Scanning Electron Microscopy

Volume 1986 | Number 2 Article 40

5-9-1986

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SCANNING ELECTRON MICROSCOPY/1986/II (Pages 703-711)
SEM Inc., AMF O'Hare (Chicago), IL 60666-0507 USA

0586-5581/86$1.00+05

PREPARATION METHODS FOR QUANTITATIVE ELECTRON PROBE X-RAY MICROANALYSIS OF RAT EXOCRINE PANCREAS: A REVIEW

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(Received for publication February 24, 1986: revised paper received May 09, 1986)

Abstract

Pancreatic acinar cells are thought to secrete a fluid containing digestive enzymes and electrolytes and use e.g. calcium as a second messenger upon stimulation. Together with their pronounced morphological polarity, they provide a model system to study the effect of different preparation methods for quantitative biological electron probe X-ray microanalysis (EPXMA) of ultrathin sections. Several preparation methods i.e., freeze-drying and plastic-embedding, freeze-substitution (2 days) and freeze-drying of ultrathin cryosections have been applied to examine the retention of sodium, magnesium, phosphorus, sulfur, (chlorine), potassium and calcium in subcellular compartments (basal cytoplasm, apical cytoplasm, mitochondria and zymogen granules). In freeze-substituted samples the phosphorus, potassium and sulfur concentrations were 2-3 times lower in all compartments compared to freeze-dried, plastic-embedded samples. Intracellular potassium-to-sodium ratios obtained on frozen substituted and frozen-dried, plastic-embedded samples were considerably lower than for cryosections. Element gradients between adjacent organelles were large in frozen-dried cryosections, smaller in frozen-dried plastic- embedded samples and insignificant in frozen-substituted samples.

KEY WORDS: Quantitative X-ray microanalysis, Exocrine Pancreas, Low Temperature Methods

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Introduction

The exocrine pancreas as a model system

Pancreatic acinar cells are thought to use calcium as a second messenger because the ionophore A23187 is able to increase enzyme release from these cells in the presence of extracellular calcium (4,6,14,51,56). Physiological secretagogues initiate a rise in cytosolic calcium and rapid increase of Ca^{2+}-efflux (5,29,36) and stimulate enzyme release even in the absence of extracellular calcium (1,5,41,52). This indicates that intracellular stores liberate calcium upon stimulation (49). Various techniques have been used in an attempt to identify these intracellular stores (8,9,10,11,13,25,29) and different organelles have been identified as calcium stores, i.e., mitochondria, microsomes and cell membranes. To which extent the different subcellular compartments are involved in calcium storage and calcium release is still controversial. Since studies of ion transport in insect salivary gland employing EPXMA have proved the validity of the technique (18,19,21), it has been used to analyse the elemental distribution in acinar cells of rat exocrine pancreas (31,43,44,45) before and after cholinergic stimulation.

Preparation methods

The purpose of conventional preparative techniques is not to leave the chemical composition of the specimen unchanged but to preserve ultrastructural details. Preparation methods maintaining the chemical integrity of the sample often result in reduced morphological information and the choice of technique depends on factors such as instrumentation, nature of the elements of interest within the sample, morphological and analytical resolution. Using wet chemical methods to prepare specimens for EPXMA it is not possible to rule out loss and/or translocation of diffusible elements. In a few cases methods used for morphological electron microscopy employing liquid fixatives, embedding media and sectioning onto a liquid surface are applicable for EPXMA studies, e.g., for the identification of precipitates (3,24,25). In order to avoid the dehydration steps and improve the preservation of electrolytes. Yarom et al.(58) used a water soluble urea/glutaraldehyde mixture as embedding medium for skeletal muscle and

myocardium.

The mixture will polymerize under specific conditions (40). Most elements of physiological importance, except for calcium and chlorine, were not adequately preserved. However, the method proved to be superior to conventional preparation methods including dehydration and embedding in Epon 812 with regard to electrolyte preservation. Other preparation techniques described for biological EPXMA comprise the microdroplet method (38) and air dried specimens (16,33,37,55) at ambient temperatures, the latter suffering from severe disadvantages such as chemical contamination from the surrounding fluids (33) and redistribution of elements (17). To overcome these limitations low temperature methods have been introduced. They include processing steps following a rapid freezing such as freeze-drying, plastic-embedding (30,57) freeze-substitution (28,34) freeze-drying of bulk specimen (42) freeze-drying of cryosections (27,48,59) and fully hydrated bulk specimen (35) or fully-hydrated ultrathin cryosections (20,22,23). All these methods have a rapid FREEZING-procedure in common, which is either achieved by bringing the sample in contact with a liquid coolant or with a cold metal surface. Under ideal conditions the cooling can be extremely fast and lead to vitrification of the sample (12). Unfortunately the poor thermal conductivity of the specimen results in vitrification of small volumes at the specimen surface only. The frozen specimen can be analysed either after removal and/or replacement of the water or in the fully hydrated state.

Freeze-substitution is based on a slow replacement of ice by cold substitution liquids (e.g., acrolein in diethyl ether, (34)). The substitution liquid is replaced by an embedding medium and the polymerized blocks are sectioned on a dry knife. The process takes up to 3 weeks and the method is used in several laboratories (28,32,34,39). The reports indicate that the loss of elements can be reduced on the cellular level, but it has not been shown yet, that the original compartmental element distributions are preserved quantitatively.

