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A COMPARISON OF THREE LOW TEMPERATURE TECHNIQUES OF
SPECIMEN PREPARATION FOR X-RAY MICROANALYSIS

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Abstract

A quantitative x-ray microanalytical comparison of intracellular ion concentrations in two tissues (chicken kidney and duck nasal salt gland), prepared as bulk frozen-hydrated, embedded freeze-dried and freeze-substituted samples, shows that there are similar losses of K⁺ and P in freeze-dried and freeze-substituted samples in both types of tissue. It is suggested that this may be due to extraction by chloride-free Spurr's resin during infiltration. There was also an indication of an increase in Na⁺ concentration in freeze-dried samples.

In chicken kidney cells there was a reduction in Mg⁺⁺ and Ca⁺⁺ concentrations in freeze-dried and freeze-substituted samples and an increase in Cl⁻ concentration in freeze-dried samples (which may have been partially due to contamination) and a decrease in freeze-substituted samples. In spite of these differences between kidney preparations, consistent differences in Na⁺ and Cl⁻ concentrations between kidneys from normal chickens and chickens infected with IBV (infectious bronchitis virus) which causes perturbations in kidney physiology were observed in frozen-hydrated, freeze-dried and freeze-substituted preparations.

KEY WORDS: Biological x-ray microanalysis, low temperature, diffusible ions, frozen-hydrated bulk samples, freeze-substitution, freeze-drying.

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Introduction

The determination of the distribution of ions in cells and extracellular space has been an objective of biological analytical electron microscopy. In recent years this objective has been realised and applied to a number of cell types largely through the use of freeze-dried cryosections (reviewed by LeFurgey et al., 1988). Whilst sections which are maintained in the frozen-hydrated state during x-ray microanalysis may be the optimum procedure, the technical difficulties are formidable and only Gupta and Hall have consistently made such analyses (Hall and Gupta, 1983). Frozen-hydrated bulk samples have also been used for analysis (Marshall, 1987).

A problem with the forgoing preparations is that they are not stable in the sense that they can be readily stored, analysed and re-analysed. It is also very difficult to locate specific areas in a sample, successful cryosectioning and fracturing being largely random in nature. Thus there are potential advantages in using freeze-dried or freeze-substituted samples which have been resin embedded prior to sectioning.

Whilst there is no doubt that diffusible ions are retained in embedded freeze-dried and freeze-substituted tissues (e.g. Van Zyl et al., 1976; Marshall, 1980a; Edelmann, 1986; Appleton, 1987; Wroblewski et al., 1987; Wilson et al., 1988), the question of whether there are losses or movements of these ions is still unresolved. The question of ion loss has been addressed by isotope experiments (e.g. Van Zyl et al., 1976) and by comparison with model systems and chemical analyses (Ingram and Ingram, 1984). There have been a number of comparative x-ray analytical attempts, of a qualitative and semi-quantitative nature, to investigate this problem (e.g. Marshall, 1980a; Wroblewski et al., 1985; Chandler, 1985; Meyer et al., 1985; Roos and Barnard, 1985), however, there has been no attempt to compare preparation methods by x-ray microanalysis in a fully quantitative manner.

The principles of freeze-drying and freeze-substitution have been discussed by a number of authors (e.g. Ingram and Ingram, 1980;

Marshall, 1980a; Harvey, 1982; Humbel and Muller, 1986; Robards and Sleytr, 1985; Steinbrecht and Muller, 1987). It is important to note that the conditions of freeze-drying and freeze-substitution can vary considerably. Freeze-drying may rely on the removal of water molecules by a vacuum pump or by trapping on a cold surface. In the latter case this is more akin to a freeze-distillation process. The optimum drying temperatures and times are still a matter of debate (see for example Linner et al., 1986). Similarly freeze-substitution for x-ray microanalysis is a quite different process to freeze-substitution for morphological purposes. Freeze-substitution for x-ray microanalysis necessitates the use of a non-polar solvent. Substitution in a non-polar solvent such as ether is very slow, thus substitution times must be very long and anhydrous conditions must be rigorously maintained from beginning to end.

It is clear that small differences in freeze-drying and freeze-substitution protocols may lead to significant analytical differences. Thus a failure to find analytical consistency between different preparations is more likely to indicate a technical error or inadequacy rather than that the preparation procedure *per se* is invalid. This should be kept in mind when the results presented here are examined.

We present here the results of an attempt to quantitatively compare bulk frozen-hydrated samples with bulk embedded freeze-dried and freeze-substituted samples. The results are sufficiently encouraging, we believe, to warrant further investigation and refinement of techniques.

The protocol of the investigation has been to: a) analyse specimens from the same tissue sample which were processed by different methods b) analyse specimens from the same tissue from different animals which were processed by different methods and c) compare tissues in different pathophysiological states which had been processed by different methods.

