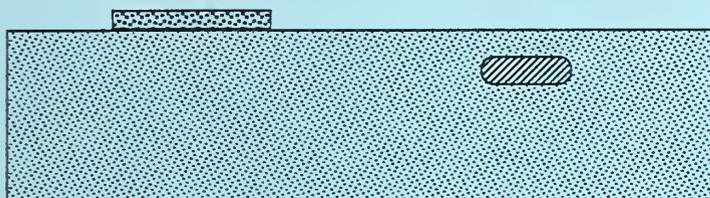


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Handbook for Evaluation of TEM Sample Preparation of Particles on Membrane Filters: Version 1.0



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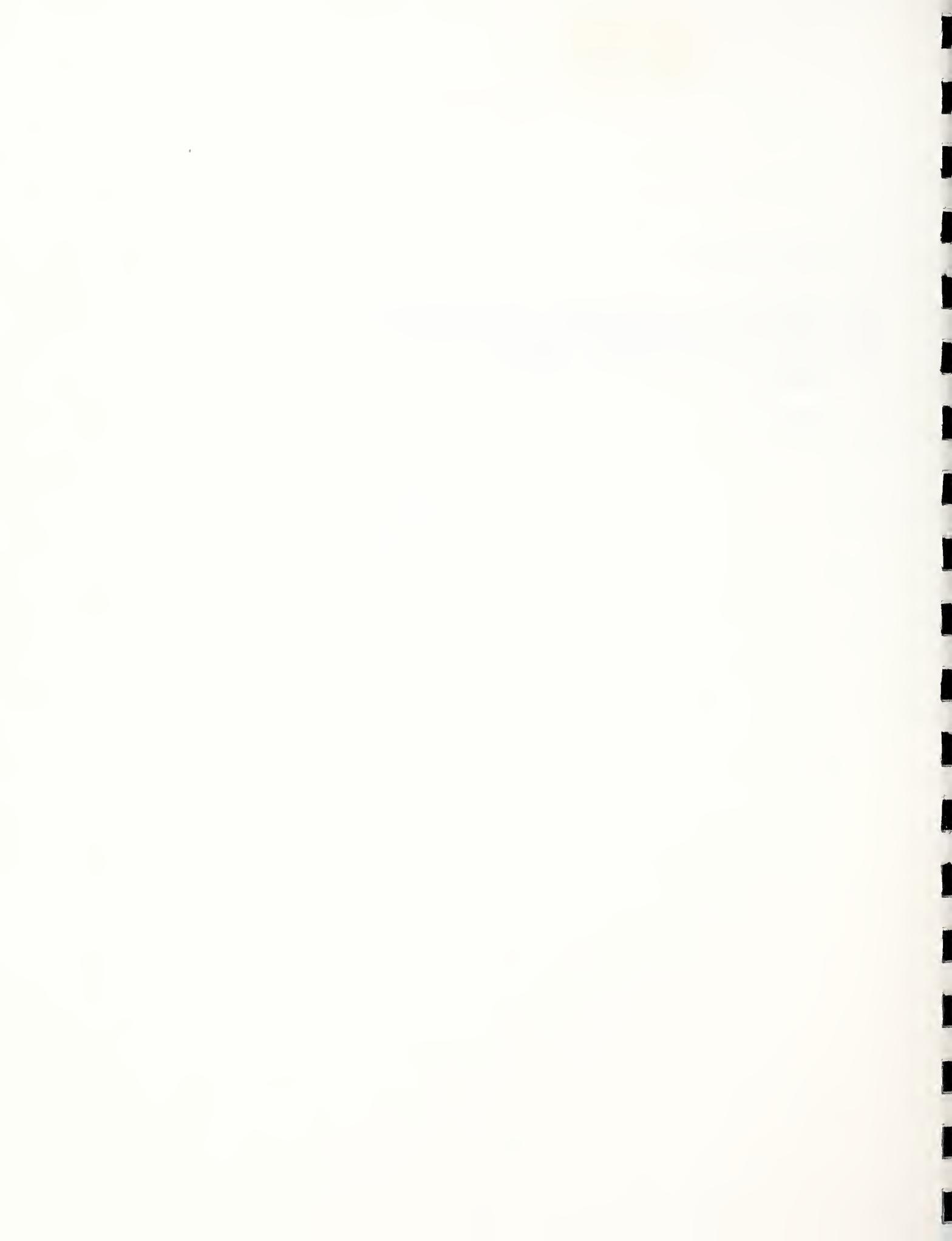
**U.S. DEPARTMENT OF COMMERCE
Ronald H. Brown, Secretary**

**NATIONAL INSTITUTE OF STANDARDS
AND TECHNOLOGY
Arati Prabhakar, Director**



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I. Introduction

The concentration and identity of particles in air or water are commonly determined by filtering the fluid and analyzing the collected particles. A necessary step for the analysis of such fluid-collected particles by transmission electron microscopy (TEM) is the preparation of a thin, carbon film containing the particles. The carbon film is obtained by preparing a replica of the filter onto which the particles are collected. An ideal carbon replica is thin, clear and coherent, so that unhindered detection and analysis of any particles are possible. Evaluation of replicas produced at the National Institute of Standards and Technology (NIST) and by laboratories participating in round robin studies, has shown that a variety of problems and artifacts can occur on replica preparations¹. The problems and artifacts have the potential of modifying the number of particles observed by an analyst leading to an incorrect determination of the particle concentration. The main purposes of this handbook are 1) to describe and define the problems and artifacts found on filter replicas and 2) to provide a procedure for analysis of replica preparations by light microscopy and TEM. The description and classification of problems and artifacts in the handbook provide a nomenclature system for interlaboratory comparisons of the evaluation of replica preparations.

