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QUANTITATIVE ANALYSIS OF PARTICULATE BURDEN IN LUNG TISSUE

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Abstract

Numerous methods have been used in the preparation and analysis of the particulate matter deposited in human lungs. Preparation techniques include those for particle isolation and for *in situ* analysis. Analytical techniques include bulk and particle-by-particle analysis. In this paper, a general discussion of many of these methods is presented along with examples of how two specific techniques have been used. In one study, individual particles from the lungs of 75 randomly selected autopsy cases were analyzed using an automated scanning electron microscopy (SEM)/ energy dispersive X-ray microanalysis (EDX) system. An average of 613 million particles, of exogenous origin, per gram of dry lung tissue were found, the major classes of particles being silica, talc, aluminum silicates, and rutile. In the second study, lungs from 50 randomly selected autopsy cases were analyzed using gravimetric and X-ray diffraction (XRD) analysis. The median total particulate material was 0.33 grams, for cases in which samples were prepared by high temperature ashing, and 0.41 grams, for those in which nitric acid digestion was used. The median amount of quartz for all cases, was 0.044 grams. Samples of eighteen of the 75 lungs previously analyzed by automated SEM/EDX were also analyzed using gravimetric and XRD analysis. A good correlation was seen between the results of the two procedures (r=0.91 for number of exogenous particles versus grams of particulate matter and r=0.97 for number of silica particles versus amount of quartz).

KEYWORDS: Lung tissue, particulate matter, tissue destruction, *in situ* analysis, bulk analysis, particle-by-particle analysis, X-ray diffraction, automated scanning electron microscopy/energy dispersive X-ray analysis, quartz, pneumoconiosis.

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Introduction

Certain types of inorganic particles have been recognized as causes of lung disease for many years. The diseases they cause are a type of pneumoconiosis and are normally associated with inhalation of toxic particles in an occupational setting. In addition, most people, regardless of occupation, are exposed to respirable particulate matter. Evidence of this exposure can be seen in nearly all adult lungs taken at autopsy as black pigment, sometimes incorrectly called anthracotic pigment. This pigment consists of different types of minute particles, including carbon, from such possible sources as cigarette smoke and industrial combustion processes.

The field of particle-induced lung toxicology has grown greatly in the twentieth century. A large amount of information has been collected concerning the gross and microscopic pathology, radiographic changes, and epidemiology associated with dust-induced diseases. In addition, many samples of inorganic particulate material, collected from the site of dust exposure or isolated from lung tissue, have been analyzed. However, even though much work has been done, more remains to be done, because of the new technologies being developed and because of the lack of data on pulmonary particulate burden from individuals who do not work in dusty occupations.

The purposes of this paper are to review some of the techniques which have been used to analyze inorganic lung particulate matter and to discuss some of the considerations involved in choosing a technique. Examples of studies in which some of these techniques are used are presented. The focus of this paper will be primarily quantitative analysis, although when quantitation is done by identification and counting of individual particles, the problem becomes one of qualitative analysis as well. Roggli, et al., (97) and Brody (15) have reviewed many of the techniques currently available for identification of inorganic particles in the lung, and Baker, et al. (8), have reviewed the use of microprobe methods for analysis of human pathologic material. In addition, data on the analysis of particulate

matter isolated from lung tissue obtained from randomly selected autopsy cases using automated scanning electron microscopy combined with energy dispersive X-ray analysis will be reviewed, and results of quantitative X-ray diffraction analysis on a similar set of lungs will be presented. These techniques have been used extensively by the authors, and these studies represent examples of how analysis of particulate material from lung tissue can be performed.

A number of techniques are available for analyzing particulate matter and for processing the tissue prior to analysis. They all have advantages and disadvantages, and the choice of technique is dependent on the amount of tissue available, the type of information needed, the nature of the particulate material, and the resources available. Ideally, a number of different techniques should be used if possible. In this next section, techniques for tissue processing and particle analysis will be discussed along with some of their advantages and disadvantages and how they have been used in the past.

Tissue Processing

A good first step in choosing a technique is to decide whether the particles should be isolated from the tissue before analysis or examined *in situ*.

In situ Analysis

In situ analysis offers the advantage of allowing the investigator to determine the location of the particles in the tissue, e.g., within macrophages, free in the connective tissue, or associated with an area of inflammation. In addition, routine processing such as for light or transmission electron microscopy can be used. It is even possible to perform multiple analyses on the same section using light microscopy, scanning electron microscopy, electron microprobe X-ray analysis, and/or ion probe microanalysis (64,72,88). The disadvantages include the small amount of tissue used, which can make getting a representative sample of the lung and hence the particulate matter more difficult, since the particulate matter may not be distributed evenly throughout the lung. Furthermore, particles are often encountered concentrated in small areas, such as lysosomes, causing particle overlap and making analysis of the individual particles difficult.

With current microbeam technology, *in situ* analysis can be quite a powerful technique. Abraham and Burnett (2,3), Funahashi, et al., (34), and Siegesmund et al., (99,100) have presented techniques for quantitation of particles in 5 μm sections of lung tissue. These will be discussed in greater detail later. One other type of *in situ* analysis that has been used involves placing a wire screen over a hilar section from a lung that had been fixed in inflation and counting the number of intersections of the wires lying over pigmented (black) tissue and the total number lying over any parenchymal tissue. Using this technique, a modification of a technique used by Weibel (118), Pratt and Kilburn (94) were able to estimate the percentage of the volume of tissue that was pigmented, i.e., that contained visible amounts of respirable black dust.

Particle Isolation

It is preferable to isolate the particles from the lung tissue before analysis if the tissue components interfere with the analysis or if the particles need to be concentrated from a large volume of tissue. This latter situation might occur when the particles are in low concentration or when tissue from several areas is pooled in order to get a more representative sample of the whole lung. In addition, particle separation is necessary if the element or compound of interest is present in the tissue as well as in the particulate matter. This would hold true for elements such as calcium and potassium. Particle isolation is generally a two step process: destruction of the tissue and separation of the particles from the destroyed tissue.

The choice of isolation techniques is based on the specific needs of the research. For instance, digestion in concentrated HCl, while very useful for the study of silica, is not generally suitable for the analysis of asbestos fibers, due to the sensitivity of certain types of silicates (e.g., chrysotile) to attack by acids (7,102). Further attention must be paid to such unique characteristics as the fibrous morphology of particles, which can be altered in processing. Consequently, no one technique is universally best.

Tissue Destruction. Nagelschmidt (83), Guest (40), and Jaunarajs and Liebling (55) have reviewed some of the techniques available for destroying lung tissue prior to particle separation. In addition, Gylseth, et al. (41,42), Pooley (90,91), and Langer, et al. (67) have reviewed techniques for isolating fibrous particles. A major concern here is that the method chosen removes the biological material without altering the particulate material of interest. Standards, representative of the particulate matter of interest, should be used to determine that the technique used is suitable.

The selection of tissue destruction methods can be divided into physical and chemical. The physical techniques used most often are high temperature (i.e., 380°C or higher) and low temperature ashing. The major advantages of high temperature ashing (HTA) are the relative simplicity and the completeness of destruction of the tissue. The technique is generally safe for most minerals, but carbonaceous materials such as coal are lost. In addition, metals that have a high vapor pressure at high temperatures, such as mercury which has a vapor pressure of 1138.4 mm. of Hg (1.50 atm.) at 380°C (117), can be lost due to volatilization (51). Kaolinite is altered by temperatures greater than 500°C in such a way that it becomes less detectable by X-ray diffraction. This can be overcome by using lower temperatures (e.g., 380°C) and longer ashing times (61). Furthermore, Gylseth, et al. (41,42), found that HTA treatment of fibrous particles at 500°C

and 600°C resulted in only a 55% and a 20% recovery, respectively. The time required for most samples, on the order of days for larger samples, can be a disadvantage.

