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PROTON MICROPROBE ANALYSIS IN BIOLOGY

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Abstract

This paper is intended as an introduction to the field of proton microprobe analysis with special emphasis on applications in biological sciences. It is mainly intended for users of electron microscopes equipped with microprobes or other analytical equipment. The basic principles of Particle Induced X-ray Emission analysis are discussed as well as the instrumental requirements for the proton microprobe. The analytical characteristics including quantification procedures are compared with those of the electron microprobe and a review is given of various analytical applications of the proton microprobe within biology and medicine.

KEY WORDS: Proton microprobe, trace element, particle-induced X-ray emission, biological microanalysis, medicine, single cell, hair, skin, mineralized tissue, brain, artery, liver, kidney, limpet, plant, wheat, pollen tube.

Introduction

In the early seventies a new analytical technique, Particle- Induced X-ray Emission (PIXE) was developed. The pioneering work by Johansson et al. (1970) demonstrated the possibility of analysis at the picogram level using protons or heavier particles of a few MeV/u from an electrostatic accelerator to induce characteristic X-rays in a thin sample. This work constituted the first step in the development of the PIXE technique which since then has been in constant progress. Three international conferences (Lund, 1976 and 1980, Johansson, 1977 and 1980; Heidelberg, 1983, Martin, 1984) on the development and applications of the technique have each attracted hundreds of delegates. At about the same time as the introduction of PIXE analysis a group at Harwell, UK (Cookson et al. 1972) was developing a micro-analytical device which they called a proton microbeam. This comprised a probe-forming optical system of quadrupole magnets intended to focus collimated ion beams to small dimensions for analytical applications using PIXE and nuclear reactions.

The analytical sensitivity of the PIXE technique is superior to that of the electron microprobe. The intense primary continuous Bremsstrahlung produced when keV-electrons impinge on a sample limits the sensitivity in energy-dispersive detection to detection limits in the interval 0.1-1 mg/g dryweight (Coleman, 1978). Due to the much lower charge-to-mass ratio of protons (and heavier ions) the intensity of the primary Bremsstrahlung is many orders of magnitude lower than for electrons. The detection limits for PIXE analysis in an organic matrix are in the interval 0.1-10 µg/g. The cross-sections for inner-shell ionization are much larger than the nuclear cross-sections. However, a combination of nuclear reaction analysis and X-ray detection for PIXE analysis constitutes a very powerful analytical tool for microanalysis of biological matter.

Other micro-analytical methods for the analysis of biological material offer various useful properties and the general conclusion that can be drawn from a comparison is that a specific analytical task is best solved by a suitable combination of different methods. As I will try to demonstrate in the following, the Proton Microprobe (PMP) is one very useful analytical technique for biomedical applications. For a detailed comprehensive description of the proton microprobe and its applications in the biomedical field the recently published book by Vis is highly recommended (Vis, 1985).

The Proton Microprobe

Particle-Induced X-ray Emission

When protons or heavier ions impinge on a target part of their kinetic energy is transferred to electrons in the atomic shells of the target atoms via the Coulomb interaction. The cross-sections for inner-shell ionizations by projectiles of a few MeV/u are high, while the continuous primary Bremsstrahlung is of low intensity. In fig. 1 K X-ray production cross-section as a function of the proton energy is given for different target materials (Gordon and Kraner, 1972). Due to the high

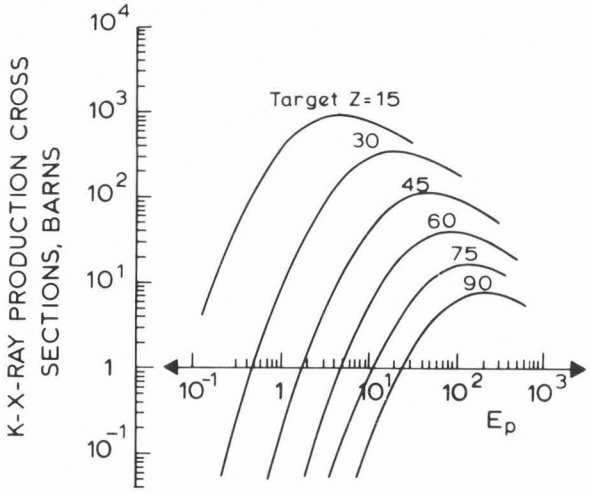

Fig.1. K X-ray production cross-sections as a function of proton energy for different target materials (Gordon and Kraner, 1972)

ionization probability secondary energetic electrons are ejected from the atoms and then decelerated in matter. During this process secondary electron Bremsstrahlung (SEB) is emitted. This is the most important contribution to the continuous background in particle-induced X-ray spectra. In fig. 2 some significant background components in PIXE spectra are shown (J. Guy, private communication). The relative importance of these components is dependent on target composition, detector

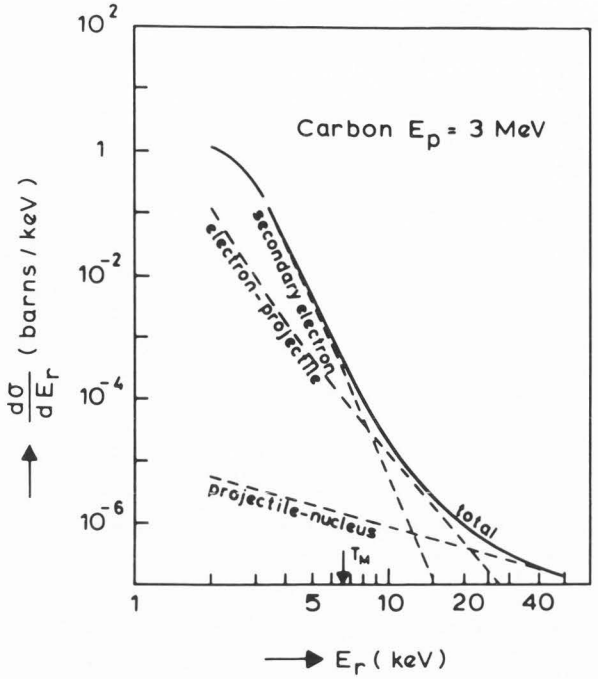

Fig.2. Important background components in PIXE spectra (J. Guy, unpublished)

material, projectile mass and energy and experimental geometry.

Classically, a particle can transfer an energy of Tm=4m/MxEp (m=electron mass, M=particle mass, Ep=particle energy) to a free electron. Consequently, the importance of SEB is dependent on the choice of energy of the projectile as are the cross-sections for the production of characteristic X-rays (see fig. 1). From this a general conclusion can be drawn that for optimum detection limits for low-Z elements one should select low-energy and for high-Z elements high-energy particles. However, when heavier projectiles are used and when the projectile energy is increased not only is the probability of producing vacancies in the inner atomic shells increased, but the cross-sections for the nuclear reactions also is increased. When the gamma rays are Compton scattered in the detector they yield a continuous background which is relatively constant over the whole energy spectrum. The contribution for the higher X-ray energies will be of relatively higher importance since the SEB is very low for energies above Tm. Consequently there is an optimum choice of energy and projectile mass which will yield the best detection limits in a "normal" analysis (Folkmann et al., 1974). Most PIXE analysis is carried out with protons of 2-3 MeV.

A common problem for all X-ray methods is the difficulty in detecting the X-rays from the lightest elements. In PIXE the problem is sometimes more pronounced (cf.

section on Detection system). The nuclear reactions induced in the target nuclei may be used for analytical purposes. Light elements, e.g., sodium, although normally possible to analyse with PIXE, may also be determined by the simultaneous detection of gamma rays (Duerden et al., 1980). In addition, projectiles which are scattered by the target nuclei lose energy in reverse proportion to the mass of the scattering nucleus. The lightest elements may then be determined by detection of the scattered particles (Chu et al, 1978).

In "normal" PIXE analysis (macroPIXE) ion beams with dimensions of a few millimetres are used to irradiate samples, which may be either thin (projectile deceleration and X-ray attenuation negligible) or infinitely thick. The samples are normally irradiated in vacuum. However, the beam may be extracted through a thin foil and particularly sensitive samples, for example biological material, can be irradiated in air or a helium atmosphere in order to promote convective cooling during the analysis (Williams, 1984).

To obtain quantitative results the total beam charge incident on the sample is monitored using a Faraday cup or a foil which "chops" the beam, the particles scattered from the foil then being counted in a surface-barrier detector (Hollis, 1972).

The spectra accumulated in PIXE analysis are often quite complex and require sophisticated computer evaluation for fitting of the characteristic peaks and the continuous background. There exist several approaches, either fully automatic, "batch mode" codes (Johansson, 1982) or interactive codes which require some manual attendance at the terminal (Maxwell et al., 1984). The choice of a suitable program is often guided by the available computing capacity. In a recent survey several different codes were compared for various analytical situations (Campbell et al., 1986). The results show very good agreement between the different codes in fitting and calculating the areas of the characteristic peaks. If very high speed is required in the fitting procedure simplified versions of the codes may be used, e.g., for a scanning proton microprobe in elemental mapping mode (see section on Data acquisition and handling).

Instrumentation

When ion beams are used for microanalysis they may be collimated or collimated and focused. For practical analytical use the probe based on pure collimation has limited use since the current density will normally be too low for trace element analysis. The collimator has to be very close to the sample and severe problems in the form of a halo around the beam due to slit scattering normally occur. However, for "semi-microprobes" (>50 μm) this approach may be feasible and offers a very simple means of obtaining a probe. Indeed there exist several probes based on pure collimation (MacArthur et al., 1982; Swann, 1982).

The other approach in the production of a micrometre beam is demonstrated in fig. 3 together with the principles of the electron microprobe. The ions are produced in an ion source and accelerated in either an electrostatic accelerator or in a cyclotron. The existing ion sources for the production of, e.g. protons (Rf-source and duoplasmatron source) for the electrostatic accelerator are of low brightness compared with the sources used, for example, in an electron microscope. This is, in fact, an important limiting factor when trying to produce a narrow beam.

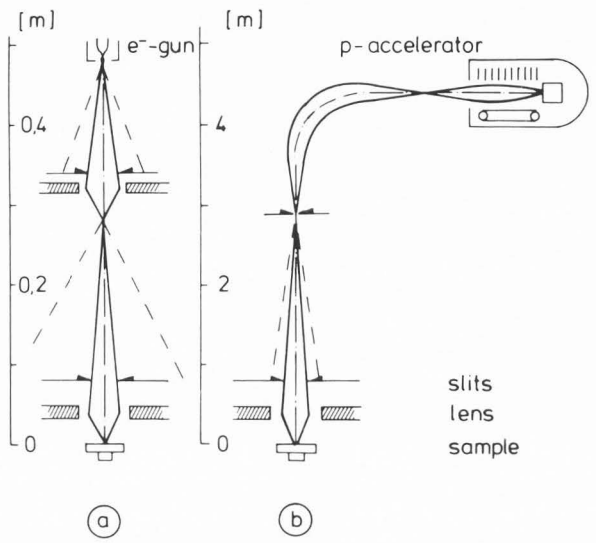

Fig.3. Schematic beam profiles in microprobes:
a) Two-stage demagnification in an EMP
b) One-stage demagnification in a PMP, (Nobiling, 1983)

Furthermore, the quality of the beam is degraded on its long way (typically 5-25 metres) through the accelerator. The relatively short and very stable vertical column of the electron microscope is superior to a normal ion accelerator in maintaining the beam quality.

