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TED HALL AND THE SCIENCE OF BIOLOGICAL MICROPROBE X-RAY ANALYSIS: A Historical Perspective of Methodology and Biological Dividends

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Abstract

This review surveys the emergence of electron probe X-ray microanalysis as a quantitative method for measuring the chemical elements in situ. The extension of the method to the biological sciences under the influence of Ted Hall is reviewed. Some classical experiments by Hall and his colleagues in Cambridge, UK, previously unpublished, are described; as are some of the earliest quantitative results from the cryo-sections obtained in Cambridge and elsewhere. The progress of the methodology is critically evaluated from the earliest starts to the present state of the art. Particular attention has been focused on the application of the method in providing fresh insights into the role of ions in cell and tissue physiology and pathology. A comprehensive list of references is included for a further pursuit of the topics by the interested reader.

[This paper is dedicated to the fond memory of Dr Vernon Ellis Cosslett who died in Cambridge on 21 November 1990 at the age of 82 years.]

Key Words: Biological microprobe analysis, history of biological microanalysis, ions in cells and tissues, review of electron probe x-ray microanalysis in biology.

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Introduction

Biologists have known for at least a century that ions of various chemical elements have a key role in a variety of biological functions. There has always been a quest for methods which will allow the elemental composition of cell and tissue components to be visualized and quantified in situ consonantly with the resolution of the available microscopic image. Prussian blue and red thiocyanate methods for tissue-iron, the ammonium molybdate method for phosphate, H_2S for mercury and "gypsum" method for Ca date back to the last century (see Pearse, 1961). In the first half of the century the histochemists and cytochemists adapted a variety of 'microchemical' methods for a microscopical visualization of the important chemical elements (Lison, 1936; Glick, 1949; Gomori, 1952; Pearse, 1953) and even attempted to quantify them (Eranko, 1955). As in other preparatory techniques for cell and tissues, these precipitation methods developed for light microscopy were adopted for the electron microscopy of biological materials: these adaptations suffered from much the same limitations. As if 'damned' by the biogenetic laws governing embryogenesis, the same process seems to be recapitulating in tissue preparation for biological microanalysis.

The idea that the loss of energy in the incident electrons could be used directly to obtain chemical information about the specimen had already been suggested at the very inception of electron microscopy (Hillier, 1943; Hillier and Baker, 1944) but the quantification of such electron energy loss spectra (EELS) from biological specimens still remains to be resolved (Hall, 1989a, b). However, the interaction of incident electrons with the atoms in the specimen also generates X-ray photons. For his Ph.D. research at the University of Paris, Castaing (1952) used flat crystal X-ray spectrometers to measure such X-ray quanta and thus constructed the first electron probe microanalyzer (Castaing and Guinier, 1949). The specimen was viewed with an optical microscope and the area of interest brought under the electron beam by mechanically shifting the stage. Ten years later, in

Washington D.C., Brooks *et al.* (1962), and Tousimis and Adler (1963) used similar instruments but with curved crystal X-ray diffractors to study, respectively, the distribution of calcium in bone-forming tissue and the accumulation of copper in Wilson's disease. Here I shall review the development of electron probe microanalysis (EPXMA) in Cambridge and the impact of Ted Hall on its biological application. Some unpublished records from the 'archives' of the Biological Microprobe Laboratory (BML) in the Zoology Department, Cambridge, are also included for historical interest. However, the account is essentially for commemorating Ted Hall's contributions as I see them and might be questioned in a global context of the history. No strict priorities are intended.

Early History of EPXMA in Biology: 1951-1962

The instruments developed by Castaing (1952), Brooks *et al.* (1962), and Tousimis and Adler (1963), had no facilities for scanning the electron beam or for focusing it into a really fine probe. In the Cavendish Laboratory at Cambridge (UK), in late 1950s, another Ph.D. student, Peter Duncumb modified a RCA electron microscope and built the first scanning microanalyzer (Fig. 1) with facilities for backscattered electron imaging (Cosslett and Duncumb, 1956; Duncumb, 1959). A finely focused probe could therefore be located with some precision on the after-glow images on the cathode-ray tube (CRT). This remains the common current practice in all biological EPXMA using SEM or STEM facilities.

[In 1974 while working on the JEOL JXA-50A in BML, Ted Hall and I discovered to our horror that when one switches from a full screen scan to a static probe mode, the apparent positions of the probe and the point of interest in the image on the CRT are not in register. The relative displacement on the CRT can sometimes be as large as 1 cm but the magnitude of this anomaly depends on the type of the instrument and on the geometrical configuration of the specimen with respect to the image forming system (Gupta *et al.*, 1977; Gupta, 1979, pp 384-385). Some of the more recent instruments do not have this defect.]

As in other instruments at that time, the X-ray photons in Duncumb's microanalyzer were recorded with a curved diffracting crystal. The quoted sensitivity of detection was 10^{-14}g in 10^{-11}g of the total mass (1 part per 1000). Lever and Duncumb (1961) used this instrument for studying the pathological accumulation of iron in rat duodenum. This work was later extended by another Ph.D. student, D.J. Marshall in Ted Hall's laboratory (Marshall and Hall, 1966, 1968) who was also responsible for the comprehensive formulation of the continuum method. Marshall obtained fully quantitative data on iron accumulation in haemosiderosis, and in the tissues of Bantu Africans who use iron cooking-utensils (relevant for the current debate on the accumulation of aluminium from cooking-utensils and Alzheimer's disease). Robertson *et al.* (1961) used Duncumb's microanalyzer to identify tin in the phagocytosed individual particles in the lung-tissue of tin-smelters and tin-baggers but found silicosis to be more common amongst the tin-miners. Thus from the start, the great potential of EPXMA for identifying environmental hazards due to heavy metal pollutants, leading to cell and tissue specific pathology was already evident (for further reviews see Hall *et al.*, 1972; Ghadially, 1979; Slater *et al.*, 1983; Abraham and Burnett, 1983; Roomans, 1989; Baker *et al.*, 1985).

In 1961 Duncumb left the Cavendish and moved to the Tube Investment Research Laboratories (TIRL) near Cambridge. Here, he produced a new scanning microanalyzer which was later manufactured as 'Microscan-1' by the Cambridge Scientific Instruments Company (CSI). A similar instrument (Figs. 2a, b) was built by V.R. Switsur in the room once occupied by the atomic physicist J.J. Thomson in the Cavendish Laboratory (Cosslett and Switsur, 1963). This microscan was the instrument that Ted Hall used from 1962 until 1971 for his classical work on biological EPXMA.

Ted Hall Era I : 1961 - 1970

The development of EPXMA had been in the hands of physicists for application to material and earth sciences. As noted by Gupta (1991), the material scientists who continue to control EPXMA facilities are generally insensitive to the analytical problems which require quantitative measurements of soft elements ($Z < 20$: Na, Mg, P, S, Cl, K, Ca etc.) in very low concentrations found in soft biological tissues. Furthermore, the conventional theory of quantification developed for materials was not really applicable to biological materials where the main mass was composed of soft elements, C, N, H, and O. The most important role of Ted Hall was to plead the cause of biologists with his colleagues in physical sciences. The first arrow in that direction was fired with the following memorandum dated 30th January 1963, sent by Ted Hall to the microprobe group in Cavendish, and reproduced here with Ted Hall's permission.

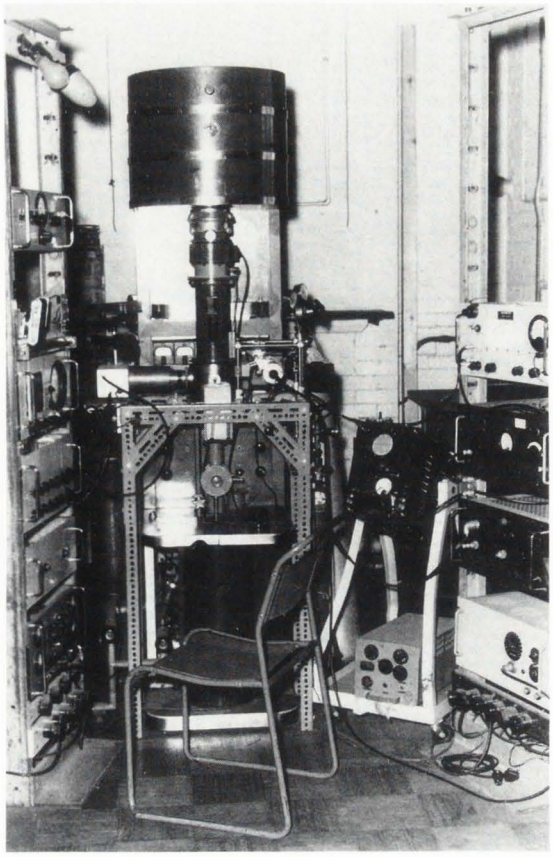
"A note on "transmission" electron-probe microanalysis for biological work

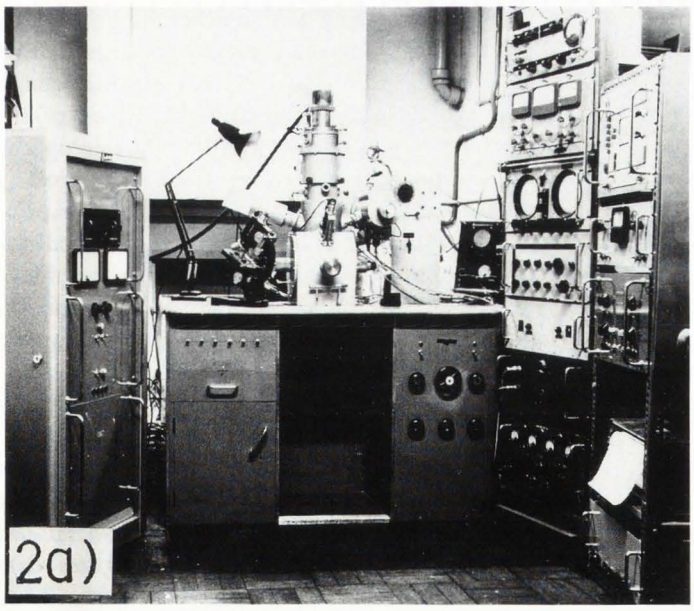
In the discussion at the Cavendish lab on Jan. 22, there did not seem to be a solid meeting of minds on the desirability or the necessity of "transmission" microanalysis for biological work. I am circulating my own viewpoint for the more leisurely consideration of the people who were at the discussion.

Fig. 1 (at right). First electron beam-scanning microanalyzer built by Duncumb (Cosslett and Duncumb, 1956) from an RCA electron microscope.

I. For many biological studies a lateral resolution in the neighborhood of 1μm will be needed, and a specimen (section) thickness not more than 5μm will be best. The dimensions of many biological objects (mammalian cells, for example), the need to correlate with the light-microscopical view of the specimen, and the need to avoid excessive lateral scattering of the electron beam, will often preclude specimens thicker than 5 or at most 10 microns.

II. Many problems call for localization of elements in the atomic number range of 25-35. In this range, element concentration is generally too low to permit non-dispersive analysis of L lines, which would suffer interference from oxygen, sodium, and magnesium K emission. Therefore the energy of the probing electrons at the very least would have to exceed the energy of the K edge, E_k. Actually, the ionization cross section is said to be of the form E^{-1} ln E/E_k (Green and Cosslett, Proc Phys Soc 78, p. 1210, 1961). This function equals zero at $E=E_k$ and peaks at $E=eE_k$, suggesting that a probe voltage of approx. twice E_k or more is desirable.





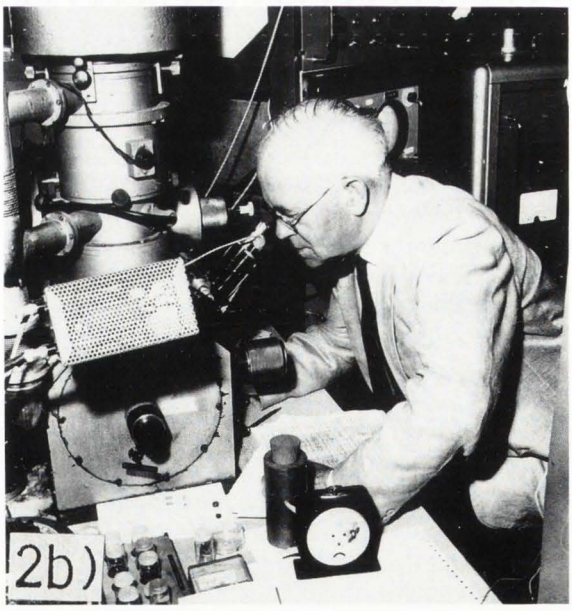


Fig. 2. (a) Cavendish prototype for 'Microscan-1', later used by Ted Hall; (b) V.E. Cosslett (now deceased) using the instrument (see Cosslett and Switsur, 1963).

III. A 5μm section of soft biological tissue, after drying, weighs only approx. 0.1 mg/cm^2. Calculation indicates that electrons of energy $2E_k$, for Z=25 or higher, will generally lose only a small part of their energy in penetrating such sections. (Because of the low average atomic number of soft tissues electron scattering is relatively weak and the electron path in such specimens should not be much greater than the thickness). Hence it must often be expected that the probing beam will pass through the specimen and impinge on the support with much of the energy unspent. In such a case, the background from the specimen support may easily be intolerable unless the support is very thin.

IV. With ashed specimens cut at 5-10μm, in general almost all of the beam will reach the specimen support, since only approx. 3% of the average soft tissue remains after ashing. Therefore it seems that signal/background will be adequate for such ashed specimens on thick support only when there is a relatively high concentration of the element of interest in the specimen. (No doubt this requirement of high concentration will be satisfied in many interesting situations.)

To sum up: While many interesting biological problems should be approachable with specimens mounted on thick supports, I believe that for the most general category of biological problems, background suppression will require very thin specimen support, with the beam passing through both specimen and support. A Formvar-coated EM grid seems suitable as a support.

(signed by) T. Hall

30/1/63

(Copies to : VE Cosslett, AJ Hale, JS Halliday, T. Mulvey, VR Switsur)."

Thus for the biological application Ted Hall in 1963 was already committed to: (1) the use of sections rather than bulk samples; (2) the use of low background, thin film mounting on hollow specimen holders (tubes or collars with or without a grid); (3) the use of high kV (> 30) electron beam; and (4) the need for a new theory of quantification suitable for analysis of soft tissues and other organic materials. All these problems continued to concern Ted Hall and dictated the direction of his future work. However, the immediate consequence of the above memorandum seems to have been that the Cavendish 'microscan' became probably the first EPXMA facility anywhere, more or less dedicated to biological work. [It was for some of the time under contract to AJ Hale in London.] However, the situation for biologists interested in microprobe work did not improve much over the next 5 years. Hall (1968, p. 270) again made a compassionate plea to his fellow physical scientists:

"*Experience has shown that probe specialists pay no attention to biological papers at probe meetings. There is mild interest when a biologist discusses technical problems which are common to most work, but when he talks about the less familiar problems which are especially urgent for biological application (as I shall), the audience automatically switches off. This is natural. I am bored stiff by the refinements of the conventional theory of quantitation because they are irrelevant to my problems; to follow these developments I have had to overcome a barrier which naturally also blocks attention from the other side. So I have to begin by persuading you to read this paper.*"

And further:

"*You should read this paper because:*

1. You owe it to the biologists who come to you for help. The techniques which have been developed for probe studies of minerals and metals will not take biologists very far on most of their problems.

2. While I am concerned only with biological specimens, much of the paper is applicable to the analysis of organic materials in general, and especially to the analysis of very thin specimens."

Even today, to many biologists seeking the help of probe laboratories in material sciences, the following comment may seem very familiar (Hall, 1968, p. 271).

"*The technical difficulties are outside of the biologist's field and outside the experience of the owners of the probe. The biologist may hang on long enough to get something that he can put into a paper, but usually decides that the promise of the probe was illusory and he better keep to more orthodox methods in the future.*"

A vast number of biological papers published today contain such a 'something' which generally has no real biological meaning but which makes good decoration and impresses those editors and referees who are equally unfamiliar with the subtleties of EPXMA. However, the blame for the biologist's disillusion with the microprobe is not laid entirely on the physical scientists controlling the facilities. Ted Hall also chides biologists for expecting too much from the microprobe methods. The following remarks from Hall (1968, pp 272-273) are almost prophetic of what has since followed, and in spite of a 20 years progress in techniques, would apply today (see for example, Zierold, 1988a, b).

"*Histological and cytological correlation: this is one of the most serious difficulties in biological probe work. The biologist wants to measure elemental concentrations within certain components of tissues, such as different types of cells, intracellular organelles like nuclei and mitochondria, extracellular protein aggregates like collagen and elastin, the extracellular ground substance. But once the specimen is inside the instrument, the interesting component may not be identifiable in either the electron-scan image or the optical microscope.*"

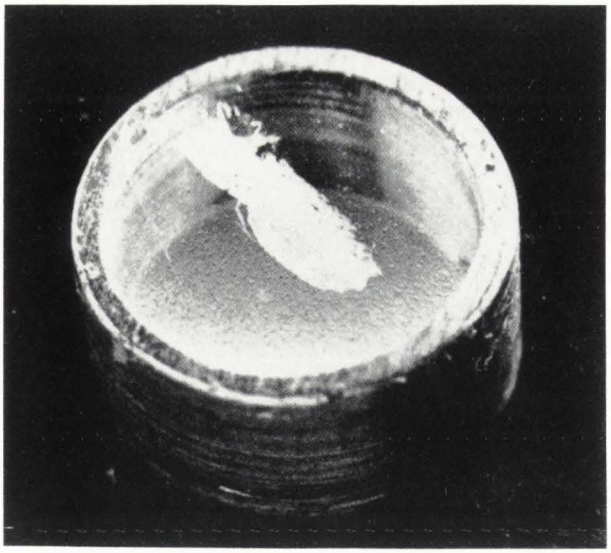


Fig. 3. Ted Hall's aluminium collar with a nylon film and a frozen-dried cryostat section without the aluminium coating.

[Optical microscopes of limited capabilities were incorporated in many microanalyzers fitted with diffracting X-ray spectrometers.]

Hall writes further on resolution: "*...there is no lack of important biological work for probes at a spatial resolution of one or a few μm. But biologists studying ultrastructure, especially electron microscopists, rapidly lose interest in probes when they learn that resolution is not better than 1 μm. ... They will grant that 0.1 μm might be quite useful, although they need 100 Å or less to be really happy. These aristocrats* (underline mine) *are doing the most fundamental biological research and a considerable effort would be worthwhile if it could provide them with a resolution of 0.1 μm.*"

A cursory survey of the major biological microprobe work today shows that irrespective of the specimen preparation and instrumentation used, the biological work is being carried out using either cryo-sections or frozen bulk specimens, with an image resolution not much better than a few microns (Wroblewski *et al.*, 1978; Roomans *et al.*, 1982a; Rick *et al.*, 1978, 1982; Zs-Nagy *et al.*, 1982; Smith *et al.*, 1983; Marshall and Condron, 1985; Morgan, 1985; Oates and Potts, 1985; Echlin and Taylor, 1986; Marshall, 1987; Saubermann and Heyman, 1987; Rick and Schratt, 1989). Even with the best available facilities using high resolution TEM/STEM and ultrathin cryo-sections the limit of analytical resolution remains at about 0.1 μm (Shuman *et al.*, 1976; Appleton *et al.*, 1979; Nakagaki *et al.*, 1984; Somlyo, 1984; Wendt-Gallitelli and Wolburg, 1984; Kendall *et al.*, 1985; LeFurgey *et al.*, 1988; Johnson *et al.*, 1988; Zierold, 1988a; Ingram *et al.*, 1987); although the feasibility of a better resolution for the 'future' has been demonstrated (Somlyo, 1984; Hall and Gupta, 1983; Hall, 1986a, 1989a; Zierold, 1988a).

Specimen mounts

A great deal of early microprobe work in Cambridge was done on conventionally prepared 4-10 μm thick histological sections of paraffin embedded blocks. In the pre-Hall period, deparaffinized sections were mounted on metal stubs or glass slides. As noted in the 'memorandum' above, Hall resorted to a thin film support. The sections were mounted on nickel EM grids with enlarged square opening of 300-600 μm width covered with Formvar film. To protect the specimens from the beam damage under an intense probe of about 1-μA, the sections were coated with about 70 nm vacuum-evaporated aluminium (Hall *et al.*, 1966). To get rid of the excessive extraneous X-rays from the grid, Hall (1966, 1968) introduced the use of Nylon films (Brown *et al.*, 1948) stretched over a "3/8 inch" (about 1 cm) diameter tube. The nylon film was coated with 20-50 nm thick aluminium. Similar coating was applied after mounting the specimen (sections or suspensions). The aluminium coating improved both the electrical and thermal conductivity of the specimen under the electron beam. Full details of the method are described by Hall *et al.* (1972; pp 162-163). The whole preparation is illustrated in Fig. 3.

This method of mounting the sections was essentially adopted for the analysis of 1-2 μm thick frozen-hydrated sections in BML. Höhling and his collaborators (Krefting *et al.*, 1988) still use an 8-mm aluminium tube but with a carbon-coated Pioloform film for analyzing 4 μm thick frozen-dried sections in a CAMECA MS46 microanalyzer. The use of aluminium top-coating was also adopted in many other laboratories for the analysis of frozen-hydrated bulk specimens (Fuchs and Lindemann, 1975). Other metals such as Be, C, Cr have also been used for this purpose (see Marshall, 1987).

Hall method of continuum normalization

Ted Hall is best known for his method of the quantification of X-ray data from thin specimens but especially for biological tissue sections. I do not propose to go into the history and the detailed formulations of this method here because it has been extensively discussed in numerous publications both by Ted Hall (see Hall, 1989b and Gupta, 1991 for references) and by others (see Roomans, 1988b). It has found extended application and is incorporated in many a commercial soft-ware for EPXMA data processing. Although the method was intended for thin specimens (not more than a few μm thick), it has been used for the analyses of frozen-hydrated bulk specimens because in such samples the

internal charging spreads the electron beam laterally to form a pancake rather than a pear-shaped geometry of the excited volume (Brombach, 1975; Marshall, 1975, 1980b, 1987; Zs-Nagy *et al.*, 1977; Fuchs and Fuchs, 1980; Oates and Potts, 1985), therefore restricting the effective thickness of the analyzed volume to a few micrometers. For some recent discussion of the continuum method, its use in practice and its limitations the reader should refer to Hall and Gupta (1979, 1982a, 1983, 1984, 1986) and Hall (1979a, b; 1986a, b; 1988, 1989b).

