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PROTON MICROPROBE AND PARTICLE INDUCED X-RAY EMISSION (PIXE) ANALYSIS FOR STUDIES OF PATHOLOGICAL BRAIN TISSUE

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Abstract

Particle Indiced X-ray Emission and proton microprobe analyses have been applied for the investigation of regional elemental distributions in connection with various pathological states in the brain. Malignant brain tumours and adjacent histologically intact tissue removed during surgery were analysed with PIXE. Systematic elemental variations, e.g., for calcium and selenium, were observed in the tumour front. The proton microprobe was applied to study the Ca and K concentrations in various cell strata in hippocampus following transient ischaemia in rat brain. Significant increases in the Ca level occurred in selectively vulnerable cells within 48 h after the ischaemia.

KEY WORDS: Microanalysis, particle induced X-ray emission, proton microprobe, brain tissue, glioma, tumour, ischaemia, hippocampus, calcium, potassium.

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Introduction

Interest in studies of the elemental composition of brain tissue has generated a demand for suitable analytical techniques. The possible connection between various diseases and regional distribution of chemical elements is of great interest in studies of the pathogenesis of, e.g., neurodegenerative states like Alzheimer's disease. Several hypotheses regarding the aetiology of this disease have been suggested and several signs indicate that aluminium is important. Using the high resolution of the proton microprobe, a recent investigation demonstrated the presence of aluminium and silicon in the core of senile plaque from the cerebral cortex of a patient with diagnosis of Alzheimer's disease (Watt et al.,1986). Another ion which has attracted considerable interest is calcium, which is supposed to be a mediator of cell death due to ischaemia and various toxins (e.g Wrogemann and Pena 1976, Katz and Reuter 1979, Schanne et al. 1979, Siesjö 1981, 1988), and which together with aluminium has been implicated in the formation of neurofibrillary tangles in dementia of Guam (Garruto et al. 1984). Another important field is the study of malignant brain tumours with possible connections to environmental factors. To further explore these fields, and others, it is important to develop suitable analytical techniques capable of probing for many elements with high analytical sensitivity in small sample masses. The multielemental capability is especially important when investigating possible correlations between elements.

The Particle Induced X-ray Emission (PIXE) technique has been shown to be a powerful and sensitive method for multielemental analysis of small samples. It is a useful tool in studies of biological material and is capable of revealing and quantifying elements heavier than sodium. The capability of determining both heavy metals and the lighter electrolytes in a

single analysis at concentrations of the order of 0.1 to 10 µg/g is very useful. Since the sample masses required are low (µg-mg) it is also possible to use a fine-needle biopsy technique without major inconvenience to the patient, and to study the regional distribution with high lateral resolution. The resolution may be improved even further by a Proton Microprobe (PMP) using a collimated and focused proton beam giving a lateral resolution of a few micrometres (Legge, 1984). The much improved sensitivity, compared with the traditional electron microprobe (EMP) (Malmqvist, 1986), allows new investigations to be carried out in order to elucidate the role of various trace elements in, e.g., pathological processes. Comprehensive reviews of the use of the PMP in biological studies have been given by Vis (1986) and by Malmqvist (1986).

In a recent study, Duflou et al. (1987) applied the PIXE technique in a systematic study of the regional distribution of trace elements in normal human brain. Earlier studies (Höck et al., 1975; Larsen et al., 1979) revealed a heterogeneous distribution of various trace elements with several elements at significantly higher levels in regions consisting of grey matter than in white matter areas. Duflou et al. took samples from 46 different regions of normal human brain from three persons who died from hypoxic-ischaemic disorders. The samples were freeze-dried, wet-digested and spotted onto thin foils which were subsequently irradiated by 2.4 MeV protons for PIXE analysis. The precision and accuracy were checked by comparison with instrumental neutron activation analysis. Good agreement was obtained between the two analytical techniques. For the elements studied, consistently higher values were found in cortical (grey) structures than in white matter. The promising results of this study inspired our group to apply the same technique in the analysis of pathological brain.

