Energy dispersive x-ray analysis in the study of pneumoconiosis¹

A. FUNAHASHI, K. A. SIEGESMUND, R. F. DRAGEN, AND K. PINTAR

From the Medical and Pathology Services, Veterans Administration Center, Wood (Milwaukee), Wisconsin; the Departments of Medicine, Anatomy and Pathology, The Medical College of Wisconsin, Milwaukee, and Midwest Research Microscopy, Inc., Milwaukee, Wisconsin

ABSTRACT Identification of inorganic substances in the lung is an important step towards the establishment of a cause and effect relationship in the study of pneumoconiosis. The conventional methods for this identification usually require an ashing which makes it difficult to study the localisation of these substances in relation to pathology. A method is described to identify foreign substances in the tissue obtained either on biopsy or autopsy without destroying them. The technique employs scanning electron microscopy together with energy dispersive x-ray analysis. This method not only allows simultaneous multi-elemental analysis of over 80 elements, but also permits detailed morphological examination while the tissue is being analysed.

The complex industrial society in which we live has increased the exposure of the human lung to a variety of substances which are potentially hazardous. When these offending agents are organic in origin, a variety of methods are available which may establish a cause and effect relationship (Fink, 1974). However, when the substances are inorganic, the diagnosis is usually based on a history of exposure, the appearance of chest radiographs and occasionally results of a lung biopsy. It is often difficult to obtain an accurate history of exposure, because an individual may be totally unaware of the hazard. Both chest radiograph and pathological changes often lack findings specific for each disease entity. As most of these inorganic substances, especially minerals and metals, are not detected by histochemical procedures, an ashing technique followed by atomic absorption spectrophotometry or x-ray diffraction analysis is sometimes employed.

The detection of foreign material by these conventional ashing techniques not only requires a relatively large amount of tissue but also makes it difficult to determine any relationship between these substances and the reaction of the surrounding tissue. The

microincineration method, when applied to silicosis, has yielded information on the site of silica deposition in relation to histopathological changes (Gross *et al.*, 1956). Recently, electron microprobe analysis has been found useful in the characterisation of individual asbestos body cores (Langer *et al.*, 1972).

Energy dispersive x-ray analysis (EDXA) is a recently developed analytical technique which permits simultaneous multielemental analysis of all elements above atomic number 8 while the object is being examined by the scanning transmission electron microscope (S-TEM) or the scanning electron microscope (SEM). We have used this technique to study lung tissue from a case of interstitial fibrosis in which numerous electron-dense materials, observed by conventional transmission electron microscopy, were identified by EDXA and S-TEM as containing iron, nickel, chromium, and cobalt (Siegesmund *et al.*, 1974). We have also studied silicosis with this technique and established the levels of silicon in lung tissue, in both normal and silicotic subjects (Funahashi *et al.*, 1975). The usefulness of this method was also demonstrated on lung tissue where conventional diagnostic criteria had failed to establish a diagnosis of silicosis (Pintar *et al.*, 1976).

The purpose of this communication is to describe the technical details for the application of this analytical method which we have developed for the study of pneumoconiosis.

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Materials and methods

SPECIMEN PREPARATION

Sections of lung tissue from well-established cases of silicosis were cut from ordinary paraffin blocks. Paraffin was removed from the sections with xylene; they were then hydrated by passage through decreasing concentrations of alcohol and into distilled water. The sections were floated from distilled water on to the surface of spectroscopically pure $1 \times 16/31$ inch carbon planchets (Ultra Carbon Company, Bay City, Michigan). The tissue sections were air-dried slowly at room temperature to prevent curling. The sample was given a $100 \text{ \AA}$ (10 nm) carbon coating by vapour deposition to reduce charging of the specimen during examination in the SEM.

SEM EXAMINATION

The carbon planchet was placed in the SEM and examined directly in the secondary electron mode for morphology. The incident primary electron beam of the SEM generates a number of signals when it interacts with the specimen. Secondary electrons are electrons in the material which are excited by collisions with the primary beam. Those which escape the surface are of low energy (0-50 eV; 0-8.0 aJ) and are generated with a thin layer at the specimen surface. These low energy electrons can be collected and converted into a relatively high resolution (10 nm) image of the specimen. This represents the conventional mode of surface imaging.