Ted Hall Era II : 1970 - 1984

The late 1960s and early 1970s saw many parallel developments which dramatically changed the course of biological EPXMA, both in Cambridge and elsewhere. Firstly, the introduction of linear track, fully focusing diffracting spectrometers with high collecting power pushed the limit of detectibility in the range of 10^{-18} - 10^{-19} g (see Wittry, 1969; Hall, 1971). Secondly, the introduction of lithium drifted silicon semiconductor (SiLi) X-ray detectors with their associated electronics, made it possible to record the entire deconvoluted X-ray energy spectrum from the same irradiated area. The energy-dispersive X-ray (EDX) spectra could be further processed either manually (by eye) or with an elaborate software programme on a dedicated computer. Apart from providing the characteristic counts for every element with atomic number $Z > 13$ (Russ, 1971, 1972), one also had 'continuum', Bremsstrahlung, or white counts as an estimate of local mass as postulated by Hall formulations. Furthermore, the X-ray collecting efficiency of EDX spectrometry was about 100 times better than that of wavelength-dispersive spectrometry (WDS) for a given radiation. Therefore much lower probe current and/or thinner specimens could be used. Improved designs of electron guns and condenser lenses on the electron microscopes could deliver sufficient current even into a finely focussed probe of 20 nm diameter. High resolution biological microanalysis now seemed feasible with a moderate electron dose of < 100 nC (nanocoulombs) i.e., 1 nA for 100 seconds (Hall, 1979a, 1986a).

Two approaches to microanalytical instrumentation emerged. One was to improve dedicated scanning microanalyzers by fitting more versatile imaging facilities, with the possibility of using several fully focusing WDS operating simultaneously and covering virtually the entire range of elements in the periodic table. An EDX spectrometer (EDXS) could be added as a further facility. The second was to fit an EDXS to an existing scanning (SEM) or transmission electron microscope (TEM). The second development became popular with the biologists and apparently 'liberated' them from dependence on the physical scientists.

Peter Duncumb (at TIRL) again took the lead by developing the first transmission microscope microanalyzer or EMMA (Duncumb, 1963). In this prototype EMMA, the X-ray spectrometer was a proportional gas-flow counter used in an energy dispersive mode. This was useful for analyzing inorganic precipitates (Duncumb, 1966) but not for serious biological work. When diffracting crystal spectrometers were fitted to EMMA (Duncumb, 1968), Ted Hall and collaborators commenced an extensive biological programme. Thus for the first time, one had a microanalyzer with a good electron microscope image, X-ray analytical facility, and if required, an electron diffraction pattern from the same micro-area of a specimen. A collaboration between the TIRL and AEI Scientific Apparatus Ltd, UK, led to the development of a 'high' resolution (0.2 μm) EPXMA working at 100 kV and fitted with two fully focusing WDS (Cooke and Duncumb, 1968). Other microanalytical instruments commercially available by the end of 1970s are listed by Hall (1971), Hall *et al.* (1972), and Chandler (1977). An enormous range of new instruments with various degrees of operative sophistication and analytical capabilities has since been added. The expansion continues. For biologists the EPXMA world to-day is an oyster but unfortunately the 'funds' for the pearls are hard to find. [Anecdotally, the prototype EMMA was built upside down with the fluorescent screen located conveniently at about 6 feet to suit Duncumb's physical stature. The lesser people needed a prop.]

In 1971, Ted Hall replaced his historic microscan with an EMMA-4 and additionally fitted it with a KEVEX SiLi spectrometer. He did not, however, have any cryo-preparative facilities in his Cavendish laboratory. Ted Hall's EMMA-4 was not therefore used much for fully quantitative analysis of cryo-sections. Tim Appleton in the Physiological Laboratory across the road had been one of the pioneers in the development of cryo-ultramicrotomy (Appleton, 1968, 1969). He too acquired an EMMA-4 in 1971 and produced probably the first semiquantitative data on Na, K, P, and Ca in an ultrathin cryo-section of chemically unmolested exocrine cell from the mouse pancreas (Table 1). It is both uncanny and amusing that his arbitrary units of elemental concentrations are surprisingly similar to the wet weight concentrations (Table 2) from a recent study (Nakagaki *et al.*, 1984). Beware of the hand of fortuitous coincidents! Hall *et al.* (1973) then confirmed the validity of the 'continuum' method for ultrathin, frozen dried sections. This paper is probably the most frequently cited reference in the literature on quantitative EPXMA. Further validation was provided by Shuman *et al.* (1976). More recently the continuum method has again been validated by quantitative X-ray elemental mapping using digital processing (Ingram *et al.*, 1989).

Table 1. Summary of semiquantitative data [(*Characteristic X-ray counts for element) / ('White' X-ray counts) x 100*] on ions from an ultrathin frozen-dried cryo-section of mouse pancreas, analyzed in EMMA-4 using 60kV, 200 nm electron spot and WDS. From Appleton (1972).

Field	Na	K	P	Ca
Free cytoplasm	4.76	115.00	121.00	-
Nuclei (light area)	1.44	41.40	59.00	-
Nuclei (dark area)	5.30	123.00	117.20	2.72
Rough ER	3.20	97.00	118.80	2.62
Vesicle (cond. vac.)	3.23	122.30	45.80	4.26

Table 2. Comparable values in mmol/kg wet wt from a recent study of frozen-hydrated cryo-sections by Nagaki *et al.* (1984). EDS X-ray data were quantified by Hall's continuum method.

Field	Na	K	P	Ca
Cytoplasm	4.8	132.00	165.00	-
Zymogen (granule)	6.00	60.00	36.00	6.00

[Nuclei were very similar to the cytoplasm: no separate data were given.]

Ali *et al.* (1977) used EMMA-4 with Appleton's approach for preparing frozen-dried ultrathin sections, for their work on calcifying cartilage. In recent years Marion Kendall and Alice Warley in London (see Kendall *et al.*, 1985; Warley, 1987) have continued to use EMMA-4 with Hall's continuum method for the quantification of data from frozen-dried cryo-sections.

Biological Microprobe Laboratory (BML) (in the Zoology Department, Cambridge, U.K.)

As noted in the accompanying biographical sketch (Gupta, 1991), the key experiment which led to the establishment of BML in Cambridge in order to develop the techniques for EPXMA of soft biological tissues in fully hydrated state and apply them to a number of biological projects, was done by Hall, Gupta and Weis-Fogh on 17 July 1970 at the applications laboratory of the Cambridge Scientific Instruments (CSI) (see Echlin, 1971; Gupta and Hall, 1981a). The instrument used was a Cambridge Stereoscan fitted with a prototype LN_2-cooled stage, a Faraday cup for measuring the probe current, and an EDAX SiLi detector. The specimens were the sections of blow-fly flight muscles cut at about 4 μm in a cryostat at -20°C and mounted on aluminized nylon films stretched over hollow brass-stubs (described later, see Fig. 5a). The sections were transferred 'hydrated' into a metal-block container in an LN_2 Dewar and transported to CSI. Before loading onto the cold stage of the Stereoscan the sections were allowed to thaw for making good contact with the substrate film. The sections were rehydrated in moist air before loading into the microscope. The 'hydrated' sections froze again on the cold stage of the Stereoscan. [Our technician Nigel Cooper (now Dr. N. G. Cooper, Associate Professor of Neuroanatomy, University of Tennessee, Knoxville, TN, USA) was stranded with the specimen Dewar in an elevator between two floors in the Zoology Department and had to be rescued by the maintenance engineers. Later at the CSI laboratory, at the end of the day, the Dewar exploded when Ted Hall was trying to drain out LN_2 from it but fortunately Ted escaped injury.] Nevertheless, in spite of the mishaps, the experiment seemed to have worked, as recorded by Hall in his unpublished report of results, reproduced here, without any editing:

Extract from a report by T A Hall dated 17/7/1970 in the files of BML

*Performance: Prep seems stable at the low current used simply to obtain a scanning image, namely 2 * 10^{-10}A. At $3*10^{-8}$A, the ice over the hole is quickly dissipated by full field scan, while the ice over the brass is also dissipated, though not so quickly. Temperature -140 to -125°C. Ice whiskers are seen to have formed around the edge of the Faraday cup which is used to measure probe current, and the stability of these under the probe is also tested. The whiskers appear to be much more stable than the ice on the brass specimen stub, with only a slow removal at $3*10^{-8}$ A.*

Afternoon. Second specimen. No dag. 1/8" hole. Preparative history same as the first specimen. But the bottom of the stub is wiped before it is screwed onto the stage. Prep goes onto warm stage and into chamber and chamber is evacuated with no cooling; the intention is to see the stability and appearance of the dry section without any interference from ice. The preparation seems completely stable at "high current" even under a scan over the reduced field. Air admitted to chamber; stage then cooled to form ice; chamber then evacuated. Ice is seen over brass and near edge of hole but not over the hole except near the edge. High current is put onto a field of ice over the hole, where ice is farthest from the edge of the hole. The ice is stable, under both full-field and restricted-field scans. The stage is warmed and the ice is removed at -95°C.

III. Back to the first specimen (the one which was studied in the morning). The first specimen is put back onto the stage, but this time the bottom of the stub is wiped first. Specimen put onto warm stage; then stage into column; then prep is cooled; then chamber is evacuated. Ice over the hole very slowly fades at high current scanned over full field; temperature -145°C.

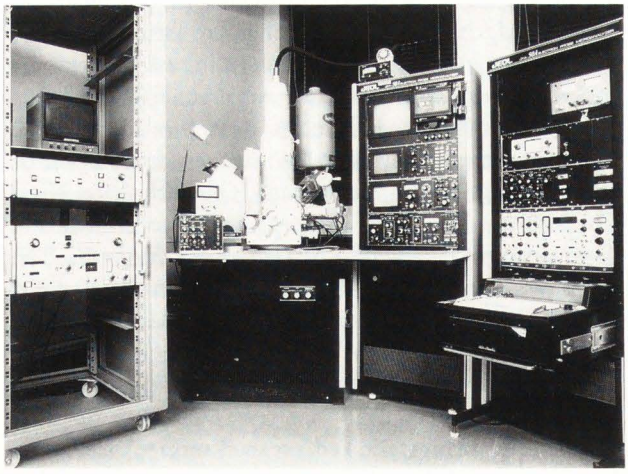


Fig. 4. JEOL JXA-50A microanalyzer with a KEVEX-LINK EDXS used in BML, Cambridge before the incorporation of a full LINK Systems data processing facility (pre-1981).

IV. Third specimen. Dag. This specimen was mounted on a stub which had spent a long time in the cryostat, and the prep then went into the Dewar and was stored at the temperature of liquid nitrogen. Approx half an hour before going into the Stereoscan, the stub was put into moist chamber at room temperature. The stub was removed from the moist chamber; its bottom was quickly wiped; then cooled; then pumped. Measurements were then made at the Faraday cup in order to be able to deliver known amounts of current to the specimen. The intention was to raise the current in known steps to see how much the prep would tolerate. The data with the cup show that the "high current" used through the afternoon was not the same as the high current of the morning, although the lens settings were the same. The "high current" referred to above seems to be $2.2*10^{-8}$ A.

Unfortunately it does not seem easy to go up in current in small steps and the next current available with selected and recorded lens current is $1.6*10^{-7}$ A. Back to the specimen: current $2.2*10^{-8}$ A. Magnification 100x. Polaroid picture 1. Mag. raised to 500x. Polaroid picture 2. (Pictures are of the ice over the hole.) 2 1/2 minutes allowed to lapse. Picture 3. Current then raised to $1.6*10^{-7}$ A. The ice disappears at once. New field, over the brass: at the same current, $1.6*10^{-7}$ A, the ice rapidly disappears (though not at once).

Conclusions and remarks:

1. The stability of the specimen in the afternoon was much better than was observed in the morning. The stability in the afternoon was still not shown to be good enough to tolerate the high current static probes wanted for X-ray analysis, but enough to give hope of achieving sufficient stability through improved technique.

2. The observed variations in rates of removal of ice might have been due to one or more of these factors:

a. "High current" in the morning is measured to be $3*10^{-8}$, and in the afternoon (at the same lens settings and beam current) $2.2*10^{-8}$ A. It is not clear how large would be the effect of this difference in current, if real.

b. In the morning the moisture was not wiped from the bottom of the stub just before putting it on the stage. The same specimen seemed more stable in the afternoon when its bottom was wiped.

c. The contact between brass stub and copper stage may be poor and variable, as neither surface is either lapped or flexible, and the copper is not even very flat. It would be better to screw these surfaces, or wedge them, tightly together, and one should be flexible.

3. The differences in phenomenon seen over the middle and near the edge of hole suggest that the conductivity of aluminized film is limiting factor. Smaller holes or grids might overcome this.

T.A. Hall"

On retrospection, the above experiment seems naive and almost an act of faith. But it provided a conceptual leap over the barrier, then prevalent, that ice even on a 'cold' stage could not withstand the intensity of electron beam in a high vacuum of an EM column. Apart from the BML group in Cambridge, Tom Hutchinson in Minnesota, USA (Bacaner *et al.*, 1973) and Thurau's group in Munich (Gehring *et al.*, 1973) also embarked on the EPXMA of frozen-hydrated cryo-sections, using the Cambridge Stereoscan fitted with EDXS.

Technical Aspects: Biological Microprobe Laboratory, Cambridge

"Oh what a tangled web we weave
When we measure electrolytes with a sieve,
(Shakespeare)" [Actually by Ted Hall, in BML Data Book I, 7 Nov., 1973]

The first phase of BML (1971-1973) was for studying the feasibility of analyzing soft biological tissues in a frozen-hydrated state with a resolution of 1 to 2 μm. The laboratory was equipped with a Cambridge Stereoscan S-4 operating at 30 kV and fitted with a standard CSI Series 200 'hot-cold' stage. This stage was modified by Roger Moreton for scanning transmission electron microscopy (STEM) images (Echlin and Moreton, 1973, 1974; Echlin *et al.*, 1973). Ted Hall selected a SiLi EDX detector for the best performance then available (Quartez-et-Silice, Ortec amplifier-model 716 A, and a Northern Scientific model NS-Econ multichannel analyzer with X-Y recorder). The fresh tissues without any cryo-protection were quench-frozen in liquid nitrogen (sic). For cutting 1-2 μm thick cryo-sections at -80°C, we followed a cryostat approach (Stumpf and Roth, 1965; Appleton, 1968) and used an instrument custom-

built by SLEE, London (see Gupta and Hall, 1981a for the advantages). The 'frozen-hydrated' sections were top-coated with a layer of evaporated aluminium in a Balzer BAE120 vacuum coating unit. All the technical developments of this period were reported at the annual meetings of the IIT Research Institute, Chicago (Echlin, 1971; Echlin and Moreton, 1973) and elsewhere (Echlin *et al.*, 1973). Moreton *et al.* (1974) reported some microanalytical results from 'frozen-hydrated' sections of the larval salivary glands of *Calliphora*. Bacaner *et al.* (1973) and Hutchinson *et al.* (1974) reported similar results from 140-nm thick 'frozen-hydrated' sections of rabbit psoas muscle. Judging from the good contrast and detail in the published STEM images in both these biological reports it is almost certain that the sections analyzed then were not fully but partially hydrated. In the report by Moreton *et al.* (1974), it is particularly noteworthy that the free lumen of the gland has no contents. The STEM images in both these reports are also free from any superficial frost, even though Cambridge Stereoscans did not have a pre-evacuated air-lock. The specimens had to be transferred to an LN_2-cooled stage at the atmospheric pressure, using rather clumsy and make-shift transfer system. Thurau's group in Munich abandoned the analysis of frozen-hydrated sections. Dörge *et al.* (1974) reported the first fully quantitative data for intracellular Na and K in frozen-dried cryo-sections of frog-skin. In this report they used Hall's continuum method for the quantification of X-ray data. Some early microprobe work on 7 μm thick frozen-dried sections of mammalian kidney was reported by Kopsell *et al.* (1974). The proceedings of the first two Symposia on biological microanalysis (Chandler and Lacy, 1972; Hall *et al.*, 1974) provide interesting insights into the 'state of the art' at that time.

How to establish that a cryo-section or a thin film of biological material is in fully hydrated state, either for high resolution imaging or for EPXMA, still remains a controversial issue and has concerned Ted Hall for 20 years (see Hall, 1986a; p.93). After the publication of an extensive paper by Saubermann and Echlin (1975), the issue became divisive amongst the principal participants in BML. It was especially painful for Ted Hall because most of the observations and conclusions about the analysis of frozen-hydrated sections published by Saubermann and Echlin were in direct conflict with the experience of the rest of the group (see Discussion after Gupta, 1979). Time has shown that Ted Hall was right (Gupta *et al.*, 1980a; Gupta and Hall, 1981a; Zierold, 1985, 1988a).

After the completion of the feasibility studies, the BML entered the second phase of biological studies. The biological programme included projects which required measurements of free-fluid spaces either outside the cells or as intracellular vacuoles (as in plant cells), which cannot be meaningfully analyzed in a frozen-dried state. Hall and his colleagues in BML thus remained committed to analyzing frozen-hydrated sections, come hell or high water. For these studies the group (mainly Ted Hall) selected JEOL-JXA 50A microanalyzer (Fig. 4) which had the following features, many based on the earlier stipulations of Hall (1971) and unique for that time.

1. An electron gun and condenser system operating at 50 kV and capable of delivering several μA of current into a focussed probe of about 200 nm in diameter.

2. A versatile imaging system with a push-button selection for secondary, backscattered, compositional and STEM imaging with dark-field facilities. Modest facilities were also available for 'image processing' on cathode ray tube (CRT) display screens. The STEM and secondary images could be obtained simultaneously on two CRT screens, with after-glow. A third CRT screen was available for photography.

3. A waveform monitor for the image density distribution which proved invaluable in checking that the probe or the reduced raster on the CRT was properly localized on the area of interest selected for analysis.

4. A good vacuum system with proper gauges for continuously monitoring the vacuum in the column. The diffusion pump system had an LN_2-cooled vapor-trap.

5. An LN_2-cooled stage with an anti-contamination cap close to the specimen. However this was replaced by our own stage built to Ted Hall's specifications (Taylor and Burgess, 1977). The stage accommodated numerous standards for the setting of WDS's.

6. A Faraday cup on the stage for accurate setting and monitoring of the probe current at the specimen level. We later adopted an innovation by Marshall (1980a) and continuously monitored the probe-current at the objective aperture level (calibrated to the Faraday cup on the specimen stage). This important facility is commonly ignored in most microanalytical instruments. The probe-current for every microanalytical measurement ("run") is entered in the comprehensive "Hall-programme" on LINK-SYSTEMS Quantem-FLS software and is important for an accurate correction of extraneous background from the substrate film etc., as well as for the correct estimation of mass-thickness of the analyzed area in the data print-out. (For further discussion on the correction of extraneous background see Heinrich, 1982; Roomans and Kuypers, 1980; Steinbrecht and Zierold, 1989; Warner *et al.*, 1985).

7. Two fully focusing WDS, each with a choice of several diffracting crystals. The superiority of WDS over EDXS for measuring very low levels of Na and Ca (in the presence of high K) has been repeatedly emphasized by Hall (see Hall, 1975; 1979a; Gupta and Hall,

1978, 1982; Hall and Gupta, 1982a, 1983) as well as by others (Lechene, 1980). CAMECA instruments with several WDS are commonly used for EPXMA of microdroplet samples (Roinel, 1988) but only in a few laboratories for the microanalysis of cryo-sections (e.g., Krefting *et al.*, 1988).

8. An efficient specimen transfer system through a pre-evacuated air-lock for loading frozen-hydrated specimens, without seriously degrading the vacuum in the column.

9. An optical 'reflecting' microscope, mainly to ensure that the specimen was at the correct focus for the diffracting spectrometers. However, this facility also proved invaluable for the preliminary examination and selection of the cryo-sections without any exposure to the electron beam. The interference colours of the sections often proved invaluable for such diagnostic purposes.

10. One KEVEX EDXS operating simultaneously with both the WDS. This EDXS was later adapted for the LINK-SYSTEM. The detector was mounted with a 35° take-off angle with respect to the horizontal specimen. The distance between the detector and the specimen could be adjusted for avoiding excessive dead-time when using high probe-current for WDS analysis. Later, micro-magnets were fitted to the nose-piece of the SiLi detector (Hall, 1977) in order to cut out backscattered electrons from entering the detector and degrading the X-ray spectra. Ultimately, a comprehensive software with dedicated computer facilities became available so that the concentration values in mmol for up to 10 elements could be printed out within about a minute of acquiring the data (see Gupta and Hall, 1982).

The programme includes a file of standards both for the elements and the mass-thickness, accommodates WDS + EDS analyses, allows for a multiple-source correction for the extraneous X-rays, can process data by two alternative methods of quantification (see Hall and Gupta, 1982a, 1983, 1984). Even with our relatively old EDX detector with an approximately 8 μm thick beryllium window, Hall and Gupta (1982a) have demonstrated that the programme could handle a virtually invisible Na peak (= about 5 mmol/kg wet wt) in an EDX spectrum over a high continuum background from a 1 μm thick cryo-section provided there were no other interfering peaks (e.g., Cu and Zn L-lines).

11. A high reliability of performance with good instrumental stability. Here Ted Hall's experience and expertise was invaluable. He generally spent 2 hours for setting up the instrument before an analytical session. Every parameter was meticulously checked, particularly for a proper management of extraneous background X-rays and for a proper setting of X-ray spectrometers. Apart from the correct setting of WDS, EDXS was set with reference to a Cu characteristic X-ray line in order to keep the integral X-ray quanta below 2000 cps and thus avoid pulse pile-up and peak-shifts in EDS spectra. It is likely that many anomalies in the biological data in other EPXMA arise from two basic questions: (1) Are all the sources of extraneous background being properly identified and corrected for? (2) What does the microprobe measure? Suitable warnings and comprehensive instructions for handling both these questions have been provided by Ted Hall in his numerous publications on the quantification procedures since the early 1960s.

Specimen preparation

During the course of his studies, first on the 'microscan' in Cavendish and later on EMMA-4 as well as in BML, Hall with his numerous collaborators experienced virtually every method of specimen preparation. After discussing all these approaches in detail, his thoughts had already crystallized in the following statement (Hall *et al.*, 1972, p. 165).

"*Hence a procedure which would be much closer to ideal, especially in the study of electrolytes, would be to freeze the tissue block rapidly, section, and analyze the sections without dehydration, maintaining at all the times a temperature so low that deterioration and sublimation would be negligible, and the water would be retained as ice, even locally under the electron beam during analysis.*"

This was the approach pursued in BML. However, for his EMMA work with ultrathin sections, the specimens were prepared by a variety of methods. Alternative methods of specimen preparation continued to be used in several other laboratories. Hall and Gupta (1984) and Hall (1986a) have surveyed the current approaches in brief, and so have many other authors (Moreton, 1981; Morgan,1979, 1985; Wroblewski *et al.*, 1988; Zierold, 1988a, b). For the description of some practical methods used for EPXMA see Warley and Gupta (1991).