Low temperature ashing (LTA) of tissue in a radio-frequency activated oxygen plasma has been used extensively in recent years. It is safe for minerals, but organics and very volatile inorganics such as some arsenic and selenium compounds can be partially lost (63,116). After the initial expenditure for the asher, this technique is fairly inexpensive; in our lab, a 244 cubic feet cylinder of oxygen lasts for one to two months with routine use. The chemical techniques discussed below, however, are generally less expensive.

A major disadvantage of both HTA and LTA is that endogenous iron, from blood, hemosiderin, etc., is converted to iron oxides which are difficult to remove and can interfere with subsequent analysis. Acids can be used to dissolve this iron, but other components of the particulate matter may be removed, as well.

Oxidizing agents are used routinely to digest lung tissue. Sodium hypochlorite can be purchased quite inexpensively in a 5% solution as commercial bleach. About 50 ml. per gram of wet tissue are required; hence the technique is better for smaller samples. Generally, in this procedure, the tissue is not completely digested and in order for the sample to be directly filtered, chemicals such as oxalic acid and ethanol are required to remove the undigested material. Alternatively, the sample can be centrifuged (see below). Procedures using sodium hypochlorite for isolation of asbestos fibers and ferruginous bodies are widely used (20,27). The technique is not well-suited for some types of samples, however. For instance, treatment of montmorillonite with sodium hypochlorite causes an increase in the size of the spacings between the layers of the phyllosilicate structure (personal observation). Hydrogen peroxide (30% solution) is more useful than sodium hypochlorite when larger amounts of tissue (e.g., greater than five grams) are used. It is relatively safe for most minerals and for high rank (anthracite) coal, but low rank (bituminous or sub-bituminous) coal is attacked somewhat (83). Organic solvents and dilute acids can be used to remove lipids and endogenous salts, respectively. A major disadvantage is the foaming that occurs, but anti-foaming agents are available to reduce this problem (107). The digestion can be made more efficient by increasing the pH of the sodium hypochlorite to about 12 with a sodium hydroxide solution or by heating the sample during digestion.

Strong alkaline solutions, e.g., NaOH or KOH, heated to 90°C can also be used to digest samples of lung tissue. The digestate is generally flocculent and not easily filterable. The sample can be centrifuged, or alternatively, it can be dialyzed several times against water, which will make the digestate filterable. Acid digestion is relatively quick and dissolves the tissue thoroughly, but dissolves metals and also attacks chrysotile (7,68,102). Furthermore, the acid is generally heated and therefore presents a danger to the investigator. Guest (40) compared a number of methods of tissue destruction and concluded that 11.3 N HCl gave the best overall results for recovery of coal and some inorganic dusts, including quartz.

Enzymatic digestion is perhaps the mildest procedure for the particles, since the digesting agent is specific for the tissue components. The original procedure reported by Cooke in 1933 (22) called for using trypsin. Nenadic and Crable studied the effectiveness of papain, trypsin, ficin, bromelin, and pronase in digesting lung tissue and presented a procedure for digestion of lung tissue using ficin (85). A disadvantage of enzymatic digestion in general is the time required, on the order of three to four days (83).

It is often desirable to have the dry weight of a lung tissue sample. This is sometimes accomplished by heating the sample to temperatures near 100°C. The elements mentioned above in connection with LTA might be lost in this process. When analyzing for these elements, freeze-drying to a constant weight should be used. It has also been shown that drying tissue samples at 80°C followed by LTA can cause fiber breakage (41,42). This has been attributed to shrinkage during drying. It is sometimes advisable to use duplicate samples, one for drying and one for analysis, when the particles may be affected by the drying process.

Particle Separation. Often procedures for destroying tissue result in the particles being in a suspension from which they must be separated prior to analysis. Filtration is in some cases the simplest way to recover the particles. Several types of filters are available. The choice depends on the analytical method used as well as the chemicals used to digest the tissue. For instance, polycarbonate filters, such as Nuclepore® brand, are excellent for scanning electron microscopy because of their even topography, but will dissolve in some organic solvents which are often needed to extract lipids. Similarly, silver membrane filters work well for X-ray diffraction because they give a low background. However, dilute acids which are sometimes needed to remove endogenous lung salts will attack the silver. Dialysis of the digested material against water can sometimes be used to remove digesting chemicals prior to filtration.

If the technique used does not dissolve the tissue or endogenous inorganic material completely, or if a large amount of particulate matter is present, filtration may be difficult or impossible. In this case, centrifugation can be used. Centrifugation is also indicated if a suitable filter is not available. The non-carbonaceous particulate matter is normally denser than the undigested debris and carbonaceous particulate matter. A disadvantage of centrifugation is that particles can be lost due to their sticking to the centrifuge tube or to disturbance of the pellet while decanting the supernatant (41,42).

It is often desirable to solubilize the particulate matter prior to analysis. Wet

ashing in acid at 600° to 700°C has been used for many years. Volatile elements can be lost during this treatment, however. For this reason, a method of acid decomposition inside a sealed Teflon container has been recommended (63).

Sample preparation, then, can involve dissolution of the tissue and the particulate matter. In most studies, both components have been dissolved simultaneously. With this type of sample preparation, one cannot be certain that the substance being measured originates from particulate matter. In order to assure that only particulate material is being analyzed, the particles must first be isolated from the tissue and then dissolved. The importance of this is suggested by the study by Vanoeteren, et al., in which fourteen elements were measured by neutron activation analysis in whole lung samples and in particulate samples isolated from the same lungs. It was found that the isolated particulate matter contained large portions of certain elements in lung (e.g., 60 % of the total Cr in the lung), but much less of other elements (e.g., 1 % of the total Zn in the lung) (112). Specific methodologic details were not given, however. It is, therefore, possible that the some particles, e.g., those containing Zn, could have been preferentially lost during tissue preparation, especially if these particles were smaller than the others.

An alternative method has been given (66,89) which avoids the need for particle separation by using small amounts of tissue, i.e., a paraffin section. The procedure, known as "carbon-extraction replication", entails ashing a deparaffinized histologic section on a glass slide, applying a thin film of polyvinyl alcohol (PVA) on the ashed section, removing the PVA film, coating the film with carbon by evaporation, and dissolving the PVA with steam. This leaves a carbon film containing the particles in approximately the same orientation as they were in the tissue. Before dissolving the PVA film, it can be cut into pieces small enough to be placed on electron microscopy grids. This technique is useful for fiber analysis, because the fibers are easily distinguished from the ashed material.

Analytical Methods

The methods for analyzing the prepared samples are also numerous. Again, the choice is based on individual needs, and the use of a combination of techniques, if possible, is recommended. The analytical methods can be divided into particle-by-particle analysis and bulk analysis.

