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ADSORPTION STAINING OF FREEZE-SUBSTITUTED AND LOW TEMPERATURE EMBEDDED FROG SKELETAL MUSCLE WITH CESIUM : A NEW METHOD FOR THE INVESTIGATION OF PROTEIN-ION INTERACTIONS

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Abstract

A new adsorption staining method for transmission electron microscopy is described by means of which cellular adsorption sites of alkali-metal ions can be visualized in freeze-substituted and low temperature embedded biological material. The main features of this staining method are : 1) the use of Cs^+-ions which are known to accumulate in living cells like K^+-ions and 2) the removal of the staining solution from thin sections of the embedded material by centrifugal force. It is shown that sections of freeze-substituted and Lowicryl embedded frog skeletal muscle which has not been treated with chemical fixatives can be stained with electron-dense Cs^+-ions: protein sites of preferential ion adsorption are visualized. These sites are similar to those accumulating monovalent ions in living cells as had been shown previously with frozen-hydrated preparations. An observed pH-dependency of the adsorption staining is consistent with the view that the ion adsorption sites are β- and γ-carboxyl groups of cellular proteins. The results obtained so far indicate that the new method can be used to investigate weak interactions between cellular proteins and different ions by electron microscopic methods.

Key Words: Ion adsorption, potassium, cesium, cesium staining, β- and γ-carboxyl groups, skeletal muscle, freeze-substitution, low temperature embedding, Lowicryl K11M, HM23.

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Introduction

Visualization of ultrastructural details of biological material by means of a transmission electron microscope is usually carried out after staining of thin or ultrathin sections of resin embedded material with heavy metal ions. For instance, electron-dense ions of uranium and lead used in the most popular staining solutions combine in high concentrations with certain components of the tissue specimen with the result of a local increase of electron opacitiy; hence different parts of an electron micrograph are recognizable because they differ in contrast. Besides the aim to visualize cellular ultrastructure, many attempts have been made to obtain cytochemical information about cellular components by using different staining solutions and procedures. However, we are confronted with the general problem that the preparative procedures used for electron microscopy may cause severe physicochemical changes of the biological material and that the binding of the electron-dense stains at subcellular sites is generally only reflecting the interaction of the modified cellular macromolecules with the used stains, hence it is usually very difficult to obtain biologically relevant information from staining experiments.

The motivation for developing a new staining method came from the idea that β- and γ-carboxyl groups of cellular proteins may possibly be visualized by using Cs^+ as a stain according to the following reasoning. Previous experiments with frog skeletal muscle have shown that K^+, Rb^+, Cs^+ and Tl^+ ions accumulate in the living cells by the same molecular mechanism and that these ions are preferentially localized within the myosin rich A bands as well as in Z lines (for reviews see Edelmann 1984, 1988). The results were seen as an experimental confirmation of the view that the ions are adsorbed to β-and γ-carboxyl groups of aspartic and glutamic acid residues of cellular proteins (Ling 1962, 1977, 1984). Now, if proteins of rapidly frozen cells maintain this adsorption property after e.g. freeze-substitution (FS) and low temperature embedding (LTE) it is expected that sections of resin embedded material can be exposed to alkali-metal ions including the electron-dense Cs^+ which then preferentially accumulate at proteins rich in β- and γ-carboxyl groups; hence an increased electron opacity of these proteins is expected (for reviews on FS and LTE see Humbel and Müller, 1986; Steinbrecht and Müller, 1987; Edelmann, 1989a).

The purpose of this paper is to show results obtained with the new staining technique and to give

suggestions for future electron microscopic investigations of the interactions between cellular proteins and ions.

Materials and Methods

Frog sartorius muscles from Northern American leopard frogs (Rana pipiens pipiens, Schreber) were cryofixed by rapid contact with a LN2 cooled copper block as described elsewhere (Edelmann 1989a). Small pieces of the frozen tissue were transferred into a Reichert-Jung CS-auto and freeze-substituted for 7 days in pure acetone at -80°C. Afterwards the muscles were infiltrated either with Lowicryl K11M or Lowicryl HM23 (70% Lowicryl + 30% acetone 5 hours, -80°C; 100% Lowicryl 17 hours -60° C, one change of the medium after 5 hours), transferred into fresh embedding medium in flat embedding moulds inside the CS-auto (see Edelmann 1989a) and polymerized by UV irradiation at -60°C for 24 hours. Samples embedded in HM23 were further irradiated by UV light for 2 days at -50°C. The polymerized specimens were warmed up to room temperature (without further UV irradiation) and removed from the moulds.

Diatome diamond knives were used for cutting 0.2 µm thick sections. The sections were picked up from the water through with a Formvar coated slot grid (2 mm x 1 mm) and mounted in a modified coffee mill as set out in Fig. 1. A drop of a staining solution was placed on the grid and left there for 5 min, afterwards the drop was removed by centrifugal force (see legend of Fig. 1). The staining solution was prepared by dissolving 100 mM LiCl, 10 mM CsCl and 0.5 mM $CaCl_2$ in distilled water (pH 7); staining experiments have also been made with glycine-HCl buffer solutions (pH 3) containing the same concentrations of LiCl, CsCl and $CaCl_2$.

Sections were examined in a Zeiss EM 902 (Egle et al., 1984) by elastic imaging in brightfield (increased contrast by filtering out the inelastically scattered electrons). The sections were photographed at magnifications up to 12 000 and the photographed section areas were exposed to less than 1000 e /nm². The used Kodak film SO-163 was developed for 4 min at 20°C in a Kodak D19 developer (diluted 1 + 2).

