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BIOMEDICAL APPLICATIONS OF PROTON INDUCED X-RAY EMISSION

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Abstract

Apart from studies on aerosols, the majority of applications of proton induced X-ray emission (PIXE) with a normal beam or a microprobe (micro-PIXE) is found in biology and medicine. Two aspects of broad beam PIXE are often decisive for the choice of this analytical technique. Compared to other techniques capable of analysis down beyond the ppm level, PIXE can be carried out with a very small amount of material and minute fractions of the composite samples, even in the scale of micrometers and quite often with minimal sample preparation, which are important requirements for biomedical investigations. Secondly, the speed of the total analysis opens the possibility to analyze large numbers of samples in a reasonable time, which is often necessary in biomedical studies in order to obtain sufficiently significant correlations between trace element concentrations and biomedical phenomena. Few, if any, techniques can compete with micro-PIXE; quantitative trace element analysis on a micrometer scale still represent a challenging problem. The electron microprobe normally lacks the sensitivity while the laser induced techniques suffer as yet from quantification problems. This paper describes recent developments especially in micro-PIXE in biomedical research.

KEY WORDS: Proton induced X-ray emission, proton microprobe, trace-element analysis, synchrotron radiation, radiation damage, biological applications.

Introduction

Since the origin of PIXE in 1970 by Johansson et al. (2) and its subsequent development, an almost exponential growing number of papers dealing with the technique and its applications have been published. The most condensed form of information can be found in the proceedings of the four conferences held on PIXE and its applications (3,4,9). In addition, a number of review papers has been published, some of which emphasise work in the biomedical field (1,14). In order not to duplicate those reviews, this paper is mainly devoted to recent developments in all stages of the analytical procedures using the PIXE technique, including aspects of sample preparation, irradiation, data collection and concentration assignment, but also to recent improvements on the instrumental side. Since in a few laboratories synchrotron radiation has been introduced as the primary source to excite characteristic X-rays and, moreover, attempts are underway to focus these sources to a microprobe, attention will be paid to these developments as well.

Sample preparation

The thickness requirements for micro-PIXE work are different from those usually valid for the EMP. This difference is caused not only by the much longer range of the proton energies between 1 and 5 MeV, normally used for PIXE, but also by the real trace technique of micro-PIXE, which necessitates samples of a reasonable thickness to maintain sufficient detection power. To give an example: detection limits of PIXE normally are in the ng/cm^2 range (13). A matrix density of around 1 g/cm^3 then means that one needs slices of 10 µm to operate at the ppm level. Since on the other hand biological structures are generally inhomogeneous in three dimensions, one should not produce much thicker slices than the lateral resolution required. This example illustrates that going down in lateral resolution to a few or even one micron will shift the detection power of micro-PIXE to lower values.

Specimen preparation is still normally done with a cryotome at temperatures between -20 and

-30^0C. The sections are mounted on a thin plastic foil and freeze-dried. The sample remaining for irradiation is very irregular, inhomogeneous and with thickness variations due to variations in water loss during the freeze drying step. Unknown thickness and matrix composition per pixel will cause inaccuracies in the quantification step (see section on concentration assignment).
A promising way out seems to be a cold stage for the specimen, as is commonly used in electron microscopy but not yet in combination with proton beams. Preliminary experiments by Lenglet (private communication) show a reduction of radiation damage when a cold stage for the specimen is used in combination with an external microprobe obtained by passing the beam through a nozzle with a pinhole of 20 μm in diameter into a He- atmosphere. With sufficient pumping capacity, there is no problem to maintain the vacuum conditions in the accelerator and beam line used. A remaining problem is that due to the requirements of a very short pathlength of the beam in the atmosphere, it is not possible to visualize the specimen in the irradiation position, which means that the selection of areas to be scanned has to be done elsewhere.

