They are not found in mitochondria (m), contrary to what would be seen with OsO_4-pyroantimonate as a primary fixative. Bar = 0.5 µm.

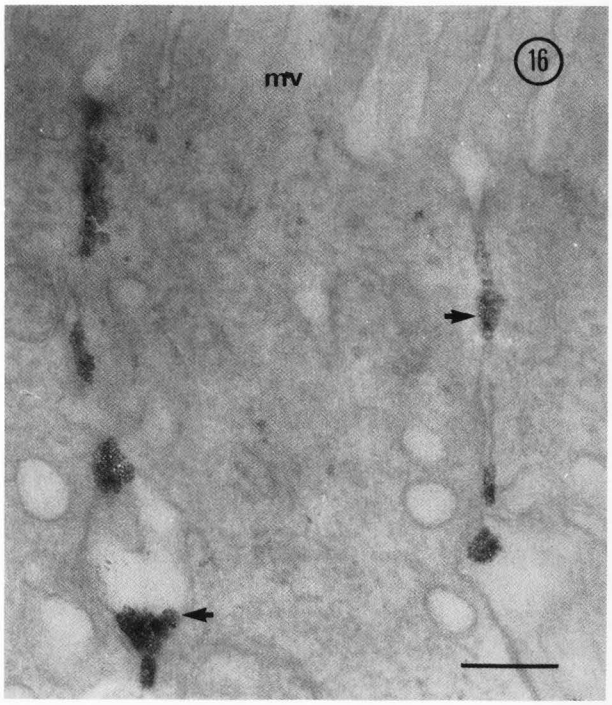


Fig. 16. Section of the calcium-transporting epithelium of the crustacean *Orchestia* posterior ceca treated with fluoride. The precipitates are localized in the intercellular spaces (arrows) where they are more reduced than those observed after oxalate, they are absent at the base of the microvilli (mv) (from Meyran et al., 1984). Bar = 0.2 µm.

X-ray microanalysis is particularly needed after pyroantimonate as it can precipitate with a variety of cations, and particularly with sodium (Lane and Martin, 1969); in his review of 1978, Chandler already listed 20 articles in which X-ray microanalysis had been used to verify the presence of Ca.

In wavelength-dispersive spectrometry it is easy to resolve the Ca Kα from the Sb Lα1 lines (Chandler, 1977; Fig. 14). In energy-dispersive spectrometry, computer processing of the spectrum (*e.g.*, Cramer et al., 1978; Van Iren et al., 1979; Jansen et al., 1982; Ueno and Mizuhira, 1983) is needed to separate the two peaks as they are only 86 eV apart and the best resolution of a diode is of the order of 140 eV.

The best specialists of subcellular Ca localization particularly recommend against the pyroantimonate method (Somlyo, 1985) and it has indeed been used in several biologically meaningless works; however it is possible to mention results which seem pertinent or were confirmed by later work with other methods.For example the distribution of Ca-pyroantimonate precipitates in muscle cells of *Mytilus* byssal retractor muscle in the resting, actively contracted, and catch states strongly suggest a translocation of Ca from peripheral stores to the myofilaments during active contraction, and a reaccumulation in the cell periphery during catch (Atsumi and Sugi, 1976). Similar findings were reported on guinea pig taenia coli (Sugi and Daimon, 1977); they are not contradicted by the better established cryotechniques which were used by the Somlyos group on other mammalian smooth muscles (see Bond et al., 1984). The effect of light on the pyroantimonate-precipitable sarcolemmal Ca stores in muscle cells of the hamster iris suggest that these cells have a photoreceptive function (Zucker and Nolte, 1978). Pyroantimonate stains glycogen particles during glycogenolysis but not during glycogenosynthesis, in agreement with the present biochemical knowledge of the role of calcium in glycogen metabolism (Mentré and Halpern, 1988; Fig. 15).

Several authors used **oxalate** or **phosphate** fixation **followed by pyroantimonate** : the first treatment is intended to immobilize Ca while rinsing Na and Mg and the second to intensify the Ca precipitate (see Wick and Hepler, 1982; Borgers et al., 1983, 1984); the two variants do not give the same results (Borgers et al., 1984). Significant data were obtained with this method on the microvesicles of neurosecretory terminals (Shaw and Morris, 1980), on the effect of experimental seizures in the hippocampus (Griffiths et al., 1983; Meldrum, 1986), on the protective effects of Ca-entry blockers in cerebral ischemia (Van Reempts et al., 1986), on the effect of tetanizing stimulation on pre- and post-synaptic organelles (Parducz et al., 1987), or on the irregular distribution of axoplasmic Ca in myelinated fibers (Mata et al., 1987).

Calcium **fluoride** is less soluble in water than Ca-pyroantimonate or Ca-oxalate, but few authors used fluoride in ultrastructural Ca cytochemistry (Neff, 1972, Meyran et al., 1984, 1986; Poenie and Epel, 1987). It seems however that this technique can be recommended, the precipitates are discrete and localized, as with oxalate (Fig. 16) but the fluoride anion presents the advantage to readily penetrate the plasma membrane. The presence of Ca in the precipitates has been verified by X-ray microanalysis (Meyran et al., 1984; Poenie and Epel, 1987). Their relatively low electron opacity can be enhanced by pyroantimonate (Poenie and Epel, 1987), as in the case of phosphate or oxalate (see above). Poenie and Epel (1987) report biologically relevant results obtained with fluoride, as the appearance of precipitate in sperm