Optical-density measurements were made with a Joyce-Loebel 3CS optical microdensitometer (courtesy of Prof. Dr. J. Gutjahr, Fachhochschule Köln, Fachbereich Photoingenieurwesen).

Results

Generally the stained sections show a regular clean contrast as demonstrated in Fig. 2. Dark Z lines and A bands particularly well stained at the outer edges are visible at low magnification, indicating that the electron-dense Cs^+ ions are preferentially localized at these places (compare Fig. 2 a with Fig. 2 c). Densitometry tracings of the negatives of the micrographs confirm the visual impression; the contrast between A and I bands actually increases after adsorption staining (compare Fig. 2 b with Fig. 2 d). Ultrastructural details are visible at higher magnifications (Figs. 3 a, b). Individual stained filaments and axial periodicities (although very faint) are visible both in K11M and HM23 preparations. For comparison a frozen hydrated cryosection of a Tl^+ loaded muscle is shown in Fig 3 c (Edelmann, 1988). Fig. 4 demonstrates two different types of artifacts. The redistribution and precipitation

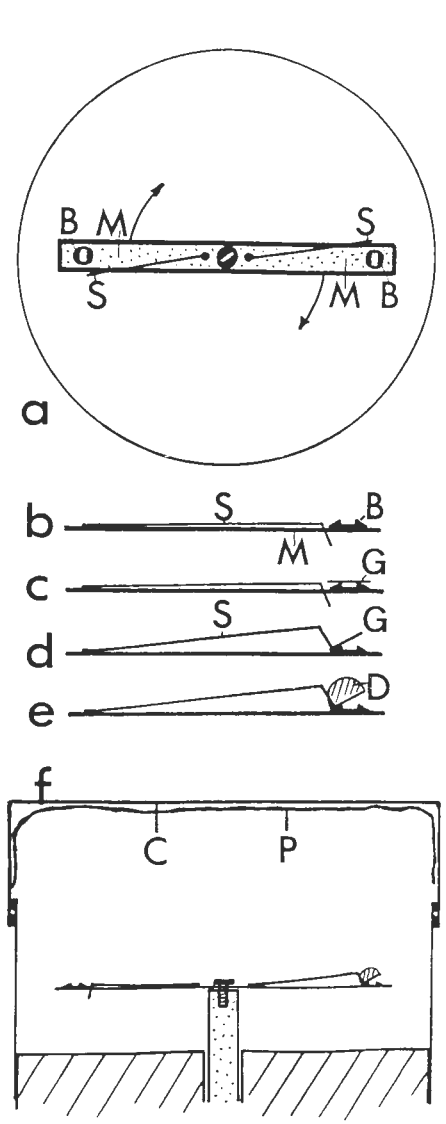


Fig. 1: Procedure of adsorption staining. **a)** Top view of an opened chamber of a coffee mill showing two metal bars M, two fixed stainless steel springs S, and two fixed aluminium bases B for supporting two grids. **b)** Enlarged side view of one M. In **c)** a grid G is placed on B, in **d)** it is fixed with S. **e)** A drop of the staining solution D is placed on the grid and **f)** the coffee mill (side view) is closed with cover C containing a wet filter paper P to prevent drying of the staining solution. After 5 min (or any other time interval) the machine is switched on, the metal bars rotate and the staining solution is removed from the grid. A main asset of this method is the fast complete removal of the staining solution from the grid without using distilled water or another liquid; concentration changes of the used stains during staining or during removal of the staining solution are avoided.