Irradiation and data collection

The specimen is normally irradiated with a few MeV protons which is a compromise between a maximal cross section for inner shell ionisation and a cut off energy of the secondary electron Bremsstrahlung background below the energy of the characteristic X-rays of interest. For scanning purposes beam deflection in the x- and y direction is used although movement of the specimen holder e.g. with piezo-electric crystals is applied as well. Fast scanning is necessary if one wants to image the sample using secondary electrons (12). To survey larger areas, specimen movement under computer control is necessary because deflecting the beam over larger areas will introduce spot enlargements due to aberrations.

Data acquisition can be done in two ways. One way uses a hardware division of the scanned area in fixed pixel sizes and collects spectra per pixel of the different detectors used during the experiment. The scanned area is divided in, e.g., a 32 x 32 matrix and 1024 spectra are built up. A routing system is used and steered by the beam position to maintain event-to-pixel-correlation. The second possibility is the use of list mode data collection. Every event is labelled with the x,y position of the beam at that time. The advantage of the latter system is that pixel size and thus lateral resolution can be chosen after the experiment. One can, so to speak, zoom in on areas of interest and improve statistics at the price of spatial resolution in less interesting areas. For list mode collection one needs a fast read out system in order not to limit the maximum count rate capability and one needs a mass storage device.

Concentration Assignment

Although it is normally claimed that one of the major advantages of PIXE and also micro-PIXE is its fully quantitative character, a number of precautions are needed to obtain really quantitative results. An obvious advantage is, of course, that the underlying physics of PIXE is described in detail but nonetheless full calibration of a PIXE or micro-PIXE set up is necessary. It is therefore very valuable that efforts are made to make standards available especially designed for microprobes (10). Glass and ceramic microparticles are made at the National Bureau of Standards by Small et al. (11) and these can be produced in a wide variety of elemental compositions and sizes.

After the complete calibration of the set up one is faced with the problem that one has to know exactly the amount of mass under irradiation. For bulk analysis with PIXE an internal standard may be helpful, but for microprobe work adding an internal standard and maintaining the biological structure at the same time is cumbersome. Taking the thickness of the cryotome slices and quoting results on a wet weight basis is in principle possible, but on one hand one is often interested in dry weight values of trace element concentrations and on the other hand not knowing the residual thickness after the freeze drying step may cause problems in calculating concentrations from X-ray spectra due to a lack of information on the stopping of the proton beam and self-absorption of, in particular, soft X-rays in the sample.

In situ measurements of target thicknesses per pixel can be done in several ways. The Bremsstrahlung background caused by secondary electrons is proportional to the target thickness, provided that the bulk concentrations do not vary too much. Although this method is attractive in that only one detector is necessary, there are disadvantages. First of all one has to be sure that no other phenomena contribute to the background, such as Compton scattering of high energetic γ-rays in the detector or charge build-up of the specimen causing a higher continuous background extending up to high X-ray energies. Secondly, especially for micro-PIXE work, the statistics in peak-free areas of the background in a spectrum per pixel are normally very low, which introduces significant statistical errors. For those reasons, a second detector is introduced. Often, a surface barrier detector is used to measure scattered protons under backward angles and in this way monitor the accumulated charge and the matrix thickness per pixel simultaneously. A procedure used by Lenglet et al. (6) to normalize current and thickness separately is illustrated in fig. 1. A thin gold foil evaporated on a hostaphan backing foil is placed downstream of the sample. Rutherford backscattered (RBS) protons from this gold foil are detected with a surface barrier detector. In this way, the number of backscattered protons is proportional to the beam current per pixel, while the peak position in the RBS spectrum shifts in the proton spectrum with matrix thickness per pixel. In this way, with detailed calibration

of the geometry, one can assign concentrations quite reliably. Moreover, following the Au-proton peak in the course of time, one is able to follow possible deterioration of the sample under bombardment (15).

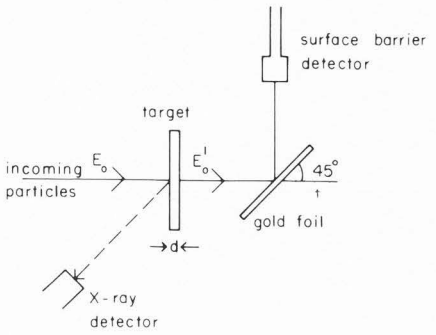


Fig. 1. Beam and target geometry used for concentration assignment.