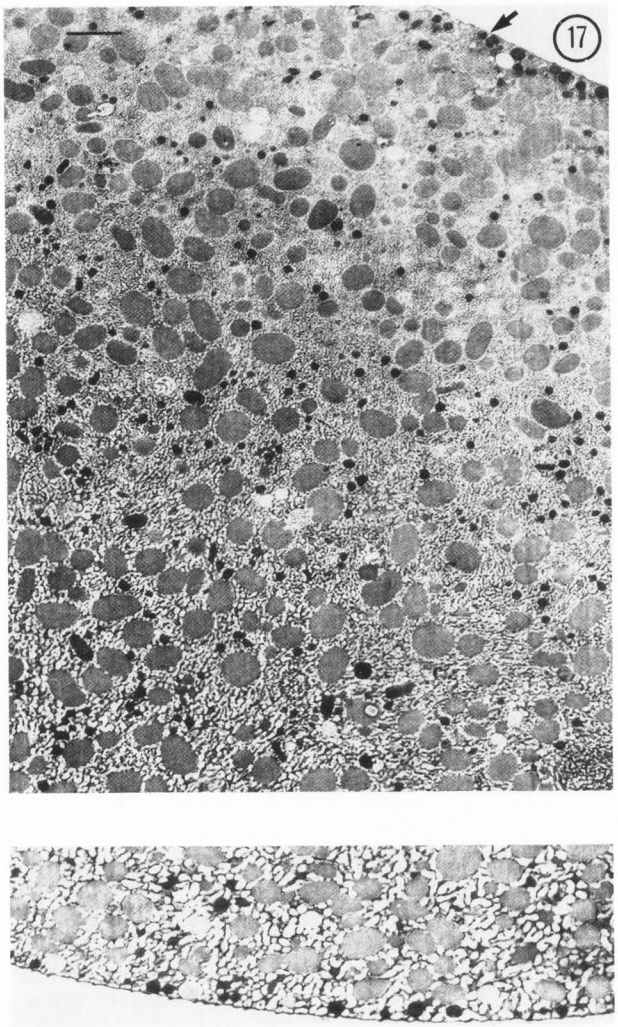


Fig. 17. Electron micrograph of a section of sea urchin egg rapidly frozen, freeze-substituted and embedded in Epon. The egg cortical zone stuck to the aluminum foil displays well preserved subcellular structures (arrow) and no ice crystals. The size of ice crystals increases with the distance from the aluminum foil. Note that the yolk granules, rich in lipids, do not show ice crystal formation, even in the deeper parts of the cell. The upper and lower part of the picture are printed from the same negative but are actually separated by a distance of 30 μm. Bar = 2.5 μm.

mitochondria at the time of the acrosomal reaction, or its temporary absence in egg reticulum during elevation of the fertilization envelope (the reticulum is presumed to be the source of Ca^{2+} during fertilization in the sea-urchin egg- Eisen and Reynolds, 1985).

Freeze-substitution

The techniques reviewed in the preceding paragraphs only reveal a fraction of the Ca content of a given subcellular compartment. However, electron probe X-ray microanalysis permits one to quantitatively measure the *total* physiological Ca content of cell organelles. The best method to measure this total Ca content is microanalysis of cryosections made from rapidly frozen tissues; this method has the advantage to allow investigation of other diffusible elements at the same time, and has given major results (for Ca, see ref. in Chandler, 1978; Gupta and Hall, 1978; Moreton, 1981; Somlyo and Somlyo, 1986). However, good frozen sections may be difficult to obtain and the image formation is often poor. Several authors, specifically interested in physiological Ca distribution, submitted the frozen tissues to freeze-substitution, often in the presence of a Ca-insolubilizing agent as first suggested by Spurr (1972). Freeze-substitution has also been used in combination with oxalate *in vivo* precipitation (Henkart et al., 1978) or with strontium and barium loading (Ornberg and Reese, 1980; Amsellem et al., 1988).

The technique. The **freezing** step is not peculiar to this method and can be achieved with any of the numerous techniques available (see Plattner and Bachmann, 1982; Robards and Sleytr, 1985; Menco, 1986; Gilkey and Staehelin, 1986); we sometimes used slamming on a helium-cooled copper block (on the Reichert intrument - see Escaig, 1982) and more often dipping by hand in a plumber's mixture of propane and butane (approximately 50/50), liquefied by a surrounding bath of liquid nitrogen. Pure propane would freeze at the temperature of liquid nitrogen ; various other additives were used to lower the melting point of propane (*e.g.*, Barlow and Sleigh, 1979). Propane is easy to liquefy in simple "home-made" systems, the risk of explosion can be limited by letting gaseous nitrogen flow over the surface of liquid propane, thus avoiding condensation of oxygen from the ambient air. The results vary, there is always in our specimens (listed in the Recent results paragraph) a depth at which the ice crystals are too large for a reliable analysis, but it depends mainly on the thickness of the layer of physiological solution surrounding the tissue : most of the time, we avoided drying or concentration by evaporation of this solution, which could interfere with the physiological distribution of Ca. When possible, glueing the cells to aluminum foil with polylysine will simultaneously provide a good freezing surface and limit evaporation of the surrounding fluids (Fig. 17)

After rapid freezing, the specimens are transported in liquid nitrogen and put into 2 ml screw-cap cryotubes which have been punctured with a hot needle to allow gaseous nitrogen to escape. They can be stored in liquid nitrogen for long periods of time without noticeable changes. We transfer the cryotubes into 50 ml screw-cap centrifuge tubes containing the substitution liquid which is in a viscous state (near solidification) induced by a surrounding bath of liquid nitrogen.

