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# CRYOULTRAMICROTOMY AND ELECTRON MICROANALYSIS OF ISOLATED BOVINE ADRENAL CHROMAFFIN CELLS

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## Abstract

Instrumentation and techniques have been developed for the cryoultramicrotomy of isolated bovine adrenal chromaffin cells frozen with a liquid helium cooled copper block method. X-ray microanalysis and electron energy loss spectroscopy were used to measure both light and heavy element content of the adrenal storage granule, the chromaffin granule. Ultrathin freeze-dried cryosections usually have a fine compression which in cellular structure compresses the cytoplasm between organelles. Nuclei, chromaffin granules, and vacuoles are relatively free of compression. The mechanism for this distortion is not known although static charge induced sticking to the knife may be the most probable cause.

The elemental composition of the chromaffin storage granules is in reasonable agreement with the known biochemical species present in granules.

Key Words: Ultracryomicrotomy, Instrumentation, X-ray microanalysis, Secretion, Adrenal chromaffin cell.

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## Introduction

Information provided by microanalytical methods are on the verge of adding a new dimension to the study of the structure and function of cells. Specimen preparation techniques aimed at preserving the natural distribution of chemicals and elements for subsequent microanalysis have flourished with new technology. Physical fixation, or quick freezing is now possible with a variety of instruments, the most notable being the liquid helium cooled metal block freezing devices (Heuser et al,1979). Adequately frozen specimens can be sectioned routinely with new second generation cryomicrotomes and frozen hydrated specimens can be transferred into electron microscopes without perturbing ultrafine structure(McDowall et al., 1983 and Hagler, 1984). In this way precise measurements by conventional analytical X-ray and electron energy loss analysis can be performed on equally precise specimens. Although this new technology can be purchased and used "off the shelf", techniques for using a given combination of equipment still requires some patient development.

To this end, I describe here the instrumentation and technique I am currently using to obtain frozen thin sections from quick frozen samples of fresh, uncryoprotected suspensions of isolated bovine adrenal chromaffin cells. In addition to technique, the need and utility of both heavy element ( $Z > Na$ ) and light element ( $Z < Na$ ) microanalysis in cell biology is discussed with respect to chromaffin cell function.

## Materials and Methods

Adrenal chromaffin cells were isolated from intact bovine adrenal glands by collagenase digestion and culture for three days prior to freezing, as previously described (Pollard et al.,1984). Cell suspensions were frozen in a balanced salt solution containing 5 % bovine serum albumin using a liquid helium cooled copper block freezing machine similar to that described by Heuser et al.(1979). The frozen suspensions were cryosectioned on a Reichert Model E ultramicrotome equipped with a model FC4 cryosectioning attachment. The ambient temperature in the region of the knife was measured to be between 109.$^{o}$K and

113 $^{o}$K unless otherwise stated. Frozen hydrated sections were transferred with a Gatan model 626 cryotransfer stage into a Hitachi H700H TEM/STEM equipped with energy dispersive x-ray analysis system and electron energy loss spectrometer. The sections were dried in the microscope vacuum at a temperature of 170 $^{o}$K for 30 - 45 min prior to analysis.

Freezing for cryosectioning

Originally, suspensions were frozen on thin aluminum disc supports which had been covered by a small disc of Parafilm followed by a smaller, 1/4 inch (6.5 mm) dia., disc of 0.22 μm Millipore filter. The filter served as a support for the suspension during the freezing and microtomy procedures. The frozen stack of cells and filter were separated from the aluminum disc and mounted in a vise type holder for edge-on sectioning. Since a large gradient of ice crystal size existed through the depth of these frozen specimens, good sections of well frozen cells were difficult to obtain presumably due to the mechanical inconsistency in the block.

Alternatively, specimens were cut enface to the well frozen surface. For this the cell suspensions were frozen on a three tier hat shape disc of aluminum machined to have a base 13.2 mm dia x 1.55 mm thick followed by a concentric space, 11.6 x 0.5 mm thick, and a central platform, 6.34 mm x 0.5 mm thick. A precut disc of filter paper was glued to this central platform to support the cell suspension. This specimen disc was mounted in the microtome with a Reichert holder that had been modified to hold a freeze etch specimen table, (Balzer part no. BB 192 210-T) attached via a threaded post tapped into the Reichert holder. The specimen disc, being designed for the Balzers specimen table, was clamped to the modified holder with the threaded ring provided by Balzers (Balzers Union, Nashua, NH). To facilitate specimen disc loading in the Reichert FC-4 cryochamber, a new chuck was made to hold the modified Reichert holder in a vertical position. This chuck was clamped in the cryochamber with the clamping mechanism used for holding the knife assembly. These pieces are shown in Figure 1 and interested readers can receive drawings upon request.

