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ELECTRON ENERGY LOSS SPECTROSCOPY AND BIOLOGY

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Abstract

Although most of the recent work concerning EELS in electron microscopy is related to instrumental developments, there are a number of papers relating to biological applications. The aim of this paper is to attempt to present in a pedagogical manner the subject of EELS and biology. The biologist will be taught about EELS and the physicist about the possible biological applications of this technique. The paper thus consists of : 1) a presentation of a minimum background on EELS required for biologists to realize what its potential is ; 2) a description of why EELS is a useful tool in biology ; 3) a description of which kind of biological problems may be resolved through EELS ; 4) finally a survey relating to the problems encountered in applying EELS to biology, i.e., specimen preparation, beam damage and artefacts.

Key words: Microanalysis, electron energy loss spectroscopy, biological specimen, chemical mapping, unconventional image generation, premortem/postmortem xenobiotics, biological components, radiation damage.

Introduction

Many authors have presented the physics of electron energy loss spectroscopy (EELS) but nevertheless few of these papers have been written with biologists in mind. For experimental details the reader should consult, for example, Johnson (46,47). For the reader who is not attracted by a mathematical presentation, the following generalities are aimed at providing a sound background.

EELS consists of the analysis of energy of the electrons that have traversed the specimen. The majority of the electrons that have lost some energy have only lost a small amount (15 to 30 eV), if the specimen is thin. A typical spectrum is shown in Figure 1. This shows a very strong elastic (i.e., no energy loss) peak, which extends only over a few tenths of eV. Following this there is a broad feature in the 15 to 30 eV range and after this the spectrum decreases with increasing energy loss, and furthermore the slope of the spectrum decreases also. Thus, an exponential law is a good approximation to fit this curve (e.g., Egerton (19)). Briefly speaking, the biologist is concerned with :

. The main inelastic signal, given by the area under the spectrum (excluding the elastic peak). This is used for contrast enhancement techniques and for mass-thickness measurements.

. The absorption edges, which occur when the energy loss has a value corresponding to the ionization of atoms of one element present in the specimen. This is thus clearly characteristic of the elemental composition of the specimen, and this signal forms the basis of EELS microanalysis.

In addition to these two major aspects, there are other features, which although seldom actually used in biology could be promising :

. Absorption edges at very low losses (0-10 eV). These correspond to ionization of a weakly bonding electron and thus reflects, in a way, the molecular organisation of the specimen. This signal can be compared to a UV absorption spectrum (Hainfeld et al. (25)).

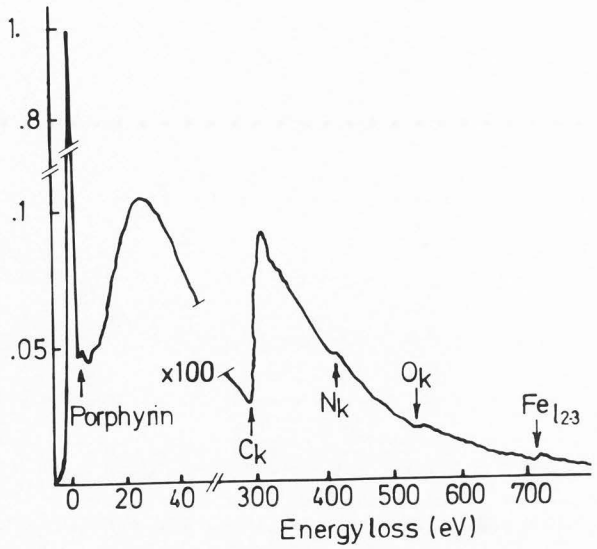

Figure 1

Electron energy loss spectrum of thin film of hematin on thin carbon foil, courtesy of F.P. Ottensmeyer (74). Three principal features are clearly seen :

1. The zero loss peak (energy loss : 0 eV ; relative intensity : 1.0).
2. The low loss region (energy range : from few to 40 eV ; relative intensity : 0.1).
3. The far loss region (energy range : few hundred eV ; relative intensity : 10^{-3}).

. For normal absorption edges (or elemental absorption edge), the shape of the spectrum both near the edge (Energy Loss Near Edge Fine Structure : ELNES) (37) and far from the edge (Extended Energy Loss Fine Structure : EXELFS) give information about the chemical environment of the atom studied, usually, in the form of a characteristic 'signature' for a given environment (32) (36).

In practice, the application of these last two signals to biological systems is at present limited by problems of radiation damage, since these are very weak signals requiring long counting times and hence a heavy dose.

Finally these various electron energy loss signals, if displayed for each pixel of the area analysed, produce a map of the corresponding information. The size corresponding to the image pixel can,in principle,be very small. This is the area from which a spectrum can be recorded. The first obvious limitation is the size of the electron probe, and for a FEG-STEM (Scanning Transmission Electron Microscope equipped with a Field Emission Gun) this can be as small as 0.5 nm. However, unfortunately both contamination and etching of the specimen set a lower limit on the area from which a spectrum can be recorded, and this is considerably larger than the smallest probe size. It is also worth mentioning that conventional electron microscopes can record energy filtered images if suitably equipped. Egerton (20) has recently reviewed the practical design of EELS for application.

Potential advantages of EELS in biology

The advantages of EELS in biology have already been the subject of a number of reviews Johnson (47-49); Egerton (20); Hainfeld (25) and appear also in more specialized papers (45) (37). The possiblity of analytical images has recently revived interest in EELS potential.

Here we will consider the following points, comparing them with other microanalytical techniques : high spatial resolution, light element analysis, high sensitivity, molecular analysis, contrast enhancement and analytical imaging.

High spatial resolution

Compared with X-ray microanalysis or Auger spectroscopy, EELS is free of fluorescence phenomena. The only physical process which could affect resolution is delocalisation, i.e., a fast incident electron can ionize an atom at a certain distance, which is typically less than 1 nm for an energy loss of 285 eV (K edge of C) ; this distance increases for decreasing energy loss, due to the nature of the inelastic process. However, this limitation is not important for biological applications where other causes of loss of spatial resolution are more severe. These are specimen preparation and beam damage. Nevertheless, despite this theoretical limitation in spatial resolution, EELS is a factor of about ten better in resolution compared to X-ray microanalysis. This factor is even more favourable for EELS compared to SIMS (Secondary Ion Mass Spectrometry) where the limitation is due to the strong aberrations in ion optics.

Light element analysis

With the sole exceptions of hydrogen and helium, all the elements can be analyzed with EELS, whereas electron probe X-ray microanalysis with a standard (windowed) Si-Li detector is limited to elements with $Z > 11$. This can be extended using window Si-Li detectors, but only to $Z > 5$.

High sensitivity with respect to X-ray microanalysis

With respect to X-ray microanalysis, EELS shows high sensitivity. This enhancement is related to two factors : (i) ionization is a primary effect, whereas X-ray emission is a secondary effect and only one of several mechanisms of de-excitation and (ii) for EELS the collection efficiency is high with value in the range of 0.5-1, while this is $\sim 10^{-3}$ for the efficiency in X-ray microanalysis.