X-RAY ANALYSIS

X-rays are also generated by the collision of the primary electron beam with the specimen. The wavelength, energy, and number of x-rays will depend on the elements present in the specimen and their abundance. Using a solid-state silicon semiconductor detector, x-rays emitted from the samples are collected and used to generate a small electrical signal with a charge proportional to the energy of the incoming x-ray photon. This signal is electronically processed and stored in a computer as a single count which is filed according to the voltage of the incoming pulse. As incoming x-rays are detected, sorted, and stored a spectrum is produced of which the x-axis represents an x-ray energy distribution and the y-axis represents the number of x-rays of a given energy. Such a spectrum is shown in Fig. 1 and represents a typical 200-second analysis of lung tissue from a silicotic patient. Each peak seen on the cathode ray screen represents an element found in the tissue. The peaks representing the given elements are superimposed on a background of white radiation (Bremsstrahlung) and a carbon background radiation. Each peak represents one of the characteristic x-rays generated by an element. To facilitate rapid identification of these peaks, a computer programme generates vertical dotted lines (K, L markers) locating peak positions in these spectra for the selected element. The peak identified in Fig. 1 is silicon (Si) of atomic number 14. Such an analysis can then reveal more than 80 elements simultaneously and in a matter of minutes. Most analyses

Fig. 1 Spectrum of tissue analysis by EDXA seen on cathode ray screen.

in this study were run for periods of 200 seconds each.

Three to five randomly selected areas of approximately 1 mm² were photographed and x-ray analysed to obtain the relative concentrations of any elements in the area of the lung photographed. An Ortec 165 eV resolution Si (Li) detector and a Tracor North Ns-880 computer base data handling system were used for the analyses. All spectra were stored on magnetic tape for future reference.

DATA REDUCTION

The relative abundance of each element can be calculated from an analysis of the counts generated for a given element by comparison with the reference spectra of the materials containing known concentrations of that element. Concentration ratios were calculated and corrected for absorption, fluorescence and atomic number effects, assuming carbon as a background. Because of the difficulties inherent in determining the exact amount of material examined, the data were presented as relative elemental concentrations by weight.

Results

SECTION THICKNESS

An earlier study (Funahashi *et al.*, 1975) has shown that when analysing lung tissue sulphur could be used as a reliable indicator of tissue mass, and that a Si/S ratio gave a more valid expression of silicon concentration than silicon alone. Sections of varying thickness were analysed to discover the relationship between elemental count and section thickness and to determine whether there was a minimum thickness which would still provide reasonable counting rates. The table shows the analysis of sections 10 to 40 μ m in thickness. The Si/S ratios were relatively

constant for 20, 30, and 40 μ m. Sections cut at 10 μ m showed a higher Si/S ratio than the thicker sections. Sections cut at 5 μ m proved too difficult to handle because they tended to curl up and failed to adhere well to the carbon stub. Figure 2 shows that a linear correlation exists between sulphur content and sec-

Fig. 2 Section thickness and contents for sulphur (S) and phosphorus (P).

X-RAY MAPPING

The technique of x-ray mapping enables single elements to be mapped according to their location on the secondary electron image. The precise position of the elements within tissue and their relative concentration can then be ascertained.

Figure 3 shows a conventional secondary electron image of a silica nodule and thickened septa. Figure 4a shows a conventional x-ray map for silicon. Silicon concentrations, shown as white dots, are seen primarily in the nodule. Figure 4b shows the same area mapped for iron, with most of the element appearing in the septa. Figure 4c was produced by making a negative of the x-ray map of silicon and printing a positive directly onto the secondary electron image. This procedure produces an x-ray map of black dots, showing precise locations of the element directly on the electron image. A more exact localisation of silicon can be determined within the nodule. Figure 4d shows the same technique, used to map iron in the same area. The iron is seen to be exactly within the septa with little occurring in the nodule itself.