Radiation damage

The technique described above is useful to establish mass loss during irradiation. Following the matrix thickness per pixel in time one can monitor mass loss. Fig. 2 gives results of such measurements on a section of a brain sample.

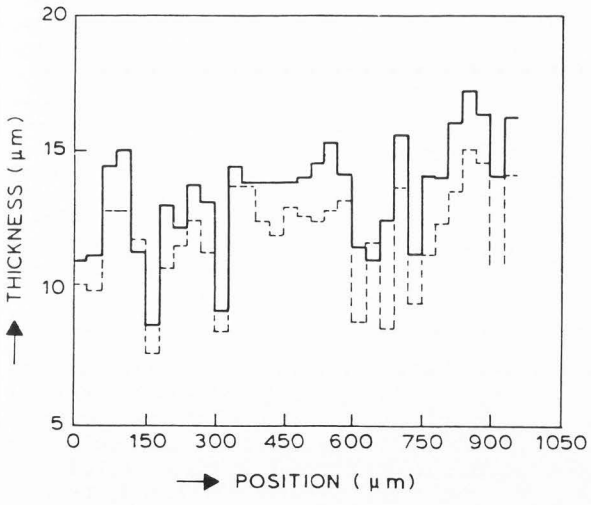


Fig. 2. Matrix thickness distribution of brain tissue before (solid line) and after (dotted line) proton bombardment for 1 hr with 1 nA beam. Results shown are from a line scan divided into 32 pixels.

The figure illustrates not only the considerable loss of material, but also the phenomenon that this loss is position dependent, and thus matrix dependent, even with the quite low current density of 1 pA/μm^2 used. To investigate what bulk elements were responsible for the mass losses, experiments were carried out with a 4 MeV deuteron beam in order to induce γ-rays via (d,pγ) reactions on C and O (see fig. 3). Results for carbon and oxygen are shown in figs. 4a and b.

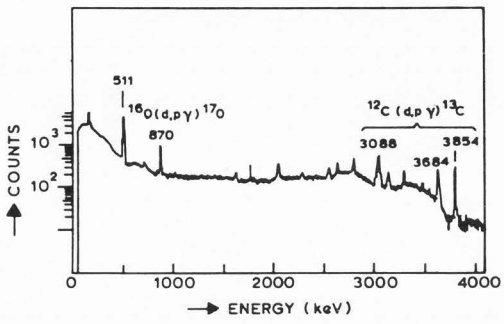
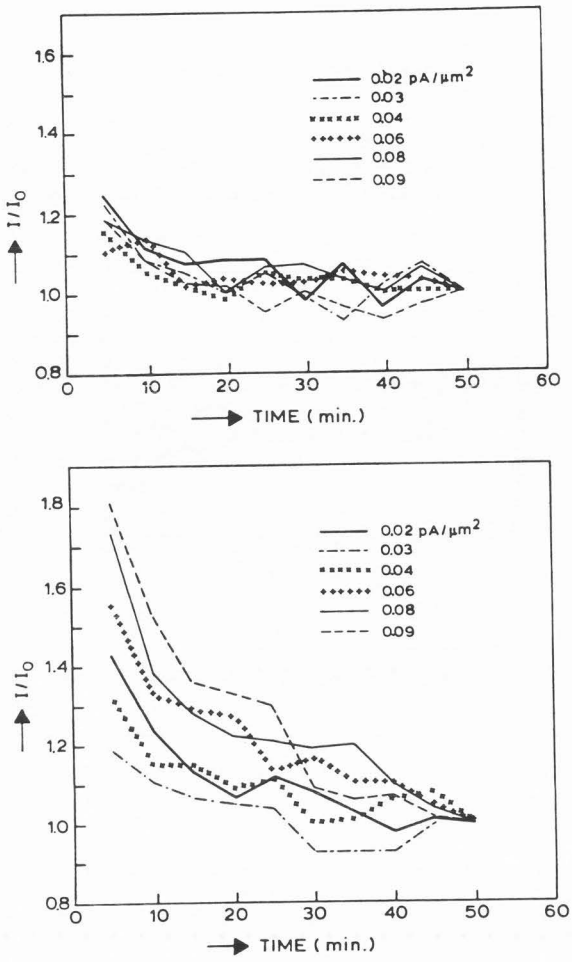