Molecular analysis

At both the very low loss regions and the edge fine structure, EELS spectra show dependence on the local environment of the atom species considered, and hence are sensitive to molecular structure. Examples of such work are papers by Hainfeld et al. (25) who have recorded the low loss spectrum of cholesterol, lecithin and spectrin, and by Isaacson et al. (37) who have recorded ELNES features. In order to reduce the effect of beam damage these spectra were recorded on large areas of pure substance. Hainfeld et al. (25) show that some of the structure of the spectrum disappears as soon as the dose is increased to values of a few C/cm^2. Nevertheless, it should be borne in mind that, with the advent of parallel recording of the spectrum, which will increase signal to noise for the same dose, these techniques may be useful in biology in the near future. Otherwise, to the author's knowledge, there have been no applications other than to pure, isolated molecules. Similarly there would appear to have been no published biological application of the EXELFS technique. In addition to these points EELS is a useful tool for the study of radiation or beam damage. The measurement of mass thickness was first used to study mass loss in the microscope and energy loss spectra showed the selective sensitivity of some elements with regards to sublimation. For example, Isaacson et al. (33) showed that fluorine, and to a lesser extent oxygen, were more sensitive to the beam while carbon and oxygen remained in the specimen even after a considerable dose.

Unconventional image generation

Images are crucial to the biologist, since specimens may present structural variations on many different levels (tissue, cell, organelle and molecular). EELS instruments can provide different means of forming different inelastic images (15) or true elastic images (30).

One of the oldest techniques presently used is the so-called "Z-contrast" method, first suggested by Crewe et al. (18). This is also called "ratio-contrast" by some authors. In this case the image is produced by the division of the annular dark field signal by the inelastic, small angle, signal. The resulting image intensity is then, to a first approximation, linearly proportional to the Z in the specimen, and is therefore sensitive to variation in atomic number. This technique has proved useful in biology not only for the imaging of heavy atoms decorating an isolated molecule, but also for the imaging of the hydrophobic pole of protein membranes (12,13,23). Mass thickness measurements (55,57,75, 76) are the basis of quantitative microscopy. Although extensively used in STEM (Scanning Transmission Electron Microscope), they can also be applied to the CTEM (Conventional Transmission Electron Microscope) case, especially with the advent of new two-dimensional detectors. The same approach is used in thickness measurements (31) by assuming that the inelastic mean-free path * is the same in all the specimens considered. Recently the possibility of obtaining thickness-independent contrast, by calculating the elastic to inelastic cross-section ratio, has been pointed out, but this is still at a preliminary state (Egerton (21), Jeanguillaume (40)).

Analytical imaging

Over the last few years considerable effort has been spent on developing techniques of chemical mapping. Absorption core edges can, in suitable circumstances, localize an element with a resolution of up to 1 nm. The major problem, which has been pointed out by many authors, is that of background subtraction. If this is not properly carried out, then, since the signal intensity is typically 1/100 or 1/1000 of the inelastic intensity, the major features of such an image are given by mass-thickness variation. Hence considerable care is needed to avoid that a possible analytical map becomes simply a morphological image. It is for this reason that we believe with other authors, that the single subtraction of two images, one before and one after the edge is a method fraught with dangers (45,46,58,61,77). These are due to the fact that the energies used must be sufficiently different to avoid overlap, and that furthermore, in the medium range of energy loss (i.e., $\sim$ 100eV), the slope is high and hence the variation between the two different energies used will be considerable (62) (Fig. 2).

To demonstrate this last point we have published slope images (15)(41)(43) obtained by calculating the slope by fitting the background with an exponential law, as first proposed by Egerton (19). Although this model is relatively complicated we feel it should be used to calculate the background extrapolation below an edge, for each pixel of the image. Using this procedure we thus obtain chemical maps which do not exhibit "false-positive" features i.e., intensity at regions where the analyzed element is absent. However, this simplified treatment is not sufficient to obtain a truly quantitative chemical map, since this procedure does not take into account the effects of multiple elastic and inelastic scattering, nor does it consider variation in incident energy. Nevertheless, although these effects may modulate the final analytical image, they cannot lead to qualitative artefacts as other methods may.

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* A measure of the probability of an inelastic event. The shorter the mean-free-path the higher is the probability of interaction. It depends on the composition of the specimen, and on the primary beam energy.

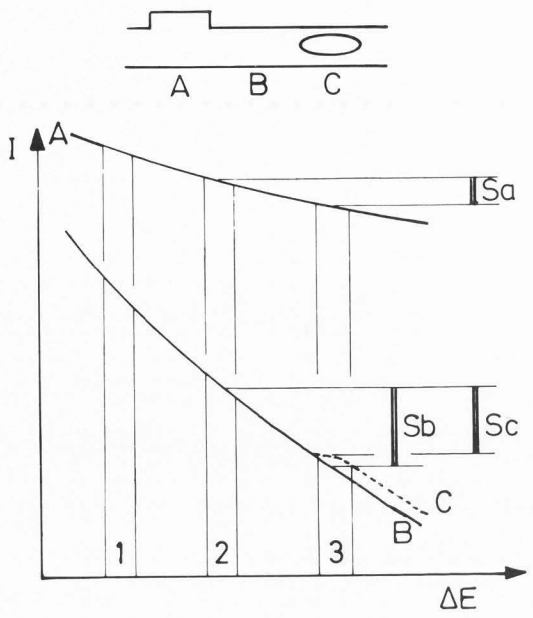

Figure 2

Schematic representation of a specimen with three different areas A, B and C. A deviates from the average matrix B section with a thickness step while C contains a small amount of the element of interest with no associated change in thickness. The problem is to discriminate chemical variations versus thickness ones. The corresponding model spectra in the energy loss of interest (around a typical edge for the C impurity) are shown below. Three energy windows of use for chemical mapping are chosen below (1) and (2) and above (3) the core edge. If one uses a two windows subtraction method between (2) and (3), one cancels to zero the pixel corresponding to the matrix area ($S_b = 0$). Consequently, the chemical signal S_c becomes a slightly positive value while the topographical one S_a becomes a strong positive one.

Biological problems that EELS has or could have resolved

Extraneous components or xenobiotics

By this, we mean chemical substances which are not normally present in the biological specimen. These could arise or be introduced either during the life of the cell or post mortem.

Pre-mortem xenobiotics

This is an important field of investigation and much work remains to be done. These types of studies are of great importance in biological and medical research, and this is unlikely to diminish. For example, these could be part either of pathological studies after pollutant exposure or quasi-physiological accumulation dependent on the environment, (e.g., some algae or bacteria are known to concentrate barium or lead), or of forensic science, therapeutic kinetics and surgical implant reactions. For many of these applications, EELS could analyze the substance of interest, localize it within the specimen at the nanometer scale and eventually link this with morphological aspects, as well as give some information concerning the time dependence of this localization and the associated substance involved in the response by the organism. Some examples of application can be found in Table 1 ; these relate to the study of biological sections.

The lung is generally the first organ exposed to pollution agents, and hence there have been many studies using X-ray microanalysis. However, beryllium, which cannot be detected by standard X-ray microanalysis is easy to detect by EELS, and was therefore the first to be analyzed by this method. (52) Another example concerns the ferrugineous sheath of iron hydroxide which surrounds asbestos fibers in the human lung and which is a typical example of response from the organism. Both the sheath (Fe and O) and the various pollutants (Si, O, C..) can be analyzed in a human biopsy (Leapman et al. (56), Jeanguillaume et al. (41)). The kidney is also often involved in concentrating xenobiotics and many elements (Be, Ag, O, F, Fe, Ga, U) have been found in tubular cells and Bowman capsules (Mignon-Conte et al. (65), Berry et al. (8)).