Freeze-drying and plastic-embedding is based on removal of the water at temperatures at or below 193K. Freeze-drying might introduce artifacts insofar, as elements in the aqueous phase will migrate towards the nearest surface(s) as the drying front proceeds.

Due to the limited spatial resolution of the electron probe, this degree of displacement is usually not a problem for intracellular EPXMA, whereas measurements in the extracellular space where no solid matrix is present and the electrolytes will migrate towards the next cell surfaces are compromised (23). The tissue will then be embedded in either Spurr's low viscosity resin (54), Epon (30) or Lowicryl (57) and cut on a dry knife. But as for the freeze-substitution procedure, possible intracellular dislocation of elements has not been excluded.

Cryosectioning for EPXMA of quench-frozen biological material is a standard procedure in many laboratories now (2,27,45,48,53,59) and after initial disagreements on sectioning temperatures (20,50) most workers consider temperatures around 150K sufficient to prevent diffusion, partial dehydration, melting and recrystallization during sectioning. The sections are transferred using cold transfer devices and analysed in the fully hydrated state (18,22,23) or after external freeze-drying (45) or freeze-drying in the column to prevent rehydration artifacts (27,48,59).

In this study 3 different preparation methods for rat exocrine pancreas cells have been compared to assess their validity for quantitative EPXMA on the subcellular level. Rat exocrine pancreas cells have been chosen as a model because of their morphological polarity which results in good identification of subcellular regions (basal cytoplasm rich in rough endoplasmic reticulum and apical cytoplasm rich in zymogen granules) their interesting physiological processes (exocytosis) and good accessibility for quench freezing with either the "helium-slammer" or copper blocks attached to pliers.

Materials and Methods

Freezing

Male rats (200g) were anaesthesized with Nembutal, dissected and perfused with Krebs-Ringer phosphate buffer (KRPB) for 10 minutes. The pancreas was carefully dissected as intact organ thus avoiding any mechanical damage, placed in a buffer bath and mounted on a frame. The sample was quench frozen against a helium vapor cooled copper block (helium slammer designed and produced by Dr. J. Escaig at the C.N.R.S. in Paris), quickly transferred to and stored in liquid nitrogen.

Freeze-drying, fixation, embedding and sectioning

In order to achieve appropriate freeze-drying several requirements have to be fullfilled. The volume of the sample has to be small, preferably smaller then 1 mm^3. The specimens are placed in a specimen holder (maximum loading 10 samples) the temperature of which can be recorded and regulated from 130K-400K. The specimen holder is placed in a glass cylinder which is immersed in liquid nitrogen, which acts as a cooling trap or condenser for the subliming water. The distance between specimen and condenser is 1.5 cm. The vacuum system of an old SIEMENS ELMISKOP IA provided a vacuum better than 10^{-5}Torr at the gauge. The freeze-drying schedule followed was proposed by Ingram & Ingram (30). Fixation was achieved by heating para-formaldehyde powder to 333K and osmium vapor fixation by introducing OsO_4 crystals. For all these operations it is not necessary to break the vacuum. In order to introduce the resin however, the vacuum was broken by leaking dry nitrogen gas into the unit. Spurr low viscosity resin was used as embedding medium. The blocks were cured overnight at 333K and sectioned at a nominal thickness of 200-400 nm using a dry knife and a Sorvall MT 5000 or an LKB ultratome V ultramicrotome. The sections were transferred with an eyelash and mounted on Formvar coated 75 mesh copper grids, carbon coated and analysed.

Freeze-substitution

Small quench-frozen tissue fragments were placed on frozen dry acetone containing 2% osmium at 90K. The vials were then placed on dry ice in an insulated box. The temperature rose from 193K to 203K in 27 hours and from 203K to 293K in 24 hours. The dehydrated tissue blocks were embedded in Spurr low viscosity resin and processed as described for the frozen-dried samples. For morphological evaluations the sections were post stained with uranyl acetate and lead citrate.

Cryosectioning

Freezing of the tissue against a helium vapor cooled copper block results in a thin sample with two flat surfaces one of which is well frozen (referred to as the freezing front). Tissue was mounted over liquid nitrogen at approximately 110K with the freezing front "en face" on an aluminium pin using butyl-benzene as glue, or transversely to the knife in a vise-type chuck. Cryosections were cut with a Sorvall MT 5000 and an FS 1000 cryo attachment, at 200-250 nm nominal thickness using glass knives. The temperature of the knife and specimen was 138-128K and 126-116K for the chamber atmosphere. Sections were transferred, using an eyelash, to 75 mesh copper grids previously coated with Formvar. The grids were moved to a grid holder and sandwiched under a second Formvar film. A precooled GATAN cold transfer stage was used to transfer the grid holder assembly into the microscope and to freeze-dry the sections at 193K in the column.

X-ray microanalysis and quantitation

Analysis was done in a JEOL 100CX column equipped with a (S)TEM unit, a free condenser lens 1 control and a LINK 860 series 2 energy-dispersive X-ray spectrometer. The detector had a resolution of 148 keV and was mounted horizontally. The details for routine measurements are described elsewhere (48). Standards for the quantitation procedure on plastic-embedded samples were prepared as described earlier (46). Cryostandards as described by Hagler et al. (26) and later by Roos and Morgan (47) were also used.

