X-Ray microanalysis of animal tissues by means of the field emission-scanning electron microscope

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Summary. With FESEM the X-ray microanalysis of unstained ultrathin sections (600 Å) free of osmium is possible. Deposits in smooth muscle cells of arteriosclerotic aortae show high calcium-peaks already after 80 sec of measurement. No contamination spots were seen after 11 min of measurement on the same point.

The application of X-ray microanalysis in biological and medical ultrastructure research is greatly hindered by the inadequacy of structure analysis of the rather thick tissue sections required by the method. By means of the field emission-scanning electron microscope (FESEM) with ultrahigh vacuum those disadvantages can largely be eliminated, as shown by our first results on experimental arteriosclerosis.

The X-ray microanalysis of biological tissues enables the localization and semi-quantitative determination of biochemically important elements in their place of action[1]. The morphological resolution of the structures of interest is, however, unsatisfactory, as the objects for investigation have to be as thick as about 2000 Å to guarantee a sufficient impulse yield[2]. Owing to the tissue preserving low primary beam energy in FESEM, its higher beam current, and the ultrahigh vacuum it should be possible to reduce this section thickness significantly even if frozen sections are used[3]; this would however result in contrast reduction. The preparation of tissues in a conventional manner, embedded for example in epon, is associated with well-known problems concerning the escape of unstable ions, but is justifiable for certain analytical though not quantitative investigations[4]. So in our case we can measure covalently bound elements in more or less mineralized deposits in the aorta. Using ordinary ultrathin sections of not more than 500–700 Å to provide better structural quality, this would be profitable only if the analysis time is considerably extended, beyond the usually preferred 100–200 sec. Otherwise the element density in the desirable conventional ultrathin tissue probe would be too small to reach concentration levels in the TEM capable of indicating clear differences from the pronounced high background produced by the organic substances. Unfortunately, during the longer time of analysis the usual vacuum conditions of 10^{-4}–10^{-6} Torr would lead to a heavy contamination or destruction of the examined region by the electron beam, not to mention the problems for routine work caused by long periods of measurement.

Finally, in the TEM at an acceleration voltage of 40–100 kV, the optimum 500–700-Å-thick sections show a contrast sufficient for adequate picture quality only if they are fixed with OsO_4 and stained with heavy metals. However, the peaks in the spectrum due to Os, Pb, U, overlap the actually interesting but weaker signals of the biologically important elements, so it is necessary to do without the aid of these heavy metals. That is a second reason why the conventional X-ray microanalysis is dependent on roughly 3 times thicker sections which produce more structure.

In the FESEM TESLA-BS350, the ultra-high vacuum (better than 1×10^{-8} Torr in the specimen chamber), however, can guarantee a contamination-free analysis even beyond a multiple of the usual measuring times. Thus a sufficient impulse yield is possible with long-term measurement also with extremely ultra-thin sections. In the BS 350

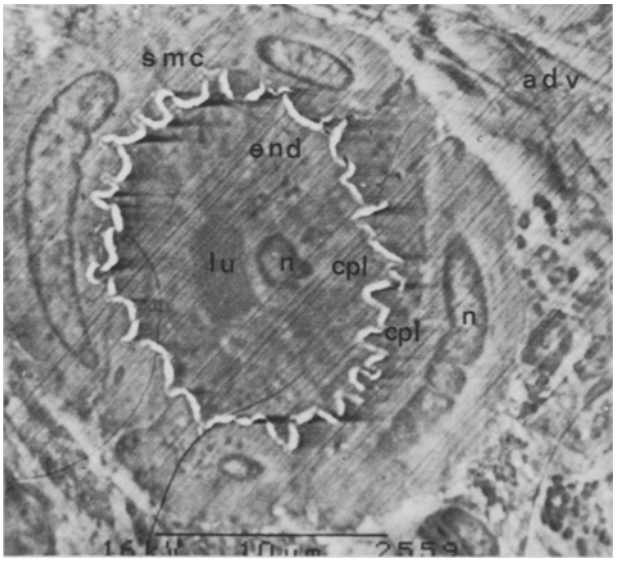

Figure 1. Cross-section of a small arteriole (thickness 600 Å) No OsO_4-fixation, no staining. The analyzed area (ca. 300 nm) in the cytoplasm of a smooth-muscle cell is not yet perceptible after a total of almost 11 min (lu, lumen; end, endothelium; smc, smooth muscle cell; cpl, cytoplasm; n, nucleus; adv, adventitia).

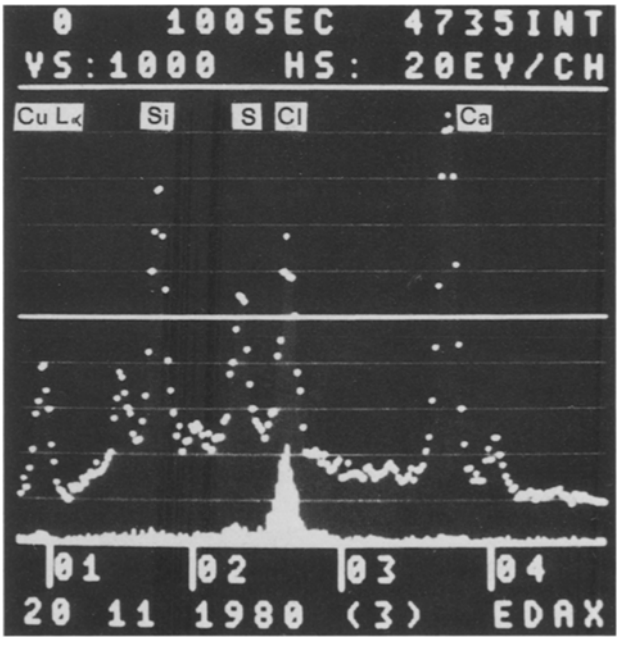

Figure 2. Analysis of an arteriosclerotic alteration after experimental diet in the aorta of a rat. In the background (dotted line) high Si, S, Cl and, in particular, Ca-content. In the foreground (white area) result of measurement in an adjacent area of the vascular wall not yet altered pathologically.