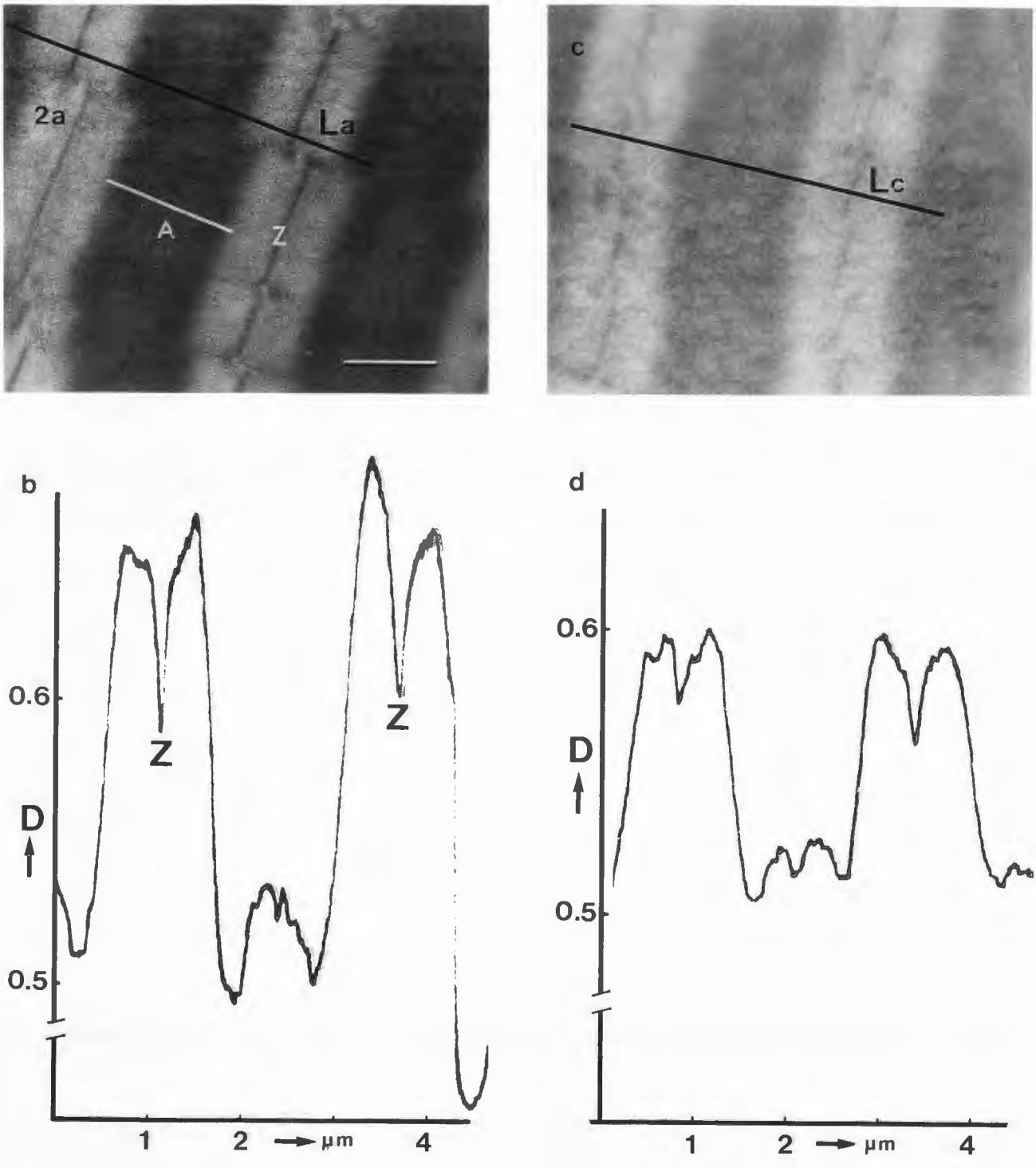


Fig. 2: 0.2 µm thick sections of frog sartorius muscle after FS and LTE in K11M (see Materials and Methods). **a)** Section after staining for 5 min with a 100 mM LiCl - 10 mM CsCl - 0.5 mM $CaCl_2$ solution (pH 7). Dark A bands (A) and dark Z lines (Z) indicate sites of preferential Cs^+ uptake. Bar: 1 µm. **b)** A densitometer tracing was taken from the negative of a) by scanning along line La. The electron opaque A bands produce areas with the lowest optical-densitiy (D) of the photographic plate. **c)** Section without any staining. **d)** Densitometer tracing from the negative of c) along Lc. The optical-density measurements confirm that adsorption staining with Cs^+ increases the contrast between I and A bands.