Various **freeze-substitution fluids** have been used by different authors (see Harvey, 1982); we initially chose ethanol containing oxalic acid because Geyer et al. (1974) had established the validity of the method for Ca with this solution, and also because of the low melting point of ethanol. We occasionally used acetone (as Ornberg and Reese, 1980) and we plan to use tetrahydrofuran (as Ornberg and Reese, 1981b; Phillips and Boyne, 1984), which was claimed to improve Ca retention (Ornberg and Reese, 1981b), perhaps because it allows a lower substitution temperature than acetone. Gielink et al. (1966) found that acetone, contrary to ethanol, did not dissolve water-soluble Ca salts but Harvey et al. (1976) mention a significant solubility of calcium nitrate and calcium chloride in acetone. To ensure a perfect dehydration of the substitution fluid, it is normally recommended to add freshly dehydrated molecular sieve : care must be taken to use calcium-free pellets (we use the Prolabo 3A). We did not use acrolein (see Van Zyl et al., 1976; Marshall, 1980; Ornberg and Reese, 1981a) in the substitution fluid and seldom osmium tetroxide. Acrolein may improve the retention of diffusible ions (Van Zyl et al., 1976); OsO_4 may be needed to improve the contrast but should be avoided when possible for microanalysis (see the above paragraph).

We tried 3 kinds of **refrigerating systems** for the substitution : i) the cryochamber of an ultramicrotome, with its peripheral attachments, makes a simple cryostat; it has the advantage of keeping the lowest temperature compatible with the melting point of the substitution fluid and can be left overnight with the normal store of liquid nitrogen (25 l) if the whole system, insulated with an additional polystyrene box, is kept in a cold room at -15°C; ii) a Dewar filled with the same solvent as the substitution fluid, refrigerated by a cold finger (Cryocool CC-100, on the advice of J. Escaig) is less demanding but more expensive; this system needs a propeller to homogenize the temperature of the bath around the centrifuge tubes; frost forms on the top of the tubes and can even block the propeller if it is left too long without inspection; iii) a low-temperature (-93°C) refrigerator is the most expensive but the most convenient of the three systems; it is limited in temperature but allows one to easily install UV light for low temperature embedding; a centrifuge rotor or a box filled with glass beads can be used both to store conveniently the 50 ml centrifuge tubes and to establish a thermic buffer around them, particularly during the warming up. The eutectic temperature of calcium chloride (-54.9°C) is sometimes evoked as critical in the cryotechniques leading to Ca localization, but this seems to be largely guesswork (see Marshall, 1980). It is not clear either if it is really advantageous to substitute at the lowest possible temperature.

The **substitution time** varies considerably with the authors, from a few hours (Ornberg and Reese, 1980) to one week (Chandler and Battersby, 1979) or even two or three weeks (Geyer et al., 1974; Marshall, 1980; see also Robards and Sleytr, 1985). This time must be adapted to the solvent, the loss of solvent-soluble dyes from agar blocks is effected in one day with acetone, three weeks with diethyl ether (Harvey et al., 1976). Ornberg and Reese (1981a) verified with tritiated water that the substitution by acetone at -83°C was practically complete in 24 h. We usually leave the specimens in our substitution fluid (ethanol) at -92°C for a minimum of 3 days, then bring them to room temperature progressively (see Marshall, 1980) in a few hours by 10°C steps with the Cryocool system, or continuously in two days with the low-temperature refrigerator (taking advantage of the thermic inertia of the centrifuge rotor).

All our results were obtained after **embedding** in Epon. The specimens were generally transferred at room temperature from the substitution fluid into a mixture of Epon and ethanol. Although we found Lowicryl (see Ornberg and Reese, 1981b) technically difficult to use (stench before and brittle consistency after polymerisation), we intend to try it again, if only to change the conditions of image formation. However, the lower electron opacity of Lowicryl may not be an advantage when the studied organelles are recognized by being less electron dense than Epon (as in Blaineau et al., 1987).

We collected **sections**, approximately 150 nm thick (at the limit between purple and gold when observed on water), either on glycerol or ethylene glycol, or on water. Sectioning on water did not decrease the Ca X-ray signal of freeze-substituted sperm embedded in epoxy (low viscosity) resin (Chandler and Battersby, 1979) when compared to air-dried or cryosectioned specimens. Neumann and Janossy (1980) found no difference in a freeze-substituted plant tissue between the elemental content of sections collected on water and on a dry knife. Similarly Simon and Heng (1988) indicate that calcium (but not sodium or potassium) is retained when resin sections are allowed to float on water. However Ornberg and Reese (1980) demonstrated a 50 % loss of exchangeable ^{45}Ca in freeze-substituted muscles cut on water while the specimens cut on glycerol retained at least 98% of their activity. Hagler et al. (1981) found that if the mitochondrial calcium deposits seen in alcohol-fixed ischemic cardiocytes are

