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THE ELECTRON MICROSCOPE DETECTION AND X-RAY QUANTITATION OF CATIONS IN BACTERIAL CELLS

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Abstract

Electron microscope autoradiography and X-ray microanalysis have been used for the detection and quantitation of cations in the bacterium Pseudomonas tabaci. These techniques differ in the information they provide (relating to either cation uptake or in situ levels), their applicability to different cations, their sensitivity and their spatial resolution. With uptake of $^{63}Ni^{2+}$, high resolution autoradiography (involving gold latensification and physical development) demonstrated a high degree of cation localisation to the central nucleoid area (glutaraldehyde-fixed cells) and within this to the constituent chromatin (acetic-alcohol preparations).

X-ray microanalysis of whole bacterial cells revealed the presence of substantial levels of K (mainly soluble cation), Ca, Mn, Ni, Cu and Zn (mainly insoluble cations) and Fe (present as major soluble and insoluble components). The use of whole cells provided a particularly useful experimental system to demonstrate the importance of cell preparation technique in relation to element detectability. The application of X-ray microanalysis to lysed cells permitted analysis of extruded contents - including cell protoplast (protoplasm without associated cell wall material) and chromatin fibrils. The microprobe detection of DNA-associated cations was most effective with freshly extracted chromatin and showed the presence of bound K, Ca and transition metals.

KEY WORDS: Autoradiography, ^{63}Nickel, X-ray microanalysis, transition metals, bacteria.

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Introduction

In recent years there has been an increased interest in the general occurrence and importance of cations in the physical and biological environment. This has been promoted in part, at least, by international concern over problems of acid rain (Babich and Stotzky, 1982) and other forms of pollution. This increased interest has been paralleled by the need to understand more about the biological role of these molecules and the need to develop improved methods of detection in biological tissue.

In the case of bacteria a substantial body of experimental work has now demonstrated that cations are important both as essential requirements (eg. Tabillion et al., 1980) and potential toxins (eg. Blundell and Wild, 1969). As essential micronutrients they are required for a number of specific metabolic processes. Nickel cations, for example, are required for the synthesis of various enzymes - including hydrogenase (Pedrosa and Yates, 1983) and carbon monoxide dehydrogenase (Hammel et al., 1984). In addition to such specific biochemical roles, cations are also important in bacterial cell surface activity (Haavik, 1976; Jarrell and Sprott, 1982) and are important structural components of macromolecular complexes such as ribosomes (Tal, 1969) and DNA (Eichhorn, 1962).

Against this background of general metabolic information, there have been various studies on the detection and quantitation of cations in bacteria, involving techniques such as scintillation counting (Diekert et al., 1979, 1980) and atomic absorption spectrophotometry (Wirsen, 1966; Webb, 1970). These techniques give information on the general levels of cations within bacterial populations, but give little information about the location of molecules within cells.

The object of this paper is to describe recent electron microscope studies carried out at Manchester on the uptake and occurrence of cations in bacterial cells using autoradiography and X-ray microanalysis. These techniques have been carried out on Pseudomonas tabaci, and a description of the analytical work involved will be preceded by a brief account of the culture and fine structure of this bacterium.

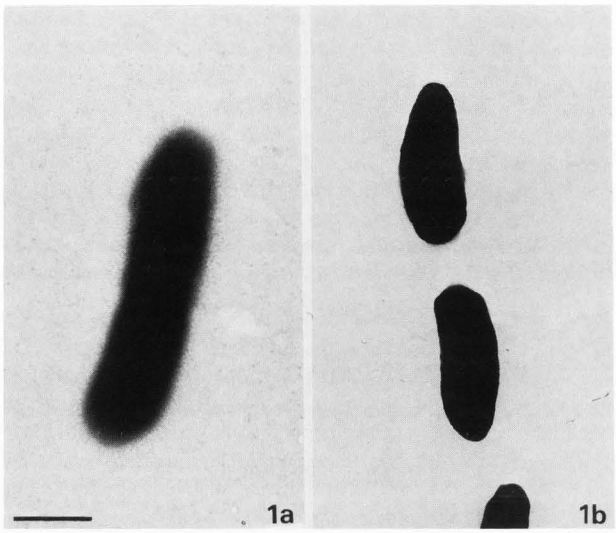


Fig. 1: Transmission electron micrographs of whole cells of Pseudomonas tabaci. (Bar = 0.5µm.
(1a) Air-dried unfixed cell. The edge of the cell appears diffuse due to a covering of fine pili.
(1b) Ethanol-dehydrated glutaraldehyde-fixed cells. These have lost the coat of pili and have a considerably reduced volume compared to 1a.

Culture and fine structure of Pseudomonas tabaci

Materials and Methods

Cultures of Pseudomonas tabaci, originally obtained from Professor R.N. Goodman, University of Missouri, were grown in Oxoid nutrient broth in a shaker at 28°C to log phase. 1 ml of bacterial culture was removed, added to 99 ml of fresh medium, and subcultured to a second log phase - as determined by making direct counts using a haemocytometer. Samples from this final culture were used for all experiments and preparations (Fig. 3).

Preparation for microscopy. Whole cell preparations were made of (a) untreated cells, and (b) chemically-processed cells, as follows. Log phase bacterial cells were collected by centrifugation, then either - (a) twice resuspended in distilled water, and a drop of bacterial suspension placed on a carbon-formvar grid which was air-dried at room temperature, or (b) resuspended in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2h at 20°C, washed in cacodylate buffer followed by distilled water then dehydrated in an ethanol series. Chemically-processed cells were then rehydrated to 25% ethanol and deposited on an EM grid as previously.

Fixed, embedded cells were prepared by 2h fixation in either buffered glutaraldehyde or acetic-alcohol (1 part acetic acid, 3 parts ethanol), followed by ethanol dehydration and embedding in Spurr resin. Sections were cut at 60 nm thickness and stained in alkaline lead citrate for direct observation (unstained for X-ray microanalysis).