Materials and Methods

Experimental animals

Eight 3 week-old specific pathogen free Webster, mini crossbred chickens (Gallus domesticus) (supplied by Animal Health Division, CSIRO, Parkville, Australia) were placed in a controlled temperature room at 12°C. After 3 days, 4 birds (normal animals) were anaesthetised, kidney tissue collected and rapidly frozen. The remaining birds (infected animals) were inoculated with T-strain of IBV (infectious bronchitis virus) as described previously (Condron and Marshall, 1986). Eight days following infection, kidney tissue was collected and frozen in the same manner.

One male Pekin duck (5 weeks) (Anas platyrhynchos) was reared on fresh water (normal animal) and one male duck was reared on saline (2 percent w/v NaCl) (adapted animal) for 1 week prior to light anaesthesia and removal of the

nasal salt glands. Pieces of salt gland were rapidly frozen.

Frozen-hydrated bulk samples

Kidney tissues (1 mm thick slices) from 2 normal and 2 infected chickens and nasal gland tissues from 1 normal and 1 adapted duck were mounted in adjustable sample holders and rapidly frozen by pushing into solid nitrogen (Marshall, 1987). The specimens were capped whilst under liquid nitrogen for transfer to a modified vacuum evaporator for fracturing at -180°C and coating with 10 nm beryllium (Marshall, 1980b; Marshall and Carde, 1983). After recapping the sample holders were stored in liquid nitrogen until transferred to the microscope.

Freeze-dried bulk samples

Kidney tissues from 1 normal and 1 infected chicken were cut into small cubes of approximately 1 mm³ and plunged into liquid propane. Tissue from the nasal salt gland of the adapted duck was treated similarly. The tissues were stored in liquid nitrogen prior to drying.

Each sample was placed in a brass cup on a Peltier stage cooled to -95°C and held under a vacuum better than 5 x 10⁻⁶ Torr for 10 days. After 10 days the temperature was slowly increased to room temperature over a period of 24 hrs. When the samples were at room temperature the vacuum was broken with dry nitrogen gas and the samples immediately infiltrated and embedded in chlorine-free Spurr's resin (Pallaghy, 1973) which had been dried over molecular sieve (Linde 3A pellets).

The polymerised blocks were prepared for analysis by trimming and polishing the surface with a dry glass knife on an ultramicrotome. After mounting on carbon stubs the specimen blocks were coated with 20 nm of aluminium. A section of tissue from the polished blocks was stained with toluidine blue and photographed in a light microscope as an aid to tissue recognition during x-ray microanalysis.

Freeze-substituted bulk specimens

Kidney tissues from 1 normal and 1 infected chicken and tissue from the nasal salt gland of the normal duck was collected and frozen as for the freeze-dried preparation. The frozen tissues were transferred into tubes of substitution media which had been cooled with CO₂/acetone slush. The substitution fluid was a 20 percent (v/v) mixture of acrolein in diethyl ether which had been dried over molecular sieve. The substitution tubes contained 8 ml of fluid and were half filled with molecular sieve (Linde 3A pellets) (Marshall, 1980a). After transfer the tubes were recapped and placed in a low temperature refrigerator at -96°C for 30 days, the temperature was adjusted over a period of 5 days to -60°C, -40°C, -20°C and then to room temperature. The specimens were transferred to a dry box (10% RH at 20°C) and infiltrated in gradations of ether/chlorine-free Spurr's resin and pure resin. The resin and ether were predried with molecular sieve (Marshall, 1980a). The tissues were finally embedded and polymerised in 'Beem' capsules. The

polymerised samples were trimmed, mounted and coated for x-ray microanalysis as described for freeze-dried samples.

Analytical procedures

X-ray microanalysis of all specimens was carried out in a Jeol JSM35 scanning electron microscope fitted with a low temperature (-172°C) specimen stage and two (standard and windowless) energy dispersive Si(Li) detectors interfaced to an EDAX PV9100 multichannel analyser. Take off angles were 40° for the standard detector and 35° for the windowless detector. The microscope was operated at an accelerating voltage of 14.8 kV (nominally 15 kV) and with a column vacuum of 1×10^{-7} Torr. The specimen stage was cooled to -172°C and the temperature was monitored continuously using a thermocouple. A Dindima FT 4011 temporal filter and frame store was used for image enhancement. This proved very effective for the interpretation of images.

Analyses of the frozen-hydrated specimens were conducted for 200 s using a static beam and a beam current of 0.2 nA. Spectra were collected with the windowless detector using a raster of $70 \mu\text{m}^2$ and the backscattered electron (BE) signal from the same area was recorded from a BE detector mounted on the X-ray detector (Marshall, 1981, 1984; Marshall and Condon, 1985). Flat areas of the fractured surface were selected for analysis using a backscattered electron image from a BE detector mounted on the standard x-ray detector (Marshall, 1981). Analyses of the resin embedded specimens were conducted for 200 s using a raster of $2.6 \mu\text{m}^2$ and a beam current of 0.25 nA.

X-ray spectra were reduced using the Edax Halo method of peak fitting. Peak intensities were then corrected for absorption and atomic number effects using factors derived from x-ray depth distribution or $\Phi\rho Z$ curves and converted to concentrations using non-matching standards (Marshall, 1982; Marshall and Condon, 1987). Estimates of intracellular water were derived from oxygen intensities measured using the windowless detector (Marshall, 1982, 1984; Marshall and Condon, 1985). Validation of these techniques has been confirmed using a variety of standards with defined organic matrices (Marshall and Condon, 1987).