Three animals were used for the plastic embedded group and four were used for the cryosectioned group. Up to 8 cells per animal were chosen and per cell three spectra of each subcellular compartment were taken and averaged. The values of the individual cells were pooled and used for the calculation of average concentration values and standard deviations within an experimental group. The subcellular compartments analysed were: zymogen granules, mitochondria, basal cytoplasm and apical cytoplasm.

Results

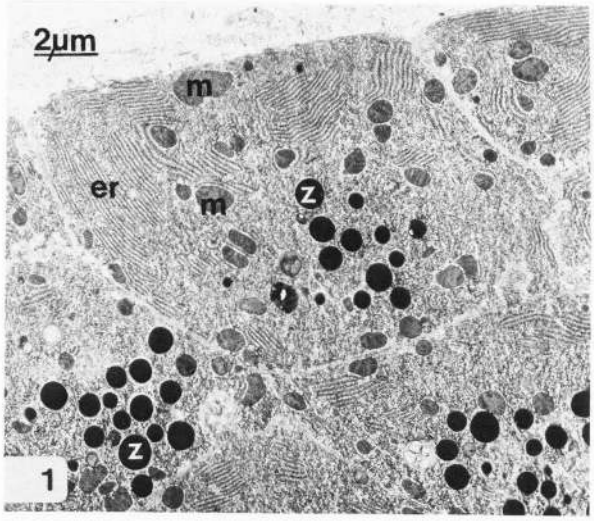
Morphology and structural details

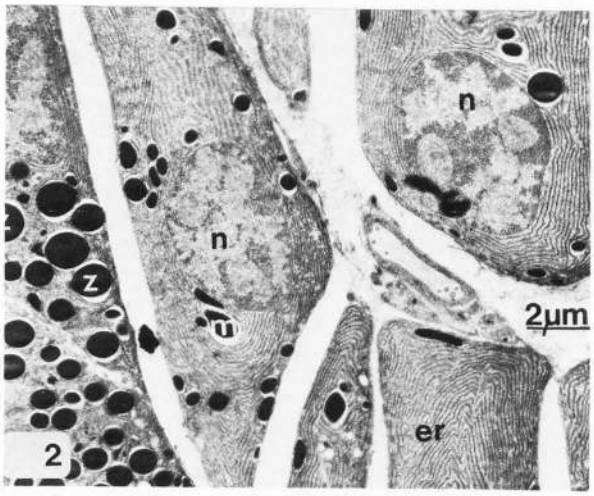
Freeze-substitution was used routinely on all samples to check for freezing quality and morphological preservation. The micrograph in Fig.1 shows the first cell layers close to the freezing front. The cells show only slight morphological signs of ice crystal formation in the nucleus. Freezing is very good in general, even though the depth of well frozen tissues varied from sample to sample. Starting from the freezing front, the layer of well frozen cells was up to 60 µm thick. Contrast in the images was good in the absence of any post stain and it was possible to identify organelles easily. Golgi regions could not be identified in any of the preparation procedures. Fig. 2 shows a micrograph of an ultrathin wet cut section of a frozen-dried, osmium vapor fixed sample. Morphological preservation was excellent and all the subcellular compartments are recognizable. Dehydration of the tissue by freeze drying and subsequent infiltration with plastic was obviously complete. The cryosection in Fig. 3 shows typical sectioning artefacts such as compression and chatter (7) which can also be observed when using dry knives to cut plastic embedded material. Contrast was very good in the absence of any stain and the morphological integrity of the cells was maintained without chemical fixation. Holes and cracks in the sections are possibly a result of handling them with an eyelash.

As a rule X-ray microanalysis has been performed on the cell layers closest to the freezing front, where the dimension of the ice crystal damage is much smaller than the probe size, suggesting that the dimension of any possible elemental displacement was smaller than the spatial resolution of the probe. Frozen-dried cryosections (CS) sections of frozen-substituted material (FS) and frozen-dried, plastic embedded material (FDPE) were all stable under the beam.

Analytical results

Since the FS samples have been substituted in an anhydrous acetone/osmium solution, the concentration ratios are compared with FDPE samples that have been osmium vapor fixed. We did not measure chlorine concentrations in plastic sections since the resin contained chlorine. We do not know the hydration of the different subcellular compartments in CS samples and to which degree the resin penetrates the subcellular compartments in FS and FDPE samples. Table 1 therefore summarizes the concentration values normalized to sulfur, the element that most likely is least affected by the different preparation procedures since it is bound to macromolecules. FS samples clearly show a loss of potassium. The K/S ratio is between 2 to 3 times lower than in FDPE samples. The P/S ratio for ER suggests, that during freeze-substitution a major part of the phospholipids gets lost or redistributed. The comparison of the P/S and K/S ratios for ER and AC in FDPE and CS samples indicates, that in FDPE samples a considerable part of the phosphorus and potassium is lost. Zymogen granules show a slight loss of potassium in FDPE samples compared to CS samples, whereas the other elemental concentrations are not affected. To compare another important parameter we summarized the potassium/sodium ratios in different compartments for all the three preparation methods in Table 2. In apical cytoplasm, basal cytoplasm and mitochondria the ratio is lower in FDPE samples. This is mainly due to a loss of potassium, whereas in zymogen