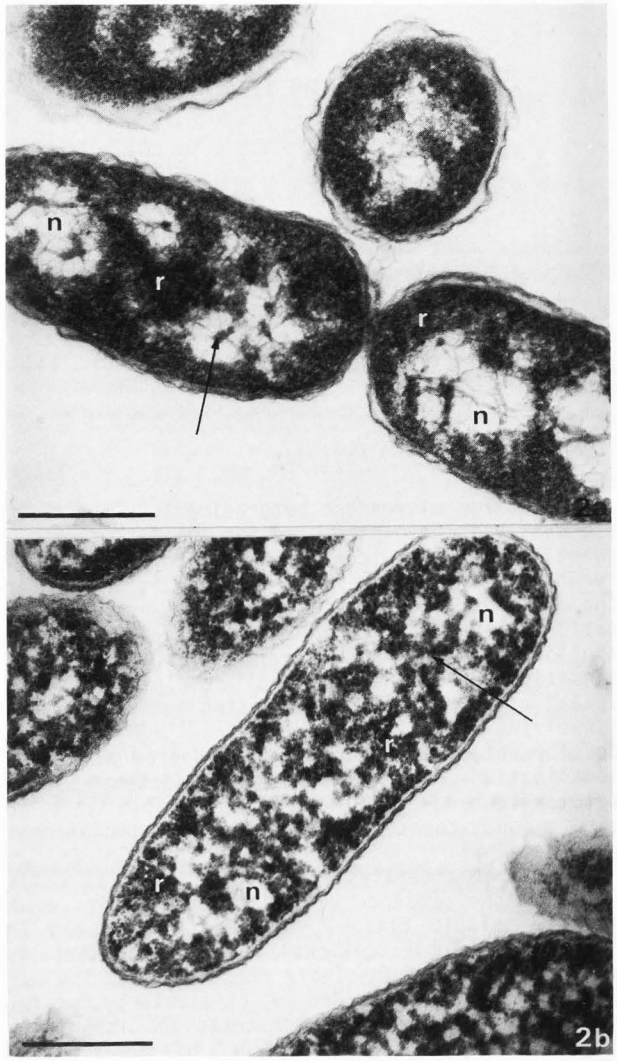


Fig. 2: Ultrathin sections of Pseudomonas tabaci, stained in alkaline lead citrate and uranyl acetate. (Bars = 0.5µm).
(2a) Glutaraldehyde fixation. The cells contain large central nucleoid areas (n) containing strands of chromatin (arrow), surrounded by dense ribosomal protoplasm (r).
(2b) Acetic alcohol fixation. The nucleoid (n) appears as a dispersed central electron-transparent region and contains indistinct regions of coagulated chromatin (arrow).

Results

Whole bacterial cells appeared as electron-dense rods by transmission electron microscopy. Fixed, ethanol-dehydrated cells (Fig. 1b) differed from fresh air-dried cells (Fig. 1a) in lacking peripheral hairs (pili) and in being considerably smaller in size.

In ultrathin section, detailed fine structure

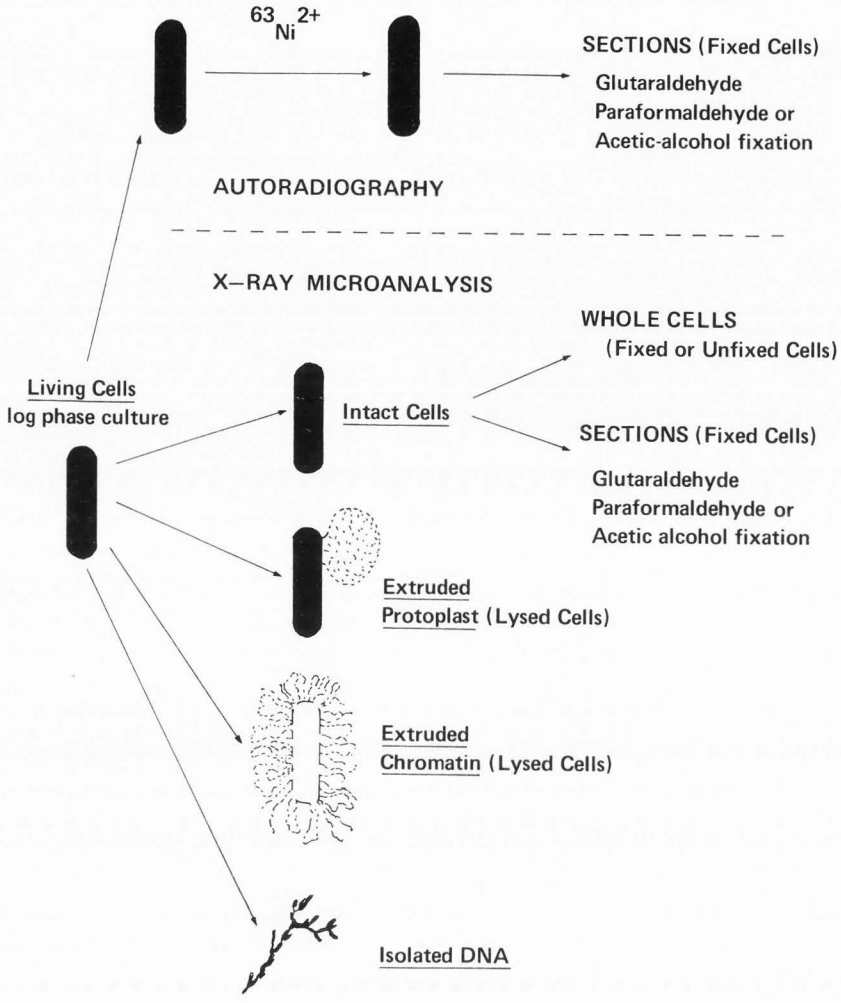


Fig. 3 Electron Microscope Detection of Cations in Pseudomonas tabaci

Fig. 3: Electron microscope detection of cations in Pseudomonas tabaci. Summary of experimental approaches adopted. These E.M. investigations have been supported by $^{63}Ni^{2+}$ scintillation counting (Al-Rabaee and Sigee, 1984) and preliminary work involving atomic absorption spectrophotometry.

showed considerable variation between cells prepared by additive (aldehyde) and coagulative (acetic-alchol) fixation. In the first case (Fig. 2a) the bacteria had clear central nucleoids containing strands of precipitated chromatin. In the second case (Fig. 2b) the electon-transparent nucleoid appeared more dispersed and contained chromatin condensed as diffuse masses rather than coarse strands. Comparison with the appearance of nucleoids in living cells (Binnerts et al., 1982) suggests that aldehyde fixation gives a more authentic preservation of fine structure.

The detection and localisation of cations

The various experimental approaches used in the application of autoradiography and X-ray microanalysis are summarised in Fig. 3. Analysis of material involved the use of whole cells, cell sections, lysed cells and extracted macro molecules. A feature of particular interest which became apparent as the investigations proceeded was the localised association of bound cations with bacterial chromatin - and specifically with bacterial DNA.

