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X-RAY MICROANALYSIS OF BIOLOGICAL TISSUES - AN EXAMINATION OF COMPARATIVE SPECIMEN PREPARATION TECHNIQUES USING PROSTATIC TISSUE AS A MODEL

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Abstract

A number of reproductive tissues have been investigated by X-ray microanalysis for a large number of elements. These elements occur both free and with many different forms of binding to tissue proteins and cellular constituents. The prostate tissue studied here represents a glandular tissue with both parenchymal and stromal components having different cell types. While no general rule can be applied to the preparative technique for all tissues, it would seem that of all the methods examined here freeze substitution or the use of dehydration fluids combined with subsequent embedding offer the most promising compromise between cryoultramicrotomy and aqueous fixation.

KEY WORDS: X-ray Microanalysis, reproductive, specimen preparation, prostate.

Introduction

Studies on soft tissues of the male and female reproductive tracts with X-ray microanalysis have been mostly concerned with glandular structures such as prostate, epididymis and uterus. Analysis of sperm cells has also been extensively investigated as well as investigation of electrolytes and 'non-diffusible' elements in blastocytes, testis and endometrium. A review of all tissues studied and the associated techniques of microanalysis that have been employed has been previously described [4].

Reliable X-ray microanalysis depends upon the successful operation of a number of factors that may otherwise provide wrong or misleading information.

A number of weaknesses occur, particularly in the analysis of thin biological samples in the electron microscope of which specimen preparation and electron radiation damage are the most severe. Methods of quantitation are limited by the unknown losses associated with these sources of error. The subject of radiation damage has been considered in these tissues in a previous publication [5] but specimen preparation remains a major problem for the satisfactory combination of adequate ultrastructural detail and reliable retention of elements *in situ*. Although much debate has been given to the virtues of comparative preparative regimes for diffusible or non-diffusible elements there is a sense in which all the elements are diffusible in that their retention depends on the stability of the structures with which they are associated. Cryoultramicrotomy has been demonstrated by a number of workers as the optimum choice for retention of elements from their *in vivo* situations, but morphological detail in ultrathin sections of glandular tissues has generally been inadequate, and most successful work has been achieved with semithin (1μm) sections. The preparation of thin sections of soft tissue by cryoultramicrotomy presents formidable problems for routine use but can be employed initially to obtain samples for comparison with other techniques. Aqueous methods of preparation involving fixation cause unavoidable losses of most elements from whole tissue despite strict controls of fixation conditions but these

may be partly limited by the use of histochemical precipitating agents. Losses in whole tissue are paralleled by translocation of elements found by analysis of subcellular regions in thin sections and can be used as a first check on the suitability of a particular preparative regime for a certain set of elements.

In this paper a comparison is made of the different preparative techniques used for the study of reproductive tissues to examine both freely diffusible and partly diffusible elements within these samples. The heterogeneous nature of biological tissue makes the choice of a perfect model system impossible since no one tissue can adequately express the tolerance to chemical stress or viability of cellular integrity for all organic specimens. The rat prostate has been taken here, however, as a model for this study since much is already known about its biochemical and ultrastructural nature and the gland provides a number of cell types within a small area of tissue which can be studied for their relative tolerance to chemical stress. A number of reviews have appeared discussing the general merits and disadvantages of various preparative techniques when used by different workers on a range of tissues. This report, however, deals with a number of preparative methods applied to a single tissue type in order to determine relative elemental displacement caused by each technique in comparison with cryoultramicrotomy.

Materials and Methods

Animal tissue

Tissues used in these studies were taken from 3 month old Sprague Dawley rats, killed by cervical dislocation. The tissues were quickly removed following abdominal incision and care taken in the dissection of the lateral prostate to sample the anterior tip which is the one rich in zinc and the subject of previous investigations [19,20]. Samples were then prepared according to the individual experiments. When being transferred from the animal to any one preparation the samples were kept in an atmosphere of water saturation to prevent surface drying.

Analytical instrumentation and quantitation

All analyses were performed on an EMMA 4 electron microscope microanalyser (Kratos Ltd, UK) as described elsewhere [2]. Accelerating voltage for these analyses was 80kV, and the electron probe current adjusted with the C1 condenser lens and measured with a Faraday cage in the viewing chamber. Crystal spectrometers were used for measurement of CaKα lines using a Lithium Fluoride (LiF) or Pentaerithrytol (PET) crystal, other elements being measured with a Kevex (USA) silicon detector of active area 10 mm^2 at a specimen detector distance of 5 cm with a resolution of 150 eV. X-ray data was processed with a Tracor Northern NS880 multichannel analyser (MCA) linked to a DEC PDP1105 computer.

Specimens were mounted on aluminium grids of 100 mesh and retained in a beryllium specimen holder producing a measurable signal of FeKα for background determination.

Thin specimen analysis quantitation was performed according to methods previously described [1] using a modification of the Hall method [8] with appropriate corrections for spurious radiation from scattered electrons. White radiation was monitored in the region 4850-7450 eV of the energy spectrum and full width half maximum values were measured for each element. Analysis of thin sections are given here as relative weight fractions since the data for each preparation are considered relative to one another and not in absolute terms.

Whole tissue analysis

In order to monitor the loss or retention of elements from tissues during processing a number (3-5) of fragments of tissue, 1-2 mm^3, were retrieved after each step in the described schedules below. The retrieved pieces were pressed flat between glass slides and allowed to dry at room temperature for a few hours. The flattened sheets of tissue (several micrometres thick) were then lifted from the glass slides and stored in glass specimen tubes until required for analysis.

Individual fragments were sandwiched between wide mesh (100) aluminium grids ready for analysis without any other supporting membranes. Although opaque to the electron beam, it was possible to determine when the specimen alone was being irradiated by the absence of, or greatly reduced, aluminium signal in the energy spectrum. For the analysis of whole tissue a probe diameter of 10μm was employed with a probe current of 10nA at 80kV accelerating voltage over an analysis time of 40 seconds. Characteristic intensities were measured by peak heights and the continuum measured in the range 4850-7450eV on the MCA with constant analytical conditions. Usually ten or more readings were made on each sample across the whole area. Calcium was recorded on the LiF crystal spectrometer because of the high K K$_\beta$ signals.

The retention or loss of elements during the processing procedures was expressed as a percentage of the control, i.e., of fresh tissue, also air dried, the figures being calculated for a constant value of white radiation in each case. All readings were taken on areas of tissue opaque to the electron beam so that bulk analysis principles applied, but no correction procedures were necessary since all readings were relative to the control.

Thin specimen preparation

Various methods of specimen preparation were investigated on prostatic tissue. The leaching or displacement of elements in each process was studied by bulk analysis and by thin specimen analysis as described above. Cryotechniques were compared with methods involving aqueous fixation, histochemical precipitation and substitution by organic media as described in each section below.

Results

Frozen dried sections

A general procedure for preparation of frozen sections of prostatic tissue has been

described elsewhere [2] but a brief account is given here.

Freshly dissected prostatic tissue was quenched in melting Freon 22, previously cooled with liquid nitrogen, by rapid ejection into the liquid while mounted on silver pins. Ultrathin sections (~100nm) were cut on an LKB cryokit with a knife temperature of -80°C and a block temperature of -90°C after much prior experimentation with temperatures of both. Only sections obtained from the surface 10 μm zone were used, to reduce the possibility of ice crystal artefacts. The sections were mounted on 100 mesh aluminium grids and freeze dried at 10^{-2} Torr for 4 hours at a temperature of -100°C. The sections were brought to room temperature under dry nitrogen gas, vacuum coated on both sides and stored in a desiccator until required for analysis.

Natural contrast in the sections was very low and made identification of organelles extremely difficult (fig. 1). However, nuclei were readily identified as was smooth muscle surrounding the acini of the gland. When a complete alveolus was sectioned in a favourable position the whole extent of the epithelium from basement membrane to luminal space could be recognised.