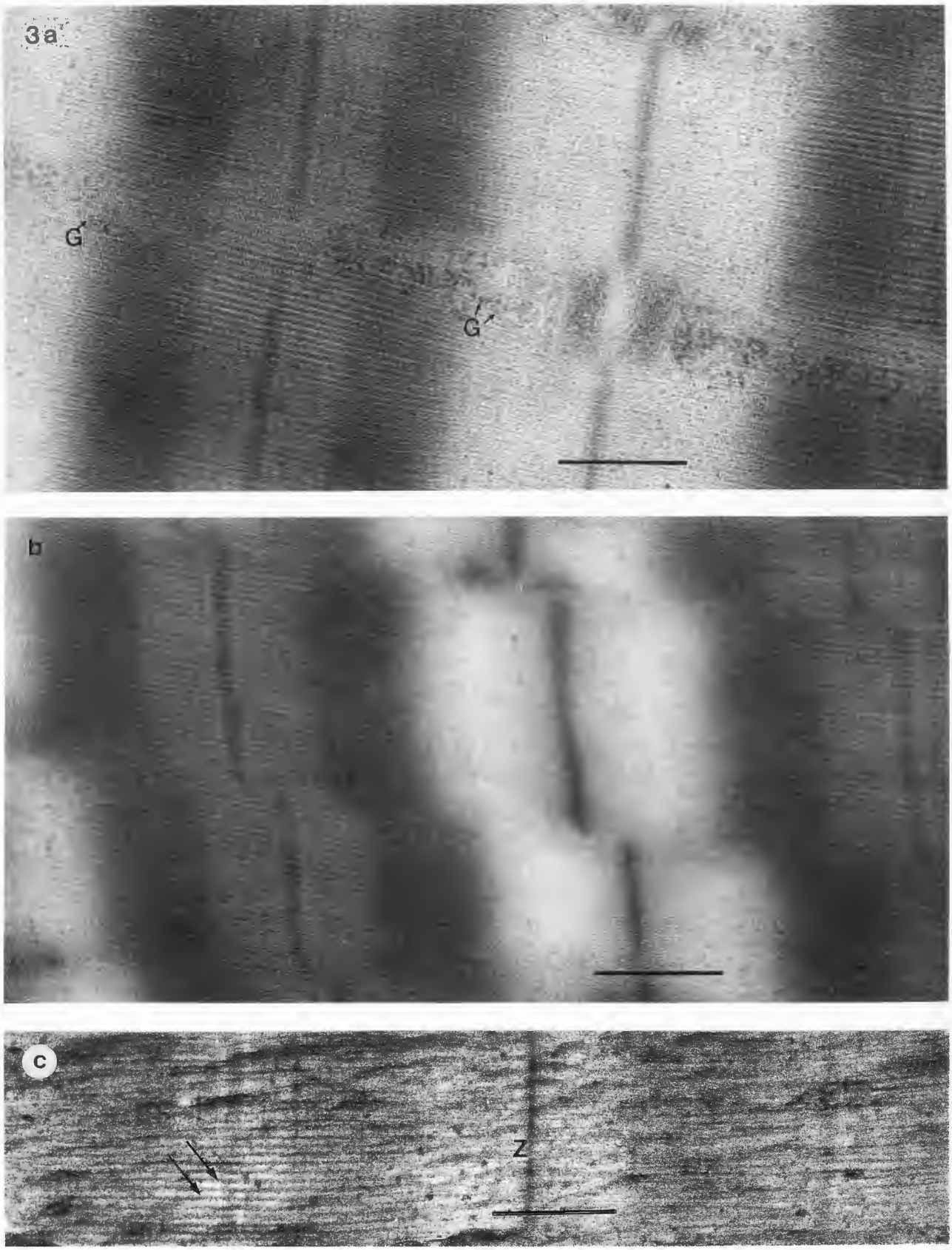
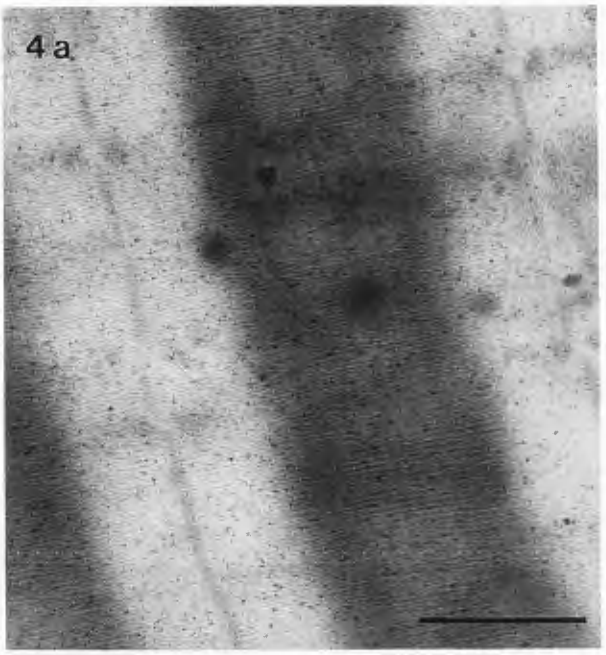
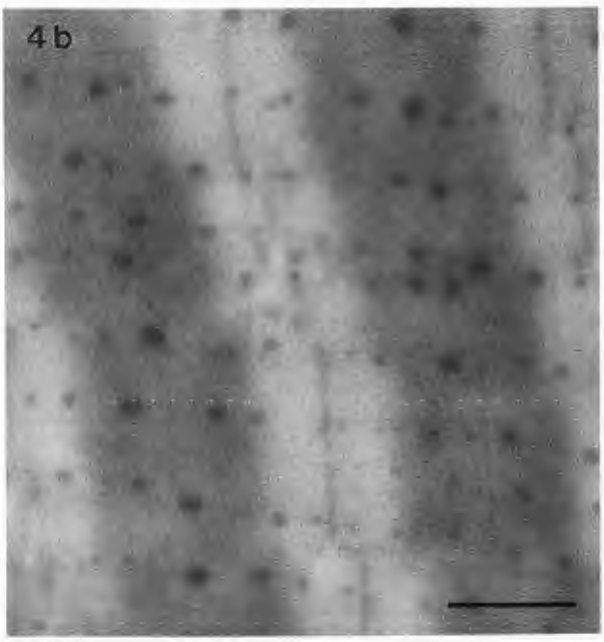

3a
G
G
b
c
Z

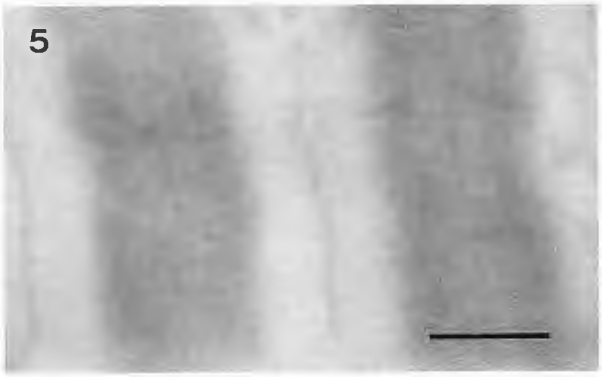
Fig. 3: Sections of frog sartorius muscle. Bars: 0.5 µm. **a)** FS and LTE in K11M, staining as in Fig. 2 a. **b)** FS and LTE in HM23, staining as in Fig. 2 a. **c)** Frozen-hydrated cryosection of a Tl^+-loaded muscle, photographed in a Zeiss EM 10 CR (see Edelmann, 1988). Dark myosin filaments (arrows) in the A bands and dark Z lines (Z) indicate sites of preferential Tl^+ accumulation in the living cell. By comparing a) and b) with c) it is evident that the distribution patterns of Cs^+ in the resin embedded preparations are similar to the distribution pattern of Tl^+ in the frozen-hydrated cryosection. Besides the main places of ion uptake (myosin filaments and Z line proteins) faint axial periodicities are visible in all three preparations. To be noted however is the different appearance of the two resin embedded muscles: the sarcoplasmic proteins seem to be more homogeneously distributed in b) (HM23) than in a) (K11M). Glycogen (G) is visible in a).

Fig. 4: 0.2 µm thick muscle sections prepared as those shown in Fig. 2 a and Fig. 3 a (K11M). **a)** The stain is irregularly redistributed after irradiation of the section with a high electron dose (>5000 e^-/nm^2). **b)** This ion redistribution artifact is observed after storage of a stained preparation for 10 days in a grid box. It is assumed that conformation changes of the proteins took place with the result that ions were released from their adsorption sites. Bar: 1 µm.

