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ENERGY DISPERSIVE X-RAY MICROANALYSIS, NEUTRON ACTIVATION ANALYSIS AND ATOMIC ABSORPTION SPECTROMETRY - COMPARISON USING BIOLOGICAL SPECIMENS.

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Abstract

X-ray microanalysis, neutron activation analysis and atomic absorption spectrometry were performed on normal and injured skeletal muscle. X-ray microanalysis of tenotomized rat soleus muscle showed significantly elevated levels of sodium and chlorine and lower potassium compared with normal muscle. Similar ion shifts could be demonstrated by neutron activation analysis and atomic absorption spectrometry. The concentrations of sodium and chlorine obtained by these techniques were somewhat higher and that of potassium lower than the values obtained by X-ray microanalysis. This can probably be attributed to the fact that in atomic absorption spectrometry and in neutron activation analysis the entire muscle biopsy contents are measured while in X-ray microanalysis only the contents of muscle cells unaffected by extracellular, non-muscular components are determined.

It can be concluded that X-ray microanalysis is a reliable technique to study the elemental content of biological tissue, especially tissue undergoing pathological changes affecting the extracellular spaces. Other types of analysis should be used when elements not detectable by X-ray microanalysis are of interest.

KEY WORDS: energy dispersive X-ray microanalysis, neutron activation analysis, atomic absorption spectrometry, biological specimens.

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Introduction

Skeletal muscle cells are very elongated cells, and can therefore be cut transversely to provide many sections which may be examined by a wide range of (histochemical) techniques. This has enabled the concurrent identification and determination of enzyme activity and substrate content of the same muscle fibre. The histochemical techniques have been extended to include microchemical methods, mainly energy dispersive X-ray microanalysis (XRMA) in the electron microscope (Nichols et al. 1974, Wroblewski et al. 1978, Horowitz and Engel 1983).

The image of the histochemical preparation is projected onto a TV screen and a corresponding part of the (adjacent) serial section processed for XRMA is examined in the electron microscope (Wroblewski et al. 1978). The distribution of the various fascicles is scrutinized and the light microscope image is rotated to coincide exactly with that of the image obtained on the electron microscope screen (Wroblewski et al. 1978, Edström et al. 1979, Wroblewski and Edström 1983). Thereafter the same muscle fibres are identified and subjected to microanalysis in the electron microscope.

In contrast to many other analytical techniques, X-ray microanalysis measures the actual intracellular composition of cells uninfluenced by the chemical content of the surrounding environment of the muscle fibres. The possibility of analyzing muscle fibres alone is of great importance in numerous pathological conditions and in aged muscle where a high content of non-muscular elements such as fat and connective tissue introduces an analytical error when the analysis is performed on the whole biopsy e.g., as with atomic absorption spectrometry (AAS) or neutron activation analysis (NAA).

In our laboratory we have used energy dispersive X-ray microanalysis in several studies concerned with the physiology and patho-physiology of mammalian muscle (Wroblewski et al. 1978, Wroblewski 1982, Wroblewski and Edström 1983, 1984, Wroblewski and Wroblewski 1984, Wroblewski et al. 1987). Since chemical fixation markedly affects the original elemental composition of the tissue, only cryofixed and freeze-dried sections were used.

Our earlier XRMA studies (Wroblewski and Edström 1984) on the soleus muscle of the rat, before and after tenotomy and denervation, revealed characteristic elemental changes. These were most pronounced in the tenotomized muscles where the sodium and chlorine levels were markedly elevated and the potassium levels were lowered. A lesser degree of elemental changes was found in soleus muscle which had been tenotomized and denervated (at the same time) and even smaller changes in the muscle which had only

been denervated.

The purpose of the present study was to evaluate the results of XRMA on the soleus muscle of rat under the conditions mentioned above and to compare them with the results on the same material obtained by AAS and NAA.

The results of AAS and NAA are based on examination of the entire contents of the biopsy material (intra and extracellular) while XRMA selectively examines the intracellular elemental composition.

Our interest was mainly focussed on following the direction of shifts in elemental distribution rather than on obtaining absolute concentration values by different analytical methods.

Materials and Methods

Rat soleus muscle fibres, 5 and 16 days after three different types of surgical alterations: tenotomy, motor denervation, or tenotomy combined with denervation (as described in detail by Wroblewski and Edström 1984) were used. Care was taken to prevent damage to the blood supply of muscle during surgery.