Methods for EPXMA of Frozen-Hydrated Sections on a JEOL JXA-50A SEM-Microanalyzer

The first fully quantitative analysis of Na and K, both inside the cell and in free fluid-spaces outside the cells in the Malpighian tubules of the blowfly *Calliphora*, were carried out by Hall and Gupta on 21, 26 and 27 June 1974 (see Gupta, 1976). Ted Hall's interest in this material as the first 'test-case' might have been stimulated by the following recollection.

Sometimes in 1963, (the late) Dr. Ken Machin, a biophysicist from the Zoology Department, came to see Ted Hall in the Cavendish Laboratory and asked him if it were possible to measure the concentrations of diffusible electrolytes (Na, K, Cl etc.) in the cells of insect Malpighian tubules with his electron-probe. Due to the

classical work of Wigglesworth (1932) and Ramsay (1953) there was a great interest then in the fluid transport by these excretory organs. Ted Hall listened to Ken's precise description of the problem and then told him to come back in ten years time. So in 1974 the time had come to answer the question.

The results of this experiment have never been published in detail and are therefore of historical interest.

The Malpighian tubules of *Calliphora* when bathed in a fortified physiological Ringers (Na: 140 mM, K: 20 mM) secrete at a fast rate an isotonic fluid in which the Na:K ratios are essentially reversed. The fine structure of the tubules has been examined (Berridge and Oschman, 1969). The solute:solvent coupling for generating an isotonic solution had been postulated by a "standing gradient osmotic flow model" (Diamond and Bossert, 1967). Although the electrolyte composition of the fluid in the tubule lumen was known from several species of insects (Ramsay, 1953), there were no direct measurements of the electrolyte composition in cells and in intercellular spaces either in these tubules or in fact, in any other animal epithelium. In insects (and in many other invertebrate), epithelia also have the advantage that the tissues in the body lie in a bath of haemolymph. Therefore, during the removal from the body, these tissues do not suffer the surgical traumas and post-mortem changes which plague the tissue sampling from vertebrate animals.

For EPXMA of 1-2 μm thick frozen-hydrated sections, short lengths of tubules were checked for normal function in vitro by M. J. Berridge. The tubules operating normally were mounted in a drop of Ringers in a high humidity atmosphere (Ramsay, 1953; Hall and Gupta, 1982a). The specimens mounted on aluminium micro-chucks for the SLEE cryostat were quench-frozen in melting Freon-22 and transferred in liquid nitrogen into the large cryo-chamber of the cryostat maintained at -85°C. 1-2μm thick cryo-sections were cut on freshly sharpened carbon/steel knives, (bevel or rake angle 12°) using an anti-roll plate and picked up on aluminized nylon films mounted on special holders (Fig. 5; also see Gupta *et al.*, 1977). The holders were directly pressed on to the sections against the steel knives. The cryo-sections did not seem to stick very well to the aluminized films. [We later discovered that as in the case of carbon-coated EM grids, the aluminium films were hydrophobic due to residual hydrocarbon contamination in the vacuum coating units. Such films can be made hydrophilic by 'glow discharge' (Dubochet *et al.*, 1970; Gupta *et al.*, 1977). As noted by Griffith *et al.* (1983, 1984), such 'glow-discharged' films rapidly lose their hydrophilicity and should therefore be used within a few hours. For further discussion see Dubochet *et al.* 1982b, 1988].

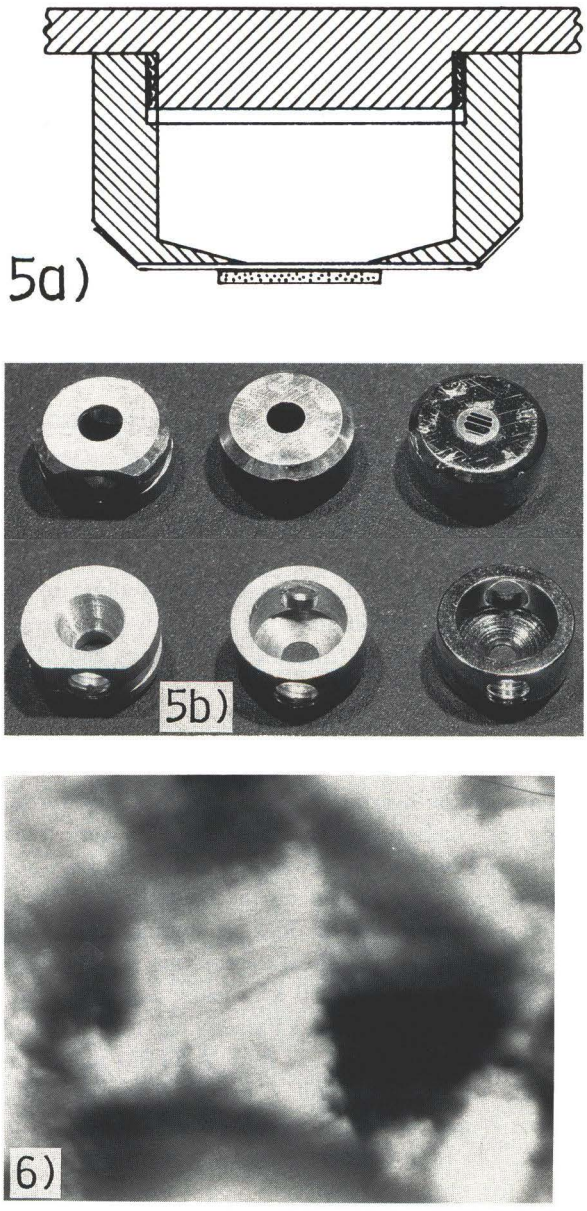


Fig. 5. (**a**) 1-cm diameter 'knife-edge' brass holder with a nylon film and a cryostat section used by Hall, Gupta and Weis-Fogh in 1970 for the experiment with CSI Stereoscan and cold stage. (**b**) Three BML section-holders (Duralium) for STEM analyses evolved from above: **left**, Saubermann-Echlin version without the knife-edge hole; **middle**, Hall-Gupta version with a knife-edge hole; **right**, Gupta-Hall holder with a heavy nickel plating and a multi-slot nickel grid in order to eliminate extraneous X-ray signals from Al, Cu etc. from the EDX spectra.

Fig. 6. First STEM image of a fully hydrated 1-μm thick cryo-section of *Calliphora* Malpighian tubule, cut at -81°C (Gupta, 1976). The stellate lumen looks transparent in this high contrast image because of lower mass-thickness (uneven section thickness).

Table 3. Raw X-ray quanta [Mean (SE)], from 1 μm thick frozen-hydrated sections of *Calliphora* Malpighian tubules studied in BML, Cambridge. Experiments by Gupta and Hall, June 1974. JEOL JXA-50A, 50 kV, 9-12 nA beam current, 1-2 μm^2 rasters, 200 s/run.

Field	n	WDS Na P-B	WDS K P-B	EDS 'White' $W-W_{film}$
Ringers(std.)	8	390 (61)	90 (19)	210 (51)
Lumen fluid	5	79 (15)	612 (109)	212 (7)
Brush-border	4	64 (11)	1158 (151)	315 (61)
Cell-body	11	194 (13)	1501 (189)	498 (99)
Basal infolds +base. memb.	6	758 (141)	1595 (201)	602 (131)

Values are Mean(SE). Low values for the white counts are because the continuum X-ray quanta were collected over a narrow window from the high-energy end of the spectrum.

The section-bearing holders, without any further coating (cf. Saubermann and Echlin, 1975) were transferred to an LN_2-cooled Dewar, transported to JXA-50A microanalyzer, and loaded on the cold stage (-150°C) through a pre-evacuated air-lock. A suitably flat section was selected under the optical microscope. The analyses were carried out with a 50 kV beam, using 9-12 nA probe current (measured on a Faraday cup near the specimen and checked after every run). A STEM image was obtained, using dark field / bright field conditions (Bacaner *et al.*, 1973) and recorded on a polaroid film (Fig. 6). The high contrast image was very poor in detail but the outline of the cells could be distinguished from the major extracellular compartments. Reduced scanning rasters, 1 to 10 μm^2 were located on the 'afterglow' image and the position recorded on the polaroid print . K_α radiation from Na were recorded with a RAP (rubidium acid phthalate) crystal and from K with a PET (penta-erythritol) crystal. The continuum radiations were simultaneously measured with a KEVEX 'SiLi' detector (nominally 8 μm thick beryllium window, detector area 30 mm^2, take-off angle 35°, distance 45 mm from the specimen center, resolution 160 eV) with a 'window' between 30 and 40 keV centered at 35 keV. Each 'run' was 40 s real time for WDS (= < 20 s for EDXS running about 60% dead time). The raw X-ray data are given in Table 3.

The X-ray data were quantified by Hall's method using the following equation:

$$f_{ion} = \frac{r_{specimen}}{r_{Ringers}} * f_{Ringers} \quad (1)$$

Where $r = (P\text{-}B)_{element} / W_{sp} - W_{film}$; P = characteristic counts for Na or K, B = background counts with diffractors - 4 mm off-peak, W_{sp} = continuum counts from the specimen, and W_{film} = continuum counts for the aluminized film.

After the analysis in a frozen-hydrated state, the sections were allowed to dry overnight in the high vacuum of the EM column, with the stage slowly warming. In confirmation of the experience of Moreton *et al.* (1974), the general contrast and details in the images from dry sections were vastly improved. The raster mark caused by the latent mass loss confirmed the position of the analyzed fields recorded on the Polaroid pictures of frozen-hydrated sections. An attempt to reanalyze sections in a frozen-dried state was frustrated. During the process of drying, the sections had lost 'good' contact with the substrate film and literally flew off the holders under the electron beam of 10 nA in a reduced raster. On retrospection, the results turned out to be remarkably accurate (Table 4). The only other comparable EPXMA data then available was from 1 μm thick cryo-sections of frog-skin, analyzed in a frozen-dried state by Dörge *et al.* (1974; Table 5). The standard used by Dörge *et al.* for the conversion of X-ray data was Ringers + 22% albumin, frozen and cut with the specimen. Hall's continuum method was used for the data conversion. The agreement for the values of the intracellular Na and K between these two studies is remarkable in spite of the different methodological approaches [we did not know each others' results until after the publications].

What Did We Learn From These Studies?

1. The results showed that Hall's method of continuum normalization is valid both for EPXMA of frozen-dried sections in an SEM using an EDXS and for frozen-hydrated sections using WDS for the characteristic X-rays and EDXS for the continuum X-rays. Our study also demonstrated that the continuum method compensated for the variations in the probe-current and in the specimen thickness.

2. Even the crude methods of cryo-preparation then used seemed to have preserved the distribution of ions, without scrambling, at least in the major compartments of the Malpighian tubules, in spite of a substantial ice-crystal damage seen in frozen-dried sections.

3. The first STEM images from truly frozen-hydrated 1 μm thick sections were devoid of all detail but still 'workable' for the analysis of the major tissue-compartments. [It turned out that the differences in the

section-thickness between the free fluid spaces and cells helped to produce such sharp contrast at the boundaries, as later demonstrated by Hall and Gupta (1982b)].

4. Even frozen-hydrated sections, when in good contact with the substrate film of aluminium could withstand the trauma of 10 nA probe-current in 1-μm^2 for 40 s without any apparent mass-loss (no obvious fall in continuum count-rate). [In later studies with improved section mounting, we often used 5 nA current in a probe of about 200 nm diameter for 80 s.]

5. The results of our experiment were in sharp contradiction of those published by our BML colleagues, Saubermann and Echlin (1975), but extended the preliminary feasibility studies by Moreton *et al.* (1974).

The technique that emerged in the subsequent 10 years is summarized in Figs. 7-10. Here I comment on those aspects which are of current interest.

EPXMA of Frozen-Hydrated Sections: Is Love's Labour Lost?

Stability and beam damage

It was established by Bahr *et al.* (1965) that under the electron beam at room temperature the ionization damage is virtually complete at a dose of about 0.2 nC/μm^2 (= 0.2 A/100 μm^2/100 s) leading to the loss of specimen mass. In all EPXMA this dose is invariably exceeded.

Commenting on his early studies in the Cavendish Laboratory, Ted Hall (1989a) states :

"In my own laboratory, with a diffracting spectrometer of very modest performance, in order to get adequate counting rates, one had to use probe currents ranging up to 1 μA. For many months, our thin specimens promptly disintegrated when exposed to such currents, but then we learnt to coat them heavily with evaporated aluminium, about 30 nm applied to both top and bottom faces. With such specimens we did not see any beam damage, so we innocently assumed that there was no beam damage (Hall et al., 1966)". (Underline mine)

After our initial experiences and the study by Hall and Gupta (1974), we too innocently assumed that there was no mass-loss. However, subsequent studies (Gupta *et al.*, 1976; 1977; 1978a, b; 1980a, b) requiring the use of highly focused static probes with a current of about 4 nA for 100-200 s, did show that there was a rapid loss of mass from the specimen under the beam. However the start of the mass-loss, as indicated by the continuum count-rate on EDS, seemed to have a lag period of about 5 s. Therefore, for such high resolution analysis we adopted the strategy of recording the initial count-rates and using these normalized averages for calculating the total mass for each run as if no mass-loss had occurred.

Table 4. Conversion of X-ray data in Table 3 into mean concentration values (mmol/kg wet wt.), using Hall's continuum method as explained in the text (from Gupta, 1976).

Field	Na	K
Ringers (standard)	140	20
Lumen fluid	26	138
Brush-border	13	156
Cell-body	26	143
Basal infolds + basement membrane	87	128

Table 5. Comparable results from 1 μm thick frozen-dried sections of the frog-skin analyzed by Dörge *et al.* (1974). X-ray data were collected with an EDXS only.

Field	Na	K
Cell-bodies (control)	35	135
Cell-bodies (+ ouabain)	120	43

Nevertheless, further experience in many other laboratories has shown that cooling the specimen to -170°C can only retard the loss of mass by a factor of less than 100 and even then the tolerable dose does not exceed 20 nC/μm^2. The problem of mass-loss in frozen-hydrated sections is especially severe (Dubochet *et al.* 1982a, 1988; Heide, 1984; Talmon, 1984, 1987). From the experience of ultrathin (< 0.2 μm thick) sections, it has sometimes been said that it is not at present possible to carry out EPXMA on frozen-hydrated sections and quantify the data by X-ray continuum method (e.g., Zierold, 1982a). This impression prompted Hall (1986b) to carry out a series of systematic studies on the question of mass-loss in frozen-hydrated sections. Hall firmly established that under the conditions used for most of our biological studies it is indeed possible to complete the analyses in 1 μm thick sections at 100 kV even with a probe size of 100 nm^2 and with an electron dose of up to 50 nC.

This is consistent with the hypothesis that the loss of water from hydrated sections occurs by surface etching (Dubochet *et al.*, 1982a, 1988; Heide, 1984). Thus even a 0.5 μm thick section, with an electron dose of 20 nC/μm^2, will only lose about 8 nm of mass-thickness (essentially water). In a 0.2 μm thick section analyzed for high resolution of 100 nm, the entire mass under the beam could disappear long before sufficient 'continuum' X-rays could be acquired (see also Talmon, 1987; Zierold, 1988a, b). Thus the initial decision of Ted Hall and Torkel Weis-Fogh to use 1-2 μm thick sections for the analyses in the frozen-hydrated state, optimistically aiming for a spatial resolution of 1-0.5 μm has been

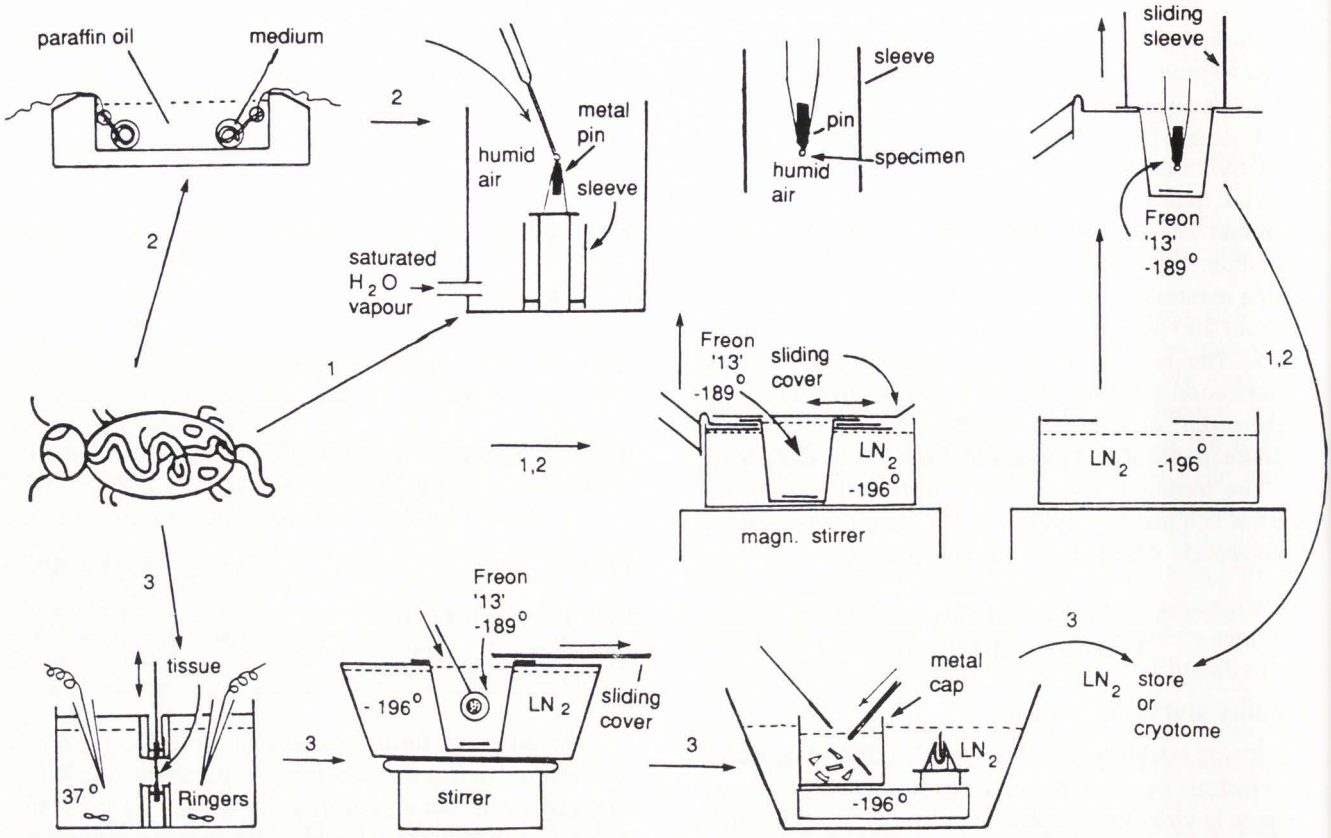


Fig. 7. A scheme of specimen preparations used in BML, Cambridge. (1) Excised tissue mounted directly on a metal pin in a humid chamber or (2) transferred first to a Ramsay preparation for monitoring the physiological function; (1, 2) a pin-mounted specimen in humid air protected with a sliding sleeve, fast frozen on a 'loyal toaster' (see Fig. 11); (3) excised tissue mounted in an Ussing-type chamber for physiological study, frozen manually in sub-cooled Freon-13, fractured into small pieces in LN_2 and either stored or mounted in indium metal loop in a vice-type cryotome chuck. Temperatures are in degrees Celsius.

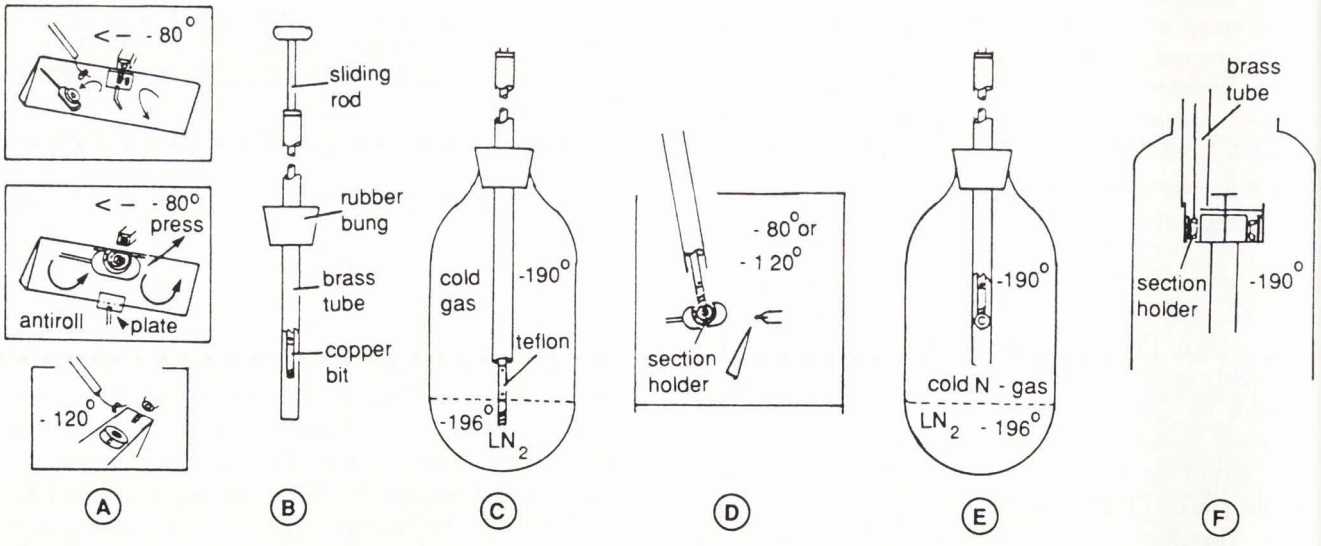


Fig. 8. Schematic flow-diagram for cryo-sectioning, mounting and storage of section-holders in BML, Cambridge. (**A**) Blocks cryo-sectioned at 0.5 to 2 μm thickness either in a SLEE cryostat at about -80°C or in a modified LKB CryoKit at about -120°C; (**B**) a sliding rod and brass-sheath device (**C**) precooled in LN_2, (**D**) for retrieving the section-holder and (**E**) protected transfer to (**F**) the storage Dewar. Temperatures are in degrees Celsius.