Sectioning

For en face cutting, a pyramid was trimmed from the well frozen face with a diamond knife blade mounted on a round brass rod. This tool was used and mounted in the knife holder in place of the tungsten carbide trimming rod provided by Reichert. Typically the cutting face was 0.5 mm X 1.0 mm with very gradual sloping sides ie. 10 $^{o}$. The shallow angled face minimized the mechanical shock incurred when the knife met the block and made for more uniformly thick sections that were free of chatter. Sections were cut on a Diatome diamond cryoknife at an angle of 2$^{o}$ and speeds of 0.2 - 0.4 mm/sec. The sections came free of the knife with very little visible compression and were placed on formvar/carbon coated folding grids with the aid of a hand drawn glass whisker, dia ~ 10 μm. An anti static device (Simco model OP, Simco Co. Hatfield, PA) was used to eliminate charging during cutting.

## Results

Morphology

Chromaffin cells are neuroendocrine cells which secrete catecholamines, epinephrine and norepinephrine, into the blood to mediate systemic sympathomimetic effects such as cardiac output and blood pressure. These amines are stored together with adenosine triphosphate (ATP), calcium, and protein in secretory chromaffin granules. The ultrastructure of the adrenal chromaffin cell provided by rapid freezing and freeze-substitution is illustrated in Figure 2. As in other endocrine cells, the granules are dispersed throughout the cytoplasm. Chromaffin granules are spherical with an average diameter of 0.3 μm and in rapid frozen cells approximately 60% of these contain small,independent membrane bound vesicles within them (Ornberg and Duong, 1984). In addition to granules, vacuoles containing a less dense retracted matrix are present. Mitochondria and rough endoplasmic reticulum are generally confined to the region of the Golgi apparatus.

Views of chromaffin cells in freeze-dried cryosections revealed these features, although the cellular morphology was usually distorted due to compression during sectioning. This fine compression, which was difficult to observe as the section was being cut, was minimized by lowering the cutting speed and dropping the knife angle to 0.2 mm/sec and 2$^{o}$ respectively. The temperature of the cryostat was kept as low as possible (109 $^{o}$K - 113$^{o}$K) to add mechanical strength to the sectioning surface. The elimination of static charge during cutting was perhaps the most important technique for reducing compression. Static charge build-up induced by turning off the charge eliminator completely ruined what would otherwise have been good sections. This finding suggests that static charge induced sticking to the knife during cutting is a major cause of section compression.

The problem of compression did not affect all structures within the cell to the same extent. Nuclei and granules were only marginally distorted, while the intergranule cytoplasm was folded or compressed. Though the granules appeared to be flattened, stereo views revealed that their oval appearance was due to their being turned on edge and, in severe cases, pushed on top of one another. Consequently the sections looked thicker than that which the microtome setting and electron energy loss measurement of 100 nm had indicated.

Although compression was a severe problem, it should be noted that, unlike Figure 3, areas free from compression were easily found for elemental analysis of individual granules or adjacent cytoplasm.

Microanalysis

Both light element ($Z < 11$) and heavy element analysis ($Z > 11$) were performed on frozen, freeze-dried sections. Sections dried in the microscope and observed cold at 170 $^{o}$K showed reasonable stability when folding grids or a two grid sandwich was used. Specimen drift and mass loss did occur if the section was not completely dry. Usually 30 minutes at 170 $^{o}$K was adequate. As expected x-ray spectra from the extracellular