Experimental pharmacology can be illustrated by the analysis of titanium in Ehrlich ascites tumor cells following a treatment with the antitumor agent titanocene dichloride (cited in Egerton (20)). The cisplatinum (cisdiamino dichloroplatinum) and other antitumor agents can also be studied by EELS (Hawkes (27)). The location of more common substances in the organism can also be studied by coupling them with xenobiotic tracers. Although the method assumes that the marked substance behaves like the non-marked one it can yield important results. Joy et al. (51)(17) have shown that the capture of dihydroserotonin by platelets can be studied by this method using a fluorinated compound.

Post-mortem xenobiotics

Following the examples of optical histochemistry, one can imagine reactions involving xenobiotics components, which may then help in the understanding of cellular architecture. Although there has been little work in this field, it seems potentially fruitful, and the considerable choice of tracers coupled with different biological activity shows that such methods have great scope. Lechene, Jeanguillaume, Trebbia, Tencé (Unpublished work) have developed a method in which they demonstrate various phosphatase activities using a cerium deposit; we have shown that EELS can readily produce good quality maps. Various stains, less characteristic than the latter, such as uranyl acetate or calcium dichloride, have been detected and again show the potential of the method (67,45). We can compare these post mortem dyes with phenomena typical of fossils, and although there are no published EELS results, other analytical techniques such as

X-ray and secondary ion emission microanalysis have produced spectacular results in this field.

Biological components

As we have seen previously, these can be detected by indirect methods and here we will consider the methods for direct detection.

Diffusible elements

These unfortunately present special problems of rapid fixation which have not yet been completely resolved. Even worse, certain elements (Na, K, Cl) have a tendency of evaporating under electron beam irradiation and both EDX or SIMS appear to be more appropriate techniques for this kind of problem. However, calcium is one element which appears to be easy to detect by EELS, and hence calcium distribution has been widely studied, notably in muscle by Shuman H., Somlyo A.V. and Somlyo A.P. (79,81,84,85,86,87,90). These authors have shown that a high concentration of calcium occurs in the terminal cisternae.

Non-diffusible elements

The main elements considered here are of course C, N and O and it is surprising that there are no extensive studies, in spite of promising results. It is also surprising that there has been no extensive work on the ratios of these elements since these could give indications on the identification of fundamental compounds (protids, lipids and glucids). Nitrogen is easy to detect and its presence is usually related to proteins or nucleic acid. This has been demonstrated in the case of secretory granules of pancreas cells (Leapman (58)), of chromaffin cells (Leapman (60)), in chromatin in the crystalloid of eosimophil cells (Jeanguillaume (42)(43) and unpublished work) and in other cases where the localization is not always clearly related to a known organelle.

Carbon and oxygen images may be more difficult to analyze since these elements are present in the embedding medium (cryosections should be of importance in this area) and carbon images seem to show smaller variations than nitrogen images. Hence possible contrast from carbon variation may be masked by residual mass thickness contrast and scattering fluctuations which may still remain in these images. In any case it is still possible to observe a reinforcement of the carbon signal in regions of high lipid concentration, for exemple (Leapman (61), Jeanguillaume (43)). Of these three major constituent elements, oxygen is the most beam sensitive. Nevertheless, the feasibility and the use of oxygen images have been demonstrated.

Paradoxically, it is other elements which are not major constituents which have been more completely studied, with iron in the ferritin molecule as the most popular. The bulk of this work has been carried out on molecules deposited on a thin carbon film (Table 2) and images from these show the iron core and the protein shell. It is however also possible to detect and image the ferritin in thin sections ($\sim$ 500 Å) of tissue embedded in epon. (Jeanguillaume (42)(43)). Phosphorus is also of particular interest, since it is assumed to be present in phospholipid and nucleic acids. In our own experience, however, it is difficult to detect these compounds due to their relatively low concentration. As we have previously mentioned, elemental mapping at high spatial resolution requires a higher dose than for spectrum acquisition. Nevertheless, many applications of elemental mapping of phosphorous have already been published, without the spectrum showing a phosphorus peak. While the biological interest of such images is obvious, the physical interpretation in terms of a real phosphorus signal remains doubtful. On the other hand, phosphorus in mineralised tissues can be detected in a more realistic manner, as demonstrated by the published spectra (Arsenault (73)). Similarly, calcium and sulfur have also been detected by many authors in such tissues. Undoubtedly, a true sulfur map should yield valuable results, since this element is known to occur in proteins like proteoglycan or collagen.

Of course, molecular analysis by low loss studies, ELNES or EXELFS should provide much information, but some progress is again needed before application.

Specimen preparation techniques

Since, up to now, EELS has not been widely used in biology, only a few methods of specimen preparation are documented. The usual techniques for preparing specimens for transmission electron microscopy (TEM) are the most used. Glutaraldehyde fixed epoxy resin embedded materials has been shown to be particularly useful (Table 1). This simple method, however, has disadvantages : many constituents are leached out during the process (diffusible ions and also organic compounds) especially during fixation. Furthermore, it would be preferable to use a more conservative method avoiding a wet phase. The cryotechnique, in this regard, appears to be very promising. However since this technique requires substantial effort and involves technical difficulties, it has not been widely used in the context of EELS applications in biology. It can also be considered that this technique has not yet reached full maturity. There are still many research papers on the technique, technical advances from mixing "cold" and "warm" methods are being made, and finally there remain points concerning artefacts and other problems still to be resolved. Concerning these difficulties, one can simply note ice crystal damage, thawing and recrystallization artefacts, thin sectioning, spreading the section on the grid and finally very low contrast in the resulting images, together with lack of reference to the morphological image. The latter point, however, should be solved through the use of EELS.

Nevertheless it should be noted that this technique has been used in the study of calcium localization in striated muscle (79,81,84,86,87).

TABLE 1 - Core Loss in Section

Specimen	Element(s)	Fixation	Embedding medium	Image spectrum	References
Murine Leukemia virus	$P(L_{23})$	glut		1 + 1	Adamson Sharpe (1) Ottensmeyer (72, 74)
Larval lobster	$Ca(L_{23})$ $P(L_{23})$	glut	TAAB	1 + 1	Arsenault (2)
Homarus americanus	$S(L_{23})$	glut			Ottensmeyer (68, 73)
Epiphyseal growth plates femur of mice	Ca, P, S	glut	Spurr	1 + 1	Arsenault (3, 4, 5) Ottensmeyer (71)
Human lung Alveolar macrophage	$Al_{(K)}O_{(K)}P_{(K)}$	glut	epoxy	S	Berry (8) Kihn (52)
Human kidney Tubular cell	$Ga(L_{23})$ $U(N_{23})$	glut	epoxy	S	Berry (8) Kihn (52)
Human kidney	$BeAg(M_{45}-M_{23})$ O,I,Fe,Si	glut	epoxy	S	Kihn (52) Mignon-Conte (65)
Cultural tumoral cell	$Ni(L_{23})$	glut	epoxy	S	Berry (8), Kihn (52)
Larval Calpodes Ethlius	P	glut + OsO_4		1 + 1	Brodie (10, 11)
Eschericia coli	Ca C,U,P	glut	epoxy		Chang (14) Jeanguillaume (45)
Pancreas α ; β cell	N	freeze dried		5 + 5	Gorlen (24) Leapman (56, 58)
House cricket Acheta domesticus	$Ga,In,Pt(M_{45})$	glut	epoxy	S	Hawkes (27)
Garden dormouse Nuclear inclusions of the epididymis Epithelial cell	$Mg_{(K)}P_{(K)}S_{(K)}$ Fe				Hawkes (28)
Schistocerca gregaria Acheta domesticus (Testis)	$CdM_{23}M_{45}$	glut + OsO_4	epoxy	S	Hawkes (29)
Human lung - ferrugineous sheath	C,Fe,O C	glut	epoxy	3 + 2 5 + 5	Jeanguillaume (41) Leapman (56)
Human bone	Ca	glut	epoxy	S	Jeanguillaume (44)
Human bone marrow	C,O,N,Fe	glut	epoxy	2 + 1	Jeanguillaume (42, 43)
Hela cells	U,P			S	Joy (50), Kihn (52)
Mineralizing enamel	Ca,N			5 + 5	Leapman (59)
Neurons	C,N			5 + 5	Leapman (60)
Mineralizing cartilage	C,Ca			5 + 5	Leapman (60)
Cultured bovine chromaffin cell Guinea pig Leukocyte	C,N Ce	freezing glut	HM20 epoxy	5 + 5 3 + 2	Leapman (61) Lechene (unpublished)
Thalassiosira pseudonana	P			1 + 1	Ottensmeyer (68)
French bean plant cell	Si,O,C			1 + 1	Ottensmeyer (69)