Beam transport After passing through the analysing magnet, which selects the proper particle energy, the ions are focused on the object collimator. The transmitted beam is then passed through the lens system which forms an image of the object collimator on the target; cf. the optical system of the electron microprobe in which the cross-over of the primary ion source is used as a probe-forming object (fig. 3). Due to the rigidity of the protons they are not as easy to focus as electrons in an electron microscope. The solenoid lenses used for

focusing of keV-electrons has to be replaced by a superconducting lens which can produce strong enough fields to focus MeV protons (Maggiore, 1980). This is a very expensive solution and therefore not very common in the design of PMP systems. The most widespread technique incorporates magnetic quadrupoles (Cookson et al., 1972; den Ouden et al., 1981, Watt et al., 1982, Legge et al., 1982, Bosch et al., 1980). Electrostatic quadrupoles are also used, in particular when different ions are routinely used and the same quadrupole excitations can be used (Augustyniak et al., 1978; Lefevre et al. 1983).

During their course in the vacuum system the ions interact with residual gas molecules and at the edges of the object collimator. The scattering events result in a halo around the core of the focused ion beam. This limits the lateral resolution of the microprobe in particular when steep concentration gradients are analysed. The vacuum should therefore be better than 10^{-6} torr in the beam line and the collimator designed to be "anti-scattering". The design by Nobiling et al.(1975) is used by several groups and the probability of slit scattering is reduced by a geometrical arrangement in which a double-scattering event is required for the ion to reach the sample. It is important to be able to set the object collimator to openings between 10 and hundreds of micrometres. When using diaphragms they can be exchanged and if slits are used the distance between the edges is therefore either manually controlled by micrometre screws or remotely,e.g., by a set of piezo-electric crystals (Heck, 1979). The material in the edges of the collimator has to be very resistive to radiation damage and high temperatures since it would otherwise gradually deteriorate leading to an increase in the slit scattering. Normally, the object collimator is preceded by a somewhat larger aperture (sometimes water-cooled) to dissipate most of the energy in the beam.

Focusing systems The choice of lens configuration for focusing the ions to a small spot on the sample depends on the accelerator, the demagnification factor, lens aberration, selection of ions, economy etc. It is not easy to tell which system is the best. For a detailed discussion on probe-forming systems the comprehensive book by Grime and Watt (Grime and Watt, 1984) is highly recommended.

A choice which may seem obvious to the experienced electron microprobe user is to use solenoid coils. The optical properties of the beam are very well understood due to the long experience gained with such systems in electron microscopes and the phase-space acceptance is large compared with other systems. However, as already pointed out, the current required for focusing MeV ions is too high for a conventional system and to realize such a system a superconducting coil has to be used (Maggiore, 1980). This is expensive and it requires a constant supply of liquid helium. However, in combination with an accelerator of high energy stability such systems have good optical characteristics.

As mentioned above the lens systems in most widespread use for proton microprobes are various configurations of magnetic quadrupoles. In fig. 4 a few examples of configurations are shown. Normally, 2, 3 or 4 quadrupole magnets are combined . For details on the characteristics of the different configurations see Grime and Watt, 1984.

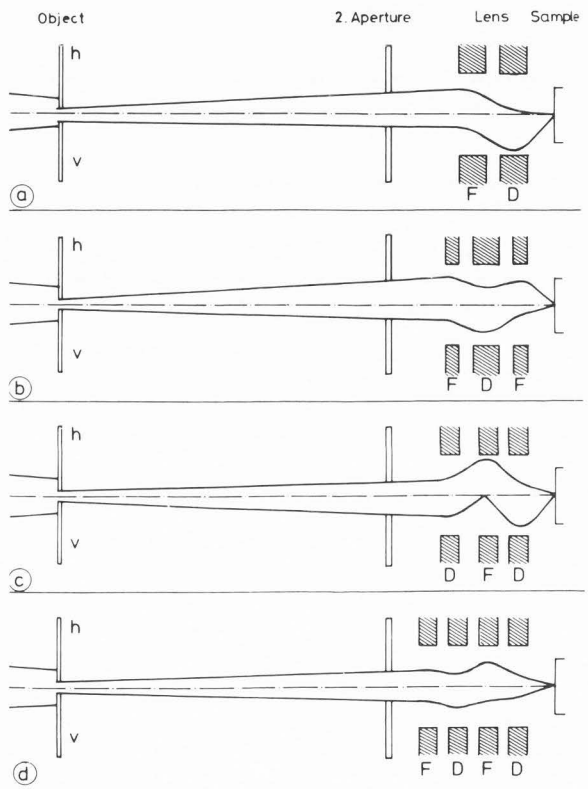

Fig.4. Quadrupole lens configurations used in existing PMP systems. Beam profiles in horizontal (h) and vertical (v) planes. Not to scale. (a) doublet, (b) symmetric triplet, (c) special triplet, and (d) Russian quadruplet (Nobiling, 1983).

Generally when focusing to micrometre dimensions one either has to use a small object aperture and suitable demagnification or a larger aperture with large demagnification factors. The disadvantage in using a small object is the increasing influence from scattering at the edges which causes deterioration of the beam quality. On the other hand, selecting very high demagnification factors increases the

lens aberration coefficients which will then limit the smallest attainable beam spot. For optimum use of a quadrupole system a compromise has to be made between high demagnification and small object collimator. The aberration coefficients normally most important for well-aligned microprobes are chromatic (small variations in ion energy), rotational (magnets rotated relative to each other) and spherical (imperfect magnetic fields). The relative importance of these varies with the exact configuration and calculations for many different configurations are given in the book by Grime and Watt, 1984. Using a ray-tracing computer code, OXRAY (Grime et al, 1982) they have compiled a lot of data into very useful graphs of each system

When comparing the performance of existing proton microprobes it is obvious that good performance is possible for both single-ended and tandem accelerators and for two-, three- and four-quadrupole systems. The care put into the design and construction of the system is very important for the end result. In the best systems of today the minimum beam dimension is about one micrometre with a beam current density of 50-100 pA/μm^2 (Watt et al., 1982; Legge et al., 1982). At present, smaller beam spots have only been obtained with very low beam currents, which are not useful for X-ray analysis, and for low proton energies. However, there are plans for second-generation PMP systems in which the beams could reach a few tenths of a micrometre (G.Grime and and G.J.F. Legge, private communications) with beam currents useful for analysis. Development of high brightness ion sources and improved optics including achromatic elements etc. are examples of important contributions required for such systems. Successful development of this kind would, of course, mean a giant step forward in trace element analysis of biological material on the subcellular level.

Detection system Normally, X-rays are detected in an energy-dispersive detector, as is often the case with the EMP (see fig. 5). Wavelength dispersive systems have also been planned, but they suffer from a very small solid angle which makes it difficult to combine them with the PMP. In order to determine the sample thickness, scattered particles are counted in a surface- barrier detector (see also section on Quantification techniques).

A special problem in PIXE analysis is the possibility of scattered projectiles entering the detector. They could after the scattering still have a substantial velocity left and deposit significant energy in the crystal. The detector window has to be thick enough to stop these protons since the X-ray spectra will otherwise be distorted. In the case of the electron microprobe the scattered electrons can easily be stopped by magnets or very thin windows (Neumann et al., 1978). In PIXE analysis deflection magnets can also be used (Musket, 1986) but to be able to deflect the particles enough the flight path in the magnetic field has to be very long which will result in a large sample-to-detector distance. This is a significant disadvantage in microprobes where a large solid angle is important. The need for a relatively thick window limits the capacity of the PMP to determine the light elements, e.g. sodium. However, exchanging the protons for alpha particles, which, in most accelerators is a rather straightforward procedure, would reduce the problems of penetration of scattered particles into the detector due to the very short distance travelled by alpha particles in matter. In addition, alpha particles give high X-ray production cross-sections even at rather low energies and for sodium it was shown by Sealock et al. (1983) that 2 MeV alpha particles are superior to 20 keV electrons in the EMP (see fig.6).

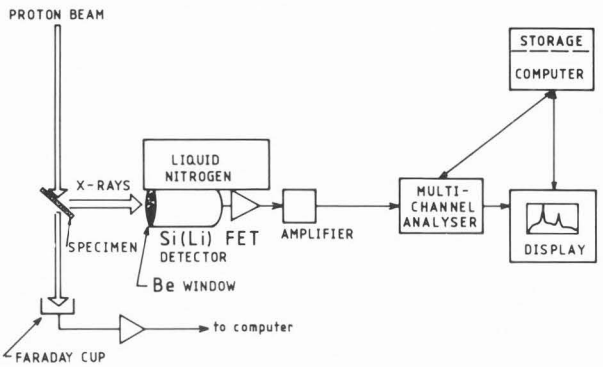

Fig.5. Schematic experimental arrangement for PIXE/PMP analysis.

Manipulation and viewing of the sample

The samples are normally mounted in a way similar to the EMP. A stage which can be moved in micrometre steps in three perpendicular directions, and in some cases also allowing for tilt, enables the user to reach any position on the sample with the beam. For special applications, e.g. channelling, goniometers may be used (Ingarsfield et al., 1981). For convenience several systems allow simultaneous mounting of several samples and change of sample by remote control. This reduces the pumping times. Depending on the measurements performed many different sample arrangement will occur; the details of which are beyond the scope of this paper.

To ensure the correct positioning of the sample, which in biological applications can be both critical and very difficult, optical microscopes are normally used. The sample may be viewed from the front or the back. Viewing from the back is normally

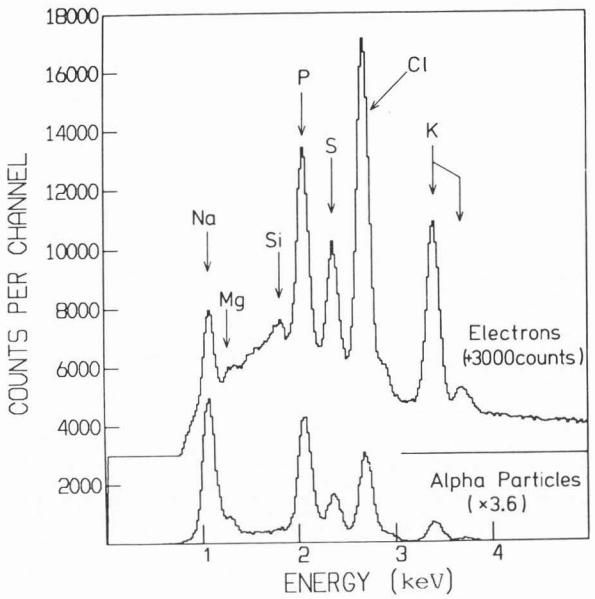

Fig.6. Energy spectra of X-rays detected during alpha particle (2 MeV) and electron (20 keV) irradiation of a freeze-dried cryosection of rat kidney tissue (Sealock and Mazzolini, 1983).

used for focusing the beam on fluorescent plates or foils. In attempts to position the beam it is preferable to use a zoom stereomicroscope with at least 120 times magnification. Another technique for orientation and focusing is to detect secondary electrons (see next section).

In remote controlling of the beam position on the sample stepping-motors or piezo-electric drivers can be used (den Ouden et al., 1981). The speed of the latter facilitates also relatively fast sample movement for scanning proton microprobe analysis (see next section).

Beam scanning In analogy to the scanning electron microscope there are significant advantages in rapidly "rastering" the proton beam over the sample. This reduces heating effects which may pose a serious problem for biological material (see section on Sample deterioration) and enables imaging or "elemental mapping" equivalent to the SEM. This scanning can either be achieved by moving the sample relative to the beam (den Ouden et al., 1981) or by moving the beam relative to the sample (Heck, 1979; Grime et al, 1984). The scanning frequency of the former method is limited but no deterioration of the lateral beam resolution will occur. For beam scanning, on the other hand, the frequency at least for electric scanning can be high and magnets without an iron core can be acceptable. The disadvantage is the problem in maintaining the optical properties (resolution) of the beam when the deflection amplitude is large (>1mm). Distortion can be avoided by deflecting the beam after the lens system. However, this solution calls for a lot of space and there is often too much equipment to fit into the available working space. Another ingenious idea is to deflect the beam twice before the lens and ensure that the beam always crosses the optical axis in the principal plane of the lens (Heck, 1982). In this way scanning could be performed with an amplitude of a few mm:s without serious distortion of the beam. With a nominal beam size of a few micrometres, for example the Oxford group is able to keep the beam size below 10 micrometres for a maximum deflection amplitude of 2 millimetres.