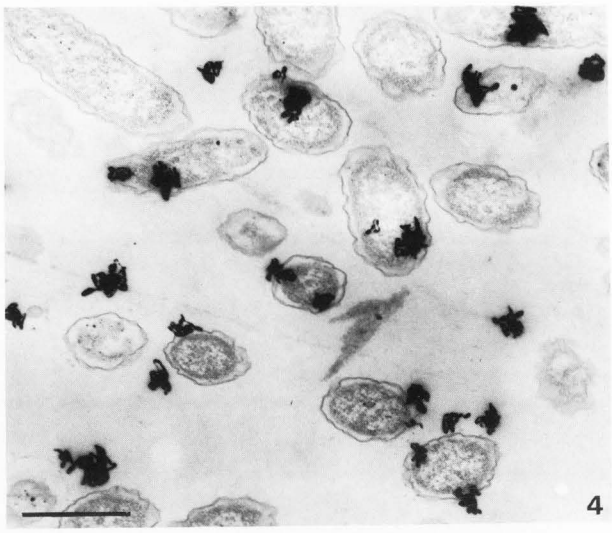


Fig. 4: E.M. autoradiograph of 2h $^{63}Ni^{2+}$-labelled bacterial cells fixed in paraformaldehyde and processed in D-19 developer. (Bar = 1μm).

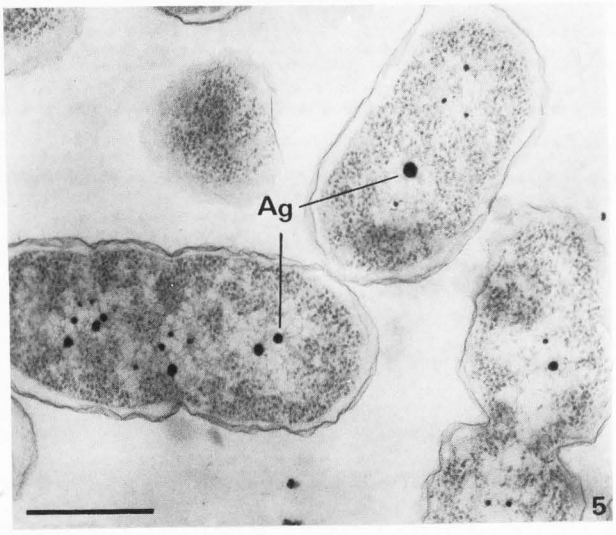
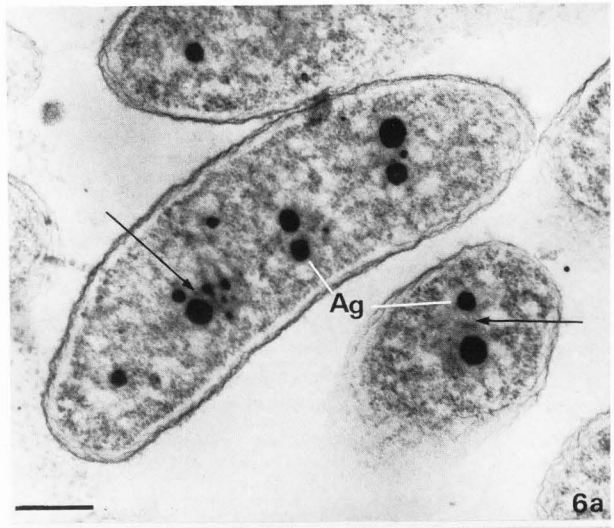


Fig. 5: E.M. autoradiograph of 2h $^{63}Ni^{2+}$-labelled cells fixed in glutaraldehyde and processed by gold latensification/physical development. Unstained preparation. The fine silver grains (Ag) are localised to the central nucleoid region. (Bar = 0.5μm).



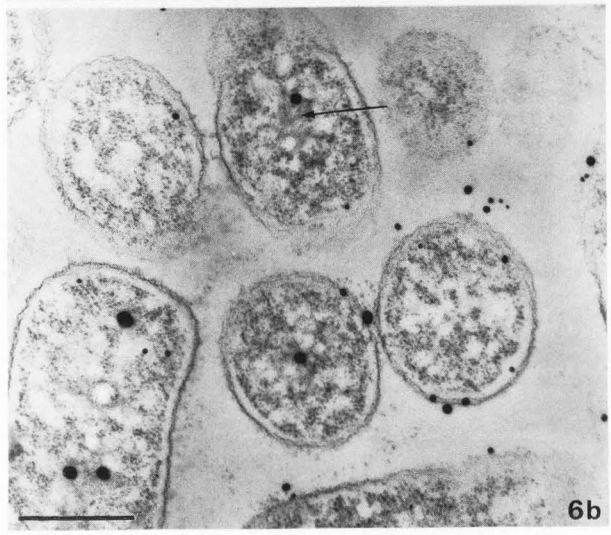


Fig. 6: E.M. autoradiographs of acetic-alcohol fixed cells, labelled and processed as in Fig. 5. Stained preparation.
(6a) High resolution (monolayer) autoradiograph, showing localisation of silver grains (Ag) to regions of condensed chromatin (arrows). (Bar = 0.25μm).
(6b) Low resolution region of the preparation, where the covering emulsion has exceeded a monolayer over the section. Fine silver grains are scattered over cells and surrounding resin, with little clear localisation to areas of condensed chromatin (arrow). (Bar = 0.5μm).

Autoradiography

This technique can be used for particular cations, where there is an isotope available of sufficient half-life and with low energy β-emission. Two such isotopes - $^{45}Ca^{2+}$ (half-life 164d, mean energy of emission 254 keV) and $^{63}Ni^{2+}$ half-life 92y, mean energy 67 keV) have recently been used in the study of cation uptake in dinoflagellate cells (Sigee 1982, 1983a) and uptake of $^{63}Ni^{2+}$ has been studied in bacteria (Al-Rabaee and Sigee, 1984). There appear to be no other reported studies on the autoradiographic localisation of radio-cations in bacteria, and the results reported here relate directly to the Al-Rabaee and Sigee investigation.

Materials and Methods

Full details on the labelling of bacteria with radioactive nickel and monitoring of isotope

uptake by scintillation counting and autoradiography are given by Al-Rabaee and Sigee (1984).

Bacterial labelling was carried out by addition of 10 ml of $^{63}Ni^{2+}$ (Amersham International Ltd., Amersham, U.K.) to 50 ml of bacterial culture (early log-phase) to give an overall concentration of 8.3 µCi/ml. For autoradiography, bacteria were sampled after a period of 2h, washed in sodium cacodylate buffer, then fixed in glutaraldehyde, paraformaldehyde or acetic-alcohol prior to ethanol-dehydration, resin embedding and sectioning.

Electron microscope autoradiographs were prepared by coating ultrathin sections with a monolayer of Ilford L4 nuclear research emulsion, using a loop technique. Preparations were left for a period of 6 months, then processed using either conventional (D19) development - followed by fixation in 10% Hypam, or using fine-grain physical developer (paraphenylenediamine) preceded by gold latensification, according to the method of Salpeter and Bachmann (1964).

Results

Both light and electron microscope autoradiographs showed heavy labelling, indicating an active uptake and high retention of label by the cells. High frequencies of silver grains were present over both acetic alcohol-fixed and aldehyde-fixed (glutaraldehyde, paraformaldehyde) preparations, indicating that retention of insoluble (bound) label could not be attributed to the type of specific fixation artefact demonstrated by Peters and Ashley (1967) in relation to amino acid uptake.

