-SPECIAL ADDRESS-

ELEMENTAL ANALYSIS OF BIOLOGICAL SPECIMENS BY ELECTRON PROBE X-RAY MICROANALYSIS

VINCI MIZUHIRA

Department of Cell Biology, Medical Research Institute, Tokyo Medical and Dental University, Yushima, Bunkyo-Ku, Tokyo 113

Electron Probe X-Ray Microanalysis:

Recently many new technical advances in histochemical and cytochemical studies have been made. The electron microscope fitted with an electron probe X-ray microanalyzer is a unique method for detecting the fine distribution of elements in fine structure by using a very small electron beam spot. The characteristic X-ray energy or wave length of the element can be ascertained by the aid of energy dispersive or a wave length dispersive X-ray detectors with an amplifier and a display system.

Analytical Electron Microscope:

We have devised a new “analytical electron microscope (AEM)” for these purposes. The AEM is basically composed of a JEM 100-C transmission electron microscope fitted with a scanning device (ASID-4), side-entry goniometer stage (SEG) and an energy dispersive type X-ray microanalyzing unit (EDAX-707A or 707B). The specimen chamber is specially shielded by a carbon plate having a goniometer and a collimeter with holes for the insertion of the specimen and for the pick-up of X-rays.

The electron images were made by the scanning secondary (SEM), scanning transmission (STEM) or conventional transmission (TEM) electron images. Then one could analyze a very small part of the image by the combination of EDAX and ASID systems, such as spot X-ray pulses (Fig. 1), line X-ray pulses (Fig. 2), scanning X-ray pulses or X-ray pulse image analysis (Figs. 3 and 4), superimposed on these original electron images. The accelerating voltages were 10, 20, 40, 60, 80 and 100 KeV for the X-ray excitation. The illuminating current to the specimens was mostly $n \times 10^{-10}$ A. The specimens were tilted at 25 to 45° degrees to the electron beam for the purpose of picking up the X-rays from the specimen at a right angle to the electron beam using a goniometer stage system. The goniometer stage was also made from a carbon plate.
FIG. 1. A frozen-dried ultrathin section of a mouse lung. A small piece of lung was fixed with 2.5% glutaraldehyde containing 0.5% tannic acid for ten min. at 0°C. Then the tissue block was touched to a copper rod at the temperature of liquid nitrogen. The ultrathin section was cut with a LKB-Cryokit and mounted on a carbon grid by Mr. T. Sakai, Akashi Seisakusho Ltd., Tokyo. Finally the grid was air-dried in a cryochamber for about 30 min. A STEM-image was taken at 40 KeV. Details of analysis are explained in the text.

FIG. 2. An air dried SEM-image of the synthesized cell membranes of "pleuroneumonia-like organismus (PPLO)" in tin (Sn)-containing culture medium on a carbon plate (the cultured sample was presented by Dr. R. J. Barnnett and analyzed by Drs. V. Mizuhira and Y. Futaesaku). The spot X-ray pulses were obtained from the membranes as shown in the lower spectra of this picture. The PPLO membrane consists of many elements, such as Na, Mg, Al, Si, P, Cl, Sn, and Ca. A part of the light elements, Na, Mg, P, or Ca may have come from the PPLO storing solution. Sn should be in the membraneous component instead of a part of the carbon molecules of the structural lipids (Barnnett). The line X-ray pulse analysis of tin was obtained as shown in this picture.
Fig. 2
Fig. 3. A silicosis of a horse lung tissue was analyzed. An Epon embedded section was cut, and mounted on a carbon film covered carbon grid, and a STEM-image was taken at 10 KeV (upper). Many silicon and carbon particles were scattered in the lung tissue. Si and Al were obtained from a silicon particle at 20 KeV with 10^-18 A, with a spot X-ray pulse analysis (spectrum), and an X-ray pulse image of silicon was obtained (lower). The material was presented by Dr. A. Koester and Mr. O. Kindig, Ohio State University.
Fig. 4. Fresh Ehrlich tumor cells were spread out on a carbon film, and an SEM-image was taken directly after air drying and carbon coating on the surface. A scanning X-ray pulse analysis showed a beautiful X-ray energy spectrum as shown in the lower part of this picture. Na, P, S, Cl, K and a large amount of Fe were detected. An X-ray pulse image of iron (K) overlapping an SEM-image was seen in some tumor cells containing a large amount of iron, but other cells were scarce.
Colour Analysis of X-Ray Data:

In some cases, we have obtained improved colour analysis of the X-ray data by using Kodak high speed Ektachrome film with a combination of coloured plastic filter plates over a display cathode-ray tube, and by using the superimposed exposure method on a scanning picture. With this simple method, it is very easy to demonstrate the distribution of selected elements at the same time in a coloured scanning picture (SEM or STEM, Fig. 5).

Basic Problems on X-Ray Microanalysis of Biological Specimens:

We have very little basic data on the X-ray microanalysis of biological specimens. For example, most of the biological samples are composed of very light elements, such as C, H, O, N, Na, Mg, P, Cl, K, or Ca. Iron (Fe) is distributed in red blood corpuscles as hemoglobin, but the density per constant square in ultrathin sections is not high. Consequently, it is very difficult to detect the iron in a plastic embedded section of the red blood cell after conventional fixation, dehydration and embedding.

The density of iron atoms is very low in ultrathin section and also there is a possibility that the embedded resin may disturb or absorb weak X-rays from the section. However, a small amount of iron (Fe) can be detected in a fresh dried or

Fig. 5. A coloured X-ray microanalysis of fresh air-dried Ehrlich tumor cells, Kodak High Speed Ektachrome Film, at 40 KeV. First an SEM-image was taken with a green plastic filter, and next it was double-exposed with Fe-Ka X-ray pulse image using a red filter. The red colour appeared as orange to red on the green cell-images.
fresh frozen-dried section of the red blood cell (Fig. 1). This means that the improvement of fresh frozen-dried sectioning technique is very important for obtaining sufficient X-ray counts and for finding the true sites of the elements in a cell.

If the illuminating current is constant, the correlation between the white X-ray intensity, accelerating voltage, and the base material nature seems very important obtaining the best S/N ratio (characteristic X-ray intensity/white X-ray intensity) (Fig. 7). We performed these basic experiments using various elements, Na, Mg, S, P, Cl, K, Ca or Fe; various base materials, bronze plate, carbon plate and carbon film deposited on a carbon grid; and various accelerating voltages, 10, 20, 40, 60,

![Diagram](image)

Fig. 6. White X-ray intensity in various experimental conditions. (A) bronze plate, (B) carbon plate and (C) carbon film deposited on a carbon grid were examined as base materials. The accelerating voltage was varied from 10 to 100 KeV, but the illuminating current was constant in each case, at $10^{-10}$ A. The accelerating voltages influenced the white X-ray intensities in the case of bronze plate (A), but the influence was negligible in the case of carbon film (C).
80 and 100 KeV. From these experiments, it was clear that the intensity of white X-rays was influenced by the value of accelerating voltages and also that carbon film was less than 1/10 of the bronze plate (Fig. 6). In the S/N ratio, the carbon film or carbon plate was excellent at lower accelerating voltages, such as 10, 20 or sometimes 40 KeV, but the bronze plate showed very high efficiency (Fig. 7).

In the biological samples, there are only small amounts of light elements scattered in the tissues, as mentioned above. Judging from our basic experiments, the biological specimen must be supported on a carbon film with a carbon grid or carbon plate (handmade, Fig. 14), and must be analyzed with low accelerating voltages in the range of 10 to 40 KeV (Figs. 6, and 10). Under such conditions,