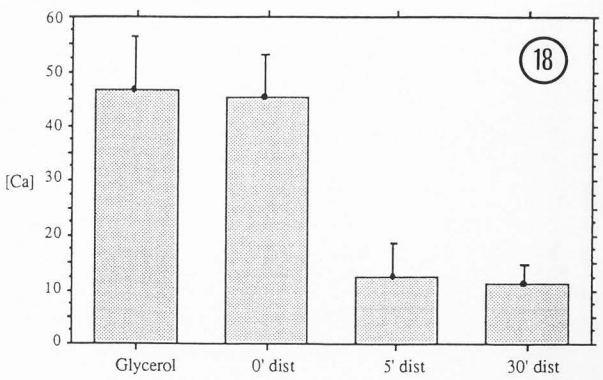


Fig. 18. Graph showing the leaching of Ca from molluscan glio-interstitial granules during flotation of the sections on distilled water. Flotation for 30 or even 5 min on water significantly reduced the Ca concentration, but if the sections were collected immediately, there was no difference between the sections collected on water and those collected on glycerol (from Blaineau et al., 1987).

sectioned on water instead of glycerol, they lose at least 50 % of their Ca, and the authors add that "with tissues fixed in phosphate-buffered osmium, sectioning on glycerol resulted in (only) little improvement... indicating that the major loss of Ca occurred during aqueous fixation".

We compared the effects of sectioning on anhydrous liquids or on water on the total Ca content of resin-embedded specimens by X-ray microanalysis (of freeze-substituted tissue). Glycerol or ethylene glycol were carefully dehydrated by an excess of molecular sieve before use and pellets of molecular sieve were even added during sectioning.

In the case of the extracellular spaces of *Orchestia* posterior ceca epithelium the sections collected on ethylene glycol contain approximately twice as much calcium as the sections collected on water (Meyran et al., 1986). In the case of molluscan glio-interstitial granules: if the sections are collected after 5 min of flotation on water, the granules have lost 75 % of their Ca but if the sections are collected without delay, their content is the same as after sectioning on glycerol (Blaineau et al., 1987; Fig. 18). It is difficult to draw general conclusions as most authors do not mention the delay between sectioning and collection of the sections, but we do not recommend collecting on water for mineral Ca salts (see Morgan, 1979) nor in general for organelles on which Ca leaching from sections has not been tested. Sectioning on glycerol may need particular and not clearly defined experimental care as Marshall (1980) reported an increase in the Ca content of sections collected on glycerol when compared to dry-cut. We did not investigate the possible increase in Ca induced by flotation on distilled water stored in glass (Mentré and Halpern, 1988).

Validity of the technique. The validity of this method to study the distribution of Ca can be assessed at different levels. It is possible to test if there is no Ca loss at the cellular or tissular level by loading the living tissue with ^{45}Ca then counting the activity of the surrounding fluids during the successive steps and/or the activity remaining in the specimen.

Winegrad (1965) loaded living frog muscle by soaking in ^{45}Ca Ringer for 8 h; after quick-freezing, the muscles were substituted in ethanol containing 1 % OsO_4, for 3 days at -75°C. Less than 5 % of the ^{45}Ca was lost during the fixation and dehydration procedure. In a subsequent work (Winegrad, 1968), the author used ethanol containing 1 mM oxalic acid and osmium tetroxide for 30 min to impregnate the freeze-dried muscle before methacrylate embedding; no ^{45}Ca loss could be detected in either the ethanolic solution or the methacrylate.

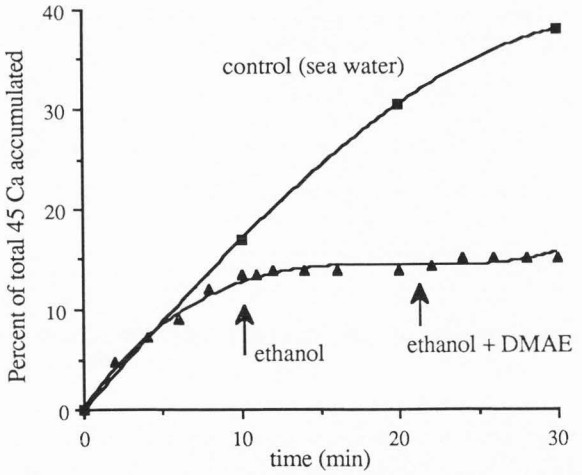


Fig. 19. ^{45}Calcium discharge from sea urchin eggs in two different media used for cryosubstitution. Fresh unfertilized eggs were loaded during 1 h in ^{45}Ca artificial sea-water and the discharge performed either in sea water (control), absolute ethanol, or ethanol + DMAE (dimethylamino-1-ethanol, suspected to induce washout of Ca during embedding in resin). The results are expressed as % of the total ^{45}Ca accumulated, corresponding to the sum of the isotope collected in the external medium plus the activity remaining in the eggs at the end of the discharge. There is no significant loss of Ca in the non-aqueous solvents.

Geyer et al. (1974) loaded glutaraldehyde-fixed erythrocytes with 1% or 0.1 % $^{45}CaCl_2$ and treated them by freeze-substitution in ethanol at -79°C for 2 to 3 weeks, with or without 1 % oxalic acid; they counted the radioactivity in the ethanolic oxalic acid supernatant and found a Ca loss of 0.02 %, freeze-substitution in ethanol alone resulting in a loss of more than 99%. They also freeze-substituted protein gels soaked with 16.5% $^{45}CaCl_2$ and showed by autoradiography that the distribution of ^{45}Ca was not affected by the technique.

Neumann and Janossy (1980) used filter paper impregnated with various radioactive saline solutions as a model. 98 % of the ^{45}Ca remained in the model specimen, 1% being lost during substitution in hexane (without precipitating agents) during 4 days at -80°C, and 1 % during infiltration by the low-viscosity resin ERL.