Scanning the beam facilitates the use of secondary electrons for imaging the sample, in analogy to the SEM. When the protons impinge on a sample secondary electrons are emitted. A traditional detector for secondary electrons can be used to intensity-modulate an oscilloscope. The horizontal and vertical deflection voltages of the scanning system are used to control the X and Y plates, respectively (see fig. 7). A secondary electron image of the sample may thus be obtained on-line (Traxel and Mandel, 1984).

A major difference in the behaviour of protons compared with electrons is the penetration depth. Protons of a few MeV will penetrate several tens of micrometres in biological matter while, for example, 20 keV electrons, excite down to a depth of about 10 micrometres (see section on Lateral and depth resolution). The large penetration depth of the protons makes it possible to "see through" semi-thick sections of biological material by detecting the secondary electrons emitted from the

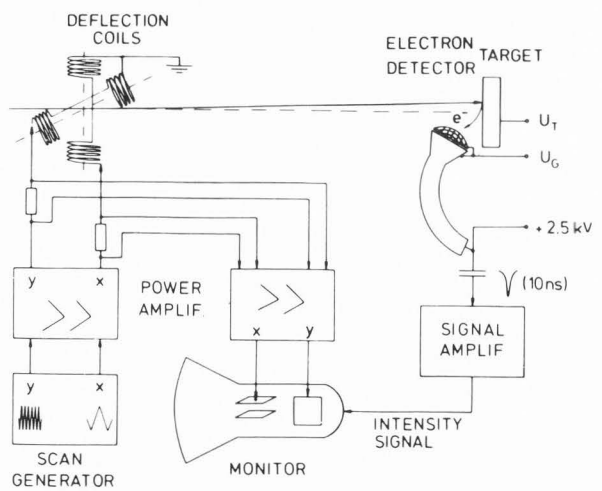

Fig.7. Secondary electron imaging system at a proton microprobe (Traxel and Mandel, 1984)

back surface of the sample (Kneis et al., 1981). In contrast to the electron currents from a SEM the current from an accelerator is rarely stable even over rather short time intervals. Consequently, the secondary electron image from a PMP can be unstable and difficult to interpret and it would be useful to be able to normalize the picture to the actual accumulated charge of each picture element (pixel). This is accomplished by accumulating the charge in each pixel and by using computer software to generate the image.

Data acquisition and handling

In a "static" PMP analysis the data acquisition is straight-forward and X-ray energy spectra are stored in multi-channel analysers and subsequently evaluated by computer programs (see section on Particle-Induced X-ray Emission). In scanning-mode analysis each pixel produces a spectrum by repeated analysis and cumulative storing of events. In a realistic analysis a square of 128x128 pixels may be analysed. The large number of spectra produced using this technique is very difficult to manage and the available computer memory capacity is seldom sufficient. This problem is solved by using the technique of event-by-event (list-mode) collection which is a standard technique in subatomic physics experiments. The principle is to assign one ADC to each kind of signal (X-rays, scattered particles, secondary electrons etc.) and one ADC each to horizontal and vertical coordinates. When an event is registered it triggers the read-out of the coordinates of the pixel. The three numbers (type of radiation and x and y coordinates) are stored together in a mass memory possibly with a flag indicating which kind of event took place (if more than one detector is used). If high enough on-line computer power is available it is possible to have a continuous graphic display of selected X-ray maps (cf. elemental mapping in the EMP). In fig. 8 an example of a data acquisition system is given (Legge and Hammond, 1979). Using computer software, the data stored in a mass memory (usually on magnetic tape) is normally sorted off-line. In this way it is possible to perform any kind of evaluation, e.g. elemental mapping (one or two dimensions), energy spectra for a single pixel or a specific one- or two-dimensional sample region, or sample mass distribution etc. (G. Grime, private communication) This is a very flexible technique which can produce very useful representations of the large data base accumulated in each PMP analysis.

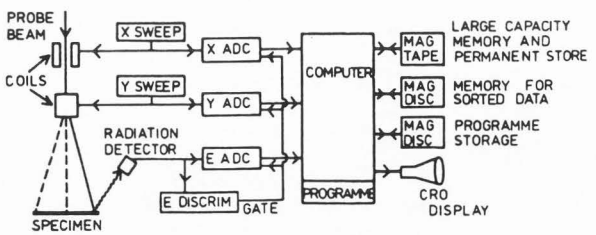

Fig.8. A computer based system of data collection, sorting, processing and display for scanning PMP analysis (Legge and Hammond, 1979)

Since the data are stored consecutively, the real-time parameter can also be used in the analysis of the data to check possible loss of material or elements due to radiation damage (cf. section on Sample deterioration).

Preparation of samples

In general it is true to say that the requirements for preparing samples for PMP analysis are very similar to those for EMP analysis. In biological applications the samples are normally thin to semi-thin sections (< 3-4 mg/cm^2) of tissue or free cells. However, in studies of mineralized or keratinized tissues, e.g. bone, teeth, hair, nails, samples infinitely thick (>10 mg/cm^2) relative to the proton range are also analysed.

The detailed requirements for preparation of thin and ultra-thin sections from biological material without disturbing, e.g. the balance in intra- and extracellular electrolytes etc., are beyond the scope of this paper. However, since the PMP can be used for analysis at lower concentration levels than the EMP new effects may be observed and even greater precautions may be necessary in the preparation of samples for the PMP in order to be able to use the increased analytical capability.

A "typical" example of preparation and mounting of a sample for analysis with the PMP will be given. The sample is quench-frozen, e.g. in heptane cooled with liquid nitrogen, cryosectioned at -30°C or ultra-cryosectioned at < - 80°C, deposited on a thin (2 μm) plastic foil, e.g. Kimfol(TM) and freeze-dried. It may then be transferred directly to the specimen holder in the irradiation chamber or before that coated with a thin layer of carbon to prevent charge build-up and enhance heat transport during irradiation. In contrast to the SEM the samples are mounted on thin films and the beam is allowed to pass through sample and backing. If the sample matrix is of low conductivity and the sample is thick a charge build-up will occur and voltages of several kV may be reached. This will not interfere with the beam, as it does in the SEM, but will create a significantly higher continuous background extending up to high X-ray energies. This will give higher detection limits and therefore charge build-up must also be avoided during PMP analysis (Ahlberg et al., 1975).

Lateral and depth resolution

In microbeam analysis the lateral resolution is a very important parameter. Due to the optical limitations and the relatively low brightness of PMP systems a lateral resolution better than about 1 μm is difficult to obtain with a beam current useful for PIXE analysis. Even in the most technically advanced systems, analysis is in fact carried out with a beam of 2-3 μm or more. This resolution is normally not good enough to allow investigations on, e.g., organelle level. In the EMP, beams of well below 100 nm can be used but to exploit this resolution ultra-thin samples have to be prepared. The extensive scattering of electrons in the sample matrix will give a "pear-shaped" excitation volume (Coleman, 1978) and the effective lateral resolution will depend on the sample thickness (see fig. 9) The depth of this excitation volume is also limited. For 20 keV electrons in a "normal" organic matrix the penetration depth is about 10 micrometres (Szökefalvi-Nagy et al., 1977).

The protons penetrate matter in almost straight lines and undergo only minor deflections in the interactions with the electrons of the matrix atoms (see fig. 9). The probability of scattering in an interaction with the nuclei is orders of magnitude less. In organic material the range, which is a well-defined entity for charged particles, for 2.5 MeV protons is about 100 μm (10 mg/cm^2). The protons will be able to maintain, say 2 μm resolution even for sections tens of micrometres thick while the electron microprobe (20 keV)in such a case would give an effective resolution of about 10 micrometres. Obviously, for samples which are not ultra-thin the lateral resolution of the PMP may be even better than that of the EMP. However, the limited resolution in depth of the PMP could pose some problems in analysing samples which are inhomogeneous in depth, e.g., single cell analysis with other cells underneath. The protons will also induce X-rays in the underlying cells and some of these may reach the detector and interfere with those of the cell under study. In this case the EMP is a better choice.

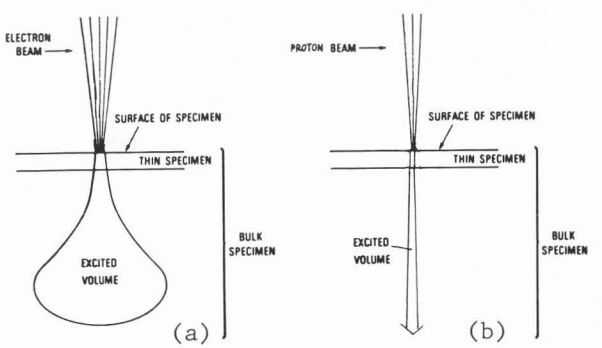

Fig.9. Beam characteristics in bulk and thin samples for (a) EMP and (b) PMP analysis.

From the discussion above it is not clear that the superiority of the SEM in lateral resolution can be fully exploited in analytical applications. To be able to keep the measurement times reasonably short the analysed sample cannot be too thin. This means that it is not always possible to exploit the ultimate resolution of the EMP and consequently the PMP is a realistic alternative in many analytical situations.

Quantification techniques

The PIXE technique allows absolute quantitative determinations with good accuracy (inaccuracy < 10%). In the analysis of infinitely thick samples corrections for attenuation of X-rays and slowing-down of the protons can be made, and by using a simple approximation of the major composition of the biological matrix the concentrations of each element can be calculated (Carlsson et al., 1981). For thin or semi-thick (<5 mg/cm^2) samples it is also necessary to accurately determine the sample thickness for calculations of corrections and the concentrations. Since this is the most common type of sample in PMP analysis it is important to make accurate thickness determinations to maintain good analytical quality. An alternative to this approach is to use techniques of the same kind as those normally used in EMP analysis. Standards with a composition similar to the samples are prepared in the same way as the samples and doped with known concentrations of some elements (Roomans, 1981). Then samples and standards are evaluated together and the concentrations calculated by a direct comparison. To correct for differences in thickness between sample and standard the continuous background can be used as in the case of EMP. However, the background in PIXE spectra is mainly due to SEB (see section on Particle-Induced X-ray Emission) which is proportional to the sample thickness (Uemura et al., 1978) but of much lower intensity than in the case of electron-induced X-ray emission, which makes the determination of the background (thickness) less precise in the PMP spectra. In a systematic comparison between the EMP and the PMP on known gelatin standards it was demonstrated using this approach for both techniques that the results obtained were very well correlated (Forslind et al., 1985a). The correlation is demonstrated in fig. 10.