Fig. 5: 0.2 µm thick muscle section after FS and LTE in K11M (same preparations as used in Fig. 2 a and Fig. 3 a) after "staining" with 100 mM LiCl, 10 mM CsCl and 0.5 mM $CaCl_2$ in glycine-HCl buffer (pH 3). The contrast of this section resembles that of an unstained section (see Fig. 2 c). Bar: 1 µm.







of the stain visualized in Fig. 4 a occured during the irradiation of the sections with a high electron dose. This artifact is reminiscent of the well-known "pepper" artifact found after lead staining and electron irradiation. The artifact shown in Fig. 4 b was observed after storage of a stained preparation for 10 days in a grid box. This artifact may be similar to the rehydration artifacts known from cryosections (Roomans and Sevéus, 1976) and from dry-cut sections of freeze-dried and embedded muscle (Edelmann, 1984; Fig. 9 a). The conditions necessary to avoid these artifacts remain to be determined. Fig. 5 shows that at pH 3 the section exposed to the LiCl-CsCl-$CaCl_2$ solution remains almost unstained. Sections of glutaraldehyde fixed muscle could not be stained by using the described method (not shown, see Edelmann, 1986, Fig. 6 b).

Discussion

The following experimental findings and theoretical considerations served as the basis for the development of the adsorption staining method: 1) numerous investigators have demonstrated the similarity of cellular uptake of K^+ and of the K^+ surrogates Rb^+, Cs^+ and Tl^+ (for review see Edelmann, 1984); 2) according to the association-induction hypothesis (AIH) most of the cellular K^+ ions (or K^+ surrogates) are

adsorbed (weakly bound) to β- and γ-carboxyl groups of cellular proteins (Ling, 1962, 1984, for a detailed discussion of the term "adsorption" see Ling 1990, Discussion with Reviewers); 3) it was proposed to test this concept with striated muscle because myosin contributes more than 60% of the β- and γ-carboxyl chains of all the muscle proteins and is found primarily within the A bands of the striated muscle (Ling, 1977); 4) the expected preferential accumulation of monovalent cations in A bands was detected in differently prepared muscle preparations including frozen-hydrated specimens (for reviews see Edelmann, 1984, 1988, see also Fig. 3 c), and 5) by transmission electron microscopy it was shown that electron-dense Cs^+ and Tl^+ ions are retained in muscle preparations even after cryofixation, FS for 7 days at -80°C in pure acetone and LTE in Lowicryl K11M at -60°C (Edelmann, 1988, 1989b, 1989c, 1991). The retained ions are preferentially localized at A bands and Z lines like in frozen-hydrated preparations. This ion distribution could be demonstrated in dry-cut sections of resin embedded muscle. The electron-dense ions are lost during wet-sectioning. These findings support the view that the ions remain adsorbed at negative sites of cellular proteins during all preparative steps.

It was now expected that sections of freeze-substituted and low-temperature-embedded muscle may be exposed to a solution containing different alkali-metal ions with the result that formerly adsorbed cations exchange with cations from the solution and that an equilibrium between free and adsorbed ions will be established. If Cs^+ ions are present in the solution and a sufficient amount of these electron-dense ions accumulates at the proteins an increased contrast of the tissue should be visible under the electron microscope. However, from the fact that sections exposed to distilled water lose alkali-metal ions the usual procedure of rinsing the sections after staining with distilled water is not applicable. Hence the method of removing the staining solution almost completely by centrifugal force from the grid was considered. After testing different combinations and concentrations of alkali-metal ions in the staining solutions it was found that the best staining results could be obtained by using 0.2 µm thick sections and solutions containing LiCl and CsCl. The results were qualitatively similar to those obtained several years ago with sections of freeze-dried and Spurr-embedded muscle (Edelmann, 1980; the staining method used at that time was not as advanced as it is now); with these preparations it was shown by using X-ray microanalysis and the LAMMA technique that addition of LiCl to a solution containing K^+, Rb^+ and Cs^+ causes a remarkable preferential uptake of Cs^+ by the muscle section (see also Edelmann, 1981). It was also found that addition of 0.5mM Ca^{++} to the staining solution yielded the most reproducible results (unpublished). In the present case microanalysis of the stained sections has not yet been carried out but it was found that in the presence of 0.5 mM Ca^{++} staining was more stable upon irradiation of the section with electrons than without using Ca^{++}.

The main information of the presented results is the following: by using FS and LTE it is possible to preserve the capability of adsorbing monovalent cations at those protein sites which adsorb ions in living cells. A comparison of a frozen-hydrated cryosection of a Tl^+ loaded muscle with the Cs^+-stained sections of freeze-substituted and resin embedded muscles shows the close correspondence of ion adsorption sites (see Fig. 3). Even without a quantitative evaluation of the accumulated ions it is evident that binding of a large amount of alkali-metal ions at proteins can be detected within a "stable" in vitro preparation of biological material.

Why is this finding important? So far, the majority of scientists has rejected the idea that K^+ accumulation in living cells may be due to ion binding or adsorption at proteins. A main reason for this rejection is probably the finding that isolated proteins and cell homogenates do not bind a significant amount of alkali-metal ions (Szent-Györgyi, 1945; Erdös, 1946; Carr, 1956; Lewis and Saroff, 1957); the argument that the ion adsorption may be dependent on a metastable conformation of the proteins which they maintain in living cells (Ling, 1962, 1984, 1990) is not yet generally accepted or taken into consideration. But now cryofixed biological material can be dehydrated and stabilized within a resin without using a chemical fixative and with this preparation ion adsorption at proteins can be detected and investigated by electron microscopic methods. It is noteworthy that such investigations cannot (yet?) be carried out with chemically fixed biological material (Edelmann, 1986).