The appearance of a typical electron microscope preparation, processed using D19 developer, is shown in Fig. 4. Although there is clear association of silver grains with bacterial profiles, the large size (diameter about 0.3 µm) and scatter of silver grains limits any possible demonstration of internal localisation.

The use of physical development following gold latensification leads to much smaller silver grains (mean diameter about 0.04 µm) and much clearer localisation. This is shown in Fig. 5, where the fine silver grains are entirely located in the central nucleoid area of the glutaraldehyde-fixed cell. In acetic alcohol-fixed cells (Fig. 6a), the silver grains are localised to regions of condensed chromatin in the centre of the cell. In both types of preparation, silver grains are largely absent from ribosomal groundplasm and cell wall. The use of two types of fixation thus provides complementary information, demonstrating that incorporated bound nickel is localised almost entirely to the nucleoid area, and within this to the chromatin.

The high degree of localisation seen in physically-developed EM autoradiographs is dependent on the initial presence of a monolayer of silver halide crystals over the ultrathin section. Fig. 6b is taken from an area of preparation where this was not the case, and the fine silver rains appear randomly scattered over cell profiles and into the surrounding resin.

These autoradiographic studies give information about the location of insoluble radioactive nickel that remains after extraction of soluble radio-isotope during cell preparation. Parallel studies using scintillation counting from whole-cell samples (Al-Rabaee and Sigee, 1984) show that approximately 50% of the nickel incorporated into living cells is lost during washing, 30% during ethanol dehydration and only about 20% is retained in the final preparation.

X-ray Microanalysis

In contrast to autoradiography, which has been used to determine the level of cation uptake, X-ray microanalysis gives information on the total in situ level of a particular element (cation). There has been little previous work on X-ray microanalysis of bacteria, though Ridgway et al. (1981) have carried out SEM-associated studies on Gallionella, and Sigee et al. (1985) have reported preliminary results from studies carried out on Pseudomonas tabaci.

Materials and Methods

Microscopy Energy-dispersive X-ray microanalysis was carried out using an AEI (Kratos) Corinth analytical microscope (CORA), fitted with a Kevex detector and Link 860 analyser (Link Systems Ltd., High Wycombe, U.K.). Specimens were routinely examined at a magnification of 20K, accelerating voltage 60kV, using a probe spot with an area diameter of 0.3 µm. Beam current was normally adjusted to 60 nA, using a Faraday Cage, and counts made over a livetime of 200 sec.. Calculation of mass fractions was carried out using a computer program based on Hall (1971) quantitation for ultrathin specimens, and contained spectral information derived from crystal standards.

Preparation of material As outlined in Fig. 3, X-ray microanalysis was carried out on three main types of material - intact cells (whole cells and sections), lysed cells and extracted DNA. All preparations were given a final carbon-coating prior to microanalysis.

i. Intact cells. Whole-cell preparations were carried out as described previously, with the addition of two extra treatments - air-dried preparations of glutaraldehyde-fixed cells, and ethanol-dehydration of unfixed cells. Estimates of bacterial volume after the four treatments were made using mean values of length and width (determined from whole-cell preparations) and regarding bacteria as cylinders with hemispherical ends. Resin sections of aldehyde and acetic acohol-fixed cells were prepared as in the preceding section.

ii. Lysed cells. Bacterial cell lysis was carried out using a technique similar to that used by Griffith (1976). 40 ml of cell suspension was collected by centrifugation at 4°C, washed in distilled water, then buffer A (0.15M NaCl, 0.05M Tris-HCl and 1mM EDTA, pH 7.5). 10 ml of egg white lysozyme solution (25 µg/ml - Sigma grade III) was added to the washed cells, and after 5 min at 0°C, cells were collected by centrifugation

and resuspended in buffer B (0.15 M NaCl, 0.01M Tris-HCl and 1 mM EDTA, pH 7.5) without lysozyme. Droplets of bacterial suspension were then placed on a glass slide at 0°C, and ionically-discharged carbon-formvar grids inserted into the droplets for 4 min. Grids were then washed in buffer B (0°C), and inserted in droplets of Triton-X 100 in buffer B at 0°C. The preparation was allowed to warm to 37°C over a period of 2 min, then grids were washed in buffer B at room temperature, air-dried, ethanol-dehydrated, and finally air-dried overnight.

iii. DNA extraction. Whole cell (chromosomal and plasmid) DNA was extracted from cells of Pseudomonas tabaci using a modified procedure of Sato and Miura (1963). In broad terms, this involved - a) breakdown of bacterial cells by freeze-thawing, lysozyme and detergent, b) centrifugation of the lysate over a layer of phenol, c) precipitation of crude chromatin in ethanol, d) removal of RNA by RNA'ase, e) final removal of protein by phenol treatment, and f) final precipitation of pure DNA in ethanol. For X-ray microanalysis, droplets of DNA in distilled water were placed on a hydrophilic carbon formvar-coated nylon grid, and the preparation then ethanol-dehydrated and air-dried prior to final carbon coating. For EM observation, the DNA sample was treated with 2% osmium tetroxide in distilled water (30 min), diluted with distilled water, and droplets placed on a carbon-formvar Cu grid. This was then processed as previously.

Results

Intact cells

X-ray emission spectra taken from whole bacterial cells are shown in Figs. 7 and 8 and mean levels of elemental mass fractions in Table 1. In all of the preparations, the cells showed substantial peaks of P (major component of nucleic acids) and S (derived from proteins), but levels of cations were highly variable.

Spectra from chemically untreated (air-dried, unfixed) cells typically showed a large peak of K, which is clearly the major cation present in these cells (mass fraction 1.7% dry weight). Apart from Fe, no divalent cations were detectable in these preparations (Fig. 7a). Fixation in glutaraldehyde, followed by air-drying resulted in a small decrease in the mass fractions of S and K, and small peaks of divalent cations - including Ca and occasionally Mn (Fig. 7b) were now apparent. Ethanol dehydration brought about large scale removal of all water-soluble constituents, resulting in the complete loss of Cl and a substantial drop in the level of K. The detection of divalent cations was considerably enhanced, and peaks of Ca, Fe and Ni were routinely present (Fig. 8a). In the final (glutaraldehyde-fixed, ethanol-dehydrated) preparation, peaks of K were infrequently observed, but the presence of Ca, Mn, Fe, Ni, Cu and Zn was routinely detected (Fig. 8b).