An estimate of shrinkage of freeze-dried tissue was obtained from comparison of measurements of micrographs of frozen-hydrated specimens taken during analysis and of the same areas after freeze-drying overnight in the specimen exchange airlock of the microscope (10^{-3} Torr, -160°C to ambient temperature). In order to investigate the possibility of contamination during freeze-drying prior to embedding, a simulated specimen was processed and analysed. The specimen was a nickel sintered disc saturated with distilled water and frozen in solid nitrogen. It was coated with beryllium and analysed prior to and after drying by the method used for freeze-dried tissues.

A paired t-test and a two-way analysis of variance was used to examine the differences

between normal and infected chicken kidneys. Comparisons between the results from the different methods of kidney preparation were analysed by two-way analysis of variance. Differences between preparation methods of duck nasal glands were examined by a one-way analysis of variance.

Results

In order to see structural detail in frozen-hydrated tissues, the frozen specimens must be fractured. An example is shown in Fig. 1. No detectable freeze-drying occurs in this type of sample. Any irregularities in the specimen surface are a result of the fracturing process not ice sublimation. If the surface of the specimen was microtomed then no detail was visible. Detail was similarly extremely difficult to discern in embedded freeze-dried and freeze-substituted specimens which had a microtomed surface. Sufficient detail for analysis could just be obtained with the aid of digital image filtering. Examples are shown in Figs. 2 and 3. These may be compared with a light micrograph of a freeze-substituted specimen (Fig. 4).

X-ray spectra collected by the standard detector from resin embedded freeze-substituted and freeze-dried embedded samples were qualitatively similar to spectra from frozen-hydrated samples (Fig. 5).

Estimates of shrinkage of freeze-dried tissue from measurements of frozen-hydrated tissue before and after drying indicated a linear shrinkage of 6% and this represents a shrinkage in volume of 17%. The values shown in Tables 1 and 2 have been corrected by this factor so the concentrations are estimates of the composition of fresh tissue and can be compared directly with the results from specimens prepared by the other techniques. It has been assumed that the resin totally replaces water in freeze-dried and freeze-substituted specimens (see Ingram and Ingram 1983).

Chicken kidneys

Measurements of element composition of proximal tubular cells in chicken kidneys from the different techniques of specimen preparation are detailed for normal and IBV infected chickens in Table 1. A two-way analysis of variance showed a significant difference between the techniques for all elements. A significant difference was observed for sodium and chloride concentrations between normal and infected birds. There was a consistent decrease in the sodium and chloride concentrations in chickens with IBV infection for all types of preparations.

Sodium and chloride concentrations were higher in freeze-dried samples. Phosphorus, magnesium and calcium were present in higher concentrations in the frozen-hydrated specimens. The levels were approximately double the concentrations of the resin embedded specimens. Potassium concentration was reduced in the freeze-dried and freeze-substituted specimens. Analysis of a sintered nickel disc, saturated with water and frozen, before freeze-drying and