An alternative approach for calculating the sample thickness is to use the number of scattered protons from the sample which is proportional to the number of scattering nuclei and to the scattering cross-sections. In a first approximation one can

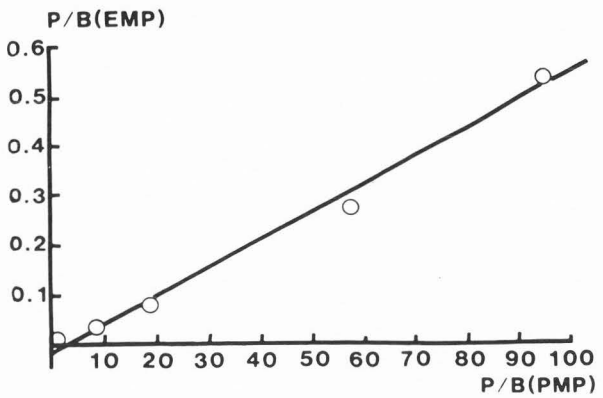

Fig.10. Electron microprobe signal versus PMP signal calculated by linear regression analysis. Results from analysis of thin gelatin standards doped with varying concentrations of nickel (Forslind et al., 1985a)

assume that the matrix composition is similar to a calibration foil of known thickness and e.g. detect backscattered protons in a surface-barrier detector and compare the results from the foil with the sample. Another approach is to introduce a compensation for a different hydrogen content in the standard foil and the organic matrix under investigation (Heck and Rokita, 1984 a) or correct for differences due to varying cross-section for several elements (Themner and Malmqvist, 1986).

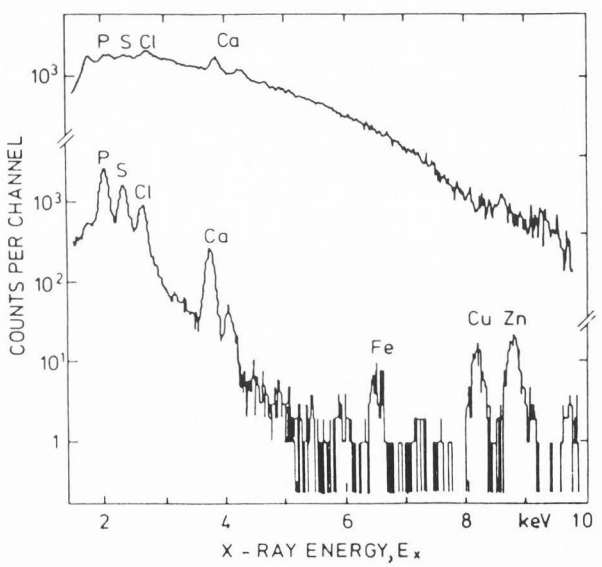

Fig.11. X-ray spectra from microprobe analysis of a freeze-dried sample (pollen tube). Upper spectrum: EMP, 10 keV
Lower spectrum: PMP, 2.5 MeV
(Bosch et al., 1980)

Detection limits (MDL)

The major reason why the rather complicated analytical procedure in PMP analysis is worthwhile is the low detection limits. In fig.11 two spectra are shown from the analysis of the same sample by EMP and PMP and from these spectra it is obvious from the much less intense continuous background radiation that PMP analysis is a more sensitive technique.

For macrobeam PIXE analysis the detection limits are in the interval 0.1 to 10 µg/g in organic material. In microbeam analysis it is, to some extent possible to scale these values with the accumulated charge and irradiated area etc. However, due to limitations in the useful current density such scaling could be misleading regarding the experimental detection limits. The normal definition used for detection of a peak in an X-ray spectrum is that the peak area should exceed three standard deviations of the background under the peak. The MDL will thus scale as the square root of the background; e.g., a hundred-fold reduction of accumulated charge in an irradition will increase the MDL by a factor of ten. This ratio (100) between charges for macro- and micro-beam analysis is quite reasonable and if we assume that the geometrical arrangement in the irradiation chamber allows a detection limit of 0.5 µg/g for a macro-beam analysis the corresponding detection limit for a PMP analysis would be 5 µg/g.

The potential of the PMP technique for trace element analysis is significant. However, it should be mentioned that the scanning mode PMP which is sometimes stated as allowing trace element determinations (MDL<10 µg/g) in biological matrices with 1 micrometre resolution, requires that this condition be fulfilled in each pixel. When considering a two-dimensional scan the number of pixels has to be kept relatively low. If we make the (optimistic) assumption that we can use 100 pA of proton current and that we analyse an area 50x50 μm^2, the total number of pixels will be 2500 and the charge required for each pixel 100 nC. Such an experiment would need about 30 days of continuous beam time! Indeed, it is necessary to decrease the beam resolution and the number of pixels if true trace element analyses are to be carried out. The technique of zooming in on an interesting area in steps has been demonstrated by the group in Oxford (Watt et al., 1984). In fact, the pixels could be enlarged by the computer when handling the event-by-event data by adding elemental counts from adjacent pixels together into larger ones which will then give better pulse statistics but poorer resolution. The technique of using line scans over interesting interfaces is another way of maintaining the trace element capability of micro-PIXE with reasonable irradiation times (Heck

and Rokita, 1984b).

The two-dimensional elemental mapping technique is useful in demonstrating the major element distribution in a sample and can be a guide for a more detailed study of a chosen segment. The static beam analysis of course allows low detection limits in that particular pixel, but then the current is limited by the risk of sample deterioration by heating, a problem which will be further discussed in the next section. Another alternative is to use a nonsymmetric beam spot. If an interface in a section is known to be composed of, for example cells aligned in one direction a beam which is narrow only in the direction perpendicular to the cell layer may be used. The beam can then be static (Malmqvist et al., 1983) or moved in a "line scan" perpendicular to the cell stratum and provide good resolution in this direction only. This can be a relevant approach from a biological point of view, if all cells in the strata are supposed to be similar and the inclusion of many cells only increases the accuracy in the conclusions regarding the results.

What is stated above regarding the limitations on the MDL with the PMP in a two-dimensional scanning mode is in part also true for the EMP and should not discourage a biological or medical researcher from applying the PMP technique in his/her specific field.

Sample deterioration

It is well known that damage and loss of major and minor elements occur during irradiation with charged particles. This is the major disadvantage in using charged particles instead of photons to create inner-shell vacancies for analytical purposes. In EMP analysis these effects are often considered and corrected for by using standard samples which are supposed to suffer from equivalent radiation/heat damage (Roomans, 1980). The current density used in the EMP is often much higher than that for the PMP and should then indicate a more severe problem in EMP. However, the area irradiated and the sample preparation (carbon coating etc.) play important roles in the estimation of these effects. Using the PMP it is possible to determine an increased number of elements due to the higher sensitivity. Unfortunately, some of these trace elements may be more likely to be lost during irradiation (mercury, lead, etc). The problem of sample deterioration must therefore be carefully considered in evaluating the potential and limitations of the PMP technique.

In broad-beam PIXE some experiments have been carried out to measure temperatures and damage due to particle irradiation (Hill et al., 1981). In a recent calculation (Cahill et al., 1986) an attempt was made to estimate the temperature increase in a thin non-conducting sample in vacuum. If it is assumed that no heat removing mechanism is present, and paper is irradiated with a 4.5 MeV proton beam with 3 mm diameter and at a current of 10 nA, for paper disastrous temperatures would quickly be reached: the temperature would rise by about 6^{o}C/s. Paper is, in principle, cellulose, a common biological material, and if the calculation is directly scaled to micrometre dimensions using a very crude approximation the temperature rise for a 10 μm beam of 0.1 nA would in this case be 3500^{o}C/s!

Few systematic experimental investigations have been carried out for microbeam PIXE. However, some calculations have been made regarding the possible risks of high temperatures caused by the beam spot on the sample. The results of such calculations seem to underestimate the temperatures reached (Maggiore, 1981). In a recent rather extensive calculation Vis (1985) estimates temperature elevations in PMP analysis under realistic conditions. In fig. 12 results are shown from these calculations and the effect of coating the

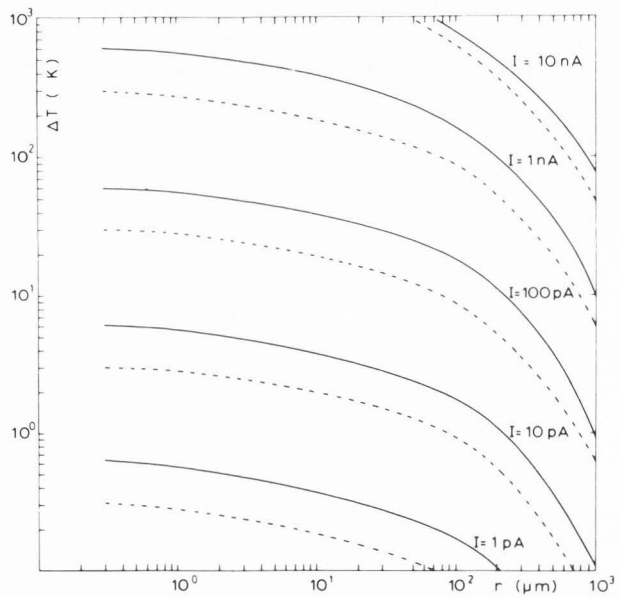

Fig.12. Increase of specimen temperature as a function of the beam radius for different beam currents assuming cooling by both conduction and radiation. The dashed lines correspond to a specimen coated with 5 nm of Al (Vis, 1985)

sample with a thin layer of a heat-conducting material is also demonstrated. His conclusions are that normally temperature effects do not play a limiting role in micro-PIXE work. Although there may be some differences of opinion on the matter of temperature rise, a natural precaution is to use rapid beam-scanning over the sample (see section on Beam scanning).

The radiation damage caused by ionization is proportional to the accumulated charge/unit area. In principle it should be equivalent to the radiation damage caused by photons of the same radiation dose and cause, e.g., broken chemical bonds in organic material, transformation of chemical compounds, etc., which may then lead to loss of matrix elements or of specific elements. The damage to biological structures does not necessarily cause any changes in the regional elemental distribution but has been demonstrated to do so in some cases. Legge et al. demonstrated a fast and slow component in the loss of chlorine from an alga sample (Legge and Mazzolini, 1980). Such studies can be made by using the time dimension in event-by-event data acquisition (see section on Data acquisition and handling).

There are a few set-ups for PMP in which the beam is extracted into air (or helium) through a small hole using differential pumping to protect the accelerator high vacuum (Swann 1982: Shroy et al., 1978). This is carried out in collimated PMP systems and allows static microbeam measurements on very sensitive samples. To further improve the protection of the sample forced cooling can be applied, e.g. with a helium jet directed at the beam spot.

Comparison with other microbeam techniques

In the sections above there are several examples of comparisons between the very widely used EMP technique and the PMP method. To complete this comparison it is important to state that although it requires very thin and carefully prepared samples, the EMP is the only microprobe which, using a high-brightness ion source, can be used for elemental analysis on intracellular (organelle) level down to a lateral resolution well below 50 nm. There are no reasons to believe that this will be possible for PMP in the near future. Consequently, the PMP analysis should be regarded as a complementary tool in biological research which can extend the range of detectable elements.

Another technique of microbeam analysis is the photon beam analysis which can be performed at synchrotron radiation facilities (Bos et al. 1984; Jones et al., 1984). This photon source offers intense beams of highly polarized X-rays which may be focused to dimensions of 10-30 micrometres using gratings or crystals (Prins et al., 1984). With still higher intensities of hard X-rays (multipole wigglers) and improved focusing techniques this will be an important instrument in the analysis of biological material. The major reason for this is the high ionization cross-sections in combination with low energy deposition and low background due to polarization. In constrast to the EMP, this technique suffers, even more than the PMP, from the "big science" installation. However, instead of using the synchrotron and storage rings, it may be possible to use a linear accelerator or a race-track microtron instead for the production of electrons or positrons of about 100 MeV. By exploiting a new technique, channelling radiation which is produced by oscillations in a crystal, intense beams of hard X-rays can be produced (Berman and Bloom, 1981). The radiation characteristics are similar to those of synchrotron radiation but the source is much cheaper and more compact. For a detailed discussion of the intrinsic and effective sensitivity of the three modes of excitation, i.e. electrons, photons and protons see Grodzins, 1983.