This handbook can be used for the general topic of evaluation of sample preparation of particles. However, specific examples are given using asbestos as the particles to be identified and quantified. The handbook is intended to be used with the methodology written by the Environmental Protection Agency (EPA) for identification and analysis of asbestos in air-collected samples². This methodology will be termed the AHERA^{*} method in this handbook. A general description of sample preparation problems and limited guidelines for evaluation of filter replicas are given in the AHERA method. Definitions for some terms related to asbestos analysis and asbestos particles (e.g., structure, fiber, bundle, cluster, matrix) are given in Appendix A of this handbook.

Several topics related to sample preparation are not addressed in this handbook. For example, the methods for filter preparation are not discussed; these procedures are discussed in the AHERA method and in other references³⁻⁴. Additionally, the causes of the problems and artifacts are not addressed in detail in this handbook. Lastly, the magnitude of the effect of problems and artifacts on the particle counts has not been quantified and is not discussed.

Problems and artifacts found on polycarbonate (PC) filters and mixed-cellulose ester (MCE) filters are described in section II of the handbook. Area determinations for selected problems and artifacts are given in section III and a replica analysis procedure is given in section IV.

^{*}This methodology was written in response to the Asbestos Hazard Emergency Response Act enacted by Congress in 1986. This act mandated that all schools in the United States be evaluated for the presence of asbestos.

II. Problems and artifacts on replicas of PC and MCE filters

This section provides descriptions and examples of the types of problems and artifacts that may be present on the replicas of PC and MCE filters. The subsections give a listing of the types of problems and artifacts that relate to the carbon replica, filter residue or particles. Most of the problems and artifacts occur on both PC and MCE filters; those features unique to only one filter type are indicated by a comment in parentheses.

For reference, replicas of PC and MCE filters that are acceptable for analysis of asbestos by TEM are shown in Figures 1a, b. The replica of PC filter, Figure 1a, shows an amorphous carbon film that contains circular holes that are approximately 0.4 μm in diameter. These holes correspond to the tubes in the PC filter that allow for the filtering of particles from gases or liquids. MCE filters, in contrast, consist of a mesh of MCE fibers. The process of preparing these filters leads to a replica that consists of an amorphous carbon film as shown in Figure 1b.

This section refers to the possible effects of many of the sample preparation problems and artifacts. The possible effects of these features include:

- 1) obscuring particles, thereby affecting the visibility and/or analysis of particles;
- 2) mimicking particles of interest, thereby slowing an analysis;
- 3) providing too much detail, thereby slowing analyses or camouflaging particles;
- 4) causing a physical loss or gain of particles;
- 5) biasing the grid squares chosen for analysis, thereby affecting the statistical validity of the analyses and
- 6) modifying the particle count.

Possible effects of the features on analyses are mentioned further in the text below, are summarized in Table 1 and are discussed in more detail in section III.

Features relating to the carbon replica

Ideally, the carbon replica of PC or MCE filters is coherent and is sufficiently thin to allow for easy viewing and analysis of particles. Some types of problems that relate to the carbon replica are as follows.

1. Overlapping replica

This feature consists of two or more layers of carbon film as shown in Figure 2. Overlapping replica occurs when pieces of a replica have broken off the main replica and are redeposited on a grid square or when carbon replica is folded onto itself.

Overlapping replica may result in a physical gain in the number of particles counted due to the increased replica area. It is commonly not clear if a particle observed on a doubled area is on the original or overlapping replica. Therefore the derived particle concentration (particles/volume of air) may be larger than the actual particle concentration. Additionally, overlapping replica may obscure particles.

2. Splitting of a replica

A. Irregular splitting of a replica:

Irregular splitting occurs in a nonsystematic fashion (as opposed to parallel splits discussed as the next item) and occurs on both PC and MCE filter replicas. An example of a replica that is split along the length of a chrysotile fiber is shown in Figure 3a. An example of a replica that is split across a fiber arrangement is shown in Figure 3b.

B. Parallel splitting of a replica

PC and MCE replicas may show a series of parallel splits that occur as one split per grid square. In addition, MCE replicas may show a series of parallel splits within a grid square. The parallel splits may occur over just a few grid squares or extend over an entire grid. An example of parallel splitting of an MCE replica within a grid square is shown in Figure 3c.

If parallel splits occur along the edge of a replica section, they may relate to the cutting of the original filter to form the filter section. Parallel splits throughout a filter section may result from the process of separating a collapsed filter section from a glass slide. To determine the cause of splitting, filter sections should be observed in an optical microscope before and after each step in the preparation process.

Irregular and parallel splitting of replicas may cause a loss of particles from a replica. The splitting may change an asbestos structure count if a fiber arrangement is separated as shown in Figure 3b. For this case, it is unclear if the fibers in the arrangement were originally touching and should therefore be counted as one cluster. A split replica may result in a significantly reduced area of the replica available for analysis. This can occur where the replica curls, thereby precluding analysis.