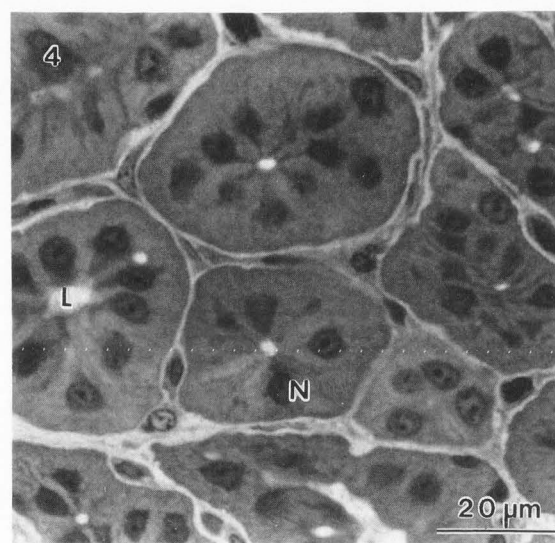
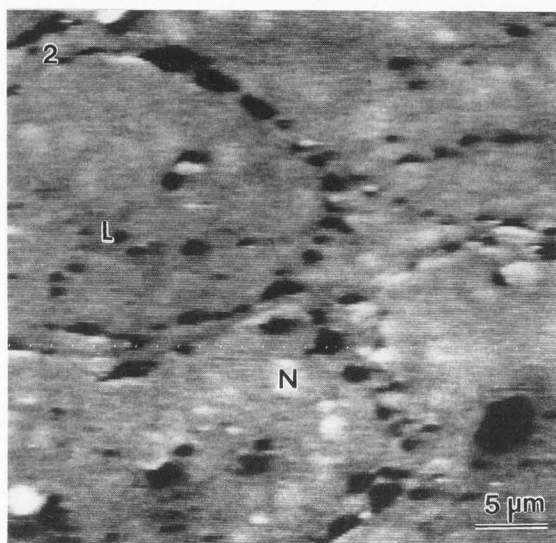
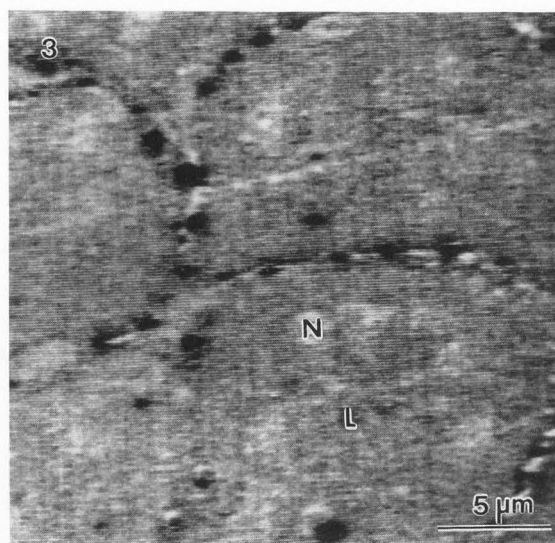
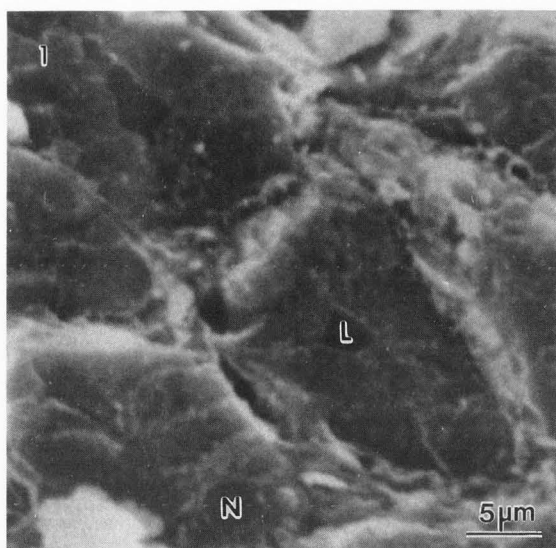


Fig.1 Secondary electron image of fractured surface of frozen-hydrated nasal salt gland showing lumina (L) of secretory tubules and nuclei (N) of principal cells.

Fig.2 Filtered secondary electron image of embedded freeze-dried nasal salt gland showing lumina (L) of secretory tubules and nuclei (N) of principal cells.

Fig.3 Filtered secondary electron image of embedded freeze-substituted nasal salt gland showing lumina (L) of secretory tubules and nuclei (N) of principal cells.

Fig.4 Light micrograph of section of freeze-substituted duck nasal salt gland stained with toluidine blue. Cross-sections of secretory tubules are visible showing lumina (L) and nuclei (N) of principal cells.

analysis after freeze-drying revealed that there was contamination with both chloride and sulphur during the freeze-drying procedure.

Duck nasal salt gland

The preparation of nasal salt glands differed from the chicken kidney preparations in that freeze-substitution and freeze-drying were each compared with frozen-hydrated samples from the same gland. The results of the analyses

are shown in Table 2.

In both freeze-dried and freeze-substituted samples there was a significantly lower potassium and phosphorous concentration compared with the corresponding frozen-hydrated samples. Furthermore in the freeze-dried samples sulphur concentration was also significantly lower whereas sodium concentration was higher.