Small muscle specimens, 2-3 mm in diameter, were dissected for X-ray microanalytical investigations. Larger specimens from the same muscle were used for atomic absorption and neutron activation analysis.

The specimens obtained 5 days after surgical treatment were used for comparison of X-ray microanalysis and atomic absorption, and after 16 days for comparison between energy dispersive X-ray microanalysis and neutron activation analysis.

Freeze-fixation

Directly after dissection, 2 mm^3 large samples were cryofixed by plunging the tissue into liquid nitrogen (LN_2) cooled liquid propane (-189°C). Prior to further processing the frozen samples were routinely stored in LN_2.

Cryosectioning

Semithin sections (2-6 µm) were cut in a conventional cryostat at -30°C (Wroblewski et al. 1983), transferred in the frozen state to Formvar film coated graphite plates (specimen holders) with a centrally drilled hole (Wroblewski and Wroblewski 1982), and freeze-dried in the cryostat overnight. The sections were then brought to room temperature and coated with carbon to avoid charging in the electron microscope.

Electron microscopy and X-ray microanalysis

Freeze-dried cryosections were analyzed in a Philips 400T electron microscope with a scanning attachment. Energy dispersive X-ray microanalysis was carried out with a Kevex 8000 analytical system. Freeze-dried cryosections were routinely observed and analyzed at an accelerating voltage of 100 kV. Both conventional transmission and scanning transmission electron images (bright field) were obtained. During all observations the anti-contamination cold trap was used.

Quantitative analysis was carried out with the help of a standard: a gelatin/glycerol matrix containing mineral salts in known quantities, frozen and sectioned in the same way as the specimen (Wroblewski et al. 1983). The relative peak intensity for an element was defined as R=P/B where P is characteristic intensity and B is the background count measured under the peak (Wroblewski et al. 1978, Statham and Pawley 1978, Wroblewski et al. 1983).

Neutron activation analysis

Neutron activation analysis (NAA) was performed using the R-1 reactor (Studsvik Energi AB) and radioactivity from the various radioactive nuclei was measured. Muscle samples were weighed before and after freeze-drying. For analysis, specimens were placed in clean high purity (polyethylene) plastic vials.

Atomic absorption analysis

Atomic absorption analysis (AAS) was performed using a Techtron AA-4 atom absorption spectrophotometer. Muscle samples were weighed after freeze-drying, fat-extracted and weighed again according to the standard procedure. The electrolytes were extracted in HNO_3 and the concentrations of individual electrolytes were determined by AAS (Na, K, Mg). Chlorine measurements were performed by means of titration (Bergström 1962).

Morphometry

Muscle fibre areas and extracellular spaces were estimated by measuring areas of single muscle fibre within hematoxylin eosin stained 6 µm thick cryosections by computer digitizer morphometry.

Results

Morphological observations

Core fibres with plasma membrane defects can be produced in the rat soleus muscle by short time tenotomy (Baker and Baldwin 1982). We could detect core fibres in muscle after 5 and 16 days tenotomy. In tenotomy combined with denervation the formation of core fibres was prevented. The morphological changes after denervation were minimal.

To evaluate changes in the volume of intracellular and extracellular components after different surgical treatments, computerized planimetry on hematoxylin-eosin stained sections was performed. Ratios representing the changes in the extracellular volumes are shown in Table 1. The most pronounced increase of the extracellular component was found in muscles after combined tenotomy with neurotomy.

X-ray microanalytical investigations

Elemental changes in rat soleus muscles after different procedures (5 days effect) are presented in Fig.1. Tenotomy thus resulted in a decrease in potassium and strong increase in sodium and chlorine concentrations. In tenotomy combined with neurotomy the increase of chlorine levels was still significant while the change in the concentrations of sodium was only slight. The concentration of potassium was increased. In denervated muscle with intact tendon an increase in potassium was the only deviation from normal.

16 days denervation of m. soleus resulted in further elevation of sodium (229 mmol/kg dw), chlorine (60 mmol/kg dw) and lowering of potassium levels (456 mmol/kg dw).

Table 1

The R values represent the ratio between total measured area in transversally cut muscles and area occupied by muscle fibres.