3. Holes in replica, missing replica

Many grids will show a loss of replica and in some cases, entire grid squares contain no replica. Holes in the replica and missing replica may affect an analysis by biasing the choice of grid squares analyzed and by reducing the number of particles observed.

4. Excessively thick carbon replica

A carbon coating of a filter that is greater than approximately 50 nm may affect an analysis by obscuring particles or by affecting the acquisition of diffraction patterns or chemical spectra. An excessively thick carbon film can be recognized in the optical microscope by a darkened replica that is partially to completely opaque.

5. Carbon flakes (PC filter replicas)

This feature refers to cylinders of amorphous carbon. The edges of the cylinders are linear as shown in Figure 4a. Carbon flakes result from carbon coating of the interior of the pores of PC filters as shown by the curved replica in Figure 4b. The carbon flakes may mimic asbestos fibers or provide excessive detail thereby slowing an analysis.

6. Convoluted replica (MCE filter replicas)

This feature refers to a replica that has a complex topography as shown in Figure 5. The topography results from carbon coating of an uncollapsed or partially collapsed filter section. Such replicas should not be used for counting asbestos.

7. Craters and holes (MCE filter replicas)

Craters and holes are topographic features that are approximately 1 to $>10\ \mu\text{m}$ in size (Figure 6). Craters and holes have been found to occur in round robin studies conducted by NIST only on MCE filters prepared using the NIOSH 7402 method⁵. The presence of sizeable holes in a replica may indicate a loss of particles or may indicate a change in the areal dimensions of the replica.

8. Replica textures (MCE filter replicas)

A variety of textures have been noted on replicas of MCE filters including:

Type 1 texture: circular features approximately $0.1\ \mu\text{m}$ in size

Type 2 texture: network of lace-like features

Type 3 texture: irregular, elongate features

Examples of these textures are given in Figures 7 a-c, respectively. Type 3 texture results from excessive ashing of a filter section. Type 2 and 3 textures may affect an analysis by mimicking asbestos and providing excessive detail thereby slowing an analysis.

Features related to filter residue

One goal of replica preparation for TEM analysis is to completely remove any filter material from a carbon coated section. This allows for optimum viewing of particles on the replica. Evidence that the complete dissolution has not occurred is given by the following features.

1. Clouded features

These features consist of an amorphous to granular material that can occur as a layer or as patches as shown in Figures 8a-c. The clouded layer can cover most of a grid square in a uniform layer, but more commonly has a wedge-shaped cross section. It can be found in the center of a grid square or around the grid bars. Patches of clouded material may occur throughout a grid square. Some patches are less uniform in contrast as shown in Figure 8c. By light microscopy, clouded features may be indicated by an iridescence of the replica. Clouded features may affect an analysis by obscuring particles or by biasing the grid square chosen for analysis if inhomogeneous in distribution.

2. Bubble-like mesh (or overlying mesh)

This feature consists of an amorphous film of material that contains circular open areas as shown in Figure 9 and Figure 10. It occurs more commonly on replicas of PC filters than on MCE filters. The mesh can be quite thin and barely noticeable or thick enough to preclude observation of the replica.

The bubble-like mesh can affect analyses by: 1) obscuring particles, 2) biasing the grid squares chosen for analysis, 3) mimicking fibers and slowing analyses (sections of the mesh where "bubbles" intersect can be close to linear, Figure 9) or 4) slowing analyses by providing excessive detail.

3. Highlighted, interconnected pores (PC filter replicas)

Highlighted pores are an opaque outlining of the holes in a PC replica. Interconnected pores is a term applied to opaque material that connects pores that are in close proximity. Examples of these features are shown in Figure 10. Interconnected pores can obscure particles.

Features related to particles

1. Displaced or missing particles

Displaced particles are recognized by holes or changes of texture in the replica that have the same shape as a nearby particle. Examples are shown in Figures 11a and b. The presence of displaced particles on a replica indicates the possibility that other particles may be missing from the replica. Missing particles can be recognized by holes or changes in texture in a replica that have shapes characteristic of the particles on the replica. An example of a missing particle is shown by a change in texture in an MCE replica, Figure 11c.

2. High particle loading

The AHERA method defines an overloading of particles to be greater than 25 percent coverage of a replica. Coverage of 10 percent of a replica by particles should be considered unacceptable and may cause a significant bias (see revised and expanded criteria, page 22). A high particle loading may obscure asbestos structures, provide excessive detail thereby slowing an analysis or bias the choice of grid squares analyzed.

Miscellaneous features

1. Round spots (MCE filter replicas)

Round spots of opaque to semiopaque material occur on some MCE replicas. Examples are shown in Figure 12.

2. Carbon tubes

This feature consists of a cylinder of carbon with irregularly shaped edges as shown in Figure 13. The aspect ratio (length:width ratio) is greater than that of carbon flakes. Carbon tubes can mimic asbestos fibers.

Table 1

A summary of the types of problems and artifacts found on filter replicas and their possible adverse effects on TEM analyses for asbestos is given in Table 1. The adverse effects are described in more detail in Section III.