Comparison of low temperature techniques

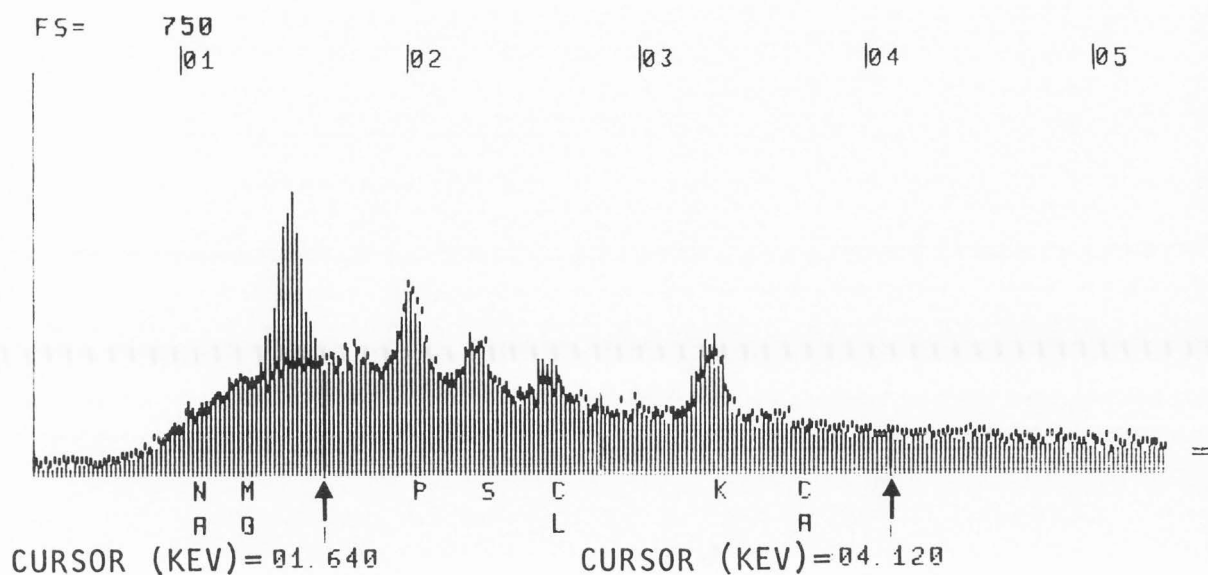


Fig. 5 Comparison of spectra from principal cells of secretory tubules in a frozen-hydrated (dots) and a freeze-substituted (hatched) nasal salt gland.

Table 1. Comparison of specimen preparation techniques for chicken kidney proximal tubule cells.

	Na	K	Cl	Mg	Ca	P	S
	(m mol l ⁻¹)				(m mol kg ⁻¹)		
Normal							
FH (105)	43±2	125±4	41±1	27±1	12±0.5	108±3	71±2
FD (126)	71±4	82±7	70±4	18±1	7±1	67±2	75±2
FS (98)	55±2	60±5	28±2	15±1	7±0.5	56±2	60±1
Infected							
FH (100)	36±3	114±2	30±2	26±1	12±0.5	107±2	70±1
FD (112)	46±2	84±5	55±3	16±1	6±0.5	68±3	74±2
FS (98)	44±2	67±9	23±1	13±1	7±0.5	58±4	60±3
t test	P <0.1	ns	<0.05	ns	ns	ns	ns
ANOVA (Normal vs Infected)	<0.01	ns	<0.01	ns	ns	ns	ns
ANOVA (FH vs FD vs FS)	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Mean ± SE, number of analyses in parentheses. FH, frozen-hydrated; FD, freeze-dried; FS, freeze-substituted.

Table 2. Comparison of specimen preparation techniques for principal cells of duck nasal salt gland.

	Na	K	Cl	Ca	P	S
	(m mol kg ⁻¹)					
Normal						
FH (6)	21±1	129±7	47±2	6±2	136±6	54±0.4
FS (11)	22±4	103±2*	49±3	4±1	99±5*	54±2
Adapted						
FH (10)	19±4	150±7	60±6	3±1	123±6	81±3
FD (29)	26±2*	94±3*	59±2	4±0.4	71±3*	50±1*

Mean ± SE, number of analyses in parentheses. FH, frozen-hydrated; FD, freeze-dried; FS, freeze-substituted.

* ANOVA P<0.05

Discussion

Normal chicken kidney tissue from different animals was processed in different ways to produce frozen-hydrated, embedded freeze-dried and embedded freeze-substituted bulk samples. Proximal tubule cells were analysed in these preparations and the results show that there were marked differences in composition associated with the different preparation techniques. Significant differences were seen for every element analysed between the three preparations. The main distinctions, however, were a marked loss of K⁺, Mg⁺⁺, Ca⁺⁺ and P and an increase in Na⁺⁺ in the freeze-dried and freeze substituted preparations. There was an increase in Cl⁻ and S in the freeze-dried tissue and a decrease in these elements in the freeze-substituted tissue. The increase in Cl⁻ and S might be attributable to contamination during the freeze-drying procedure since some small contamination by these elements was observed when a sintered nickel disc was freeze-dried. A similar pattern of elemental differences was observed in tissue from infected kidneys which was representative of a further four animals.

In the duck nasal gland, tissue samples from a gland from one animal were prepared as a frozen-hydrated specimen and as an embedded freeze-dried specimen. Similarly tissue from another animal was prepared as a frozen-hydrated specimen and as a freeze-substituted specimen. Again as in the chicken kidneys the distinctive differences between the preparations were a decrease in K⁺ and P. In the freeze-dried preparation there was also a decrease in S and an increase in Na⁺. The decrease in S is contrary to the result for freeze-dried chicken kidneys and suggests that the increase in S in

the kidneys could be a consequence of contamination. No significant differences were seen in Cl⁻ and Ca⁺⁺ concentrations, Mg⁺⁺ concentrations were not recorded.

The differences seen in the nasal salt gland preparations were less marked than in the chicken kidney preparations. Because these were comparisons between tissue samples from the same organ which had been prepared in different ways, biological variation could be expected to be less and this may account in part for the smaller differences in comparison to the chicken kidney analyses. The possibility must be considered that there could be a critical dimension for preparing kidney tissue prior to freezing below which ion redistribution occurs. It should be noted, that kidney for frozen-hydrated samples was cut into 1 mm thick slices whereas for freeze-substitution and freeze-drying the samples approximated cubes of 1 mm side. It seems unlikely that such differences would produce the observed differences in ion distribution.