Printing such maps from the original negative produces white dots which are less satisfactory when viewed on top of the secondary electron image.

Table Section thickness and elemental count

Section thickness (μ m)	Elements			
	S	P	Si	Si/S
10	10	2	15	1.5
20	17	3	11	0.9
30	23	5	14	0.7
40	29	11	27	0.9

S = sulphur; P = phosphorus; Si = silicon.

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Fig. 3 Scanning electron micrograph of silica nodule and thickened alveolar septa (original magnification: $\times 100$).

COLOUR MAPPING

Simultaneous viewing of two or more elements is possible by printing x-ray maps for these elements on the same piece of colour photographic paper. Each element map is printed separately, using a different colour filter in front of the enlarger lens.

Discussion

In the study of pneumoconiosis, it is important to detect all inorganic materials in lung tissue, because individuals are often exposed to more than one element. On the other hand, the mere detection of foreign elements may be of no significance in terms of tissue injury because different substances may cause quite a different tissue reaction. For example, iron oxide is thought to be relatively innocuous, while silica is known to be highly cytotoxic. For this reason, the relationship between the location of a foreign substance and tissue pathology becomes very important, especially in cases where more than one dust is involved. Conventional methods of analysis usually do not permit a study of this relationship. A microincineration method has been used in silicosis to study tissue reaction and the presence of silica, and has yielded valuable information concerning its site of deposition (Gross *et al.*, 1956; Gross and Smith, 1959). This indirect method, however, is not only destructive of the sample but is also extremely tedious and time-consuming. Electron microprobe analysis, which uses wavelength detectors, provides a sensitive nondestructive means for analysing tissue structure (Robertson, 1968). This technique, however, does not allow for simultaneous multielement

analysis and requires rather long analysis times. Energy dispersive x-ray analysis (EDXA) is non-destructive like electron microprobe analysis but has the additional advantage of being more rapid and of providing simultaneous multielemental spectra. This capability of EDXA for simultaneous multielemental analysis is particularly important in the study of pneumoconiosis, where workers are commonly exposed to more than one element.

Morgenroth *et al.* (1973) used EDXA and SEM to identify silica in macrophages, in semi-thin sections of guinea pig lung embedded in Epon. Ruttner *et al.* (1973) analysed by EDXA and SEM lung dust residue sprayed on a collodion-coated copper grid, and observed peaks of aluminum, silicon, potassium, and iron. These investigators demonstrated the feasibility of EDXA and SEM in the identification of silica deposited in lung tissue. We have used specimens from ordinary paraffin blocks and analysed them at a much lower magnification than in previous studies (Morgenroth *et al.*, 1973; Ruttner *et al.*, 1973). Our method not only permits the study of a much larger area (1 mm^2 for each analysis) but the thicker sections reduce the sampling error which is an inherent problem in electron microscopic studies. The method also permits a much closer correlation with an ordinary light microscope. When ordinary deparaffinised pathology specimens are used however, poor resolution is noticeable at higher magnifications. Epon-embedded thin sections may therefore be necessary when one wishes to study morphology at an ultrastructural level with simultaneous analysis of EDXA. In cases where elements such as heavy metals have a relatively low concentration but

a high electron density, the use of the scanning transmission electron microscope (STEM) together with EDXA will allow a detailed ultrastructural examination to be carried out, as demonstrated in a case of interstitial fibrosis reported previously (Siegesmund *et al.*, 1974).

The present study confirmed the validity of using sulphur as an indicator of tissue mass, as described previously (Funahashi *et al.*, 1975). There was a linear correlation between section thickness and the amount of sulphur and very little variation of silica content when the silicon/sulphur ratio was applied

Fig 4a

Fig 4b

Fig. 4 X-ray maps for silicon (a, c) and iron (b, d). See text.

Fig 4c

Fig 4d

in a range of 20 to 40 μm . Why the 10 μm thickness gave a higher Si/S ratio is not entirely clear. It is possible that with this thickness a small change of distribution of silica in the specimen may have had an unduly large effect on the Si/S ratio.

