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PREPARATION OF BIOLOGICAL SAMPLES FOR TRANSMISSION X-RAY MICROANALYSIS: A REVIEW OF ALTERNATIVE PROCEDURES TO THE USE OF SECTIONED MATERIAL

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Abstract

Although transmission X-ray microanalysis of biological material has traditionally been carried out mainly on sectioned preparations, a number of alternative procedures exist. These are considered under three major headings - whole cell preparations, analysis of cell homogenates and biological fluids, and applications of the technique to microsamples of purified biochemicals. These three aspects provide a continuous range of investigative level - from the cellular to the molecular.

The use of X-ray microanalysis with whole cell preparations is considered in reference to eukaryote (animal) cells and prokaryotes - where it has particular potential in environmental studies on bacteria. In the case of cell homogenates and biological fluids, the technique has been used mainly with microdroplets of animal material. The use of X-ray microanalysis with purified biochemicals is considered in relation to both particulate and non-particulate samples. In the latter category, the application of this technique for analysis of thin films of metalloprotein is particularly emphasised.

It is concluded that wider use could be made of the range of preparative techniques available - both within a particular investigation, and in diverse fields of study. Transmission X-ray microanalysis has implications for environmental, physiological and molecular biology as well as cell biology.

KEY WORDS: X-ray microanalysis, whole cells, bacteria, tissue fluids, metalloproteins, Deoxyribonucleic Acid.

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Introduction

The development of electron probe X-ray microanalysis by Castaing (1951) laid the foundation for the microscopical determination of elemental composition in a wide range of specimens in both materials and biological sciences. A major aspect of this technique lies in the ability to combine conventional microscopy with the analytical procedure, so that the position of the probe can be accurately determined.

In the field of biology the majority of microscopical studies have traditionally been carried out on sectioned material, and it is therefore not surprising that some of the earliest X-ray microanalytical investigations involved the use of this type of preparation. Brooks et al. (1962), for example, studied the distribution of Ca in the epiphyseal cartilage of sections of rat tibia, and Tousimis & Adler (1963) investigated the accumulation of Cu in sectioned material taken from patients suffering from Wilson's disease. In both of these studies the sections used were relatively thick (2 μm) and the position of the probe was determined by light microscopy. Early emphasis on the use of sectioned material for the biological application of transmission X-ray microanalysis has continued to the present day, with contemporary studies being carried out on fixed dehydrated material (Kearns & Sigee, 1980), freeze-dried resin-infiltrated cells (Pfaller & Rovan, 1978; Sigee & Kearns, 1982), freeze-substituted material (Harvey et al., 1976; Zingsheim, 1984), freeze-dried cryosections (Somlyo et al., 1981; Zingsheim, 1984) and fully hydrated cryosections (Saubermann & Echlin, 1975; Hall & Gupta, 1982). A recent review of this area has been published by Hall (1986).

Although the majority of studies have involved sectioned material, other types of specimen preparation have also been used. The object of this paper is to consider these alternative procedures, and to emphasise the diversity of techniques available in specimen preparation for X-ray microanalysis of biological material. In general, these procedures fall into three main categories - whole cell techniques, analysis of cell homogenates and biological fluids, and use of purified cell extracts - including isolated macromolecules and biochemical samples.

Whole Cell Preparations

Analysis of whole cells provides a rapid and very useful technique for the determination of total cell elemental levels, where the investigator is not concerned with the intracellular location of the elements under study. The technique has been used with both eukaryote and prokaryote cells -

Eukaryote cells

The use of X-ray microanalysis with whole eukaryote cells has been reviewed by Wroblewski and Roomans (1984) and has been applied particularly to mammalian cells - where it has been used both for cells that occur naturally in suspension (e.g. sperm cells, blood cells) and cultured cells derived from tissues. In practice, the cells are deposited onto an electron microscope grid, where they are analysed in an air-dried or freeze-dried state. Two particular problems that occur with this type of preparation relate to the size of the specimen and the presence of external medium.

For quantitative work, the continuum method can only be used where ZAF corrections are not applicable - with a specimen thickness limitation of the order of 5 μm (Hall, 1971), and where external medium deposited around cells does not contribute significantly to background. A large amount of extracellular medium may also be a major disadvantage if it covers the cells - since the cells will be difficult to see in the transmission image, and the increased continuum will reduce the concentration (and detectability) of cellular elements in the specimen. Removal of this medium by washing the grid in distilled water is difficult since it may displace cells from the surface, and resuspension of the cells in water prior to deposition may cause severe damage and elemental loss. In the case of lymphoma cells, for example, Hook et al. (1986) found that exposure to distilled water caused severe lysis within 5 min. This was avoided in this particular case by washing cells in pH neutral isotonic NH_4NO_3. The final procedures of cell collection and dehydration may also be important. The high g forces required to sediment larger cells in a mixed population may cause some cell damage and elemental loss (Hosseini et al., 1983) and it is important to restrict analysis to undamaged (healthy) cells. Air-drying on the electron microscope grid may also cause some elements to leak out of the cells (Moreton, 1981) - particularly diffusible elements and those located at the surface.