Fig. 3. Deuteron induced γ-ray spectrum used for measurement of C- and O-losses under bombardment.

Results are normalised to the final concentrations. Note the very low current densities compared with usual values in microprobe work.

Clearly demonstrated is the major loss of oxygen and the relative smaller loss of carbon, supporting the theory that the usual blackening of samples under the beam is carbonisation of the sample and not the result of extra carbon delivered by the beam in the usually not very clean vacuum conditions in particle accelerators. Not shown in these experiments is the loss of hydrogen; nevertheless, it is also very likely that considerable amounts of hydrogen will leave the sample, to a large extent in the form of water. In conclusion, it is obvious that real quantification is not an easy task. The problem of quantification can be solved in different ways. Either one expresses the area density of a trace element concentration relative to the thickness of the original ice slice, or one rapidly determines in the beginning of the measurement the matrix thickness per pixel using techniques comparable to those mentioned above, and expresses area densities of trace elements relative to those values, in order to obtain dry weight based concentrations. In both ways subsequent loss of matrix under bombardment is ignored, assuming of course that the trace elements themselves are not removed by radiation damage.



Figs. 4a(top) and 4b(bottom) Losses of C and O in the course of deuteron bombardment with current densities as indicated in the figures. Ordinate is normalized on the final peak intensity. Lines are drawn between data points only to guide the eye.

Applications

A comprehensive review of biological applications of proton microprobes may be found in ref. 7 and 14. Here only a few recently obtained results will be mentioned. Wensink et al. (16) studied the effect of dietary zinc deficiency on the zinc content of mossy fibers of the rat hippocampus. A typical result of a Zn distribution together with a schematic drawing of the area of interest is shown in figs. 5 and 6. The authors found that during Zn deficient diets the Zn concentration in the mossy fibers drops considerably despite the fact that the average concentration of Zn in brain tissue remains constant but this decrease only appears after a long period of time (90 days).

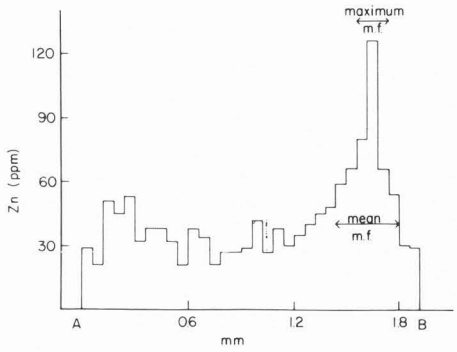


Fig. 5. Zn distribution obtained from the scan given in fig. 6. Shown is a typical result of a hippocampal section from a rat with a control diet of 50 mg Zn. The position of the mossy fibers is indicated. Frozen tissue was sectioned in 30 μm slices, mounted and freeze-dried. Beam size was about 50 μm and the beam current 0.1 nA.

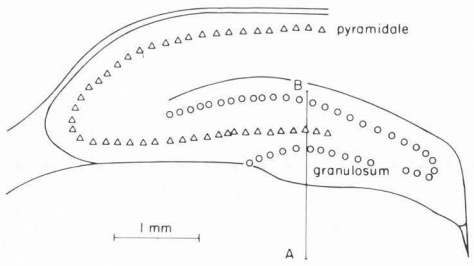


Fig. 6. Drawing of the rat hippocampus with an indication of the scan used. Stratum pyramidale and stratum granulosum are indicated.