	R
Control muscle	1.08
Denervation	1.08
Tenotomy	1.20
Tenotomy/Denervation	1.24

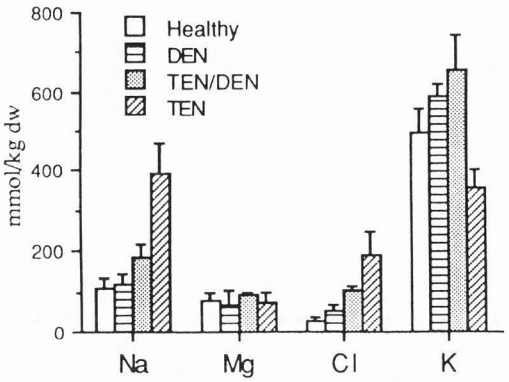

Fig. 1: Absolute concentrations (mmol/kg dw) of sodium, magnesium, chlorine and potassium in rat soleus muscle after 5 days tenotomy (TEN), neurotomy (DEN), combined tenotomy/neurotomy (TEN/DEN), and in healthy muscles as revealed by means of X-ray microanalysis on semithin freeze-dried cryosections. Thin bars represent standard deviation.

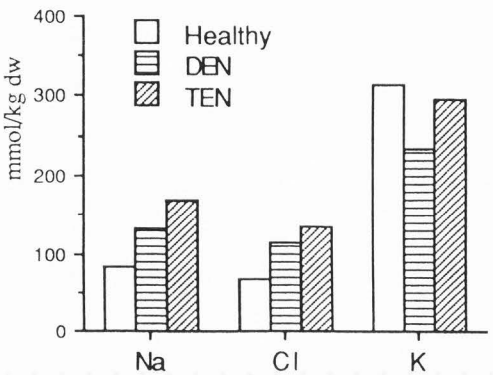

Fig. 2: Absolute concentrations (mmol/kg dw) of sodium, chlorine and potassium in rat soleus muscle after tenotomy and neurotomy (16 days) as revealed by means of neutron activation analysis.

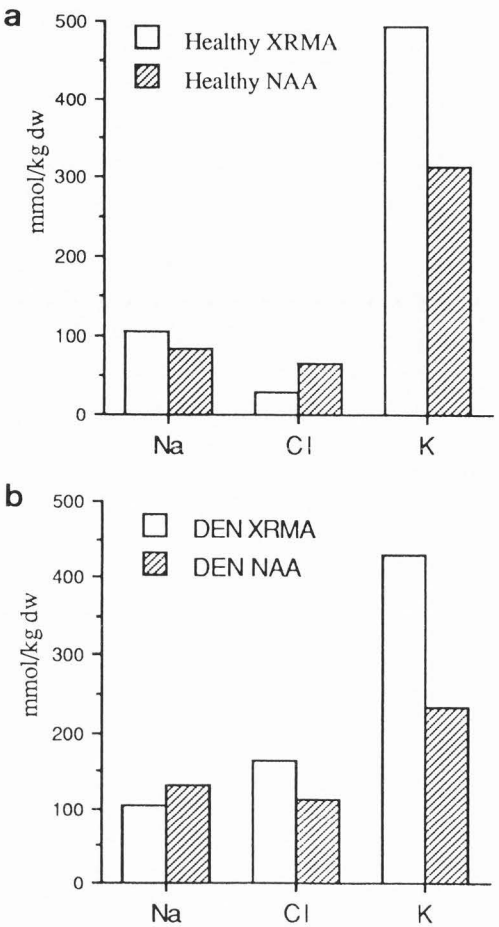

Fig. 3a-b: Comparison between XRMA and NAA. a. healthy soleus muscle, b soleus muscle after 16d denervation. Concentrations are expressed in mmol/kg dw.

Neutron activation analysis

The comparison between X-ray microanalysis and neutron activation analysis was performed on denervated (16 days) and tenotomized muscles. The results obtained by NAA (Fig. 2) were in good agreement with those of XRMA. The elemental data of neurotomized muscles show that elemental changes appear first after 16 days post surgery and that changes are of lower magnitude than changes found in tenotomized muscles. A comparison between XRMA and NAA on healthy and denervated soleus muscle is presented in Fig. 3a-b.