Quantitative analysis of whole cells, as with other biological specimens, is best carried out with standards of similar thickness and elemental composition to the specimen. In the analysis of red blood cells, for example, several workers have used similar cells of known elemental composition. This approach was first adopted by Roinel and Passow (1974), using standard cells of known K and Na concentration, and has been subsequently used by Lechene et al. (1977) and Zierold (1981). Elbers (1983) used a standard matrix of similar dry matter and elemental content to the egg cells being analysed, and Hook et al. (1986) used glass microsphere standards of similar size and average atomic number to the dried lymphocyte cells under analysis. Correlated atomic absorption spectrophotometry (e.g. Elbers, 1983; Hook et al., 1986) has generally shown that the use of such standards for X-ray microanalysis gives accurate mean values for whole cell elemental composition. In cases where absolute quantitation cannot be achieved, semi-quantitative data can be obtained by comparing elemental levels in the specimen relative to a reference element in the standard.

Prokaryote cells

Although a number of X-ray microanalytical studies have been carried out on frozen (Chang et al., 1986) or fixed, resin embedded (Scherer & Bochem, 1983; Sigee et al., 1985b) sections of bacterial cells, most work has been carried out on whole cells - either with the scanning electron microscope (Scherrer & Gerhardt, 1972; Ridgway et al., 1981; Richter & Banwart, 1982) or the transmission electron microscope.

Bacterial cells provide ideal specimens for transmission X-ray microanalysis. They are easily collected from suspension and deposited on electron microscope grids, and their high electron density and regular shape make them readily distinguishable - without the need for contrast enhancement. This is illustrated in Fig. 1, which shows the typical appearance of cells of Pseudomonas syringae - without any shadowing or negative staining. The small size of bacteria (cell diameter normally about 1 μm) qualifies them for analysis by the Hall (1971) continuum method.

A further major advantage of whole cell preparations is that the presence of soluble elements can be readily determined in chemically-untreated, air dried preparations - without the need for technically demanding freezing and other cryopreparative procedures that are required with sectioned material. Recent studies on laboratory-cultured bacteria (Sigee et al., 1985a; 1985b) have shown that chemical fixation and dehydration of bacteria leads to a major loss of soluble constituents and a marked change in the X-ray emission spectrum. This is shown in Fig. 2, where the air-dried cells have a major peak of K (the main detectable soluble cation) and Ca, but no other detectable cations, while chemically processed cells have lost their K peak, but have enhanced peaks of Ca and transition metals. In these chemically treated preparations, where elemental mass fractions can be defined as the mass of insoluble element per unit mass of insoluble matrix, the degree of extraction of soluble components depends on the particular treatment used (Sigee et al., 1985b). Elemental mass fractions in these preparations will therefore vary considerably in relation to the procedures used for cell processing, in contrast to air-dried cells - where mass fractions should have an absolute value. In spite of the uncertainties surrounding the quantitative data obtained from chemically-dehydrated specimens, this approach is useful for two main reasons - (1) It allows major soluble cations to be distinguished from insoluble

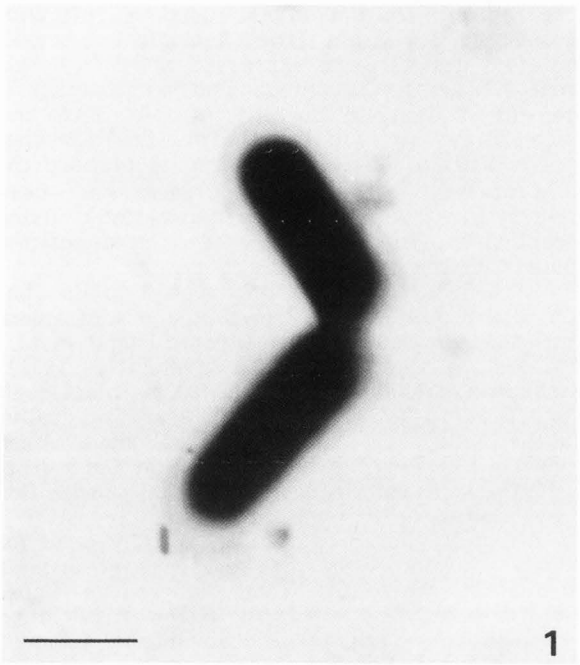

Fig. 1. Transmission electron micrograph of air-dried (unfixed) cells of Pseudomonas syringae. The electron dense cells are surrounded by an electron transparent capsule. Bar = 0.5 μm.