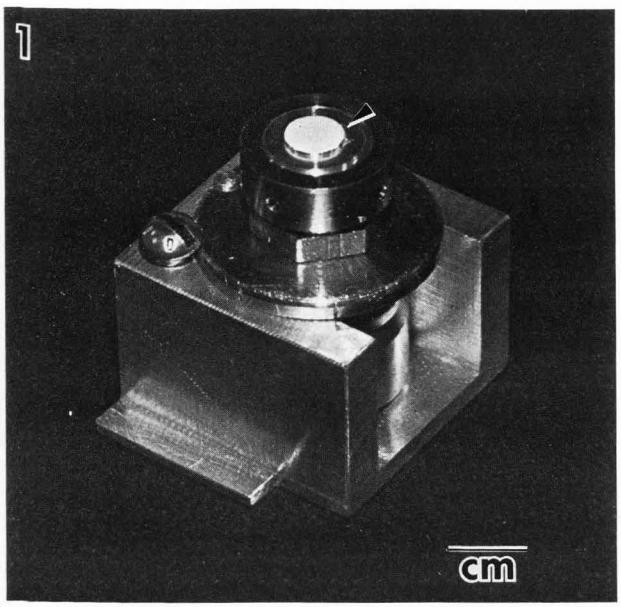


Figure 1. Modified Reichert specimen planchet holder and loading block assembly. The holder was remachined to mount a Balzers four position, freeze-etch specimen table to which the aluminum freezing disc (arrow) is held. The loading block is clamped in the knife position for specimen loading.

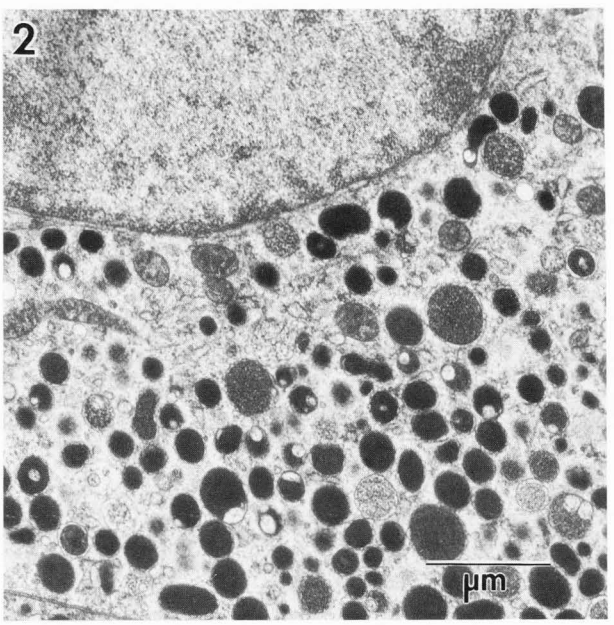


Figure 2. Rapid frozen, freeze-substituted, isolated adrenal chromaffin cell. The dense granules, chromaffin granules, are spherical (round in cross section). The clear round spaces within the granules are intragranular vesicles which are seen only in rapid frozen cells.

space (Figure 4a) indicated the high sodium (155 mM) and chlorine(135 mM) content of the saline solution and was henceforth used as an internal standard for quantification. The cytoplasm (Figure 4b) contained potassium, phosphorous and small amounts of chlorine. The silicon peak and some of the sulfur peak were from the formvar/carbon support. The granule contained predominantly phosphorous and some potassium and calcium. From preliminary quantitative analysis the wet weight concentration of phosphorous was ~430 mM. Since all of this phosphorous is in the form of ATP and not inorganic phosphorous these results suggested an ATP concentration of ~143 mM. From biochemical measurements, the granule ATP concentration has been reported to be 150 mM (Winkler and Carmichel, 1982).

Light element analysis on frozen sections by electron energy loss spectroscopy has been regarded as impossible because frozen sections were not sufficiently thin to prevent multiple scattering. At accelerating potentials of 200 kV or higher, plural scattering decreases and spectra (Figure 5) from chromaffin granules contained visible core-loss edges from the K shells of carbon, nitrogen, oxygen and phosphorous. These elements can be quantified by removing the plural scattering contributions from the edge peaks and then subtracting the background from the edge with an equation determined from the pre-edge spectrum. The mathematical procedure and physical validity of the method has been described (Swyt and Leapman, 1982).

In addition to elemental analysis, electron energy loss spectroscopy, EELS, was used at very low doses to measure the thickness of the