The detectability of cations in whole bacterial cells thus directly related to the preparation procedure. The four major types of whole cell preparation used in this investigation

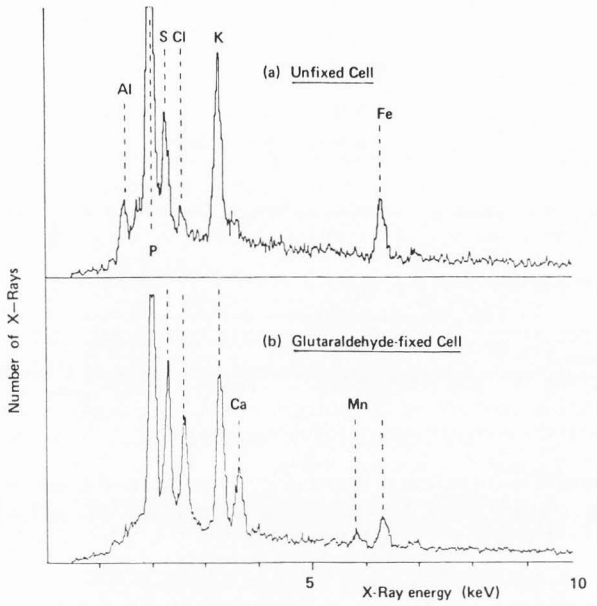


Fig. 7: X-ray emission spectra from air-dried whole bacterial cells. The Al peak in spectrum a is an extraneous contribution from the gridholder. All other peaks are derived from the bacterial cells (see control spectrum in Fig. 8).

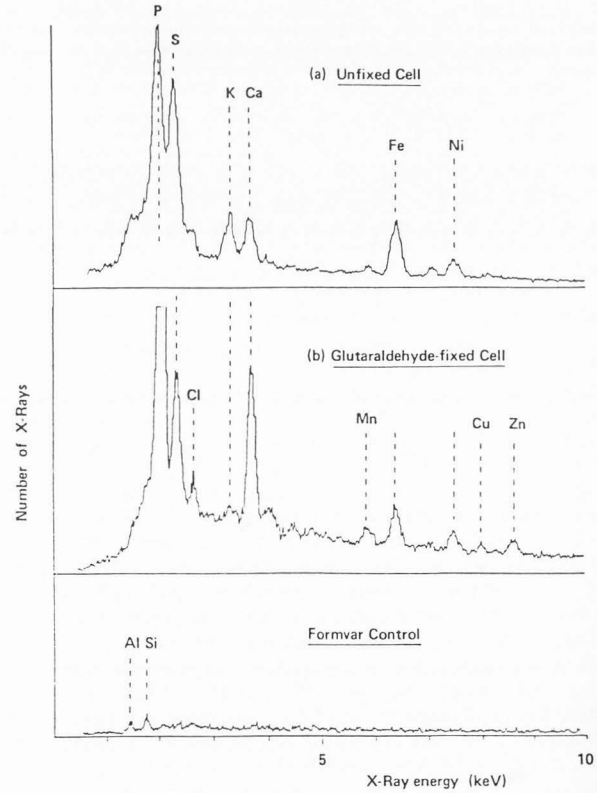


Fig. 8: X-ray emission spectra from ethanol-dehydrated whole bacterial cells.

Cations in bacterial cells

Treatment	P	S	Cl	K	Ca	Mn	Fe	Ni	Cu	Zn
Air-Dried Unfixed	5.1±0.8	1.0±0.4	1.3±0.3	1.7±0.3	-	-	0.3	-	-	-
Air-Dried Glut Fixed	3.5±0.9	0.7±0.4	1.7±0.8	1.4±0.6	0.5±0.2	-	0.3±0.1	-	-	-
Ethanol-Dehydrated Unfixed	5.0±0.8	1.1±0.5	-	0.5±0.2	0.7±0.2	-	0.8±0.1	0.2	-	-
Ethanol-Dehydrated Glut Fixed	5.0±0.7	0.6±0.2	-	0.3±0.1	0.7±0.1	0.1	0.3±0.1	0.1	0.1	0.1

Table 1. Elemental Mass Fractions (% Dry Weight) in Whole Bacterial Cells

% Dry weights are expressed as mean levels in a total of fifteen analyses. In each sample, elements detected in less than 2/3 of spectra are excluded. Confidence limits are at the 95% probability level.

represent a sequence in which there is increased removal of soluble cell components, including both soluble cations (particularly K and Fe) and soluble proteins. This loss is paralleled by a decrease in cell size, with estimated mean volumes decreasing in the sequence - unfixed air-dried cells (mean volume 1.18 μm^3), glutaraldehyde-fixed air-dried cells (mv 0.66 μm^3), unfixed ethanol-dehydrated cells (mv 0.4 μm^3) and glutaraldehyde-fixed ethanol-dehydrated cells (mv 0.27 μm^3).

In fresh, chemically untreated bacterial cells, the full range of divalent cations cannot be detected by X-ray microanalysis because their concentrations are below the level of detectability. Their presence in these cells can, however, be demonstrated by atomic absorption spectrophotometry. Removal of soluble cell components renders the insoluble cations detectable by microanalysis for two main reasons. Firstly, the loss of cell constituents - particularly the protein matrix, brings about an increase in the mass fraction of insoluble cations (increase in peak/continuum ratio). Secondly, the decrease in cell volume results in an increase in insoluble cation mass per unit volume (increase in total characteristic signal in probe area).

The experiments on whole cells give information on the overall cation content of individual cells, but provide no information on the location of cations within cells. One possible means of investigating this is by using ultrathin sections. This approach is limited, however, by the small size of bacteria (diameter 0.5 - 0.8 µm) in relation to the size of the microprobe (diameter 0.3 µm), and also by the low levels of cations within the mass of the section. Thus, although a clear Ca peak was routinely obtained in resin sections of acetic-alcohol, paraformaldehyde and glutaraldehyde-fixed cells, other cations were not generally detected - though Zn was occasionally apparent in glutaraldehyde-fixed material (Fig. 9).

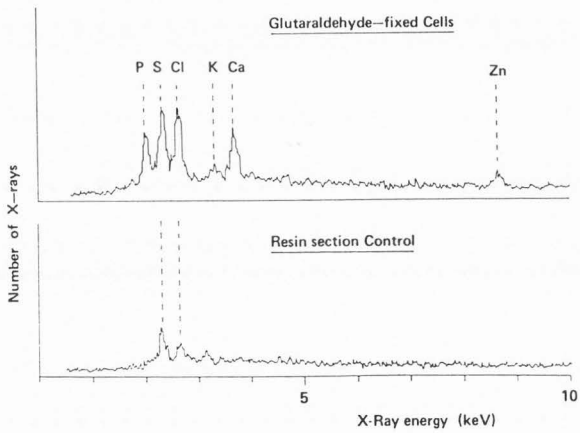


Fig. 9: X-ray emission spectra from resin sections.