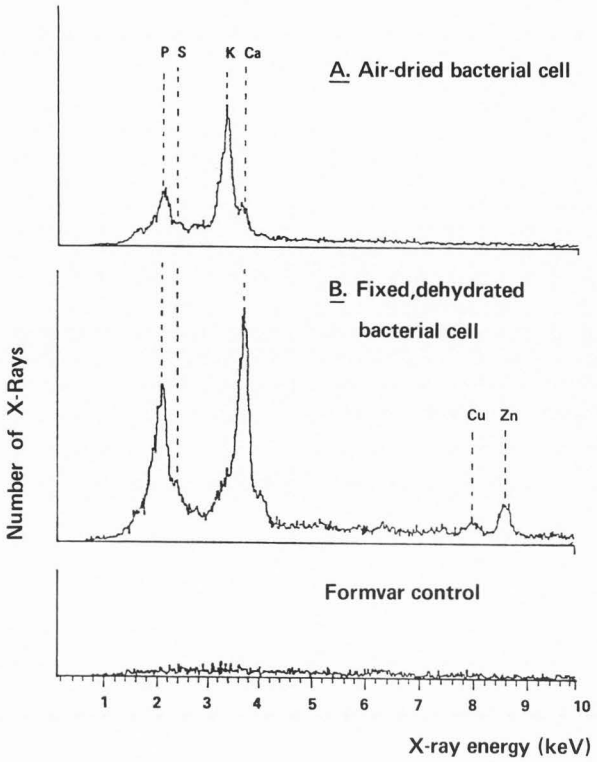

Fig. 2. X-ray emission spectra from whole bacterial cells. Spectra are from air-dried (A) and fixed, ethanol-dehydrated (B) cells of Pseudomonas syringae. The Formvar control is taken from an area of Formvar close to the cell analysed in A.

cations, (2) It increases the detectability of insoluble constituents, since the mass fractions of these components show an increase (due to loss of soluble matrix) and the concentration of the element per unit volume increases (due to cell shrinkage). As a result of this change in detectability, insoluble divalent cations, which are frequently not observed in fresh preparations (Fig. 2A) are now seen as clear peaks in the chemically processed cell spectrum (Fig. 2B).

The deposition of bacteria onto electron microscope grids is most effectively carried out on glow-discharged (hydrophilic) surfaces, and is a relatively rapid (1-2 min) operation. In the case of laboratory-cultured bacteria, as with cultured animal cells, it is important to remove the extraneous medium. This can be achieved by resuspension of bacteria in tap-water or distilled water prior to deposition on the grid. Care must be taken to avoid excessive washing, however, since this may lead to loss of soluble monovalent (Chang et al., 1986) and divalent (Al-Rabaee & Sigee, 1984) cations. Scintillation experiments on the uptake and retention of $^{63}Ni^{2+}$ by bacterial cells (Al-Rabaee & Sigee, 1984) have shown that there is a rapid loss of label with successive washes. This loss may be either of cell surface (adsorbed) cations, or by outward diffusion of internal soluble constituents. Where bacterial cells are not surrounded by concentrated medium, it is simpler to avoid washing and carry out deposition directly from the original medium. This approach was used in the analysis of bacteria from lake water (Booth et al., 1987) - where the lack of contamination from the lake water was shown by the absence of elemental peaks from probe sites of blank Formvar close to the bacterial cells.

The application of X-ray microanalysis to whole bacterial cells has particular potential in an environmental context. In this connection, studies have been carried out on marine fouling bacteria (Heldal et al., 1985), halophilic bacteria (Hovind-Hougen et al., 1981) and freshwater (lake) bacteria (Heldal & Tumyr, 1983; Booth et al., 1987). Studies by Booth et al. (1987) have shown that X-ray microanalysis can be used to detect a range of cations in freshwater bacteria, and that the level of cations in bacterial cells shows some relationship to the cation changes in the surrounding lake water. This fact, together with the ability of bacterial cells to concentrate cations to a high degree, suggests that X-ray microanalysis of bacteria from freshwater may provide a rapid monitoring technique to detect unusually high levels of cation pollutant in the environment. The effects of heavy metal pollution on the elemental composition of bacterial cells have been investigated in the laboratory by Sigee & Al-Rabaee (1986) using nickel as the pollutant cation. In this study, the highest internal levels

of nickel occurred in non-toxic rather than toxic medium, suggesting that nickel toxicity does not primarily relate to internal concentration. The results also showed that toxic levels of nickel in the external medium result in a range of ionic changes in the bacterial cells - involving K, Mn, Fe and Cu as well as Ni. The experimental data were consistent with a primary toxic effect of nickel at the cell surface - possibly affecting the transport activity of the plasmalemma - resulting in an efflux of K and secondary changes in the internal levels of divalent cations. The ability of X-ray microanalysis to monitor a broad range of elements (within a single spectrum) is particularly useful in an experimental situation such as this, where a single external effect can induce a whole sequence of internal cell changes.