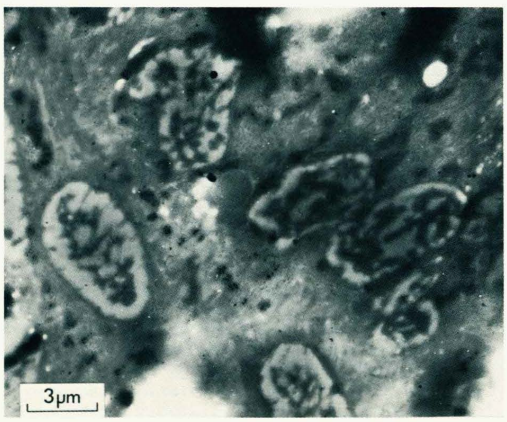

Fig. 1. Frozen dried sections of rat prostate. Nuclear and cytoplasmic regions are clearly defined, precipitates containing calcium may be contained in mitochondria.

Results of the analysis of frozen dried sections of prostate are shown in fig. 2 in which the weight fraction of each element for 3 subcellular regions is shown relative to the nuclear value (taken as 100). Large standard deviations of the mean values occurred for each element in every region. In all cases but for Zn, Cl and S, the nuclear dry weight concentrations were the greatest, especially for P and K. The relative concentrations found for each element in each subcellular region were used for comparison with those determined from other preparations.

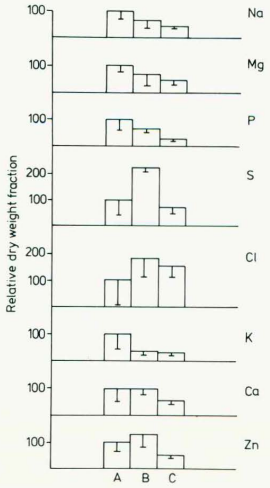

Fig. 2. Subcellular elemental distribution in a frozen-dried thin section of prostate. Dry weight fractions relative to nuclear value (100). (A) Nucleus; (B) Cytoplasm; (C) Smooth muscle. Bars represent 1 s.d.

Aqueous fixation techniques

The effect of various fixation procedures was investigated to determine the retention of elements in whole tissue and at subcellular locations during the processing. The air drying technique described in Materials and Methods was employed for analysis of whole tissue by X-ray analysis, samples being taken at each stage in the procedure as indicated. The analysis of thin sections was compared with that of frozen dried sections and the data shown in figs. 3, 4.

Elemental losses after an aqueous soak. Rat prostate was dissected into 1mm³ pieces and subjected to an aqueous wash at pH 7.0 for 30 mins. The pieces were then flattened and air dried for analysis together with those from untreated control samples.

The change in elemental content is seen in fig. 5 for P, S, Cl, K, Ca and Zn. Chlorine was totally lost from the samples together with major reduction of potassium. Significant losses occurred also for Ca but P and Zn were only marginally affected while an apparent increase ($p=0.1$) was seen in S. This increase may have been explained by the possibility of selective loss of organic components not containing sulphur, thus leaving behind a sulphur rich matrix.

Elemental losses during a conventional preparative procedure. Rat prostate was dissected into 1-2 mm³ fragments and processed by a standard method to determine elemental changes during each step. Between 3-5 pieces of tissue were taken after each step and air dried for bulk analysis as previously described. Control, untreated tissue was examined in a similar way. The steps of the procedure were as follows: (i) Control untreated tissue,

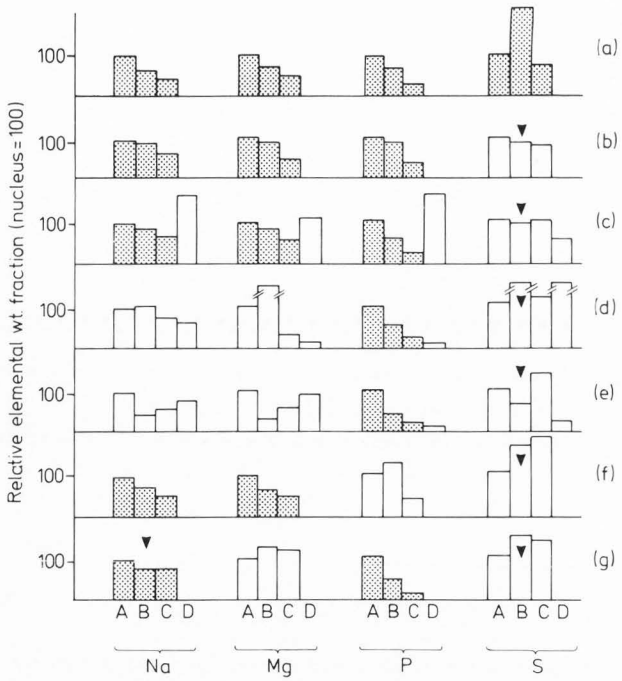

Fig. 3. Subcellular elemental distributions of Na, Mg, P and S in thin sections of prostate prepared by the following methods:
(a) Frozen-dried, unembedded
(b) Ether substituted at 20°C
(c) Freeze substituted at -70°C
(d) Potassium pyroantimonate fixed
(e) Potassium pyroantimonate + glutaraldehyde
(f) Potassium pyroantimonate + osmium tetroxide
(g) Glutaraldehyde alone (pH 7.0)
Subcellular regions: (A) Nucleus
(B) Cytoplasm
(C) Smooth muscle
(D) Extracellular deposits
▼ indicates possible contamination from resin, buffer, fixative.
▒ indicates similar distribution to frozen dried sections.

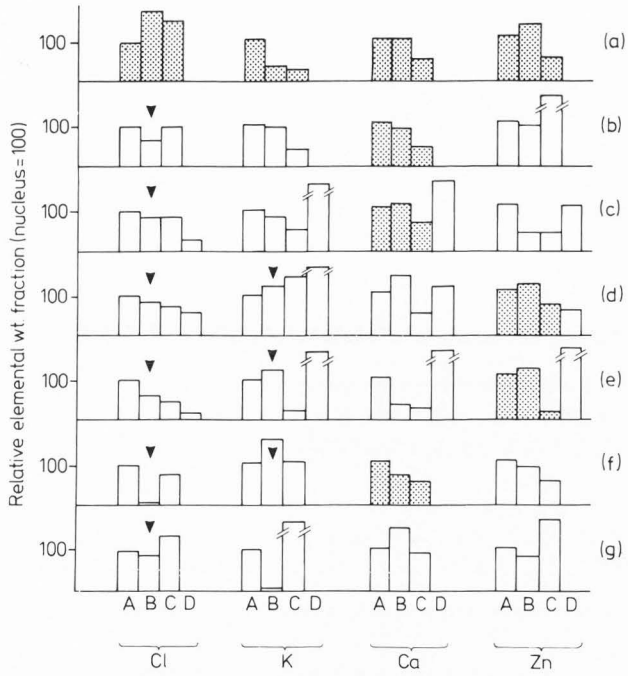

Fig. 4. Subcellular elemental distribution of Cl, K, Ca and Zn in thin sections of prostate prepared by the following methods:
(a) Frozen-dried, unembedded
(b) Ether substituted at 20°C
(c) Freeze substituted at -70°C
(d) Potassium pyroantimonate fixed
(e) Potassium pyroantimonate + glutaraldehyde
(f) Potassium pyroantimonate + osmium tetroxide
(g) Glutaraldehyde alone (pH 7.0)
Subcellular regions: (A) Nucleus
(B) Cytoplasm
(C) Smooth muscle
(D) Extracellular deposits
▼ indicates possible contamination from resin, buffer, fixative.
▒ indicates similar distribution to frozen dried sections.