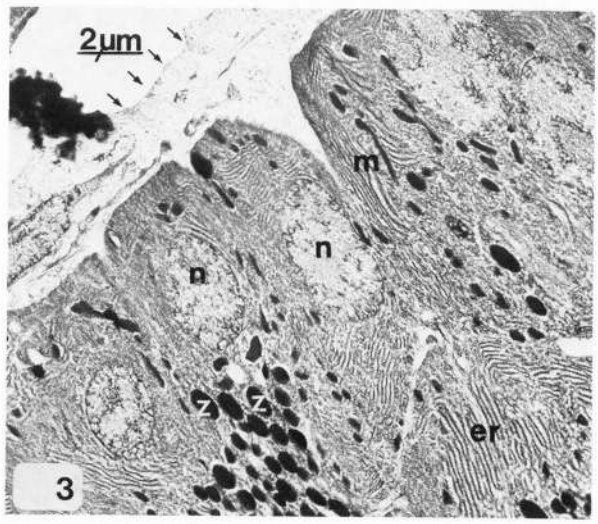


FIG. 1. Ultrathin section of quench-frozen, freeze-substituted rat exocrine pancreas. Section poststained.
z = zymogen granules, er = basal cytoplasm, m = mitochondria.

FIG. 2. Ultrathin section of quench-frozen, frozen-dried, osmium-vapor fixed, plastic-embedded rat exocrine pancreas.
z = zymogen granules, n = nuclei, er = basal cytoplasm, m = mitochondria.

FIG. 3. Frozen-dried ultrathin cryosection of quench-frozen rat exocrine pancreas. Arrows indicate freezing front.
z = zymogen granules, er = basal cytoplasm, n = nuclei.

granules potassium is slightly elevated, indicating a redistribution within the cell. The situation for FS samples is that most of the potassium is lost and the original potassium/sodium ratios are gone.

Discussion

In order to perform meaningful EPXMA on biological soft tissue, extreme care has to be taken of each step in the preparation chain. These steps are: (i) specimen treatment before physical fixation (freezing), (ii) freezing, (iii) dehydration, (iv) vapor fixation, (v) sectioning, (vi) transfer of the specimen to the microscope and (vii) exposure to the beam. Different treatments of the pancreas prior to freezing have been reported (43,45). Unlike those studies we tried to avoid additives such as cryoprotectants in the perfusion medium and stress exerted on the tissue by cutting it into small pieces. Even though no cryoprotectants were used, the freezing was good and reproducible, due to the very low temperature of the metal mirror (15). We did not check our samples for amorphous ice, but examined all the frozen samples routinely for freezing quality by freeze-substitution. Freeze-substitution takes place above the devitrification temperature for water and one might expect ice-crystal growth to occur. Freeze-substitution will represent a "worst case" situation and we are therefore confident that our freezing method does not produce gross ice crystal damage which would lead to redistribution of the analysed elements. Further processing of the quench-frozen samples includes a dehydration step, either by replacing water with anhydrous acetone (FS) or subliming water at low temperatures before (FDPE) or after (CS) sectioning. Whereas partial rehydration is usually not problematic for FS samples, the very hydrophilic, frozen-dried samples are more difficult to handle. For FDPE samples we made sure, that there was no contact with the atmosphere (breaking the vacuum with dry nitrogen gas, etc.). The fully-hydrated cryosections were transferred into the microscope and frozen-dried in the column ("internal freeze-drying").

TABLE 1

Elemental concentrations as measured on FS, FDPE and CS samples normalized to sulfur (= 100). ZG = zymogen granules, AC = apical cytoplasm, ER = basal cytoplasm and M = mitochondria.

		COMPARTMENT			
		ZG	AC	ER	M
	ELEMENT				
FS	Na	40	65	116	74
	Mg	10	34	39	7
	P	45	166	48	202
	K	36	56	87	57
	Ca	6	2,4	6,4	4,8
FDPE	Na	16	46	100	41
	Mg	5	23	50	16
	P	19	209	531	216
	K	31	121	288	142
	Ca	6,8	4,0	4,8	2,2
CS	Na	15	89	168	49
	Mg	6	41	102	25
	P	18	480	1324	294
	K	18	304	732	198
	Ca	7,7	9,4	9,4	4,0

TABLE 2

Intracellular potassium-to-sodium ratios in different subcellular compartments after different preparation methods. AC = apical cytoplasm, ZG = zymogen granules, ER = basal cytoplasm, M = mitochondria. FDPE = frozen-dried, plastic-embedded, FS = frozen-substituted, CS = cryosectioned.

	COMPARTMENT			
	AC	ZG	ER	M
FDPE	2,61	1,81	2,88	3,45
FS	0,85	0,91	0,75	0,77
CS	3,39	1,15	4,36	4,00

In our laboratory "external freeze-drying" (transfer of the cryosections to a carbon evaporation unit and subsequent drying and coating) did not prove useful. The values for Na and especially Cl were much higher than after "internal freeze-drying" (data not shown). Freeze-drying and plastic-embedding (30,57) and freeze-substitution (28,34) have been claimed to be useful preparative methods for EPXMA, but quantitative measurements have mainly been done on the cellular level. Since we are interested in intracellular electrolyte shifts we tested the three methods on our biological model system. Except for calcium all elements of interest could be detected in all compartments. The loss of elements in FS samples is probably due to the use of osmium and exposure to acetone at room temperature in the last step of the substitution procedure and is in contradiction to what has been reported for plant tissue. Retention of elements might improve by using more sophisticated substitution protocols. But since we were interested in finding a preparation method which provides us with reliable results in the shortest possible time, we did not consider a 20 days freeze-substitution schedule practical for our purposes.