Table 1. Problems or artifacts on replicas of filters and their possible adverse effects on analysis for asbestos by TEM

	Obscure asbestos structures	Provide excessive detail	Mimic fibers	Bias choice of grid squares (if nonrandom)	Physical loss or gain of particles	Modify structure counts
<i>Features related to the carbon replica</i>						
Overlapping replica	X			X	X	
Splitting of replica				X	X	X
Holes in replica, missing replica				X	X	
Thick carbon replica	X					
Carbon flakes (PC)		X	X	X		
Convoluted replica (MCE)	X	X				
Craters and holes (MCE)					X?	
Type 2, Type 3 texture (MCE)		X	X	X		

Table 1. continued

	Obscure asbestos structures	Provide excessive detail	Mimic fibers	Bias choice of grid squares (if nonrandom)	Physical loss or gain of particles	Modify structure counts
<i>Features related to filter residue</i>						
Clouded features	X			X		
Bubble-like mesh	X	X	X	X		
Interconnected pores (PC)	X			X		
<i>Features related to particles</i>						
Missing or displaced particles				X	X	
High density of particles	X	X		X		X
<i>Miscellaneous features</i>						
Round spots (MCE)	X			X		
Carbon tubes			X			

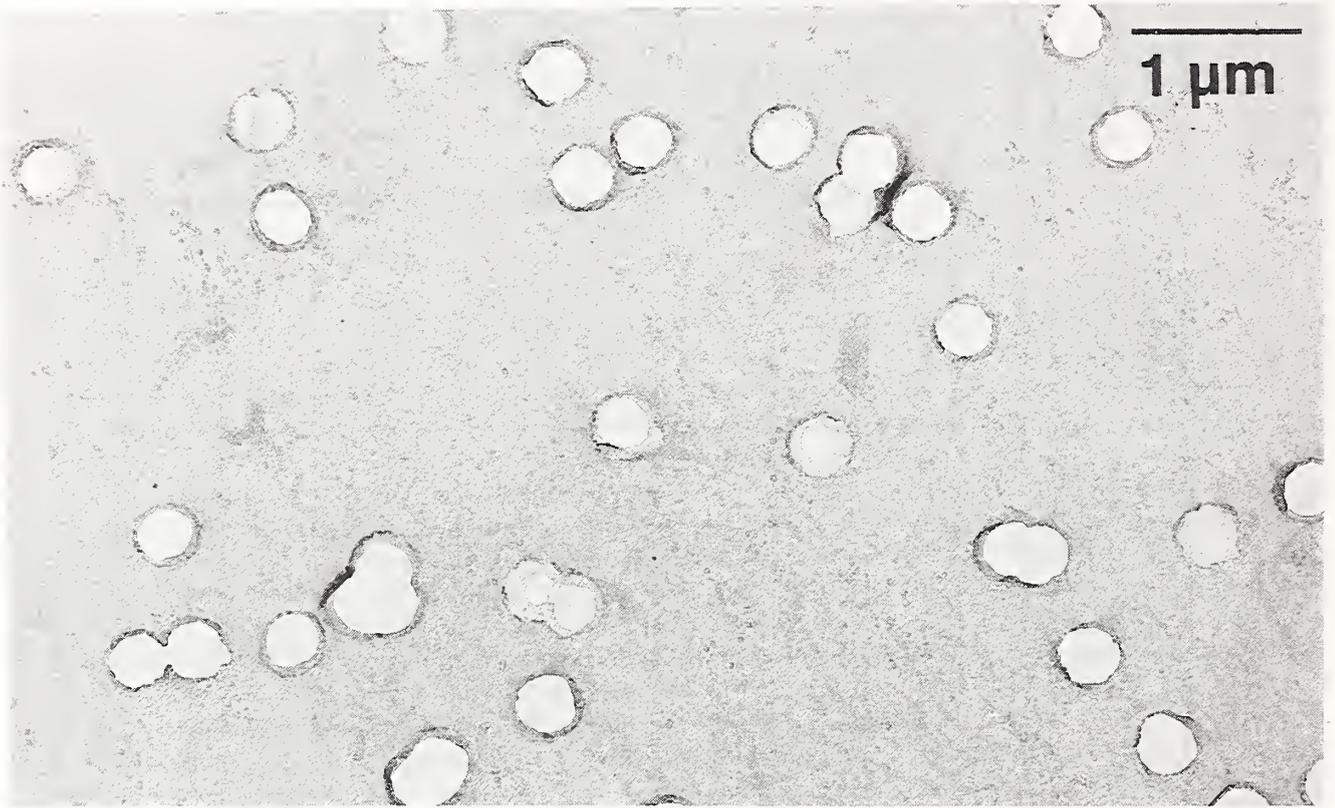


Figure 1a. (top) Example of an acceptable PC preparation.
Figure 1b. (bottom) Example of an acceptable MCE preparation.