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**Fig. 7.** The S/N ratio, which is the characteristic X-ray energy intensity/white X-ray intensity was examined under various conditions. (A) S/N ratio of some elements (S, Cl, Ca and Fe) under various accelerating voltages on a bronze plate. An accelerating voltage of approximately 30 KeV shows one of the highest S/N ratios. (B) In the case of carbon plate, accelerating voltages of 20 to 40 KeV gave a good S/N ratios. (C) In the case of carbon film also, accelerating voltages of 20 to 40 KeV gave a good S/N ratios.
one should obtain a higher S/N ratio, and the identification of elements should be very easy. As an example, we studied liver sections obtained from a human with chronic hepatitis. First, we tried to analyze Epon embedded sections using an accelerating voltage of 60 KeV, and we detected Fe, Cl, P, or Os(M-line) X-ray energy spectra from various lysosomes in the sections after taking a STEM image without electron staining (Fig. 12, c). Next we tried analysis using a lower accelerating voltage of 10 KeV to the same sections and the same portion. In this experiment, we succeeded in finding other metallic elements besides iron, that is, cadmium(Cd) and sometimes silver (Ag) (Fig. 12, a, b). In the case of cadmium, iron was also distinguished very clearly, but in the case of silver, iron was not detectable (Fig. 12, a, b).

Embedding Media for Biological Samples:

As mentioned above, Epon seems not to be the best embedding media for X-ray microanalysis. It is very important to find good embedding media as well as to find fixation methods for soluble ions including the fresh frozen dried sectioning methods for this purpose.

Glutaraldehyde-urea resin quick embedding method (Pease, 1972; Yarom 1974), glutaraldehyde-amonium resin (Pease, 1972), resorcinol-formaldehyde resin (Hildebrand, 1974) and glycomethacrylate (GMA) resin (Ashley, 1966; Ashford, 1972) were tested without dehydration procedures with organic solvents. In particular, the glutaraldehyde-urea resin quick method is very simple and requires only two hrs. after fixation to finish the polymerization. Furthermore, glutaraldehyde-urea resin in contrast to Epon resin does not contain chlorine and other elements which might disturb the elemental identification of the biological sections under the NDS type X-ray microanalysis. Since the procedure is very short and simple, a part of many light electrolytes, such as Na+, Mg++, Cl-, K+, or Ca++, still remains in the embedded tissue sections, even though some technical problems are still encountered (Figs. 8 and 11, 2).

Fresh Air-Dried and Fresh Frozen-Dried Sections:

The best results with electrolytes in biological samples can be obtained with fresh air-dried (Figs. 4 and 5), or the fresh-frozen and dried sections (Figs. 1 and 11 -1, -2). However, there are still some technical difficulties in the fresh-frozen and dried sectioning method at the ultrastructural level. A speedily fixed frozen-dried ultrathin sectioning method was nearly as good as the fresh air-dried or fresh frozen-dried methods (Figs. 1, 2, 4, and 11, -1, -3).

Only a small amount of elements should be lost during the fixation procedure in this speedily fixed frozen-dried method (Figs. 1 and 11). We used 2.5% glutaraldehyde containing 0.5% tannic acid solution for this method. In this case, the chemical nature of the buffer solution is very important. If one uses physiological

Fig. 8. Glutaraldehyde-urea resin embedded mouse skeletal muscle. The block was fixed with a mixture of 1% osmium tetroxide and 2.5% glutaraldehyde, then moved to 50% glutaraldehyde, and embedded in a mixture of 50% glutaraldehyde and saturated urea at pH 4.2 adjusted with saturated oxalic acid. An ultrathin section was mounted on a carbon film coated copper grid. First a STEM-image was taken, and then the section was analyzed with 20 KeV at 10^-10 A for 100 sec. Calcium was clearly observed in the Z-band and the tubular systems with other elements, such as Mg, Al, S, and Os (M).
Fig. 9. A glutaraldehyde-Urea resin quick-embedded section of Ehrlich tumor cells. The specimen was fixed with 2.5% glutaraldehyde and embedded in a glutaraldehyde-urea resin mixture, without osmium tetroxide treatment. The STEM-image did not have enough electron contrast, but a great amount of elements still remained in the tumor cells. Many kinds of fine granules are scattered in the tumor cells and have different kinds of elemental composition, as shown in the different energy spectra in this picture.
Fig. 10. This figure shows a comparison of the characteristic X-ray intensities with energies distributed from 1 to 40 KeV under various accelerating voltages. In this experiment, some metal elements which are very important in the identification of elements overlapping with light elements were selected. They were compared at various accelerating voltages, intensities and type of X-rays, which are the M, L and K lines. For example, the highest intensities of the U-M line and the Cd-L line were obtained at an accelerating voltage of 20 KeV. However, the highest intensity of the Cd-K line was obtained with 100 KeV. This figure also shows that one can easily find adaptable correlation between the characteristic X-ray and accelerating voltage. For example, in the case of P-Kα the voltage is 10 KeV; for Ca-Kα and Fe-Kα are 40 KeV; for Na, it is 10 KeV; for Hg-L, it is 40 KeV; for Hg-M, it is 10 KeV. Thus, this graph is very useful for the biological X-ray microanalysis. The illuminating current was constant, 10^-10 A.