Cell homogenates and biological fluids

X-ray microanalysis of cell homogenates and biological fluids is typically carried out as a microdroplet technique - and provides a close analogy to whole cell microanalysis, since in both cases a discrete mass of heterogeneous material is being analysed within the probe area. The use of X-ray microanalysis for elemental determination of small liquid samples was first proposed by Ingram & Hogben (1967), and has recently been reviewed by Roinel & de Rouffignac (1982) and Hyatt & Marshall (1985).

The microdroplets may be deposited either singly - using a constant volume picolitre (Garland et al., 1973; Roinel, 1975) or as a number of discrete droplets on a grid surface using a nebulizer (Morgan et al., 1975; Marshall, 1977).

The major advantage of single droplets is that very small amounts of liquid can be analysed, so that elemental composition of samples below the volume required for conventional bulk analysis can be determined. The use of a constant volume picolitre pipette permits deposition of sub-nanolitre volumes, which are then lyophilised (Roinel, 1975) and analysed as a circular dried deposit. A 0.3 nl microdroplet gives a circular deposit size of about 50-80 μm diameter, the whole of which is irradiated by the electron beam during analysis (Roinel, 1981). The method of sample dehydration is important in producing crystals of minimal size within the droplet - to avoid electron and X-ray absorption, and achieve proportionality between X-ray intensity and elemental concentration. Under adverse dehydration conditions, where larger (2-6 μm) crystals are formed, proportionality between X-ray intensity and elemental concentration can only be achieved by increasing the accelerating voltage - which reduces the sensitivity of the technique (Morel & Roinel, 1969). For quantitative information to be obtained from specimen microdroplets, standard microdroplets are normally prepared, analysed, and standard elemental curves constructed (Roinel, 1981). An alternative procedure to using reference microdroplet standards is to use internal standards within the specimen microdroplet. Morgan (1983) used a reference element (Co) within the biological sample, permitting results to be expressed as mM/l. This approach has the advantage that quantitation is independent of beam current, droplet size and duration of count, but has the disadvantage that large volumes of fluid are required to prepare the microdroplets. A similar approach has been adopted by Hyatt and Marshall (1985) using micropipettes, but without the need for depositing constant volume specimens.

X-ray microanalysis of microdroplets has been widely used for electrolyte analysis of animal exudates - including renal fluids (Garland et al., 1973; Greger et al., 1978; Roinel, 1981), haemolymph (Weidler & Sieck, 1977), blastocoele fluid (Borland et al., 1977), sweat and saliva (Quinton, 1978). Fewer studies have been carried out on plant exudates, though Gartner et al. (1984) have examined the elemental composition of xylem exudate with this technique.

Although microdroplets are typically used for analysis of electrolytes, the presence of organic components should not be ignored. These constituents may be important in the stabilisation of more volatile elements such as chlorine (Roinel, 1975; LeRoy & Roinel, 1982), and the presence of high levels of protein may interfere with crystal formation and may also result in high absorption of X-rays (Bonventre et al., 1980). Where an organic matrix is present, mass loss from this part of the specimen may be much greater than from the crystalloid component (Rick et al., 1977).

Purified biochemicals

X-ray microanalysis of pure biochemicals represents a logical extension from the previous section, where complex mixtures of biologically important chemicals were being analysed. In this situation, the heterogeneity of the specimen required that a relatively large area (the whole of the microdroplet) was used for microanalysis. In the case of the purified biochemical sample, where the specimen is essentially homogeneous, a much smaller probe area can be used.

Analysis of purified samples involves two major types of specimen - particulate samples (comprising discrete molecules or molecular complexes) and non-particulate samples.

Particulate samples

In the case of high molecular weight specimens, the molecules (or molecular complexes) may form a clearly visible structure under the transmission electron microscope - which can be directly analysed by micro- or nanoprobe.

In some cases the visibility of the molecule is determined by a specific electron-dense component. In the case of ferritin, Shuman & Somlyo (1976) were able to visualise individual molecules on the electron microscope grid due to the presence of an electron-dense Fe core - and to carry out microanalysis. The main purpose of this study was to use the molecular sample as a