(ii) Fixed in 3% glutaraldehyde (aqueous, pH 6.0) for 2h, (iii) Washed in distilled water - 10 min, (iv) Dehydrated in 70% ethyl alcohol - 10 min, (v) Dehydrated in 90% ethyl alcohol - 10 min, (vi) Dehydrated in 100% ethyl alcohol - 10 min, (vii) Infiltrated with propylene oxide - 10 min, (viii) Infiltrated with propylene oxide + Araldite - 10 min, (ix) Embedded in Araldite at 60°C for 30h.

After embedding the tissue pieces were cut out of the block and freshly exposed with a glass knife. Flat faces of the tissue were presented to the electron beam for analysis. Each air dried sample, and the embedded tissue, were analysed with an electron probe current of 2 nA at 80 kV accelerating voltage with a probe diameter of 200μm. The counting time was 100 seconds.

Characteristic and continuum intensities were recorded for each sample and the results shown in Fig. 6, the weight fractions of the treated samples being shown as a percentage of

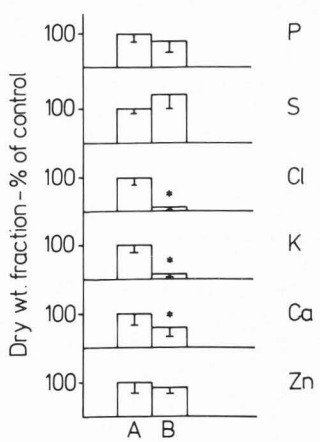

Fig. 5. Elemental changes after washing prostate in distilled H_2O for 30 min. (whole tissue analysis). Dry wt. fractions relative to unwashed control (100). (A) Control; (B) Washed tissue. Bars represent 1 s.d. Asterisk indicates $p<0.05$.

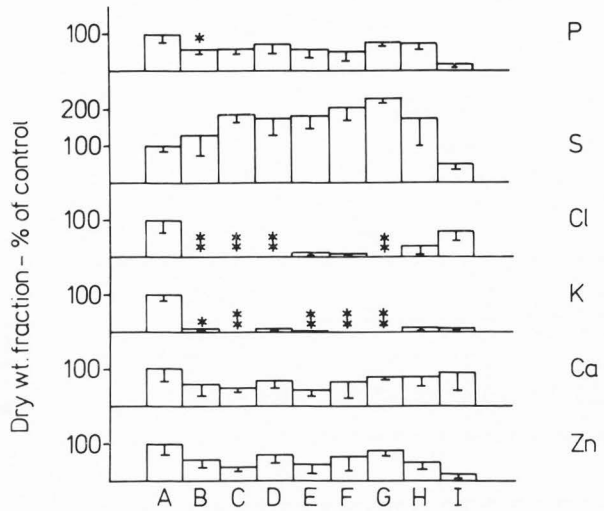

Fig. 6. Elemental changes in rat prostate during conventional fixation and embedding (whole tissue analysis). Dry weight fractions relative to untreated control (100).
(A) Control; (B) 3% glutaraldehyde 2h; (C) H_2O 10 min.; (D) 70% alcohol 10 min.; (E) 90% alcohol 10 min.; (F) 100% alcohol 10 min.; (G) Propylene oxide 10 min.; (H) Propylene oxide + Araldite 10 min.; I. Araldite embedded.
Bars represent 1 s.d. Single Asterisk indicates $p<0.05$ compared to control following fixation. Double asterisk represents <5% of control.

the controls. Losses of all elements except S occurred after initial fixation with glutaraldehyde, the most severe being for Cl (totally displaced) and for K. P, Ca and Zn were reduced to about 60% of control levels and thereafter remained fairly constant. Final reductions occurred in the embedded tissues, due to the diluting effects of the resin, except for Ca (large s.d.) and Cl (contaminant).

The increase of S in the samples following fixation was not readily explained. It may have been due to the contamination of tissues with this element from the fixative, but may also have arisen through the selective extraction of non-sulphur containing components of the tissue. Its apparent reduction following embedding was again due to the dilution by the resin.

Analysis of the thin sections of tissue prepared by glutaraldehyde fixation is shown in figs. 3,4 in comparison with other preparations. Fig. 7 shows the morphological preservation of the prostate fixed in glutaraldehyde alone compared with double fixation (fig. 8) in glutaraldehyde and osmium tetroxide and double staining.

Histochemical techniques

Methods for precipitating diffusible ions in situ in prostatic tissue were investigated. The potassium pyroantimonate technique, devised for the retention of cations in tissue, was studied by in vitro precipitation of varying molarities for a number of elements to test for potential efficacy in tissue. Combinations of the potassium pyroantimonate salt or the potassium oxalate salt with glutaraldehyde or osmium tetroxide were investigated.

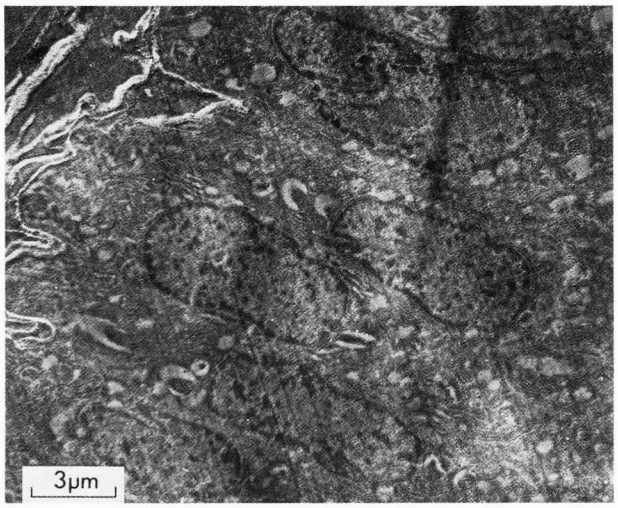

Fig. 7. Glutaraldehyde fixed prostate, embedded in Araldite. Subcellular detail is clearly seen.

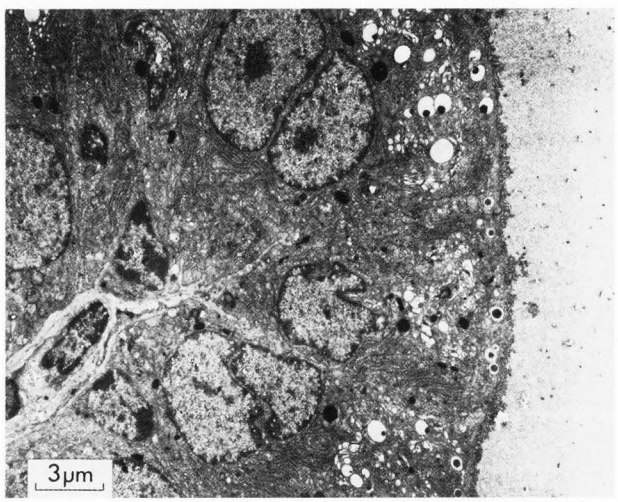

Fig. 8. Rat prostate fixed in glutaraldehyde and osmium tetroxide, embedded in Araldite, and double stained.

In vitro studies with pyroantimonate. The pyroantimonate method has been used to precipitate a number of cations in tissue although it was first proposed as a histochemical demonstration for sodium. Several authors have examined the effects of various parameters (pH, buffers, postfixation, dehydrating agents, infiltration media) on the nature and efficacy of the reaction in tissue sections. Torack and Lavalle [22] have also described the way in which, according to the molecular configuration of the pyroantimonate salt, many monovalent, divalent and trivalent ions could be added. In this investigation the different types of precipitation and the competition between cations when reacted in vitro were briefly studied. A similar investigation has been performed by Simson et al. [17].