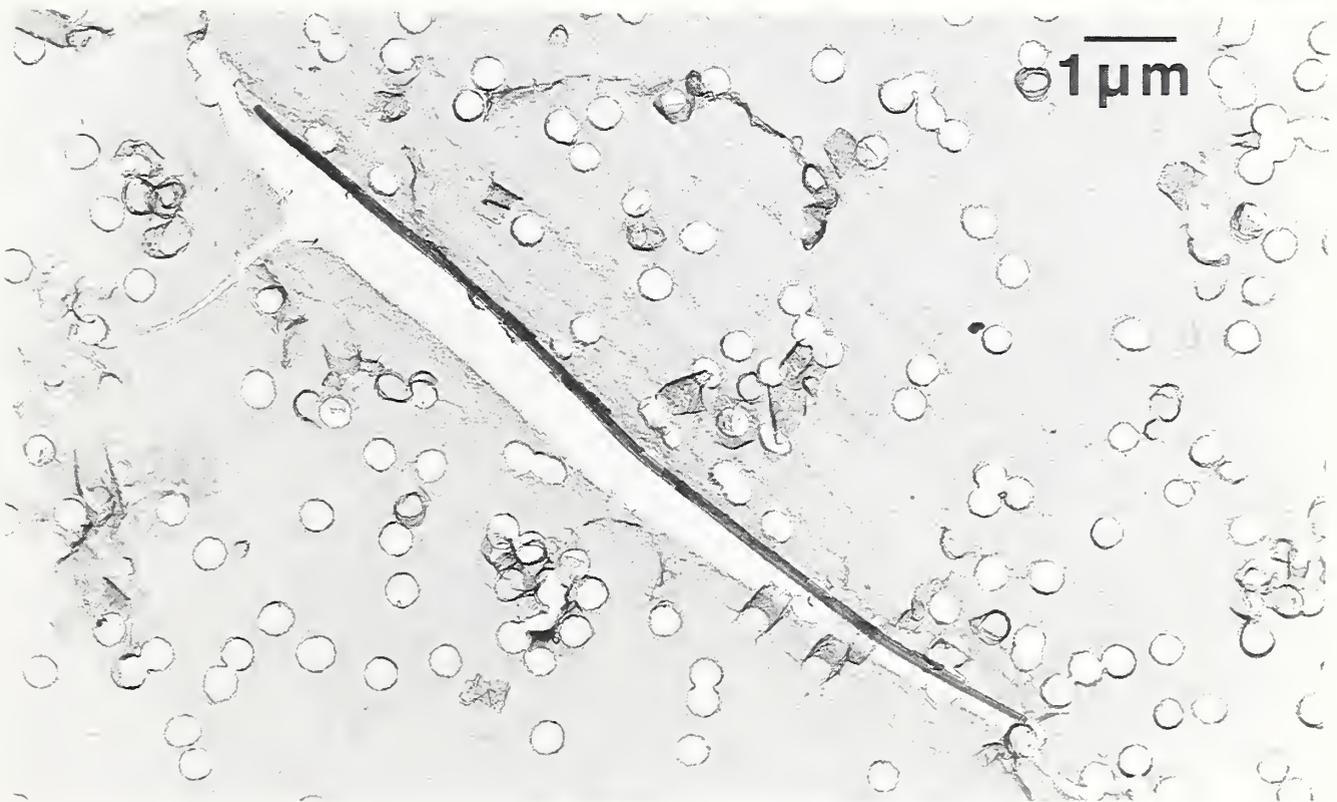
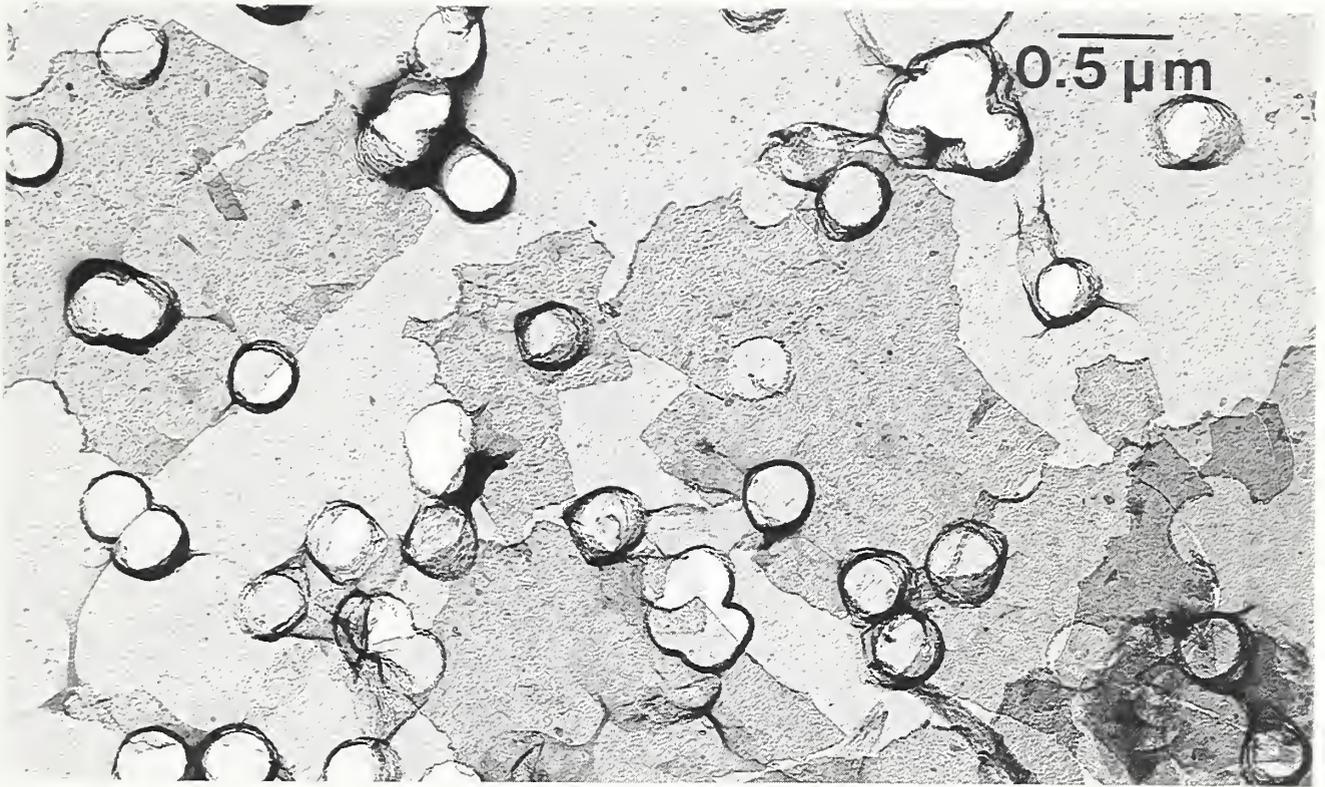