saline solution with fresh tissue or free cells, the X-ray counts of sodium and chlorine will be increased; in the case of phosphate buffer, phosphor counts will be increased; in the case of cacodylate buffer, arsenic (As) will be detected in the section; and in the case of veronal acetate buffer, sodium will be increased. For these reasons, ammonium-acetic acid buffer may be one of the best buffer solutions for the X-ray microanalysis of biological samples.

Recently, we studied mouse lung tissue in frozen-dried sections fixed in glutaraldehyde and tannic acid mixture for ten min. at 0°C (Fig. 1). The STEM-image was recorded at 40 KeV and spot analyzed at some locations, such as the red blood cell, the nuclear chromatin of the alveolar epithelium, and the granules, at 40 KeV with 10^-10 A. We found Na, Mg, Si, P, S, Cl, K, Ca and a small amount of Fe in the red blood cell (Fig. 1, R); Na, Mg, Si, P, Cl, and Ca in the nuclear chromatin (Fig. 1, N); and a large amount of K, Si and a small amount of Na were found in the
cellular granules (Fig. 1, G).

**Calcium in the Nucleus:**

It was discovered in 1971 that calcium is accumulated in the chromatin and the nucleoles in a nucleus using an electron probe X-ray microanalyzer after fixing with osmium tetraoxide containing potassium antimonate (Mizuhira et al, 1971). The same results were obtained with fresh frozen-dried tissue sections using an AEM. We observed the same results in various tissue cells including liver, kidney, skeletal

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**Fig. 11.** (1): Small pieces of mouse liver with acute cadmium poisoning were frozen with liquid nitrogen, and a freeze-dried section was mounted on a carbon film coated carbon grid with no fixation and electron staining. The section was analyzed with scanning X-ray pulses at 20 KeV and $10^{-10}$ A for 400 sec. A large amount of elemental counts can be obtained including a small amount of Cd (L). Na, Mg, Al, Si, P, S, Cl, K and Cd were detected. The light spectrum at the bottom of this spectrum shows background noise from the carbon film, under the same analyzing conditions. Si is the common element and almost the same height from the base of these spectra, so that this may have been caused by contamination during the carbon evaporation procedure. (2): A thin section of cadmium-poisoned mouse liver, embedded with glutaraldehyde-urea resin after fixing with 2.5% glutaraldehyde and tannic acid mixture for 60 min. The block was postosmificated, and the sections were mounted on a copper grid without staining. The section was analyzed at 10 KeV for 200 sec. with $10^{-10}$ A. A large amount of S and a small amount of Mg and Cd was obtained. The Cu (L) is reflected from the copper grid, and the Os (M) is also caused by the fixation procedure. It seems easier to demonstrate the cadmium in contrast to that of Epon embedded section. (3): An analysis of a purified cadmium induced metallothioneine. The sample was obtained from a cadmium-poisoned mouse kidney by courtesy of Dr. H. Tsunoo, Tokyo Medical and Dental University. Diluted metallothioneine was dropped on a carbon plate, then dried. The sample was analyzed at 10 KeV for 100 sec. with $10^{-10}$ A. A typical X-ray energy spectrum of the metallothioneine has been demonstrated here, from A to C. A large amount of S (K) and Cd (L), and a small amount of Cu (K) and Zn (K and L) can be identified.