Particle-by-particle analysis

This type of analysis has some advantages over bulk quantitative analysis. In addition to allowing a detailed analysis of the particles, particles that are present in small numbers, and hence might be missed with bulk analysis, can be analyzed. Particle-by-particle quantitation, however, has the disadvantage of often being slow and tedious, both because the individual analysis can be slow and because a large number of particles is usually needed to get statistical validity. Use of computer-controlled analysis has been developed to help lessen this problem. (See below.)

As mentioned in the Introduction, this type of analysis involves at least two analytical problems--identification and enumeration. It is often desirable to have particle size information as well. Although there are ways of determining the size distribution of a population of particles without identification of the particle types, this kind of analysis is of little value given the heterogeneous nature of the particulate matter in most lungs, especially human lungs.

Particle identification. Identification of particles is generally based on morphology, elemental composition, and/or crystallography and involves direct visualization of particles in a microscope either by the investigator or a computer. The technique chosen depends, again, on the type of information needed. However, since the maximum number of particles should be analyzed, the speed with which data are collected is also important. The most commonly used imaging techniques are light microscopy and electron microscopy. Images can also be produced by spatial analysis of X-rays emitted from a sample during irradiation by an electron beam (X-ray mapping) and by mass spectroscopy analysis of charged species produced by sputtering a sample with a charged particle beam (secondary ion mass spectroscopy) (64). These techniques have not been used as extensively for quantitative analysis of lung particulate burden as the light and electron beam techniques.

The light microscope has been used for identification of particles based on morphology, refractive index, polarized light analysis, dispersive staining, or by staining with certain element-specific dyes (79,108). The chief disadvantage of this technique is that many respirable particles are too small to be resolved. Light microscopy is routinely used to identify ferruginous bodies, which are normally large enough to be seen in the light microscope, both in tissue sections and isolated from the tissue (20,27,39).

The electron microscope offers many advantages over the light microscope. The limit of resolution of the electron microscope can be up to 1000 times better (82). The multiple interactions that occur between the electrons of the incident beam and the atoms of the sample are perhaps even more important for particle analysis. A detailed description of these interactions can be found elsewhere (37). The physical events that occur as a consequence of these interactions can be studied to obtain information from a sample.

Transmission electron microscopy (TEM) is analogous to conventional light microscopy in that the image is formed by differential absorption of the radiation by the sample. TEM is used for morphologic analysis of particles.

Secondary electrons (SE) and backscattered electrons (BSE) are most often used to obtain morphologic information. Secondary electrons are produced when electrons from the incident

beam eject electrons from the outer orbits of the atoms in the sample. They are of low energy, and only those emitted near the surface of the sample escape and are detected. The SE image is, then, one of surface detail. Back-scattered electrons are primary beam electrons that strike the nuclei of atoms in the sample and are scattered without losing energy, i.e., are elastically scattered. The image that the BSE produce is based not only on the surface topography of the sample, but also on the average atomic number of the atoms in the sample. A back-scattered electron image is useful for locating particles of higher atomic number in a matrix of lower atomic number elements, such as silica or metal particles in tissue sections.

In general, morphology is an essential tool in particle analysis for distinquishing broad types of particles, such as fibers and platy mineral particles like talc and kaolinite, as well as for making more rigorous identifications, such as identifying chrysotile fibers based on the presence of the central capillary. However, other information, such as elemental and crystallographic data, is usually needed to more fully characterize particles. Because of the different types of information that can be gathered simultaneously and the speed with which data can be collected with electron probe techniques, the electron microscope has been the instrument most often utilized for this type analysis.

Characteristic X-rays are produced when an electron from the incident beam ejects an inner orbital electron from an atom. As an outer orbital electron drops to the lower energy level to replace the ejected electron, energy is emitted as a photon of X-radiation. The energy (and wavelength) of the emitted photon is characteristic of the species of element from which it was emitted. Analysis of the wavelengths of the emitted radiation (wavelength dispersive X-ray analysis; WDX) gives good elemental resolution but is relatively slow and multiple spectrometers are needed for simultaneous multi-element analysis. Analysis of the energies of the emitted radiation (energy dispersive X-ray analysis; EDX) is faster and multi-elemental analysis is possible. Analysis of a particle by EDX can be performed in as little as five seconds. EDX can detect elements with atomic numbers of eleven, e.g., sodium, and higher with conventional detectors with beryllium windows, although newer detectors without beryllium windows can detect elements with atomic numbers as low as five, e.g., boron (76). Similarly, WDX can be used to detect elements with atomic number of five and above.

Two problems encountered with particle analysis using either EDX or WDX are particle overlap and secondary fluorescence, in which X-rays produced by the bombardment of a particle by the electron beam can themselves induce the production of characteristic X-rays in nearby particles. Both of these problems can make it difficult or impossible to assign X-ray peaks to the individual particles and can make it appear that an element is present in a particle when in fact it is not.

A characteristic X-ray can be reabsorbed by an atom resulting in the emission of an outer orbital electron. This is known as the Auger effect. The production of characteristic X-rays and Auger electrons are competing processes. As the atomic number of an element increases, the ratio of characteristic X-rays to Auger electrons increases. Auger spectroscopy can, therefore, be used for lower atomic number elements for which techniques that analyze characteristic X-ray production are not very sensitive. Elements with an atomic number of three (lithium) and greater can be detected with Auger spectroscopy. An Auger microprobe has been used to analyze inorganic particles in lavaged alveolar macrophages for the elements S, C, N, O, Fe, Na, Al, and Si (73,74).

Electrons can collide elastically with the atoms of the samples. These diffracted electrons radiate in all directions. However, if the sample is crystalline, at certain angles there will be constructive interference of the beams diffracted by the parallel planes of the crystals. If a piece of photographic film is exposed by a properly focused beam that is transmitted through a crystalline substance, a "diffraction pattern" can be obtained. If the sample is a single crystal, the pattern will consist of a two-dimensional array of dots. Analysis of the positions of the dots can be done to determine the sizes of the spaces between the planes of the crystalline lattice. This technique is known as "electron diffraction", and the data obtained from this type of analysis, if combined with morphologic and EDX data, can be used to make fairly complete identifications of particles. Churg, et al., for example, have used this combination of techniques to identify mineral particles, including asbestos and non-asbestos fibers (20,21).

Particle Counting. The second part of particle-by-particle quantitative analysis is particle counting. The simplest and most often used technique is manual counting. There are two advantages that this method has over computer-controlled analysis. First, the investigator can better discriminate between, for instance, particles and undigested or unashed debris. Secondly, certain analytical techniques, e.g., electron diffraction and ion microprobe analysis, which are not currently available in an automated system, can be used.

Manual counting procedures have been applied to particles analyzed *in situ* and particles isolated from the tissue. Below are examples of studies which use these types of analytical procedures. The first is presented because it gives a novel method for *in situ* analysis. The second study uses methodologies commonly employed in the analysis of air-borne dusts; i.e., particles, isolated by filtration, are transferred in a carbon film to TEM grids for analysis. These are given as examples, and whereas we believe them to be very useful methods, they do not necessarily represent the only methods or even standard methods.