Figure 2. (top) Overlapping replica on a PC replica.
Figure 3a. (bottom) PC replica split along a chrysotile fiber.

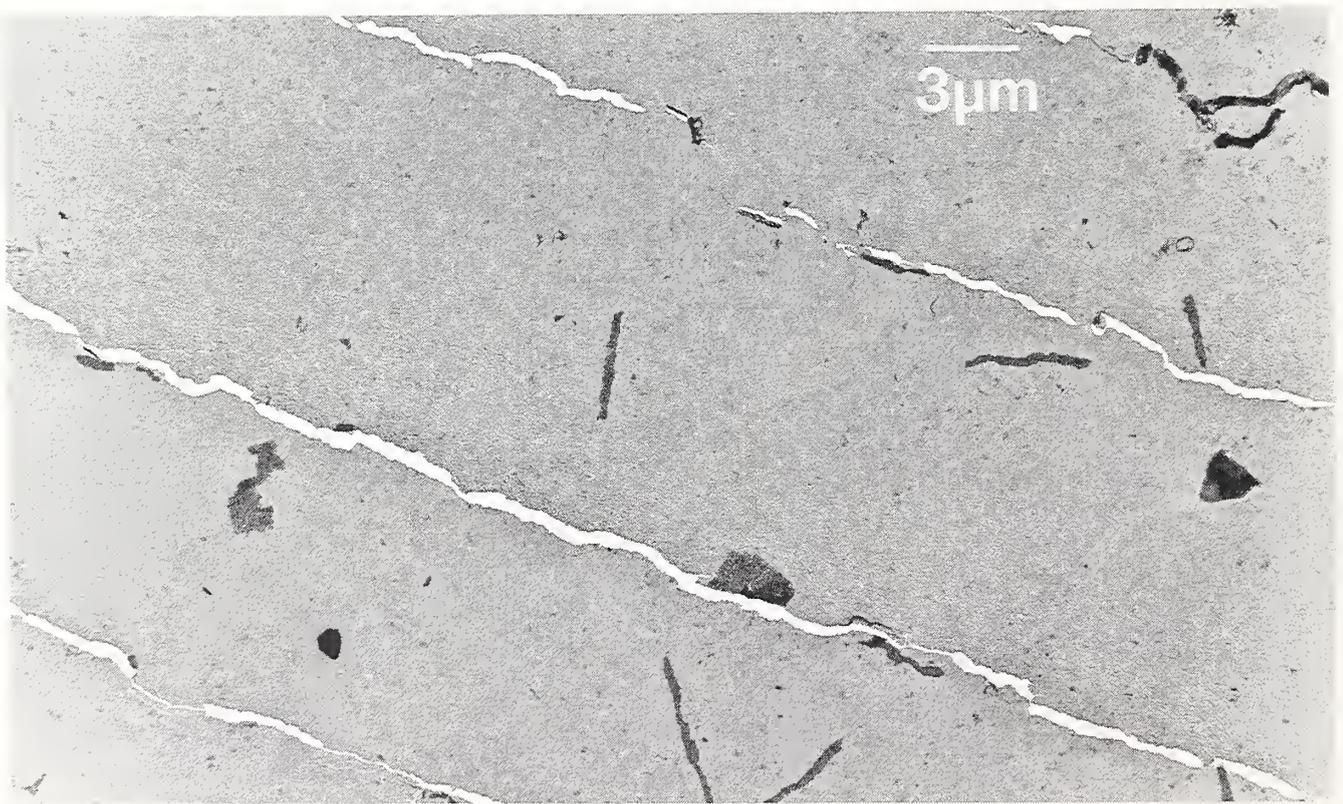
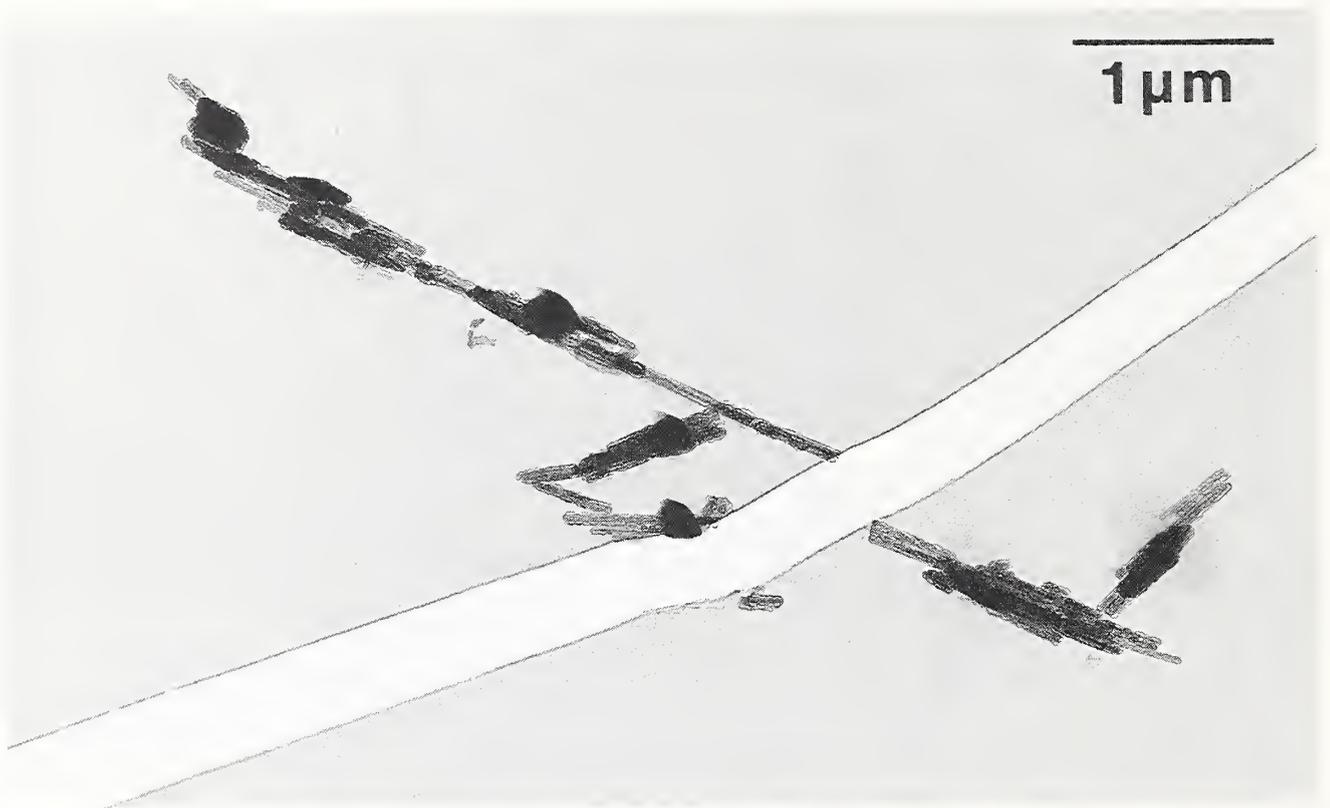