**Fig. 12.** STEM-image of a human liver section, obtained from a chronic hepatitis patient by the biopsy method. The tissue block was fixed with osmium tetroxide buffered with phosphate, and embedded in Epon after dehydration. The sections were mounted on a copper grid, and analyzed with various methods. The upper curve in this picture indicates a photointensity crossing to the middle of this picture. The middle curve indicates an X-ray pulse intensity of Os-M line, and the lower one indicates an X-ray pulse intensity of Fe-Ka line. A large amount of ferritin molecules were accumulated in the lysosomal granules (Lys, a and b). If one analyzes such a granule at 60 KeV or more high accelerating voltages, there can be obtained Fe (K) very clearly, but no other metallic elements were observed in this picture, (c). However, if one uses lower accelerating voltages for example, 10 or 20 KeV, there is Cd (L) or sometimes silver (Ag) in the same section (a and b).

**Fig. 13.** An example of the elemental identification using a computer system with microanalyzer (EDAX-707B-EDIT). (1): A combined spectrum with the Sb (L) and Ca (K) (A). (B) It is impossible to identify the Sb (L) and the Ca (K) in each energy spectrum. But EDIT can do it very easy and also can subtract the base noise from the combined spectrum. (2): (A) The X-ray energy spectrum with white line is the combined spectrum of the Cd-L and the U-M lines. The dotted peak shows the computed original spectrum of the Cd-L line. This figure shows important information, that is, the combined peak shows an *energy shift* approximately 40 to 60 eV to the right compared to the standard peak of Cd-L at (highest dotted peak). (B) U-M line energy counts were subtracted from the combined spectrum. There appears the original Cd-L spectrum and the original U-M spectrum (light). (3): (A) is the combined X-ray energy spectrum of a mixture of mercury chloride and ammonium ferri sulfate. The X-ray energy spectrum of the M line of Hg is superimposed on the energy spectrum of S-K line so perfectly that one can not be identified to each other. (B) But the EDIT does it very easy within a few seconds. The dotted spectrum indicates the K-line of sulfur, and the light-lined spectrum indicates the original Hg-M line.
Fig. 12
FIG. 14. This picture shows the method of making carbon grids for the X-ray microanalysis in biology. (1) A used carbon rod was used as material. First, make a smooth surface on the rod-cut surface. Then make small holes with a very slender dental drill (0.2 to 0.5 mm in diameter) using a dental engine, parallel to the long axis. (2) With a dental engine and a carborundum disk cut a thin carbon slice approximately 0.3 mm in thickness having small holes. (3) Grind both faces of the carbon slice (grid) with a fine grinder with the tip of the finger. (4) Clean the grid with aceton or ether. (5) Coat a thin layer of chloroprene rubber on the carbon grids with spray. It is necessary to stick the carbon film on a carbon grid. (6) Cover the carbon grids with a carbon film, then dry them in an oven.

and heart muscles, and chromosomes in a dividing cell. But in the DNA synthesizing nucleus, the content of calcium seemed very low. Yarom (1974) also ascertained the presence of calcium in the muscle cell nucleus by the antimonate method combining with an AEM.

It is very important that we have observed conspicuous decrease of the calcium content in the nuclear chromatin and nucleolus of cancer cells by both X-ray microanalysis and potassium antimonate fixation.

Ehrlich Tumor Cells:

In the air-dried Ehrlich tumor cells spread out on a carbon film deposited on a carbon grid, the SEM-images were taken on colour film using a green filter, then an overlapped exposure with X-ray pulse images was made using various kinds of
coloured plastic filters; and black and white pictures were also made (Figs. 4 and 5). For example, Fe was recorded with a red filter, P with purple, K with green, Cl with yellow and Na with orange.

Iron (Fe) was distributed distinctly in some tumor cells, but not in others. Cl, P, K and Na were also distinct in air-dried tumor cells (Fig. 4).