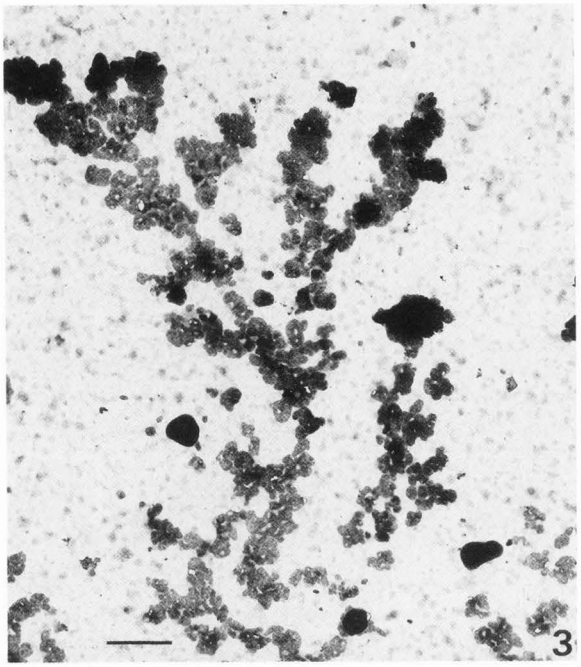

Fig. 3. Transmission electron micrograph of DNA sample extracted from Pseudomonas syringae, deposited on hydrophilic Formvar grid, and stained with osmium tetroxide. The preparation shows highly condensed molecular supercoils. Bar = 1 μm.

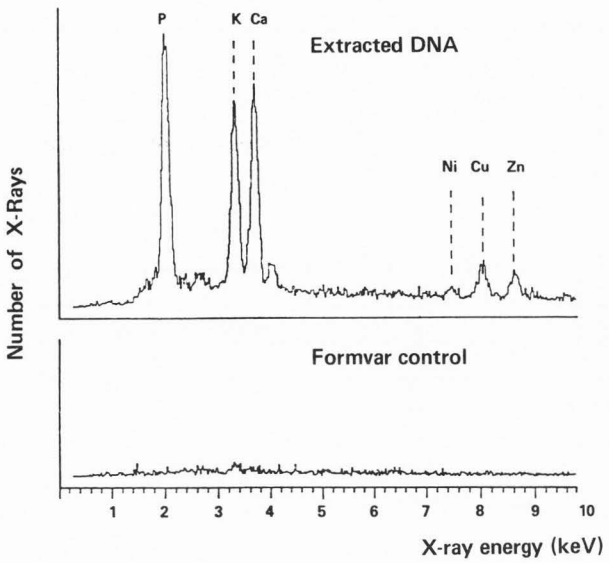

Fig. 4. X-ray emission spectrum from extracted DNA. The spectrum is derived from an electron dense supercoiled region of the molecule. The Formvar control spectrum is taken from an unused grid derived from the same batch as used for the extracted DNA.

standard for the determination of minimal detectable mass. Significant Fe K_{α} peaks were generated from single molecules over a 100 sec count time, using a probe current of 0.35 nA with a spot size of 60 nm diameter. The results were consistent with a minimal detectable mass of 0.9×10^{-19} g of Fe under the experimental conditions specified.

In other cases, molecules can be visualised due to their overall electron density rather than a specified spatial component. This applies to the electron microscope observation and analysis of bacterial DNA (reported in Sigee et al., 1985b), where extracted purified nucleic can be isolated as intact supercoiled molecules, and deposited and analysed on a Formvar coated grid. The DNA molecules typically occur as large, highly condensed structures - possibly corresponding to individual bacterial nucleoids or nucleoid aggregates. Detail from one of these structures is shown in Fig. 3. Although the contrast in this particular preparation was enhanced by treatment with OsO_4, sufficient detail could be seen in unstained preparations to position the probe for X-ray microanalysis. The emission spectra derived from these preparations showed clear peaks of monovalent and divalent cations (Fig. 4). The presence of K as a bound cation is of particular interest, since analysis of whole cells had suggested that this is generally present as a soluble cell component.

Non-particulate samples

With the majority of purified biochemical samples individual molecules cannot be seen as discrete structures - due to limitations of size, specimen contrast or loss of structure during isolation and processing.

Protein films constitute such a non-particulate specimen, and have been the subject ot particular interest - largely in relation to the preparation of standards for transmission microanalysis. Eshel (1974) has considered the theoretical aspects of sample composition and film thickness on corrections for backscatter, electron penetration and X-ray absorption. His calculations, based on a hypothetical sample with a composition similar to gelatin, have shown that small changes in either thickness or density of the protein film can lead to several-fold changes in X-ray yield.