Atomic absorption spectrometry

The results obtained by means of AAS and titration of muscle samples from the same experimental animals (5 days neurotomy and tenotomy) as analyzed by means of XRMA (see above) showed similar elemental shifts. In tenotomized muscles both XRMA and AAS methods showed elevated chlorine and sodium and lowered potassium. In the denervated muscles there were only minor changes. (Fig. 4 a-b).

The elemental composition of normal muscle can be studied with great accuracy by using analytical methods (NAA and AAS) based on whole biopsies. There are only minor differences in the concentrations of sodium, chlorine and potassium depending on the quantitation procedure, giving somewhat lower concentrations with NAA than by using AAS (Fig. 5). The intracellular values of chlorine and potassium measured by means of XRMA are related to the high intracellular potassium and low chlorine content.

Discussion

There is a good agreement between the data on elemental composition obtained by means of XRMA and by NAA and AAS, although there exist differences in the absolute concentrations mainly caused by the contribution of extracellular space in chemical analysis. The range of the changes in the extracellular content and volume largely depends on the type of the injury and its duration. In the present study differences in the shape of the muscle and the extracellular space were detected already after 5 days.

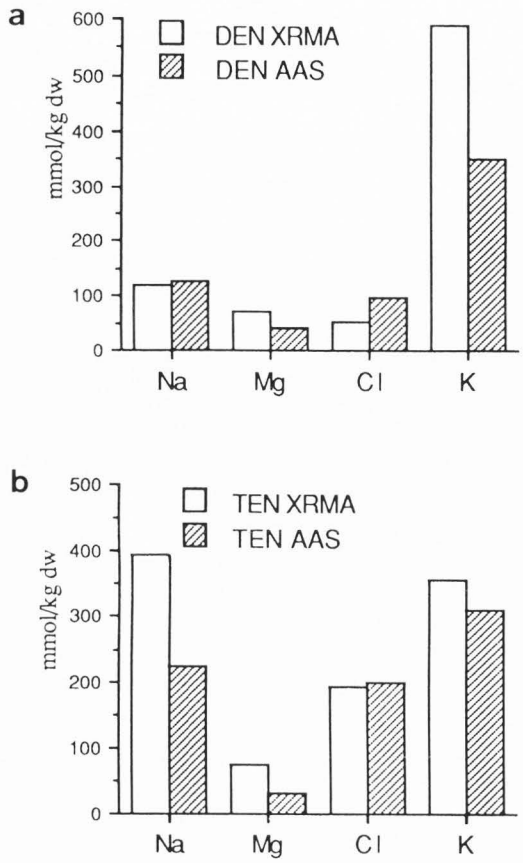

Fig. 4a-b: Elemental composition of rat soleus muscle of the same experimental animals (5 days neurotomy and tenotomy) analyzed by means of AAS and XRMA. Concentrations are expressed in mmol/kg dw. Similar elemental shifts are detected in the denervated muscles and in the tenotomized muscles by using XRMA and AAS methods.

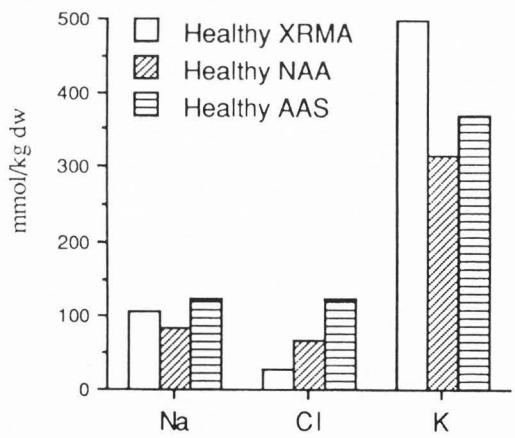

Fig. 5: Elemental composition of normal soleus muscle analyzed by means of XRMA, NAA and AAS. Concentrations are expressed in mmol/kg dw.

In numerous neuromuscular disorders the proportion of non-contractile tissues increases and there are no direct methods to dissect out or dissolve selectively this part of the sample without loosing the ions from the muscle fibres. In several pathological conditions changes are unevenly distributed in the tissue. In such conditions it is likely that elemental concentrations obtained by chemical analysis might not be significantly changed.

X-ray microanalysis

X-ray microanalysis should be the method of choice for the analysis of individual muscle fibres free of extracellular components. By analysis of central areas of transversally cut muscle fibres it was possible to acquire data that were not influenced by adipose tissue, connective tissue , capillaries and other structures which may vary for reasons unrelated to the state of the contractile apparatus.