Because of the obvious difficulties involved in performing in vivo experiments with human brain, animal models are often used. An early investigation of rat brain was performed by Wenrink et al. (1987) on hippocampus using the PMP technique. In this study the distribution of zinc in rat hippocampus was of particular interest, but the multielemental capability of the PMP method makes it feasible to investigate the regional distribution of several elements. Histochemical techniques are specific to single elements which make them unable to reveal more than one element in a particular sample, while the PMP method can reveal several elements with accurate quantitative results. In the work presented below, lateral resolution of some tens of micrometres was sufficient to study various structures in the human brain.

PIXE Analysis of Brain Tumours

Benign brain tumours are often curable by operation while malignant (invasive) tumours are hitherto incurable. In spite of all known therapy these tumours invade the surrounding, apparently normal, brain tissue. The mechanisms behind the growth of these tumours are essentially unknown and it is of utmost importance to collect all possible information about the changes occurring in the boundary layer between the tumour and "normal" tissue. The trace elemental changes may be studied by sampling during normal surgery since both the tumour and some of the adjacent normal tissue have to be removed for therapeutic reasons. However, obviously a minimum of normal tissue should be removed and the small mass of samples available makes PIXE analysis a suitable technique. In this study we have examined specimens of malignant brain tumours (astrocytomas grade III-IV). The samples were divided into central tumour (often necrotic), tumour front and surrounding "normal tissue." Samples were taken from 12 patients during surgery and from two persons at autopsy (Tapper et al., 1987).

Material and Methods

After sampling the specimens were deepfrozen and stored at -40 °C. The samples were classified histopathologically into the three groups discussed above. From each sample 50-100 mg were taken and freeze-dried and then wet-digested in 100 µl ultrapure nitric acid per 10 mg of dry matter. Yttrium and vanadium were added as internal standards and 40 µl of the solutions were spotted onto thin polycarbonate foil (Kimfol (TM)). The procedure was carried out entirely in a clean-room and was checked by preparing bovine liver (NBS reference material, SRM 1577) similarly. Each sample was irradiated with 2.5 MeV protons for PIXE analysis. Two targets were prepared from two different pieces from each brain tissue sample. One target was irradiated with an accumulated charge of 40 µC and the second with 180 µC in order to lower the detection limits.

Results

The concentrations found in normal tissue in this study in both surgery and autopsy samples were in good agreement with the results of the systematic study performed by Duflou et al. (1987) on autopsy material. Twelve elements were determined in the samples, ten of which are listed in table 1 together with values from Duflou et al. Due to high attenuation of soft X-rays in the present

Table 1

The mean values and relative standard deviations of the measured elemental concentrations in normal human brain tissue, tumour front and tumour centre. In addition, the elemental concentrations in normal brain measured by Duflou et al. (1987) are given.

Element	x ± s (µg per g dry weight)			
	Normal brain n=7	Normal brain Duflou et al. n=64	Tumour front n=14	Centre n=2
P	10900±1400	-	7500±2500	4600±800
S	6000±700	-	7000±900	6000±500
K	13700±3200	12300±2400	11600±4400	7600±5600
Ca	350±70	320±110	750±300	620±210
Mn	1.7±0.22	1.57±0.47	2.0±1.6	1.2±1.4
Fe	260±110	270±170	390±150	340±140
Cu	19±4.5	25±10	20±12	12±7.0
Zn	54±20	53±13	100±45	67±14
Se	0.57±0.28	0.69±0.17	1.0±0.3	0.78±0.15
Rb	17±7.1	14±2.9	19.1±10	10±12

analytical set-up sodium is not given in the table. Elevations of iron, calcium, zinc and selenium concentrations were found in the tumour front. Three samples were taken in apparently "normal" brain very close to the tumour front. In this tissue a possible decrease in the zinc level was seen. The decrease in phosphorus within the central tumour is probably explained by cell necrosis. Bromine is partly lost during sample preparation and hence not included in table 1, but the bromine concentration in the tumour is about three times higher than in normal tissue. This is in agreement with what has been reported by Maenhaut et al. (1980) in studies of liver tumours.

The results from this study of brain tumour confirm the conclusion of Duflou et al. that the PIXE method is highly suitable in investigations of regional elemental distributions in brain tissue. Very small samples can be used and the multielemental capability facilitates correlation of elements which may be very helpful in the further evaluation of malignant tumours in brain.