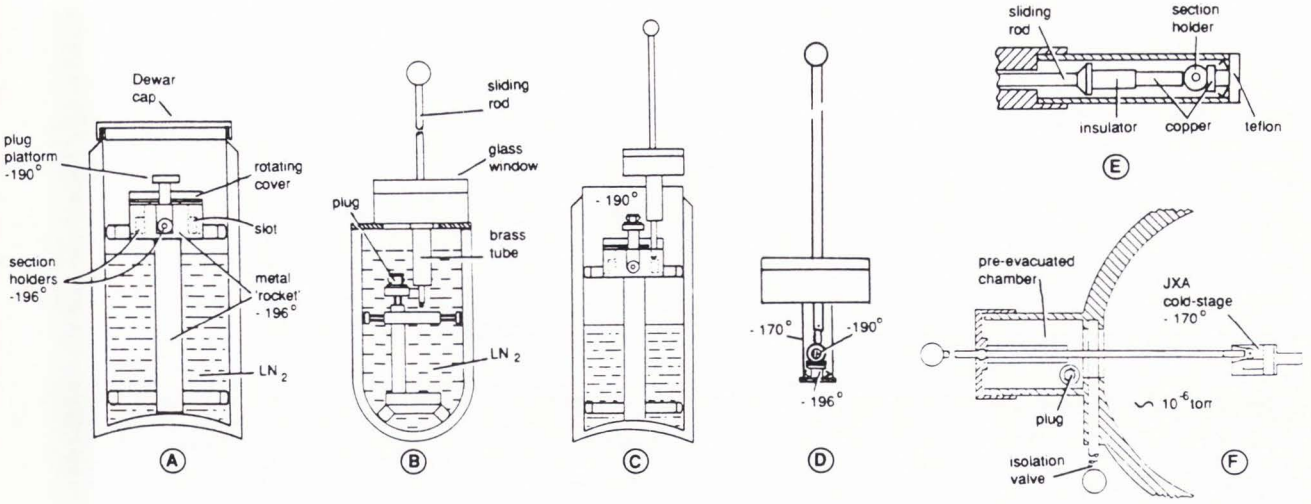


Fig. 9. Schematic flow-diagram for retrieving the stored section-holders and loading them on the cold stage of JXA-50A through a pre-evacuated chamber. (**A**) BML section-storage Dewar (capacity 5 liters); (**B**) pre-cooling JXA specimen loader and protective plug; (**C**) retrieving a section-holder; (**D**) transfer through air; (**E**) details of the protected holder; (**F**) loading on to JXA-50A cold stage. Temperatures in degrees Celsius.

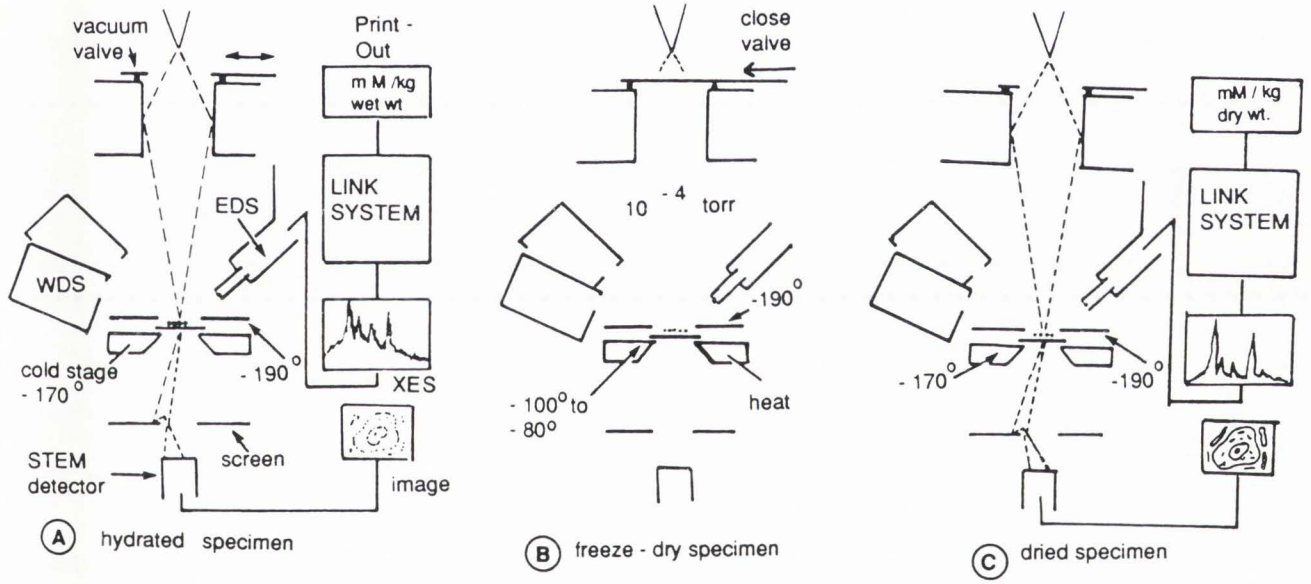


Fig. 10. Schematic diagram of JXA-50A microanalyzer in BML (post-1981); (**A**) during analyses of cryo-sections in a frozen-hydrated state; (**B**) during freeze-drying; and (**C**) during analyses of frozen-dried sections. Temperatures in degrees Celsius.

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fully vindicated, even though the original choice was dictated by the generation of sufficient X-ray quanta with the limited collecting power of WDS rather than by the considerations of mass-loss and the retention of hydration.

We were never successful in top-coating our frozen-hydrated sections either with aluminium or carbon without causing intolerable dehydration, in spite of Ted Hall's early experience that such a top-coat should improve specimen stability and possibly prevent a loss of mass under the beam for EPXMA. Zierold (1988a) has now demonstrated that a 20 nm thick coat of evaporated carbon on 200 nm thick frozen-hydrated sections reduces the rate of mass-loss substantially but not enough to allow quantitative EPXMA using continuum method to be completed. It may be that if the problem of low image-contrast can be resolved by some new method of image processing, EPXMA of even ultrathin frozen-sections at a resolution of < 100 nm may be possible by using alternative rapid method for estimating the mass before any

significant loss begins (Hall, 1988).

Cryo-fixation

For most of our studies on animal epithelia it was necessary to quick-freeze samples up to 1 mm or even more in thickness. Since 1974, we used Freon-13 (dichloro-trifluoromethane, m.p. -181°C, b.p. -81°C) cooled with liquid nitrogen. We did not use propane or ethane because our BML was located in the basement of the building and we did not have a 'safe' fume-cupboard. We always used 25-30 ml of cryogen in a 'Tripour' polythene beaker filled up to the brim. Rigorous stirring with a fast magnetic stirrer lowers the temperature of the cryogen without freezing to -189°C, continuously monitored with a thermocouple. The container was kept tightly covered with a sheet of compressed polystyrene until the moment of immersing the specimen. This excluded the layer of cold gas (Leidenfrost) on the top of the container which is probably the most common cause of poor freezing. Following the practice of Somlyo and Silcox (1979) we subsequently constructed and used a gadget (Fig. 11a, b) which can be mechanically triggered to move the cryogen vessel upwards and freeze the sample (Barnard, 1982). Ryan and Purse (1984, 1985) have now shown that if during cryo-fixation one can exclude the zone of cold gas, one can obtain a good preservation of biological tissues to the depth of some 250 μm without any apparent ice-crystal formation as judged by freeze-substitution and TEM. Fig. 12a-e illustrate the quality of cryo-preservation we often achieved in our laboratory even without the use of any macromolecular cryo-protectants. Since the practical spatial resolution in most biological EPXMA is generally not better than about 100 nm, the cryo-fixation of even large biological tissues is not a limiting factor (for further discussion see Roomans *et al.*, 1982a; Morgan 1985; Sitte *et al.*, 1987; Elder and Bovell, 1988; Zierold, 1988a, b; Zierold and Schäfer, 1988). [The technique of measuring ultra-rapid cooling efficiencies of various liquid cryogens for biological materials was introduced by Loyet and Gonzeles (1951). This method was used by Costello and Corless (1978) for a systematic study of cryo-fixation. More recent assessments show that the cooling efficiency of Freon-13 is only slightly lower than that of liquid propane (Roomans *et al.*, 1982a; Sitte *et al.* 1987).]

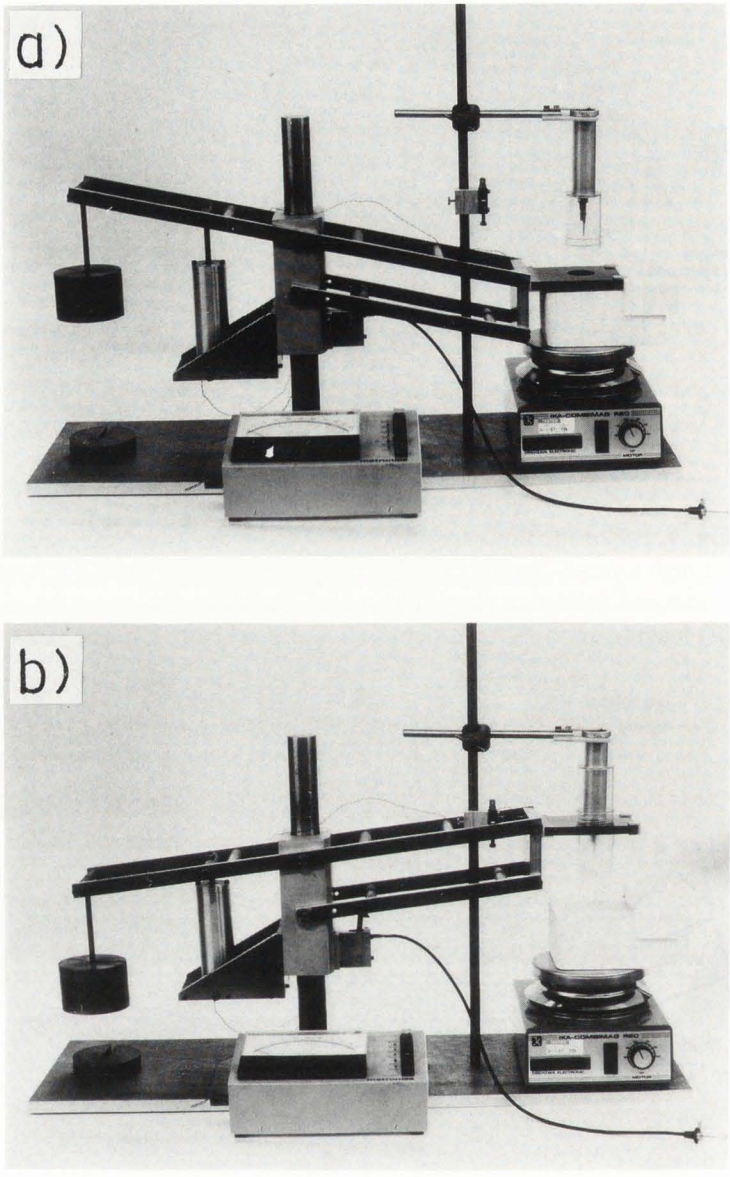

Fig. 11. BML 'loyal toaster', a mechanically triggered quick-freezing apparatus (**top**) resting and (**bottom**) freezing the specimen.

Peripheral standard

Following the suggestion of Pallaghy (1973) and Dörge *et al.* (1974), we used the bath Ringers, usually containing 10-20 % Dextran (molecular weight, MW, about 250,000), frozen and cut with the specimen, both as a peripheral standard for the quantification and for assessing the level of hydration in the sections. When for physiological considerations the samples had to be studied without such a peripheral medium (Dow *et al.* 1981, 1984) we used alternative strategies for the quantification of X-ray data. However, it should be emphasized that in Hall's continuum method, incorporated in LINK Analytical Quantem-FLS software, it is not necessary to use

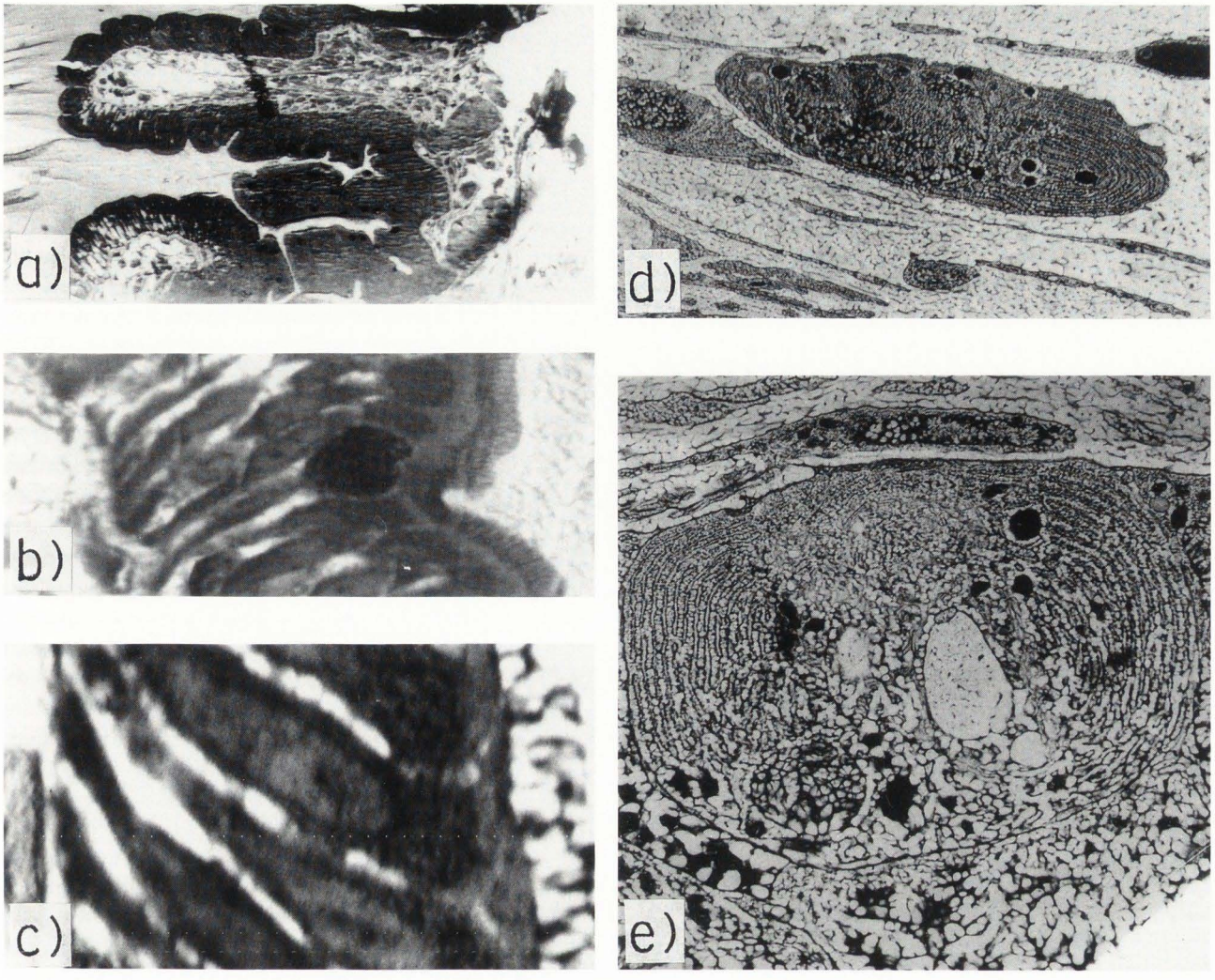

Fig. 12. (**a-c**) STEM images of a 1-μm thick frozen-dried section of rabbit ileum, quick frozen as a 1 cm diameter and 1.5 mm thick piece of tissue in the regime 3 of Fig. 7, to show the quality of preservation: (**a**) the whole length of a villus with a 100μm thick layer of mucosal mucus and serosal connective tissue (partly torn); (**b**) details of an enterocyte. The lateral intercellular spaces had been analyzed before in the frozen-hydrated state of the section and show latent mass-loss. (**d, e**) A part of the block had been freeze-substituted in acetone-osmium at about -80°C, embedded in Araldite, thin-sectioned, stained and examined in a Philips EM 300 TEM to show the degree of fine structure preserved: (**d**) in a serosal fibroblast and (**e**) in a crypt cell.

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a peripheral medium as a standard for quantification (Hall and Gupta, 1983; Morgan 1985). Various schemes for the quantification of data have been discussed by Hall in many recent publications (Gupta and Hall, 1982; Hall and Gupta, 1982a, 1983, 1984, 1986; Hall, 1986b, 1989b). The efficacy of Dextran-Ringer as a standard has been confirmed by Zierold (1988a, p. 68).

It has been suggested in the past that the use of Dextran in the peripheral medium in our studies might have caused serious alterations in the physiological function of the tissues and therefore would invalidate our EPXMA results. We always tried to ensure that the addition of Dextran has no adverse effect on physiological functions (Gupta and Hall, 1981b, 1982; Barnard *et al.* 1984). In a more recent study of rabbit ileum (Gupta 1989a, b) we have confirmed our earlier observations (Gupta *et al.*, 1978b; Gupta and Hall, 1979, 1981b) by using samples from in vitro preparations, cryo-fixed without any Dextran.

State of the specimen before freezing

Ted Hall and coworkers have repeatedly emphasized that unlike many other physiological methods, EPXMA measures an element in the specimen as it is under the

electron beam in the microanalyzer. It is therefore critical to ensure that until the specimen has been frozen, it is in the desired physiological state (Gupta *et al.* 1977; Gupta, 1979). Ramsay (1953) in our Department had already noted that tissues in small samples of a physiological medium can undergo rapid changes in the dry atmosphere of the laboratory. We followed Ramsay's practice of maintaining a humid atmosphere surrounding the samples up to the moment of their immersion into the cryogen (Fig. 7; Gupta and Hall, 1982). Ted Hall made a quantitative assessment of the problem (Hall and Gupta, 1982). Under normal atmospheric conditions in our laboratory, with no air-conditioning and with 77% relative humidity, a water droplet with a diameter of 0.5 μm and weighing 33 μg, lost 20% of its weight in 10 seconds. A similar droplet of 100 mM KCl + 20% Dextran (w/w) lost 20% of its water in 20 seconds. When such standard droplets were frozen, cryo-sectioned and analyzed, the K and Cl concentrations were found to be about 120 mmol kg/wet weight as compared with 99 mmol kg/ wet weight in 'properly' frozen controls. Such a change would disturb the native physiological state of the cells and tissue samples. If used as a 'peripheral' standard for X-ray data reduction, the change would degrade the results. Many recent articles on the sample preparation for EPXMA also emphasize such necessary precautions (Zglinicki *et al.* 1986; Zglinicki, 1989).

Cryo-microtomy

The instrumentation and methodology for cryo-microtomy has been extensively reviewed by Robards and Sleytr (1985) with more critical assessment by Gupta and Hall (1981a), Sitte (1984) and Zierold (1986a, 1987). With the notable exception of Saubermann *et al.* (1981a, b; Saubermann, 1989) in the USA and Nakagaki *et al.* (1984) in Japan, the EPXMA in all laboratories other than Cambridge BML has been performed on cryo-sections which have been completely dried before analysis. The EPXMA of cryo-sections in a frozen-hydrated state imposes an additional, rather critical, requirement: the entire preparative procedure should ensure that the cryo-sections neither significantly lose nor gain any water. It was this imposition which directed us to opt for an isothermic cryostat approach in 1971 (Appleton, 1968, 1969, 1972, 1974; Gupta *et al.*, 1977). For most of our work, we relied on our custom-built SLEE cryostat for cutting 0.5 to 2 μm thick frozen sections. The original cryostat was cooled with two cascade refrigeration units, allowing a maximum cooling to -82°C. In 1977 this cryostat was modified to the specifications of SLEE TUL (Appleton, 1978; Robards and Sleytr, 1985) with radiator-type liquid nitrogen cooling. A stable atmosphere of a large cryo-chamber (0.2 m^3) maintained around -80°C with the LN_2-cooled heat-exchangers located 25-30 cm away from the cutting area of the microtome ensured good sectioning and retrieval of section with the retention of full or nearly full hydration and with a minimum of frost-contamination (Gupta and Hall, 1981a). Following the experience of Stumpf and Roth (1965), we used low bevel angle (12 to 14°) carbon-steel knives, freshly sharpened before use. The advantage of using sturdy steel knives for cutting approximately 1-μm thick cryo-sections has been demonstrated by Dörge *et al.* (1978). More recently, Lickfeld (1985) has argued on theoretical grounds that deformation of cryo-sections cut at low temperatures (< -70°C) would be greatly reduced by using knives with a bevel angle considerable below 40° that is commonly used for glass knives. A metal surface earthed through the cryostat body also generates less static, although additional measures for reducing the static charge are often necessary (Robards and Sleytr, 1985): we used an antistatic 'pistol' originally marketed for cleaning gramophone records (Gupta *et al.*, 1977). The cryo-sectioning qualities of glass knives are critically dependent on the methods used for breaking the glass to make them : the standard commercial knife-makers have proven inadequate for this purpose (Griffith *et al.*, 1984). The cryo-sectioning qualities are further improved by a thin coating of tungsten (Roberts, 1975; Griffith *et al.*, 1984; Dubochet *et al.*, 1988) or possibly carbon (Seveus, 1980) applied to the cutting edge in a vacuum-coating unit.

There has been a considerable debate about the mechanics of cutting frozen-sections at low temperatures, mainly stimulated from the claims of Saubermann and coworkers. Saubermann and Echlin (1975) also used a SLEE cryostat in BML for cutting 1-2 μm thick cryo-sections for their EPXMA of frozen-hydrated cryo-sections. Subsequently Saubermann *et al.* (1977) applied the physics of machining metals and adapted a Sorvall Porter-Blum MK II ultramicrotome with a custom-built cryo-attachment (Saubermann *et al.*, 1981a), using single-edged razor-blades (Sjöstrand, 1956; Wigglesworth, 1959) for cutting 0.5-1 μm thick cryo-sections. They claimed: (a) that smooth 0.5-1 μm thick sections with a good preservation of tissue morphology cannot be cut at temperatures much cooler than -40°C. It was claimed that apparently smooth cryo-sections cut below -70°C are the result of substantial melting caused by the workload. These claims do not seem to be substantiated by subsequent findings from several other laboratories (see Zierold, 1987). For some of our studies, Ted Hall and I also used an LKB Ultratome III with a modified 'CryoKit' (Gupta *et al.*, 1977; Gupta and Hall, 1982; Barnard, 1982), and on one occasion a Reichert-Jung Ultracut E with FC-4 attachment (Gupta and Hall, 1984a) for cutting 0.5 to 1 μm thick cryo-sections below -90°C. The tissues used (Malpighian tubules of

Rhodnius, Gupta *et al.*, 1976; salivary glands of *Calliphora*, Gupta *et al.* 1978a; salivary glands of *Periplaneta*, Gupta and Hall, 1983) contained many tissue compartments with distinct ionic compositions. The tissues also had large fluid-filled spaces (lumina) with little organic matter. No evidence of melting, which would have resulted in the scrambling of ionic distributions and degradation of morphology, could be found. This is in accord with the seminal analyses of Thornberg and Mengers (1957) who predicted that as the knife temperature is lowered (in cryo-microtomes), an increasingly larger fraction of energy of cutting would be conducted directly into knife and therefore the melting-zone would decrease and may ultimately disappear.