In practice, protein films have normally been prepared by freezing solutions of protein or protein mixtures, cutting ultrathin sections, and analysing these in the frozen or hydrated state. Hagler (1983), for example, prepared cryosections of gelatin-glycerol-salt mixtures as standards for cryoultramicrotomy. An alternative approach to cryosections is to deposit protein films by evaporation from solution. Maroudas (1972) prepared thin films of protein (with $CaSO_4$) by freeze drying on quartz slides - while other investigators have carried out deposition directly onto electron microscope grids. Transmission microanalysis has been carried out by Shuman et al. (1976) on bovine serum albumin, bovine insulin and phosvitin - using either thin crystals or thin evaporated layers. Serum albumin films

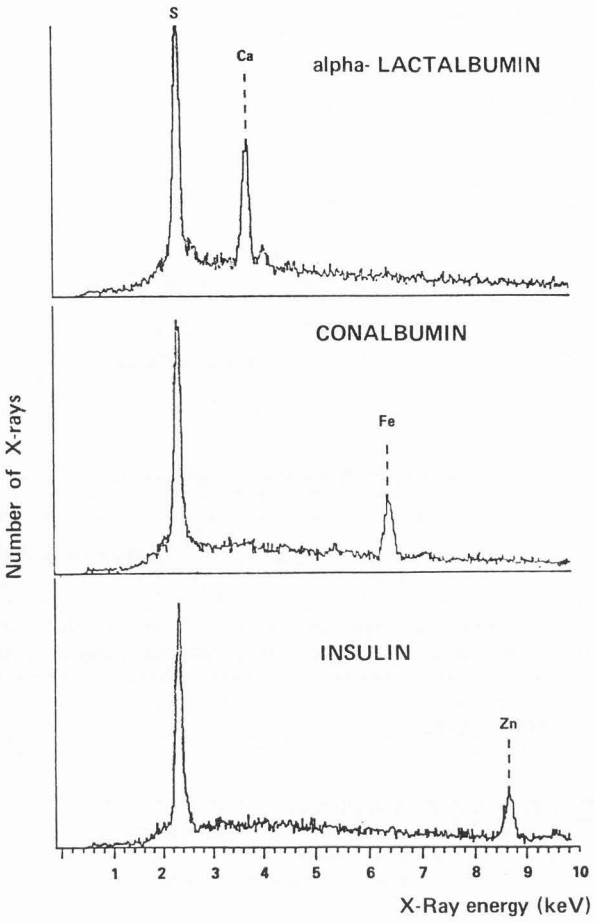

Fig. 5. X-ray emission spectra from metalloproteins. Spectra are derived from ultrathin layers of protein, deposited on an electron microscope grid by air-drying from solution. The samples shown here contain only the major (structurally-bound) cation.

showed a mass loss of 13% at electron doses up to 760 C/cm^2, which compares with previous estimates of mass loss from protein films of 15% (Stenn & Bahr, 1970) and also compares with a mass loss of about 50% from a layer of sucrose at 0.03 C/cm^2. Shuman et al. (1976) pointed out that the proteins contained stoichiometric levels of S (albumin, insulin) or P (phosvitin) which could be adjusted in concentration by altering the proportion of different proteins in mixtures.

The use of metalloprotein films as standards for transmission X-ray microanalysis has also recently been considered by El-Masry and Sigee (1986). Specific metalloproteins typically contain one major cation structurally bound within the polypeptide chain, plus, in some cases, electrostatically bound ions - depending on the purity of the sample. Where only the structurally bound cation is present, as in the samples analysed of lactalbumin, conalbumin and insulin (Fig. 5) X-ray emission spectra show a single

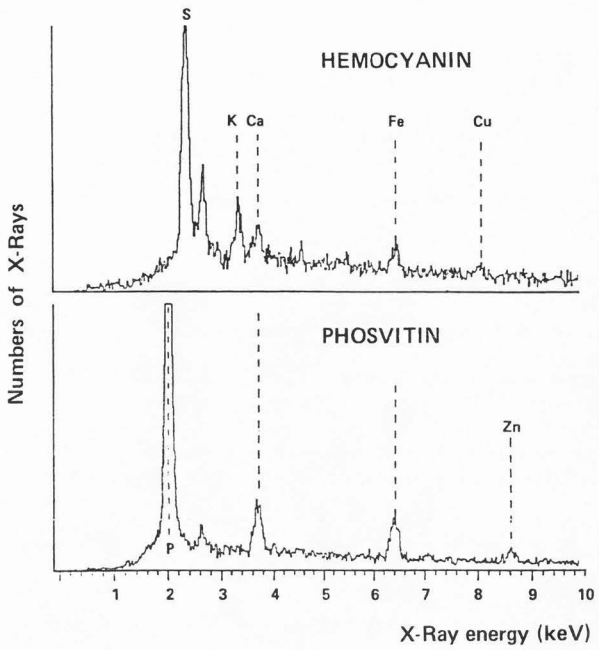

Fig. 6. Emission spectra from metalloproteins. Legend as in Fig. 5, except that electrostatically bound cations are present in addition to the structurally bound cations of hemocyanin (Cu) and phosvitin (Fe).