Proton Microprobe Analysis of Ischaemic Brain

Ischaemia/hypoxia (lack of cellular oxygen) leads to neuronal necrosis affecting certain vulnerable regions in the brain and, within these, certain neurons (Siesjö, 1981). Calcium is known to play an important role in intercellular communication and in the transformation of external stimuli into intracellular responses (Rasmussen and Waisman, 1983). Such responses may be a modification of the lipid and protein constituents of the cell. The messenger role requires that Ca^{2+} is maintained at 10^{-7} mol/l which is 10^4 times the extracellular concentration, and also 10^4 times lower then the total cell calcium content. Accordingly, most of the calcium is bound or sequestered. In response to ischaemia, loss of Ca-homeostasis occur with an uncontrolled rise in the Ca^{2+} level. Such a rise is supposed to damage cells by mitochondrial overload and/or by activation of proteases and lipases (Wrogemann and Pena 1976, Katz and Reuter 1979, Schanne et al. 1979, Siesjö 1981, 1988). Following short periods of transient ischaemia cell death may be conspicuously delayed, occurring 1-3 days after the initial insult. It has been proposed that loss of cell viability is due to gradual calcium overload due to increased calcium cycling across cell membranes (Desphande et al. 1988). The hypothesis predicts that the total cell content increases before signs of cell necrosis develop. In order to test this hypothesis, the Ca concentrations in cellular and dendritic regions of hippocampus have been investigated as a function of time after transient ischaemia. Hippocampus contains both vulnerable (e.g. CA1) and resistant cells (e.g. CA3) in different regions which are well separated laterally (Themner et al. 1987 and 1988).

The lower relative detection limits of the proton microprobe (PMP) compared with the electron microprobe (Malmqvist, 1986) facilitates the detection of calcium at low enough levels and of other elements of interest, e.g., iron. In these particular samples the detection limits of PMP for calcium are at least an order of magnitude lower than those of the EMP

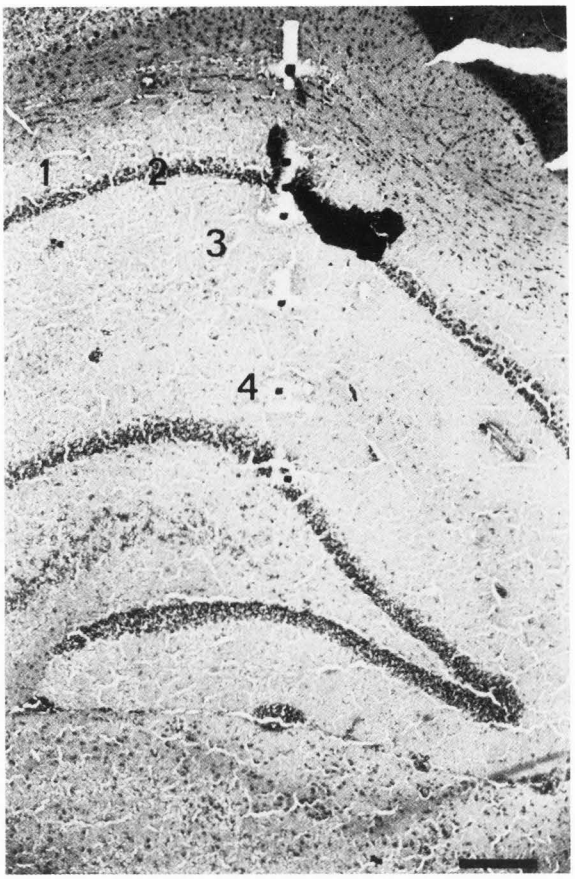

Fig.1. Micrograph of a section of the dorsal hippocampus stained after irradiation. The formation of cracks in the section originates from the staining procedure after irradiation. Bar is 300 µm. The numbers 1, 2, 3 and 4 refer to stratum oriens, CA 1 pyramidal cells, stratum radiation and stratum moleculare, respectively.

technique. Since the requirements in lateral resolution are rather moderate (tens of micrometres) the analytical task appears to be ideal for the PMP.