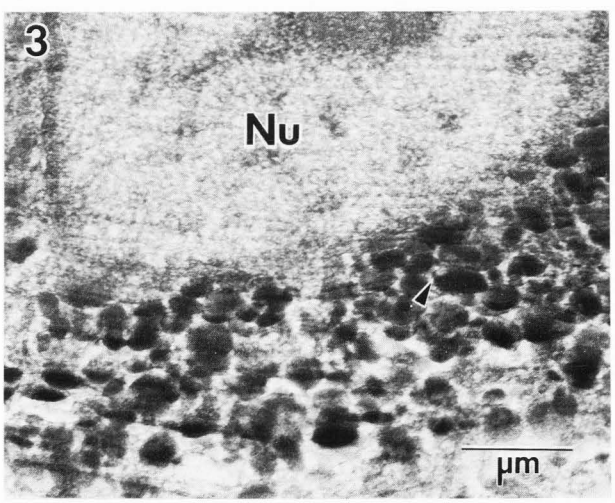


Figure 3. Freeze-dried unstained cryosection view of rapid frozen chromaffin cell. The fine structure of the cell is distorted by compression artifact. The nucleus (Nu) and granules are not as distorted as the cytoplasm. Many of these granules have been turn on edge as a result of compression during sectioning. The small intragranular vesicles can be seen in some granules (arrow).

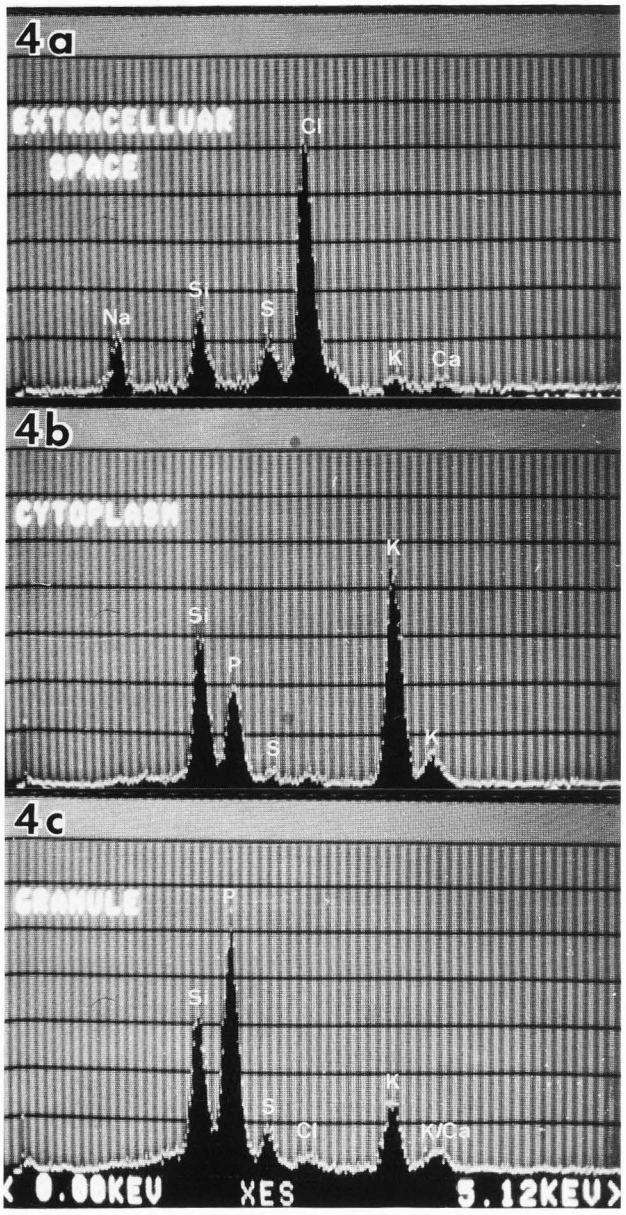


Figure 4 a-c. Energy dispersive X-ray spectra obtained from a) extracellular space, b) cytoplasm, and c) chromaffin granule. Spectrum 4a reflects the high NaCl concentration of the saline solution. In spectrum 4b, the small peak to the right of the K peak is the $K_B$ peak. The spectrum from the granule (4c) however has a significant calcium peak which slightly overlaps the $K_B$ peak. All spectra were obtained with a 1 nA beam current in the scanning reduced raster STEM mode. Vertical scale 1024 counts.

hydrated section. This measurement determines the thickness of the irradiated section in terms of the mean free path of incident electrons (Leapman et al., 1984). A typical value for hydrated cryosections of chromaffin cells in the uncompressed extracellular space was 1.6. An appropriate value for one mean free path in ice is