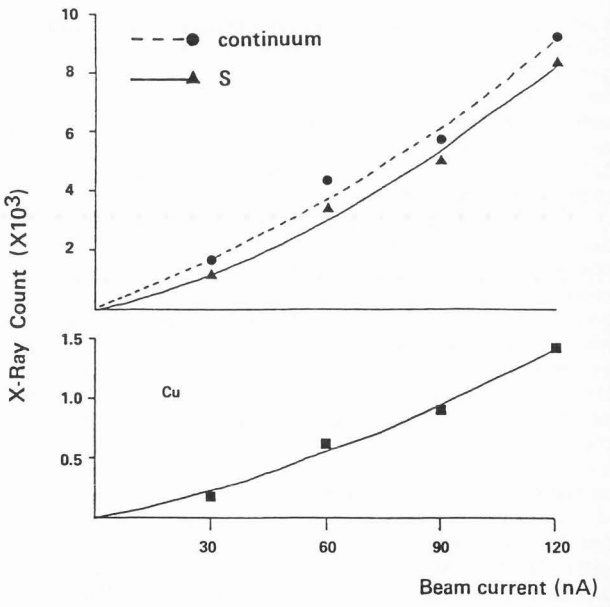

Fig. 7. Electron dose - X-ray emission characteristics of hemocyanin. Mean X-ray counts, derived from 15 spectra, are given for continuum (15-20 keV) and characteristic Kα peaks of S and Cu. Spectra are taken from a standard probe area (diameter 0.5 μm) at beam currents of 30-120 nA.

peak in addition to sulphur. Where electrostatically bound cations are also present, as in the samples analysed of hemocyanin and phosvitin (Fig. 6), the spectrum appears much more complex.

Two very important aspects of these metalloprotein films - in terms of their suitability both as test specimens and standards - are their stability under the electron beam and their homogeneity. Within the statistical limits of our experiments the metalloprotein films appeared to be relatively stable up to a beam current of 120 nA. This is shown in Fig. 7, for hemocyanin, where there was no detectable fall off in proportionality of X-ray emission with electron dose. The converse effect shown by the data (i.e., an apparent over-proportional rise in X-ray emission with dose) may relate to contamination of the specimen (in the case of continuum) or re-distribution of metalloprotein in constituents under the beam (in the case of S and Cu) - as suggested by Shuman et al. (1976). The absence of any detectable differential loss of elements over a range of beam currents is also indicated by a constancy (within experimental limits) of the ratios of elemental characteristic/continuum counts (El-Masry & Sigee, 1986).

Metalloprotein films appear fine structurally homogeneous under the transmission electron microscope - unlike evaporated salt-protein mixtures (El-Masry & Sigee, 1986). This homogeneity at the micro-level is also indicated by the X-ray emission data, where there is a significant (99.5%) correlation between elemental characteristic counts and continuum. In a more sensitive test for sample homogeneity, the correlation between characteristic counts of cations and S or P (phosvitin) also proved highly significant (at 97.5-99.5% level) over the complete range of metalloproteins examined. In this test, the level of S or P provides a reference for the total level of polypeptide present in the sample.

As potential standards for transmission analysis, evaporated metalloprotein films have the advantage of ease of preparation, ability for independent analysis and suitable features of stability and homogeneity under the electron beam. They also have the advantage that they typically resemble the chemical composition of the biological specimen under investigation. Metalloprotein films may either be used as primary standards (in the direct determination of specimen mass fractions) or as secondary standards (corroborating a system of quantitation using other primary standards). In the studies of El-Masry and Sigee (1986) metalloprotein cation mass fractions determined using inorganic microcrystals as primary standards showed close agreement with values obtained by atomic absorption spectrophotometric analysis of bulk metalloprotein samples.

Although this section on the application of transmission analysis to purified biochemical samples has considered mainly protein films, other types of thin layer samples have also been investigated. The work of Shuman et al. (1976) on sucrose has already been mentioned, and recent studies on thin layers of synthetic chemicals also have considerable potential. An example of this is the work of Lupton and Saubermann (1986) using aminoplastic salt mixtures.

General Conclusions

Although sections of biological material have traditionally provided the major type of preparation for transmission X-ray microanalysis, other types of preparation are available. This review considers three major types of alternative, and gives a broad overview of the potential and applications that they provide.

The diversity of techniques available suggests that a range of approaches can often be usefully considered with any one type of material or within a single research project. The review also suggests that transmission X-ray microanalysis may be used in very diverse types of investigation, and has implications for environmental, physiological and molecular biology as well as cell biology.

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Discussion with Reviewers

A. Warley: The author mentions the washing of bacterial cells with tap water. Does this introduce elements into the spectrum?

Author: Emission spectra from bacteria washed in tap water do not differ from those of bacteria washed in distilled water, suggesting that tap water does not introduce detectable levels of elements.

A. Warley: What precautions does the author take to ensure that chemical treatment does not introduce contaminants when chemically fixed bacteria are examined?