Materials and Methods

Male Wistar rats were selected for the study of induced ischaemia. They were divided into 5 groups: a sham operated group (n=8), and ischaemia followed by 24 h (n=10), 48 h (n=11), 72 h (n=10) and 96 h (n=4) recirculation. The rats were exposed to cerebral ischaemia by clamping both common carotid arteries and hypotension, to a mean arterial blood pressure of 50 mm Hg, maintained by phlebotomy for 10 minutes (Smith et al., 1984). The brains were then recirculated by removal of the carotid clamps and by reinfusion of the blood. The animals were frozen in situ after designated intervals according to the experimental grouping. The brains were exposed to methyl-butane and liquid nitrogen and were then chiselled out during irrigation with liquid nitrogen and sectioned (12-20 µm) in a microtome at -20 ^{0}C. After deposition on thin polycarbonate foils (Kimfol (TM)) they were freeze-dried for 12 to 16 h at 10^{-2} torr.

The proton microprobe analysis was performed using 2.5 MeV protons and a 20x20 μm^2 beam size. Since the beam rested on each spot for a couple of minutes the beam intensity was limited to a few $pA \cdot \mu m^{-2}$ to reduce beam induced damage of the samples. Each spot was exposed to total charge of 200 nC. The characteristic X-rays were detected in Si(Li) detector (active area: 60 mm^2, resolution: 160 eV). Quantification of the PIXE results was performed by spectrum evaluation using the HEX code (Johansson, 1982) and by the detection of backscattered protons in a surface-barrier detector for determination of the sample mass in each irradiated spot (Themner and Malmqvist, 1986).

Irradiated slices, as well as adjacent slices, were stained using a modification of the celestine blue-acid fuchsin method for visual inspection in a light microscope. In Fig 1 a section of the dorsal hippocampus is shown stained after irradiation. The cracks are caused by the staining procedure.

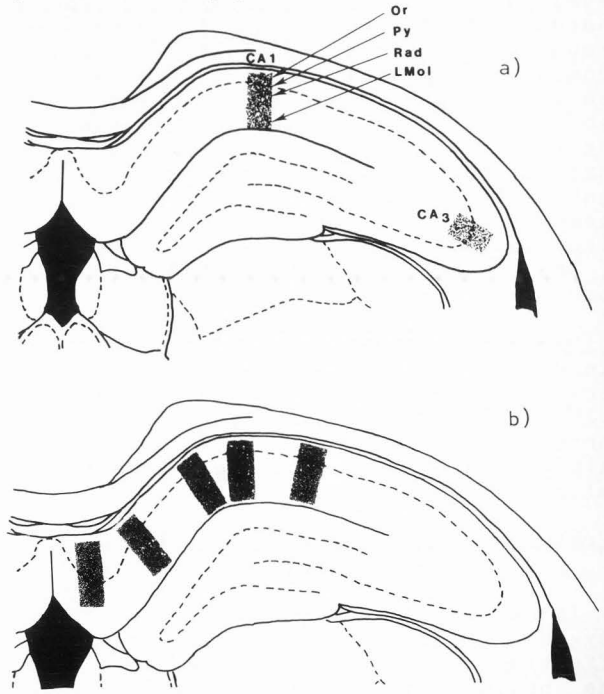

Fig.2. a) Sampling areas CA1 and CA3 in hippocampus selected for PMP analysis with identification of the cell layers discussed in the text.

b) Indications of sampling sites in CA1 which were shown to give essentially equivalent elemental results.