Elemental loss is less pronounced in FDPE samples, but still considerable. Especially the phosphorus and potassium values are lower in basal and apical cytoplasm. One explanation could be that membrane phospholipids get dissolved and redistributed by the highly hydrophobic resin. As a result the membranes get leaky for potassium, thus leading to potassium loss from the cell. The intracellular potassium-to-sodium ratio is an important parameter if one is interested in stimulation of exocrine glands. A preparation method for such tissue must therefore preserve the original elemental ratio. We found that the potassium-to-sodium ratio is most pronounced in cryosections and least pronounced in FS samples. We conclude, that plastic-embedding disturbs the potassium-to-sodium balance within intracellular compartments mainly by redistribution and/or loss of potassium. Cryosectioning is a standard procedure in our laboratory and not more time consuming than any of the described "plastic"-methods. The results from cryosections seem to be more reproducible as indicated by the lower standard deviation of the measurements. This is probably due to the fact that dry cut plastic sections are thicker than cryosections and the analysed volume might include partly other structures than the subcellular compartment one seems to analyse.

Acknowledgements

We would like to thank Dr. J.Escaig (C.N.R.S., Paris) for collaborating on the use of his helium-slammer and Ulla Kinde for skilled cryosectioning. Norbert Roos is recipient of a NAVF fellowship.

References

1. Argent BE, Case RM, Scratcherd T (1973). Amylase secretion by the perfused cat pancreas in relation to the secretion of calcium and other electrolytes and as influenced by the external ionic environment. J.Physiol.(Lond.) 230,575-593.

2. Barnard T (1982). Thin frozen-dried cryosections and biological X-ray microanalysis. J.Microsc. 126,317-332.

3. Barnard T, Roos N, Stenmark ST (1984). X-ray microanalytical cytochemistry of calcium in secretory cells. Proc. VIIth Intern.Congress of Histochem. and Cytochem. Lunkka, Helsinki. pp. 28.

4. Bonting SL, DePont JJHHM, Jansen IWCM (1980). The role of sodium ions in pancreatic fluid secretion in the rabbit. J.Physiol. 309,533-546.

5. Case RM, Clausen T (1973). The relationship between calcium exchange and enzyme secretion in the isolated rat pancreas. J.Physiol. (Lond.) 235,75-102.

6. Chandler DE, Williams JA (1977). Fluorescent probe detects redistribution of cell calcium during stimulus-secretion coupling. Nature 268,659-660.

7. Chang J-J, MacDowell AW, Lepault J,Freemann R, Walter CA, Dubochet J (1983). Freezing, sectioning and observation artefacts of frozen-hydrated sections for electron microscopy. J.Microsc. 132, 109-123.

8. Cecarelli B, Clemente F, Meldolesi J (1975). Secretion of calcium in pancreatic juice. J.Physiol. (Lond.) 245,617-638.

9. Clemente F, Meldolesi J (1975). Calcium and pancreatic secretion. I. Subcellular distribution of calcium and magnesium in the exocrine pancreas of the guinea pig. J.Cell Biol. 65,88-102.

10. Clemente F, Meldolesi J (1975). Calcium and pancreatic secretion dynamics of subcellular calcium pools in resting and stimulated acinar cells. Br.J.Pharmacol. 55,369-379.

11. Dormer RL, Williams JA (1981). Secretagogue-induced changes in subcellular Ca^{2+} distribution in isolated pancreatic acini. Am.J. Physiol. (g) 240,130-140.

12. Dubochet J, Lepault J, Freeman, F, Berriman JA, Homo J-C (1982). Electron microscopy of frozen water and aqueous solutions. J.Microsc. 128, 219-237.

13. Edelmann L (1978). A simple freeze-drying technique for preparing biological tissue without fixation for electron microscopy. J.Microsc. 112, 243-248.

14. Eimerl S, Savion N, Heichal O, Selinger Z (1974). Induction of enzyme secretion in rat pancreas slices using the ionophore A-23187. J.Biol.Chem. 249,3991-3993.

15. Escaig J (1984). Control of different parameters for optimal freezing conditions. In: The Science of Biological Specimen Preparation. (eds) JP Revel, T Barnard, GH Haggis. Scanning Electron Microsc. Inc., AMF O'Hare, IL. 117-122.

16. Fischer E, Tromlitas K (1980). X-ray microprobe analysis of chloragosomes of untreated and of EDTA treated Lumbricus terrestris by using fresh air dried smears. Acta Histochem. 66,233-242.

17. Gullasch J, Kaufmann R (1974). Energy-dispersive X-ray microanalysis in soft biological tissues: Relevance and reproducibility of the results as depending on specimen preparation (air-drying, cryofixation, cool-stage techniques). In: Microbe Analysis as Applied to Cells and Tissues. (eds) TA Hall, P Echlin, R Kaufmann. Academic Press London New York. 175-190.

18. Gupta BL, Berridge MJ, Hall TA, Moreton RB (1978). Electron microprobe and ion-selective microelectrodes studies of fluid secretion in the salivary glands of Calliphora. J.Exp.Biol. 72, 261-284.