The fixative solution used throughout the studies was a mixture of 4% glutaraldehyde and 2% $K_2Sb_2O_7.4H_2O$. The antimonate was first dissolved by boiling in distilled water, cooled to 40°C and added to the glutaraldehyde, also at 40°C, to prevent spontaneous precipitation. The pH of the solution, when allowed to cool to 20°C, was 7.4

Aqueous solutions of CaCl, $ZnCl_2$ and $CdCl_2$ were prepared and added to the fixative in the following proportions:

A 0.01 MCa + fixative (equal volumes)
B 0.01 MZn + fixative (equal volumes)
C 0.01 MCd + fixative (equal volumes)
D 0.01 MCd + 0.01 MZn + fixative (1:1:2)
E 0.01 MCd + 0.1 MZn + fixative (1:1:2)
F 0.01 MCd + 0.01 MZn + 0.01 MCa + fixative (1:1:1:3)

Cloudy precipitation formed in each case and droplets of each solution were dried onto carbon coated grids for examination in EMMA. Samples of precipitation A, B and C were also washed thoroughly by dilution in distilled water and centrifugation four times, and further samples prepared for analysis.

CaKα, CdLα, SbLα and KKα were detected with the wavelength dispersive detector (WDS) using a PET crystal, and ZnKα with a LiF crystal. Analysis of precipitation was performed at 80 kV with a beam current of 0.01 μA and varying beam diameter after prior determination of specimen stability. X-ray counting time was 40 secs.

The analysis of elemental ratios is shown in Table 1. The figures are taken from the mean of a number of analyses for each specimen covering an area of about 1 μm^2 of precipitation (i.e., several hundred particles). Values for each elemental ratio (atomic) were corrected for detection efficiency in the WDS by methods similar to those already described.

In all reactions potassium was found to be precipitated within the antimonate salt. Although in the original complex, $K_2Sb_2O_7.4H_2O$, the atomic ratio of K to Sb was unity, no such simple relationship existed in any of the precipitates for those elements. Washing the precipitation A, B and C in distilled water thoroughly caused the potassium content to decrease by about half in each case. The ratio of each cation Ca, Zn and Cd to antimony, however, was unaffected by the washing indicating the insolubility of the salts.

In mixture B (zinc alone) the ratio of Zn to Sb as well as the amount of precipitation (++), was increased from that of Ca to Sb in A. This was to be expected since chelators have a greater affinity for heavier elements. When Cd alone was used (mixture C) the ratio to antimony was again increased above that of Ca, as was the precipitation (+++).

Equal molarities of Cd and Zn (in mixture D) demonstrated equal competition between these elements, the ratio of each element to Sb being reduced from that in B or C. With a tenfold increase in molarity of Zn (mixture E) a rise in the Zn to Sb ratio occurred at the expense of Cd, the ratio of Zn to Cd now becoming 10:1. When all three elements were mixed in equal proportions there was a good correlation in the elemental ratio produced in the complex, and the levels of Cd, Zn and Cd were all lower than in any of the other individual complexes.

Table 1. Atomic ratios of elements precipitated in vitro with antimony

	Cation mix (molarities)	Ca/Sb	Zn/Sb	Cd/Sb	K/Sb
A	0.01 Ca+	0.36 (0.36)	-	-	0.12 (0.04)
B	0.01 Zn++	-	0.47 (0.47)	-	0.43 (0.13)
C	0.01 Cd+++	-	-	0.50 (0.50)	0.35 (0.14)
D	0.01 Cd 0.01 Zn+	-	0.36	0.38	1.04
E	0.01 Cd 0.1 Zn++++	-	0.69	0.07	0.29
F	0.01 Ca 0.01 Zn 0.01 Cd++	0.11	0.10	0.11	0.47

Figures in parentheses represent atomic ratios after washing
+ = Degree of precipitation

There would thus seem to be a definite relationship between the ionic concentrations of each element in solution and the amount of that element bound in precipitation with antimony. When competition between elements occurs, as would be the case in organic material, the total antimony within the complex is increased, provided there is an excess of fixative. In addition, there is a relationship of cations found within the precipitates which exactly reflects the ratio of free cations in the original solution [25]. The precipitate of potassium with the cations does not appear to affect this ratio, the potassium being freely washed out of the complex without affecting the rest of the insoluble precipitate.

Fixation of prostatic tissue with pyroantimonate. Potassium pyroantimonate has been widely used in attempts to reduce translocation of diffusible cations during fixation. In the reaction the soluble potassium salt dissociates and insoluble complexes are formed with a number of different elements [25]. In the experiments below the efficacy of the fixation technique on retention of elements within the prostate was examined. Potassium pyroantimonate has been used in a wide range of combinations with different fixatives [3]. The studies described here examine its use in combination with osmium tetroxide, glutaraldehyde, or alone as both a fixative and precipitating agent.

Potassium pyroantimonate alone. A 2% solution of potassium pyroantimonate was made up by dissolving 2g of $K_2,Sb_2O_7.4H_2O$ in 100 ml of 0.01N acetic acid at 80°C. The solution was cooled and brought to pH 8 with 0.01N acetic acid or

0.1N KOH to avoid the spontaneous precipitation found at more acid values.

Potassium pyroantimonate in osmium tetroxide. A 2% solution of potassium pyroantimonate was made up as above, the pH brought initially to 10. A vial containing 0.1g of osmium tetroxide was broken into 10 mls of this solution to make a 1% concentration. The acidity was then adjusted to 0.1N acetic acid or 0.1N KOH.

Potassium pyroantimonate in glutaraldehyde. A 3% solution of glutaraldehyde was made from the 2% solution of potassium pyroantimonate prepared as above. The acidity of the fixative was adjusted to pH 8 with 0.1N acetic acid or 0.1N KOH. Some spontaneous precipitation occurred even at this pH value and the clear supernatant solution only was used for fixation.

Rat prostate was dissected to 1-2 mm² pieces and fixed in each of the three solutions for 1h. The tissues were then transferred immediately to 100% alcohol for 10 mins, infiltrated in propylene oxide for 10 mins, and in propylene oxide-Araldite for 10 mins and embedded in Araldite. Polymerisation was for 30h at 60°C.

Tissue pieces were retrieved before and after fixation for bulk analysis. Sections of the embedded tissue were cut with glass knives onto distilled water, collected on Al grids and carbon coated for analysis. Subcellular analysis of the sections is shown in figs. 3,4 in comparison with other preparations.

Fig. 9 shows an ultrathin section of prostatic tissue treated with osmium and potassium pyroantimonate. Precipitation containing largely calcium and zinc occurred within the nucleus, at the microvilli and along the basement membrane and cellular membranes. Secretory granules were high in zinc concentration.

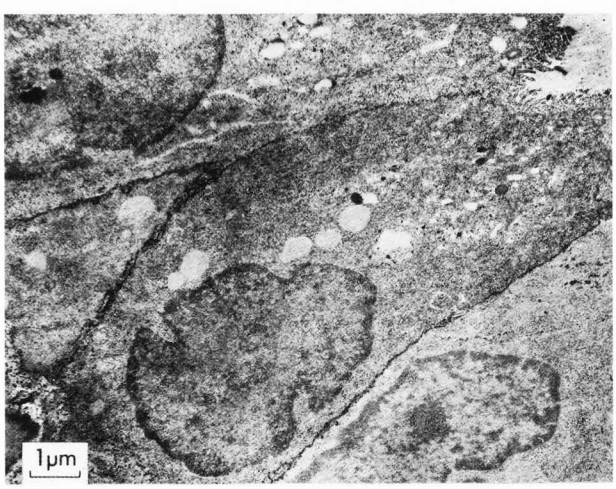

Fig. 9. Ultrathin section of rat prostate treated with potassium pyroantimonate and osmium tetroxide and embedded in Araldite. Precipitation contains a number of cations, largely calcium and zinc within the nucleus and at the cellular membrances.