	Treatment	P	S	K	Ca	Fe	Ni	Cu	Zn
a.	Unburst Whole Cell	6.2±0.4	1.0±0.1	0.1	1.4±0.2	0.1	0.3	0.2	0.2
b.	Burst Whole Cell	5.1±0.6	0.9±0.2	0.2	1.1±0.2	0.1	0.1	0.1	0.1
	Protoplast	7.0±2.4	-	0.4±0.2	2.4±1.0	-	0.1	-	0.1

Table 2. Elemental Mass Fractions (% Dry Weight) in Lysed Bacterial Cells

% Dry weights are expressed as mean levels in a total of fifteen analyses. In each sample, elements detected in less than 2/3 of spectra are excluded. Confidence limits are at the 95% probability level.

The limited success in investigating ultrathin sections of bacteria lead to the use of other techniques to determine cation localisation - including the use of lysed cells and extracted molecules.

Lysed cells

Treatment of bacterial cells with lysozyme and Triton-X resulted in a heterogeneous preparation containing three main types of cell - entire cells with no evidence of extrusion, cells with extruded protoplasmic contents (protoplasts) and cells with extruded chromatin.

The appearance of lysed bacterial cells with extruded protoplasts is shown in Fig. 10. In the absence of shadowing (Fig. 10a) the protoplast has a reticulate appearance, while in shadowed preparations (Fig. 10b) the general matrix can be seen. The spatial separation of a mass of protoplasm permits microanalysis of bacterial contents free of cell wall material, while analysis of the residual bacterial cell should provide information on the elemental composition of the bacterial envelope plus remaining protoplasm. Intact, unburst cells have broadly similar spectra (low levels of monovalent cations, clear presence of Ca and transition metals) to the fixed, fully-dehydrated whole cells analysed previously. Interpretation of the spectral information from protoplasts and residual burst

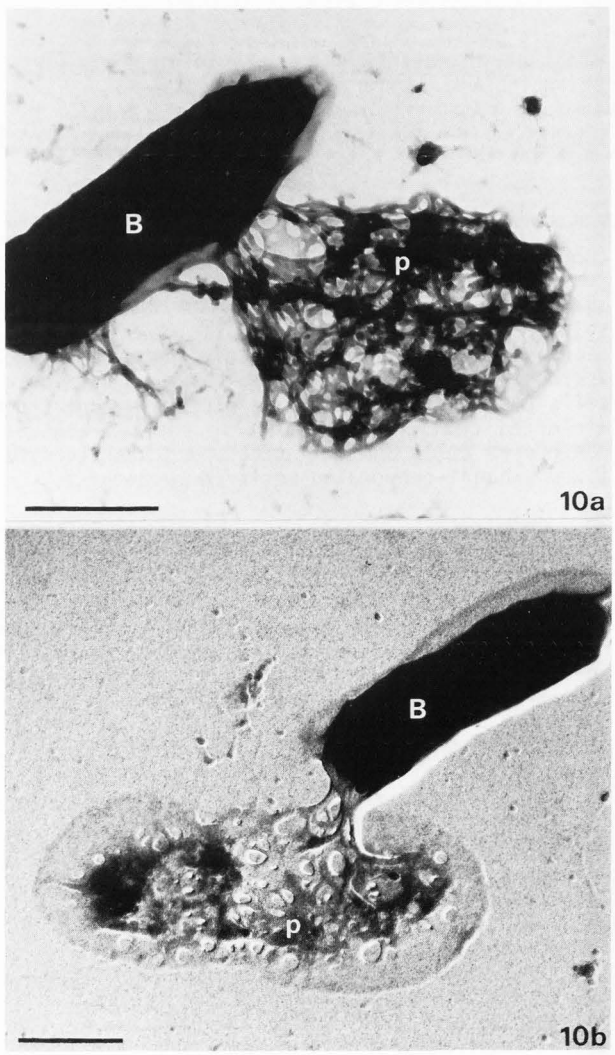


Fig. 10: Transmission electron micrographs of extruded protoplasts (lysed cells). (Bars = 0.5µm).
(10a) Unshadowed preparation. The extruded protoplast (p) has a reticular appearance.
(10b) Shadowed preparation. The extruded protoplast (p) appears as a flattened mass of protoplasm. The original bacterial cell (B) does not show any other extrusions.

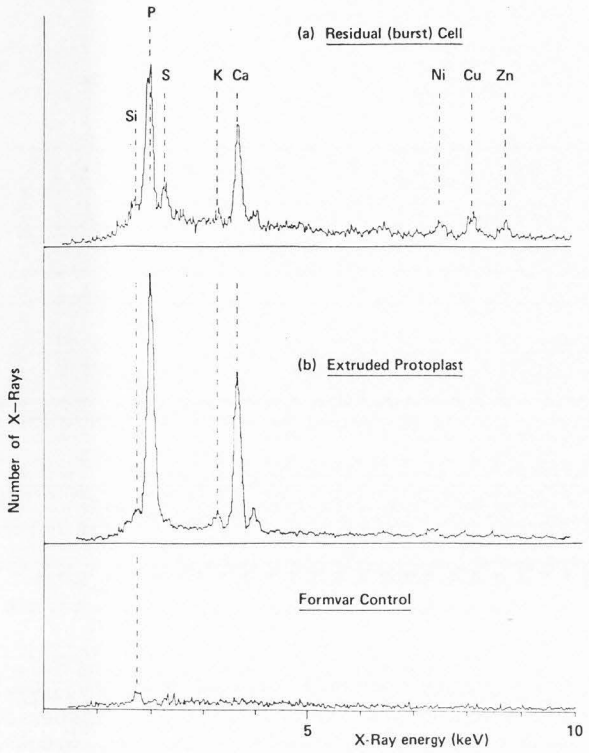


Fig. 11: X-ray emission spectra from lysozyme-treated cells - Residual cells and extruded protoplasts.
(a) Residual cell - spectrum taken from a cell with an extruded protoplast (similar to B in Fig. 10a) b. Protoplast - spectrum taken from an extruded mass of protoplasm (similar to p in Fig. 10a).

cells (Fig. 11b,a and Table 2b), in comparison to the whole cells, is rendered difficult by the change in mass of material analysed (the protoplast forms a thin layer of material on the EM grid) and also by the greater loss of extractable material from these broken-up cells. Emission spectra from protoplasts (Fig.11b) suggest almost complete loss of protein (no detectable S) but substantial retention of P, and bound (K) and divalent (Ca) cations. Analysis of the residual burst cell (Fig. 11a, Table 2b) demonstrates retention of the full range of cations, suggesting that these may be directly bound to the cell wall.