Ornberg and Reese (1980) used live frog skeletal muscle, loaded by bathing in ^{45}Ca-containing saline during repetitive nerve stimulation. They counted the radioactivity in the freeze-substitution solution (4 % OsO_4 in acetone with or without 20 mM oxalic acid) but also in subsequent acetone washes, propylene oxide washes and propylene oxide-Araldite mixtures; less than 1 % of the ^{45}Ca was lost, but this loss raised to 10 % if oxalic acid was omitted. This 10 % loss occurred mainly during infiltration with epoxy resin. The concentration of oxalic acid could be as low as 2.5 mM without detectable change.

We loaded freshly collected sea urchin eggs with ^{45}Ca during 1 h and, after 10 min in cold sea water, soaked them in ethanol during 10 min, then 10 more min in ethanol plus dimethylaminoethanol (DMAE - as the amine accelerators have been suspected to induce washout of Ca during embedding, Ornberg and Reese, 1980). The radioactivity of the medium was counted by sampling every 2 min during the 30 min of the experiment (Fig. 19). The unloading regularly observed in sea-water was completely and immediately stopped in ethanol (a record of 0.5 % loss in one run, none in others), even if DMAE was added in the first bath of ethanol.

The very small exchangeable ^{45}Ca losses from a piece of tissue or a collection of cells (see above) as well as the fact that external Ca does not seem to cross the plasma membrane during the process (Winegrad, 1968; Ornberg and Reese, 1980) suggest that freeze-substitution preserves the actual distribution of Ca but does not demonstrate unequivocally that there is no Ca translocation *within* the specimen. We found little evidence in the literature of translocations due to freeze-substitution; the successive refinements of the technique adopted by Ornberg and Reese were obviously improving Ca retention in the reticulum (Ornberg and Reese, 1980; 1981b). We were unable to reliably identify the reticulum on our unstained freeze-substituted specimens but we compared the Ca concentration in membrane bound glio-interstitial granules in freeze-substituted Epon sections and freeze-dried cryosections with success (Blaineau et al., 1987). Of course this comparison postulates that freeze-drying of cryosections is better than freeze-substitution, but this assumption is not necessarily made by all authors (see Marshall, 1980).

The good use of mmoles of Ca per kg of resin. The microanalytical results obtained with resin embedded specimens are normally expressed in mmol/kg of resin-embedded tissue. It is of course desirable to compare these units to the more familiar mmoles/kg of wet weight, or to mmoles/kg of dry weight, when evaluating the results of freeze-substituted and embedded specimens with those of freeze-dried cryosections. To establish this equivalence, one must :

i) know the water content of the specimen or to assume it from bibliographical data,

ii) assume that the water eliminated before embedding is exactly replaced by resin,

iii) assume that no other component than water is replaced by resin,

iv) know the mass of polymerized resin per unit volume.

It is often possible to find in the literature the water content of a given tissue, particularly when working with laboratory rodents, but this content may vary from one cell or one organelle to the next. For example, Saubermann and Scheid (1985) report in leech ganglia a water content of 55 % in the neurons and of 90 % in a lacunar zone of glial cells; in the adrenal medulla, Ornberg et al. (1988) use the chromaffin granule (66 % of water) as an internal standard to estimate a water content of 89 % for the extracellular space, of 88 % for the nucleus, and 70 % for the mitochondria. In our study of molluscan glio-interstitial granules (Blaineau et al., 1987) we gave an example of equivalence by adopting the water content given in the literature for the whole muscle, but this choice very likely overestimated the actual water content of the granules. Ingram and Ingram (1983) used bromine as a quantitative marker of the epoxy embedding medium to estimate the water space of the specimen, assuming that all tissue water is exactly replaced by the resin (see next paragraph).

With the second condition listed above, one assumes that there is no shrinking nor swelling of the tissue during the procedure : with aldehyde fixation, volume changes may occur, linked to the osmotic pressure of the fixative, but it is generally true that freeze-substitution preserves the dimensions of the tissues (*e.g.*, Wilson et al., 1983) at least in the well frozen parts of the block.

The third assumption is more hazardous as it postulates for example that no lipid is removed by the non-polar solvents which are used before embedding in resin. A high lipid content in sea urchin eggs is reflected by the low water content of 53 %, measured with tritiated water; the total Ca concentration obtained by destructive spectrophotometric analysis is equal to 32.3 ± 1.3 nmol/mg of protein (n = 19); 1 mg of sea urchin egg protein corresponds approximately to

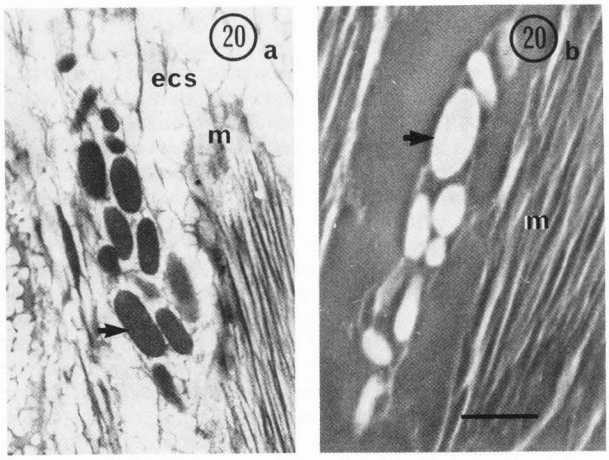
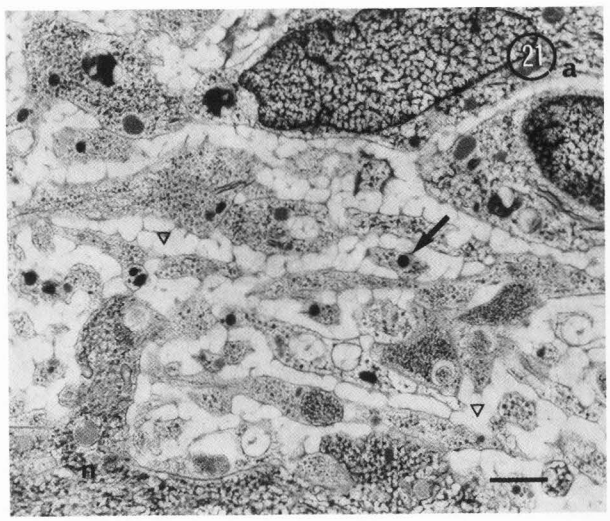