The results of whole tissue analysis are shown in Fig. 10. Large statistical deviations were found for Na concentrations due to the difficulty of detecting this element in the whole tissue. Nevertheless, a significant reduction of this element was found for treatment of the tissue in potassium pyroantimonate alone compared with control untreated prostate. Large reductions occurred for Mg, P, S and especially Cl after treatment with all three fixatives. The elevated levels of K were explained by the introduction of this element into the tissue from the fixatives.

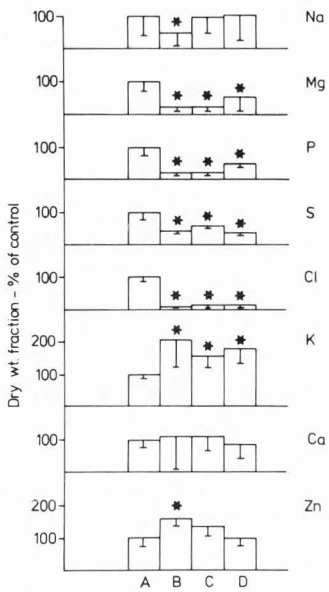

Fig. 10. Elemental changes in rat prostate following 1h fixation with aqueous potassium pyroantimonate (KSb) (whole tissue analysis). Dry wt. fractions relative to untreated control (100).
(A) Control; (B) 2% KSb; (C) 2% KSb in 3% glutaraldehyde; (D) 2% KSb in 1% osmium tetroxide.
All at pH 8.
Bars represent 1 s.d. Asterisk represents $p<0.05$ compared with control.

No significant losses were seen for either Ca or Zn after treatment with any of the 3 solutions and there was an apparent increase in zinc after treatment in pyroantimonate alone. Such observations do not, however, eliminate the possibility of translocation of these elements at the cellular level and the rise in observed Zn concentration may be due to diffusion of this element in surface layers.

Substitution techniques for prostatic tissue.

The use of organic media to substitute the water content of prostatic tissue was investigated. Ether and acetone were used at 20°C while freeze substitution at -70°C was performed with ether. Substitution offers a possible alternative to conventional fixation and dehydration described above while obviating the difficulties of cryoultramicrotomy.

Room temperature substitution in acetone or ether. Acetone and diethyl ether have been suggested as freeze substitution fluids for de-

hydration of soft tissues at -80°C. In order to determine the potential of this method for elemental retention, samples of rat prostate were fixed for different times in the two fluids and examined by bulk analysis.

Rat prostate was dissected into 1-2 mm^3 pieces and separated into 5 groups. One group was air dried as a control group while the others were washed for 10 min. or 1h in acetone or sulphur-free diethyl ether at 20°C. These samples were then immediately air dried for bulk analysis.

The results of whole tissue analysis are shown in Fig. 11. Soaking the tissue in either

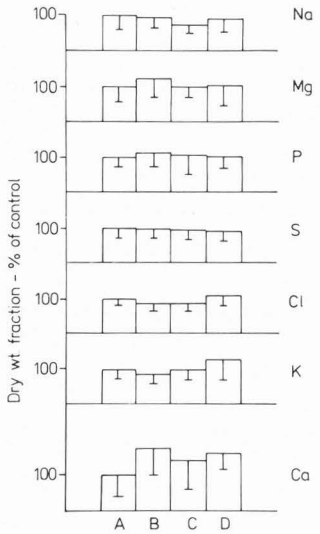

Fig. 11. Elemental changes in rat prostate following substitution of tissue water at 20°C in different fluids (whole tissue analysis). Dry weight fractions relative to untreated control (100).
(A) Control; (B) Acetone 10 min.; (C) Acetone 60 min.; (D) Diethyl ether 10 min.
Bars represent 1 s.d.

fluid caused rapid fixation, as observed by the hardness of the samples, but produced no significant change of the elemental concentrations. Calcium was apparently increased after treatment but the standard deviations of the mean values were very large, due to the low concentrations of the element, and there was no significant difference between levels.

Thus washing or fixing the tissue at 20°C in either solution caused no overall change in elemental composition but these observations gave no indication of possible translocations at the subcellular level.

Ether substitution and embedding at 20°C. Rat prostate was dissected into 1-2 mm^3 pieces and fixed in diethyl ether at 20°C for 100 mins. The samples were then infiltrated with 1:1 ether/Araldite and finally embedded in Araldite. Polymerisation was for 30h at 60°C. Samples of tissue were retained at each step for bulk analysis.

Thin sections of embedded tissue were cut on a dry knife mounted on Al grids and carbon coated. The results of subcellular analysis are

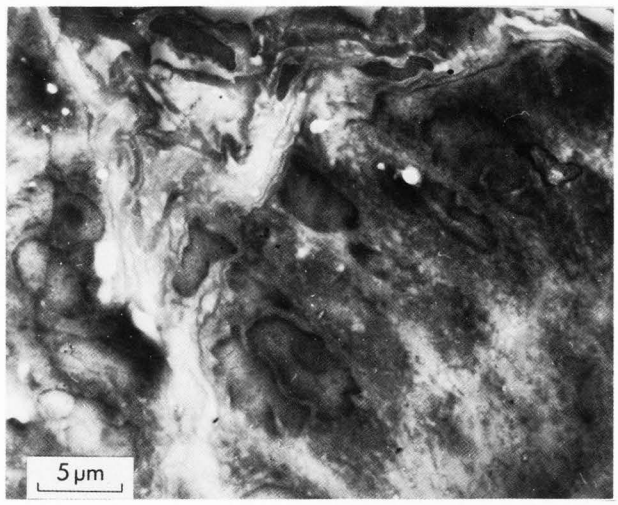

Fig. 12. Ultrathin sections of prostate substituted in ether at 20°C and embedded in Araldite resin. Stromal and epithelial regions are clearly defined.

shown in Figs. 3, 4 in comparison with other preparations. Fig. 12 shows the morphology of ether substituted prostate demonstrating the ready identification of stromal and epithelial components.

Fig. 13 shows the results of whole tissue analysis from samples retained after each step

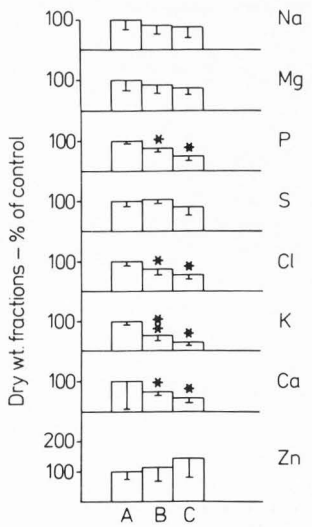

Fig. 13. Elemental changes in rat prostate following substitution of tissue water in diethyl ether at 20°C (whole tissue analysis). Dry wt. fractions relative to untreated control (100).
(A) Control; (B) Diethyl ether, 100 min.; (C) Diethyl ether/Araldite, 30 min.
Bars represent 1 s.d. Single asterisk represents $p<0.1$ compared to control. Double asterisk represents $p<0.05$ compared with control.

in the procedure. Losses occurred for Na, Mg, P, Cl, K and Ca after immersion in the ether but

to a much lesser extent compared with aqueous fixation. An apparent small increase was seen for Zn. Following infiltration in propylene oxide and Araldite, further reductions of these elements were seen to occur, again with an apparent increase in Zn. This final reduction in most elemental levels could be partly explained by the increase in density that would have occurred following infiltration with resin.

Thus substitution in diethyl ether, while causing some elemental changes, did not produce the large reductions seen for all elements (particularly K and Cl) following aqueous fixation.