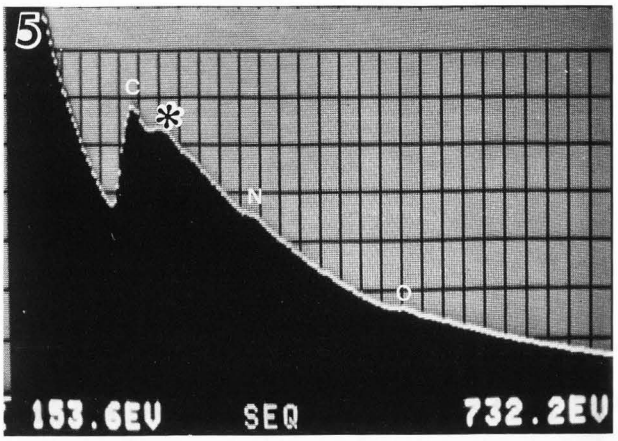


Figure 5. Electron energy loss spectrum obtained from a freeze-dried chromaffin granule. The various elemental K edges are indicated by their elemental symbol. The extra peak on the carbon edge (*) results from plural scattering of the carbon K core-loss electrons. Vertical scale is 262,000 counts.

60 nm. Therefore the sections were typically 100 nm thick. This was in good agreement with the 100 nm microtome advance settings used for sectioning.

## Discussion

In this paper I have described my technique and routine experiences in preparing cryosections from rapid frozen cell suspensions for analytical electron microscopy. From this I have drawn the following conclusions.

1) Frozen sections are most easily obtained from well frozen specimens rather than from those which have large or inconsistent ice crystals in them. The quick frozen chromaffin cells described here are probably vitrified rather than frozen since Dubochet and colleagues have demonstrated that metal mirror freezing can vitrify water in liver tissue (McDowall et al.,1983). Furthermore, electron diffraction studies currently in progress have indicated that at least the first 10.0 $\mu$m of 10% gelatin solutions are vitrified with our freezing method.

2) Compression on a very fine scale can occur in cells without visible compression in the section. Zierold (1984) has described similar results. In chromaffin cells this compression appears to be restricted to the cytoplasm. Nuclei and chromaffin granules are relatively free of compression.

3) A number of technical adjustments can minimize compression. These include a) slow cutting speed,(<0.5 mm/sec) b) low knife clearance angle (<3°), and c) static charge elimination. These conditions also optimize the performance of the microtome in that sections have thicknesses close to that of the microtome advance settings.

4) X-ray microanalysis and electron energy loss analysis can be done on freeze-dried frozen sections. Quantitative analysis with EELS does require that plural scattering, even at 200 kV accelerating potential, be accounted for and removed from core loss edges.

Biological Relevance

The chromaffin granule has served as the model secretory granule for studying mechanisms of exocytosis and membrane recycling in secretory cells. The apparent stable physical existence of chromaffin granules *in situ* is one of the more intriguing problems for study in modern cell biology, since large quantities of membrane permeant molecules are stored against large concentrations gradients (Winkler and Carmichel, 1982). Attempts to define macromolecular complexes within the granule core using magnetic resonance methods have failed (Sen and Sharp,1981). The contents show little interaction with one another in studies on isolated granules. Since changes in the content undoubtedly occur during granule isolation, it is important for the understanding of granule function to know the composition of the granule content inside the cell.

If one begins with our current knowledge of the biochemical content of chromaffin granules, two very important points regarding the elemental distribution among stored molecules emerge. First, all of the phosphorous in the granule should be in adenosine triphosphate. Second, the nitrogen content should be divided among protein (65 % of total nitrogen), ATP (22% of total nitrogen), and catecholamine (13% of total nitrogen). Therefore having determined the phosphorous content, the catecholamine concentration may be determinable by subtracting the protein and ATP nitrogen contribution from the total nitrogen. Consequently the biochemical composition of the granule core may be able to be determined with the analytical electron microscope.

Secretory granules are likely to undergo tremendous physical state changes prior to exocytosis. One current hypothesis for exocytosis, the chemiosmotic hypothesis, proposes that the granules experience an increase in internal osmotic pressure following cell stimulation (Pollard et al.1979). The raised osmotic pressure is transferred to the membrane as intramembranous tension which drives membrane rearrangement during exocytotic membrane fusion. Several lines of evidence from studies with chromaffin granules suggest that permeant chloride ions move into the granule in response to a stimulus induced increase in the granule membrane potential. The osmotic pressure increases due to compensatory water flow associated with chloride influx. Whether or not such elemental redistribution occurs within the cell is not clear. However, it is reasonable to expect that microanalytical methods done on adequately prepared specimens described here will be valuable in answering these questions.

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