The extrusion of bacterial chromatin from multiple sites on the cell surface results in the presence of a halo of fibrils surrounding the bacteria (Delius and Worcel, 1973; Griffith, 1976). These are clearly observed in shadowed (Fig. 12, Fig. 13b) but not unshadowed (Fig. 13a) preparations. The residual body of the bacteria typically appeared electron-dense (Fig. 12), but in some cases - referred to as 'ghost cells' - the bacterial cell had been severely degraded and appeared electron-transparent. These ghost cells invariably had extruded fibrils when observed in

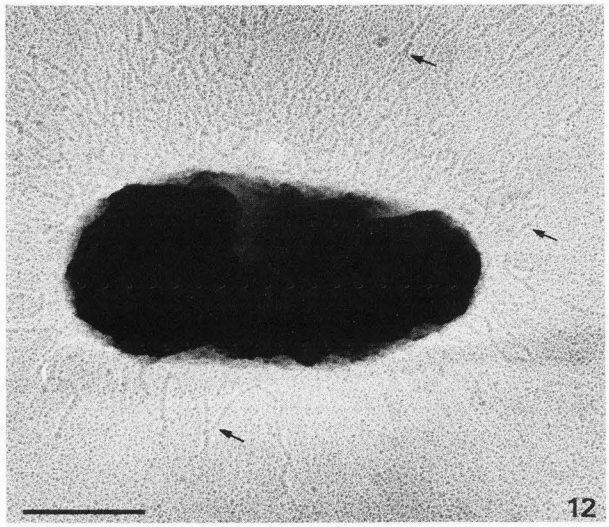


Fig. 12: Transmission electron micrograph of electron-dense bacterial cell with extruded chromatin (lysed cell) - shadowed preparation. The cell is surrounded by a web of dispersed extruded chromatin fibrils (arrows). (Bar = 0.5µm).

shadowed preparations (Fig. 13b) and were therefore particularly useful for microanalysis since the presence of surrounding fibrils in unshadowed preparations could always be inferred. X-ray microanalysis of ghost cells and surrounding dispersed chromatin (Fig. 14) met with only limited success, however, due to the very small mass of material in the preparation. This was particularly true of the dispersed chromatin, where count rates were close to the formvar background, and where only small peaks of P and Ca were detectable.

Successful X-ray microanalysis of isolated bacterial chromatin clearly requires a more substantial layer of macromolecules, such as would be obtained by direct extraction of bulk DNA from bacterial cells.

Extracted DNA

The appearance of a preparation of extracted DNA is shown in Fig. 15. The molecules of DNA are seen as highly compact nucleoids with a supercoiled configuration, and are closely similar to the molecules of extracted DNA from E. coli observed by Masterman and Van Gool (1978).

The emission spectra from these preparations (Fig. 16) invariably had large peaks of P, but no detectable S - in line with the high degree of purity (absence of proteins) in the cell extract. The emission spectra had high levels of K and Ca, and showed the full range of transition metals observed previously in intact cells. The results obtained from extracted DNA would thus suggest that a wide range of cations are directly bound to the DNA in the living cell, and that these cations are largely retained by the macromolecule during the extraction process.

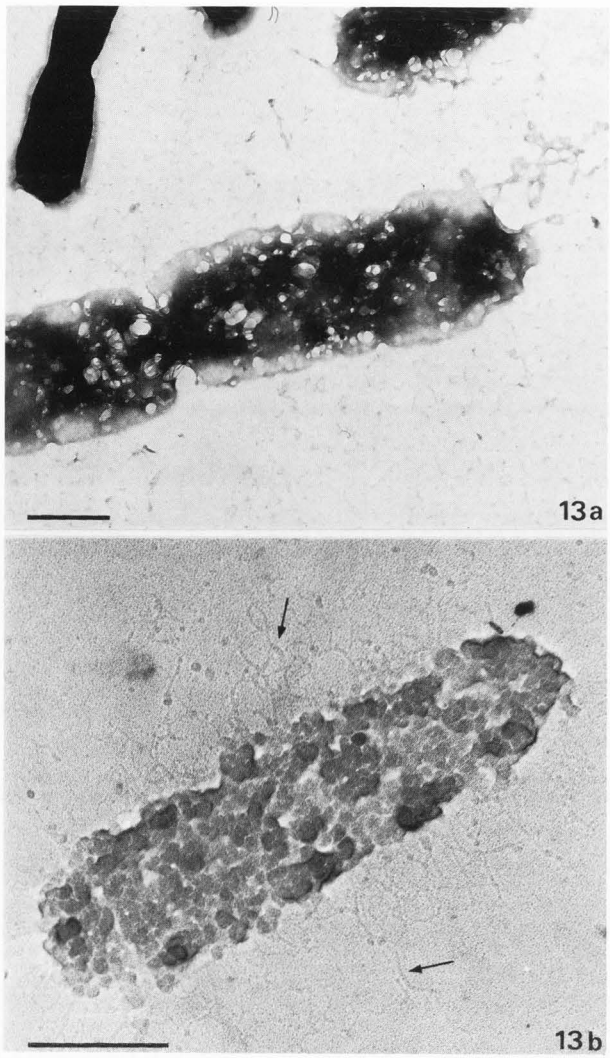


Fig. 13: Transmission electron micrographs of electron-transparent bacterial cells (ghost cells) with extruded chromatin. The low electron density in these cells is probably due to extensive wall degradation and loss of cell contents. (Bars = 0.5μm).
(13a) Unshadowed cell. The presence of extruded chromatin around the cell is indicated by a faint reticulum.
(13b) Shadowed preparation. The cell wall appears to be broken down, and numerous chromatin fibrils (arrows) can be seen emanating from the cell periphery.

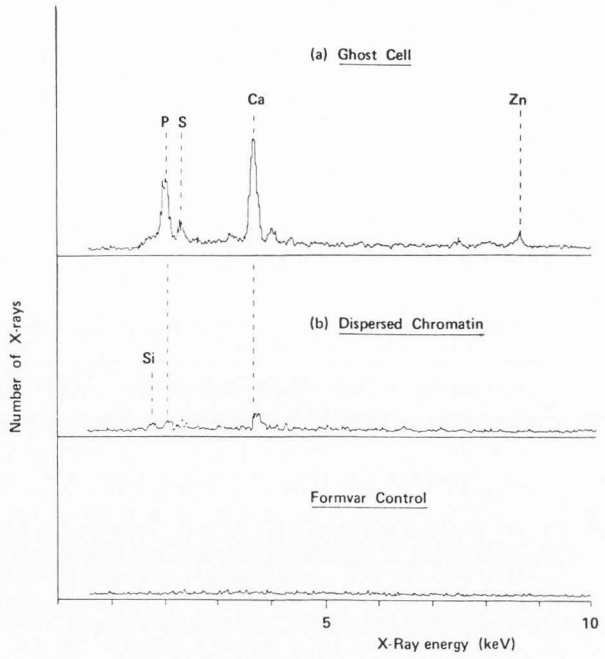


Fig. 14: X-ray emission spectra from lysozyme-treated cells - ghost cells and dispersed chromatin (unshadowed preparations). Spectra obtained over 300 seconds livetime at a beam current of 60 nA.