TABLE 1 - Core Loss in Section - continued from facing page

Specimen	Element(s)	Fixation	Embedding medium	Image* spectrum	References
Insect fat body ribosome	P			1 + 1	Ottensmeyer (69)
Cultured osteoblast	C,O,P,S,Ca	glut + OsO_4		1 + 1, S	Ottensmeyer (70, 72)
Chondrocytes	P				Ottensmeyer (70)
Human peripheral nerve	P			1 + 1	Ottensmeyer (72)
Frog atrial muscle	Ca			1 + 1	Ottensmeyer (72)
Frog semitendinosus muscle	Ca,N,C P(K,L_{23})	freezing			Shuman (79, 81, 84, 86) Somlyo (87)
Bacillus coagulans	Ca				Shuman (83)
Portal anteriormesenteric vein smooth muscle	Ca,C			1 + 1	Shuman (83)
Bacterial platelet dense bodies	C,Ca,N,O,P				Leapman (64)
Rabbit Lens	Ca	glut	epoxy	2 + 1	Wroblewski (unpublished)

*S = Spectrum 1 + 1 = one filtered image taken before the edge and one after 5 + 2 = five images before the edge and two after

Table 2 - Sample deposited on very thin support films

Specimen	Element	Preparation	Image / Spectra*	References
Chromatin of calf thymus	P	glut + c.p.d.**	1 + 1	Bazett-Jones (6, 7) Harauz (26) Ottensmeyer (68)
Ribosomes	P	uranyl acetate + c.p.d.	1 + 1	Boublik (9)
	P		1 + 1	Korn (53)
	P		1 + 1	Ottensmeyer (68, 70)
	P		1 + 1	Shuman (86)
	P		1 + 1	Somlyo (89)
5 fluoro cytosine 5 trifluoromethyl uracil	C, N, O, F		S	Isaacson (33)
Ferritin	Fe (M_{23})		S	Isaacson (37)
	Fe (M_{23}) C		1 + 1	Shuman (82, 86)
	Fe (M_{23}) O		1 + 1	Somlyo (89) Hainfeld (25)
Nucleic acid bases	C, N, O		S	Johnson (48) Isaacson (37, 39) Misra (66)
Blood Platelet	F	Air dried	+ 1 S	Costa (17) Joy (51)
Amino acid	C, N, O, S		S	Misra (66)
Influenza virus	Cl (L_{23})	$CaCl_2$	2 + 1	Oikawa (67)
Hematin	C, N, O, Fe		S	Ottensmeyer (69, 74)

**c.p.d. = critical point drying *see foot note to table 1

Deposition of molecules or organelles on very thin support film has the advantage of reducing multiple scattering (Table 2). This has been widely used in particular in the study of ferritin molecules. The technique of deposition could also involve (at least in theory) other preparative techniques such as cryofixation or critical point drying methods in order to reduce surface tension artefacts.

Radiation damage

EELS techniques require greater electron doses than the usual methods of morphological observation. The minimal dose required to detect a given element is high, due to the very small ionization cross-section (typically 10^{-22} cm^2) and because of the statistical fluctuations in the background (16). For example Shuman (82) used 1.8×10^5 c/m^2 to detect 10^{-20} g of Fe ($\sim$ 5000 atoms). This dose will be greater if the element to be detected is localized in a small area or if we have to detect fluctuations in concentration. It should be noted, as a rule of thumb, that to increase the resolution of chemical mapping by a factor 10, the dose has to be increased by a factor 100.

As a result of this, many biologists are distrustful about EELS, even more so since fading of electron diffraction patterns occurs for smaller doses typically, e.g. $D_{1/e} = 5.4$ c/m^2 for the fading at 0.44 $Å^{-1}$ of a valine crystal (66). ($D_{1/e}$ = dose required for reducing by a 1/e factor a representative signal of the specimen disappearing under the irradiation). However, a crude comparison of these values is not justified, and microscopists who are convinced that such a dose must not be exceeded are not always right. One must bear in mind that electron microscopy is a necropsic method and the specimen observed will reflect, to a greater or lesser extent, the living organism. The danger which must be avoided is to interpret the changes occurring during preparation or observation as biological features. Hopefully as the methods all require transformation of the specimen, they may also provide the means of assessing the effect of the transformation (22,34,35, 38,78) EELS, like all other microanalytical methods, has its own limitation depending both on the element analyzed and the specimen. In other words, chemical maps would be totally uninterpretable if the electron beam irradiation had led to drastic translocation. In our experience, and in agreement with published work, this is not the case since only mass-loss has been demonstrated, on the scale studied up to now. Thus for quantitative analysis, it is necessary to take this into account. Fortunately, the dose necessary to lead to observable mass loss is greater than required to lead to fading of the diffraction pattern. It should be emphasized that EELS provides an easy method of measuring mass loss and moreover allows the measurement of a specific mass loss.

Organic fluorine is one of the most volatile elements, i.e. :

$$D_{1/e} \sim 1 \text{ C/m}^2 \text{ at } 43.5 \text{ keV} \qquad (1)$$

as measured by Isaacson et al. (33) on an evaporated film of 5-fluorocytosine. Nitrogen would appear to be more resistant with :

$$D_{1/e} \sim 2400 \text{ C/m}^2 \qquad (2)$$

from the work of Misra-Mano and Egerton (66) on thin films of adenine at 80 kV. However the nature of the sample strongly influences these values. For instance, in cytosine the value of $D_{1/e}$ falls to 192 c/m^2 for nitrogen loss. However, it has been shown that decreasing the accelerating voltage of the incident beam and decreasing the specimen temperature leads to increased stability. Furthermore it has been demonstrated that a thin covering layer of carbon increases the value of $D_{1/e}$.

Finally, parallel recording of the EELS spectrum will improve the detection efficiency for a given dose considerably (54,80).

Artefacts

The most important effects are due to the specimen preparation techniques, as mentioned previously. EELS as such, is not fraught with numerous artefacts, for example, the technique is completely free of any fluorescence effect. The effects of delocalized interaction are slight as we have seen. Spurious peaks in the EELS spectra can be caused by reflections of the electron beam on the microscope column walls, but this effect can be controlled with a suitably smaller aperture.