Fig. 20. Sections of frozen and freeze-substituted anterior byssal retractor muscle of *Mytilus* observed in regular transmission electron microscopy.
Ice crystals are larger in the extracellular spaces (ecs) than in the muscle cells (m) and the glio-interstitial granules (arrows) are always well preserved from ice damage, even in the deeper parts of the muscle (not shown). Bar = 1 µm.
20a Stained section.
20b.Unstained section, used for X-ray microanalysis: the calcium-rich granules are less electron dense than the embedding medium (Epon).



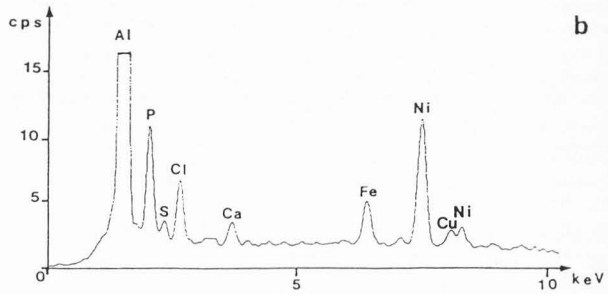


Fig. 21 : 21a. Electron micrograph of conventionally stained ultrathin section showing the periphery of a neurone (n) in freeze-substitued Aplysia ganglion. Ice crystal formation is mostly observed in the extracellular spaces (Δ) (presumably rich in water) and less developed in the cytoplasm ; the characteristic glial granules (arrow) are the best preserved organelle. Bar = 1µm.
21b. Energy dispersive spectrum of a glial granule obtained from a dark gold unstained section. In addition to calcium, glial granule analysis shows the presence of phosphorus, sulphur and chlorine (the section is collected on an Al grid, the Fe, Ni and Cu signals come from the column).

4.3 µl of cell water; assuming that the water is replaced by Epon, the result obtained by spectrophotometric analysis is practically identical (6.15 ± 0.24 mmol/kg of resin embedded tissue) to that recorded with a large (5 µm) probe diameter by X-ray microanalysis of freeze-substituted and resin-embedded eggs, *i.e.*, 6.24 ± 0.71 (Gillot et al., 1988). It is possible that some lipids were substituted and that the X-ray method overestimated the Ca concentration, the two errors compensating each other exactly, but it is more likely that the two methods were correct and that the lipids are not significantly removed.

The fourth condition is easy to meet. In our hands, 19.739 g of polymerized Epon displaced 16.2 (± 0.2) ml of distilled water, we can thus consider that one dm^3 of Epon weighs 1.22 kg. For a water content of 78 %, 1 mmol/kg of Epon-embedded tissue therefore equals approximately 5.3 mmol/kg of dry weight and 1.172 mmol/kg of wet tissue (or 1.5 mmol/l of cell water). For a water content of 53 %, 1 mmol/kg of Epon-embedded tissue will equal 1.116 mmol/kg of wet tissue, which means that the estimation in mmol/kg of wet tissue is only moderately affected by variations in water concentration.

Recent results. We applied cryofixation by propane followed by freeze-substitution in oxalic acid-containing ethanol (or acetone) and embedding in Epon as a routine preparatory technique for quantitative X-ray microanalysis of Ca or related cations in several cell types (Figs. 17, 20, 21).

The **interstitial cells** which form a net in most tonic molluscan muscles (see Nicaise and Amsellem, 1983) are characterized by large oval membrane-bound granules (0.2 to 0.7 µm in diameter). The best known slow-contracting molluscan muscle is certainly the byssal retractor muscle (ABRM) of the edible mussel *Mytilus*, a good model to investigate the role of interstitial cells and their granules (Fig. 20). It has been speculated that these granules would be a Ca store, used to regulate the concentration of calcium ions $[Ca^{2+}]$ in the immediate vicinity of the muscle cells (Hemming and Nicaise, 1982; Julliard and Nicaise, 1984). The interstitial granules of *Mytilus* ABRM contain variable amounts of Ca, up to 180 mmoles/kg of embedded tissue (Nicaise et al., 1984). This Ca content is significantly lower in the tissues of animals submitted to high potassium artificial sea water (Blaineau et al., 1987), a finding which we had predicted.

The molluscan interstitial cells form a continuous tissue with the glial cells of the smaller nerves. The **glial cells of central ganglia** contain similar (slightly smaller) granules; it has been proposed that the role of glial cells in supporting neuronal function would be parallel to that of interstitial cells in muscle (Nicaise, 1973). The Ca content of *Aplysia* glial granules varies from 3 to 90 mmoles/kg of Epon-embedded tissue (Keicher and Nicaise, 1988; Fig. 21). The freeze-substitution technique allowed us to measure precisely the lacunar spaces surrounding the glial cells; it can thus be speculated that this store could raise the $[Ca^{2+}]$ of the perineuronal spaces by 1 mM (*ibid.*).