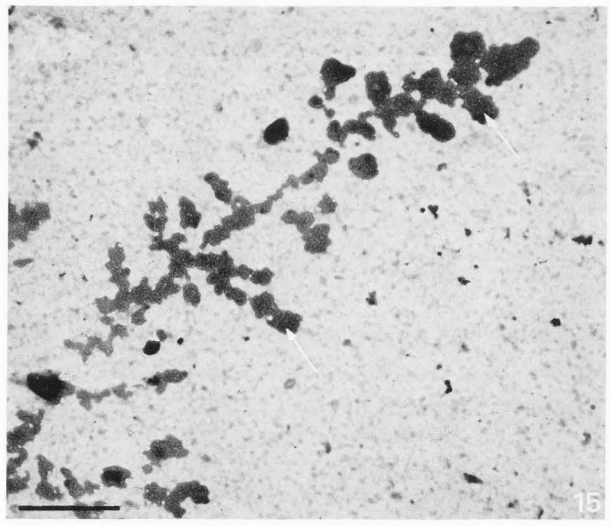


Fig. 15: Sample of extracted bacterial DNA, deposited onto an ionically-discharged carbon-formvar grid, showing highly condensed supercoiled branches (arrows). Unshadowed preparation. (Bar = 2μm).

Discussion

Autoradiography and X-ray microanalysis provide very different experimental approaches to the problem of cation localisation. Autoradiography has the disadvantages of restriction to particular cations and long incubation period, but has advantages (under optimum conditions) of high sensitivity and good spatial resolution. In terms of sensitivity, it is interesting to note that in the studies reported here, radioactive nickel was being detected in sections taken from bacteria with mean

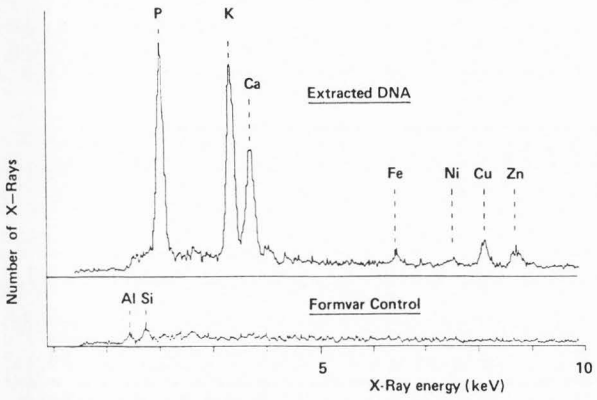


Fig. 16: X-ray emission spectrum from extracted DNA. The control spectrum b is taken from an unused grid derived from the same batch as used in a.

levels of incorporated nickel as low as 8.8×10^{-19} g (1.5×10^{-11} nmol) per cell (Al-Rabaee and Sigee, 1984). X-ray microanalysis carried out on the same sections did not reveal any detectable nickel, emphasising the difference in sensitivity between the two techniques. With regard to spatial resolution, the use of autoradiography permitted spatial analysis within ultrathin sections, while microprobe analysis did not.

The majority of transmission EM studies in biology using X-ray microanalysis have involved the use of sectioned material. In this investigation, the use of ultrathin sections appeared to be of limited value, and the application of X-ray microanalysis to other types of preparation such as whole cells, lysed cells and extracted DNA was a necessary development.

X-ray microanalysis of whole bacterial cells provides a particularly useful model system to investigate the importance of cell preparation techniques in relation to element detectability, since the cells contain high levels of cation elements, they are self-contained (no problems of element migration within the cell or tissue) and they are sufficiently small to qualify for analysis by Hall thin film quantitation (Hall, 1971; Chandler, 1975). The results suggest that although examination of fresh (chemically untreated) material has advantages in providing information on total (soluble and insoluble) element levels and in avoiding potential artefacts during preparation, it has the disadvantage that full retention of soluble components may obscure the presence of certain elements. In these preparations, demonstration of the presence of bound cations became progressively more effective as soluble cell constituents were removed.

The autoradiography and X-ray microanalysis lead to the conclusion that a major part of the cell's complement of insoluble cations are associated with the chromatin, and specifically with DNA. This is of considerable interest in view of the known structural importance of cations in stabilising this macromolecule (Eichhorn, 1962). Although there is a considerable amount of published information on the structural (eg. Borochov et al., 1984; Makarov and Dimitrov, 1982) and biochemical (eg. Dod et al., 1981; Duerre et al., 1982; Goel and Modak, 1984) importance of cations in eucaryote chromatin, there has been much less attention paid to the chromatin of procaryote cells. The presence of substantial levels of cations in isolated bacterial chromatin is of particular relevance to previous studies on dinoflagellate (eucaryote) cells - where the chromatin resembles bacteria in having an absence of histones and an apparently primitive type of cationic stabilisation system (Sigee, 1983b, 1984, 1985). Studies are currently in progress to fully characterise the ionic composition of bacterial and other types of extracted DNA, and to further exploit the use of X-ray microanalysis in relation to isolated macromolecules.

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Discussion with Reviewers

L. Kunst The data presented in Figs. 7 and 8 and Table 1 do not indicate a significant loss of Fe in relation to the preparation procedure. However, you point out that there is an increased removal of Fe besides K from the cell. Could you

please comment on the possible evidence for such a conclusion?

Authors Evidence for the removal of K and Fe during preparation is inferred from comparison of elemental mass fractions (mass of element/mass of biological material). The extraction of soluble components will have a dual effect on the mass fraction of any particular element. If that element is soluble then its loss from the cell will tend to reduce its mass fraction (decrease in mass of element). The loss of soluble cell matrix will, conversely, tend to increase the elemental mass fraction (a decrease in mass of biological material). In the case of K, which is mainly present as a soluble cation, the elemental loss far outweighs the loss in general cell matrix, and there is a decreased mass fraction. In the case of Fe, which is present as a major soluble and insoluble component, there is no decrease in mass fraction. Its maintenance at constant level (0.3% in both fresh and fixed/dehydrated cells) implies substantial element loss, however, in view of the removal of soluble matrix from the chemically-processed cells.

G.M. Roomans Have you considered the presence of contaminants in the various fixatives and extracting chemicals used?

Authors We have tested for the presence of contaminant transition metals in the aldehyde fixatives and ethanol dehydration series, and have not been able to demonstrate their presence at limits of detectability of 1 ppm. For this reason (and also for reasons given in the text) we reject the possibility that the presence of transition metal peaks in the emission spectra of fixed, dehydrated (but not fresh cells) could be attributed to preparation artefact - such as adsorption of cations from the processing solutions.