Unfortunately, the new staining technique has major disadvantages as compared to conventional staining techniques: due to the weak electrostatic interaction of Cs^+ (and other ions) with cellular components the stain is unstable both under the beam (Fig. 4 a) and under exposure to room air (Fig. 4 b), precluding easy storage as well as their investigation under harsh conditions (e.g., high electron exposure of the specimen) as is usually the case in routine electron microscopy. These disadvantages may be seen as an indication for chemical and physical processes taking place within the chemically unfixed metastable biological material despite its locking within a solid resin. Fortunately, the experience with freeze-dried cryosections and with frozen-hydrated preparations (storage and low dose electron microscopy) enable us to avoid or minimize changes within the sections and to investigate properties of cellular proteins captured by cryofixation and LTE which cannot yet be maintained by other in vitro methods.

This optimistic view does not mean that we have already a method which is able to capture the precise ion-binding properties of proteins in living cells. But we may start to tackle some problems and test hypotheses which have been developed in the past. A few feasable investigations are discussed next.

Determination of the ion-adsorbing sites

As mentioned already the AIH postulates that free β- and γ-carboxyl groups carried respectively on aspartic and glutamic acid residues of cell proteins function as the fixed anions for the adsorption of K^+ or other alkali-metal ions in living cells. Experimental support for this postulate has been collected and discussed in detail by Ling (1990, see in particular Discussion with Reviewers); the most direct experimental evidence for the correctness of this postulate is provided by two different kinds of experiments: a) Ling and Ochsenfeld (mentioned by Ling, 1990) demonstrated that muscle cytoplasm exposed to a modified Ringer's solution maintains selective ion accumulation for several hours and that the amount of accumulated alkali-metal ions depends on the pH of the medium. The concentration of accumulated ions decreases sharply as the pH falls from 5 to 3. The pH of the inflection point of the ti-

tration curve is about 4 which is characteristic of protein carboxyl groups (Tanford, 1969). b) Experiments with the same preparation shows an expected sensitivity of ion accumulation to the carbodiimide EDC (1-ethyl-3-dimethylaminopropyl-carbodiimide). EDC interacts with the free protein carboxyl groups producing modified carboxyl groups that no longer bear negative electric charges (Hoare and Koshland, 1967), hence ion adsorption is reduced.

The described similarity of localized ion uptake in living cells (as visualized in frozen-hydrated cryosections) and after adsorption staining suggests that the visualized adsorption sites may be carboxyl groups as postulated. Unfortunately, there are no staining procedures in conventional electron microscopy by means of which a specific staining of carboxyl groups could be achieved. Therefore, even a comparison of the staining patterns obtained after conventional staining and after adsorption staining is most likely not very helpful to see whether carboxyl groups or other charges are responsible for an observed staining of different structures (most cellular structures such as membranes, glycogen, nuclei etc. contain proteins which carry β- and γ-carboxyl groups). An example should be given: in sections of conventionally prepared biological material glycogen is strongly stained by lead; "the surface coats containing protein and polysaccharides are stained, but which component achieves this is not known" (Hayat, 1970, p. 258). In living cells Cs^+ accumulates at glycogen to such an extent that it can be seen in dry-cut sections of a Cs^+-loaded, freeze-dried and embedded muscle (Edelmann, 1977, Fig. 1). Accumulation of Cs^+ at glycogen is also observed after adsorption staining of freeze-dried embedded muscle (Edelmann, 1984, p. 884) and of freeze-substituted and K11M embedded muscle (Fig. 3 a). This example shows again that we can observe a similar uptake of Cs^+ at subcellular structures of living cells and after adsorption staining of chemically unfixed embedded biological material; but we cannot yet determine the sites responsible for ion adsorption just by comparison of staining patterns.

At the present state of our knowledge it appears easier to investigate first the mechanism of ion adsorption in the myofibril in which different proteins carrying different concentrations of β- and γ-carboxyl groups are periodically arranged. One possibility of testing the assumption that these carboxyl groups are responsible for ion adsorption during adsorption staining is - as has been done with muscle cytoplasm - to determine the amount of adsorbed ions at different pH values. First experiments of this kind have been carried out; at pH 4 the muscle is already poorly stained with Cs^+ and at pH 3 adsorption staining with Cs^+ is almost ineffective (Fig. 5); this supports the postulation that the ion adsorbing sites are carboxyl groups. The quantitative determination of the ion uptake as a function of pH and as a function of carboxyl-blocking agents (e.g., carbodiimides) may help to solve the discussed problem. In this context, the question whether phosphate groups on proteins or nucleic acids are able to adsorb alkali-metal ions is most interesting. This possibility has been discussed by Ling (1990, Discussion with Reviewers) and his conclusion that K^+ adsorption at phosphate groups, may be small compared to that at carboxyl groups, may be checked in future studies.

Selectivity of ion adsorption

A main goal of future studies of the interaction between alkali-metal ions and proteins in sections is the quantitative determination of accumulated ions. This can be done with X-ray microanalysis or other analytical methods. Such studies may provide answers to the following questions: is the amount of alkali-metal ions accumulated at cellular proteins similar to that found in living cells? Is it possible to detect a selective uptake of different ion species? Studies aimed to answer the latter question are most important because of the following reason: at present a generally accepted theory on the molecular mechanism of selective K^+ accumulation in living cells does not exist. The majority of scientists believes that this phenomenon is due to membrane properties: a minority assumes that adsorption sites in the cytoplasm are responsible (see Edelmann, 1989c, Appendix). Although the localized accumulation of alkali-metal ions (and Tl^+) at cellular proteins of living muscle is an established fact it is still debated as to what extent the fixed charges in the living cell adsorb ions with a high selectivity as concluded by Ling (1977, 1990) or if different cellular alkali-metal ions only hover near the fixed charges as "free" counter-ions without specificity (von Zglinicki, 1988).