Conclusions

The EELS technique has been a research tool for a long time now but is increasingly becoming useful to the biologist. The current efforts by many groups to enhance the sensitivity of the method, notably through the development of parallel spectrum detection should, in the near future, make this technique truly promising.

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References

(1) Adamson-Sharpe K.M., Ottensmeyer F.P. (1981) - Spatial resolution and detection sensitivity in microanalysis by electron energy loss selected imaging. J. Microscopy 122, 309-314

(2) Arsenault A.L., Castell J.D., Ottensmeyer F.P. (1984) - The dynamics of exoskeletal-epidermal structure during molt in juvenile lobster by electron microscopy and electron spectroscopic imaging. Tissue and Cell 16, 93-106.

(3) Arsenault A.L., Ottensmeyer F.P. (1984) - Stereoscopic representation of complex overlapping elemental maps in electron spectroscopic images. J. Microscopy 133, 69-72.

(4) Arsenault A., Ottensmeyer F.P. (1984) - Visualization of early intramembranous ossification by electron microscopic and electron spectroscopic imaging. J. Cell Biol. 98, 911-921.

(5) Arsenault A.L., Ottensmeyer F.P. (1983) - Quantitative spatial distribution of calcium, phosphorus and sulfur in calcifying epiphysis by high resolution spectroscopic imaging. Proc. Natl. Acad. Sci. USA 80, 1322-1326.

(6) Bazett-Jones D.P., Ottensmeyer F.P. (1982) - DNA organization in nucleosome. Canadian J. Biochemistry 60, 364-370.

(7) Bazett-Jones D.P., Ottensmeyer F.P. (1981) - Phosphorus distribution in the nucleosome. Science 211, 169-170.

(8) Berry J.P., Galle P., Kihn Y., Zanchi G., Sevely J., Jouffrey B. (1984) - Application de la spectroscopie de pertes d'énergie d'électrons à haute tension à l'analyse de microparticules dans des coupes de tissus biologiques. IXCOM 10, Toulouse, 1983. J. de Phys. Coll. C2 suppl. n° 2, 45, 581-584.

(9) Boublik M., Oostergetel G.T., Frankland B., Ottensmeyer F.P. (1984) - Topographical mapping of ribosomal RNAs in situ by electron spectroscopic imaging. Proc. 42nd Ann. Meet. EMSA, G.W. Bailey (ed.), San Francisco Press, pp. 690-691.

(10) Brodie D.A., Locke M., Ottensmeyer F.P. (1982) High resolution microanalysis for phosphorus in Golgi complex beads of insect fat body tissue by electron spectroscopic imaging. Tissue and Cell 14, 1-11.

(11) Brodie D.A., Huie P., Locke M., Ottensmeyer F.P. (1982) - The correlation between bismuth and uranyl staining and phosphorus content of intracellular structures as determined by electron spectroscopic imaging. Tissue and Cell 14, 621-627.

(12) Carlemalm E., Colliex C., Garavito M.R., Villiger W., Acetarin J.D., Kellenberger E. (1982) - Imaging of unstained thin sections from resin embedded biological material using Z-contrast. Electron Microscopy 1982, vol. I, 605-606.

(13) Carlemalm E., Kellenberger E. (1982) - The reproducible observation of unstained embedded cellular material in thin sections : visualisation of an integral membrane protein by a new mode of imaging for STEM. The EMBO J. 1, 63.

(14) Chang C.F., Shuman H., Somlyo A.P. (1984) - Electron probe analysis, X-ray mapping and electron energy loss spectroscopy of elemental distribution in escherichia coli B. 42nd Ann. Proc. EMSA, G.W. Bailey (ed.), San Francisco Press, 400-401.

(15) Colliex C., Jeanguillaume C., Mory C. (1984)- Unconventional modes for STEM Imaging of Biological Structures. J. Ultrastructure Research 88, 177-206.

(16) Colliex C., Trebbia P., (1979) - Quantitation and detection limits in electron energy loss spectroscopy (EELS) of thin biological sections. in Microbeam analysis in biology (ed.) Lechene C.P., Warner R.R., Academic Press, 99, 109.

(17) Costa J.L., Joy D.C., Maher D.M., Kirk K.L., Hui S.W. (1978) - Fluorinated molecule as a tracer : difluoroserotonin in Human Platelets mapped by Electron Energy Loss Spectroscopy, Science 200, 537-539.

(18) Crewe A.V., Langmore J.P., Isaacson M.S.(1975) Resolution and contrast in the STEM. In Siegel: Physical Aspects of Electron Microscopy and Microbeam Analysis, p. 47-62, Wiley, New-York.

(19) Egerton R.F. (1975) - Inelastic scattering of 80 keV electrons in amorphous carbon. Phil. Mag. 31, 199-215.

(20) Egerton R.F. (1982) - Electron energy loss analysis in biology. Electron Microscopy 1982, vol. I, 151-158.

(21) Egerton R.F. (1982) - Thickness dependence of the STEM ratio image. Ultramicroscopy 10, 297-299.

(22) Egerton R.F. (1980) - Chemical measurements of radiation damage in organic samples at and below room temperature. Ultramicroscopy 5, 521-523.

(23) Garavito R.M., Carlemalm E., Colliex C., Villiger W. (1982) - Septate junction ultrastructure as visualized in unstained and stained preparations. J. Ultrastruc. Res. 80, 344-353.

(24) Gorlen K.E., Barden L.K., Del Priore J.S., Fiori C.E., Gibson C.C., Leapman R.D. (1984)- A computerized analytical electron microscope for elemental imaging. Rev. Sci. Instrum. 55 912-921.

(25) Hainfeld J., Isaacson M. (1978) - The use of Electron Energy Loss Spectroscopy for Studying membrane architecture : a preliminary report. Ultramicroscopy 3, 87-95.

(26) Harauz G., Ottensmeyer F.P. (1984) - Nucleosome reconstruction via phosphorus mapping. Science 226, 936-940.

(27) Hawkes F. (1984) - Platinum, gallium and indium distribution in insect testi. An EELS study in the house cricket acheta domesticus. IXCOM 10, Toulouse, 1983, J. de Phys. Suppl. n° 2, 43, 589-593.

(28) Hawkes F., Kihn Y., Sevely J. (1984) - An EELS study of the intranuclear inclusions of the epididymis principal cells in the garden dormouse elionys quercinus. IXCOM 10, Toulouse, 1983, J. de Phys. suppl. n° 2, 43, 585.