The value of the x-ray mapping technique deserves emphasis. It permits visualisation of a specific substance at its location within the tissue, and

enables a detailed study of the site of deposition and tissue reaction to be carried out. The site of deposition of an element, however, does not necessarily indicate the place where the tissue injury had occurred, for it has been suggested that mobilisation of substances may take place after the initial tissue reaction (Gross and Smith, 1959).

The use of EDXA has been described in the study

of organs other than lung (Russ and McNatt, 1969; Fowler and Parker, 1973). Recently, silicon was found in a kidney biopsy specimen from a patient with a history of heavy exposure to silica (Saldanha *et al.*, 1975). Phagocytosis by alveolar macrophages is one of the key mechanisms involved in the clearance of foreign dusts from the lung, and it is therefore quite conceivable that many foreign materials other than silica may be found in organs distant from the lung and may produce tissue reaction at the site of deposition. EDXA should prove useful in the study of such cases, although technical details are yet to be determined.

A disadvantage of EDXA is that the method takes no account of chemical form. For example, EDXA will not discriminate between free silica, silicate, or amorphous silica. For this reason, we believe that EDXA will not replace the conventional analytical methods but will complement them, and further expand our ability to identify elements in the tissue. It will be of particular use in the study of their location in relation to tissue structure.

References

- Fink, J. N. (1974). Hypersensitivity pneumonitis due to organic dusts. *Clinical Notes on Respiratory Diseases*, **13**, 3-8.
- Fowler, B. A., and Parker, P. (1973). Observation on the preparation of epoxy embedded tissue samples for energy dispersive x-ray analysis. *Stain Technology*, **18**, 333-335.
- Funahashi, A., Pintar, K., and Siegesmund, K. A. (1975). Identification of foreign material in lung by energy dispersive x-ray analysis. *Archives of Environmental Health*, **30**, 285-289.
- Gross, P., and Smith, K. W. (1959). The topographic distribution of mineral dusts in some pneumoconiotic lungs. *Diseases of the Chest*, **35**, 140-154.
- Gross, P., Westrick, M. L., and McNerney, J. M. (1956). Silicosis: the topographic relationship of mineral deposits to histologic structures. *American Journal of Pathology*, **32**, 739-775.
- Langer, A. M., Rubin, I. B., and Selikoff, I. (1972). Chemical characterization of asbestos body cores by electron microprobe analysis. *Journal of Histochemistry and Cytochemistry*, **20**, 723-734.
- Morgenroth, K., Blaschke, R., and Schlake, W. (1973). Energy dispersive x-ray analysis of semi-thin section in the scanning transmission. *Beiträge zur Pathologie*, **150**, 406-411.
- Pintar, K., Funahashi, A., and Siegesmund, K. A. (1976). Diffuse form of silicosis in foundry workers. *Archives of Pathology and Laboratory Medicine*, **100**, 535-538.
- Robertson, A. J. (1968). The electron probe microanalyzer and its application in medicine. *Physics in Medicine and Biology*, **13**, 505-522.
- Russ, J. C., and McNatt, E. (1969). Copper localization in cirrhotic rat liver by scanning electron microscopy. In *Proceedings of the 27th Annual Meeting of the Electron Microscopy Society of America*, St. Paul, Minnesota, pp. 38-39. Claitor's Publication Division: Baton Rouge, Louisiana.
- Ruttner, J., Spycher, M. A., and Sticher, H. (1973). The detection of etiologic agents in interstitial pulmonary fibrosis. *Human Pathology*, **4**, 497-512.
- Saldanha, L. F., Rosen, V. J., and Gonick, H. C. (1975). Silicon nephropathy. *American Journal of Medicine*, **59**, 95-103.
- Siegesmund, K. A., Funahashi, A., and Pintar, K. (1974). Identification of metals in lung from a patient with interstitial pneumonia. *Archives of Environmental Health*, **28**, 345-349.