Adsorption staining experiments with sections of freeze-dried and embedded muscle have already shown that the alkali-metal ions accumulate with a high specificity at cellular proteins (Edelmann, 1980, 1981). Preliminary experiments with sections of freeze-substituted and low-temperature-embedded muscle yielded similar results; it is expected that adsorption staining can be used to test in detail the ion exchange model of the AlH. This implies that "staining" solutions may be used which do not contain Cs^+ as a stain for transmission electron microscopy but ions which can only be detected by other analytical methods. The simultaneous use of electron-dense ions, however, has the advantage that artifacts caused by preparative procedures are immediately evident in the transmission electron microscope.

Conclusion

Adsorption staining of freeze-substituted and low-temperature-embedded biological material with cesium can be used for the visualization of subcellular structures which are rich in negative fixed charges (probably β- and γ-carboxyl groups). The method is suited for the electron microscopic investigation of weak interactions between alkali-metal ions (or other ions) and cellular macromolecules which have been captured in a life-like position without the use of chemical fixatives.

Acknowledgements

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Discussion with Reviewers

G.M. Roomans: Your finding that the Cs staining needs the presence of high concentrations of Li ions and is stabilized by Ca is intriguing, also in the context of the theoretical background for the staining method. Li is sometimes considered as a surrogate for Na, and the composition of your staining solution (high Li/Cs ratio and presence of Ca) resembles that of the extracellular fluid rather than that of the intracellular milieu. Could you comment of this? Have you tried a reverse Li/Cs ratio? Have you tried to see whether Mg stabilizes the staining?

Author: It was indeed intended to use a "staining" solution which resembles that of the extracellular fluid. The first experiments carried out with high Na^+ and low Cs^+ concentrations and sections of freeze-dried and embedded muscle (Edelmann, 1980), however, yielded in most cases poorly stained sections obscured by many crystals (not published). Replacing Na^+ by Li^+ yielded clean sections and better staining results. With sections of freeze-substituted and low-temperature-embedded muscle one obtains similar results. It appears that addition of Li^+ favours the preferential uptake of Cs^+ (Edelmann, 1980). The use of Li^+ is, therefore, recommended for pure "staining" experiments. On the other hand, we are still looking for a better "extracellular" solution containing high Na^+ and low K^+ concentrations from which the embedded proteins take up reproducibly much K^+ and less Na^+.

We have tried a reverse Li/Cs ratio (100 mM CsCl, 10 mM LiCl) but the staining with Cs^+ was not enhanced compared to that shown in this paper. The effect of Mg^{++} has not yet been investigated; it is expected that Ca^{++} is more effective than Mg^{++} (or other alkaline earth ions) in favouring K^+ or Cs^+ adsorption at cellular proteins (see Ling, 1984, pp. 362-363).

Th. von Zglinicki: Is there any difference in adsorption staining with Cs^+ compared to staining with complex forming cationic reagents? Is there any prevalence of Cs^+ to either carboxyl group (myofibrils) or phosphate group (nuclei) negative charges?

Author: The main difference between adsorption staining with Cs^+ and conventional staining with complex-forming reagents can be seen after rinsing the stained sections with distilled water. As already mentioned, rinsing of adsorption stained sections causes the loss of alkali-metal ions; as a consequence the sections appear unstained. An example is given in Fig. 6 a (compare with Fig. 6 b) which demonstrates that under the described conditions Cs^+-staining does not lead to complex formation or to an irreversible binding of Cs^+ to anionic fixed charges. Fig. 6 c shows that staining with uranyl acetate is much more stable, the stain cannot be removed by distilled water.

Fig. 6 b and Fig. 6 c reveal similar staining patterns of myofibrils and nuclei after adsorption staining with Cs^+ and after conventional staining with uranyl acetate. However, this result does neither signify that identical sites are occupied by the different electron-dense particles nor that we can answer the question whether there is a prevalence of Cs^+ to either carboxyl group or phosphate group negative charges. A review of the literature on uranyl acetate staining shows that we do not know the degree of staining of the different negatively and positively charged components of a biological specimen after exposure to the different uranyl complexes found in an uranyl acetate solution (see Hayat MA., 1989. Principles and Techniques of Electron Microscopy. Biological Applications. Macmillan Press, Houndmills, Basingstoke, Hampshire RG21 2XS and London, 305 - 313); in particular, "the role of nucleic acid-associated proteins in the contrast (of nuclei after uranyl staining) is not clear (p. 308)". In order to answer your second question we need methods by means of which the subcellular distribution of negatively charged carboxyl groups and that of negatively charged phosphate groups can be detected separately. To the best of my knowledge such methods are not yet available.

G.N. Ling: (1) If the adsorption staining method is truly so simple and rapid as described, is the result highly reproducible? Are different muscle cells in a single section stained the same way? Are different muscle cells in different sections but from the same block stained the same way? Are different muscle cells in different sections prepared from different block stained the same way? (2) If the answers to (1) are positive, or largely positive, then the adsorption staining method has made it possible to use electron microscopy to achieve goals hard to achieve before. Thus, by exposing muscles to low concentration of metabolic poisons (e.g., iodoacetate, 2,4-dinitrophenol), one can control the level of ATP and other key components essential for the maintenance of the living state and through the use of the adsorption staining method, one can observe the parallel changes in the ability of selective adsorption of Cs^+ and other indicators of the living state and compare the results with theory. Please comment.