A substantial number of analyses have been carried out on freeze-dried cryosections from a variety of tissues (for a review see Le Furgey et al., 1988). A comparison of frozen-hydrated and freeze-dried sections has also been made by Civan et al., (1980). There can be little doubt that ion concentrations are reliably maintained in freeze-dried cryosections, particularly when they are dried in the microscope column (Hagler and Buja, 1984). Cryosections dry rapidly, the drying time for larger bulk samples is less certain (e.g. Robards and Sleyter, 1985; Edelmann, 1986; Steinbrecht and Muller, 1987). The possibility of ion movement through incomplete drying is therefore a real possibility. In this context it is noteworthy that Inaram and Inaram (1984)

include a drying step of +50°C to remove water not removed by low temperature drying.

Ingram and Ingram (1984) made a careful study of ion retention in muscle during freeze-drying and embedding. They concluded that ion distributions were reliably retained. Wilson et al. (1988) have made quantitative analyses of freeze-dried embedded samples and their results seem to have a physiological validity. A major difference between the procedures of these authors and that used here was in the nature of the resins used. Ingram and Ingram (1984) used Epon 826 and Wilson et al. (1988) used Araldite whereas chlorine-free Spurr's resin (Pallaghy, 1973) was used in the present work. It is also noteworthy that Roos and Barnard (1985), who found discrepancies between freeze-dried cryosections and embedded freeze-dried sections, also used Spurr's resin.

The nature of the resin may be of considerable importance. Chlorine-free Spurr's resin was used for embedding freeze-dried and freeze-substituted samples. In both freeze-substituted samples and freeze-dried samples there were significant losses of K⁺ and P compared to frozen-hydrated samples. Since the only common feature between the two procedures is resin embedding this strongly suggests that extraction of these elements is a consequence of infiltration by the resin. Certainly it can be expected that epoxy resin will dissolve lipids (Luft, 1973) and a decrease in P may be in part a result of phospholipid removal. The apparently greater loss of P in freeze-substituted tissues compared to freeze-dried tissues may be the result of exposure to ether which presumably will also tend to remove lipids. The possibility that the higher viscosity resins have less tendency to remove elements from both freeze-dried and freeze-substituted samples should be investigated.

It must be stressed that the results described here should be considered to be encouraging. The attainment and maintenance of optimal conditions for freeze-drying and freeze-substitution is extremely difficult and any failure in achieving these conditions must inevitably result in disturbances to the intracellular ion distributions. With rigorous attention to detail and improvement in technique it may well prove possible to obtain analytical results for diffusible ions from embedded freeze-dried and freeze-substituted tissues which are indistinguishable from analyses of frozen-hydrated tissues.

Whilst there were discrepancies between absolute values for ion concentrations in the three types of preparation, it must be pointed out that in the chicken kidney relative differences seen in the frozen-hydrated samples as a result of infection with IBV were faithfully maintained in the freeze-dried and freeze-substituted samples. We conclude that for comparative studies the latter preparation techniques may be satisfactory without further modification.

Acknowledgements

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References

- Appleton J (1987) X-ray microanalysis of growth cartilage after rapid freezing, low temperature freeze drying and embedding in resin. *Scanning Microsc.* **1** (3) : 1135-1144.
- Chandler JA (1985) X-ray microanalysis of biological tissues - an examination of comparative specimen preparation techniques using prostatic tissue as a model. *Scanning Electron Microsc.* 1985, II : 731-744.
- Civan MM, Hall TA, Gupta BL (1980) Microprobe study of toad urinary bladder in absence of serosal K⁺. *J. Membrane Biol.* **55** : 187-202.
- Condron RJ, Marshall AT (1986) Pathogenesis of infectious bronchitis nephritis. 1. Morphometric analysis of kidney proximal tubular epithelium in chickens. *J.Comp. Path.* **96** : 47-61.
- Edelmann L (1986) Freeze-dried embedded specimens for biological microanalysis. *Scanning Electron Microsc.* 1986; IV : 1337-1356.
- Hagler HK, Buja LM (1984) New techniques for the preparation of thin freeze-dried cryosections for x-ray microanalysis. In : *Science of Biological Specimen Preparation for Microscopy and Microanalysis*. Revel JP, Barnard T, Haggis GH (eds), Scanning Electron Microscopy, Inc., AMF O'Hare, IL, pp161-166.
- Hall TA, Gupta BL (1983) The localization and assay of chemical elements by microprobe methods. *Quart. Revs. Biophys.* **16**: 279-339.
- Harvey DMR (1982) Freeze-substitution. *J. Microsc.* **127** :209-221.
- Humbel B, Muller M (1986) Freeze-substitution and low temperature embedding. In : *The Science of Biological Specimen Preparation for Microscopy and Microanalysis* 1985. Muller M, Becker RP, Boyde A, Wolosewick JJ (eds), Scanning Electron Microscopy, Inc., AMF O'Hare, IL, pp175-184.
- Ingram FD, Ingram MJ (1980) Electron microprobe quantitative bulk sample analysis of intracellular electrolyte distributions. In : *X-ray Microanalysis - An Introduction*. Hayat M.A. (ed), University Park Press, Baltimore, pp367-399.
- Ingram MJ, Ingram FD (1983) Electron microprobe calibration for measurement of intracellular water. *Scanning Electron Microsc.* 1983; III: 1249-1254.
- Ingram DF, Ingram MJ (1984) Influences of freeze-drying and plastic embedding on electrolyte distributions. In : *The Science of Biological Specimen Preparation for Microscopy and Microanalysis*. Revel JP, Barnard T, Haggis GH (eds), Scanning Electron Microscopy, AMF

O'Hare, I1, pp167-174.