Abraham and Burnett (2,3) have presented a

method for manual analysis of particles in situ in tissue sections using SEM and XES or ion microprobe mass analysis (IMMA). A light micrograph is made of the section to be analyzed or of an adjacent serial section. In the SEM, particles are located by BSE imaging and analyzed by EDX. The particles are then classified as endogenous (containing Ca and P or Fe and P as the main constituents.), silica (only Si), silicates (Si and other cations), and other exogenous particles. Analysis of lung tissue from 40 cases using this method showed a range of particle concentrations from 3×10^6 to 2×10^9 particles per cm^3 of tissue, which corresponds to approximately 20 million to 1.3 billion particles per gram of dry weight. [The number of particles per gram dry weight is calculated by dividing the number of particles per cm^3 by 0.15. This latter value represents an average dry to wet weight ratio for a sample of uninflated lung tissue (personal observation)]. Silicates represented, on the average (median), 51.5% of the total exogenous particles counted while silica represented 7.4%, and metals represented 32.3%. These cases included pneumoconiosis cases as well as tissue from a normal adult lung and from a still-born infant. The particles were located mostly in the interstitial and perivascular areas.

Churg and Wiggs (21) analyzed particulate matter from the lungs of 28 men, 14 with lung cancer and 14 without. Hydrogen peroxide digestion was used to isolate the particles. Particle identification was done using TEM morphology, EDX, and electron diffraction. They found 542 million and 266 million exogenous particles per gram of dry tissue for patients with and without lung cancer, respectively. Kaolinite, talc, mica, feldspar and silica were the major exogenous particle types found. In the cancer patients, silica represented 10% of the total exogenous particles, aluminum silicates (kaolinite, mica, and feldspar) 56.5%, talc 21.7%, and titanium 4.3%. In the patients not having lung cancer, silica represented 16%, aluminum silicates 53.6%, talc 11%, and titanium 3.0%.

With the use of rather sophisticated computers, automated particle analysis has become possible with the light or electron microscope. However, since the light microscope has the limitations discussed earlier, the electron microscope has more applicability in computer-controlled analysis of pulmonary particulate matter. Lee and Kelly (69) have provided an overview of the topic of automated SEM analysis.

Stettler, et al., have analyzed particles from 75 lungs of randomly selected individuals from the Cincinnati area (103). The instrumentation used consisted of a Hitachi S-570 scanning electron microscope equipped with a LeMont backscatter detector, a Kevex 7000 energy dispersive X-ray analysis system, and a LeMont DA-10 image analyzer. The description of the instrumentation and procedure used has been given elsewhere (104). Briefly, homogenized, freeze-dried lung tissue was ashed in a low temperature plasma asher and the ash suspended in water and filtered onto a 0.1 μm pore size Nuclepore® filter. The filter was mounted on a carbon planchet and analyzed uncoated. A backscatter image of a field of particles was obtained at 1000X magnification and this image converted by the computer to a binary image. The computer-controlled sizing and counting were done from this binary image. Automated EDX analysis was also done on each particle. The data were stored on either a hard disk or on floppy disks. After analysis, particles were classified by the computer using a chemistry definition file which defines various particle classes on the basis of their major elemental components and the net fractional intensities for these components. The classification scheme used is given in Table 1.

Table 1. Major particle classes of chemistry definition file(103)

Class	Requirements
Silica	0.90 / Si / 1.00
	0.00 / DF*/ 0.10
Aluminum Silicate	0.10 / Al / 0.50
	0.40 / Si / 0.90
	0.00 / DF / 0.50
Magnesium Silicate	0.05 / Mg / 0.50
	0.00 / Fe / 0.50
	0.50 / Si / 0.90
	0.00 / Al / 0.05
	0.00 / DF / 0.001
Iron Oxide-like	0.90 / Fe / 1.00
	0.00 / P / 0.001
	0.00 / DF / 0.10
Rutile-like	0.90 / Ti / 1.00
	0.00 / DF / 0.10
Alumina-like	0.90 / Al / 1.00
	0.00 / DF / 0.10
Combination-Endogenous	0.01 / P / 1.00
	0.00 / Zr / 0.001
	0.00 / Y / 0.001
	0.00 / DF / 0.99
Silicon-rich	0.50 / Si / 0.90
	0.00 / Zr / 0.001
	0.00 / DF / 0.50
Iron-rich	0.50 / Fe / 0.90
	0.00 / DF / 0.50
Titanium-rich	0.50 / Ti / 0.90
	0.00 / DF / 0.50
Other Aluminum Silicate	0.10 / Al / 0.50
	0.10 / Si / 0.50
	0.00 / DF / 0.70
No X-rays	0.00 / DF / 0.001
Aluminum-rich	0.50 / Al / 0.90
	0.00 / DF / 0.50
Miscellaneous	No requirements. All remaining particles are placed in this class

*DF refers to the fractional X-ray contribution of all remaining elements not previously specified in a class.

Table 2 shows the results of the analysis of the 75 lungs. The mean concentration of all exogenous particles was 613 million per gram of dry tissue and the median value was 417 million particles per gram. Churg and Wiggs (21) reported a mean value of 525 million and 261

Table 2. Summary of Exogenous Particle Data from 75 Urban Lungs. Note: Total Exogenous results are in particles per gram dry tissue and the other categories are in percent total exogenous particles (104).

Particle Type	Mean	Std. Dev.	Min.	Max.	Med.
Total Exogenous ($X\ 10^6$/g dry lung)	613	601	107	3127	417
% Silica	19.8	7.0	8.9	40.8	19.3
% Aluminum Silicate	36.0	10.7	13.9	60.8	36.0
% Magnesium Silicate	2.3	2.8	0.0	17.1	1.4
% Iron Oxide-like	7.4	5.9	0.7	34.4	5.9
% Rutile-like	8.4	7.7	0.6	38.6	5.7
% Alumina-like	0.7	1.2	0.0	8.7	0.4
% Exogenous-Endogenous Combination	7.5	7.3	0.6	35.6	4.7
% Silicon-rich	3.1	2.1	0.7	12.9	2.6
% Iron-rich	4.1	1.9	0.9	11.0	3.8
% Titanium-rich	2.7	1.0	0.4	5.9	2.6
% Other Aluminum Silicates	1.3	0.6	0.0	3.1	1.2
% Aluminum-rich	4.0	2.2	0.5	10.4	3.5
% Miscellaneous	2.7	1.2	0.6	7.7	2.5

million exogenous particles per gram of dry tissue in lungs from men with lung cancer and without lung cancer, respectively. The median value for particles per gram of dry tissue in the Abraham and Burnett study (2) can be calculated to be 710 million. One possible reason for the higher median value in this last study is that the sample population included individuals with occupational histories of exposure to dusts. Silicates represented the largest group in all these studies. The percentages for silica were 19.8% in the Stettler study, 10.4% and 16.1% for patients with and without lung cancer, respectively, in the Churg and Wiggs study, and 7.4% in the Abraham and Burnett study.

We have found that image analysis of fibers, using the automated particle counting system described above, is not very reliable because inorganic fibers often have very small diameters and are composed of elements having low atomic numbers, e.g., magnesium and silicon. As a result, parts of the fibers may not be visible on the backscattered electron image, and consequently on the binary image, which is taken from the backscatter image. The computer, therefore, "sees" a series of discontinuous particles, rather than a continuous fiber.

In the study described above, the number of fibers, although not counted, appeared low compared to the number of non-fibrous particles. This is in agreement with the study by Churg and Wiggs (21) in which it was found that the ratio of non-fibrous to fibrous particles was 30:1 for patients with lung cancer and 52:1 for patients that did not have lung cancer.

Bulk analysis.

Analysis of bulk samples has many advantages over analysis of individual particles, especially when quantitative information is needed. Two of these are speed and ability to get samples that are more representative of the whole lung, since a larger sample can often be used. However, information on particle species that are present in low concentration, but still of toxicologic importance, can be lost.