Figure 3b. (top) Split in an MCE filter that separates a chrysotile cluster.
Figure 3c. (bottom) Example of parallel splits in an MCE replica.

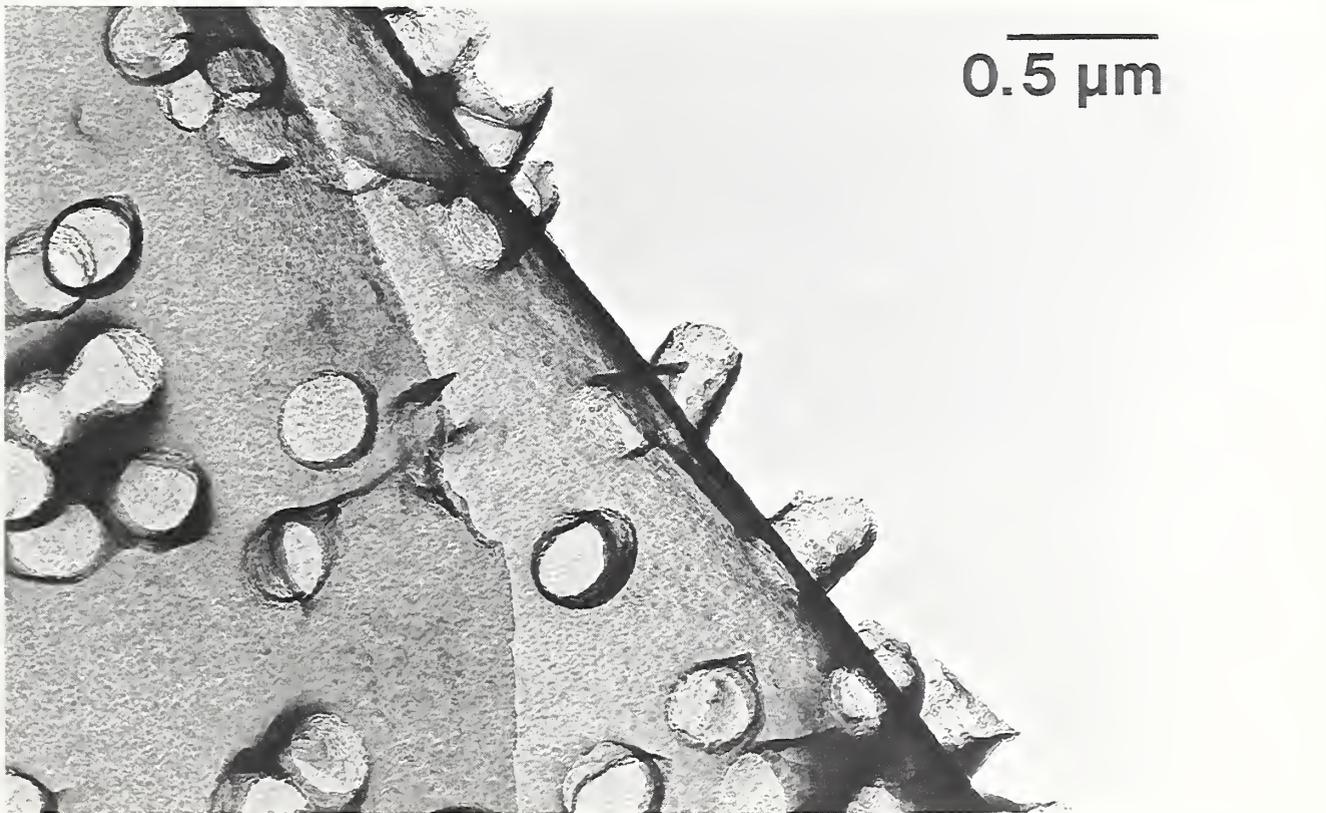