The LAMMA-technique is another alternative in which high-power lasers are used to disintegrate the sample spot-wise and analyse the fragments in a mass spectrometer. High lateral resolution and high sensitivity are attainable but the quantification is poor and the technique is destructive.

Applications in biology and medicine

Microprobe analysis is normally used in biology and medicine to study elemental distributions in cells and cell strata. The elements of relevance for the specific investigation may be elements at high concentrations or in trace concentrations. In the latter case there is no alternative method to PMP which can quantitatively determine trace elements with a lateral resolution of a few micrometres. Since trace elements have attracted a growing interest in biology and medicine and since the PMP can be used to analyse traces as well as high concentrations, the technique of proton microprobe analysis is a very interesting complement to the established electron microprobe technique in research within biology and medicine.

In the following sections various applications within the biomedical field are presented and some results are presented and discussed. It is not intended to fully cover all possible biomedical applications, but some applications have been selected in order to demonstrate some of the advantages of PMP analysis.

Applications in botany

In this field not very many examples of PMP analysis can be found in the literature. Most plant material is very well suited for use with the PMP, to study the elemental distribution, as the importance of trace elements is as great as in the case of zoology. The sample preparation is simple and often more distinct cell strata are found. In addition, botany is a field in which many studies have been made using the EMP technique. Since the heavy metals on trace element levels are very important in botanical processes one could expect a significant growing interest from, e.g., plant physiologists in the

future.

Pollen grains One example which represents the exception to the rule in that extensive studies have been devoted to it is the pollen grain of Lilium longiflorum. In the Heidelberg group (Bosch et al, 1980; Reiss et al, 1983) the growing pollen tube was studied with particular reference to calcium, an element of interest in many fields of biology. If several pollen grains get stuck on the stigma of the pistil the pollen tube which is fastest-growing fertilizes the egg. The growing velocity is very fast (several mm/hour). The pollen grain and tube being part of one single cell represent a very interesting biological system. In artificially grown pollen grains the distribution of zinc and calcium was studied with PMP analysis on fixed and freeze-dried samples. It was demonstrated that a distinct intracellular tip-to-base distribution of calcium exists with a high concentration at the growing tip of the tube (see fig. 13). In one hypothesis it is claimed that there exist channels for calcium ion influx through the plasma membrane (Reiss et al., 1985). A pronounced increase in the zinc concentration is observed in a region just before the tip in which large amounts of RNA occur. Similar correlations between zinc and messenger RNA have been observed in other biological systems. The concentration of calcium in the tip is high enough to be studied with the EMP but the detailed distribution would be difficult to confirm. In the case of zinc, the PMP is the only method capable of determining the distribution. In a recent study following the pollen studies an attempt was made to find and localize calcium channels in the tube by treating the pollen tube with $CoCl_2$, which binds to calcium channels, and to then analyse the tube for calcium and cobalt using the PMP (Reiss and Traxel, 1986). There is a close resemblance between calcium and cobalt distributions and this is an indication that the cobalt-binding sites are the same as for calcium. A possible interpretation of these results is confirmation of a predicted polar distribution in calcium channels located in the plasma membrane of the pollen tube.

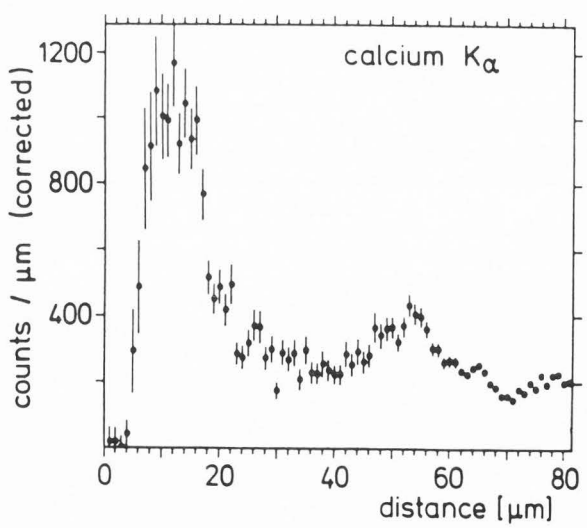

Fig.13. Calcium concentration from PMP analysis along the longitudinal cell axis in the pollen grain and growing tube (Lilium longiflorum) starting at the tip of the tube (Reiss and Traxel, 1986)

This pollen grain was also studied by the Oxford group (Reiss et al., 1986) using their two-dimensional elemental mapping technique. They arrived at the same conclusions regarding the distribution of calcium and zinc and demonstrated this in very illustrative colour-coded pictures.

Mineralization of silicon in plants In plants, macrohairs are often used to protect the developing seed. The silicification process was studied in the lemma of the grass Phalaris canariensis by EMP and PMP analyses (Perry et al., 1984). From the biological point of view these hairs are very interesting since they consist of only one or possibly two cells. In the early stages of the development of the hairs silicon is found at the tip and potassium behind the tip while in the mature hair the concentration of silicon increases by a factor of twenty, while potassium and phosphorus almost disappear. This demonstrates that the cellular activity which is linked to P and K is withdrawn during the silicification process. It was only possible to obtain the complete picture of this process by complementing the EMP measurements with the determination of traces of inorganic elements by the PMP.

Leaf tissue A few studies have been made on leaf tissues using the PMP. In Melbourne Mazzolini et al. (1982) analysed green and chlorotic tissue from Eucalyptus obliqua. The chlorosis seemed to correlate with high levels of phosphorus, sulphur, potassium and calcium. The high level of P and an imbalance in the ratio between P and the trace element Fe seemed to be of particular importance. The sample preparation was relatively easy including only simple air-drying in a desiccator for 72 hours of the leaves from seedlings grown under controlled conditions. A scanning beam of approximately 10 µm diameter was used during the analysis. In fig. 14 the irradiated area and corresponding elemental maps are shown.

Another type of "leaf" is pine needles which have attracted growing interest during the recent years in discussions on the death of forests in western Europe and Scandinavia. This is a serious environmental as well as economical problem and

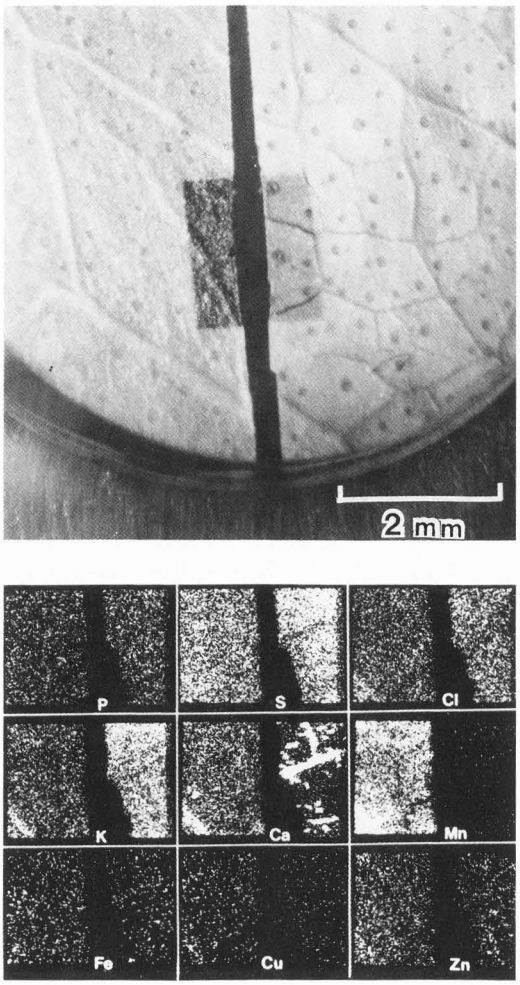

Fig.14. Upper: Photo of sample of eucalyptus leaves after irradiation with a scanning PMP. The dark field in the photo is the irradiated area. The normal leaf is to the left and the chlorotic to the right.

Lower: Corresponding elemental maps with normal tissue to the left.

several approaches have been made to solving it. In an attempt to investigate whether preferential deposition of aerosol particles at the pores would interfere with the biological processes the PMP was used to analyse the pores (approximately 10 μm) and areas between. The needles were collected on different occasions to see whether the season was important for the aerosol deposition. Preliminary results show only very minor concentrations of heavy elements, e.g., lead (S.A.E. Johansson et al., private communication). Further work is in progress.

Wheat grain Studies using the electron microprobe can reveal the distribution of many elements in the mature wheat grain. Some trace elements, e.g., manganese, are of interest for biological processes. The scanning PMP analysis demonstrated the Mn distribution which plays an important role for the root formation in regulating the growth hormone auxin. Analysis was also performed on wheat leaf epidermis but the concentrations of trace elements were not high enough to add any more information to that which could be observed with an EMP (Mazzolini et al., 1981).

Sea algae Using a semi-microprobe (about 35 μm beam diameter) samples of brown sea algae (Hizikia fusiforme) were analysed in a scanning mode (Ishikawa et al., 1984). Part of the background to the study is that this alga is an important part of the Japanese diet and it is therefore important to study it in detail for elemental content. More than ten elements were studied including some trace elements, Cu, Zn and Fe. In the medullary layer Mn and Zn were accumulated and in the epithelial layer Fe, Cu, As and Br. The elemental variations are large and in some regions the concentrations are very low and would definitely not be observable with the EMP.

Fungus In Australia the die-back of many plant species has been associated with attack by Phytophthora fungus. Spores and cysts of several kinds of Phytophthora have been analysed with the scanning PMP in Melbourne in order to investigate the process of encystment. It has been found that the process is accompanied by changes in the elemental levels within the cells (G.E. Legge et al., unpublished results). It is thought that the metamorphosis from zoospores to cysts of Phytophthora Cinnamomi depends on the concentration of calcium and that Ca may be taken up from the environment upon encystment. The cysts and zoospores were mounted on nylon foils after freeze-drying and analysed with a 200 pA beam for 20 minutes. Detectable amounts of Ca were found in the cysts although the interference from high potassium content meant increased detection limits. Further work is in progress.

Zoology

The vast majority of studies of biomedical samples with PMP analysis are performed on tissues from mammalians. So far not very many studies have been applied to purely zoological problems but rather medical investigations (see the section Applications in medicine). Several studies on animal material (test animals) have been carried out with the aim of solving medical problems. In this section studies of all kinds of zoological material except human tissues will be dealt with.

Animal bone tissue The analysis of zinc content in tissue from rabbit Achilles tendon and rat femur was carried out with the collimated microprobe in Brookhaven (Doty et al. 1981). In the Achilles tendon a scan was made from cartilage

(very rudimentary "bone") to fully mineralized tissue at the bone end of the structure. In tendons from young rabbits, the calcification is low and the zinc concentration was very low compared with that in mature bone. For older rabbits the zinc concentration was significantly higher and increased towards the more mineralized end. In rat femur it was demonstrated that the zinc concentration is correlated to the mineralization intensity. When completely mineralized the tissue contained significantly lower levels of zinc than in the growing phase. The levels of zinc were 100 to 400 µg/g which makes it very difficult to detect zinc with an EMP analysis (energy dispersive) and thus these studies would not have been possible without access to the PMP. In another study rat incisors were analysed for P, Ca, Fe, Zn and Pb with the PMP (Shroy et al., 1978).

Rat kidney The analysis of lighter elements with the PMP technique presents some problems as was discussed in the section on Detection systems. A study has been carried out by the Melbourne group in order to improve detection limits for sodium which is an important element. Using alpha particles of 2 MeV the X-ray production in Na can be maintained while the problems of charged particles into the detector is significantly reduced. In fig. 6 a spectrum from such an analysis of a cryo-section from rat kidney papilla is shown. The peak-to-background ratio for Na is one order of magnitude better than for 20 keV electrons in EMP analysis (Sealock and Mazzolini, 1983) as is demonstrated in the figure.