the preparation-dependent insufficient contrast is improved by using an accelerating voltage of only 16 kV and the possibility of increasing the primary current to 10^{-10}A without damage for the object (increase of the X-ray emission). With this acceleration voltage sufficient contrast is provided (fig. 1). A sample stage tilt for increasing the number of impulses is not necessary as in consequence of the higher specimen current in the present samples even shortening of the measuring time to 80 seconds yielded good results (table).

The comparison of the element peaks in the polaroid pictures shows that with a conventional TEM-X-ray microanalysis using 2000-Å tissue sections in several TEM systems (JEM 100B[5] and C, Elmiskop 2A[6], equipped with EDAX- and KEVEX-systems) in Szeged, Hungary; Brno, ČSSR; and Berlin, GDR as well as with the FESEM with field-emission gun and ultra-high vacuum (Brno, ČSSR) using 600-Å tissue sections typical and similar element spectra can be demonstrated, e.g. in the smooth muscle cell plasma of the arterial wall of different rats. So in rats with experimental arteriosclerosis, among others, the expected increase in elementary calcium could be proved even after conventional epon-embedding (fig. 2). Despite the fact that the time of measuring the same spot with a diameter of 300 nm has been experimentally prolonged up to 11 min, no tissue destruction or black contamination point was discernible (fig. 1).

Therefore FESEM with ultra-high vacuum - which, as far as we know, has not been applied before for X-ray microanalysis of biological and medical objects for a comparable purpose - shows a number of advantages:

a) ultra-thin section thickness of the object for analysis (between 500 and 2000 Å), b) shorter analysis time (between 80 and 320 sec) for 16 kV primary beam energy, c) higher beam current (about 10^{-10}A) and small analysis area (20-300 nm dependent on the biological problem). Moreover, d) the analysis is contamination-free (ultra-high vacuum), e) contrast in transmitted electrons is high also for conventional, i.e. relief-free ultra-thin sections.

The typical peaks of smooth muscle cell plasma of the arteriolar wall (Si, S, Cl) are recognizable already after 80 sec (left). Cu Lα, Fe and Cr derive from grid and instrument

A) 80 sec			B) 320 sec		
	0.645	7.		0.672	168.
	0.790	2.	Cu Lα	0.950	385.
Cu Lα	0.910	112.	Mg	1.260	19.
Al	1.470	9.	Si	1.745	591.
	1.510	20.	P	2.010	55.
Si	1.765	266.	S	2.322	482.
P	2.046	63.	Cl	2.634	4382.
S	2.322	213.		2.835	197.
	2.440	9.	K	3.310	6.
Cl	2.630	1525.	Ca	3.790	6.
	2.830	75.		4.270	6.
K	3.370	2.		4.300	9.
Ca	3.400	4.	Cr	5.440	42.
Fe	6.400	16.		5.640	18.
			Mn	5.775	7.
				5.950	2.
				6.030	6.
				6.120	1.
				6.270	2.
			Fe	6.415	158.

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Incorporation of the antitumor alkaloid thaliblastine in liposomes enhances its cytotoxic activity in vitro[1]

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Summary. The incorporation of the antitumor alkaloid thaliblastine in liposomes greatly enhances its cytotoxicity in Walker S and TLX-5 cell cultures.

The antitumor agent thaliblastine (USA, thalicarpine) derived from the roots of several species of thalictrum (Ranunculaceae), is a dimeric aporphine benzylisoquinoline alkaloid with N-O-O triangulation in its molecule[3,4].

Thaliblastine exhibits cytotoxic activity in vitro on KB cells, L_{1210} cells, human embryonal fibroblasts, and SAg cells[4-6], and also antitumor action in vivo on several transplanted tumors[7,8]. The absence of myelosuppressive and immunosuppressive actions have allowed thaliblastine to enter phase I clinical trials in the USA[9] and more recently in Bulgaria.

Liposomes, phospholipid vesicles, have gained recognition as promising drug carriers, firstly because of their composition and secondly because the flexibility of their composition may offer the possibility of selective cellular interactions[10,11]. Liposome-incorporated antitumor drugs are more efficient in prolonging the survival time of tumor-bearing animals[12,13], escape premature inactivation[14], prevent cardiotoxicity[15] and act on cells resistant to the free drug[16,17].

This communication describes the interaction of liposome-incorporated thaliblastine with tumor cells, and the resulting effect on the ability of these cells to grow in an in vitro tissue culture assay system.

Material and methods. Thalicarpine (National Cancer Institute, Bethesda, MD, USA) and the identical Thaliblastine (IOC, Bulgarian Acad. Sciences, Sofia) is a white crystalline powder with a m.p. 158-161 °C, $(a)_D^{23} + 79.1°$ (1% in chloroform) UV absolute maximum at 282 nm, 23,400 and at 302 nm, 18,400. Tritium (^{3}H)-labeled thaliblastine (sp. act. 4.8 mCi/mmole) was prepared by refluxing thaliblastine with tritiated water, resulting in the exchange of hydrogen with tritium. The labeled compound was tested chromatographically (R_f-values: 0.46 in benzene-methanol 7:3, 0.42 in chloroform-methanol 9:1, on fluorescent silica plates)