(29) Hawkes F. and Wu Ming Jun (1984) - Cadmium distribution in insect testis after a single injection. An EELS study. IXCOM 10, Toulouse, 1983, J. de Phys. Suppl. n° 2, 43, 587.
(30) Henkelman R.M., Ottensmeyer F.P. (1974) - An energy filter for biological electron microscopy. J. Microscopy 102, 79-94.
(31) Hosoi J., Oikawa T., Inoue M., Kokubo Y., Hama K. (1981) - Measurement of partial specific thickness (net thickness) of critical point dried cultured fibroblast by energy analysis. Ultramicroscopy 7, 147-154.
(32) Isaacson M. (1979) - Electron Energy Loss Spectroscopy within the electron microscope. Where are we ? in : Microbeam analysis in biology (eds.) C.P. Lechene and R.R. Warner, Academic Press, 53, 61.
(33) Isaacson M. Collins M.L., Listvan M. (1978) - Electron beam damage of biomolecules assessed by energy loss spectroscopy. Proc. Microscopical Society of Canada, Toronto, vol. III, 61-69.
(34) Isaacson M.S. (1975) - Inelastic scattering and beam damage of biological molecules, in : Physical aspects of electron microscopy and microbeam analysis. Siegel B. and Beaman D. (eds), John Wiley and Sons, chap. 14, 247-258.
(35) Isaacson M. (1977) - Specimen damage in the electron microscope, in : Principles and techniques of electron microscopy, (ed.) Hayat M.A., Vol. 7, 1, 78.Van Nostrand,NY.
(36) Isaacson M. (1977) - Some thoughts on Electron Energy Loss Spectroscopy (EELS). Within the Electron Microscope : where does it stand and where is it going ? Proc. Microbeam Anal. Soc. Mtg, Boston Mass., Henrich K.F., National Bureau of Standards, Washington D.C., pp. 18a-18e.
(37) Isaacson M. and Johson D. (1975) - Low Z Elemental analysis during energy loss electrons. Ultramicroscopy 1, 33-52.
(38) Isaacson M.S., Johnson D. and Crewe A.V. (1973) Electron beam excitation and damage of biological molecules ; its implications for specimen damage in electron microscopy. Rad. Res. 55, 205-224.
(39) Isaacson M. (1972) - Interaction of 25 keV electrons with the nucleic acid bases, Adenine, Thymine and Uracil I - II - Outer and inner shell excitation and inelastic scattering cross section. J. Chem. Phys. 56, 1803-1818.
(40) Jeanguillaume C. (1986) - Mean free path ratio: a new approach of no thickness dependence contrast in STEM. Proc. XIth ICEM, Kyoto, The Japanese Society of electron microscopy, Tokyo, 433-434.
(41) Jeanguillaume C., Berry J.P., Colliex C., Galle P., Tencé M. and Trebbia P. (1984) - Recent results in EELS : elemental mapping of thin biological sections. J. Physique 45, C2-577.
(42) Jeanguillaume C., Tencé M., Trebbia P., Colliex C. (1983) - Electron energy loss chemical mapping of low Z elements in biological sections. Scanning Electron Microsc. 1983 ; II : 745-756.
(43) Jeanguillaume C. (1982) - Quelques développements récents de la spectroscopie de pertes d'énergie en STEM. Thèse de spécialité, Orsay.
(44) Jeanguillaume C., Krivanek O., Colliex C. (1981) - Optimum design and use of homogeneous magnetic field spectrometers in EELS. Inst. Phys. Conf. n° 61, chapt. 4, 189.
(45) Jeanguillaume C., Colliex C., Trebbia P. (1978) - About the use of electron energy loss spectroscopy for chemical mapping of thin foils with high spatial resolution. Ultramicroscopy 3, 137-142.
(46) Johnson D.E. (1979) - Energy loss spectrometry for biological research, in : Introduction to Analytical Electron Microscopy, Hren J.J., Joy D.C. (ed.), Plenum, New-York, London, 245-258.
(47) Johnson D.E. (1979) - Electron energy loss microanalysis of biological material, in : Microbeam analysis in biology (ed.) C.P. Lechene, R.R. Warner, Academic Press, 99,109.
(48) Johnson D. (1972) - The interactions of 25 keV electrons with guanine and cytosine. Rad. Res. 49, 63-84.
(49) Johnson D.E. (1977) - The uses of electron energy loss spectrometry in biology. Proceedings of the 35th EMSA Mtg, 236-237, G.W. Bailey (ed.), Claitor's Publ. Div., Baton-Rouge, LA.
(50) Joy D.C. (1982) Summary 9th Annual Meeting Micr. Soc. Canada, Microscopical Society of Canada (ed.).
(51) Joy D.C., Maher D.M. (1979) - A practical electron energy loss spectrometer, in : Microbeam analysis in biology. (eds.) C.P. Lechene, R.R. Warner, Academic Press, 87-97.
(52) Kihn Y. (1985) - Contribution à l'exploitation qualitative des spectres de pertes d'énergie des électrons à très haute tension. Thèse d'Etat n° 1211, Toulouse.
(53) Korn A.P., Spitnik-Elson P., Elson D., Ottensmeyer F.P. (1983) - Specific visualization of ribosomal RNA in the intact ribosome by electron spectroscopic imaging. Eur. J. Biochem. 31, 334-340.
(54) Kruit P., Shuman H., Somlyo A.P. (1984) - Detection of X-rays and electron energy loss events in time coincidence. Ultramicroscopy 13, 205-214.
(55) Lamvik M.K. and Langmore J.P. (1977) - Determination of particle mass using scanning transmission electron microscopy, Scanning Electron Microsc. 1977 ; I : 401-409.
(56) Leapman R.D., Fiori C.E., Gorlen K.E. (1987)- Elemental imaging by EELS and EDXS in the analytical electron microscope : its relevance to trace element research, Biological Trace Element Research, in press.
(57) Leapman R.D., Fiori C.E., Swyt C.R. (1984) - Mass thickness determination by electron energy loss for qualitative X-ray microanalysis in biology. Journal of Microscopy 133, 239-253.
(58) Leapman R.D., Fiori C.E., Gorlen K.E., Gibson C.C., Swyt C.R. (1984) - Combined elemental and STEM imaging under computer control. Ultramicroscopy 12, 281-292.

(59) Leapman R.D., Gorlen K.E., Swyt C.R. (1985) - Digital processing of electron energy loss spectra and images, Scanning Electron Microsc. 1985 ; I : 1-13.

(60) Leapman R.D., Gorlen K.E., Swyt C.R. (1984) - Background subtraction in STEM energy loss mapping. 42nd Ann. Proc. Electron Microsc. Soc. Am. (ed.), G.W. Bailey, San Francisco Press, 568-569.

(61) Leapman R.D., Ornberg R.L. (1983) - Low atomic number imaging of cells by electron energy loss spectroscopy. Proc. 41st EMSA Mtg, Claitor's publ. Div., Baton-Rouge, LA, 590-591.

(62) Leapman R.D., Swyt C.R. (1983) - Electron Energy Loss imaging in the STEM : systematic and statistical errors,in : Microbeam Analysis 1983 (ed.) R. Gooley, San Francisco Press, 163-167.

(63) Leapman R.D., Swyt C.R. (1981) - Electron Energy Loss spectrometry under conditions of plural scattering, in : Analytical Electron Microscopy, 1981 (ed.) R.H. Geiss, San Francisco Press, 164-172.

(64) Leapman R.D., Swyt C.R. (1981) - Microanalysis of calcium and phosphorus in biology using electron energy loss spectroscopy. Proc. EMSA, 1981, 636-637, G.W. Bailey (ed.), Claitor's Publ. Div., Baton Rouge, LA.

(65) Mignon-Conte H., Carentz F., Pourrat J., Conte J.J., Kihn Y., Sevely J. (1984) - Utilisation de la spectroscopie de pertes d'énergie d'électrons à l'identification de dépôts intra-rénaux. IXCOM 10, Toulouse, 1983. J. Physique suppl. n° 2, 43, 603-606.

(66) Misra-Mano J., Egerton R.F. (1984) - Assessment of electron irradiation damage to biomolecules by electron diffraction and electron energy loss spectroscopy. Ultramicroscopy 15, 337-344.