Freeze substituted prostate. Rat prostate was prepared for analysis by a freeze substitution procedure as a variation of the method described by Spurr (18). Tissue was dissected into 1-2 mm^3 pieces and quenched in Freon cooled with liquid nitrogen to just above melting point. It was then transferred to diethyl ether, also cooled in liquid nitrogen to -70°C in a polypropylene bottle. The bottle was sealed with an air tight screw cap and placed in a deep freeze at -70°C for 35 days to freeze substitute the water from the tissue. Following substitution the ether was decanted off and replaced at -20°C with Spurr low viscosity resin made up in the follow proportions: ERL - 4206 (vinyl cyclohexene dioxide):10g; DER 736 (diglycidyl ether of polypropylene glycol):6g; NSA (nonenyl succinic anhydride):26g; S-1 (dimethyl amino ethanol):0.4g. The tissue pieces were infiltrated for 24h in the resin at -20°C and then brought to room temperature. They were then placed in a shallow aluminium tray and embedded in fresh resin by polymerisation at 60°C for 15h.

Ultrathin sections were cut on dry knives and over distilled water for comparison. The dry sections, collected without difficulty and judged by interference colour to be 100 nm thick, were sandwiched between Al grids (100 mesh) and compressed. They were carbon coated for analysis. Sections collected over water were also mounted on Al grids, carbon coated and analysed.

Analysis of whole tissue pieces indicated no significant difference ($p>0.05$) in Na, Mg, P, S, Cl, K, Ca or Zn between control and freeze substituted samples (fig. 14) after 35 days at -70°C. This, however, gave no indication of possible translocation at the subcellular level.

Analysis of sections collected dry compared with those collected over water are shown in fig. 15 for the nuclear regions of epithelial cells. It was seen that flotation on water caused large losses in Cl and K with significant reductions in Na and P. Mg, S, Ca and Zn remained unaffected by the water.

Comparative analysis of dry-cut sections of freeze substituted prostate with other preparations at a sub-cellular level are shown in figs. 3,4. Fig. 16 shows the morphology of the freeze substituted sections of prostate. Nuclear and cytoplasmic regions were clearly seen as were smooth muscle components in the underlying stroma in other

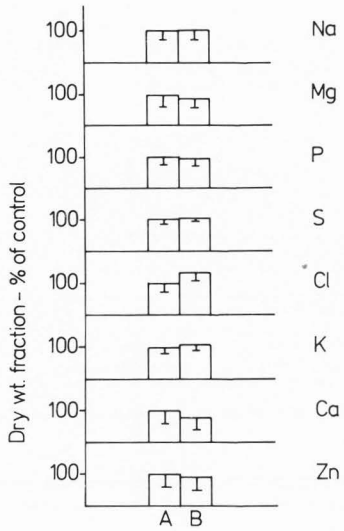

Fig. 14. Elemental changes in rat prostate following freeze substitution at -70°C in diethyl ether whole tissue analysis. Dry wt. fractions relative to untreated control (100).
(A) Control; (B) Diethyl ether, 35 days at -70°C.
Bars represent 1 s.d.

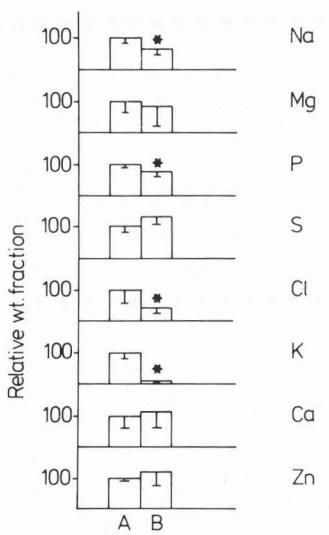

Fig. 15. Changes in elemental concentrations in thin sections of freeze substituted resin embedded rat prostate after flotation on water. Wt. fractions relative to dry cut sections (100). Analysis of epithelial cell nuclei.
(A) Sections cut on dry knife; (B) Sections cut onto water.
Bars represent 1 s.d. Asterisk represents $p<0.05$.

regions. Ice crystal damage was apparent in some cytoplasmic areas.

Comparative morphology and analysis of thin sections of prostate for various preparations.

The analyses of all the preparations for thin sections of prostate described above were compared together with frozen-dried sections.

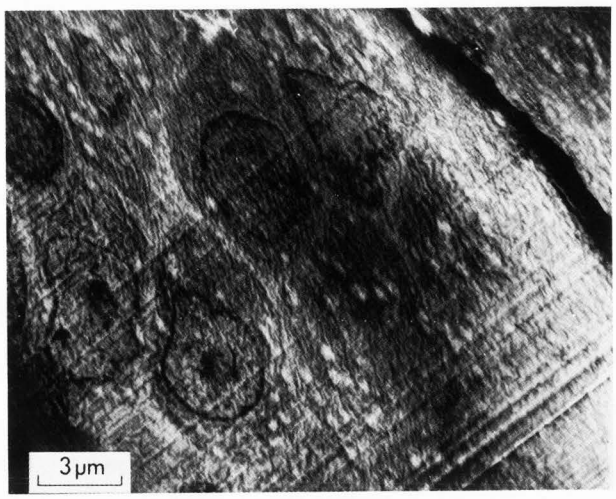

Fig. 16. Freeze substituted rat prostate embedded in Spurr resin and cut dry. Nuclei and nucleoli are clearly seen together with cytoplasmic regions.

In each preparation analysis was performed in the epithelial cell nuclei and cytoplasm (supranuclear region, large area) together with smooth muscle cells of the stroma.

Morphology. Ultrastructural details of thin sections of prostate prepared by the foregoing techniques are described in the legends to figs. 7,8,9,12,16.

Subcellular elemental distributions. The results of elements found in the three regions are given in Figs. 3 (for Na, Mg, P, S) and 4 (for Cl, K, Ca and Zn). The results are presented here for each organelle relative to nuclear concentrations (normalised to 100), and grouped for individual preparations (a-g). Indications are given (stippled bars) where the subcellular distribution was similar to that found in frozen-dried sections.

When compared with frozen dried sections, the preparations giving the most similar subcellular distributions of elements were: (b) ether substitution at 20°C, and (c) freeze substitution at -70°C. Other preparations gave valid distribution for fewer of the elements.

The most regularly preserved distributions occurred for P and Ca compared with frozen sections. Apart from S, which was a constant contaminant from the resin, very poor preservation occurred for Cl and K in all of the preparations. Zinc was retained in proportionate subcellular locations only by fixatives containing potassium pyroantimonate (d and e).

Of the fixative chosen specifically for the retention of Ca in tissue (d,e,f), only potassium pyroantimonate with osmium tetroxide (f) gave similar distributions to the frozen sections.

The observations above were treated with some caution because of the biological variation which may have existed between samples.

Because of sampling problems and normal biological variation it was not possible to accurately compare all the methods described here. The results did indicate, however that techniques involving substitution of tissue water with ether at room temperature or at -70°C may provide the best elemental integrity compared with frozen dried sections.

Discussion

The adequate preparation of biological tissue to retain elemental integrity, withstand electron irradiation and provide sufficient image detail for ultrastructural studies is undoubtedly the area of greatest weakness in the whole analytical procedure. While it is often sufficient to produce a thin specimen with only adequate image detail necessary for recognition of subcellular organelles, in other cases the morphology must be of high quality to allow correlation of local variations in fine structure with elemental analysis. Many approaches have been adopted to achieve optimum specimen preparation and have been described in a number of reviews (3,9,14,15). It is now generally agreed, however, that due to biological variation every tissue and each element requires an intensive investigation of its own rather than the adoption of a general or universal technique.

A possible exception to this individual approach lies in the use of cryotechniques where the in vivo elemental distribution and tissue structure may, in some cases, be transferred even into the microscope. Fully hydrated frozen sections of tissue have been investigated by only a few groups to date and then mostly for relatively thick sections observed by scanning transmission electron microscopy. Whereas this technique provides the greatest potential for retaining elemental integrity it has suffered until now from relatively poor image detail and requires sophisticated techniques largely out of reach of the average biological analyst. To date very few successful preparations of frozen hydrated material have been analysed as ultrathin sections and it is to alternative comparative techniques that this study has been addressed.