The **juxtaligamental cells** of Echinoderms are another granule-containing tissue from a marine invertebrate ; they are supposed to play a role in the tensility of collagen by regulation of extracellular $[Ca^{2+}]$ (see Wilkie, 1984). In a

preliminary study, we demonstrated that the granules of these cells contained appreciable amounts of Ca (10 to 45 mmoles/kg of Epon-embedded tissue) and that Ca concentrations of the order of 20 mM could be detected on the collagen in state of catch.

Important intracellular [Ca^{2+}] changes follow fertilization in the **sea urchin egg**, presumably resulting from release of intracellularly sequestered Ca (see Swann et al., 1987). We compared eggs frozen before (Fig. 17) and at various times after fertilization and were able to show a significant transient increase of Ca in mitochondria (Gillot et al., 1989); the mitochondria are presumed to be the sink of calcium during fertilization (Eisen and Reynolds, 1985).

The use of Sr^{2+} or Ba^{2+} is frequent in physiological studies of contraction or secretion, for example Ba^{2+} ions enter the **frog skeletal muscle** cell through Ca channels, but have a slowly-developing inhibitory effect on contraction. These ions may therefore be used to test to what extent the Ca^{2+} ions which activate contraction are released from the terminal cisternae or simply invade the sarcoplasm via the Ca channels of the plasma membrane. After moderate Ba loading (1.8 or even 10 mM external Ba^{2+}), rapid freezing and freeze-substitution of frog muscle, it has been shown by X-ray microanalysis that, contrary to what had been published by other authors after simple observation of chemically fixed frog muscle, Ba^{2+} ions were primarily accumulated by mitochondria ; a (delayed) uptake by sarcoplasmic reticulum terminal cisternae could only be observed with high (60 mM) external Ba^{2+} concentrations (Amsellem et al., 1988).

Freeze-drying and plastic embedding. Although we did not use this technique (mainly because few laboratories had tested it, particularly on Ca) it is necessary to mention freeze-drying followed by resin embedding as an alternative to freeze-substitution. This method has long been advocated by Ingram and Ingram for preserving diffusible ions (see ref. in Ingram and Ingram, 1984) and performed in a few other laboratories (*e.g.*, Winegrad, 1968; Fritz, 1980; Hargest et al., 1985; Wroblewski and Wroblewski, 1986). It appears to present at least the same advantages as freeze-substitution (probably better for lipid preservation) and is a promising technique.

Conclusions

The physiological (total) Ca concentration within cell organelles is important information, routinely obtained by quantitative X-ray microanalysis, which can only be performed after rapid freezing of the specimen; the rapid freezing need not necessarily be followed by cryosectioning, resin embedding is less demanding and can be used after freeze-substitution in the presence of oxalic acid. Ca is perhaps a particularly favorable element for freeze-substitution and resin embedding because it is already trapped in cell compartments *in vivo* and the ionized Ca^{2+} is essentially localized in the cytosol. The main difficulty with such techniques is that they do not permit identification of readily damaged cells (see Somlyo, 1985) because of sodium loss. This problem is open for future investigations which would more clearly extend to animal tissues the promising data obtained by botanists on sodium (see Van Zyl et al., 1976; Harvey, 1982).

Various cytochemical techniques can be used with profit to gain qualitative or semi-quantitative information on Ca accumulations and translocations between subcellular compartments, particularly when a difference is recorded between control and experimental or pathological states. Cytochemical methods are complementary to the methods of quantitation of total Ca since :

i) They may reveal previously unsuspected Ca stores, particularly in poorly investigated cell types. It is clear that the contribution of cytochemical methods is of little but historical interest for the students of vertebrate muscles in physiological conditions, but in many other cell types the knowledge of calcium movements can still be improved by cytochemistry, as shown by recent examples of sea-urchin eggs (Poenie and Epel, 1987) or *Orchestia* calcifying epithelium (Meyran et al., 1984, 1986).

ii) They may give information on the fate of recently introduced or freshly translocated (as opposed to total) Ca, particularly when heavier elements as Sr or Ba are used as tracers, or in the case of skinned cells loaded with oxalate in controlled pCa conditions.

iii) They represent the main approach to study Ca distribution in interesting tissues, in which it is extremely difficult to obtain a good sampling of well frozen intact cells.To our knowledge, only the very superficial layer of mammalian brain could be satisfactorily frozen for X-ray microanalysis (see Somlyo et al., 1985b), but biologically interesting data were collected in deeper parts with cytochemical methods (Griffiths et al., 1983; Meldrum, 1986).

iv) They give a visual pattern (along a membrane for example) which is not usually seen in analytical electron microscopy, except with X-ray or electron mapping (Somlyo, 1984, 1985; Leapman and Ornberg, 1988; Simon and Heng, 1988) or at a lower spatial resolution in analytical ion microscopy (Galle et al., 1983).

The main example of false conclusion drawn from "wet" cytochemistry of calcium is the question of mitochondrial Ca stores. Mitochondrial Ca accumulations were demonstrated to be a rare and generally pathological event in several cell types (see Somlyo et al., 1985a). But most cases of mitochondrial Ca accumulations were observed in cytochemical studies in which the method induced massive Ca influx; they are indicative of a potentially large Ca buffer in these organelles, playing a role in cases of occasional Ca overload, an overload which may be the normal state of certain cell types in calcifying tissues, in the case of calcium phosphate (*e.g.*, mammalian bone) as well as calcium carbonate (*Orchestia* posterior ceca) elaboration. In non-calcifying tissues, the mitochondrial precipitates are not seen in modern variants of the precipitative techniques (*e.g.*, Borgers et al., 1984).