The simplest form of bulk analysis is gravimetric analysis, which can entail just isolating and weighing the particulate matter. This should be done, if possible, even if more sophisticated analytical methods are to be used. Lewis and Coughlin (70) determined the amount of acid-insoluble dust in lungs obtained at autopsy from 146 men. The mean amount was 1.2 mg./gram tissue (wet weight) and 1.7 grams total for both lungs.

Discussion of the types of bulk quantitative chemical techniques available can be classified, according to the type of information they afford, into elemental and molecular.

Elemental. The main advantage of elemental analysis is the large number of techniques available with a range of sensitivities for various elements. The main disadvantage is the limited nature of the information in certain cases. For instance, equal amounts of silicon in a particulate sample can be of different toxicologic significance depending on the nature of the siliceous material, e.g., silica versus aluminum silicate. The types of bulk elemental analysis that have been used to analyze particulate material from lung tissue include wet chemical techniques, atomic absorption and emission spectroscopy, X-ray emission spectroscopy, and neutron activation analysis. With the exception of wet chemical techniques these methods have mostly been used in analyzing for metals in lung tissue. In addition, in most of these studies, analysis was done on lung samples without isolating the particulate matter. The data, therefore, probably include information from non-particulate as well as particulate material.

King, et al., reviewed several gravimetric, titrimetric, and colorimetric techniques for the analysis of silicon in biological material and concluded that the colorimetric was the best procedure (60). Standard analytical chemistry books can be consulted for procedures to do similar analyses for other elements. However, easier, more sensitive instrumental techniques have largely replaced the wet chemical techniques.

Atomic absorption spectroscopy (AAS) takes advantage of the fact that a free atom in an excited state will absorb light of a wavelength that is characteristic of that atom (101).

In practice, a sample is heated to a high temperature using a flame or electrically heated graphite furnace and irradiated with light of the appropriate wavelength for the element of interest. The amount of that element present is then ascertained by determining the amount of light absorbed by the sample. Flame AAS has been used to measure copper, iron, magnesium, zinc, chromium, and manganese in a series of

coal miners' lungs (24,58,105). Cadmium and lead (112) were measured in normal human lung samples using flameless AAS.

Free atoms in an excited state will also emit light of wavelength that is characteristic of those atoms. In atomic emission spectroscopy (AES), as in AAS, the atoms in a sample are raised to a higher energy level, e.g., by heating, but light of characteristic wavelength emitted from the sample is measured to determine the amount of that element present. Energizing of the sample can be accomplished in several ways. A flame can be used, as in AAS, but more commonly the sample is energized electrically using a continuous DC arc of 1-30 amps and 10-100 V, an AC arc, a high voltage spark (15-40 kV), an inert gas plasma, or a laser beam (75). Arc source AES was used to measure metals in the series of coal miners' lungs mentioned above (23,24,58,105) and in a series of lungs from individuals without an occupational history of dust exposure (109,110).

Increasing use is being made, in certain areas of analytical chemistry, of plasma emission spectroscopic techniques in which the samples are excited in an inert gas plasma, activated by a high-frequency magnetic field, a direct current, or microwave radiation (32,119,121). These techniques are termed inductively-coupled plasma (ICP-AES), direct current plasma (DCP-AES), and microwave-induced plasma (MIP-AES), respectively. Even though plasma emission spectroscopy has been used fairly extensively for analysis of similar samples, such as environmental particulate samples, it has had limited use in the analysis of particulate samples from lung tissue. In one study, using ICP-AES, Vallyathan found a larger amount of aluminum and silicon in smokers' lungs than in nonsmokers' in a population of individuals without histories of work in dusty environments (111).

In addition to emitted light, a sample energized using a DC arc, as described above, may also emit ions which can be identified based on their mass/charge ratio, i.e., using mass spectrometry. Using spark source mass spectrometry (SSMS) 100 lungs from normal individuals have been analyzed for twenty-one elements (16).

As mentioned earlier, X-rays can be generated from a sample by bombarding it with charged particles or electromagnetic radiation. The energies (and wavelengths) of these X-rays are characteristic of the elements present in that sample. These X-ray emission techniques are categorized according to the type of incident beam used. X-ray fluorescence (XRF) uses X-rays, generated by bombarding a metal target with an electron beam in a vacuum, to elicit X-rays from a sample. Accelerated protons will also produce characteristic X-rays from a sample. This technique is known as proton-induced X-ray emission analysis (PIXE).

XRF has been used to semi-quantitatively analyze thin slices of freeze-dried lung tissue. This technique allows correlation of elemental data with gross morphology (6,26). It was shown, for instance, that nodular silicotic lesions gave higher X-ray counts for silicon than normal areas (6). PIXE has been used for analysis of lung tissue (9) and lavage fluid (73,74).

Analysis of X-rays generated by an electron beam has been used mostly as a microprobe technique to analyze individual particles and was discussed in the previous section on particle-by-particle analysis. Funahashi, et al. (34), and Siegesmund, et al. (99,100), however, used this technology for a bulk-type analysis of particles *in situ*. A 1 mm^2 area of lung tissue was irradiated with an electron beam and the X-rays produced were analyzed. The size of the characteristic X-ray peak for an element of interest was used as a measure of the amount of that element present. The size of the sulfur peak was also determined as a measure of tissue mass. The Si/S ratio for normal lung was 0-0.2, whereas for silicotic lungs, it was greater than 0.3. The Si/S ratio also correlated well with septal thickening, determined morphometrically.

With neutron activation analysis (NAA), radioactivity induced in a sample by bombardment with accelerated neutrons is examined (101). Similarly to the X-rays produced in XRF and PIXE, the energy of the radiation produced by an isotope is characteristic of that element. By measuring the amount of characteristic radiation produced, one can determine the amount of an element present in the sample. NAA has been used to perform elemental analysis of lung samples from a population of coal miners (33), and from two series of lung samples from individuals without occupational histories of exposure to dusty environments (112,120).

Some of the considerations when choosing an elemental analytical technique include sensitivity, ease of sample preparation, instrument availability, and ability to perform simultaneous multi-element analysis (SMEA). No one of the techniques discussed is best in terms of all of these considerations. AAS gives good sensitivity, but samples generally need to be solubilized. In addition, although instruments capable of SMEA have been designed (86), none are commercially available (48). XRF analysis requires less sample preparation but is less sensitive for elements of lower atomic number (71). PIXE similarly is less sensitive for low atomic number elements. NAA has good sensitivity and can require little sample preparation but requires a neutron beam source. The "cool down" period prior to analysis can also be lengthy -- up to twenty days (112). ICP offers good sensitivity, comparable with that of AAS, and SMEA capability. However, as with AAS, samples generally need to be in solution, thus lengthening the preparation time and increasing the chance of sample contamination. The individual needs of an investigation therefore, determine which technique should be used. Comparisons of the various elemental analytical techniques, along with detection limits, can be found elsewhere (10,11,12,13,14,31,36,43,50,52, 53,57,81,87,96,114).

Molecular. As previously mentioned, information on just the elemental composition of

a sample can be of limited value, especially when dealing with mineral particles. For that reason, techniques have been employed for particle analysis that give more specific molecular information on a sample. Techniques used for this type of analysis of bulk samples include gravimetric and colorimetric analysis, infra-red spectroscopy, and X-ray diffraction spectroscopy.