Although whole tissue analysis has been used here to provide an estimation of the potential of a particular technique, the success of its execution depends on a more rigorous approach. Cryoultramicrotomy, using frozen-dried sections, was therefore employed in these investigations in an attempt to provide a comparative technique for the various tissues and methods adopted. Although an examination of cryoultramicrotomy was not the central part of this study, extensive investigations [21] were made in an attempt to obtain the most reliable samples from different tissue types. These samples were then used as comparative standards by which other preparative methods could be assessed.

Some investigations [6,7] have indicated that frozen hydrated samples really provide the only reliable material for the study of diffusible ions in tissues. As mentioned above, the techniques required for their production and examination are limited to only a few laboratories and as yet are concerned mainly with thick specimens.

More readily available is equipment for the production and examination of frozen-dried ultrathin sections with conventional transmission electron microscopy as used here. As with frozen hydrated samples, frozen dried sections provide certain difficulties for analysis. These problems arise ultimately in the poor image detail and unreliability of the technique. This unreliability is a major concern where tissue is to be processed and examined following a lengthy or vital experiment.

In the frozen sections that were obtained, certain structures were readily identified. Nuclei appeared to have greater inherent contrast, while in rare circumstances endoplasmic reticulum was visible in epithelial cells and smooth muscle cells were regularly identified in the stroma surrounding the epithelium. The sections provided enough detail to allow the subcellular regions of epithelial nuclei, cytoplasm and smooth muscle to be analysed in sufficient numbers.

A large number of preparative procedures have been adopted by many workers in which 'wet chemistry' is involved. In a previous review [3] it was revealed that almost half of all thin specimen preparations had used conventional fixation and embedding procedures for subsequent analysis. Morgan et al. [15] have listed the findings of other workers who have investigated the losses of elements from tissues treated by these aqueous methods and compared them with the more promising freeze substitution techniques.

It has been suggested by Hayat [12] that the aim of fixation is rapid preservation of structure with minimum alteration from the living state as well as protection during embedding, sectioning and subsequent handling. Hopwood [13] however, includes the added criterion of minimum loss of tissue constituents. A problem discussed by this author is the slow penetration of glutaraldehyde into tissue, allowing physiological changes to occur at greater depths before fixation is complete. This is of critical importance to subsequent analysis and, as with frozen tissue, the best regions of tissue fixed in such a way would be the surface layers. In the analyses performed here this was the choice for all tissues examined and especially for those involving precipitation techniques.

If, as is known to be the case, elements are bound to different degrees in tissue then that fraction of each element which is 'free' or at least very weakly linked to structure, will be extracted in the fixative regardless of concentration, pH, osmolarity or even type of fixative or buffer. For the remaining moderately to tightly bound fractions, variations in fixation conditions may well have some effect, as reflected in the differences seen here, but these may be quite small compared with the large initial losses. Thus the best that can be achieved with aqueous fixation techniques in the absence of precipitating media is a minimisation of the losses of strongly bound elements with no real hope of retaining those that are free or weakly bound.

Several authors have reported on the extraction of elements from tissues under specific preparative regimes [15]. Whereas those results, together with those reported here, may indicate the potential of a certain technique, the conclusions cannot be extended to similar elements in other tissues or even to the same tissue under different physiological conditions. Thus, even if elements are considered from biochemical evidence to be tightly bound to macromolecular components, the possibility still exists that those molecules themselves may be extracted or relocated.

In the review by Morgan et al. [15] of elemental losses in various treated tissues it was indicated that by far the greatest flux of elements occurred amongst the electrolytes Na, K and Cl, corresponding with the observations here. In addition, heavier elements, possibly occurring as accidentally or deliberately introduced metals, may be typical of those which bind strongly to large proteins in nuclear or cytoplasmic sites and are hence less easily displaced. Of the heavier metals occurring naturally many function as catalysts in enzyme systems where they are firmly associated with the protein and there is a fixed number of atoms per protein molecule which cannot be removed by dialysis [23]. If however, these elements are not firmly bound to tissue structures they may be readily displaced altogether from the tissue either mechanically during handling or simply by flushing out during washing. These losses would then be in addition to those produced by the free passage of diffusible ions and to those chemically displaced by bond rupture or by displacement of organic components.

It would thus seem that for the electrolytes Na, K and Cl at least, and for other elements in part, initial aqueous fixation with glutaraldehyde displaces substantial quantities of these elements if not totally extracting them from the tissue. Any subsequent treatment to try and retain these elements would thus be irrevelant and methods for hopefully achieving this by avoiding organic solvents would have little value.

Much has been made of the various histochemical techniques used to precipitate cations during initial fixation of tissues for analysis. It has been suggested, however, that precipitation of ions at this preparative stage is no guarantee that they will remain in situ in tissue during subsequent dehydration and embedding [27]. Such losses or translocations may occur at any stage after fixation according to the potential solubility of ions or organic components binding those ions. This is especially so in the case of proteins which, in the absence of glutaraldehyde fixation (as with the osmium-pyroantimonate treatment) are likely to remain unstable.

Simple experiments in vitro with the potassium pyroantimonate salt revealed that insoluble salts may be formed from elements even in competition with each other and in proportion to their relative concentrations. Although not truly representing the conditions in vivo where other factors such as local pH variation, PO_4 ions, organic components, etc., may affect precipitation these observations indicated that, in an excess

of fixative, all cations may be precipitated, although not necessarily stoichiometrically. In addition the precipitates were seen to be insoluble, possibly conflicting with suggestions that subsequent aqueous staining of tissues may affect the final result in thin sections [27], but still allowing possible solubilisation during alcohol dehydration [26].

There appears to be conflicting evidence concerning the efficacy of the pyroantimonate method for the retention of cations in various tissues. This would further underline the fact that biological variation in one tissue and differences in elemental binding between tissue types makes the prediction of a particular fixative procedure impossible. The results here do indicate, however, that while strongly bound elements may be at least partly retained in tissues treated with conventional fixatives, the addition of a precipitating agent may further enhance this retention by stabilising those ions which are free either to migrate or to precipitate.

Freeze substitution as a technique for analysis has been suggested as a suitable compromise between aqueous fixation with embedding, in which there is an undoubted loss of organic components and electrolytes, and preparation of frozen sections which yield poor morphology and are unreliable. Spurr [18] and Pallaghy [16] in early work with thin sections, used the technique to retain and localise electrolytes in plant cells and suggested it as the best choice for optimising both analysis and ultrastructure. Whereas the initial hazards of fixation are avoided by this method the use of solvents subsequent to freezing and during the substitution and infiltration process may be possible sources of extraction of lipids in particular. Harvey et al. [11] suggested however, that at the low temperatures employed (-70°C) lipid extraction should be kept to a minimum. The same authors also demonstrated the minimal ability of acetone and diethyl ether as substitution solvents to dissolve salts at these temperatures.

Many substitution solvents have been employed in preparation of tissues for analysis [10,15] and results of different workers are conflicting. Harvey et al. [11] concluded that the choice of solvent is important to minimise elemental losses even though morphological preservation is less affected. The leaching of elements has been studied extensively [10,11,25], many of the authors indicating that losses are restricted by the maintenance of anhydrous conditions during substitution and embedding. A comparison of the various substitution fluids [10,15], shows that retention of cations may be very high for both electrolytes and non-electrolytes, and certainly much higher than that seen in most aqueous preparative methods. An analysis of much of the literature dealing with freeze substitution indicates that diethyl ether is the best substitution fluid for the retention of labile elements.