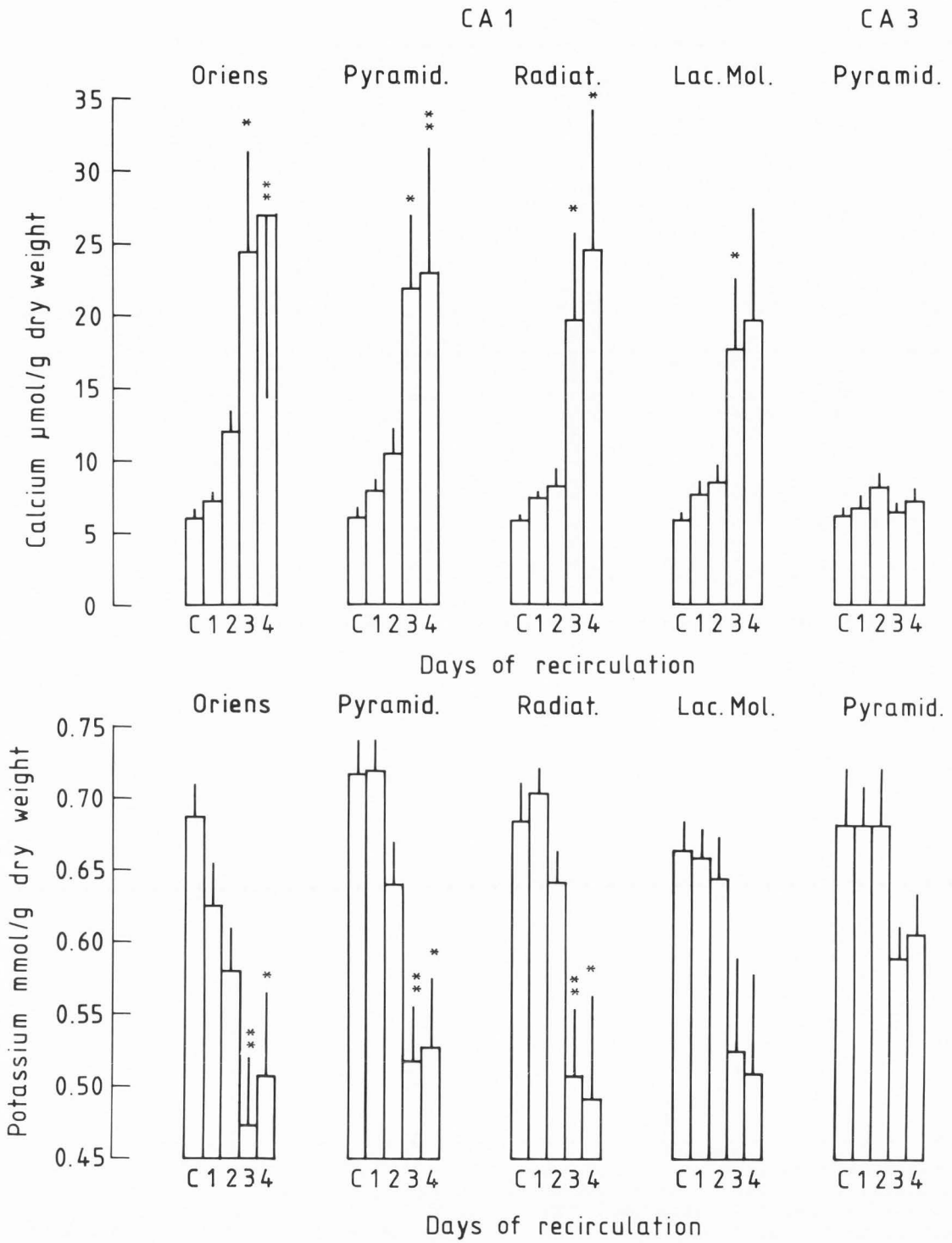

Fig.3. Calcium (a) and potassium (b) concentrations in various cell structures with different days of recovery after transient ischaemia. The number of rat brains analysed in each group is given in each column. C stands for controls (n=8). The columns of exposed animals represent n=10, n=11, n=10 and n=4, respectively. The asterisks indicate the confidence levels in the deviation from the control value of the Ca concentration. (* < 0.05, ** < 0.01). The data given are averages and error bars represent standard errors of the mean. (Martins et al. 1987)

Results

The results concern elemental changes in five regions of hippocampus: 1) oriens (basal dendrites), 2) CA1 pyramidal cell layer, 3) radiatum (apical dendrites), 4) the molecular layer and 5) CA3 pyramidal cell layer (see Fig 2a). The selection of the precise sampling area in CA1 was based on a comparison of several regions (see Fig 2b). The differences between the analytical results of these regions were insignificant. As discussed above, the most important element is calcium, but very interesting changes in the potassium level were also observed after ischaemia.