Several gravimetric and colorimetric procedures are available for measurement of silica in lung tissue. These generally involve dissolving the non-silica silicate minerals and weighing the isolated silica or dissolving the isolated silica with, for instance, hydrofluoric acid, and measuring the silicon present by complexing it with molybdenum (24,58,80,105, 106).

Most molecules have asymmetric chemical bonds which will absorb electro-magnetic radiation, usually in the infrared region, as a consequence of the constant rotational and vibrational motion of these bonds. This absorption is the basis of infra-red spectroscopy (IRS) which can be used to identify molecular species as well as to measure the amount of the molecular species present in a sample. Particulate samples are generally analyzed as a powder mixed with a matrix material such as KBr or a heavy hydrocarbon oil. IRS and X-ray diffraction (next section) give different types of information on the molecular composition of a sample. Therefore, use of both techniques can allow more complete characterization of a sample than either technique alone. Hayashi (45,46) analyzed particulate matter from lung tissue quantitatively for silica, using IR spectroscopy and found the results to be comparable to results of quantitative X-ray diffraction of the same material.

It is often desirable to have information on the different crystalline polymorphs of a particular molecular species present in a sample. The importance of this is seen when the toxicities of the polymorphs of silica (SiO_2) are compared. Tridymite was found to be more fibrogenic than cristobalite, which was in turn more fibrogenic than quartz, even though all have the same chemical formula (59). X-ray diffraction is generally used when this type of analytical data is needed on a bulk particle sample. In addition to qualitative information, quantitative information is also obtainable with XRD. The disadvantage of XRD is that interferences can occur between some crystalline substances. Many investigators of coal workers' pneumoconiosis and silicosis have used XRD to measure mineral species, most often quartz, in lung tissue (45,46,61,83,84,95,113).

Some of the major considerations when using quantitative XRD are 1) particle size, 2) the degree to which the sample absorbs the incident and diffracted X-ray beam, and 3) differences in crystalline structure between samples of the same mineral species from different sources, such as those caused by impurities in the crystal lattice. The effect of particle size is lessened by the fact that particles that reach the alveolar regions of the lung are generally less than five micrometers in diameter. The effect can also be diminished by measuring the integrated area rather than the height of the peak (38). Correction for the absorption of the X-ray beam by the sample can be accomplished by using an internal standard (62) or by actually measuring the absorption and using a correction factor (18,19,25). However, if the sample is relatively thin, the small amount of absorption can be neglected (4). Correction for differences in crystalline structure between samples from different sources is usually difficult to make and must be mentioned as a possible source of error in the analyses.

An example of how XRD can be used for analysis of particulate matter is given below. Some of the early results of this study have been reported (77).

Lung tissue was obtained from 50 randomly selected autopsy cases from the Duke University and Durham, North Carolina, Veterans Administration Medical Center autopsy services. In every case, the tissue selected was excess tissue not needed for diagnostic studies.

Two methods of particle isolation were used as described below.

Method 1: A known amount of beryl was added as an internal standard to weighed aliquots of tissue and the samples dried to determine dry weight. Beryl was used as the internal standard because it gives strong peaks in the area of the quartz peak used in the analysis, it is resistant to the ashing procedure used, and it was not present in the samples. The samples were ashed at 450-500°C in a muffle furnace, and the ash was suspended in 2.4 N HCl, to dissolve endogenous lung salts. The suspension was filtered onto a pre-weighed 0.4 μm pore size silver filter and the weight of the particulate matter ("dust") determined gravimetrically.

Method 2: Samples of lung tissue were weighed, dried, reweighed as above, and digested in concentrated HNO_3 for 30 to 45 minutes. The particulate material was filtered onto a pre-weighed 0.4 μm pore size Nuclepore polycarbonate filter and reweighed to determine dust weight.

The average dust-to-dry weight ratios were calculated along with the total amount of dust in both lungs, which was calculated as:

Total dust = (total wet lung weight)
X (dust weight/wet tissue weight).

Pratt has shown that total silica correlates better with the severity of disease in silicotic patients than does the dust/dry weight ratio (93).

Table 3 shows the results of the gravimetric analysis of the dust from these Durham area lung samples. The mean and median values for dust/dry and total dust were similar for Method 1 and Method 2.

Qualitative XRD analysis was done on 38 of the 50 cases using a Norelco/Philips X-ray diffractometer equipped with a copper target tube, a graphite crystal monochromator, a

scintillation counter, and a count rate computer. Identification of substances in the samples was made by comparing the d-spacings obtained with d-spacings for standard substances listed in the ASTM or JCPDS powder diffraction files. All 38 cases contained detectable amounts of quartz and talc. In addition, kaolinite was detected in all 17 cases prepared by Method 2. As mentioned earlier, kaolinite peaks are reduced or eliminated in samples heated to over 380°C for a prolonged period. It is probable, then, that kaolinite was also present in some or all of the lungs prepared by Method 1, as well. Analysis of all but two of the cases gave a peak at about 9.9Å . This probably represents a mica.

Quantitative XRD was done using the equipment described above. Calibration standards were made from mineral samples which were analyzed either untreated or after high temperature ashing treatment or treatment with nitric acid, as done in Methods 1 and 2. Results of XRD analysis from treated standards were compared with those from untreated samples to determine the effect of the particle isolation procedure. The treatments used did not cause any significant changes in the standard minerals.

Duplicate aliquots of tissue from four cases were treated, one with Method 1 and the other with Method 2, and the amount of quartz determined by quantitative XRD. The results did not indicate a difference in the two techniques. Results from analyses of tissue prepared using both methods have therefore been pooled.

Table 3. Results of gravimetric analysis of particulate matter isolated from lungs collected at Duke University and Durham V.A. Medical Centers.

	METHOD 1		METHOD 2	
	DUST/DRY (mg/gm)	TOTAL DUST (gms)	DUST/DRY (mg/gm)	TOTAL DUST (gms)
NUMBER	22	22	27	23
MEAN	1.94	0.476	2.39	0.580
STANDARD DEVIATION	1.45	0.466	1.98	0.493
MEDIAN	1.82	0.325	1.74	0.409
MINIMUM	0.320	0.0727	0.408	0.0705
MAXIMUM	6.43	1.84	9.53	1.97

Table 4. Results of quantitative XRD analysis of particulate matter from randomly selected lungs from Duke University and Durham V.A. Medical Centers.

	QUARTZ/DRY (mg./gm.)	TOTAL QUARTZ (grams)
NUMBER	38	34
MEAN	0.266	0.0650
STANDARD DEVIATION	0.303	0.0729
MEDIAN	0.178	0.0444
MINIMUM	0.017	0.00318
MAXIMUM	1.45	0.339

Table 5. Results of gravimetric and quantitative XRD analysis of particulate matter from randomly selected lungs collected in Cincinnati.