In glutaraldehyde-urea resin quick-embedded Ehrlich tumor cells, various small shaped granules are scattered in the cytoplasm. These granules contain various kinds of elements in this case (Fig. 9). Some granules contain a large amount of iron with other light elements; some granules contain a large amount of potassium, and others contain magnesium, zink, lead or bromium with other light elements (Fig. 9). In the fresh frozen dried ultrathin sections of cadmium-poisoned mouse liver, the sections were mounted on a carbon coated carbon grid in a freezing box of the LKB-Cryokit. The small pieces of fresh tissue were frozen at the temperature of liquid nitrogen by touching them to a liquid nitrogen cooled bronze bar. The grids were dried by Sakai's method (1974) using an empty aluminum 35 mm film case filled with silica gel. If the fresh frozen-dried section is good, the contrast of the electron image is very poor even when one has used the STEM-image system. However, one can obtain a large amount of characteristic X-ray energy counts from the section as shown in Fig. 11, -1. That is, one can obtain a large amount of P, S, Cl, and K, and a small amount of Na, Mg, Al or Cd. There can be found some amounts of Si in the energy spectrum, but almost the same counts can be observed in the background spectrum from the supporting carbon film. This may be considered to be the reflection spectrum from the background (lower white spectrum in the figure).

These data obtained from the fresh frozen-dried section mean that a great amount of cell physiological electrolytes, such as P, S, Cl, K, Na, Mg and some exogenous element, Cd, in this case, may be coming out during the conventional electron microscopic preparation procedures from fixation to embedding, and also during sectioning and staining.

However, in the case of glutaraldehyde-urea resin embedded cadmium-poisoned mouse liver after fixing with glutaraldehyde containing tannic acid for one hr., one can observe the cadmium L-line very clearly with other elements, but no potassium was obtained (Fig. 11, -2). In contrast to the results obtained by the fresh frozen-dried section (Fig. 11, -1), most electrolytes, such as P, Cl, and K except S were lost during the preparation procedure (Fig. 11, -2).

In the direct analysis of a small amount of purefied Cd-metallothioneine obtained from a cadmium-poisoned mouse kidney, one can obtain a typical X-ray energy spectrum pattern as shown in Fig. 11, -3. In this spectrum, a large amount of S (K), and Cd (L), and a small amount of copper (K) and Zn (K and L) energy spectra can be identified.

Identification of Superimposed X-Ray Energy Spectra:

There is another big problem in this study, which is the need to separate the overlapping X-ray energy spectra, such as the L-line of cadmium (Cd) and the M-line of uranium (U); the K-line of calcium and the L-line of antimony (Sb); and the K-line of sulfur (S) and the M-line of mercury (Hg) or lead (Pb) (Figs. 10 and 13).

These lines overlap each other so perfectly that they can not be identified. We succeeded in identifying the overlapping spectra using a computer system, EDAX-
707B-EDIT on the X-ray luminescence analyzer with EDAX X-ray energy detection system. In this experiment, we used mixed standard samples, cadmium chloride and uranyl acetate mixture (Fig. 13, -1); potassium antimonate and calcium chloride mixture (Fig. 13, -2); and mercury chloride and ferric ammonium sulfate mixture (Fig. 13, -3). We found that an energy shift of approximately 40 to 60 eV to the right side was observed in the combined spectra, in the case of the Sb-L line and the Ca-K line, and also in the case of the Cd-L line and the U-M line (Fig. 13, -1 and -2). But the original value was recovered after the subtraction procedure of the mixed elements.

We have succeeded in separating and identifying each element from the combined X-ray energy spectrum with the aid of a computer system, using an X-ray luminescence analyzer and standard samples. We expect that this success in the basic experiments will produce the same results in the electron probe X-ray microanalysis of biological samples, but the counts of characteristic X-ray energy must be high enough for statistical calculation.

From these basic biological experiments, we succeeded in obtaining much good data. This data will be described in our next report in detail.

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REFERENCES