The behavioral abnormalities observed may be caused by the production of excess glucocorticoids under conditions of Zn deficiency. Another interesting area for application of the proton microprobe is the measurement of single blood cells. In a comparison between blood cells of children with Down's syndrome and a control group Annerén et al. (1) analysed three different types of blood cells, erythrocytes, thrombocytes and neutrophils. They arrived at the conclusion that significant differences exist between both groups, especially in the erythrocytes where Cu and Ca were elevated in the patients while Zn, Mn, Fe and Mg were depleted. Moreover, Ti was detectable in the erythrocytes of patients with Down's syn-

drome. Although the reasons for those observations are not known in detail, the work represents a good demonstration of the analytical potential of the proton microprobe, because in my opinion no other instrument up to now is able to reproduce those results in a quantitative way. Malmqvist et al. (8) carried out studies on the physiological role of zinc in human epidermis which also included a comparison with the electron microprobe.

Preliminary measurements were reported on a comparison of Ca distribution in the normal and psoriatic skin, respectively. Finally, the work done on teeth by the Amsterdam group should be mentioned; distribution of F and correlation of those distributions with the Ca/P ratio were measured in order to see if F displaces the OH group in hydroxyapatite, rather than being in the CaF_2 form. For the detection of F, the nuclear reaction $^{19}(p,p\gamma)^{19}F$ was used. The 197 keV γ-ray was detected. Due to the half life of 122 nsec of the nuclear energy level it is possible to detect the radiation in time between bursts of protons accelerated by the cyclotron. In this way all prompt background in the γ-spectrum is suppressed and consequently the detection limit for F is lowered considerably. The technique will also be used to measure the influence of F on the growth of hamster teeth.

Future developments and Discussion

As is to be expected, attempts are made to decrease the spot sizes obtainable with proton beams. The development of high brightness ion sources is the main issue, immediately followed by the construction of achromatic lens systems. Although such developments will represent a major step forward, e.g., in trace element analysis of biological material at the subcellular level, the problem of radiation damage will become much more serious once these submicron beams become available.

A rather new development is represented by progress in attempts to focus synchrotron radiation, opening the possibility to perform XRF on a small scale. At the Synchrotron Radiation Source at Daresbury (U.K.) Van Langevelde et al. (5) used toroidal shaped Si (111) crystals to focus a 20 keV synchrotron beam to several tens of microns. The experimental set up they used is given in fig. 7; the first scans on biological material have been made and after the implementation of a high brightness lattice in the Daresbury storage ring, focussing with two crystals in combination with collimation with suitable pinholes will make beams of tunable X-ray energies with beam sizes down to 10 μm available.

Conclusions

The research field in which proton beams for microanalytical work are in use is growing and very active. Major improvement in beam quality

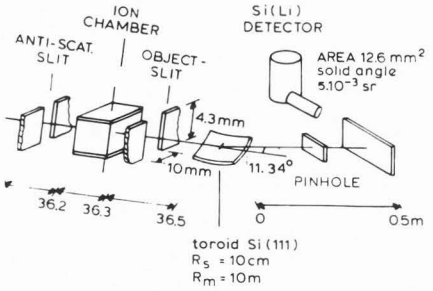


Fig. 7. Schematic layout of the set up used for the development of a synchrotron microprobe

and beam handling, resulting in smaller beams has already been obtained but further improvement is te be expected. Also, a better understanding has been reached of all processes involved in the interaction of the beam with sample material. This has resulted in a better quantification of the analytical results with improving accuracy and precision. Nevertheless, only relatively few applications of PIXE in the area of biomedical research have been published. The reason for this may be twofold; first, the number of proton microprobes in operation is as yet limited and secondly, the development of the microprobes is mostly controlled by physicists for whom daily interaction with biologists to discuss and solve these problems is not self-evident. In the future major progress is only guaranteed if multidisciplinary teams are formed using the proton microprobes to its full potential It remains to be seen at what stage radiation damage starts to limit further developments of smaller and higher intensity beams for biomedical work.