	DUST/DRY (mg./gm.)	QUARTZ/DRY (mg./gm.)
NUMBER	18	8
MEAN	6.45	0.615
STANDARD DEVIATION	4.48	0.708
MEDIAN	5.08	0.365
MINIMUM	1.82	0.116
MAXIMUM	18.3	2.38

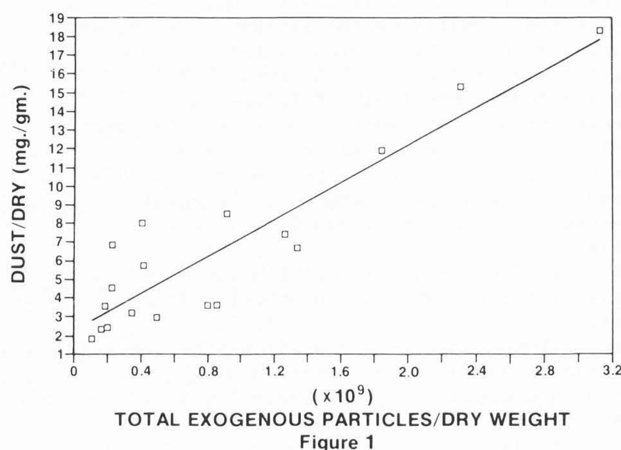

Figure 1

Plot of dust weight/dry weight (determined gravimetrically) vs. total number of exogenous particles (determined by automated SEM/EDX). Pearson's correlation coefficient = 0.91; $p < 0.001$.

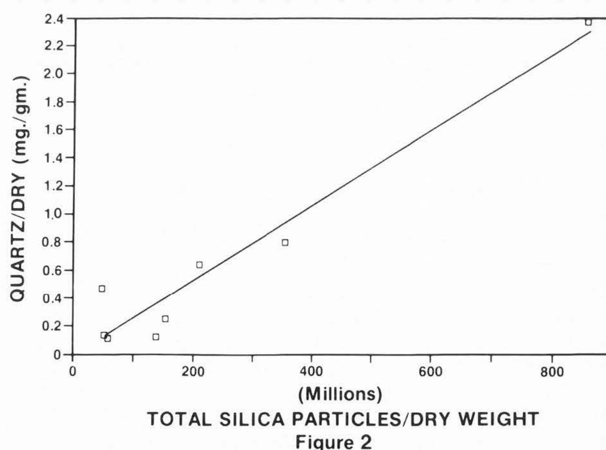

Figure 2

Plot of quartz weight/dry weight (determined by quantitative XRD) vs. total number of silica particles (determined by automated SEM/EDX). Pearson's correlation coefficient = 0.97; $p < 0.001$.

Table 4 shows the results of the quantitative XRD analysis of quartz. The mean values for quartz/dry and total quartz were 0.226 mg./gm. and 0.0650 grams, respectively. The range of values, however, are rather large, the maximum values being two orders of magnitude larger than the minimum values.

In addition to the 50 lungs described above, eighteen of the Cincinnati, Ohio, urban lungs analyzed by Stettler, et.al. using automated SEM/EDX analysis (103,104) were prepared for gravimetric analysis, and eight of these were prepared for X-ray diffraction using the nitric acid digestion method (Method 2). Table 5 shows the results of the gravimetric and quantitative XRD analysis of quartz in the series of lungs collected in Cincinnati and previously analyzed by automated SEM/EDX. The mean of the dust/dry values in this series of lungs is about three times higher than that for the 17 lungs from Duke which were also prepared by Method 2. Similarly, the mean quartz/dry for the Cincinnati lungs was about three times higher than that for the Duke lungs. Figure 1 is a plot of the number of exogenous particles per gram dry weight (determined by automated SEM/EDX) versus dust/dry weight (determined gravimetrically) for the 18 Cincinnati cases. Results from the two techniques correlate very well ($r=0.91$; $p < 0.001$). Figure 2 is a plot of total silica particles per gram dry weight (determined by automated SEM/EDX) versus quartz/dry (determined by quantitative XRD). A good correlation was seen here, too ($r=0.97$; $p < 0.001$).

Discussion

The purposes of this paper have been to review the techniques that have been used to quantitatively analyze particulate matter from lung tissue as well as some techniques that have been used to prepare the tissue and particulate material prior to analysis. Tissue preparation can be for *in situ* analysis or for analysis of the particles isolated from the tissue. Destruction of the tissue can be accomplished by physical techniques, such as ashing, or chemically, such as with acids, bases, oxidizing agents, or enzymes. Separation of the particles from the decomposed tissue, if necessary, can be accomplished either by centrifugation or filtration. The techniques that have been used for bulk analysis of the particles include wet chemical techniques, atomic emission and absorption, infra-red spectroscopy, and X-ray diffraction. The analytical techniques most often used for identification when doing particle-by-particle quantitation involve use of electron microscopic techniques, e.g., EDX. Although EDX analysis, counting, and sizing are most often done manually, computer-controlled analytical systems are available which can perform all of these functions. It is desirable to use multiple techniques to analyze a sample. This idea was illustrated in the study discussed earlier in which lung samples were analyzed by automated SEM/EDX and XRD.

Newer techniques, which have been used extensively in related fields, such as environmental analysis, but have been used infrequently for quantitative analysis of pulmonary particulate matter, have promising potential for future applications. Inductively-coupled plasma emission spectroscopy (ICP-AES) offers excellent sensitivity over a wide range of elements and concentrations, as well as simultaneous multi-element analytical capability. Laser Raman spectroscopy, like X-ray diffraction, has the capability of distinguishing the polymorphs of silica (quartz, cristobalite, and tridymite) (35). Raman microprobe analysis, which gives molecular information on small volumes of material (30), has been used to study inclusions in post-mortem lung tissue (17). Ion microprobe analysis can yield elemental or molecular data on particulate samples and has been used for analysis of particles from lung tissue (2,72). Laser microprobe mass analysis (LAMMA) has been used to obtain molecular data on the organic and inorganic components in particles from refinery emissions (28). The molecular optics laser examiner (MOLE) uses a laser to generate Raman spectra from small volumes of material, and hence, has utility as a technique for gathering molecular chemical data on particles (5). MOLE has been used to identify particles in tissue sections (1). Electron energy-loss spectroscopy (EELS) can be used to determine the elemental composition of specimens by measuring the energy lost from an incident beam of electrons when it collides inelastically with inner orbit electrons of atoms in the specimen (29). A review of the use of EELS for analysis of biological samples has recently been presented in this journal (56).

In addition to the quantitation of particles from lung tissue from pneumoconiosis patients, additional work is needed to provide similar data for individuals without history of exposure other than normal environmental exposure. Baseline data for "normal" lung particulate burdens is needed before any meaningful quantitative conclusions can be reached concerning the effects of specific particles on lung pathology. In addition, this "normal" particulate burden might affect the health of the lungs of these individuals. Several studies have looked at the association between pulmonary particle levels and pathology. Associations that have been reported include emphysema (92) and lung cancer (21), as well as cerebrovascular hemorrhage or thrombus (70) and liver disorders (105). (Note: The authors did not analyze brain or liver tissues in these studies.) In the X-ray diffraction study discussed earlier, medical and demographic data are available for analysis. In addition, quantitative assessment of emphysema is being done by the method of Weibel (118) and quantitative assessment of chronic bronchitis by the method of Jamal, et al. (54). Histologic sections will also be available for examination of the smaller airways. Statistical analysis will then be done to determine if correlations exist between the analytical and pathological data. Ambient air is the most likely source of particulate matter in the lungs of individuals

without histories of occupational exposure. Airborne particles include soil particles as well as particles produced by combustion processes. Tobacco smoke has also been shown to be a source of inorganic particles (44,47,49,65,78,98).

One important aspect of the topic of analysis of pulmonary particulate burden is inter-laboratory variability. Given the different techniques used by different laboratories, a program to compare the results of analysis of standard samples by different laboratories would be of great value.