19. Gupta BL, Hall TA (1979). Quantitative electron-probe X-ray microanalysis of electrolyte elements within epithelial tissue compartments. Federation Proc. 38,144-153.

20. Gupta BL, Hall TA (1981). The microanalysis of frozen-hydrated sections in scanning electron microscopy: an evaluation. Tissue and Cell 13, 623-643.

21. Gupta BL, Hall TA (1981). Microprobe analysis of fluid transporting epithelia: Evidence for local osmosis and solute recycling. In: Water transport across epithelia: Barriers, gradients and mechanisms. Alfred Benson symp. 15. (eds) HH Ussing, N Bindslev, NA Lassen, O Sten-Knudsen. Munksgaard Copenhagen Denmark. 17-35.

22. Gupta BL, Hall TA (1981). Ions and water in cells and tissues studied by microprobe analysis of frozen-hydrated sections in the SEM. 39th Ann.Proc. Electron Microscopy Soc.Amer., Atlanta, Georgia. (ed) GW Bailey, Claitor's Publ.Div., Baton Rouge, LA. 654-657.

23. Gupta BL, Hall TA (1984). X-ray microanalysis of frozen-hydrated cryosections: Problems and results. Proc. 8th Europ.Cong.Electron Microsc. (eds) A Csanady, P Roehlich, D Szabo. Budapest, Programme committee, 1665-1675.

24. Gylseth B, Mowe G, Wannag A (1983). Fibre type and concentration in the lungs of workers in an asbestos cement factory. Brit.J.Ind.Med. 40, 375-379.

25. Haase W, Friese W, Heitmann A (1984). Electron- microscopic demonstration of the distribution of calcium deposits in the exocrine pancreas of the rat after application of carbachol, atropine, cholecystokinin and procaine. Cell Tissue Res. 235,683-690.

26. Hagler HK, Lopez LE, Flores JS, Lundswick RJ, Buja LM (1983). Standards for quantitative energy- dispersive X-ray microanalysis of biological cryosections: Validation and application to studies of myocardium. J.Microsc. 131,221-234.

27. Hagler HK, Buja ML (1984). New techniques for the preparation of thin freeze-dried cryosections for X-ray microanalysis. In: The Science of Biological Specimen Preparation. (eds) JP Revel, T Barnard, GH Haggis. Scanning Electron Microsc. Inc., AMF O'Hare, IL. 161-166.

28. Harvey DMR (1980). The preparation of botanical samples for ion localization studies at the subcellular level. Scanning Electron Microsc. 1980;II:409-419.

29. Heisler S, Fast D, Tenenhouse A (1972). Role of Ca^{2+} and cAMP in protein secretion from rat exocrine pancreas. Biochim.Biophys.Acta 279, 561-572.

30. Ingram FD, Ingram MJ (1984). Influence of freeze-drying and plastic-embedding on electrolyte distributions. In: The Science of Specimen Preparation. (eds) JP Revel, T Barnard, GH Haggis. Scanning Electron Microscopy Inc., AMF O'Hare, IL, 167-174.

31. Kuijpers G (1984). Transport mechanisms of pancreatic fluid and enzyme secretion. Part of the Doctoral Thesis, University of Nijmegen, Netherlands.

32. Laeuchli A, Spurr, AR, Wittkopp RW (1970). Electron probe analysis of freeze-substituted epoxy-resin embedded tissue for ion transport studies in plants. Planta 95,341-350.

33. Lechene CP, Bronner C, Kirk RG (1976). Electron probe microanalysis of chemical elemental content of single human red cells. J.Cell.Comp.Physiol. 90,117-126.

34. Marshall TA (1980). Sections of freeze-substituted specimens. In: X-ray Microanalysis in Biology. (ed) MA Hayat. MacMillan Publishers LTD (London). 207-239.

35. Marshall AT (1980). Frozen-hydrated bulk specimen. In: X-ray Microanalysis in Biology. (ed) MA Hayat. MacMillan Publishers LTD (London) 167-196.

36. Mathews EK, Petersen OH, Williams JA (1973). Pancreatic acinar cells: Acetylcholine-induced membrane depolarization, calcium efflux and amylase release. J.Physiol. (Lond.) 234,689-701.

37. Morgan JA (1981). A morphological and electron microprobe study of the inorganic composition of the mineralized secretory products of the calciferous gland and chloragogenous tissue of the earthworm Lumbricus terrestris: The distribution of injected strontium. Cell Tissue Res. 220,829-844.

38. Morgan JA, Davies TW (1982). An electron microprobe study of the influence of beam current density on the stability of detectable elements in mixed-salts (isoatomic) microdroplets. J.Microsc. 125,103-116.

39. Pallaghy CK (1973). Electron probe microanalysis of potassium and chloride in freeze- substituted leaf sections of zea mays. Austr.J.Biol.Sci. 26,1015-1034.

40. Pease DC, Peterson RG (1972). Polymerizable glutaraldehyde-urea mixtures as polar water-containing embedding media. J.Ultrastruct.Res. 41,133-159.

41. Petersen OH, Ueda N (1976). Pancreatic acinar cells: The role of calcium in stimulus secretion coupling. J.Physiol. (Lond.) 254,583-606.

42. Roomans GM, Boekstein A (1978). Distribution of ions in Neurospora crassa determined by quantitative electron microprobe analysis. Protoplasma 95,385-392.