Author: The staining method is really very simple and the results obtained so far justify a positive answer to the questions of (1) under the condition that identical preparative procedures are used. Different results may

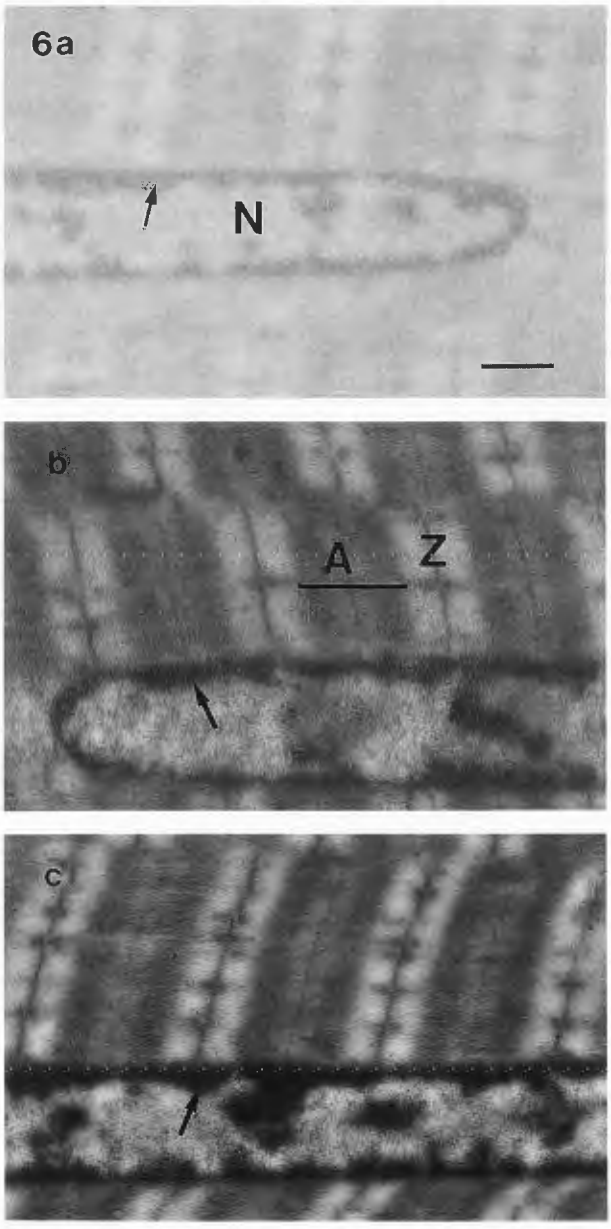

Fig. 6: 0.2 µm thick sections of frog sartorius muscle after FS and LTE in HM23 (identical photographic processing on Agfa Brovira-Speed soft paper). **a)** Staining for 5 min with a 100 mM LiCl - 10 mM CsCl - 0.5 mM $CaCl_2$ solution followed by rinsing with distilled water (5 min). The contrast is similar to that of an unstained section. The highest electron opacity is found at the condensed heterochromatin (arrow) beneath the surface of the nucleus (N). Bar : 1 µm. **b)** Same procedure as in a) without rinsing in distilled water. **c)** Staining with uranyl acetate (4 % uranyl acetate in water, pH 4) and rinsing in distilled water (5 min). In b) and c) a preferential staining of A bands (A), Z lines (Z) and heterochromatin (arrows) is observed. Staining of heterochromatin - relative to the staining of the sarcomeres - is probably more intense in c) than in b); this impression has to be checked by future microanalytical studies.

be obtained if sections are compared which do not show the same quality of structure preservation or if different FS (or freeze-drying) and embedding procedures are used. It has been reported (Schwarz H. and Humbel BM., 1989, Influence of fixatives and embedding media on immuno-labelling of freeze-substituted cells. Scanning Microsc. Suppl. 3, 57-64) that even proteins may be relocated during wet-cutting of freeze-substituted and low-temperature-embedded biological material. One may expect that the relocation artifact may influence the result the more the thinner the section. Limitations of the method and the degree of reproducibility of results have to be evaluated by quantitative microanalysis. But even without rigorous quantitation one should be able to see a difference in the staining patterns of healthy and poisened (as suggested) muscles. A similar experiment already yielded promising results (see Edelmann, 1989c). The theoretical background of this experiment is as follows: It was postulated that during muscle contraction K^+ ions are desorbed from carboxyl groups of myosin heads and that salt linkages are formed between actin and myosin heads (Ling, 1984, p. 576). A logical consequence would be that adsorption staining is reduced at a section of a muscle which has been cryofixed during contraction and subsequently dehydrated and embedded as described. The experiment was carried out as follows: A frog sartorius muscle was cut transversally with a razor blade and cryofixed. After FS and LTE ultrathin sections were prepared for conventional staining with uranyl acetate and lead citrate and 0.2 µm thick sections for adsorption staining with cesium. The ultrathin sections showed a local contraction of the muscle fibers near the place of the damage. Adsorption staining of the contracted part of a cut fiber yielded a very poor staining whereas the intact resting part of the same fiber showed a normal staining i.e., a preferential uptake of Cs^+ at A bands and Z lines as described in this paper.

G.N. Ling: Have you considered marketing your coffee-mill centrifuge? Consider the great potential use the adsorption staining method offers for the future. I think you ought to, if you have not already done so. It is not infrequently that small things like that which requires experience to make one that works and failure to do so often prevents others from adopting new and good methods.
Author: Thank you for this advice.