(67) Oikawa T., Sasakai H., Matsuo T., Kokubo Y. (1982) - Elemental filtergrams obtained by means of electron energy analyzer combined with image storage system. 40th EMSA Mtg. 736-737. G.W. Bailey (ed.), Claitor's publ. Div. Bâton Rouge, LA.

(68) Ottensmeyer F.P. (1984) - Electron spectroscopic imaging : parallel energy filtering and microanalysis in the fixed beam electron microscope. J. Ultrastr. Res. 88, 121-134.

(69) Ottensmeyer F.P. (1982) - Scattered electrons in microscopy and microanalysis. Science 215, 461-466.

(70) Ottensmeyer F.P. (1984) - Energy selecting electron microscopy, in : Electron Optical Systems, 245-251, SEM Inc. AMF O'Hare (Chicago), Proc. of the 3rd Pfefferkorn Conference, Ocean City, MD, J.J. Hren et al. (eds.).

(71) Ottensmeyer F.P. (1982) - Quantification in elemental distributions of light atoms by EELS image analysis in biological sections. 40th EMSA Mtg, 420-423, Claitor's publ. Div. Bâton-Rouge, LA.

(72) Ottensmeyer F.P., Andrew J.W. (1980) - High resolution microanalysis of biological specimens by electron energy loss spectroscopy and by electron spectroscopic imaging. J. Ultras. Res. 72, 336-348.

(73) Ottensmeyer F.P., Arsenault A.L. (1983) - Electron spectroscopic imaging and Z contrast in tissue sections. Scanning Electron Microsc. 1983 ; IV : 1867-1875.

(74) Ottensmeyer F.P., Bazett-Jones D., Adamson-Sharp K. (1981) - Electron energy loss microanalysis with high spatial resolution. Energy resolution and sensitivity. in : Microprobe analysis of biological systems. T.E. Hutchinson, A.P. Somlyo (eds.), Academic Press, 309-319.

(75) Reichelt R., Carlemalm E., Villiger W., Engel A. (1985) - Concentration determination of embedded biological matter by scanning transmission electron microscopy, Ultramicroscopy 16, 69-80.

(76) Reichelt R., Engel A. (1984) - Monte Carlo calculations of elastic and inelastic electron scattering in biological and plastic materials. Ultramicroscopy 13, 279-294.

(77) Rez P., Ahn C. (1982) - Computer control for X-ray and energy cross line profiles and images. Ultramicroscopy 8, 341-350.

(78) Schnabel H. (1980) - Does removal of hydrogen change the electron energy loss spectra of DNA bases ? Ultramicroscopy 5, 147.

(79) Shuman H. (1981) - Parallel recording of electron energy loss spectra. Ultramicroscopy 6, 385-396.

(80) Shuman H., Kruit P. (1985) - Quantitative data processing of parallel recorded electron energy loss spectra with low signal to background. Rev. Sci. Instr. 56 (2), 231-239.

(81) Shuman H., Kruit P., Somlyo A.P. (1983) - Quantitative electron energy loss spectroscopy of low concentrations of calcium in carbon containing materials, in : Microbeam analysis 1983, (ed.) R. Gooley, San Francisco Press, 247-451.

(82) Shuman H., Somlyo A.P. (1982) - Energy filtered transmission electron microscopy of ferritin. Proc. Natl. Acad. Sci. USA 79, 106-107.

(83) Shuman H., Somlyo A.P. (1981) - Energy filtered "conventional" transmission imaging with a magnetic sector spectrometer, in : Analytical Electron Microscopy, Geiss R. (ed.) 202-204, San Francisco Press.

(84) Shuman H., Somlyo A.V., Somlyo A.P. (1981) - Electron energy loss analysis in biology : application to muscle and a parallel collection system, in : Microprobe analysis of biological systems, (eds.) T.E. Hutchinson, A.P. Somlyo, Academic Press, 273-286.

(85) Shuman H., Somlyo A.V., Somlyo A.P. (1980) - Electron energy loss analysis and its biological applications with special reference to muscle. Wittry D.B. (ed.) Microbeam Analysis (1980) San Francisco Press, 275-280.

(86) Shuman H., Somlyo A.V., Somlyo A.P., Frey T., Safer D. (1982) - Energy loss imaging in biology. 40th Ann. Proc. Electron Microscopy Soc. Amer., Bailey G.W. (ed.) 416-419. Clairtor's publ. Div. Bâton Rouge, LA.

(87) Somlyo A.P. (1985) - Cell calcium measurement with electron probe and electron energy loss analysis. Cell Calcium 6, 197-212.

(88) Somlyo A.P. (1984) - Compositional mapping in biology : X-rays and electrons. J. Ultrastruc. Res. 88, 135-142.
(89) Somlyo A.P., Shuman H., Somlyo A.V. (1982) - X-ray mapping, electron energy loss analysis and quantitative electron probe analysis in biology. Proc. 10th ICEM, Hamburg, 17/24 August. Deutsche Gesellschaft Elektronenmikroskopie, 143-150.
(90) Somlyo A.V. and Shuman H. (1982) - Electron probe and electron energy loss analysis in biology. Ultramicroscopy 8, 219.

Discussion with Reviewers

G.T. Simon : Could you explain how the main elastic signal is used for contrast enhancement and for mass thickness measurements ?
Author : This is a very good question because many people seem to be confused about it. The different signals available in an electron microscope can be used in several ways, according to their intensity and to their possible combinations. To give a brief account of the subject, the main elastic peak which should rather be called "unscattered signal" can be used for contrast enhancement when compared to the whole unfiltered bright field signal which simultaneously contains an inelastic contribution. With a few more assumptions (such as linear mass dependence of the mean free path, knowledge of the efficiency factor), it can be used alone or combined with another signal to perform mass thickness measurements.

G.T. Simon : Could you explain how absorption edges at very low losses (0-10 eV), reflects molecular organization of the specimen. Are you referring to the low loss spectrum or are you referring to an energy loss image ?
Author : I refer to well-defined features in the low energy loss region (0-10 eV) of the spectrum. They are associated to molecular-type excitations from given bonds (C - C, aromatic ones...). Their interpretation is at the present time very limited and lies in our extensive comparison with ultraviolet absorption spectra, though the connection is not as direct as for atomic excitations (one must use Kramers-Kronig transformation and a dielectric constant description).

Very restricted use of this signal has been made in an image mode. It is due to two major reasons : the beam damage is very important for this type of excitation (this is one of the currently used techniques for estimating radiation damage doses, see for instrance (34) and (35). The other factor is that it is difficult to have a good model representation of the background in this energy range, to achieve satisfactory and meaningful image subtractions.

G.T. Simon : You mentioned that radiation damage is presently a limiting factor of EELS. Could this limitation be over come in using electron spectroscopic imaging as developed by the group of Ottensmeyer in Toronto ?
Author : Absolutely not!! Only a STEM operated with a parallel detection system can theoretically bring a substantial improvement in terms of beam damage for EELS. If you refer to the Electron Spectroscopy Imaging technique of the Toronto Group, the important effect is that during the image recording time, all electrons which have not been transmitted through the selection slit are lost for the detection. A parallel detection system would avoid this dramatic loss in efficiency but unfortunately it is impossible to incorporate it in a CTEM for image recording.