43. Roomans GM, Barnard T (1982). Calcium and magnesium in exocrine secretion. An X-ray microanalytical study. Scanning Electron Microsc. 1982;I:229-242.

44. Roomans GM, Wei X, Ceder O, Kollberg H (1982). The reserpinized rat in the study of cystic fibrosis: X-ray microanalysis of submandibular gland and pancreas. Ultrastruct.Pathol. 3,285-293.

45. Roomans GM, Wei X (1985). X-ray microanalysis of resting and stimulated rat pancreas. Acta Physiol.Scand. 124,353-359.

46. Roos N, Barnard T (1984). Aminoplastic standards for quantitative X-ray microanalysis of thin sections of plastic-embedded biological material. Ultramicroscopy 15,277-286.

47. Roos N, Morgan JA (1985). Aminoplastic standards for electron probe X-ray microanalysis (EPXMA) of ultrathin, frozen-dried cryosections. J.Microsc. 140,RP3-RP4.

48. Roos N, Barnard T (1985). A comparison of subcellular element concentrations in frozen-dried, plastic-embedded, dry-cut sections and frozen-dried cryosections. Ultramicroscopy 17,335-344.

49. Rubin RP (1980). Calcium and cellular secretion. Plenum Press, New York.

50. Saubermann AJ, Riley WD, Beeuwkes III R (1977). Cutting work in thick section cryomicrotomy. J.Microsc. 111,39-49.

51. Schreurs VVAM, Swarts HGP, DePont JJHHM, Bonting SL (1976). Role of calcium in exocrine pancreatic secretion. III. Comparison of calcium and magnesium movements in rabbit pancreas. Biochim.Biophys.Acta 436,664-674.

52. Schulz I, Ullrich KJ (1978). Transport processes in the exocrine pancreas. In: Membrane Transport in Biology, Vol. IVB, (ed) G Giebisch, Springer Berlin. 811-852.

53. Somlyo AV, Shuman H, Somlyo AP (1977). Elemental distribution in striated muscle and the effect of hypertonicity. J.Cell Biol. 74,824-857.

54. Spurr AR (1969). A low viscosity epoxy resin embedding medium for electron microscopy. J.Ultrastruct.Res. 26,31-43.

55. Werner G, Gullasch J (1974). Roentgenmikroanalyse an Rattenspermien. Mikroskopie 30,95-101.

56. Williams JA, Lee M (1974). Pancreatic acinar cells: Use of a Ca^{2+} ionophore to separate enzyme release from the earlier steps in stimulation secretion coupling. Biochim.Biophys.Res.Commun. 60,542-548.

57. Wroblewski R, Wroblewski J (1984). Freeze-drying and freeze-substitution combined with low temperature embedding. Preparation techniques for microprobe analysis of biological soft tissues. Histochem. 81,469-475.

58. Yarom R, Peters, PD, Hall TA (1974). Effect of glutaraldehyde and urea embedding on intracellular ionic elements. J.Ultrastruct.Res. 49,405-418.

59. Zierold K (1984). Experiences in X-ray microanalysis of freeze-dried cryosections. Scanning Electron Microsc. 1984;IV:1867-1874.

Discussion with Reviewers

A.T. Marshall: I find this paper disturbing in that you are implying that cryosectioning is a superior method to freeze-substitution and freeze-drying. What the paper may show is that your procedures for freeze-substitution and freeze-drying were inadequate. I am not convinced that you took adequate precautions with these two procedures. The freeze-substitution procedure which you employed has been long known to be inadequate in comparison to other methods (see Van Zyl et al. Micron 7:213-224, 1976.)

H.Y. Elder: For freeze drying you can place the specimen in a holder, the temperature of which can be recorded or regulated from 130K-400K. Give reference to this or give further details please. You can introduce OsO_4 crystals without breaking the vacuum. Is this as described in reference 30? - or give more details please. Why do you have to break the vacuum to introduce resin (c.f. reference 30)?

H.Y. Elder: You state that there has been a redistribution of K in the cells in the FDPE samples. Do you think that this is caused entirely by movement in the non-polar resin? Despite your stated precautions, could your tissue have been exposed to water vapor at the time of removal from the drier? Is the glass outer cryo-trap removed from the LN_2 Dewar at this time and if so what happens to the cryo-trapped water? Do you include "molecular sieve" in the chamber? And if the specimen holder is removed while the outer chamber is kept in the Dewar, how do you keep the specimens dry until resin embedded?

L. Edelmann: By comparing Fig.1 with Fig.2 it is immediately evident that large shrinking artifacts are present in the FDPE sample (large free spaces between the cells). According to our experience this may be due to incomplete drying at a sufficient low temperature; upon warming subcellular redistribution of ions is then conceivable. I suggest to try longer freeze drying times and also to avoid OsO_4 vapour fixation.

Authors: Building the freeze-dryer we followed the description given by F.D. Ingram & M.J. Ingram (30). However, we found that it is more convenient and easier to introduce the resin with a partly open lid, using dry nitrogen gas to flush the chamber continuously. The temperature of the specimen was always kept at least 30 degrees above the condenser temperature. We therefore assume that the tissue was not exposed to water vapor. We do not use molecular sieves (for details see ref. 48).