In Figs 3 a and b the calcium and potassium concentrations in the different cell strata are given for controls and ischaemic rat brain. In regions 1 to 4 it is clear from the diagrams that calcium concentrations become elevated in the recirculation period after ischaemia. During the first two days the elevation is moderate but increases significantly on the third and fourth days. In the CA3 region no such effects are seen. The reverse situation is observed for potassium which decreases significantly as calcium increases. However, a reduction in potassium also occurs in CA3, although the change is less significant. The confidence levels in the observed changes are indicated by asterisks in the diagrams.

The results observed for the control animals are in good agreement with expected concentrations for both potassium and calcium. Thus in a recent study on the same rat strain the potassium and calcium concentrations of the cerebral cortex were 509±6 and 5.6±0.2 mmoles/µg of dry weight (Warner et al. 1987). The decrease in potassium levels in the high calcium cells probably reflects a suboptimal cell membrane function. The calcium results agree well with the results of previous studies using atomic absorption spectrometry (Desphande et al. 1988) which show that calcium accumulation in these structures occurs after between 24 and 48 h of recirculation. The results demonstrate that calcium accumulation may occur in soma and dendrites before light microscopical signs of neuronal necrosis can be observed. Since the accumulation of calcium is accompanied by loss of potassium the results suggest that membrane dysfunction with partial loss of ion homeostatis precedes cell death.

The results so far seem very promising and we have initiated further studies of other pathological states which are known to induce necrosis in specific regions of the brain. Consequently work is in progress to perform similar investigations for experimentally induced hypoglycaemia and status epilepticus.

Discussion

The properties which have been demonstrated above for the PIXE and proton microprobe analysis will inevitably lead to an increasing use of the techniques in studies of the central nervous system. The unique information on highly resolved trace element distributions in cell strata or even in single cells (Johansson and Lindh 1987) when using beams of only a few micrometres diameter will help in the understanding of the very complex mechanisms behind the onset of various diseases. In studies of metal toxicology, the very good detection limits attainable with PMP analysis for heavy metals makes it a very useful tool in studies of the kinetics of metals on the cellular level. The proton microprobe thus complements the electron microprobe which may be used subcellularly to detect metal rich granules in ultrathin sections. An illustration of the use of PMP in brain is a systematic study of lead toxicology in the primate foetus. Since lead is especially toxic to the foetus the uptake mechanisms and effects in early brain development are very interesting. In a preliminary study brain tissue from the foetus of a lead exposed monkey has been analysed using the proton microprobe technique to investigate the distribtion of lead in the brain cortex (Lögdberg, private communication). No other technique except radiolabelling can be used to detect lead at the low exposure levels used in this investigation. Using the PMP/PIXE technique, the drawbacks of very long exposure times used in autoradiography and radiation burden to the experimental animal can thus be avoided, and complementary elements can be determined simultaneously.

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Discussion with Reviewers

R. Nobiling: A calcium^{2+} level of 10^{-7} mol•l^{-1} (normal resting level in cells, mass fraction ~ $4 \cdot 10^{-8}$) will barely be detectable with a proton microprobe. The results of $3 \cdot 10^{-4}$ (controls) demonstrate that mainly extracellular and/or bound intracellular calcium was detected. What are the arguments for attributing the enhanced calcium levels to be "intracellular"?

Authors: We wish to emphasize that whereas a decrease in the total potassium concentration of the tissue must reflect a corresponding decrease in the free intracellular K^+ concentration, the results on calcium cannot be interpreted similarly. This is because virtually all the calcium measured is either extracellular (about 20%) or bound sequestered in the cell (about 80%). However, any increase in total cell calcium content must reflect calcium which has entered the cell, and primarily sequestered by mitochondria. Presumably, any such increase reflects a preceding rise in the free cytosolic calcium concentration.

R. Nobiling: Are there EM-micrographs available, that could illustrate the assumed cell membrane dysfunction (p. 8/9) more precisely than light micrographs can?
Authors: No such micrographs are available at present.

U. Lindh: Do you think that the cryosections (-20°C) were freeze-dried and not air-dried? Working at quite high temperatures and depending on how the samples were transferred to the freeze-drying apparatus, the thin sections might at least partly, be air-dried.
Authors: The samples were quickly transferred from the cryomicrotome to a thermos flask and transported to the freeze-drying apparatus. When transferring the samples to the liquid nitrogen cooled stage in the dryer there is probably a risk that the thin sections may be partly air-dried. To our experience this does not interfere with the elemental distribution at the lateral resolution used in this investigation.