G.T. Simon : Does electron spectroscopic imaging have potentially a much better resolution than using scanning transmission electron microscope equipped with a field emission gun ?
Author : No. The low systems CTEM and STEM have nearly equivalent spatial resolution if one takes into account such limitations as the statistical considerations and the associated beam damage, or the microscope point resolution due to the objective or the focusing lens... The only clear advantage is for the STEM which does not suffer from chromatic aberration over the energy width of the filtered image because it has no imaging lens operating after the specimen. The great advantage of the CTEM lies in its shorter acquisition time for an image with many pixels and only one or a few energy windows, as opposed to the STEM which is better for the acquisition of many energy filtered images consisting of a small number of pixels.

G.T. Simon : It appears that you do not emphasize enough the necessity to use very thin specimens for EELS to reduce successive scattering of the electrons. This phenomenon decreases considerably the amplitude of the specific signal and the sensitivity of the technique. Could you please elaborate on this subject ?
Author : It is an important point and the thickness limitation is really one of the major problems for an increased use of EELS analysis. Thickness effects are responsible for (63) :
.) a reduction of the signal because an enhanced fraction of the characteristic core loss electrons also suffers large angle elastic processes which prevents them to go through the entrance aperture of the spectrometer ;
.) a spreading of the characteristic signal over a larger energy width as a consequence of multiple inelastic events ;
.) an increase of the background before the edge and consequently a reduction of the relative edge jump.

All these contributions cooperate to decrease the signal to background ratio, and the obvious solutions consist in using higher primary voltages or thinner specimens when possible.

G.T. Simon : Are Z contrast and ratio contrast really the same thing ?
Author : Yes.

G.T. Simon : Single subtraction of two images obtained by electron spectroscopic imaging have successfully performed by Ottensmeyer and his group. It is true that the method might be dangerous if one does not normalize the images when digitizing them before the subtraction is performed.

Author : I do not think that the major danger is the accuracy of the normalization during digitalization. It is important for quantitation where it could, in worst conditions, induce a uniform "bias" of the whole image. In the present paper, I make the point that a more important risk lies in the use of a plain subtraction. Mass, thickness or density variations of the specimen could yield a positive answer for the detection of a given element which is not present in the specimen (figure 2). This is called a "false positive", i.e. risk of finding something which does not exist !! These false contrastsare to be expected in many cases close to the detection limits, except for very uniform specimens.

G.T. Simon : Due to the thickness of cryosections I have some doubts that in the near future this type of preparation will be used routinely for EELS. This is a drawback in the analysis of diffusable elements. For the time being, alternate techniques have to be developed.
Author : I am very confident that progress can be made concerning the preparation of good cryosections. Many good people work on this problem. On the other hand, I think that the study of diffusible elements is not a problem to be solved by EELS, for many reasons mentioned in the text (low typical concentrations in biological materials, mass loss under the beam, limited sensitivity of EELS in terms of mass fraction...). SIMS (Secondary Ion Mass Spectroscopy) equipped with a cold stage should be a better alternative, the more as quantitative measurements could eventually be possible in the future.

G.T. Simon : Any analytical technique should not only be qualitative but also quantitative. It appear that there are some difficulties to interpret quantitatively the results obtained by EELS. Could you make some comment about it ?
Author : I do not think that quantitative measurements are more difficult with EELS than with other microanalytical methods. When it is compared to EDX, one must make the point that the basic cross section calculations are equivalent. For EDS, one has to add different factors such as fluorescence yield, weight of the different photon lines, and corrections for absorption. On the other hand, EELS is still to be improved regarding the accuracy of the background subtraction, of the signal measurement and of the influence of multiple scattering.

M.K. Lamvik : Higher voltage (1 MeV) may be very helpful for avoiding the problem of multiple scattering in biological materials. Could you give more information about EELS at high voltage ?
Author : Higher voltage is effectively supposed to be very helpful to reduce the problem of multiple scattering, notably in biological materials. A general review of the problem can be found in Sevely J. (EMAG's 1985, pp. 155-160). But to my knowledge no decisive work has been done till now in this field. It is perhaps due to limiting experimental factors : poor energy resolution due to the energy width of the primary beam, difficulty of matching good spectrometers and important problems of detection partly due to stray X-rays. Moreover both experimental and theoretical considerations seem to converge towards a consensus that an optimum voltage would be of the order of 400 kV.

M.K. Lamvik : While discussing the sensitivity of EELS, you say that the collection efficiency is in the range of 0.5 to 1, which is only true if an effective parallel detection system is in use. This statement would be confusing for many, because later you say that EDX may be better than EELS for certain elements (Na, K, Cl) because of the radiation damage problem ; this is hard to understand if the EELS collection efficiency were really much higher than that of EDX. Please explain this point.
Author : The sensitivity limit of EELS, as compared to EDX, is a rather complex subject and in this short answer, it is only possible to point out a few major considerations. The probability of ionization is the same in the two techniques for the same level, but EELS considers levels at lower energies (i.e. typically 100-2000 eV) than EDX (i.e. typically 1000-20000 eV) and consequently the relevant cross sections are one or two orders of magnitude higher. The efficiency of detection is the superposition of several contributions : the angular one measures the proportion of characteristic events which can be detected with the instrumental conditions used. The EELS, one collects of the order of 0.1 to 0.5 of the total signal, while in EDX the small solid angle intercepted by the detector compared with the isotropic emission is responsible of a value of a few 10^{-3}. But in the present state, the parallel acquisition in energy of the X-ray events is a factor favourable to EDX with respect to EELS, where most work has been made in serial collection. A good estimation must take into account the balance of all these contributions. Finally, I want to add that all edges in EELS are not easily usable for analysis : a few of them (Au, Ag, Cu...) are delayed, that is they cover a wide range in energy and appear as weak modulations on the slope of the spectrum. Consequently, one has to develop appropriate techniques involving filters or derivations to reveal them clearly.

G.M. Roomans : Please elaborate on the different detection systems for EELS.
Author : It is a wide subject and,speaking of detection without speaking of the other field of the instrumentation,could be quite unclear. For instance, there are two sorts of dispersive systems : 1) the spectrometer which can only give spectra ; 2) the filter (Castaing-Henry-Omega-Alpha and so on) which gives spectra and filtered images (made with electrons in a given energy window). The detection system for both images and spectra can be sequential or parallel ranging from a simple scintillator crystal associated with a photo-multiplicator to semiconductor devices such as the charge coupled device (CCD), the diode area and also to the standard photographic plate. Among these many possibilities one can summarize the best outfit for a given purpose: for quantitative analysis of a very weakly beam

sensitive specimen, the serial detection system with pulse counting mode is still the best choice (good linearity and high signal-to-noise ratio). For a quick acquisition of many informations with a poor linearity, use the photographic plate and for the intermediate range of number of information recorded and linearity, the solid detector area fills the gap.

G.M. Roomans : Since in EDX of thin sections fluorescence is assumed to be negligible, how can the absence of fluorescence in EELS be the reason for the better spatial resolution of EELS compared to EDX.
Author : As a matter of fact, the absence of fluorescence is not the only reason for the better spatial resolution of EELS compared to EDX. In EELS one has a better control of the volume of signal generation than in EDX. Large angle scattered electron can produce significant X-rays event but are not generally detected through the collection aperture by the electron energy loss detector. While not a true insurmountable limiting factor, the sensitivity is another parameter to take into account. As it offers a greater collected signal, EELS leads to better chemical mapping with high spatial resolution.