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Discussion with Reviewers

G.M.Roomans: One of the basic rules in quantitative electron probe X-ray microanalysis is, that the standard should resemble the specimen in its physical and chemical characteristics, so that e.g. mass loss in specimen and standard is comparable. We have also shown (Forslind et al., Histochemistry 82, 1985, 425-427) that quantitative correlative proton and electron probe microanalysis should be possible with the help of standards such as cryosections of gelatin containing known amounts of salts. Although "standardless" analysis still appears to be common in proton microprobe analysis, don't you think that your findings of extensive mass loss may lead to use of standards also in quantitative PIXE?
Author: In general, relative procedures using standards are more accurate than absolute procedures, due to the elimination of errors in determination of the geometry of detectors used and due to errors in the physical parameters describing the X-ray production and detection processes. However, since we cannot rely on the stability of most proton accelerators, one has to be sure that the flux monitoring procedure, very often proton scattering from the specimen or the use of a gold foil as described in this paper, is adequate for both standard and sample which sets high demands on the resemblance of standard and sample, especially regarding chemical composition. A very elegant way out of the flux monitoring problem is a specimen holder containing standard and specimen immediately adjacent to each other in order to scan this combination in one run averaging out proton beam fluctuations. From the sample preparation point of view, this is not an easy task. The demonstrated mass loss in this paper indeed underlines the need to develop relative procedures for concentration assignment.

G.M. Roomans: When you propose to relate the elemental concentration to the thickness of the cryosection in the frozen state, how do you determine this thickness? The nominal setting on the cryostat is not reliable; do you have an independent way of verifying section thickness?
Author: We did calibrate the settings on the cryostat by slicing cylindrical bars of araldite and determination of the weight of those slices with a micro-balance. Knowing the density of araldite and the diameter of the bars leads to figures for the thickness. During every series of preparations a few of these "calibration slices" are incorporated in the procedure.

G.M. Roomans: PIXE analysis at higher resolution will make an accurate morphological localization of the probe even more necessary. What progress do you see in the visualization of the sample during analysis?
Author: In the geometry in use, in most proton microprobes high quality optical microscopy is very difficult, if not impossible. People use high quality objectives with long working distances or stereomicroscopes. Very often, the illumination is far from perfect and to position the beam with micro precision is in most cases an illusion. We are developing a twin set of x-y tables with a precision read out system using optical rulers and calibrated with respect to each other. The tables are reproducibly mounted or in the microprobe or under a high quality optical microscope. In the latter position the track to be scanned is defined, tables are exchanged and one is able to find the coördinates of the track in the microprobe even without seeing the sample at all. We expect to be able with this twin set to correlate morphological structures and results of the scans with accuracies of 1 µm.

R. Nobiling: How can one be sure that loss of matrix hydrogen is mainly as water? How serious is the problem of radical formation due to proton

irradiation?
Author: The only indication of water losses is the observed ratio of the losses of H and O, remaining 2 : 1 at least for some time during the irradiation. This ratio is measured in a direct way using forward scattering and in an indirect way by following the precise shape of the Bremsstrahlung background in the X-ray spectra from which one is able to derive the change in average Z of the matrix in the course of time. Radical formation is probably the main problem and the initial step in the process of radiation damage.

R.Nobiling: You emphasize loss of matrix material as the main problem for quantification. Is the problem of loss of the trace elements sufficiently studied? Which role plays the matrix e.g. by its heat conductivity?
Author: To study loss of trace elements under proton bombardment is not easy. To follow trace element concentrations in time necessitates reasonable statistics during the time intervals one chooses to measure. Very often, these intervals are too long to draw solid conclusions. For the Zn measurements reported here we did not observe any loss of Zn even during quite extended irradiations. On the other hand, losses of Cl, Br and also Cd are reported in the literature. Assuming a two step process of radiation damage with initial breaking up of chemical bonds followed by diffusion and evaporation of volatile elements, the second step will be influenced by heat conductivity of the matrix. In my opinion, poor heat conductivity leading to more elevated local temperatures will enhance the primary effects of radiation damage.