However, we generally found it more difficult to maintain full hydration of cryo-sections in cryo-attachments. The cold and dry nitrogen gas flowing over the sections before their retrieval can act as a cryo-sorption pump (see pp 395-396 in Hutchinson and Somlyo, 1981). Similar reasoning had, much earlier, led the Munich group (Gehring *et al.*, 1973) to abandon the analysis of frozen-hydrated sections (see Discussion after Gupta, 1979). In the current models of Reichert-Jung FC4, this flow can be stopped for brief periods with only a negligible change in temperature. However, Zingsheim (1984) has demonstrated that in a Sorvall Porter-Blum 'CryoCut I' (Saubermann *et al.*, 1981a) the rate of sublimation of ice vitrified on a quartz crystal monitor is negligible at -50°C but rises with the temperature and with the flow of nitrogen gas. It is not clear whether this observation will also hold for sections full of ice-crystals, either loosely resting over the knife-edge or in the process of transfer! Nevertheless, such measurements have never been published before.

Gupta *et al.* (1977) recorded that the cryo-sections of unfixed and uncryo-protected tissues with large fluid-filled spaces are often 'corrugated' due to sequential, multiple folding, as was predicted by Thornberg and Mengers (1957). Linderström-Lang and Mogensen (1938), who first developed a cryostat microtome, introduced the use of a glass-plate in front of the knife for preventing the rolling and curling of sections. Pearse introduced an improved design for Pearse-SLEE (London) cryostat (Pearse, 1961) which was also incorporated in our SLEE cryostat. Numerous other workers have made anti-roll devices for cutting ultrathin cryo-sections and these have been reviewed by Robards and Sleytr (1985). Such devices are not as a rule supplied with the commercially available instruments. Dörge *et al.* (1978) devised their own for the Reichert-Jung Ultracryotome. Zierold (1987) has re-examined the various theoretical models which have been applied to the process of cryo-sectioning. On all the evidence available he rules out the chip-formation model (Merchant, 1945; Saubermann *et al.*, 1977). Both the folding-model and friction-sliding model would be consistent with our experience in cutting semi-thick cryo-sections. These models would also be consistent with the experience of many others for cutting ultrathin cryo-sections (Frederik *et al.*, 1984; Sitte, 1984; Zierold, 1984a, b, 1987; Dubochet *et al.*, 1988). Zierold (1987) has also argued that according to the physical theory of phase transitions in frozen water, local melting as the cause of truly sectioning ice at temperatures below -25°C, is not possible. He has cautioned that all previous studies on plastic deformation and fracturing of paracrystalline ice have been performed at temperatures warmer than -73°C. Not only are the well-frozen biological tissues quite different from pure crystals, they are far from uniform in their cutting properties. Hall and coworkers have repeatedly emphasized that large fluid-filled compartment (organic component $<2\%$) either present extracellularly as lumina of secretory and excretory epithelia, or intracellularly as vacuoles, often freeze to form much larger ice-crystals than are formed in organic matrices inside and outside the cells. The contents of such large spaces either 'chip' or crumble during cryo-sectioning at low temperatures, causing additional problems in the acquisition and conversion of X-ray data (see Gupta and Hall, 1982; Hall and Gupta, 1982a for further discussion). EPXMA of such intercellular compartments is the most important reason for using frozen-hydrated samples (Zierold and Steinbrecht, 1987). Cryo-sectioning of biological materials, therefore, remains at present as empirical an approach as it was 30 years ago (Pearse, 1961). Our theoretical understanding of the process has not made much progress since Thornberg and Mengers (1957).

The choice of cryo-sectioning temperature for EPXMA is also important for two other considerations. Firstly, the frozen water is believed to undergo phase transitions leading to coarse crystal formation at warmer temperatures (Dubochet *et al.*, 1988). Re-formation of coarse ice crystals in otherwise well-frozen specimens may lead to the degradation of fine-structure and could also cause redistribution of solutes (Gupta *et al.*, 1977, 1980b). However, some recent work has shown that in biological materials containing polyelectrolyte matrices (organic matter) recrystallization is exceedingly slow and therefore need not be a worry (Steinbrecht, 1985; also see Dubochet *et al.*, 1988). The second question relates to the rates of diffusion of ions in ice. Classically it was shown that ions can diffuse at an appreciable rate even in deep-frozen water at temperatures higher than their eutectic temperatures (Meryman, 1966). For most ionic species this eutectic temperature was thought to be above -40°C although ions such as Ca^{2+} may remain mobile even at -55°C (Mazur, 1970). In fact it was this belief which had originally prejudiced Ted Hall (and others)

against the possibility of meaningfully analyzing diffusible electrolytes in cryostat (-30°C) sections by EPXMA. Now it seems that a large scale dislocation of ions by diffusion in ice does not occur at a significant rate even in pure NaCl solutions (Lechene, 1980). The presence of polyelectrolytes would further retard the process. In theoretical predictions for this phenomenon the factor commonly overlooked is the viscosity of the frozen medium. Even at a high temperature of -10°C the viscosity of frozen water is said to be several orders of magnitude greater than that of liquid water at room temperature. Such a high viscosity alone should virtually stop all diffusive changes in deep-frozen tissues for all practical considerations (further see Bachmann and Mayer, 1987).

Nevertheless there have not been many systematic studies actually carried out with EPXMA on the same biological sample cryo-sectioned at different temperatures. Saubermann *et al.* (1981a, b) compared ionic gradients artificially created in gelatin blocks and sequentially cut at -70°C and at -30°C. EPXMA at 1 μm resolution did not reveal any significant differences in the gradients measured. However the ionic concentrations used in these model samples were unrealistically high (Gupta and Hall, 1981b). It is generally assumed that even when the sections are cut at -20°C, diffusive movement of ions does not traverse cell-membranes. 2-4 μm thick cryo-sections cut at -20°C (Dick, 1978) or at -30°C (Cameron *et al.*, 1977; Krefting *et al.*, 1988) and 16 μm thick sections cut at -20°C (Wroblewski *et al.*, 1978) have been extensively used for EPXMA studies in biology and pathology (also see Moreton, 1981; Roomans *et al.*, 1982a, Roomans, 1988 a, b). But, now there is a general consensus that such cryo-sections are not suitable for analysis at sub-cellular level requiring sections < 1 μm in thickness. The common practice now is to cut cryo-sections for EPXMA at temperatures at or below -70°C (Steinbrecht and Zierold, 1989; Zierold, 1987, 1988a, b; Dubochet *et al.*, 1988).

One serious difficulty in assessing the effect of sectioning temperature on the native ionic distributions is that EPXMA are most commonly performed on cryo-sections which have been frozen-dried outside the analytical microscope. Using permealized erythrocytes and hepatocytes, Cameron *et al.* (1983, 1986) found a large influx of Na and Cl into cells in cryo-sections cut and frozen-dried at -40°C as compared to those prepared at -100°C. Curiously, a parallel change in cell-K was not noticed. The process involved therefore does not seem to be osmotic but is thought to be electrostatic (Cameron *et al.*, 1990). It is not clear whether the drastic change in -40°C samples took place during cryo-sectioning or during freeze-drying. The conditions used for freeze-drying and the subsequent handling of cryo-sections for EPXMA without introducing serious artifacts seem to be critical (Gupta and Hall, 1982; Hagler and Buja, 1986; Zglinicki and Zierold, 1989).

Section-holders and section-support

The initial experiments by Hall and coworkers had shown that the stability of frozen-hydrated sections under the beam is critically dependent on sound electrical and thermal contact with the substrate on which the sections are mounted. In Cambridge BML, Saubermann and Echlin (1975) adapted Ted Hall's metal collar for use in the Cambridge Stereoscan S4 and later for the AMRay, AMR 900 SEM (Saubermann *et al.*, 1981a, b). Hall and coworkers adapted this duralium cylinder or collar, 10 mm in diameter, for use in the JXA-50A. Several versions of the collar were produced for different usages (see Gupta *et al.*, 1977). In order to minimize extraneous X-ray background in EDX spectra, it was found important to carefully remove metal from inside the collar, leaving a 'knife-edge' surrounding the 3-mm hole (Fig. 5b). The cryo-sections were mounted on alumininized nylon films stretched over the hole, as had been done before by Hall. Aluminized nylon films seem to be better suited for using high (5-10 nA) probe current required for WDS. Hall and Patricia Peters (Hall, 1979b) systematically established that the continuum X-rays from such section-holders could be reliably corrected for and that the corrections provided uniform film-counts virtually over the entire 3-mm hole. The practical procedures for the correction were described by Gupta and Hall (1979), Hall and Gupta (1979, 1986), and Roomans (1980). Uncertainties about the corrections for the X-ray quanta from the extraneous background continue to plague many microanalytical laboratories (Steinbrecht and Zierold, 1989; Roomans, 1988b).

As our methods of quantifying X-ray data refined, we found that thick aluminized nylon films generated intolerably excessive background and made the correct estimations of continuum X-ray quanta from < 1 μm thick sections less accurate, especially after the sections had been dried (see below). We then resorted to the use of multislot nickel grids (Fig. 5b) mounted on a Duralium collar that had also been heavily electroplated with nickel (Gupta and Hall, 1983). The grids were either covered with a thin nylon film or with a Formvar film, stabilized with 10-20 nm thick carbon. Grids were attached to the holders with carbon-dag sparingly applied to the edges. On no account should one use organic glues or plastic tapes because they often are a rich source of contamination. For example, cellotapes are loaded with Cl. Apart from providing more uniform and flatter films, this methodological change removed irritating extraneous peaks of Al, Cu and Zn (the latter from Duralium) from the EDX spectra. The characteristic X-ray peaks from nickel are in a much higher

energy range and therefore do not interfere with the elemental peaks commonly seen in soft biological specimens. So, the experience of Ted Hall in mounting specimens for EPXMA had turned full circle.

The transfer of cryo-sections to a suitable holder or to a grid (for ultrathin sections used in high resolution analyses) has remained a problem (Gupta *et al.*, 1977; Zierold, 1987, 1988a, Dubochet *et al.*, 1988). Conventionally, the cryostat sections used in histochemistry and in immunocytochemistry are picked up while thawing on warm slides or on cover-slips, which have been coated optionally with albumin, gelatine, polylysine or rubber solutions (Pearse, 1961). Cryo-sections from prefixed and cryo-protected tissue used in immunocytochemistry are picked up on sucrose droplets and transferred to EM grids or to cover-slips (Tokuyasu, 1973, 1980; Griffiths *et al.* 1984; Zierold, 1987). None of these methods are suitable for EPXMA of diffusible elements. Originally we transferred frozen-hydrated sections by directly pressing metal collars against the sections resting on the steel knife (Moreton *et al*,. 1974; Echlin *et al.*, 1973). Gupta *et al.* (1977) designed a gadget for the Duralium collars (Fig. 5b) which, for picking up sections replaced the anti-roll plate in the SLEE cryostat. The collar could be aligned and pressed over the sections while observing through a stereo-microscope. With our bulky Duralium collars, it was not possible for us to use the vacuum device of Appleton (1972) that was provided as a standard fitting with the SLEE cryostat. We did adapt this device for the glass-knives used in LKB 'CryoKit' and found that a slight positive electrical charge applied with a DC circuit to the tip of the vacuum needle improved the performance (Cooper NGF and Gupta BL, unpublished; Gupta *et al.*, 1977). Many other workers have similarly modified Appleton's vacuum device to suit their own needs (see Robards and Sleytr, 1985). Rick *et al.* (1982) pick up their cryo-sections directly on to nickel grids using an electromagnetic device first introduced by Appleton (1969).

Most commonly however, the sections are transferred loose to the cold metal-holders for SEM or to the cold EM-grids for TEM, using some variation of a thin wire or an eye-lash probe introduced by Christensen (1969, 1971). Saubermann and Echlin (1975) in Cambridge BML used this method for transferring sections in SLEE cryostat. In most of our studies, the frozen-hydrated sections were transferred similarly (Gupta and Hall, 1981a, 1982). For microanalysis this method of transferring sections remains hazardous. Apart from the loss of sections and the difficulty of transferring very small or very large sections (about 1 mm in diameter; Gupta, 1989a, b), debris of tissue on the knife can contaminate good sections. Folding of sections and overlapping is an additional problem. Some adaptation of Appleton's electromagnetic device, successfully used by the Munich group (Rick *et al.*, 1982) seems most desirable.

The frozen-hydrated sections do not stick well to the metal-coated plastic films. Again, the pressing devices introduced by Christensen (1971) and by Seveus (1980) are frequently used. Hall and coworkers used a copper disc 'thumper' (10 mm in diameter, 2-3 mm thick) with a mirror-smooth surface and precooled in the cryostat for pressing down cryo-sections. The cold thumper is gently dropped on the sections sitting on the Duralium holder in a horizontal position and left for a few minutes. It is critically important to clean and polish the front face of the thumper after every operation; otherwise the cryo-sections tend to stick to it. As noted above, the metal-coated plastic films must be made hydrophilic before use. For ultrathin cryo-sections mounted on EM-grids, numerous alternative methods of 'sandwiching' have been described (Roomans *et al.*, 1982a; Rick *et al.*, 1982; Robards and Sleytr, 1985; Hagler and Buja, 1984; Zierold, 1986a, 1987). The use of chemical adhesives for sticking cryo-sections is not permitted for quantitative EPXMA.

Retrieval, storage and transfer of cryo-sections

In the first experiments in BML, frozen-hydrated cryo-sections directly picked up on aluminized nylon films mounted on small Duralium collets, were immediately immersed in liquid nitrogen (Echlin and Moreton,1973; Moreton *et al.*, 1974). This procedure ensured that the sections neither dried nor frosted from the atmospheric moisture. Further handling of such liquid nitrogen-stored preparations on to the cold stage of Cambridge Stereoscan, without a pre-evacuated airlock for the specimen chamber, proved clumsy and unreliable, and resulted in frequent loss of the cryo-sections. However, the liquid nitrogen-immersion procedure for the storage and transfer of ultrathin frozen-hydrated specimens is now commonly used for structural studies in TEM (Robards and Sleytr, 1985; Dubochet *et al.*, 1988; Zierold, 1986a, 1987, 1988a, b; Zierold and Schäfer, 1988).

The introduction of cold metal-tubes as 'shrouds' for protecting frozen specimens in cryo-preparations for EM is generally attributed to Steer (1957). Tom Hutchinson, then at the University of Minnesota, used a cold copper-tube for transferring sections from the cryo-ultramicrotome to the cold stage of a Cambridge S-2 Stereoscan (Bacaner *et al.*, 1973; Hutchinson *et al.*, 1974). At Ted Hall's request he sent his unpublished drawings (dated 22/10/1971) to Cambridge. Saubermann and Echlin (1975) essentially adapted Hutchinson's 'sliding rod and cold tube device' in BML. We continued to use this simple, reliable, and efficient gadget for transferring

section-holders from the cryostat to the 'parking and storage' Dewars. JEOL's device for loading specimens through the pre-evacuated air lock (Nei *et al.*, 1973) was similarly adapted for transferring the specimen from the storage Dewar on to the cold stage of JXA-50A (Gupta, 1976; Gupta *et al.*, 1977; Gupta and Hall, 1982). This procedure did not involve any of the complications of the 'satellite' equipment used for bulk-frozen specimens (see Robards and Sleytr, 1985), and also proved highly reliable. Rigorous tests showed that during the entire procedure of retrieval from the cryostat, storage (sometimes for weeks) and transfer into JXA-50A, the temperature of the section-holders did not rise above -145°C.

BML storage-Dewar

A thorough microanalysis of a single holder bearing good frozen-hydrated sections from a biological tissue can often take 8-10 hours. It is therefore essential that the section-bearing holders can be stored reliably at temperatures below -150°C. The atmosphere surrounding the stored cryo-sections should also be chemically clean and free from moisture in order to avoid contamination by condensation. Such contamination at such low temperatures would be structurally amorphous (vitrified) and would not be detected in the electron images of the cryo-sections. The minimum analytical consequence would be an undetected gain of the native mass (corrected continuum) of the specimen in the X-ray data. For our relatively bulky section-holders, we constructed a storage container from a commercial 2-liter LN-Dewar (Figs. 9a-c, 13). The phage-shaped metal insert is kept at near -196°C by conductive cooling with liquid nitrogen. The section-holders stand vertically in slots in the bulky head and are further protected with a rotating metal disc as the cover. During storage the sections are not in direct contact with the liquid nitrogen, nor is there a flow of cold nitrogen gas over them. Even when the level of liquid nitrogen drops to a few centimeters from the bottom, the temperature of the holders in the slots still measured at -190°C. With a regular refilling every 24 hours, we had often stored frozen-hydrated sections in this Dewar for up to four weeks, without any apparent differences in results as compared with the unstored sections from the same batch. Care has to be taken that during the refilling of the Dewar, the liquid nitrogen does not splash over the stored sections.

Frozen-dried cryo-sections

The EPXMA of frozen-hydrated sections with the use of suitable standards provides concentration values for elements directly in mmol/kg wet weight (Hall's continuum method). For the data to be of maximum value in physiological studies, it is also essential to obtain the dry mass fractions of the sub-fields previously analyzed in a hydrated state. The cryo-sections are therefore frozen-dried within the microanalyzer (see below) and the comparable sub-fields reanalyzed for obtaining the elemental concentrations in mmol/kg dry weight (wt). Provided that the exact level of section-hydration can be established (for example with reference to a peripheral standard), then the local concentrations of H_2O in the tissue compartments can be directly obtained by comparing with the elemental concentrations in mmol/kg wet wt with the values in mmol/kg dry wt (Gupta *et al.*, 1977; Hall and Gupta, 1979, 1986; Hall 1989b). Vastly improved images from the frozen-dried sections also allow one to confirm directly the identity of the cell- and tissue-compartments tentatively recognized in the poor images from the cryosections in the frozen-hydrated state. In almost all our studies in BML we consistently followed this procedure.

In our practice, freeze-drying of the sections was accomplished by warming the section-holder on the cold stage of the microanalyzer (about 10^{-5} torr) for up to an hour. The holder was then recooled to <-150°C for reanalyses. A complete drying was assumed if the elemental concentrations in the peripheral standard-medium changed as expected. Even in those cases where the poor images from frozen-hydrated sections precluded the desired aims of the study (Forer *et al.*, 1980), the cryo-sections were generally frozen-dried within the SEM column by the above procedure. If Hall's continuum method is used in X-ray data conversion, care has to be taken that all the water has been removed from the cryo-sections. It has been shown that all the cell- and tissue-water does not sublimate during freeze-drying at temperatures between -80°C and -100°C, commonly used for freeze-drying cryo-sections under high vacuum (Wildhaber *et al.*, 1982; Gross, 1987). The remaining water fraction, which can be up to 30% (in some cases even more) of the total water, is probably 'structured water' associated with biological macromolecules and organelles, and only sublimates at temperatures warmer than -50°C under high vacuum. In a reexamination of some unpublished data from the mucus granules (Gupta and Hall, 1978) we have also found that in cryo-sections of rabbit ileum, frozen-dried at the stage temperature of about -100°C for a short time, the mucus granules in the goblet cells had a water fraction of about 60% of the wet mass. After the same sections had been dried overnight within the high vacuum of the SEM column, when the stage was allowed to warm up to the room temperature, the water fraction was about 70% of the wet mass (Gupta, 1989a, b). Which of these two values represent the solvent water for physiological considerations becomes a question. Intuitively one would think that the 'total' water-fraction estimated after a complete freeze-drying of the cryo-sections is not likely to represent the

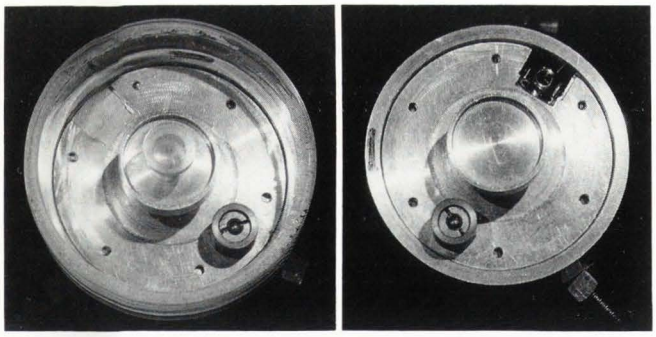

Fig. 13. Photographs showing the top of BML cryo-section storage Dewar (Fig. 9A) with (**left**) the rotating shutter closed and (**right**) the shutter rotated for withdrawing a section-holder.

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truly solvent water needed for obtaining ionic concentrations in mmol/l H_2O. But then all the elemental concentrations measured in EPXMA are not likely to represent the real values for ions acting as free-solutes. That is where the parallel data obtained by other methods of analyses, such as ion-selective microelectrodes (Thomas, 1978) becomes valuable (for some examples see Gupta, 1989b).

In most other laboratories EPXMA is commonly performed on cryo-sections frozen-dried before storage and/or transfer to an EM for analysis. Appleton (1972, 1974) in Cambridge, and other workers elsewhere (Sjöström, 1975a, b; Roomans and Seveus, 1976; Barnard and Seveus, 1978; Ali *et al.*, 1977, 1978; Roomans *et al.*, 1982a) dried their cryo-sections either by lyophilization or by cryo-sorption within the cryo-chamber of the microtomes. It was even claimed that if sections are dried under a rough vacuum from a rotary pump (about 10^{-2} torr), there is a gross distortion of the fine structure (Sjöström, 1975a, b), perhaps due to surface tension during rapid sublimation (Appleton, 1978). In BML, we never obtained satisfactory results from similarly dried cryo-sections. In one particular study of calcium stores in the mitochondria of *Calliphora* salivary glands, ultrathin cryo-sections of rapidly frozen, fresh glands were cut with our LKB 'CryoKit', frozen-dried under vacuum and analyzed in Ted Hall's EMMA-4. Some of the mitochondria showed clusters of electron-opaque granules of foamy texture containing Ca, P, S, Cl, and K (Oschman, 1977 in Gupta and Hall, 1978). Similar results from other tissues had been obtained in other laboratories but were never consistent and reproducible. All such observations have proved artefactual. Cryo-sections frozen-dried in this fashion show considerable variations in cell ultrastructure. The changes from the native state, such as the formation of calcium-rich granules in mitochondria, seem to depend either on inadvertent melting occurring during freeze-drying, or on rehydration in the subsequent steps (Frederik and Busing, 1981).