The subcellular location of elements in the tissue treated at 20°C was surprisingly good considering the obvious movement of tissue water during the substitution process. Whether translocation of elements between subcellular locations occurred is difficult to determine but there appeared to be a defined compartmentalisation similar to that of the frozen-dried sections. It was not found difficult to cut dry sections of embedded tissues substituted in ether at either temperature. Both methods gave the best elemental retention both in whole tissue and at the subcellular level compared with any of the other methods adopted, and the ultrastructural preservation was quite acceptable compared with that obtained from frozen dried sections.

A wide variety of reproductive tissues representing a range of cell types have been investigated by X-ray microanalysis for a large number of elements [4]. These elements occur in the free form and with many different forms of binding to tissue proteins and cellular constituents. It is thus impossible to predict the efficacy of any one preparative technique for the wide range of elements and binding forces that will be encountered since not only the elements but the tissue components themselves may be subjected to chemical and osmotic stresses. The prostate tissue examined here represents a glandular tissue with both parenchymal and stromal components having differing cell types. While no general rule can be applied to the preparative technique for all tissues it would seem that of all the methods investigated here, freeze substitution or the use of dehydration fluids combined with subsequent embedding offer the most promising compromise between cryoultramicrotomy and aqueous fixation. Further investigations are required to determine optimum freezing and infiltration parameters that will successfully combine satisfactory morphology and elemental integrity.

Acknowledgements

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Discussion with Reviewers

Reviewer II: Why have you ignored the freeze-dried, plastic embedded preparation? This preparation has proven itself in a few different laboratories using more rigorous methods than have been described in this paper. Like freeze-substitution, it has advantages arising from being a stable, embedded sample, but unlike freeze-substitution, it does not require exposing tissue to solvents. In fact, with materials that are polymerized with UV, such as Lowicryl, embedding and polymerization can be accomplished at -50°C so that the entire preparation can be accomplished at low temperature.
Author: Certainly the freeze-dried plastic embedded preparative method has its advantages. It would be well worth extending this study to a number of low viscosity resins for freeze dried material, especially the Lowicryl types.

Reviewer II: Were samples analysed at room temperature, or were they cooled with a cold stage during analysis?
Author: All analyses were performed at room temperature.

Reviewer II: The increase in S signal in Figure 4 is indeed puzzling. How was quantification attempted? Were the samples assumed to be uniform and thin to the 80 keV electron beam? Could the lack of control on sample size in air dried tissue have resulted in error? Could it be that there was mass loss under the electron beam that complicated measurements on dried tissue?
Author: Quantification was performed as described in materials and methods. They were bulk samples, as already described. A suggestion for the increase in sulphur has been given but mass loss may have artificially increased the sulphur content locally.

Reviewer II: How were the mass losses treated? Are mass loss dynamics different for embedded tissue from those of dried material?
Author: An account of mass loss in the electron beam in both freeze dried and embedded tissues is given in a previous SEM publication [28]. Embeddment appears to confer a stability on organic tissue mass in the electron beam but losses must inevitably occur.

Reviewer II: There are resins that do not have sulfur as a contaminant such as Spurr's plastic or EPON 826. The only element in these materials that is heavier than oxygen is Cl. Spurr's plastic can be prepared free of Cl and it is present only in low levels in EPON 826. Have you had experience using any of these materials?
Author: I have not used the sulphur-free resins.

Although it was of little importance in the study here, it has been of value in other investigations on natural sulphur content.

Reviewer II: Why is glutaraldehyde fixation necessary to stabilize proteins in the osmium-pyroantimonate method? Is the osmium not sufficient as a fixative?
Author: The osmium is used as the fixative. The pyroantimonate was used to precipitate the cations. Glutaraldehyde acts as a better fix of proteins than osmium tetroxide which primarily is an oxidising agent, but little success has been found in precipitating cations when used with glutaraldehyde.

Reviewer II: Do you advocate preparing tissue by substitution at +20°C? If not, is this indication that the criterion used in this paper for evaluating the various methods were sufficient to see only gross distortions, but not adequate to evaluate methods for analytical work?
Author: I have clearly stated in the discussion that the bulk analysis method gives an estimate only of the total retention of elements. At the subcellular level the local analyses on ultrathin sections give greater information regarding retention. I believe further examinations should be made on the usefulness of ether as a substitution fluid at room temperatures where the hazards of freezing are avoided.

A.T. Marshall: The validity of your comparisons depends on the accurate characterisation of your samples as frozen-dried sections. It is therefore of critical importance that the freeze-drying procedure be beyond question. In this context could you elaborate on the conditions for freeze-drying? Is a vacuum of 10^{-2} Torr adequate in terms of the vapor pressure of ice at -100°C for example? Also exactly what is meant by "the sections were thawed under dry nitrogen gas..."? What is the point of this seemingly drastic step? As judged from Fig. 1 the sections really did thaw since the mitochondria seem to be filled with Ca granules which is characteristic of thawed or rehydrated frozen-dried sections. This casts some doubt on the validity of your base line conditions.
Author: The sections were not thawed but freeze dried as you correctly point out. It is possible that the granules seen in the freeze dried sections may have been artefacts of ionic diffusion. It was considered, however, that adequate freeze drying had occurred under these conditions since it has been suggested by Frederik et al (29) that cryosections freeze dry completely in 10 minutes at atmospheric pressure and 193°K. Those workers estimate that freeze drying occurs at 22.5 nm/min vapour pressure at 193°K. There is bound to be some finite movement of ions during the initial process and during displacement of water molecules while freeze drying. Differences in the forms of ice, vitreous or in crystals of varying sizes, will result in differences in the rate of evaporation. During freezing the form of ice will depend in part on the electrolyte content locally. Frederik and colleagues have calculated that frozen thin sections should dry in 10 minutes in the cryochamber even without vacuum. The Ca rich granules would thus seem to be due to freezing rather than freeze drying artefacts.

A.T. Marshall: What steps did you take to maintain anhydrous conditions during freeze-substitution or substitution at 20°C and during the subsequent embedding procedure? This seems to be very critical to the success of the freeze substitution procedure.
Author: The ether was anhydrous throughout and changed during freeze substitutions. At 20°C it was not changed but the volume ratio of tissue to substitution fluid was approximately 1 to 20,000. It seems unlikely that much water would be left in the tissue following this substitution by ether.

B. Panessa-Warren: From the looks of some of your micrographs, you seemed to have some infiltration problems when using plastic embedded tissue (freeze substituted). Have you found any ways to avoid this problem, which is especially common when precipitation techniques such as lanthanum and pyroantimonate are used?
Author: The infiltration was not so great a problem as the initial freezing which is probably responsible for the relatively poor morphology. It has been shown that good ultrastructure can be achieved following freezing, thawing, fixation and embedment, where the structures are allowed to relax back from the frozen state, so masking distortions from ice crystal formation. With strictly frozen tissues, however, the morphology remains poor. The resin chosen was of relatively low viscosity at -20°C (Spurr). There are, however, newer resins such as Lowicryl which may be more suitable for this approach.

B. Panessa-Warren: In a tissue with most of the cations in an unbound (diffusible) state, do you think that freeze substitution could be safely used to obtain valid intracellular and intra-organellar trace element analysis?
Author: Shock freezing will theoretically retain the elements. If they are not soluble in the freeze substitution fluid then movement may occur through surface tension forces as the fluid moves into the tissue to replace ice. Although specimen contact with a solvent is bound to result in some redistribution of elements, the slow dehydration of the specimen by freeze substitution reduces the possibility of collapse of cell structures and does not cause translocation of water soluble ions to membrane surfaces as would occur in freeze drying of whole tissues. It is therefore preferred over freeze-drying and vacuum embedding of whole tissues but must rank below freeze drying of frozen sections in terms of elemental retention, even with better morphology.

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