Le Furgey A, Bond M, Ingram P (1988) Frontiers in electron probe microanalysis : application to cell physiology. *Ultramicrosc.* 24 : 185-220.

Linner JG, Livesey SA, Harrison DS, Steiner AL (1986) A new technique for removal of amorphous phase tissue water without ice crystal damage : a preparative method for ultrastructural analysis and immunoelectron microscopy: *J. Histochem. Cytochem.* 34 : 1123-1135.

Luft JH (1973) Embedding media - old and new. In : *Advanced Techniques in Biological Electron Microscopy*, Koehler JK (ed), Springer-Verlag, New York, pp1-34.

Marshall AT (1980a) Freeze-substitution as a preparation technique for biological x-ray microanalysis. *Scanning Electron Microsc.* 1980; II : 395-408.

Marshall AT (1980b) Quantitative x-ray microanalysis of frozen-hydrated bulk biological specimens. *Scanning Electron Microsc.* 1980; II : 335-348.

Marshall AT (1981) Simultaneous use of EDS, windowless EDS, BE and SE detectors and digital real-time line scanning for the x-ray microanalysis of frozen-hydrated biological specimens. *Scanning Electron Microsc.* 1981; II : 327-343.

Marshall AT (1982) Application of \emptyset (pz) curves and a windowless detector to the quantitative x-ray microanalysis of frozen-hydrated bulk biological specimens. *Scanning Electron Microsc.* 1982; I : 243-260.

Marshall AT (1984) The windowless energy dispersive x-ray detector : prospects for a role in biological x-ray microanalysis. *Scanning Electron Microsc.* 1984; II : 493-504.

Marshall AT (1987) Scanning electron microscopy and x-ray microanalysis of frozen-hydrated bulk samples. In : *Cryotechniques in Biological Electron Microscopy*. Steinbrecht RA and Zierold K (eds), Springer-Verlag, Berlin, pp 240-257.

Marshall AT, Carde D (1983) Beryllium coating for biological x-ray microanalysis. *J. Microsc.* 134 : 113-116.

Marshall AT, Condron RJ (1985) Normalisation of light element x-ray intensities for surface topography effects in frozen-hydrated biological bulk samples. *J. Microsc.* 140 : 99-108.

Marshall AT, Condron RJ (1987) A simple method of using \emptyset (pz) curves for the x-ray microanalysis of frozen-hydrated biological bulk samples. *Micron Microsc. Acta* 18 : 23-26.

Meyer R, Schmitz M, Zierold K (1985) The influence of different cryopreparations on the distribution of ions in bullfrog myocard cells. *Scanning Electron Microsc.* 1985; I : 419-431.

Pallaghy CK (1973) Electron probe microanalysis of potassium and chloride in freeze-substituted leaf sections of *Zea mays*. *Aust. J. Biol. Sci.* 26 : 1015-1034.

Robards AW, Sleytr UB (1985) Low temperature methods in biological electron microscopy. In : *Practical Methods in Electron*

Microscopy, Vol. 10, Glauert A.M. (ed), Elsevier, Amsterdam, pp 1-551.

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

Steinbrecht RA, Muller M (1987) Freeze substitution and freeze-drying. In : *Cryotechniques in Biological Electron Microscopy*. Steinbrecht RA and Zierold K (eds), Springer-Verlag, Berlin, pp149-172.

Van Zyl J, Forrest OG, Hocking C, Pallaghy CK (1976) Freeze-substitution of plant and animal tissue for the localization of water-soluble compounds by electron probe microanalysis. *Micron*, 1 : 213-224.

Wilson SM, Elder HY, McEwan J, McWilliams SA (1988) The effects of thermally induced activity *in vivo* upon the levels of sodium, chlorine and potassium in the epithelia of the equine sweat gland. *J. exp. Biol.* 136 : 489-494.

Wroblewski R, Wroblewski J, Anniko M, Edstrom L (1985) Freeze-drying and related preparation techniques for biological microprobe analysis. *Scanning Electron Microsc.* 1985; I : 447-454.

Wroblewski R, Wroblewski J, Roomans GM (1987) Low temperature techniques in biomedical microanalysis. *Scanning Microsc.* 1 (3) : 1225-1240.

Discussion with Reviewers

T. von Zglinicki: Freeze-drying protocols for X-ray microanalysis and for the measurement of shrinkage appear considerably different. The amount of shrinkage might be different in both preparations. Moreover, there seem to be no data to support the assumed constancy of sample dimensions during freeze-substitution. Could you give some estimation of the bias introduced by these effects?