Most of the time, more information will be obtained in tissue sections which are not stained by heavy metals ; if X-ray microanalysis is needed, they are likely to displace Ca deposits (see Morgan, 1979) and they will excessively increase the continuum. There are examples of subcellular calcium accumulations which are electron dense without staining, particularly when calcium ions have been added to the aldehyde fixative. The addition of Ca^{2+} to the primary fixative raises questions of whether the calcium accumulations are physiological or were deposited in the process of fixation, but the omission of Ca^{2+} from the OsO_4 post-fixation step has often been shown to cause a loss of the calcium deposit. It must be noted that OsO_4 stored in glass may contain unsuspected amounts of Ca. When possible, it will be necessary to compare chemically-fixed with quick-frozen tissues.

Acknowledgements

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Discussion with Reviewers

R. Wroblewski : Did you use the supporting film only because it was needed in the (quantitative X-ray microanalysis) program ?

Authors : When using the CAMEBAX (WDS spectrometry), we do the calculation "by hand" and do not use any supporting film. We thus obtain clearer images but need more frequent grid bars to support the section, which can be easily achieved without excessive noise by using aluminum grids. However, copper grids are better than aluminum for the analysis of cryosections and large mesh grids with a supporting film give a better peak to noise ratio than small mesh grids without film.

R. Wroblewski : I think that dissolution (redistribution) of Ca can only be slowed down, but not prevented by adding calcium to the fixative.
Authors : The published observations of many authors (see text) suggest that the time needed to dissolve calcium accumulations in the presence of calcium is long enough to keep calcium *in situ* during classical electron microscopic processing. But heterotopic (artifactual) deposits of externally introduced calcium are probably very rapid during chemical fixation.

R. Wroblewski : Your statement (that the potassium is washed off during freeze-substitution) is incorrect. In properly performed freeze-substitution K should be retained (see Wroblewski et al. Scanning Electron Microsc. 1985/I, p. 447-454). If K is lost during the freeze-substitution procedure also other ions (including Ca) will be washed away or redistributed.
Authors : We agree that some authors are successful in retaining K during the freeze-substitution procedure and therefore that their technique is probably better than ours. The spectra of Figs 4, 7 or 21 obviously demonstrate that we are losing potassium. We believe that if we were not using oxalic acid in the substitution fluid we would risk the loss of calcium as well, although the non-cytosolic Ca which we are looking for is probably more firmly bound than potassium (K is presumed to be mainly in ionic form). We are not aware of significant Ca loss or redistribution when oxalic acid is used, except during prolonged flotation of the sections on water.

P. Mentré : Is the risk of calcium to be washed out by pure water liberated from ice microcrystals during cryosubstitution, and to be redistributed on neighbouring calciophilic structures negligible ? What is known about water polarity under the particular conditions of cryosubstitution ?
Authors : In the ideal case, the water from the ice crystals would immediately be dissolved in a much larger volume of organic solvent so that pure, fluid water would not exist during freeze-substitution. Under such conditions, the loss of ions would be negligible. It is possible that near ideal conditions are actually met when potassium is preserved during freeze-substitution (see preceding question). The use of oxalic acid in the organic solvent may be considered as an answer to the risk of redistribution of calcium. We do not know the answer to the second question on water polarity.

R. Wroblewski : Cutting on glycerol or on ethylene glycol may cause ion loss from the section, due to the fact that both trough liquids are hydrophilic and therefore the uppermost layer (on which sections are floated) will have relatively high water content. This might be the case, because apparently you do not find any differences in calcium content between sections floated on water and collected without delay, and sections floated on glycerol.
Authors : It would certainly be interesting to know the water content of the superficial layer when glycerol or ethylene glycol are exposed to ambient air, particularly as a function of time. Without this information we are left with the comparison between our method and frozen dried cryosections (see Blaineau et al., 1987). If one assumes that there is no Ca loss or redistribution after quick freezing and cryosection, it is reasonably safe to conclude that (at least in the organelles which were tested) there is no Ca loss at all with the resin-embedding technique, neither during substitution nor during sectioning, provided that the sections are collected rapidly. But we do not know if this is also true for other structures like endoplasmic reticulum or inclusions containing loosely bound or mineral calcium.

R. Wroblewski : XRMA of sarcoplasmic bodies in distal myopathy showed high S signal and small Na, P, Cl or K, as analyzed on cryosections (Edström and Wroblewski, 1981). The same bodies when analyzed in double fixed and epoxy-embedded samples showed only S, but no Na, P, Cl or K which were detectable in cryosections. Additional to S, Os and Ca were found. It is likely that sarcoplasmic bodies were osmiophilic. Ca signal can be explained by affinity of calcium to osmium which results in development of divalent osmates (Gilloteaux and Naud 1979, Histochemistry 63, 227-243).
Authors : Our experience in mammalian heart tissue (Blaineau et al., 1989) indicates that the tissue Ca content can be much higher after osmium treatment, if the OsO_4 solution has been prepared in glass but not if it has been prepared in a plastic container. This strongly suggests that Ca enrichment can occur without the experimenter being aware that calcium has been introduced with the fixative, and the hypothesis of a Ca-Os complex may not be needed.