In many laboratories cryo-sections for EPXMA are frozen-dried at low temperatures under high vacuum (Somlyo *et al.*, 1977; Hutchinson, 1979; Rick *et al.*, 1982, Rick and Schratt, 1989; Hagler and Buja, 1984; Nassar *et al.*, 1986; Ingram *et al.*, 1987; Krefting *et al.*, 1988; Cameron *et al.*, 1990; reviews by Zierold, 1988a, b; Zglinicki, 1989). Before removal from the vacuum, the frozen-dried sections are coated with evaporated carbon (Shuman *et al.*, 1976), unless they are already sandwiched between two carbon-coated plastic films (Rick *et al.*, 1982; Rick and Schratt, 1989; Hagler and Buja, 1984; Zierold, 1987). Such sections are either analyzed on a low temperature stage in a TEM (Shuman *et al.*, 1976; Variano-Marston *et al.*, 1977; Nicholson *et al.*, 1982; Nakagaki *et al.*, 1984; Hagler and Buja, 1984; Wendt-Gallitelli and Wolburg, 1984; Cantino *et al.*, 1986; Zierold, 1986a, b; Ingram *et al.*, 1987, 1989; Ornberg *et al.*, 1988) or more commonly at room temperature either in a TEM or an SEM (Appleton, 1972, 1978; Ali, 1976; Ali *et al.*, 1978; Roomans and Seveus, 1976; Dörge *et al.*, 1978; Rick *et al.*, 1982; Kendall *et al.*, 1985; Izutsu and Johnson, 1986; Kendall and Warley, 1988; Zglinicki and Uhrik, 1988; Warley, 1989). We often retrieved good frozen-dried sections from the JXA-50A for future reference, or for demonstration.

Before removal, the cold stage and the cold anti-contamination plate (see below) were allowed to warm up to the room temperature and the sections checked for good structural detail in STEM images. The sections were then stored, with or without a top-coat of carbon, in desiccators at room temperature and atmospheric pressure. We were often mystified to find that when re-examined, the fine structure in such sections was degraded even when the distribution of ions appeared not to have been generally affected. In some cases however, it was found that some of the tissue components had highly increased levels of chloride and sulphur (Dow *et al.*, 1981). Presumably, the additional sulphur and chloride had been gained by a selective adsorption of chlorine and sulphur dioxide from the residual gases in the column when the anticontamination plate had warmed up. It is therefore possible that similar selective contaminations could arise in other methods of freeze-drying cryo-sections. We never carried out a systematic study of this question.

Because of the wide usage of frozen-dried cryo-sections in biological microanalysis, several recent studies have concentrated on investigating: (a) the best way to freeze-dry cryo-sections for microanalysis ; (b) the best way for subsequent handling for storage, transfer etc.; and (c) whether the frozen-dried cryo-sections

should be analyzed at room temperature or at a low temperature on a cryo-stage (Barnard and Seveus, 1978; Barnard, 1982; Barnard and Hagler, 1984; Hagler and Buja, 1984, 1986; Zglinicki and Uhrik, 1988; Zglinicki and Zierold, 1989; Zierold, 1984a, b, 1988a, b). Collectively, the findings of all these studies show that the general practices developed empirically by Hall and coworkers in BML are probably the most sound.

When the cryo-sections have been frozen-dried with a complete removal of all the water and are otherwise handled with due care, they show excellent images and seem to be very stable under the electron beam (Gupta *et al.*, 1977; Zierold, 1984a, b, 1987, 1988a). Such sections nevertheless remain very hygroscopic and therefore vulnerable to even a slight exposure to the ambient moisture. If transport through the room atmosphere is necessary, (Zierold, 1986b) the frozen-dried sections can be protected from the moisture by keeping them slightly warmer then the ambient temperature (Gupta *et al*,. 1977; Frederik and Busing, 1981; Gupta and Hall, 1982; Zglinicki and Zierold, 1989). Hagler and Buja (1986) have found that one cause of structural degradation as well as of elemental redistribution in frozen-dried cryo-sections might be the melting and smearing of cell and tissue lipid. When frozen-dried sections are analyzed without cooling to below < -120°C in an EM, the loss of mass (Hall and Gupta, 1974) is generally accompanied by a loss of sulphur (see Zglinicki, 1989). In the tissue compartments which are rich in polyanionic (especially the sulphated) sugars, a loss of chloride might also occur (Quinton, 1978). It would therefore seem that good cryo-transfer procedures and a cold stage, indispensable for frozen-hydrated sections, are also beneficial for the microanalyses of frozen-dried cryo-sections (Zierold, 1988a, b).

Cold stage

The cryo-system supplied with JEOL JXA-50A (Nei *et al.*, 1971, 1973; Nei and Fujikawa, 1978) included a cryo-transfer system and a low temperature stage with an anticontamination plate located above the stage. In BML this system was used for our initial studies on isolated spasmonemes (Routledge *et al.*, 1975; Gupta and Hall, 1978) and on isolated nuclei (Gupta *et al.*, 1977; Jones *et al.*, 1979). This cryo-system was found inadequate for analyzing frozen-hydrated sections and was replaced by a new stage and an anticontamination plate of Ted Hall's design (Taylor and Burgess, 1977; Robards and Sleytr, 1985). Our stage was cooled with a heavy copper braid for providing high mechanical and thermal stability with smooth translatory movements in all the three axes. The temperature of the stage and of the anti-contamination plate were continuously monitored with fixed thermocouples. However, several disadvantages remained. With a column vacuum of 5.10^{-6} torr and with the anticontamination plate cooled to near -196°C, the lowest temperature on the cold stage, close to the specimen holder was never much below -170°C. Whenever measured, the temperature of the specimen holder was not colder than -155°C. While the anti-contamination plate cooled down to < -190°C within a few minutes, the stage needed about an hour to reach the lowest stable temperature. The liquid nitrogen Dewar feeding the stage needed re-filling every 90 min. Even with the electric heater built into the design, the stage needed equally long time to warm up. During this warming the temperature of the stage could not be conveniently 'arrested' at a desired level (say at -100°C) for freeze-drying the cryo-sections: recooling the stage for a re-analysis of the sections was also time-consuming. In consultation with Ken Oates of Lancaster University (UK), E.M. Technology-Hexland Ltd (Oxford, UK) adapted the Taylor and Burgess design for direct liquid nitrogen cooling (Oates and Potts, 1985; Robards and Sleytr, 1985). However, the Oates-Hexland stage was designed and has been used only for frozen-hydrated bulk specimens: it does not seem to provide for STEM imaging which is necessary for analyzing cryo-sections.

The anti-contamination device in our cryo-analytical system was a 250 x 400 mm copper plate. Carefully shaped holes were machined through the plate for the electron beam and X-rays to pass through. The edges of the holes were carefully profiled in accordance with a 35° 'take-off' angle for EDXS and also to avoid restriction of X-rays reaching the two WDS (Taylor and Burgess, 1977). Thorough periodic checks were made by Ted Hall for ensuring that neither the specimen stage nor the anti-contamination plate contributed spurious X-ray quanta to EDX spectra. Low atomic number materials such as beryllium and carbon (Chandler, 1973; 1977) are often used for constructing 'low background' analytical holders. For an accurate quantification of X-ray data from thin biological specimens with low elemental concentrations, even a relatively small contribution from unidentified sources (Heinrich, 1982) would degrade the analytical results (see e.g., Steinbrecht and Zierold, 1989).

Our anti-contamination plate was located about 1 mm above the specimen and could be withdrawn into an 'out' position from the entire analytical configuration within the SEM column. Repeated tests on frozen-hydrated sections were conducted with the plate 'in' and 'out' positions. The comparison of the data showed that not only did the plate efficiently eliminate contamination of the sections, it also vastly improved the stability of the specimen under the electron beam provided that the temperature of the stage was kept well below -150°C. An efficient anti-contamination plate 'shrouding' the

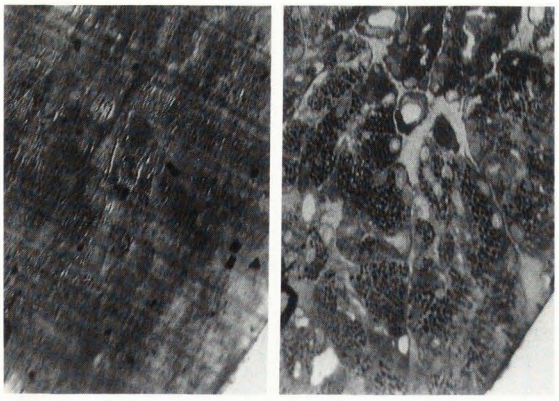

Fig. 14. STEM images from a 1 μm thick cryo-section of cockroach salivary gland (**left**) in a fully hydrated state and (**right**) after freeze-drying within JXA-50A.

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specimen is not always included in the design of cryo-systems for electron microscopes (Robards and Sleytr, 1985). It is deliberately avoided in some designs (Saubermann *et al.*, 1981a, b) with several possible disadvantages (Gupta and Hall, 1981a, 1982). The anti-contamination traps commonly fitted to TEM's for routine examination of warm specimens, are not suitable for cryo-work and EPXMA. A 'low background' analytical stage for JEOL TEM's, designed by Nicholson *et al.* (1982) incorporates an anti-contamination copper plate immediately above the specimen. A tuning-fork type, retractable aluminium anti-contaminator has also been found essential for a contamination-free high resolution imaging of thin frozen-hydrated specimens in TEM (Homo *et al.*, 1984; Dubochet *et al.*, 1988). Advantages of an efficient anticontamination device in biological microanalysis have also been emphasized by many other workers in recent years.

It should be noted that the sections on our cold stage in JXA-50A were examined flat and not tilted at 30 to 45 degrees, as is common in most other instrumental configurations. The choice of a horizontal specimen configuration was insisted upon by Torkel Weis-Fogh for ensuring distortion-free images of 1-2 μm thick cryo-sections. A horizontal configuration also aided in keeping the specimen in focus of WDS. A specimen heavily tilted with respect to the incident electrons must (a) cause distortions in the images, (b) reduce the spatial resolution of analyses by increasing the effective thickness of the specimen, and (c) increase the problem of extraneous X-rays generated by the electrons scattered underneath the specimen. However, in an ultrathin specimen the practical effects may be difficult to assess and might not be analytically significant.

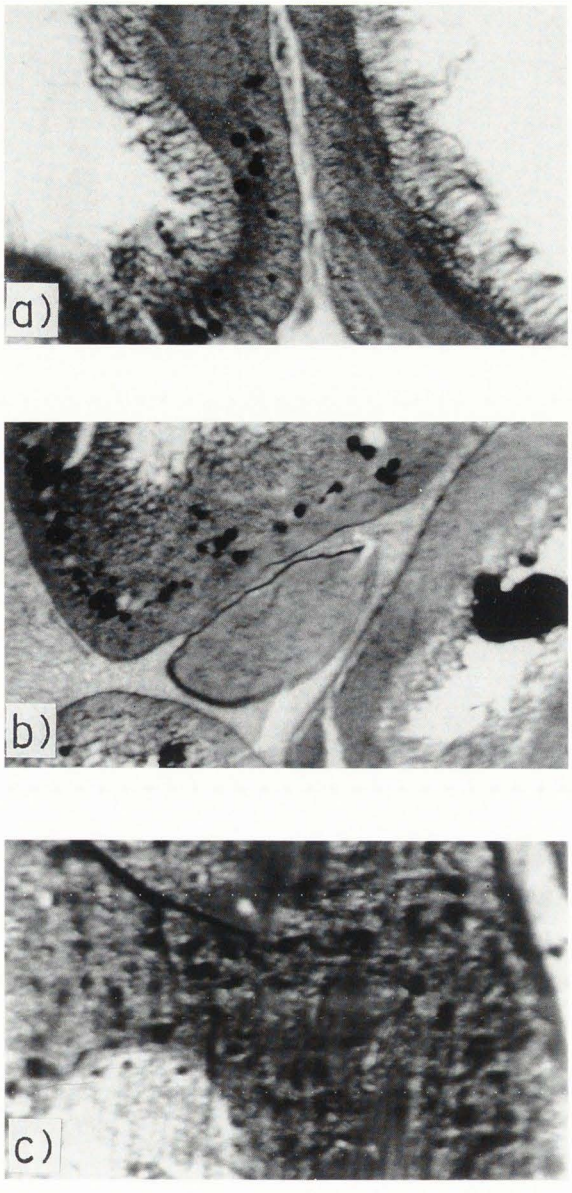


Fig. 15. STEM images of some of the insect-tissues analyzed in BML, Cambridge: (**a**) from a partially hydrated 1 μm thick section of *Rhodnius* Malpighian tubule cut at about -120°C; (**b**) from a nearly frozen-dried cryo-section of *Calliphora* Malpighian tubule; and (**c**) a partially hydrated 0.5 μm thick cryo-section of *Calliphora* salivary gland cut at about -130°C in a Reichert Cryocut ultratome; note the transparent nucleus, dense mitochondria in the cytoplasm, basal infolds and the basal lamina: large dark-gray areas in the cell-body are secretory canaliculi.

Imaging

It has now been generally accepted that the STEM images from fully hydrated cryo-sections and bulk specimens show very poor contrast and structural details (Fig.

14). Somewhat improved images can be obtained by using a mixed dark field/ bright field geometry of the beam (Bacaner *et al.*, 1973). This generalization is true for 0.5 to 2 μm thick sections examined in an SEM (Gupta and Hall, 1981a) as well as for ultrathin cryo-sections examined in TEM/STEM-type high resolution instruments (Somlyo and Shuman, 1982; Hutchinson *et al.*, 1974; Hutchinson,1979; Zierold, 1984a, b, 1985, 1988a; Hagler and Buja, 1986; Wroblewski *et al.*, 1988). During our study of Rhodnius Malpighian tubules (Gupta *et al.*, 1976) we obtained STEM images of frozen-hydrated sections which had enough contrast and detail for allowing extensive EPXMA of many fine components of the tissue (Fig. 15). However, by comparing the X-ray data with the peripheral standard (Ringers solution + 10% Dextran, molecular weight 237,000), it was found that the frozen-hydrated specimens with such good images only had about 80% or less of their 'native' water content. The issue remained a subject of considerable debate for a long time (Gupta 1979; Gupta and Hall, 1981a; 1982). The rationale of such good images in STEM configuration using dark field/bright field illumination and some rudimentary electronic image processing was then experimentally demonstrated (Hall and Gupta, 1982b). We adopted this strategy of using partially hydrated cryo-sections in all our EPXMA work.

The same phenomenon of image improvement has now been confirmed for the ultrathin (about 200 nm thick) frozen-hydrated sections by others (Dubochet *et al.*, 1982a, 1988; Zierold, 1985, 1988a). As discussed by Hall (1980, 1986b), by Dubochet *et al.* (1988) and by Zierold (1988a), the water from frozen-hydrated sections is lost by surface etching. A 10-20% loss of total water (hence the specimen mass) need not seriously disturb a 1-2 μm thick cryo-section or a similar volume in a frozen-hydrated bulk specimen. The same thickness of ice lost from a 200 nm thick section would lead to a complete dehydration. Therefore EPXMA of ultrathin cryo-sections in a hydrated state has not generally been possible (for an exception see Nakagaki *et al.*, 1984). This argument however has so far been applied only to the loss of mass (H_2O) under a high electron dosage needed for EPXMA. If the beam-induced loss of mass can be sufficiently retarded in ultrathin cryo-sections by carbon coating (Zierold, 1988a, b), it may then be possible to exploit for EPXMA the improved images from partially hydrated ultrathin cryo-sections. It might be possible to achieve such partial (about 20% of the total) dehydration by a controlled timing of the process. The importance of this approach for EPXMA clearly lies in the ability to analyze free-fluid compartments in and around the cells at a high resolution. If an accurate quantification of the data were to prove difficult in such partially hydrated ultrathin sections, even a relative distribution of various elements estimated from the characteristic peaks of the EDX spectra of free-fluid spaces could be highly informative for many biological problems (Gupta, 1989b).

Very high resolution TEM images with excellent contrast have now been obtained from 'fully' hydrated ultrathin cryo-sections as well as from very thin vitrified suspensions of viruses and biological macromolecules (Dubochet *et al.*, 1988). Such imaging only seems possible with low electron dosage technique in a TEM configuration using phase contrast (by underfocusing) or Z-contrast but not with STEM mode (Dubochet *et al.*, 1988). The method has not so far proved useful for microanalysis (Zierold, 1988a) but the possibility cannot be ruled out for the future.

Biological Studies

Zinc

As was noted in an earlier section, the importance of zinc in biological systems (Vallee, 1959; O'Dell, 1984; Prasad, 1969, 1984) is what attracted Ted Hall into microanalysis. In discussing the role of zinc in biology, Williams (1984) notes that "zinc is everywhere in biology" and unlike other trace elements, its function is not confined to enzyme catalysis. The presence of zinc in nucleoli has been known since early 50s (Fujii, 1954). In rat spermatozoa, one mg of zinc is found in one g of dried cells (= about 15 mmol/kg dry wt; Tipton and Cook, 1963). For the microanalyses of spermatozoa Ted Hall wrote (Hall *et al.*, 1966): *"Sperm cells are delightful specimens. For a good preparation one only need to lay a drop of diluted ejaculate onto a Formvar-coated grid and allow it to dry".*

Hall *et al.* (1966) attempted to map the distribution of zinc in rat sperm in an X-ray image (Fig. 16). Subsequently, Hall (1966) used spermatozoa from rat, rabbit and man, washed them in clean water, and mounted them on aluminized nylon films on Duralium sleeves for obtaining one of the earliest quantitative analyses with the 'microscan'. He found that the mass-fraction of zinc was highest (0.15) in the sperm-head and lowest (0.08) in the tail with a 'hot spot' at the junction of the head and the mid-piece. Similar air-dried preparations of spermatozoa have since been used by other workers (Chandler and Battersby, 1976; Chandler, 1980; Roomans *et al.*, 1982b; Björndahl *et al.*, 1986; Kvist *et al.*, 1987) for studying the distribution of zinc by EPXMA. These studies have also used cryo-sections for comparisons and Roomans *et al.* (1982b) have provided fully quantitative data. High contents of zinc were found in the nuclei of the spermatozoa from healthy fertile men while low levels of zinc were found to be associated with male infertility.

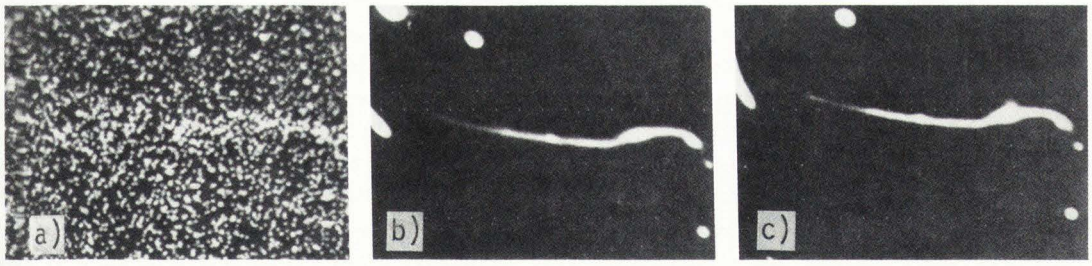


Fig. 16. Rat sperm-cell analyzed by Hall *et al.* (1966); (**a**) zinc K_α X-ray image, (**b**) secondary electron image, and (**c**) as for (b) but with a bright spot added at the site of a strong zinc signal.

Much other early work on zinc with EPXMA, especially in tissue pathology is discussed by Andersen (1967) and by Hall *et al.* (1972). In Ted Hall's EMMA laboratory, Peters *et al.* (1976) and Yarom *et al.* (1977) investigated the distribution of zinc in injured myocardium in dogs, induced by myocardial ischemia. The tissue was prepared by glutaraldehyde/osmium fixation with and without pyroantimonate. Nuclei, mitochondria and 'granules' (probably secondary lysosomes) in several types of blood cells were identified as local sites of high zinc.

Appleton and Newell (1977) used EMMA-4 and ultrathin frozen-dried sections of the mantle epithelium in aestivating desert snails *Otala lactaea*. Osmo-regulating cells had higher concentrations of zinc (and iron) than the normal controls.

Gupta and Hall (1984b) measured approximately 100 mmol/kg wet wt of zinc (and Cu) in the barbed filaments coiled inside the undischarged nematocysts from the sea anemones, *Anthopleura elegantissima* and *Rhodactis rhodostoma*. During the process of nematocyst discharge, Zn but not Cu was lost from the filament. These authors suggested that the Zn may have a zipper-type structural role in keeping the filament tightly coiled inside the undischarged nematocysts. It is interesting to recall that Fujii *et al.* (1955) reported the presence of zinc in the acrosome and middle-piece of quiescent spermatozoa in sea-urchin. When histidine (and some other substances) were added to the medium, the spermatozoa instantly became highly motile and concomitantly lost zinc into the suspension medium (presumably from the middle-piece). The implication again is that the binding of zinc structurally constraints sperm-tails from moving. Similarly, Zn in the neck-region of human spermatozoa is implicated in maintaining the attachment of the head to the tail (Roomans *et al.*, 1982b).

The structural importance of zinc in controlling the conformation of macromolecules is also exemplified in recently discovered 'zinc fingers' involved in DNA replication and transcription (Klug and Rhodes, 1987). The importance of Zn as a key element in biological systems is being increasingly appreciated (Prasad, 1984). Williams (1984) has speculated that zinc as a regulatory ion in biological systems may turn out to be as important as Ca^{2+}, and that the location of an element reveals much about its function. He further proposes that while Ca^{2+} triggers fast reactions in cells by binding to proteins, Zn^{2+} functions in a complementary fashion: the release of bound-Zn activates the protein function.

It is therefore surprising that although the study of zinc motivated a lot of early work in biological microprobe, not much attention seems to have been paid to zinc in recent EPXMA work using cryo-preparations. A likely reason is that with the exception of those who are interested in heavy metal pollutions, the other microprobe analysts working on cell and tissue physiology tend to regard Zn as a nuisance in their spectra and often dismiss it as an embarrassing, unexplained peak from some extraneous X-ray sources. If Zn is not entered as an element into the itinerating programme of the X-ray data processing for the EDXS software, the Zn L-line could make a serious contribution to the Na K-line. One should always look at the complete X-ray energy spectrum from a specimen under the beam, before instructing the computer for the processing of the X-ray data.

Sulphur

The importance of absolute quantification with the continuum method in sectioned biological specimens was demonstrated by Sims and Hall (1968). They showed that during the process of keratinization, as the cells become denser by accumulating progressively more keratin, the amount of sulphur (in keratin) per unit volume of the tissue increases. However, there is no change in the mmol of sulphur per unit specimen mass (i.e., mmol/kg wet wt). Therefore, if sulphur in keratin was quantified by the method of characteristic intensity per unit area (Dörge *et al.*, 1975; Rick *et al.*, 1982; Hall and Gupta, 1982a, 1983), one might draw the wrong conclusion about the physiological significance of the

sulphur in skin tissue (Hall, 1988). Similar considerations apply to comparing changes in other ions in different physiological states of cells and tissues, as well as to the alternative methods of specimen preparation (e.g., freeze-drying or freeze-substitution followed by plastic embedding: see Hall, 1989b, 1991). Problems with the loss and gain of sulphur in frozen-dried sections used for EPXMA have already been discussed above.