Neutron activation analysis

Neutron activation analysis was introduced by de Hevesy and Levi in 1936 and used to study composition of normal and diseased skeletal muscle by Frenk et al. (1957), Reiffel and Stone (1957), Bergström (1962), Nichols et al. (1968), Edwards et al. (1975) and Batra et al. (1976). An advantage of neutron activation analysis is the high sensitivity. Bergström (1962) analyzed muscle tissue obtained by needle biopsy from normal subjects, from kidney patients and from patients with chronic diarrhoea and described ionic changes accompanying those disorders. Broyer et al. (1974) used neutron activation analysis in the study of muscle biopsies from uremic children. The authors have found NAA more reliable than plasma determination since measurements of plasma are difficult to interpret in severe uremia.

However, the technique also involves some problems mainly concerned with specimen preparation. In small tissue samples, adhering blood, adipose and connective tissue may easily be overlooked. This may give rise to unexpected variations in the results. Only tissues visually free from blood and non-muscular components should be used. This is also of importance as the dry weight estimate of tissue obtained by weighing is used in the final calculations. The variable ratio between muscle cells and non-muscular tissues seems to be the major complication in this method in the study of intracellular electrolytes, especially in pathological conditions where the non-muscular element often increases. Since sodium and chlorine are present intra- and extracellularly, the extracellular part must be subtracted from the total amount of ions for calculation of the quantity present in the cells. This correction can be done by using the the volume of extracellular water in calculations (Bergström 1962). This correction is of minor importance in calculating phosphorus and potassium. The sensitivity of method makes it very useful for the analysis of trace elements. However, not all biologically significant elements can be successfully analyzed by NAA. The necessity of more than one irradiation of the same sample prolongs the period of obtaining the analytical results to several days. Neutron activation analysis has the distinct advantage over atomic absorption analysis of being nondestructive and offers the possibility of reanalyzing the same sample or to use it for measurements of other substances (enzymes or substrates) in the same sample by other methods. Many samples can be easily analysed as the method does not involve many manual steps of analysis. However, the cost of analysis is a considerable disadvantage of this method.

Atomic absorption spectrometry

The method is very sensitive and ideal for studies of trace elements. Chlorine cannot be analysed by AAS and titration methods have to be used. Also small fluid samples can be satisfactorily measured by AAS. In contrast to XRMA and NAA, simultaneous multielement analysis is not possible at the moment. AAS is a destructive method and samples cannot be reanalyzed. The problems with regard to the estimation of contribution from the extracellular space are similar as in NAA. However, the cost of purchasing and running of an AAS system is very low in comparison to the instrumentation needed for XRMA and NAA.

There are only few studies concerned with the comparison of X-ray microanalytical techniques with other types of chemical analysis. Leader et al (1984) studied *in vitro* ionic composition of denervated muscles by means of microelectrodes and by means of flame photometry, and found a decreased membrane potential after 3 days denervation resulting in an increase in sodium and chlorine and unaffected potassium levels. The authors point out the problems associated with the studies of intracellular ion composition, such as assumptions concerning ionic activity coefficients for ions. The data for sodium and potassium were found to be in good agreement when microelectrodes or flame photometry were used. Sjogaard (1983) found that potassium concentration is higher in type II muscle fibres than in type I fibers. At the same time she could demonstrate differences in the proportion of the extracellular space in different muscle types. Hook et al (1986) compared magnesium concentrations in individual lymphocytes obtained by X-ray microanalysis with data from atomic absorption analysis and found no difference between the two methods of analysis.

The results obtained by XRMA and ionsensitive electrodes or by flame photometry in cervical ganglia are discussed by Galvan et al. (1984), who found that XRMA revealed the lowest intracellular sodium and highest potassium concentrations in comparison with data obtained by chemical analysis. Elbers (1983) compared X-ray microanalysis and atomic absorption spectrometry in studies of single egg cells of *Lymnea stagnalis*., and found no difference between results obtained by the used methods. However, he was not able to detect sodium by XRMA. In this particular investigation single cells were analyzed and therefore arising errors from the contribution of the extracellular space to the analyzed volume were omitted.

Conclusion

We have found that comparable results can be obtained by using three different analytical methods on the normal and injured skeletal muscle. The most accurate values of intracellular elemental composition were obtained by X-ray microanalysis as the error from nonmuscular tissue and extracellular fluid can be avoided.