Shrinking artifacts in Fig.2 are indeed relatively large. We did not measure to which extent shrinkage occurred, but it varied from sample to sample, which might reflect the difference in sample size. If shrinkage is, as you suggest,a sign for incomplete drying, we agree that redistribution might have occurred. With the protocol employed (48) we are, however, confident that our freeze-drying procedure was adequate. As for the FS samples we agree that more sophisticated protocols might improve the results. But as discussed, we do not find preparation methods very useful that last for three weeks and longer and therefore tried a shortened version, especially since no quantitative data on the subcellular level for FS samples have been reported so far.

H.Y. Elder: Approximately what were the dimensions of your "small tissue fragments" used in freeze substitution? Do you know what the time course of the "overnight slow warm-up" in acetone was? (in our laboratory we have found that 1-2 weeks in acetone at $-80^{\circ}C$, with periodic agitation are required to dehydrate mm^3 tissue blocks to the center and that diethyl ether, at twice the dehydration time, is a much superior substitution fluid for EPXMA). Do you think that when your tissues warmed above $0^{\circ}C$ any remaining water in the center might come out sufficiently rapidly to cause an acetone/water mixture capable of causing the observed electrolyte losses?

Authors: The tissue was quench-frozen against a cold copper block. The result is a very thin sample ≤ 200 µm. The fragments used were approximately 1-2 mm^2 large and had a volume of ≤ 0.4 mm^3. Tissue fragments with such a small volume and a high surface to volume ratio should be dehydrated even with relative short substitution times.

K. Zierold: The ice crystal size in the cryosections (Fig. 3) seems to be larger than after freeze-substitution (Fig. 1) and freeze-drying followed by plastic embedding. How do you explain this phenomenon?

Authors: The variation in ice crystal size is not due to the preparation procedure, but to the freezing. We are looking at different samples in Fig.3 and Fig.1.

G.M. Roomans: On one hand you use freeze-substitution to check the quality of your freezing (which is very good), but on the other hand you point out that freeze-substitution takes place above the devitrification temperature and that ice-crystal growth might be expected. Isn't that contradictory?
Authors: Not really. We state that checking the freezing quality using FS represents a worst case situation because it is not possible to freeze-substitute below the devitrification temperature. Besides, our samples are probably not vitrified to start with. A recent study (R.A. Steinbrecht (1985), J.Microsc. 140, 41-46) shows that ice crystal growth is a much slower process than anticipated before.

K. Zierold: Your main result is that intracellular element gradients are better preserved in cryosections than after freeze-substitution or freeze-drying followed by plastic embedding. Is this result specific for the studied tissue type or does it generally hold for cells?
Authors: Since there is only a few if any quantitative EPXMA studies with FDPE and FS samples at the subcellular level, the results reported have to be considered to be specific for the studied tissue.

L. Edelmann: It would be interesting to know what percentage of ions (especially K^+) is lost from the cells into the embedding medium after freeze drying and embedding. Did you determine elemental loss by radioactivity measurements or did you analyse large areas in cryosections and in sections of FDPE tissue? Did you find K in the embedding medium?
Authors: We do not know the percentage of ions that is lost into the resin and we did not determine the loss by means of radioactivity measurements. Since the resin has been changed once before polymerization and the tissue volume is very small compared to the volume of resin it was not possible to detect potassium in the embedding medium using EPXMA.

H.K. Hagler: Have the authors noted any morphological changes in their externally freeze-dried cryosections that might be attributed to lipid melting at room temperature? This would be in addition or superimposed on any rehydration changes which might also be encountered when using external freeze-drying.
Authors: Externally frozen-dried cryosections showed signs of rehydration (blurred details). We do not know to what extent these morphological changes can be attributed to lipid melting.

H.K. Hagler: Have you had a chance to compare freezing results obtained from the helium vapor cooled metal block versus nitrogen cooled copper blocks?
Authors: We could compare freezing results and Fig.3 shows the tissue frozen with nitrogen cooled copper blocks attached to pliers. The cell layers close to the freezing front are well frozen, but the depth of well frozen tissue is reduced compared to "helium freezing". The freezing with the helium-slammer seems to be more reproducible (90% of the samples are very well frozen) because it is independent of the operator.

G.M. Roomans: How do you envisage a regulatory role for phospholipids in transport of K^+ ions across the cell membrane in a completely dehydrated cell?
Authors: One explanation for the loss of potassium in resin embedded samples might be, that the phospholipids of the membranes get dissolved in the hydrophobic resin. The membrane would then be a physically disrupted barrier.

K. Zierold: The comparison of element concentrations found in cryosections and after freeze-drying followed by plastic embedding shows differences not only for the diffusible ions such as sodium and potassium, but also for elements mainly bound in macromolecules such as phosphorus. How do you explain this result?
Authors: We think that especially the membrane phospholipids might be partly dissolved in the hydrophobic resin, leading to redistribution of phosphorus within and loss out of the cell.

K. Zierold: Why are high intracellular gradients in the element concentration closer to the native state than lower ones? Can you exclude element specific segregation or precipitation artifacts caused by freeze-drying?
Authors: Upon freeze-drying solutes will be deposited at the next solid surface as the drying front proceeds. We can not exclude precipitation and segregation. However, we are analysing whole organelles (e.g. mitochondria) and relatively large areas (e.g. basal cytoplasm), so that the analysed area is much larger than segregation and precipitation artifacts. If they really do exist, they will affect the FDPE samples in a similar way, so that a comparison of FDPE and CS samples still makes sense.