Jessen *et al.* (1974) used ultra-thin sections from glutaraldehyde-fixed and Araldite-embedded material and EMMA-4 for a quantitative analysis of sulphur in keratohyalin granules of lingual and oesophageal epithelium. They found that different types of keratohyalin granules in cells had different compositions: one type had 3.6-3.8 % of sulphur. Another type had sulphur-rich and sulphur-poor components. It was suggested that sulphur-rich granules are involved in the deposition of the envelope proteins of cornified cells.

Much of the early microprobe work on sulphur was related to the metabolism of proteins such as keratin. Not much attention was paid to analyzing sulphur as a part of sulphated glycosaminoglycans in cell surfaces, in mucus secretions and in extracellular matrices, where the polyanionic sulphates have specific ion-sequestering and ion-buffering properties (Gupta, 1989a, b) . Many studies in BML on transporting epithelia (Figs. 12, 14, 15, 17) revealed that the sulphur-rich structures such as basal laminae (basement membranes), glycocalyces, and intestinal mucins show relatively higher concentrations of K and Ca but lower Na and Cl than in the bulk extracellular fluids (Gupta *et al.*, 1977; Gupta and Hall, 1979; Gupta, 1989a, b). A similar relationship of sulphates and cations is found in the synthesis, concentration into secretion granules and rehydration by exocytosis of mucus in the goblet cells in rabbit ileum (Gupta, 1989a, b), and elsewhere (Hunt and Oates, 1977). Because of an enormous current interest in the role of mucoid substances in biology and medicine, there seems to be an urgent need for quantitative elemental analyses of these structures using EPXMA and cryo-preparations.

Interestingly, a very high concentration of Ca and K was found in the basement membranes of many insect epithelia. This observation generated a lot of skepticism and has been generally ignored in physiological literature. It now transpires that the basement membranes in the fruitfly Drosophila contain a newly discovered acidic sulphated glycoprotein named "glutactin" (Fessler and Fessler, 1989). This glycoprotein preferentially binds Ca in the presence of excess Mg: the preference of K over Na was not tested but is likely, as is the case in other sulphated glycoproteins.

Mineralized concretion bodies

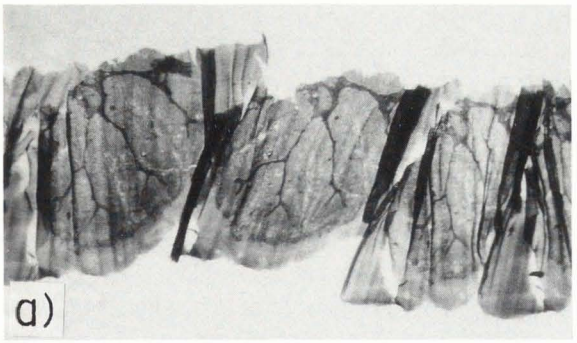
Intracellular 'concretion' bodies are found in virtually every eukaryotic organism. These bodies are usually compared with the residual bodies (= tertiary lysosomes) and seem to have a matrix of glycosaminoglycans (Brown, 1982). They serve for sequestering toxic metal ions, either as a part of normal excretory metabolism, or as a consequence of environmental pollution. Early EPXMA work on such metal 'granules' carried out in the Cavendish laboratory is summarized by Hall *et al.* (1972). Moreton (1981), Brown (1982), and Morgan and Winters (1987) have provided more recent reviews.

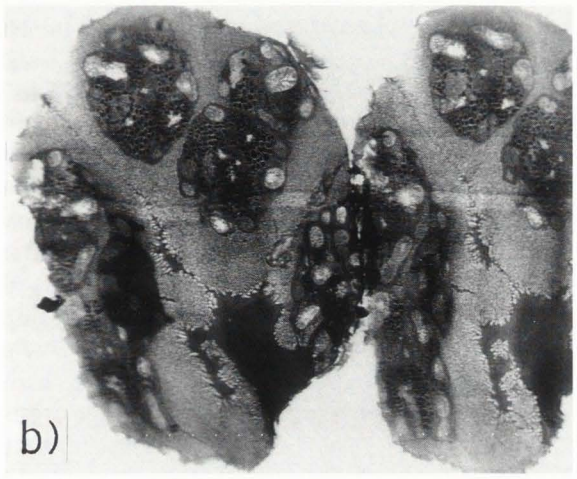
Tapp (1975) used Appleton's EMMA-4 with WDS and EDXS for analyzing Cu-rich concretions in the midgut epithelium of *Drosophila*. Sohal *et al.* (1976, 1977) in Ted Hall's EMMA laboratory found high concentrations of P, S, Cl, Ca, Fe, and Cu in an age-dependent fashion in the concretion bodies of the Malpighian tubules of *Musca domestica*. The tissues for these EMMA studies were prepared by chemical fixation and plastic embedding.

In the concretion bodies of the Malpighian tubules from *Calliphora* and *Rhodnius*, (Figs. 15a, b) analyzed in frozen-hydrated sections, Gupta *et al.* (1976, 1977) found high concentrations of Ca, P, S, and Zn (Gupta and Hall, 1978, 1981a) but less Na, K, and Cl than in the surrounding fluids.

Recently Wessing *et al.* (1988) found relatively high levels of Na and Mg but low P, S and K in the osmiophilic "vacuoles" of the Malpighian tubules in *Drosophila*, analyzed in frozen-dried ultrathin cryo-sections. Dow *et al.* (1984) noted that extremely dense bodies in the basal (undifferentiated) cells of the larval midgut in *Manduca sexta* are almost crystalline magnesium phosphate but also had high K and Ca (see also p. 426).

One category of concretion bodies has attracted considerable attention. The so-called 'chloragosomes' in certain types of cells in the intestine of earthworms accumulate metals in high concentrations. The metals are present in the soil that the earthworms eat. Therefore the analysis of chloragosomes can be used for biomonitoring the environmental pollutants in soils in a sensitive fashion. Much of the early work on these structures has been done either on chemically fixed materials or on isolated and air-dried samples and must be considered suspect. Wroblewski *et al.* (1979) used 16 μm thick cryo-sections cut at -20°C from worms which had been frozen in isopentane. They found very high levels of Ca and P in chloragosomes. Other previous workers had additionally found Zn and Mg (see Moreton, 1981). Unlike the other workers, Morgan and coworkers (Morgan, 1984; Morgan *et al.*, 1989) have now embarked on a fully quantitative EPXMA study of this material in ultrathin cryo-sections. They have identified four types of spherites in chloragenous tissue, each with its characteristic





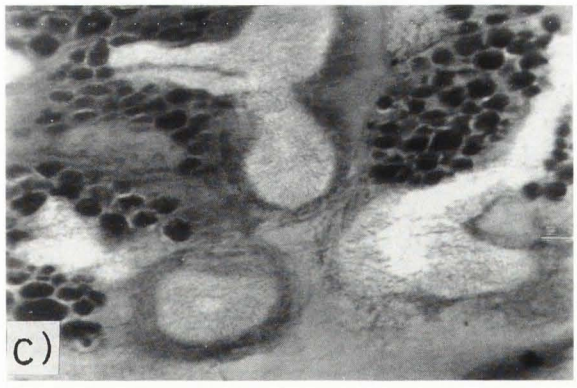


Fig. 17. STEM images of 1 μm thick frozen-dried sections to show the quality of cryo-sections cut in a SLEE cryostat at about -80°C: (**a**) from a 1 mm wide block of locust caecum encapsulated in 20% Dextran (MW, 237,000) + Ringers; (**b**) as in (a) but from cockroach salivary glands; and (**c**) details of NaCl-fluid secreting cells and of zymogen cells.

elemental 'finger print' of Mg, P, S, K, Ca, Cd, Pb, and Zn. From these rather few observations, it appears that the physiological functions of 'concretion' bodies or vacuoles in cells is highly variable and much remains to be learnt about these structures by using EPXMA in cryo-sections. Because of their high elemental and mass concentrations, such mineralized 'concretions' are readily identified even in fully hydrated cryo-sections (Hall and Gupta, 1982b; Dow *et al.*, 1984). The potential of quantitative EPXMA of cryo-prepared samples in monitoring the other environmental pollutions is obvious. The technique is already been applied for analyzing samples of polar ice from the Antarctica (Potts *et al.*, submitted).

Calcium

The microanalysis of calcium had been a continuous interest in Ted Hall's laboratories. Early work in Cavendish was concerned with the calcification of aging arteries (Hall *et al.*, 1966). The 'plaques' were shown to contain equimolar ratios of calcium and phosphorus (similar to mineralized apatite). Much lower concentrations of Ca and P with a highly variable Ca:P ratios were found in the developing or 'prestage' plaques. The attempt to study the possibility that sulphated glycosaminoglycans might be involved in Ca sequestration were frustrated by the inadequate methods of specimen preparation (Hale *et al.*, 1967). The study of Ca in epiphyseal cartilage of bone by EPXMA dates back to Brooks *et al.* (1962). A series of studies on the calcification of dentine and cartilage were carried out by Hall in collaboration with Hans Höhling and others, using a variety of common histological and cytological procedures for the preparation of tissue-sections. The general conclusion emerging from these early experiences was that even for mineralized tissues the conventional methods involving wet chemical processing and embedding are not suitable for reliable EPXMA: the use of cryo-methods was considered desirable. On his return to Münster, Höhling continued his studies on calcium mineralization of cartilage in the formation of bone. In some recent EPXMA work it has been pointed out that even some cryo-preparation methods can lead to a redistribution of both the intra- and extra-cellular electrolyte elements in the epiphyseal plates (Krefting, 1985). The most recent study by using Sr as a tracer for Ca^{2+} transport (Krefting *et al.*, 1988), casts serious doubts on all the previous models involving a transcellular movement of Ca^{2+}, with mitochondria serving as calcium reservoirs. The main Ca^{2+} transport appears to follow an extracellular route.

Ali *et al.* (1977) used thin frozen-dried cryo-sections and EMMA-4 for their microanalytical studies of calcifying cartilage. Interestingly they found that even 1 mm^3 pieces of cartilage could be frozen by plunging directly into liquid nitrogen without suffering any apparent ice

crystal damage! Presumably, the very high concentrations of glycosaminoglycans in the matrix of this tissue act as a very efficient 'native' cryo-protectant. Ultrathin cryo-sections were cut in an LKB 'CryoKit' at temperatures between -140°C and -100°C, and frozen dried by lyophilization in the cryo-chamber. These workers (Ali *et al.*, 1978) also found spongy dense granules in the mitochondria of chondrocytes with a Ca/P ration of 1.2. However, the 'dense' matrix granules previously investigated by Hall *et al.* (1971) as sites of Ca-mineralization, had a Ca/P ratios of 2.15. As noted above, the method of freeze-drying cryo-sections used in this study is likely to have caused artefactual redistribution of ions.

Emerging from the biochemical studies (Lehninger, 1970) there has been a long-standing belief that the dense osmiophilic granules in mitochondria, commonly seen in conventionally prepared transmission electron micrographs, are intracellular Ca-stores involved in the regulation of cell function (Hall, 1975). As noted above, a similar function was postulated in the salivary glands of *Calliphora* from EPXMA study on chemically fixed and plastic embedded material (Berridge *et al.*, 1975). The subsequent EPXMA work with frozen-hydrated cryo-sections (Gupta *et al.* 1978a; Gupta and Hall, 1978) revealed that the Ca-levels in the mitochondria of normal (Fig. 15c) cells are not higher than the average levels in the adjoining cytoplasm (about 1 mmol of total Ca/kg wet wt of tissue). Only the mitochondria in cells morphologically identified as damaged, had high Ca (upto 10 mmol/kg wet wt). However, in normal cells 'hot spots' of high Ca (2-6 mmol/kg wet wt) were found in the nuclear envelope and in some 'dense bodies' (presumably stacked ER) in the circumnuclear cytoplasm (Gupta and Hall, 1978). This was the first direct observation that implicated nuclear envelope (continuous with RER) in the intracellular Ca^{2+} homeostatis for cell-function. This observation is now finding support from more recent studies with techniques using biochemical methods, enzyme histochemistry (Ca^{2+}-ATPase) and immunocytochemistry (Ghosh *et al.*, 1989; Ross *et al.*, 1989). Subsequent EPXMA work with cryo-sections of carefully frozen cells and tissues has also shown that ER and not the mitochondria appear to store Ca that is mobilized for the regulation of cell function (Somlyo *et al.*, 1985; Berridge and Irvine, 1989). Even in calcifying tissues only the mitochondria in damaged cells have high calcium (Krefting *et al.*, 1988).

A novel role of calcium was discovered by Lubbock *et al.* (1981) in the acrorhagus isorhizal nematocysts of sea anemones, *Anthopleura elegantissima* and *Rhodactis rhodostoma*. The capsular contents of undischarged nematocysts had some 500-600 mmol/kg wet wt of Ca. After electrically stimulated exocytotic discharge the Ca in the capsules escaped and the contents were replaced with sea water. Lubbock *et al.* (1981) proposed that Ca in undischarged capsules forms salt linkages between highly anionic proteins and renders them osmotically inactive. The stimulation induces some unknown changes (possibly pH), causing the removal of Ca links, leading to an osmotic discharge of the organelle. It has recently been suggested that a similar osmotic mechanism might operate in the dispersal of matrix contents from the structured secretion granules in other systems such as the cortical granules of oocytes (Chandler *et al.*, 1989).

More recent work on nematocysts has exquisitely demonstrated the importance of EPXMA on cryo-sections of living tissue fast-frozen in situ. Most of the previous work on the mechanism of discharge of these structurally most complex products of cell secretion has been done on isolated nematocysts (Gupta and Hall, 1984b; Weber *et al.*, 1987). The EPXMA on isolated nematocysts has produced highly conflicting results on the ionic contents of the component structures and their role in the discharge mechanism (Hessinger and Lenhoff, 1988). In a recent study (Zierold *et al.*, 1989; Tardent *et al.*, 1990), several different types of nematocysts from several species of Cnedaria have been analyzed in frozen-dried cryo-sections. The data show that different types of nematocysts even in the same animal contain, in molar concentration, one or the other of different cations such as K or Ca or Mg. These 'native' ionic compositions are grossly altered when the organelles are isolated for physiological studies and for microanalysis, as has been the salutary experience from the previous works on isolated nuclei and mitochondria. Tardent *et al.* (1990) have, however confirmed the observations of Lubbock *et al.* (1981) that acrorhagous nematocysts in *Anthopleura elegantissima* do contain inordinately high Ca, even though the nematocysts from the food-capturing oral tentacles had only Mg. These observations suggest that the organisms deploy different ionic species for the nematocysts performing different biological functions in vivo. Such unequivocal quantitative data from a range of biological species could not have been obtained so expeditiously and reliably from samples in situ by any other method of chemical analyses available at present.

A similar variation in the usage of cationic counterions also seems to exist in other analogous systems. For example, Hunt and Oates (1977) analyzed mucus secretion in a marine snail, *Buccinum undatum* using bulk-frozen and fractured specimens in JEOL SEM-EDXS analytical system similar to the one in BML, Cambridge. They found that presumptive mucous granules inside the cells had very high K (and S) but no Ca and Mg. On release and expansion, the mucus lost K but acquired Ca and Mg. These results are in contrast with the vertebrate mucous (goblet) cells (Gupta 1989a, b) where the gran-

ules inside the cells have high Ca and Mg but the released mucus has low Ca and Mg. Clearly, a highly selective binding of ions to polyanions reflects stereochemical variations in the negative charges of the macromolecules adapted for different biological application even in the same organism. Constructing universal paradigms in biology from the study of one or a few cell-types and organisms can often be misleading.

As an example of specialized preparatory procedures, Zierold and co-workers designed elegant freezing equipment for capturing ultrafast (< 1 msec) events such as the discharge of trichocysts in *Paramaecium* (Schmitz *et al.*, 1985) and of nematocysts in *Hydra* (Zierold *et al.*, 1989).

The role of calcium in a novel mechanism for the ultrafast contraction of the spasmoneme (=myoneme) was revealed in the first study on JXA-50A in BML (Routledge *et al.*, 1975; Gupta and Hall, 1978). The analyses were performed on isolated spasmonemes (the proteinceous contractile elements), extracted from glycerinated specimens of *Zoothamnium*. These isolated spasmonemes can be made to contract and extend by controlling the free Ca^{2+} between 10^{-6} and 10^{-8} M with EGTA buffer. The spasmonemes are strongly anisotropic (birefringent) in polarized light in a relaxed state but become isotropic in the contracted state. Routledge *et al.* (1975) found that the ultrafast contraction is caused by the uptake of about 7 mmol of Ca^{2+} per kg wet wt of spasmoneme. It was proposed that the spasmonemes are maintained in an extended confirmation by the electrostatic repulsion of the negative charges on the polyanionic protein fibers. Ca^{2+} ions neutralize the negative charges, thus causing the protein to change from an extended confirmation to a random-coil. This work needs to be repeated for the analyses in situ, by methods used on trichocysts and nematocysts (Zierold, 1989). A much wider implication of the protein spasmin (Amos *et al.*, 1975) in other ultrafast contractile systems in cells was predicted by Gupta and Hall (1978). The discovery (see Goodenough, 1989) that the Ca-sensitive protein 'centrin' associated with the basal bodies (ciliary rootlets) and centrioles is immunocytochemically related to spasmin (Salisbury *et al.*, 1984) bears testimony to this prediction.

Very little work on the analysis of Ca in muscle contraction was done in Cambridge, except for some studies by Somlyo *et al.* (1974) and Yarom *et al.* (1974) on EMMA-4. The sections used were from chemically fixed and plastic embedded materials and the analytical results have now been proved artefactual (Hall, 1989a). However, EPXMA of muscle seems to occupy most other microprobe laboratories in Europe and in the USA. Earlier data on this topic are reviewed by Moreton (1981). The subject is too vast for a coverage here. The need to freeze muscle in a physiologically defined state in vitro and for obtaining reliably frozen samples for conditions in vivo has led to numerous ingenious technical innovations (see Zierold and Hagler, 1989).

Intracellular distribution of ions

The major ions inside most cells are high K and low Na and Cl. This ionic composition is generally in contrast with the extracellular milieu of high Na and Cl and very low K: it is generally believed to be maintained by the primary, active ion pumps in the plasma membrane. Before the emergence of EPXMA and cryo-methods in early 70's, the actual concentrations of these ions with any degree of certainty were only known in a few cell-types. There was much controversy about the intracellular concentrations and activities of Na, K and Cl, especially in ion and water transporting epithelia (Macknight, 1980; Diamond, 1982; Civan, 1983). Most cell physiologists also believed that for all practical considerations, cells are membrane-bounded bags of ions in which all the electrolytes are freely dissolved in all of the cytosolic water. Therefore there are no gradients of ionic concentrations and osmotic activities inside the cells even in those epithelia which generate large transepithelial fluxes of ions and water and support large differences in osmotic concentrations and ionic compositions between the extracellular compartments they separate.

The earliest EPXMA results from frozen-hydrated cryo-sections of the fluid secreting epithelia of *Rhodnius* (Gupta *et al.*, 1976) and *Calliphora* Malpighian tubules (Gupta, 1976; Gupta *et al.*, 1977, 1978a) and *Calliphora* salivary glands (Gupta *et al.*, 1977, 1978a) indicated that Na, K, and Cl are not uniformly distributed in the cytoplasm. There were gradients of total ionic concentrations in the basal-apical direction. When hormonally stimulated, these insect epithelia secrete at a very fast rate an isotonic KCl-rich fluid from an NaCl Ringers on the serosal side. The intracellular gradients of ions were also revealed in the epithelial cells of *Calliphora* rectal papillae, absorbing water from the lumen against an osmotic gradient (Gupta *et al.*, 1977,1980b; Hall, 1979a).

Large gradients of K, Cl and dry mass were also found in the osmoregulating cells of the mantle in aestivating desert snail, *Otala lactaea*. In this study Appleton and Newell (1977) in Cambridge used ultrathin frozen-dried sections and EMMA-4. The intracellular osmotic gradients deduced from the microprobe data were confirmed by microscopic osmometry of frozen-hydrated tissue-slices (Appleton *et al.*, 1979).

The mammalian ileum absorbs isotonic NaCl fluid together with the dissolved nutrients (amino acids, sugars etc.) from the gut-lumen, and in preparations in vitro, this absorption is stimulated by glucose (or other sugars) in the mucosal bath (luminal). The enterocytes

nevertheless maintain on average the normal intracellular concentrations of high K and low Na and Cl. This tissue (Fig. 12) was investigated in BML over several years in collaboration with Richard Naftalin of King's College, London (Gupta *et al.*, 1977; Gupta *et al.*, 1978b; Gupta and Hall, 1979, 1981b; Gupta, 1984, 1989a, b). The intracellular distributions of Na, K and Cl were found to be complex. There were distinct concentration profiles of Na, K and Cl in the actively absorbing enterocytes but the gradients of each ion were different in the organelle-rich core (endoplasm) from the profiles in the organelle-free peripheral cytoplasm (ectoplasm including the terminal web). It was proposed that Na, Cl and water being transported through the cell into the lateral intercellular spaces (LIS) preferentially moves via 1-2 μm wide zone of peripheral cytoplasm and does not readily mix with the core cytoplasm. Measurable intracellular gradients were essentially absent from the enterocytes both in unstimulated control preparations and from the tissue where the transport was inhibited with ouabain. These microprobe data were consistent with an earlier study of rabbit ileum in vivo, using ion-selective multi-barrelled microelectrodes (Zeuthen and Monge, 1975). The apical to basal gradient of Na in the peripheral cytoplasm of sugar stimulated enterocytes (Gupta, 1984) also resembled a similar gradient measured with EPXMA in the Na-absorbing epithelial cells of the frog-skin by Rick *et al.* (1984). The principle that has emerged from these EPXMA studies of transporting epithelia is that contrary to the popular belief, neither the ionic nor the osmotic concentrations are homogeneous in those epithelial cells which support large unidirectional fluxes of ions and water through them.

Further confirmation of this principle has come from some more recent work in relatively short (< 10 μm tall) cells in the urinary bladder of the toad, *Bufo marinus* and in the cortical collecting ducts of rabbit kidney (Rick and DiBona, 1987; Rick and Schratt, 1989). In this EPXMA work, ultrathin cryo-sections were cut at -140°C in a Reichert FC 4D cryo-ultramicrotome and frozen-dried at -90°C at 10^{-7} mbar before analyses in a Cambridge S250 SEM fitted with a newly designed transmission stage and a LINK Systems EDS.