The measurement of sodium and magnesium by means of XRMA is not highly accurate, especially at low concentrations, due to the low detector efficiency for these elements. XRMA is much more time consuming than NAA and AAS. In AAS and NAA, measurements of sodium and chlorine which are predominantly "extracellular ions" are directly affected by changes in the extracellular space. Similarly, (intracellular) potassium measurements are affected.

AAS and NAA will thus have a tendency to exaggerate actual differences between the elemental composition of the cells under conditions where the non-muscular and extracellular tissue component increases, e.g., in (chronically) affected muscles.

Only X-ray microanalysis in the electron microscope offers the unique possibility of selective analysis of muscle fibres with reference to the fibre type, degree of atrophy, substrate and enzyme content.

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Discussion with Reviewers

L. Edelmann: The spatial resolution obtainable by means of XRMA should permit analysis of A- and I-band selectively within the striated muscle. What was the size of analyzed area? How many fibres within the section were analyzed?
Authors: In the present study and in previously performed studies on 6 μm thick cryosections, only transversally cut sections were analyzed. Serial cross-sectioning allows us to detect changes in fiber diameter (atrophy, hypertrophy) and also some structural defects such as ring fibers commonly found in dystrophia myotonica. Further histochemical characterization of the fibers analyzed by XRMA can be performed on subsequent histochemically stained serial sections. Cryosections with a nominal thickness of 6 μm include 2-3 sarcomers within the entire section depending on the contraction state of muscle prior to freezing. XRMA of the central area of transversally cut muscle provides well defined analytical volumes uninfluenced by the nuclei or subsarcolemmal mitochondria. The selective analysis of A- and I-bands within the striated muscle is of course of interest and we hope to perform the study suggested by you using longitudinally cut muscle fibres.

Reviewer I: Standard error/deviation and statistical analysis of differences should be given in Figs. 3-5. Values for Na by XRMA are not consistently lower than values by AAS or NAA.
Authors: The statistical comparison between results obtained by XRMA and AAS or NAA is not straightforward. This is due to the different standards used and to the fact that by XRMA 15-20 measurements are made on each muscle sample compared to one by AAS or NAA. The values of sodium to which you refer are in Fig. 5. One can explain this discrepancy by the low sensitivity of the Si/Li detector with the beryllium window for sodium. The detector sensitivity for sodium is 60% lower than for example for Cl and K for which the concentrations obtained by means of XRMA were lower for chlorine and higher for potassium, respectively, than the results obtained by AAS or NAA. This indicates that XRMA was preformed exclusively in the intracellular milieu.

M. Engel: I found it useful to make tables for each element, recording the results with each method. There were some substantial differences. The authors attribute these changes to the inclusion of extracellular elements in NAA and AAS analysis. Could the defatting step used in AAS affect the result?

I am puzzled by the very low values (as compared to my own experience) for Cl in healthy myofibers and the high values for K.
Authors: The defatting step is a standard procedure used in AAS. Therefore, even if the muscle samples were visually fat free, we decided for the sake of standardization to include defatting step in our preparation procedure.

The values for all elements are absolute concentrations expressed in mmol/kg dw. The difference in concentrations between our results might be caused by the method of background determination, accelerating voltage used and the thickness of the specimens. The background is much lower when semithin cryosections and a high accelerating voltage are used. We should compare the original spectra from our analysis to find out possible differences.

K. Zierold. How much mass is necessary for measurements of the element content in XRMA, NAA or AAS? Is NAA or AAS possible on cryosections or single cells? Which elements not or poorly detectable by XRMA can be measured better by NAA or AAS?
Authors: The comparison between the methods above mentioned was made to compare analytical results concerning Na, P, S, Cl and K which occur in different concentrations in the intra- and extracellular spaces. We also wanted to follow by these three methods the elemental changes caused by pathological processes. The analytical data obtained on whole tissue basis (NAA and AAS analysis) were compared with data obtained intracellularly unaffected by extracellular components and we were not trying to perform any trace elemental analysis. The sensitivity of AAS and NAA is in orders of magnitude better than that of XRMA. The minimal tissue sample size that can be accurately analyzed in NAA is about 50 μg. Similar conditions apply to AAS. This would exclude single cells and thin cryosections.