Sea-urchin egg The egg of the sea-urchin is about 50 µm in diameter and is therefore a suitable subject for studies with a lateral resolution of a few micrometres. The distribution of Ca was demonstrated not to be homogeneous in a scan perpendicular to the spindle. The average concentration was about 600 µg/g which is relatively high and would make it possible to use an EMP instead (Kneis et al., 1982).

Rat brain The Amsterdam group (Lenglet et al., 1984) studied the hippocampus of rat brain. This is a region with well-defined cell structures and the rat is normally used as a model for the evaluation of different states in the human brain. They combined PMP analysis with traditional histochemical staining techniques to reveal elemental distributions. It is not possible to use very specific staining techniques on trace element concentration level. With a less specific method, e.g. Timm-sulphide-silver stain, it could be demonstrated that relatively low concentrations of metals which were found by staining were confirmed by the PMP analysis. However, the Timm method indicated the presence of metals in many regions where, e.g. no zinc could be detected by the PMP. The Timm technique is therefore favourably complemented by PMP analysis for elemental specificity.

In a collaboration with a group carrying out experimental brain research our own laboratory is at present performing PMP analysis in selected regions of rat hippocampus (B. Siesjö, 1986 private communication). Artificially induced ischaemia, hypoglycaemia and status epilepticus cause effects on special cells and cell strata in the hippocampus which are impossible to detect with traditional histochemical methods applied directly after the alteration has been performed. A few days later specific cell death is observed histochemically and the theory is that this necrosis is manifested through an increased calcium level in the cells. With particular interest in, e.g., calcium and iron, we are analysing freeze-dried sections (30 µm) mounted on thin Kimfol (TM) foils. Work is in progress and the preliminary results demonstrate good reproducibility in the PMP analysis.

Rat arteries In the pathogenesis of hypertension the important role of the element calcium has been confirmed in numerous investigations during the last decade (Zidek et al, 1982). In two groups of rats, one normotensive and one hypertensive, the lumbar aorta was removed and sectioned on a cryo-microtome into 6-µm slices (Spieker et al.,1985). The main aim of this study was to investigate the Ca distribution in smooth arterial muscle using the Karlsruhe PMP facility (Heck, 1982). The results indicate that the concentrations of Ca in aortic smooth muscle cells in hypertensive rats were higher than in the normotensive group. The analyses were performed as line scans perpendicular to the artery wall using backscattered particles as a means of monitoring sample thickness (see section on Quantification techniques). Similar studies were also performed for humans (see section on Applications in Medicine).

The alternative method of studying calcium distribution in the artery walls is the ion-selective electrode which measures the free Ca^{2+} ions while the PMP measures the total Ca content. The total Ca seems to be less correlated to the degree of hypertension than that of the free ions.

Limpet teeth In the common limpet, Patella vulgata, the mature teeth consist of hard mineral phases of iron and silicon oxides in a hard matrix. Each tooth is about 200 µm long and the radula contains about 200 rows of teeth in various stages of development. The Oxford group investigated such teeth in different stages of development using a 2 micrometre lateral resolution in the PMP. The results show that in the early stages the teeth are an organic structure without any obvious organized distribution of the elements.

The elemental mapping method was used and several elements were studied. Phosphorus, iron and calcium were all readily analysed with an EMP while silicon and copper could not be analysed without the PMP. By choosing teeth in different stages of development the PMP analysis offered means of following the mineralization process in detail. In following the copper distribution it was found that at first copper could only be found at the base while in the old tooth it is highest in the cusp. Copper oxidases seem to have been transported into the tooth at this late stage of development to activate cross-linking of the organic matter. Presumably, much of the organic material remained flexible until the inorganic matrix of crystalline goethite and amorphous silica had impregnated the tooth (Grime et al., 1985).

Honey-bee brain The brain of the honey-bee was studied with the scanning PMP in Oxford (Joseph et al., 1986). This organ was an excellent example of the information the PMP can yield. An overall analysis with a rather large beam helped in "zooming" in on an interesting part in two steps with gradually decreasing beam dimensions.

Applications in medicine

The medical field has so far attracted most interest from workers in the field of proton microprobe analysis. Part of the explanation for this may be the funding situation which is more favourable than in zoology and botany. Many of the results presented below are of basic biological interest and many of the conclusions regarding the advantages and disadvantages of the PMP technique are indeed applicable to biology in general. The discussion is divided into different parts of the human body and into different kinds of tissues.

During the last decade there has been a growing interest directed towards the role of trace elements in the life processes and in the causes of disease in the human body. Various analytical techniques have been developed for clinical applications. Deficiencies or elevated concentrations of, e.g., Cu, Zn and Se in various parts of the human body have been of particular interest. In cases where high lateral resolution is required none of the established methods of analysis is adequate. Therefore, once the proton microprobes started producing interesting results, the technique attracted great interest from medical researchers. The PMP instrumentation is normally rather complex and "bulky" for the already heavily equipped medical field, so in order to justify the running costs for such an instrument the expected results have to be quite spectacular.

Central nervous system In one of the animal studies presented above hippocampus of rat was analysed with the PMP technique after inducing various abnormal states, e.g., ischaemia in the rat brain. By studying human autopsy material the same methodology could be used in studying the effects on brain of ischemia, hypoglycaemia etc. The cells are normally quite large in the brain and therefore the rather limited lateral resolution does not present too great a problem. In our laboratory we are planning studies of various regions of glioma tumours in samples removed during brain surgery. The intention is to find any substantial elemental or trace elemental changes occurring in the growing region of the tumour (L. Salford, 1986, private communication). In a study on induced tumours in rat leg, Heck and Rokita (1984b), investigated the necrotic outer parts and the interior of the tumour and found interesting differences in elemental distributions. In the necrotic part the potassium level was very low while in the inner parts there was a very constant iron level indicating absence of any blood-vessel system. Normally each capillary is observed as a distinct increase in iron concentration.

In human cerebellum, white and grey tissue are easily recognized by staining techniques. In order to check whether it would be possible to obtain similar information without staining a study was carried out on thin sections of cerebellum (Ilgren et al., 1984). As well as plots of two-dimensional distributions of several elements similar maps of elemental ratios were also produced. In the maps of the sulphur/phosphorus ratio the same structure was produced as in stained sections. A further extension of this work would be to decrease the beam diameter and repeat the experiment on the resolved single cells and produce such maps without any staining.

In Alzheimer's disease serious mental retardation occurs. Recently this state has been suggested to be, in part, linked to high concentrations of aluminium in the brain. Aluminium, formerly regarded as a rather harmless and inert element, has attracted interest also in connection with acute cases of encephalopathy due to aluminium poisoning during dialysis. The PMP technique offers a means of studying the distribution of aluminium in brain tissue. By choosing, e.g., 2 MeV alpha particles as exciting particles instead of protons the sensitivity is optimized for elements such as Na, Mg and Al (see fig. 6). This would improve the probability of localizing Al compared with EMP analysis.

In the early stages of its development the pre-natal brain is very sensitive to exposure to heavy metals, e.g., lead and mercury. In systematic studies of monkeys radioactive tracers and autoradiography have been used to measure the distribution after exposure by sectioning the embryonic brain in different stages of development

(B. Lögdberg, private communication). To complement these investigations preliminary attempts have been made to use the PMP technique to analyse the distribution of these elements in brain cortex by line scans perpendicular to the brain surface. Since the concentrations of the heavy elements are low (ppm range) the high sensitivity of the PMP is required. Performing the experiments on monkey foetus makes the results applicable to humans.

Eye Human lenses with cataracts which have been removed by surgery have been compared with normal lenses obtained at autopsy. The samples were prepared by quench-freezing and cryosectioning and were then scanned with a semi-microprobe (100x200 μm^2) at atmospheric pressure. The results indicate very small differences between elemental distributions in different types of cataractous lenses. The elemental distributions in rat lenses were also compared before and after incubation in various media to study the effects of the different treatments. The lenses were scanned in two dimensions to study the elemental distribution over the whole structure (Koyama-Ito et al., 1984).

Liver In a special state of human liver, primary biliary cirrhosis, it is known that the copper concentration is elevated but the exact localization was not known. The progress of this illness leads to degeneration of the bile ducts and eventually to the patient's death due to hepatic failure. The Oxford group (Watt et al., 1984) investigated 7-micrometre sections from a shock-frozen biopsy mounted on thin Mylar films. To facilitate orientation, adjacent sections were stained with haemotoxylin and eosin which allowed optical viewing of the structure. Using two-dimensional scanning a large-scale image was obtained and then a particularly interesting area was selected and analysed at a higher resolution. Copper was found to be deposited at specific locations within the tissue and in addition, it seemed to be correlated to the sulphur distribution (see fig. 15). Looking in more detail at the ratio between copper and sulphur it was found that the stoichiometric ratio was about 1:1. To explain this finding the deposits must either be an inorganic salt or a sulphur-rich copper-binding protein, e.g., metallothionein (Kojima and Kagi, 1974). The organic compound is more probable from a biological point of view. The results confirm those of Sherlock and colleagues (Epstein et al., 1981) based on electron microprobe measurements but the PMP results add new details and higher sensitivity which enable the user to interpret the results more easily. This is true in general in the field of biomedicine.

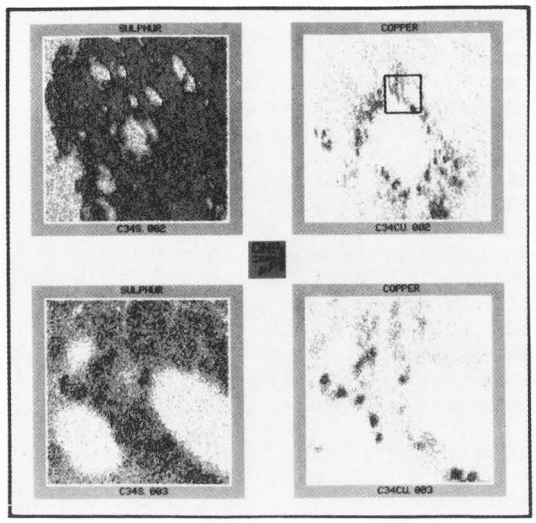

Fig.15. Elemental maps of sulphur and copper in section of liver with primary biliary cirrhosis. The images cover 1x1 mm^2 and the lower pair 200x200 μm^2 (cf. frame in upper right figure).

Lung tissue In studies using macro-PIXE investigations have been made on wet-ashed samples from lungs to correlate environmental factors with elemental increases (Bartch et al., 1982). The regional distribution is however very important and an attempt has been made in Brookhaven to study uranium-rich particles deposited in the lungs using an external collimated microprobe (Paschoa et al., 1983). Although the performance of this microprobe was not very good at the time of analysis uranium-rich hot-spots were found. In our laboratory we are planning a similar study to localize heavy metals in lungs from autopsy samples collected from exposed workers, and to search for a possible correlation between primary tumour sites and elemental concentrations (K.R. Akselsson, private communication).

Dermatological material The concentration and distribution of elements in skin and its appendages have been extensively investigated during the last years. Several studies have been made in order to investigate the potential of hair analysis in, e.g., environmental and forensic studies. Hair is a popular biopsy material since it is easy to obtain and it is often used in forensic work. Analysis is made on single hair strands or on bundles of many hairs. To be able to interpret the results from such measurements it is important to know whether the elemental concentrations found reflect body status or external contamination. In an elegant study Bos et al. (1984b) used the PMP technique to measure elemental distributions over hair

cross sections taken at different distances from the hair growth zone in the hair root (see fig. 16). The results indicate that, in addition to the growth zone, there is an extra contribution of zinc and copper along the root sheath which would mean that elements in the blood would have to be transferred in a very complex manner. Another possible implication is that this transcellular transport would be easier if Cu and Zn were bound to low-weight molecules which could more easily pass this transcellular barrier. The low molecular weight of albumin would then explain why rather high concentrations of zinc are found compared with copper which is more abundant in the larger molecule ceruloplasmin. A similar conclusion can be drawn regarding external vs. internal concentrations of elements if the hair is analysed at different distances from the hair root. The use of PMP analysis makes it also possible to follow sources of trace elements, e.g., lead and mercury.