EPXMA observations on a non-uniform distribution of ions in the cytoplasm are not confined to epithelial cells. An early Russian study (Burovina *et al.*, 1978) on frozen-dried cryo-sections cut at -20°C also reported considerable variations in the concentrations of Na, K and P in different parts of the giant neurones (50-100 μm in diameter) of the snail *Planorbarius*. K was generally associated with P, a correlation which has since then also been noted by others in many different cell-types. In the frog sartorius muscle at rest, K^+ ions are thought to be concentrated mainly in the A and Z bands but the observations are mostly based on either frozen-dried or freeze-substituted tissues embedded in plastics and cut dry (Edelmann, 1988). Cs and Tl were used as 'surrogate' ions for K in the muscle in order to improve visualization.

Although direct images of frozen-hydrated cryo-sections seem to support the results (Edelmann, 1988, 1989) no quantitative data from EPXMA of cryo-sections is provided (Edelmann, 1989). However such quantitative data has been obtained by Zglinicki (1988) from freeze-dried cryo-sections of the heart muscle: the results are in general agreement with Edelmann's qualitative observations.

It would therefore seem, that the general concept that for the physiological function, a living cell is a bag of ions freely dissolved in cell-water has been debunked. The question of the physical processes involved in determining the intracellular distributions remains unresolved. Ling (1984, 1989) maintains that most if not all the ions (especially K^+) in living cells are adsorbed on the fixed negative charges of beta and gamma carboxylated groups on proteins: the membrane and its associated transport functions have little role in maintaining intracellular ionic composition. An alternative view is that ion-pumps and channels in cell membranes are primarily responsible for the overall ionic composition of cells but the local concentrations and activities of ions and water, resulting in focal osmotic gradients within the cells, is determined by Donnan effects due to 'fixed' charges and the structured water present on the cell components (Bernal, 1965; Gupta, 1976; Rick and Schratt, 1989). Only more future work will resolve this issue.

Cell nucleus

Arising from the classical observations of Naora *et al.* (1962) by autoradiography and from the chemical analyses of isolated nuclei (see Jones *et al.*, 1979), there has been a long-standing belief amongst biologists that nuclei in eukaryotic cells are a repository for some hundreds of mM of Na as well as of other cations. Elaborate biophysical models involving a direct ionic communication between the nucleus with the extracellular Na-rich fluids have been proposed (Moore and Morill, 1976). The earliest EPXMA observations from Dörge *et al.* (1974) on the frog skin and from BML on insect tissues and chick red blood cells (Gupta *et al.*, 1976, 1977) questioned this common belief. All the subsequent EPXMA results from a large variety of cell-types in numerous laboratories have now shown that nuclei in normal, undamaged, interphase cells have Na, K, Ca, Mg, Cl levels comparable to those in the surrounding cytoplasm. Large influx of Na into the cytoplasm, either induced experimentally as with ouabain, or as a conse-

quence of cell-stimulation, cell-transformation and disease, can lead to Na levels in the nucleus which may far exceed those in the cytoplasm (Gupta *et al.*, 1978a). In general, but not always, interphase nuclei (Fig. 18) show somewhat higher levels of K than that in the cytoplasm (see Table 3 in Gupta and Hall, 1982). This higher K could, at least partly, be explained by the recently discovered K-binding heparan sulphates located in the nuclei (see Gupta, 1989a, b).

Pericellular Environment

Extracellular interspaces in epithelia

Solute-solvent coupling for generating transepithelial flows of ions and water is commonly believed to involve extracellular compartments such as lateral intercellular spaces, apical and basal infolds, secretory canaliculi etc. (Berridge and Oschman, 1972). The most commonly accepted model for explaining such transport is the 'hypertonic interspace' concept formalized by Diamond and Bossert (1967). EPXMA measurements in frozen-hydrated sections in BML provided the first direct evidence that the total ionic concentrations in the relevant interspaces in several types of epithelia are indeed higher than in the adjoining compartments (see Gupta and Hall, 1979, 1981b, 1982; Gupta, 1984, 1989a; Hall and Gupta, 1981). However, the translation of these EPXMA-measured ionic concentrations into effective osmolarities is complicated by the rediscovery that all the relevant sites are not structurally 'empty' but contain measurable quantities of organic molecules (residual dry mass). This organic matter corresponds to the extracellular matrices and the 'cement substance' of classical histology which in the past was implicated in transepithelial transport. The components of these matrices are predominantly polyanionic glycosaminoglycans which show preferential sequestration of cations such as K^+ and Ca^{2+}. They also have anomalous colligative and water-structuring properties. EPXMA of frozen-hydrated cryo-sections in BML has provided new insights into the key-functions of such polyanionic matrices in epithelial transport. This subject has recently been reviewed (Gupta, 1989b). All the available EPXMA data together with the electrophysiological observations in other laboratories (Engelhardt *et al.*, 1986; Rechkemmer *et al.*, 1986) reveal that such matrices are involved in maintaining site-specific ionic and pH microenvironments which are characteristically different both from the bulk fluids in serosal and mucosal compartments and from the intracellular fluids. Perhaps the most important consequence is recycling of K^+ leaking out of the cells, and therefore reducing the energy load for maintaining a high-K^+ intracellular environment (Civan *et al.*, 1980; Gupta, 1989a, b). However the data available so far are

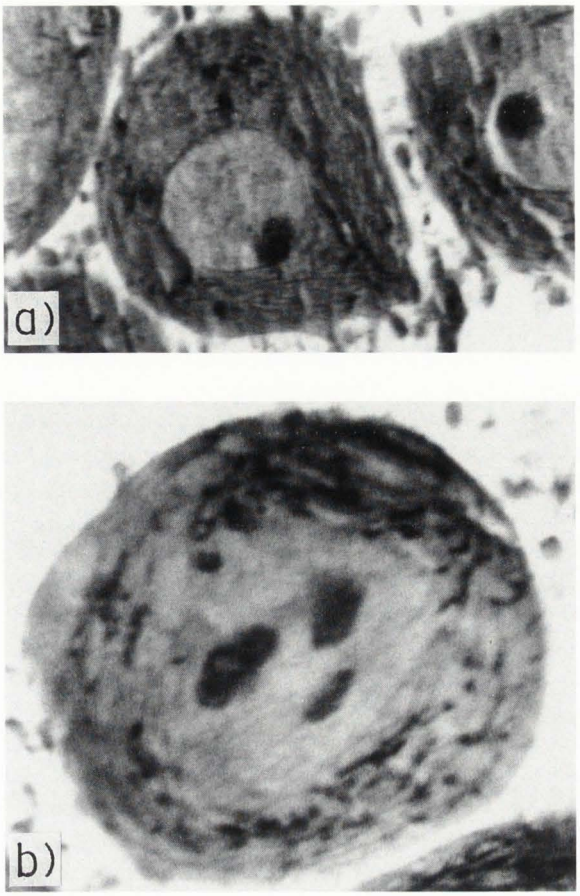


Fig. 18. STEM images from frozen-dried cryo-sections of a whole testis from a cranefly (Forer *et al.*, unpublished): (**a**) an interphase spermatocyte, and (**b**) a spermatocyte in meiosis I anaphase.

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very limited. The subject is of primary importance in general physiology and in pathology (e.g., cystic fibrosis, ulceration of the digestive tract etc.) and is ripe for more EPXMA work on cryo-preparations, preferably analyzed in a frozen-hydrated state.

Ion-exchange properties of the glycocalyx in maintaining a specific pericellular ionic environment may also be important in single-cell systems such as amoeba, lymphocytes etc. The classic studies of Marshall and Nachmias (1965) focused attention on the ion-exchange properties of glycocalyx in amoeba, with selective binding of some cations over the others. Hendril (1971) found that the concentration of monovalent cations is at least 2.1 times their concentration in the external medium. It is believed that the cationic agents induce pinocytosis in amoebae by displacing cations (K, Ca) bound to the cell surface and thereby altering the physiological properties of the cell membrane. Prusch and coworkers

(see Prusch, 1980) have found that some 18% of the total cell-Ca may be associated with the cell surface. Influx of Ca, released from the glycocalyx, into the cell in *Amoeba proteus* is thought to be an essential step in the induction of pinocytosis (Prusch, 1980). No microprobe work seems to have been done in order to address these important issues.

Epilogue

In his opening remarks for the Symposium on "History and Status of Zinc in Nutrition", O'Dell (1984) stated:

"History plays a useful role in science as well as in politics despite the fact that some persons find it difficult to justify the time spent in reviewing the past. Besides the questionable benefit of allowing the more experienced scientists to reflect on the accomplishments of colleagues, it provides a valuable analytical perspective for younger scientists entering the field of research".

I hope that this review of Ted Hall's achievements serves the purpose stated in this quotation! The seminal lesson emerging from this historical perspective is that a new technical approach can only gain popular acceptance if it can provide answers to scientific questions not obtainable by any other method. The main reasons for Ted Hall's success in making electron probe X-ray microanalysis an acceptable method in biological research were to recognize: first that the chemical elements and their ions are intimately involved in almost every biological function; second that this involvement is focalized at the cell and subcellular levels and therefore the distribution of ions needs to be known at a submicroscopic (and ultimately at a molecular) resolution; and third that for appreciating fully the physiological significance of such ionic distribution, the data need to be fully quantified in the accepted currency of biological sciences. As a physicist, he realized that it is not enough to develop physical methods with elaborate mathematical formulations and let the interested biologists grapple with them. He applied the methods, as they developed, to answering the urgent biological questions. Such attempts revealed serious limitations in the methods which required technical solutions. It is this continuous interplay between the technical developments and their immediate biological application which remained the basic approach in Ted Hall's researches. This is also the message he has delivered to all other followers of the technique. From the very modest starts some 30 years ago, the practitioners of EPXMA have witnessed spectacular advances in the instrumentation and methodology, both for analysis and specimen preparation. This has inevitably led to the explosion of technical as well as biological information. However the advances in techniques for the cryo-preservation and cryo-microtomy of cells and tissues has been much more effectively capitalized for the high resolution EM of the structures and for immunocytochemistry. X-ray microanalysts still seem to be grappling with the proper understanding and management of the X-ray data. Again the biologists seem to be hypnotized by the dazzling possibilities of computed automation and colorful images generated by pressing buttons. The new biological information in recent years does not seem to match the resources and the efforts being invested. Ted Hall's early warnings both to the physicist and the biologists come back to mind.

Increasing sophistication in the data acquisition has also raised the standards of critical appraisals. In retrospect one may look at the first location of S, Ca or P by EPXMA in histochemical precipitates as rather trivial, provoking the question so what? But nobody could have foreseen either that the tiny black dots (Palade's particles) in the electron micrographs of early 50s would turn out to be the ribosomes and a key mechanism in protein synthesis. Nor could one have foreseen that a rather ill-defined stack of membrane sacs, tentatively and controversially identified as the 'artefactual' Golgi apparatus of the classical light microscopists would come to occupy the center of the stage in cell function (See Gall *et al.*, 1981). It is also noteworthy that the special commemorative issue of The Journal of Cell Biology (Gall *et al.*, 1981) while reviewing the historic development of the subject, does not contain a single article on the distribution of chemical elements and ions in cells! To this goal of wider recognition, I have attempted to bring the observations by Ted Hall and his research collaborators into current perspectives of cell and tissue physiology. In the case of Palade's 'small particulate component' (Siekevitz and Zamecnik, 1981) it was the distribution of the structures in the electron micrographs from the liver and exocrine pancreas cells which first suggested their involvement in protein synthesis. As noted by Williams (1984), it is the distribution of an element in cells and tissues which provides important clues about its functions. Electron probe X-ray microanalysis is eminently suited for providing such information in situ in a fully quantitative fashion, provided it is remembered that it is the critical biological thinking and the not the computer-printed data which leads to correct answers.

Finally, the review is essentially focussed on the work of Ted Hall and his colleagues. It is by no means a comprehensive coverage of the field. I am fully aware of numerous important contributions by other workers to the progress of biological microanalysis. The restricted coverage in this review is not designed to belittle these contributions, many of which will be or have been published in this journal.

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Discussion with Reviewers

A. Warley: The author comments on the use of dextran-containing, known concentrations of salts as standards for quantitative X-ray microanalysis. Can such standards be recommended if the cold stage is not to be used during analysis? Surely differences in the loss of mass between dextran (a carbohydrate polymer) and the usual specimen (largely protein) will lead to inaccuracies if a cold stage is not available.

Author: A simple answer would be that I do not know because we never analyzed our sections with the stage at the ambient temperatures. However, the problem of differential loss of mass from different sub-fields of a specimen analyzed at ambient temperatures remains an open question for the quantification. Thurau's Munich group, who have habitually analyzed their 1-μm thick frozen-dried cryo-sections at ambient temperatures in a SEM, do not use the continuum normalization for the X-ray data conversion and therefore avoid the problem of mass loss. In any case the issue can only be addressed properly by directly comparing the results from the same specimen analyzed on a stage at warm and cold conditions. Zglinicki and Uhrik (1988) attempted this with 0.1 μm thick cryosections of rat's liver, analyzed in a JEOL JEM 1200 EX at 80 kV. However, they introduced a number of variables in the freeze-drying and handling of cryo-sections. They found significant differences in the values for Na, K, and S between the analyses at the ambient temperature and with the stage cold.

Unfortunately, no data, either for the continuum counts or for the dry-mass fractions were presented. Ideally, this problem should be examined by loading a batch of frozen-dried cryo-sections on the stage, analyzing some sections at the ambient temperature, cooling the stage to $< -150°C$, analyzing other sections not previously exposed to the electron beam, and then

comparing the results of similar fields. To my knowledge such systematic comparisons have not been published. It is always assumed that the loss of mass from the specimens analyzed at ambient temperatures is approximately 20% of the initial mass and is uniform for the components of the sample.

Your specific question presumably refers to the fact that sugar molecules are generally thought to be much more prone to the beam-induced loss of mass than other molecules (except perhaps sulphur). This belief again is based on some experience with sucrose added to the medium surrounding the biological samples. There is no direct evidence that very high molecular weight (MW > 250,000) dextran or other carbohydrate polymers are more sensitive than either proteins or nucleic acids (also polymers of pentose sugar compounds) or mucoids.

It is also not strictly valid to assume that the there are no differences in the proportional loss of mass in the "usual specimen (largely protein)", especially for the analyses at the organelle level. How about carbohydrate rich components, both inside the cells and around the cells and tissues? How about hydrocarbons in the membranes etc.? Only a systematic comparison can answer your question.

T. von Zglinicki: Is the main reason for the usefulness of Hall method in bulk samples (especially as background-under-the-peak) really the pancake distortion of the analyzed volume?

Author: No, but if the incident beam does take a pancake geometry, restricting the excited volume to 1-2 μm, then Hall's formulation for 'thin' specimens can be directly applied to the bulk specimens. Furthermore, the analytical spatial resolution would also be comparable to the sections of similar thickness although the lateral resolution as well the image resolution in bulk specimens may be more limited. Hall's continuum method remains valid even when the excited volume in bulk specimen takes a pear-shaped geometry but the equations become much more complicated due to a gradual loss of energy of the impinging electrons, multiple scattering, differential absorption of the generated X-rays, secondary fluorescence etc. A number of workers (e.g., Statham and Pawley, 1978; Zs.-Nagy *et al.*, 1977) have provided empirical solutions to these problems. Zs.-Nagy and coworkers have used 'continuum' method with equations similar to number (40) in Hall and Gupta (1983) for analyzing frozen-dried bulk specimens of mammalian tissues and cells (Pieri *et al.*, 1977; Zs.-Nagy *et al.*, 1981). The application of Hall method in quantification is discussed by Roomans (1988b, 1990).

T. von Zglinicki: How far does a peripheral dextran-containing standard act as a cryo-protectant during cryo-fixation?

Author: This remains a debated point and no clear answer is available. We started using a 10-20% dextran (MW about 250,000) added to the bath Ringers in the belief that it was an effective cryoprotectant (Gupta *et al.*, 1976; Skaer *et al.*, 1978). Tubular epithelia of insects in a droplet of dextran-Ringers seemed to freeze better than in Ringers without dextran. A controversy ensued regarding the adverse physiological effects of dextrans on cells and tissues. This controversy has been partly due to various workers using dextrans of very different molecular sizes (MW from 10,000 to 500,000, but commonly about 70,000) as compared to ours (Barnard *et al.*, 1984). However, we continued the use of dextran firstly for the use of dextran-Ringers as a peripheral standard and secondly because such solutions form an excellent matrix for the handling and cryo-sectioning of delicate tissues and of cells in suspension. How various non-penetrating agents act as effective cryo-protectants still remains obscure (Skaer, 1982) but would seem to exclude extensive binding and structuring of water (Barnard *et al.*, 1984).

T. von Zglinicki: Could the apparent absence of ice crystal damage in the cryosections prepared by freeze-drying in LKB CryoKit by Ali *et al.* (1977, 1978) mainly be due to partial dehydration and subsequent melting of sections? The original LKB CryoKit had a high partial pressure of water due to easy entrance of moist air into the cryo-chamber and should not be very well suited for hydrophilization.

Author: You are, of course, right about the problem of atmospheric control in the earlier versions of LKB (and many other) cryo-attachments for ultramicrotomes. However, if the residual 'ice' in a partially dehydrated section had melted and the sections subsequently dried by the sublimation of water in a liquid form, one would expect the fine structure of the tissue to be completely degraded and the distribution of ions completely scrambled. One should bear in mind that this work was done during the early formative days of the methodology when few of the modern facilities of specimen preparation, imaging in the TEM and X-ray data processing were available. It is therefore difficult to judge that work by the current standards. The TEM images of frozen-dried cryo-sections (30-70 nm thick) taken in AEI-EMMA do not have sufficient contrast but the structures such as endoplasmic reticulum (ER) in the chondrocytes seem reasonably preserved and readily identified. The background cytoplasm appears smooth. Ali *et al.* only measured Ca and P as peak/background X-ray counts and did not measure Na, K, Cl etc. However, Ca/P ratios were quite distinct between the cytoplasm, intramitochondrial granules, cartilage matrix and matrix vesicles.

Nevertheless, the possibility that you suggest cannot be ruled out.

As for the apparent high quality of cryo-preservation one should bear in mind that during cryo-fixation all cells and tissues need not behave like rat liver, vertebrate muscle or HeLa cells in suspension! Numerous organism have natural cryo-protectants in their tissues. The chemical composition, relative dry weight fractions, amount of solvent water (unstructured) and other factors are likely to significantly determine the freezing properties of the biological specimens. A very high concentration of glycosaminoglycans and glycoproteins in the cartilage matrix may cause extensive structuring of water, thus preventing a ready formation of ice crystals during freezing. Why not try freezing some cartilage and examine the cryosections? The tissue is of primary interest for research in medicine and pathology (e.g., research in arthritis) and very little reliable information is available on the concentration and distribution of ions in this tissue. Ions do seem to have a critical role in determining the mechanochemical properties of mucoids (see Chantler and Ratcliffe, 1989).

T. von Zglinicki: It should be mentioned that the preservation of biological structures up to a depth of 250 μm as stated in Ryan and Purse (1985) appears to be an extremely exceptional case. Moreover, Ryan and Purse (1985) did not publish any electron micrographs of the specimens in support of their experimental results. Today there is general agreement that tissues can be frozen to a depth of only about 20 μm at best, without any ice-crystal damage detectable in TEM (see for instance Robards and Sleytr, 1985, p. 127). Your statement that "cryofixation of even large biological tissues is not a limiting factor" [for microanalysis] seems far too optimistic, especially if subcellular resolution is required.

Author: Ryan and Purse were making the point, earlier noted by Gupta *et al.* (1977) that all other factors being equal, the quality of cryo-fixation critically depends on eliminating a layer of cold gas above the cryogenic vessel through which the specimen has to pass. This seminal point is commonly overlooked.

In most studies the purpose of cryo-fixation in order to achieve a complete vitrification of tissue-water is to obtain high resolution images of structures either by TEM of frozen-hydrated and frozen-dried sections or of freeze-fracture replicas. To this aim the generally quoted limit (with the caveats mentioned above) may well be applicable. My statement applies to the quality of cryo-preservation required for microanalysis where the practical resolution is rarely better than 100 nm. Furthermore, vast number of analytical questions in cell and tissue physiology can be addressed even with a resolution not much better than 1 μm, as was noted by Hall long time ago. As long as the ice crystals are substantially smaller than the desired (or achievable) resolution, why should the formation of ice crystals prohibit microanalytical work, provided of course the structure of the tissue is not grossly disrupted?

By subcellular you probably mean microanalysis at the single organelle level (excluding the nucleus). Such analyses would require cryo-preservation free from 'visible' ice crystals but does this necessarily mean complete vitrification? In any case how many laboratories are practicing such high resolution analyses where one can reliably measure ionic composition exclusively say within a single cisterna of ER or Golgi?

As for the practical aspects, the process of cryo-fixation still remains unpredictable and poorly understood in spite of impressive advancements in general methodology. For example, how would you explain the images such as in Fig 12e where the illustrated cell is at the base of a crypt in rabbit ileum. The sheet of unfixed, uncryo-protected frozen tissue from an in vitro preparation was about 1-mm thick and 1-cm in diameter. There was a layer (about 200 μm thick) of connective tissue plus dextran-Ringer (added immediately before freezing), and some 0.8 mm thick layer of epithelial tissue on the mucosal side? The preservation of ER etcetera seems good but the apical cytoplasm and vacuoles are badly preserved. Similar variabilities could be found from cell to cell and within cells throughout the length of the whole villi. If you now consider that we were using such samples for microanalysis in 1-μm thick cryo-sections in our JEOL JXA-50A SEM microanalyser, aiming at an analytical spatial resolution of about 0.5 μm at best, why should such samples not be useable for microanalysis? (For some results from such samples see Gupta 1989a, b). Images in Figs. 17b, c are even more intriguing.

In my statement I was recommending to the cell and tissue physiologists not to be discouraged by sweeping generalizations about the limits of useful cryo-preservation in literature. Be judiciously pragmatic and use microprobe analysis for seeking answers to problems. We did.

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Authors note added in proofs related to text on page 406.

As pointed out on page 405 above, if zinc is not entered correctly into the standards file of the X-ray data reduction programme, then the zinc L-line could be confused with the sodium K-line, leading to completely anomalous results. This is what seems to have happened in the study of Wessing *et al.* (1988). Zierold and Wessing (1990) have now corrected this error in a reinvestigation and have shown that the osmiophilic vacuoles in the larval Malpighian tubules of *Drosophila* have very high concentrations of zinc and not sodium, as was reported by Wessing *et al.* (1988). Such serious anomalies could very well exist in other studies.

Zierold K, Wessing A (1990) Mass dense vacuoles in *Drosophila* Malpighian tubules contain zinc and not sodium. A reinvestigation by X-ray microanalysis of cryosections. Eur J Cell Biol **53**, 222-226.