Authors: Freeze-drying in general seems to result in a reduction in volume of approximately 20% (Boyde, A., 1978, Pros and cons of critical point drying and freeze-drying for SEM. *Scanning Electron Microsc.* 1978; II: 303-314). Differences in techniques of freeze-drying do not appear to make a great deal of difference to the amount of shrinkage observed. In any event a difference of $\pm 5.0\%$ in volume will mean a difference in $\pm 5\%$ in estimates of concentration. This is too small to affect the general conclusion for freeze-dried kidney.

Freeze-substitution is generally considered to be free of shrinkage artefacts, there are however, no confirmatory measurements of this as far as we know.

K. Zierold: There are far more assumptions than proofs for the maintenance of ion redistribution by freeze-drying. Therefore, my question: Have you also analyzed freeze-dried and/or freeze-substituted specimens without embedding? By this experiment the portions of dehydration and embedding steps on ion

redistribution could be clearly separated.

Authors: It would be possible to compare unembedded freeze-dried samples with unembedded freeze-substituted samples if the latter were freeze-dried from the substituting solvent. However, the excitation volume would be so large that comparison would be difficult since this volume could hardly be confined to even a single cell.

K. Zierold: How accurate are your measurements of the intracellular water content by recording the oxygen peak with the windowless detector? Have you found changes in the sensitivity by contamination on the detector surface?

Authors: The use of the oxygen intensity for estimating intracellular water content is validated in text reference Marshall and Condron (1987). The accuracy of the technique can only be assessed by analysis of model systems such as gelatin solutions and by comparing our results on various tissues with results obtained by other methods on the same tissues. These are limited in number but the agreement has so far been reasonable (see for example Marshall A.T., Hyatt A.D., Phillips J.G. and Condron R.J., 1985 Isosmotic secretion in avian nasal salt gland: X-ray microanalysis of luminal and intracellular ion distributions. *J. Comp. Physiol. B* 156: 213-227).

Contamination of the detector has not been a problem since the detector surface is only exposed to the sample chamber environment when the chamber is effectively cryopumped by a very large anticontaminator and the sample stage itself. The sensitivity can be periodically checked on a quartz standard.

G. Roomans: Could you speculate on the source of S and Cl contamination during freeze-drying?

Authors: Sulphur may be derived from pump oils during freeze-drying. Chloride contamination may have arisen from PVC insulation of the electrical leads to the cascade thermoelectric stage in the freeze-drier (see Love G., Scott V.D., Dennis N.M. and Laurenson L., 1981, Sources of contamination in electron optical equipment. *Scanning*, 4: 32).

L. Edelmann: If ion redistribution occurs during dehydration or embedding it should be possible to detect K⁺ increase and Na⁺ loss in regions outside the analysed areas (e.g. in the extracellular space or in the embedding medium). Did you find such regions?

Authors: We did not look for changes in extracellular space or evidence of ion diffusion into the surrounding resin. The latter question has been addressed previously in a qualitative manner and no evidence of such a diffusion was found (Marshall 1980a).

L. Edelmann: Do you obtain different results when using pure diethyl ether for freeze-substitution instead of a diethyl ether-acrolein mixture?

Authors: We have not made this comparison on these tissues but on some invertebrate tissues

which we have been recently investigating we observed no differences when the acrolein was reduced from 20 to 10 percent.

G. Roomans: It is interesting to note that the changes in P are paralleled by changes in Mg and K. Could this imply that Mg and K are "associated" (not to say "bound") to intracellular phosphate groups?

Authors: This is an interesting idea but we know of no biochemical evidence to support it. It may be possible that selective extractions by judicious choice of freeze-substitution media could be used to investigate this further.

G. Roomans: Is resin as stable under the electron beam as ice? Differences in stability might cause systematic errors in the quantitative results.

Authors: We are not entirely certain what you mean by 'stability'. Mass loss and radiation damage appeared to be insignificant in both frozen-hydrated and embedded samples particularly when it is considered that the depth resolution of the analysis is about 2 μ m. Note that all samples were metal coated and analysed at -172°C.

G. Roomans: What is the biological significance of the lower Na and Cl in the kidney cells of the infected chicken?

I. von Zglinicki: Are ion concentrations, especially Na and Cl concentrations in proximal tubule cells, as measured in frozen hydrated samples within the physiological range? If not, this could indicate a redistribution of ions during the prefreezing treatment, which might artificially decrease the probability of redistributions during the further processing steps.

Authors: The biological significance will be discussed in detail elsewhere. Briefly we interpret the reduction of Na and Cl concentrations as indicating a pathogen induced reduction in Na⁺ and Cl⁻ reabsorption.

In normal kidneys, analysed in the frozen-hydrated state, elemental concentrations are similar to those reported in freeze-dried sections of rat proximal tubules except that Na⁺ concentrations are higher. We believe this to be a real difference between species since analysis by us (unpublished) of rat proximal tubules, using bulk frozen-hydrated samples, gives considerably lower Na⁺ concentrations than in chicken proximal tubules.