R.Nobiling: Does the problem of element dislocation arise within your lateral resolution?
Author: Dislocation by direct beam impact (recoil energy) is much smaller than our lateral resolution (>2 μm). Dislocation caused by temperature induced diffusion out of the beam track is a difficult problem. During our experiments we did not observe such diffusion but we do not have experience with very mobile elements like the electrolytes.

G.Legge: For the biological specimen, the loss of individual light elements can be measured efficiently by backscattering and even hydrogen loss has been measured by forward scattering. The (d,p) studies may be more useful for thicker specimens, but is there a problem with counting statistics?
Author: The cross section of the exothermic nuclear reactions of the (d,p) type on C and O have relatively high values (>100 mb).
In combination with the high concentration of these elements, counting rates, both for emitted protons and the 870 and 3088 keV γ-rays, are sufficient to determine losses in time intervals of about 10 min. using about 10 pA beam current. If you assume a strict dose dependent relationship, the statistics allow one to measure in intervals of 6 nC of accumulated charge, which is sufficient to follow the process for these two elements.

G.Legge: Is there any advantage in measuring the beam scattered from a foil placed after the specimen rather than measuring the beam scattered at forward angles directly from the specimen? The beam scattered by the specimen also shows an energy shift proportional to specimen thickness. At extreme forward angles, where STIM measurements are performed, the energy loss measurements may be performed with negligible beam charge and hence negligible beam damage.
Author: We wanted to measure losses in the standard experimental conditions used for trace element work. For that reason, the forward scattering as used by Lefevre is less suitable since the very high cross section at forward angles necessitates a decrease in beam current. An advantage of the gold foil is that the scattering process on gold is more easy to describe than the scattering on a complex sample with unknown chemical composition. Moreover, no influence of proton absorption from sample to detector besides the beam track is present as is the case during forward scattering from protons underway to the detector.

G.Legge: Many, if not most, modern particle accelerators have clean, metal sealed, oil free vacuum systems, in contrast to most electron probes. However, the darkening of most organic materials is still observed and has been attributed to specimen damage. Since it is associated more with beam charge than with beam current density, and is sometimes reversible, this damage is probably due to ionisation rather than thermal effects. Hydrogen has been shown to come off rapidly -in seconds- and your observations on oxygen are therefore interesting but not surprising. Presumably, from these statistics, you would not attempt to extrapolate the oxygen curves back in order to attempt a prediction of the initial oxygen content. There is still the possibility of normalizing elemental counts to the carbon content and this is sometimes done. Have you any comment on the usefulness of such a technique?
Author: Biologists are used to concentrations on a weight to weight basis. Since the C-content of most tissues is known, the procedure you indicate is possible. If you use it per pixel you have to assume a homogeneous C-distribution. Nevertheless, material loss still represents a problem due to the change in self absorption in the sample, especially for the low Z elements.

K.Malmqvist: You indicate that use of a cold stage could reduce radiation damage. Is this applied to a freeze dried target or a frozen hydrated one? The effect of such a cold stage implies that the damage observed is mainly a temperature effect rather than dose dependent.

Could you comment on this?
Author: We did measurements on a cooled freeze dried target. The effect of cooling is that the effect of the second step in the damaging process (see answer to Nobliling's 2nd question), that is the diffusion and/or evaporation of the atoms/molecules formed in the initial dose related step is decreased.

K.Malmqvist: You indicate a beam size of > 10μm for a synchrotron radiation microprobe. Is this the ultimate resolution or do you or any other groups foresee a significant improvement?
Author: By focussing alone it will be very difficult to obtain smaller spots in the near future due to crystal imperfections and shape inaccuracies if crystals are used and surface roughnesses if mirrors in total reflection mode are used. For that reason, very often collimation is performed after the focussing step by placing a pinhole directly in front of the specimen. Provided that the flux density after the focussing action is sufficient, one is able to go for smaller beams although the production of such small openings in material of sufficient thickness to stop the primary photon beam represents still a challenging problem.