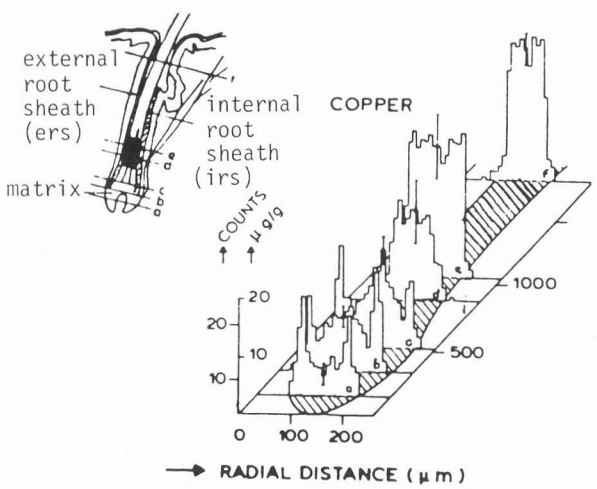

Fig.16. Radial distribution of copper along the proximal end of a hair (root) (Bos et al., 1984b)

Another type of keratinized material is skin. The PMP technique has been applied to skin studies by two groups. The Bochum group made the first attempt to scan human skin perpendicular to the surface (Wilde et al., 1981). They demonstrated how the PMP could be used to scan sections of human skin and analyse elements which cannot be detected with EMP analysis, e.g. Fe and Zn. In one scan the passing of a hair follicle could be observed by a distinct increase in sulphur where the hair strand was situated. In a second study using the PMP gold deposits were found in skin from patients suffering from rheumatism who had been treated by gold therapy. This finding suggests that gold is deposited in granules (Enderer et al., 1984).

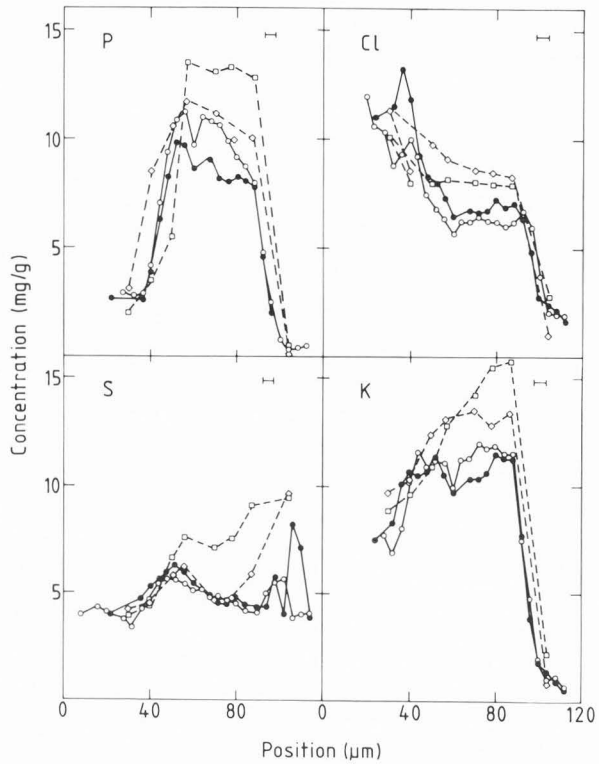

Fig.17. Comparison of the elemental distributions in human epidermis determined with PMP analysis (solid lines) and the EMP (broken line). The skin surface is at the position 95 μm. The error bars show uncertainty in positioning the EMP at exactly the corresponding sample spots.

In order to investigate the physiology of the epidermis several studies have been or are being performed in our laboratory on elemental profiles in the epidermis (Malmqvist et al., 1984; Forslind et al., 1984). Using a beam of 5x100 μm^2 several cells in a row in the various strata of the epidermis are included in each irradiation. The sample is then moved step-wise and an effective resolution of about 5 micrometres is obtained. The results for elements of higher abundance were in relatively good agreement with simultaneous EMP analyses (see fig. 17). The distributions of the elements P, K, S and Cl might as well have been analysed with the EMP but for Ca, Fe and Zn this would not have been possible (see fig. 18).

The trace element determination capability of the PMP has also been used in a study of the penetration of Cr-VI and Ni ions into human skin (Malmqvist et al., 1986; Forslind et al. 1985b). This is a very important problem since the frequency of hypersensitivity and allergy reactions due to these ions is rapidly increasing. Further work is in progress to study the barrier mechanism in detail by in vitro

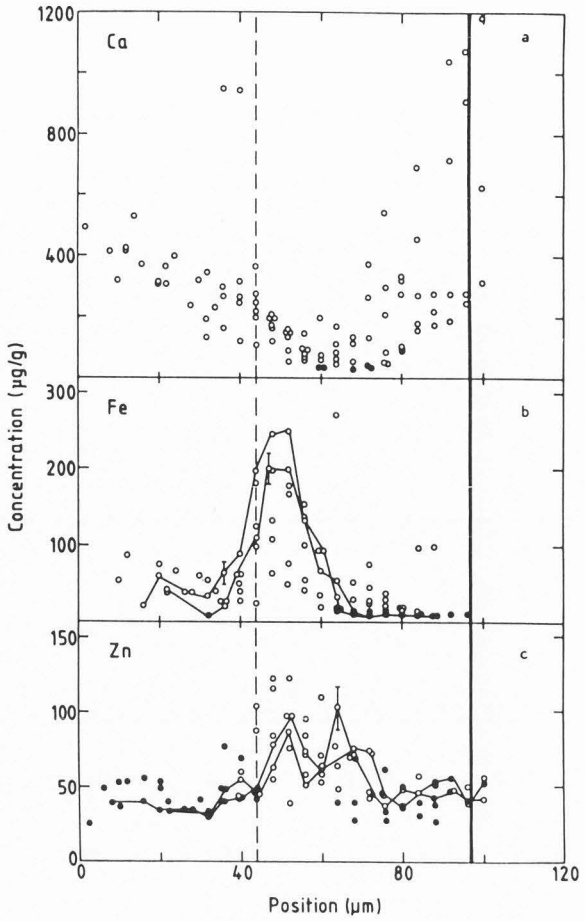

Fig.18. Trace elemental distributions in epidermis in six sections of human skin. The solid lines connect values which correspond to duplicate sections from the same skin biopsy. The skin surface (thick solid line) at about position 95 µm (Malmqvist et al., 1984)

experiments on human skin and in vivo experiments on animals.

In several skin diseases the trace elemental profiles are important in the understanding of the mechanisms behind the disease. The usefulness of PMP analysis in studies of psoriasis has been demonstrated by two groups (K.Kurz private communication, B.Forslind unpublished material). In the second study preliminary results show increased concentrations of Fe and Zn in outer parts of the epidermis compared with normal skin.

Mineralized tissue These tissues are characterized by a high abundance of calcium. The PMP technique has been used to investigate trace elements in the matrix in bone and tooth. The implications in toxicological work are obvious since, e.g., lead is known to be stored mainly in the skeleton and exposure to lead at an early age will lead to an accumulation in dentine which can be used as an index of exposure (Möller et al., 1982).

Measurements with the PMP technique have been applied to study corrosion products from fillings on dental surfaces (Brune et al., 1982). The elements used in the filling material could be traced to the root surfaces, e.g., Zn and Hg. The problems of corrosion products, especially from amalgam, have recently attracted strongly increasing attention and further studies along these lines with the PMP method would be very useful.

Bridge bones from the human ear have been analysed for deposits which could be correlated with deafness (Krmpotić-Nemanić et al., 1985). The Studsvik PMP has been applied to studies of the human femur (Lindh, 1983). The elemental distributions were measured in a scanning mode and in each individual osteon in the femur a ring-like distribution of lead was found (see fig. 19). The levels of lead were surprisingly high (up to 200 µg/g) and the distribution of lead varied between different osteons. A third study on human bone is being performed at present in our laboratory (S.Skerfving, private communication). Biopsies are taken from workers in a lead refinery who have just been temporarily relieved from work in direct contact with lead due to too high concentrations of lead in their blood. This biopsy sample (1 mm in diameter and 5 mm long) is punched from the vertebrae under local anaesthesia. Autopsies were taken in the same way from the bodies of former lead workers. These samples are difficult to analyse directly since they have a rough surface. Scans were made both with a small beam spot and a relatively large one. Results are very preliminary but the lead concentrations vary from a few tens of µg/g in the biopsy to several hundreds of µg/g in some of the autopsies. Comparisons are planned with atomic absorption spectrophotometry in which the whole biopsy/autopsy is analysed for lead.

Single-cell analysis This is a very interesting application of microprobe techniques which at present is most important for blood cells. These cells vary from erythrocytes (5-10 µm) to the larger leukocytes. Heck and Rokita (1983) scanned an area of 80x80 μm^2 of a sample with several erythrocytes deposited on a Formvar (TM) backing. The absolute sensitivity in each pixel (3x3 μm^2) was about 0.004 pg Fe. In another study the Melbourne group analysed one single erythrocyte deposited on a nylon foil (Legge, 1984). Using a 1.5 µm beam diameter of 3 MeV and 150 pA this cell was analysed for 3 hours. The results illustrate both the potential and disadvantages of the scanning PMP analysis. Such long irradiation times are required for this limited area scan in order to achieve trace-element sensitivity. On the

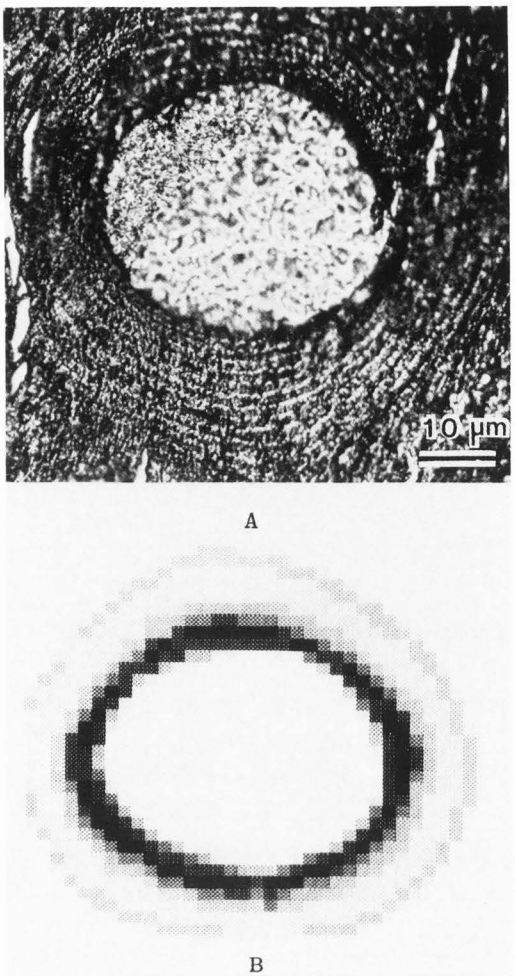

Fig.19. Micrograph of an osteon from a human femur(A) and corresponding lead distribution determined with PMP analysis (B),(Lindh, 1983).

other hand trace element analysis can be performed in a way which would not be possible with any other microprobe technique yet known.