U. Lindh: It is encouraging that the staining method employed produces staining in the irradiated areas of the sections. My experience of other methods for staining of freeze-dried sections is that they do not produce staining in irradiated parts.
Authors: We agree that this would have been very encouraging. However, in the parts of the sample actually struck by the proton beam the staining does not work (see Fig. 1). The parts in-between the irradiated regions are stained also after analysis.

U. Lindh and G.M. Roomans: In the study on ischemia-induced changes, only K and Ca data are given. Were there no concurrent alterations in other elements? Chlorine would be a very interesting element to monitor, and what about Mn, Fe, Cu and Zn?
Authors: Alterations occurring in other elements than K and Ca have been beyond the scope of the present study. Due to the multielemental properties of PIXE analysis some other data do exist, e.g., for Cl, and may be evaluated separately. However, since limited beam time is available a choice was made to give priority to analysing many samples rather than to analyse fewer with better detection limits. This choice has restricted the possibility to study trace elements, e.g. Cu and Zn.

G.M. Roomans: Many of the elemental changes found in this PIXE study after ischemia and in tumors have been found in other tissues by electron microprobe analysis. It is important that the "walls" that seem to exist between the proton and the electron microprobe literature are broken down and it would there-fore be useful if the authors could refer to some key references from electron microprobe work.
Authors: Yes, we wish to emphasize that some of the problems addressed in this study have previously been successfully studied with electron probe X-ray microanalysis (EMP). For example, it has been convincingly demonstrated that mitochondrial accumulation of calcium occurs in damaged cells only (Somlyo 1985, Somlyo et al. 1985) and that neurons which contain neurofibrillary tangles in dementia accumulate both calcium and alu- minium (Garruto et al. 1984). We anticipate that PMP analysis becomes useful for many such applications because of the superior relative sensitivity of the technique.

G.M. Roomans: Regarding sampling of brain tumors: the elemental composition of cells changes markedly after death (Roomans and Wroblewski 1985). Autopsy material cannot, normally, therefore be pooled with surgery samples, but must be tabulated separately.
O. Castejon: Which were the differences observed in the PIXE analysis in the samples taken during surgery and after autopsy?
Authors: We agree that elemental changes certainly occur in autopsy material, but at the sample size and elements investigated in the present study we do not observe any particular differences between our surgery material and the autopsy material of Duflou et al. (1987). If a rapid change occurs during transfer from surgery to the quick freezing this could partly explain the small differences between the two groups of brain tissue.

R. de Estable-Puig: Concerning the tumour material analysis: When discussing the Zn level of the adjacent "normal" tissue, please explain what you mean by a "possible" decrease. Why technique and results do not allow you to be more precise?
Authors: We have only very few samples (n=2) from the particular region you are referring to in your question.

R. de Estable-Puig: Are you able to evaluate the influence of the in situ freezing in the distribution of trace elements?
Authors: In situ freezing should by arresting circulation and preventing diffusion, maintain the in vivo distribution of trace elements.

W. Maenhaut: I would think that stainless tools were used for obtaining the brain samples. If so, was there no contamination introduced for certain elements?

I am particularly thinking of elements such as Cr, Ni, Fe and Mn.

Authors: During surgery this was certainly the case. Before preparing the samples the tissue pieces were cut in a frozen state also by stainless tools. Since all material was prepared similarly the observed difference between various regions could not be explained by external contamination.

W. Maenhaut: It is hard to believe that beam damage could be avoided by limiting the beam current density to a few pA/μm^2. At best, the beam damage could be limited or restricted. One pA/μm^2 corresponds to 100 $\mu A/cm^2$, which would be an enormous current density in macrobeam PIXE and would result in complete destruction of the target. I agree that one cannot predict microbeam induced damage by extrapolating from macrobeam conditions, but some damage will certainly occur.

Authors: We agree with you that the proton beam will inevitably cause some damage to the sample as can be seen in Fig. 1. The choice of current density is based on studies of elemental alterations during PMP analysis of freeze-dried sections of biological samples. With the beam dimension and current density in this study we have observed no significant elemental losses or migration.

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