Conclusions

The field of pulmonary particle analysis will continue to be an active area of research. Continued application of commonly used techniques, such as SEM/EDX and XRD, and more focused applications of the newer techniques, such as ICP, LAMMA, and Raman microprobe analysis, will lead to a greater understanding of the relationships between lung particulate burden and pathology.

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Discussion with Reviewers

James Millette: In the preparation of samples for automated SEM/EDX particle analysis, does the author have any information of the shapes of particles found, platelike, acicular, equant, etc.?

Frank Green: It is possible to calculate a variety of shape factors, aerodynamic diameter, etc. based upon image analysis of particles. Do you consider this information to be useful and, if so, which specific parameters?

Authors: The program being used for the automated analysis does measure four diameters for the particle. An aspect ratio is calculated for each particle as the ratio of the largest to smallest of these four measured diameters. The two dimensional shapes of the particles may be implied from this aspect ratio. However, no information concerning the particle's third dimension, height is determined. Hence,

platelike particles cannot be distinguished from non-platelike particles. One may assume a single, three dimensional shape for all particles, e.g., spherical, based on the particle's circular area equivalent diameter, and then calculate individual particle volumes and masses using assumed densities. Particle mass distributions and aerodynamic diameters may then be determined from the mass data. However, because of the gross assumptions used in determining mass, we feel that none of this data is very useful.

Joseph Krewer: Do the authors have any data on the breakup of particles during the various digestions and how this would affect the reporting of particle sizing?
Authors: We have collected no data on this subject. As mentioned in the text, other investigators have looked at the effects of tissue preparation on fiber breakage.

Frank Green: It is possible to make an estimate of mass on an individual particle based on particle dimensions, X-ray chemistry, and density information. Have you done this, and, if so, how does this correlate with gravimetric estimates of lung dust?
Authors: Because of the gross estimations inherent in calculating individual particle masses based on two dimensional data, we have not carried out a systematic study comparing individual particle analysis data with bulk data. However, as shown in Figures 1 and 2, there is a good correlation between total exogenous particle concentration determined by SEM-EDX and the gravimetrically determined dust/dry weight ratio as well as total SEM-EDX silica concentration versus quartz/dry weight as determined by XRD.

Frank Green: It has been mine and other's experience that low temperature plasma ashing frequently fails to break down particle aggregates. How do you deal with the problem and, secondly, have you tried a combination of lung digestion, followed with low temperature plasma ashing?
Authors: We certainly agree that low temperature plasma ashing frequently fails to break down particle aggregates. Unfortunately, particle aggregation is a problem we have not completely solved. We have found that prolonged (1-2 hours) mild sonication using a ultrasonic bath is sometimes useful in breaking down particle aggregates. However, even extra sonication is not always successful. We have not tried a digestion-LTA combination.

Frank Green: Do you have any information regarding changes in lung particulates with age, sex, smoking, status, etc.?
Authors: At this time we have no more data to report beyond what is included in this paper. We are currently working on that question and hope to have something to report in the near future.

Jerrold L. Abraham: What is the effect of using a 0.1 micrometer pore filter and only 1000 X magnification on the minimum particle size analyzed? Should the geometric mean or arithmetic mean be used? Is the data most likely log normal?
Authors: The ability of the image analyzer to find particles is directly related to the SEM magnification and to the point density used to scan the field. The combination of magnification and point density used in our analysis is such that all particles greater than 0.2 μm in diameter will be found by the image analyzer. Particles smaller than 0.2 μm may be missed. Because of the filter pore size used, particles smaller than 0.1 μm may pass through the filter.

In reporting particle size data, the geometric median diameters and geometric standard deviations should be given since these two parameters define the log-normal distribution. It has been our experience that the particle size data from most of the lungs we have analyzed fit the log normal distribution.

Jerrold L. Abraham: If HTA and LTA cause hemosiderin transformation into iron oxides, how is this detected and controlled?
Authors: We are unaware of any effective method for removing endogenous iron compounds without removing exogenous iron as well or for determining the relative contributions of exogenous and endogenous iron to the total iron oxide content of ashed samples. Therefore, classification of all iron particles as exogenous or endogenous is arbitrary.

Jerrold L. Abraham: Why was a 0.4 micrometer filter used? Is there a question of loss of more particles using this filter size?
Authors: A 0.4 μm filter was used for XRD analysis to facilitate filtration. Originally, a 0.1 μm filter was tried, but the filtration rate was too low to make use of this type filter reasonable. There probably was some particle loss with the 0.4 μm filter, since size analysis of particles from lung tissue usually indicates many particles less than 0.4 μm. The choice of this pore size filter was a compromise between filtration rate (and ability to analyze more samples) and trying to retain as many particles as possible on the filter.

A.M. Langer: What is the sensitivity of Auger spectroscopy?
Authors: The sensitivity is, or course, element-dependent as well as dependent on the conditions of analysis. The relative sensitivity factors for a number of elements can be found in Reference 115. In the study reported in References 73 and 74, scanning Auger spectroscopy was used to analyze particles obtained by pulmonary lavage. The results for most of the elements studied were in the range of parts per thousand. This range is considered to be the "rule-of-thumb" sensitivity for Auger spectroscopy.

J.A. Small: How many particles were actually counted to arrive at the various concentrations which are given in millions of particles per dry weight?
Authors: A total of at least 1000 particles in a minimum of 20 fields of view at 1000X are analyzed in the standard SEM-EDXA-IA procedure. Thus, the overall particle concentration for each lung is based on a minimum 1000. The concentrations for the various categories are based upon their actual fraction of the total particles counted.

J.A. Small: In Table 2, many of the standard deviations are large. What do the standard deviations refer to? Are they in reference to the total particle counts for the class, the uncertainties in the class assignments, chemical analysis, etc.?
Authors: The sample standard deviations in Table 2 are calculated using the percentage values obtained for each class of particle for each case; the "n" value, therefore, is 75 for each calculation. The standard deviations then refer to the spread of the data within each class for the entire population. We agree that the spread of the data is large but this is not unexpected since we are dealing with 75 different subjects.

J.A. Small: How many additional particle analyses would it take to make a significant decrease in the standard deviations? Could the standard deviations be decreased by using fewer groupings?
Authors: Given the large range of values in this population, we do not feel that performing more particle analyses would significantly decrease the standard deviations, although the error associated with these sample standard deviations would be decreased. It would be difficult to determine the effect of reducing the number of groups. We feel, however, that such a change would make the categories less meaningful.

J.A. Small: What improvement, if any, in the class groupings could be made by doing a cluster analysis on the data?
Authors: The major definitions given in Table 1 were formulated after inspection of the raw data from several lungs with the idea being to minimize the overall number of categories. Many of the particles fell naturally into groups. For instance, spectra for many particles contained only a silicon peak. These particles were then categorized as "silica". Many others contained aluminum and silicon and various other cations and were categorized as "aluminum silicates". In this same way, most of the other categories were fairly obvious. The actual chemical definitions for the classes are unavoidably somewhat arbitrary, but in our view represent the best compromise for including all particles in the fewest groups. In a sense, then, the philosophy used to set up the classes is similar to cluster analysis. We do not believe that much would be gained by performing a formal cluster analysis. However, it should be noted that all of the raw data collected by the image analyzer is stored on disks. Cluster analysis could therefore be done and the data reprocessed using new chemistry files at any time.