Author: I am very concerned that the use of chemical treatment carries with it the potential danger of introduction of contaminants. The precautions that we take are to use only ultra-clean glassware and to use contaminant-free processing solutions. We have tested for the presence of contaminant transition metals in the aldehyde fixing solution and ethanol dehydration series, and have not been able to detect their presence at limits of detectability of 1 ppm. Various lines of evidence also suggest that detected cations in chemically processed samples are not simply derived as contaminants from processing solutions. The detection of divalent cations in chemically processed bacterial cells relates directly to the loss of soluble constituents and not to the nature of the processing solutions (Sigee et al., Scanning Electron Microscopy/1985/ III/pp 1151-1163). Previous studies with dinoflagellate cells (Sigee & Kearns, 1982, Cytobios 33, 51-64) showed that prolonged fixation in glutaraldehyde resulted in a progressive loss of divalent cations - which would not be expected if this were the source of contaminants.

N. Roinel: Could you comment on the possibility that 'soluble' cations diffuse out of the cells by leakage during the air-drying process?

Author: Evidence suggests that there is minimal leakage of diffusible cations during the air-drying process. This is indicated by the fact that background spectra taken from formvar in the region of the cells have no cation peaks - which would be expected if leakage had taken place. The high level of retention of soluble elements by the cells would tend to corroborate this.

N. Roinel: Could you comment upon the magnitude of sample volumes required by the different types of preparation for analysis considered in your paper?

Author: With each type of specimen, two volumes are important - the volume of sample used to load onto the electron microscope grid, and the volume of specimen irradiated by the electron probe. In whole cell preparations, the volume of suspension placed on a single grid would be about 0.2 µl (10^{-7} l), while the volume of individual cells would range from about 1 µm³ (10^{-15} l) in the case of bacteria to higher levels in the eukaryote range. The volume of individual lymphocyte cells, for example, has been estimated (Hook et al., 1986) at 555 µm³ (10^{-12} l). With analysis of biological fluids, the use of individual microdroplets permits sample volumes down to about 0.07 nl (10^{-11} l), and the dehydrated volume irradiated by the beam will be considerably less than this. In the case of spray preparations of biological fluids, the minimum capacity of the nebulizer has been quoted (Morgan et al., 1975) as 2 ml (10^{-3} l), and the size of individual droplets at about 10 µm³ (10^{-14} l). With purified biochemicals, such as metalloproteins, sample volumes of about 0.2 µl (10^{-7} l) are normally applied to the electron microscope grid, though micropipette volumes (10^{-11} l) could be used. Analysis of metalloprotein films with an estimated thickness of 0.1 µm and a probe diameter of 0.3 µm, would involve a directly irradiated volume of specimen of about 0.007 µm³ (10^{-17} l). The use of a 60 nm probe, such as that used for analysis of individual molecules of ferritin (Shuman & Somlyo, 1976) would take the irradiated volume down to about 0.001 µm³ (10^{-18} l).

Reviewer 3: Fig. 2A/2B is difficult to understand. Was the same scale used?

Author: The same scale was used for 2B and the Formvar control, but the vertical scale of 2A was slightly reduced to include the full P peak.

Reviewer 3: I cannot agree with the argument of the author that fixation would make Zn detectable. Let us assume that we have a tissue containing 0.05% Zn, which would be at the limit of detectability under standard conditions. Let us further assume that the tissue contains 3% P and 3% K, which leaves 94% for the organic matrix. After fixation, we have lost all K and nearly all P, so we have 1% P and 99% matrix. Because of the loss of P and K, the Zn concentration has increased to 0.0525%. In addition, we may expect a decrease in the background because of a decrease in mean atomic number. In the unfixed specimen, $Z^2/A = 3.46$,

whereas in the fixed tissue, the value has decreased to 3.19. Together with the 5% increase in Zn concentration, this 10% decrease in background will result in a 15% increase of the P/B ratio of Zn. This is not sufficient to explain the effects seen by the author.

Author: The increased detectability of Zn (and other transition metals) due to fixation is a direct observation rather than an argument. The most likely explanation for this, and the one that is proposed here, is that the increase in detectability arises due to an increase in the mass fraction of the element originally present in the specimen. This explanation assumes that the zinc is present largely in an insoluble form, and that the soluble constituents lost during fixation include a high proportion of the protein matrix. This situation might arise if the Zn was largely bound to DNA, for example, or if it were present mainly in an inorganic form. The figures quoted by the reviewer do not take matrix loss into account. If half of the 94% matrix were lost, then the level of Zn would increase to 0.106% - which would bring it into the realm of detectability. We do not propose that the increased detectability of certain elements noted in these laboratory-cultured bacteria after cell processing is a universal phenomenon. Similar procedures with freshwater bacteria (Booth et al., 1987), for example, do not give a consistent increase in transition metal detectability.