In a later systematic investigation Johansson and Lindh (1986) have analysed single erythrocytes from blood taken from a control group and a group of patients exhibiting nonspecific symptoms which were suspected to be caused by the toxic action of mercury emanating from dental amalgam fillings. A whole-blood analysis did not reveal any enhanced concentrations of Hg. However, in some but not all of the individual erythrocytes from the patients elevated levels of Hg were found. In the control group no Hg was found. The detection limits in the dry erythrocytes were a few µg/g Hg. In another study by the Studsvik group various kinds of blood cells were analysed, e.g., leukocytes. Neutrophil granulocytes were separated from the leukocytes and analysed for comparison between four patients with different kinds of leukaemia. In comparison with a control group the patients' cells revealed significant irregularities in the microelemental profile (Johansson and Lindh, 1984).

Other human tissues In several smaller investigations various types of human tissue have been analysed with the PMP technique. In patients with gastric cancer biopsies were taken of stomach mucosa. The samples were prepared by microtomy after quench-freezing or embedding in paraffin. Fifteen slices were cut from each biopsy and every other slice was used for staining and optical investigation and the adjacent slice for PMP analysis. According to the findings in the light microscope the slices were characterized as cancerous or normal. The samples were analysed with a scanning-mode PMP in patterns which can be seen in fig. 20. Since irradiated parts of the tissue will not be easily stained the irradiation patterns are chosen to enable staining of the regions in between the beam traces after the irradiation. The sample cannot be stained prior to the

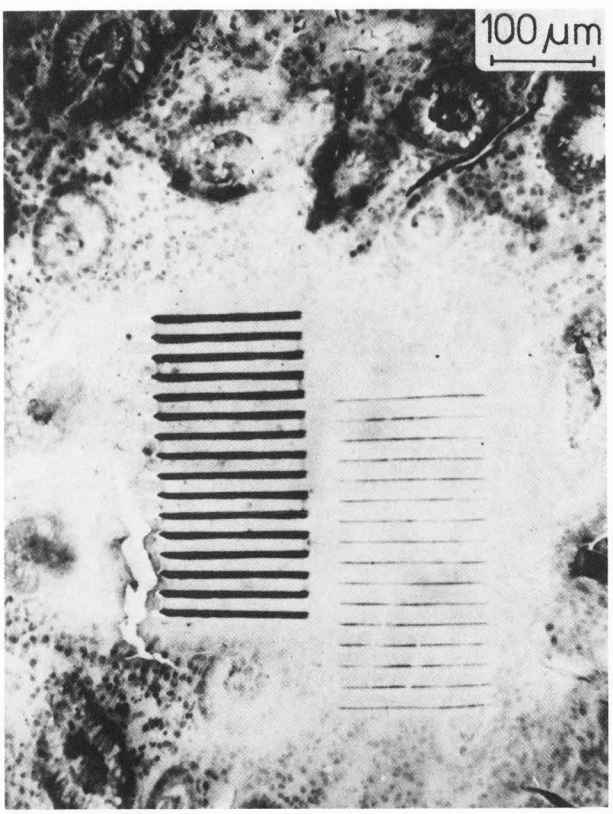

Fig.20. Micrograph of normal stomach mucosa prepared by cryofixation. The tracks of the scanning proton beam are visible as dark parallel lines. Irradiation times: 30 h (left), 30 min.(right) (Heck and Rokita, 1983).

analysis since the staining chemicals will interfere with the elemental analysis. The analysis of cancerous and normal mucosa did not show any significant differences in the trace element concentrations (Heck and Rokita, 1983).

In studies of cancer in the prostate gland it has been found that elevated levels of cadmium occur in the tumour. In studies with EMP analysis cadmium could not be localized due to the too low sensitivity. However, using the PMP technique Vis et al. (1985) demonstrated that cadmium was present in a heterogeneous distribution showing distinct peaks in some specific structures. This clearly shows that valuable extra information can be yielded by using the PMP method as a complement to the traditional electron microprobe.

Another way of utilizing staining in combination with PMP analysis is demonstrated in fig. 21. A section of kidney tissue surrounding a tubule was stained with lanthanum prior to analysis. In the photomicrograph of the stained section the cell structure can be seen and when comparing with the elemental map from the PMP analysis of the La distribution the agreement is excellent. If the staining does not interfere with the other elemental distributions the La map can be used for orientation on the sample (G.J.F. Legge, unpublished material).

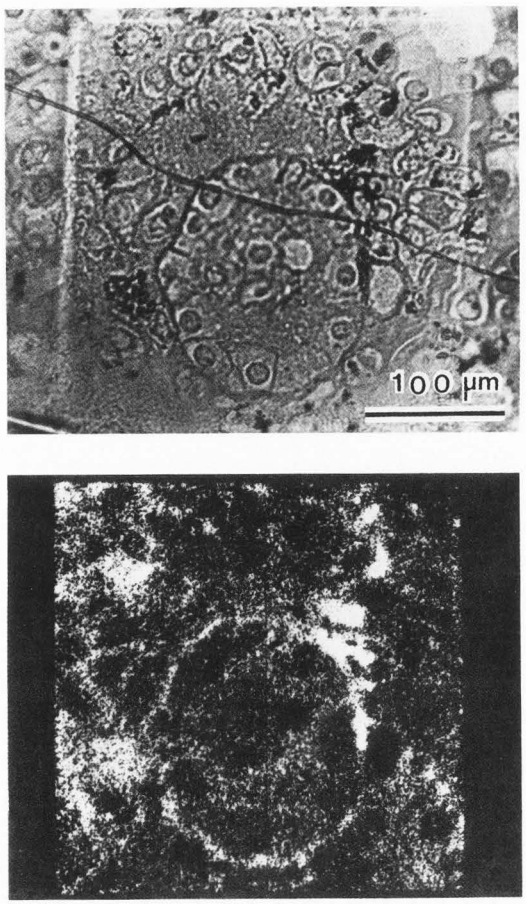

Fig.21. Upper: Micrograph of a La-stained section of kidney around a tubule
Lower: Corresponding elemental distribution from "elemental mapping" for lanthanum with a scanning PMP
(G.J.F. Legge, unpublished results).

In the section on the analysis of animal tissues artery walls of normotensive and hypertensive rats an investigation on using the PMP technique was described. A similar project was concerned with the analysis of atherosclerotic artery walls in humans (Cichocki et al., 1985). Eight-micrometre sections were cryosectioned from quench-frozen tissue. Line scan-analysis was performed with a 3x10 μm^2 beam. Elements found were Cl, K, Ca, Fe, Zn and Br and in some cases Cu and Pb. The concentrations of Ca and Fe varied in different parts of the tissue. The profiles of each element were characterized by sharp narrow peaks and in some cases the only explanation for the narrowness of the Fe and Ca peaks is the presence of crystals. The distribution of Ca, which is important in this study, correlated with those of Fe, Zn and Br.

Concluding remarks

It was the intention in this paper to demonstrate, mainly to electron microprobe users in the biomedical field, the potential offered by the proton microprobe in extending the analytical capability further when used separately or preferably in combination with an electron microprobe.

The technical development of the equipment for the PMP is still in full swing with people working on the second-generation probes with smaller beam spots and on computer software for evaluating the massive flow of information produced by e.g., a scanning PMP in a single irradiation. However, due to physical limitations discussed above trace element analysis, which is the most important characteristic of the PMP technique, requires either relatively large beams or very long irradiation times. Therefore my belief is that many of the future applications will be carried out with probes of moderate resolution (5 -15 µm). The sample preparation techniques will be further developed to ensure that localization of the trace elements is not affected and better ways of viewing the biological structures during analysis need to be developed.

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Discussion with Reviewers

G.J.F.Legge: Could you give figures for the spatial resolution and the elemental sensitivity achievable with the laser microprobe and compare these with the figures achievable with the proton microprobe?
Author: The laser microprobe and the proton microprobe are rather similar in both resolution and sensitivity. The best spatial resolution for both methods is about 1 micrometre. The laser microprobe has a concentration detection limit of about 0.5 μg/g while the proton microprobe gives a lower limit of detection between 1 and 10 μg/g. When comparing these figures one should keep in mind that the laser microprobe normally produces quantitative results which are less accurate than those from the proton microprobe.

G.J.F.Legge: In autoradiography the elemental sensitivity achievable in a given experimental time depends on the half-life of available tracers and the spatial resolution depends on the related energy and hence range of the emitted particles. Could you give some comparisons of the sensitivity and spatial resolution achievable for specific elements in the studies of brain cortex for the two techniques - autoradiography and proton microprobe?
Author: This is an interesting but complex question. The selection of a suitable radiotracer depends on many factors, e.g., time scale of body kinetics, lateral resolution, access etc. In studies of lead in brain cortex two isotopes have normally been used, Pb-203 and Pb-210. The high energy of the Pb-203 radiation limits the lateral resolution but by using the alpha particle tracks from Po-210 which results from radioactive decay of Pb-210 (through Bi-210) it is possible to increase the resolution. In microautoradiographs it is possible to localize the tracks and obtain a resolution of the order of tens of micrometres. The lower level of detection is difficult to define but seems to be below 1 μg/g (Hacket PL, Hess JO, Sikov MR (1982). Distribution and effects of intravenous lead in the fetoplacental unit of the rat. J. Toxicol. Environ. Health 9 1021-1032). A direct comparison with the PMP technique is difficult to do but generally when studying lead the detection limits of PMP are rather good (a few μg/g, dryweight) and the lateral resolution (for trace element determination) is 3 to 5 micrometres. The proton microprobe will give a more accurate quantification and the very long measurement times (exposure times of 5 to 10 weeks) can be avoided.

G.M.Roomans: Would lowering the specimen temperature decrease the extent of thermal damage to the specimen? Are cold stages for proton microprobes being considered?
Author: A direct cooling of the sample at a cold stage has been discussed and is in fact also prepared for in some proton microprobe arrangements. This would allow PMP analysis in the frozen-hydrated state. However, one basic difference between the EMP and PMP techniques is the penetration depth of the primary radiation. While electrons only penetrate the first few micrometres of the sample the protons will in an organic matrix reach 50 to 100 micrometres. To avoid unnecessary background radiation and deposition of energy, thin samples (<3 mg/cm^2) are normally preferred in PMP analysis and the beam is dumped in a Faraday cup. However, the use of a cold stage would to my knowledge require the use of thick samples to enable cooling of the specimen from underneath. The possible advantages of such an arrangement have to be tested experimentally.

F. Watt: In elemental mapping it is in my opinion not necessary to obtain good statistics in each pixel since the eye will average over several pixels when studying a distribution. Could you comment on this?
Author: It is true that to visualize an elemental distribution it is not necessary to have good statistics in each pixel. My point, however, was that if you want to visualize trace element distributions with a very high resolution very long irradiation times are required. The imaging of elements occurring in slightly higher concentrations is normally enough to select a particular region for, e.g., a line scan with trace element determinations.

F. Watt: Perhaps the author could clarify the advantages of line scanning.
Author: The use of a beam spot with a line shape, i.e., high resolution only in one dimension is common in doublet configurations where this type of focus is easily obtained. It is also true that this approach is useful only when analysing an "edge" region. However, in many biological systems aligned cells of equal type form cell strata which can be analysed with this type of beam. The significantly increased beam current shortens the irradiation times and for a set-up with a very low beam current when using very small symmetric beam spots this approach may be a good solution to perform trace element analysis with the PMP.

E.T. Williams: It would be of interest to the readers to give a table of existing PMP facilities.
Author: I fully agree that such a table could be useful. However, I would like to limit the answer to presenting an address list of the members of the Microprobers mailing system organized by Dr. G.J.F. Legge. Only a contact person and the address are given:

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*EINDHOVEN Dr. M. Prins, Eindhoven Univ. of Technology, Dept. of Physics, Eindhoven, The Netherlands.
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