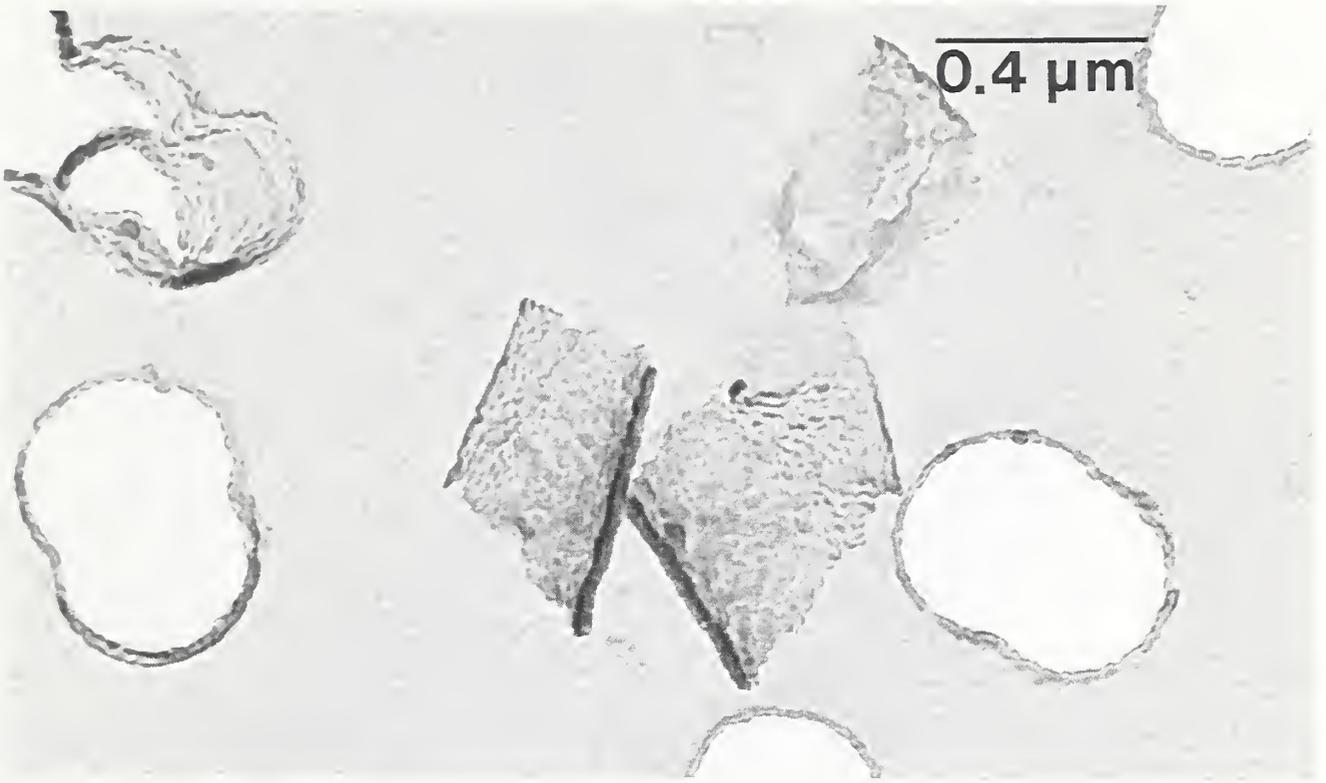


Figure 4a. (top) Carbon flakes on a PC replica.
Figure 4b. (bottom) Curved PC replica showing tubes of amorphous carbon.

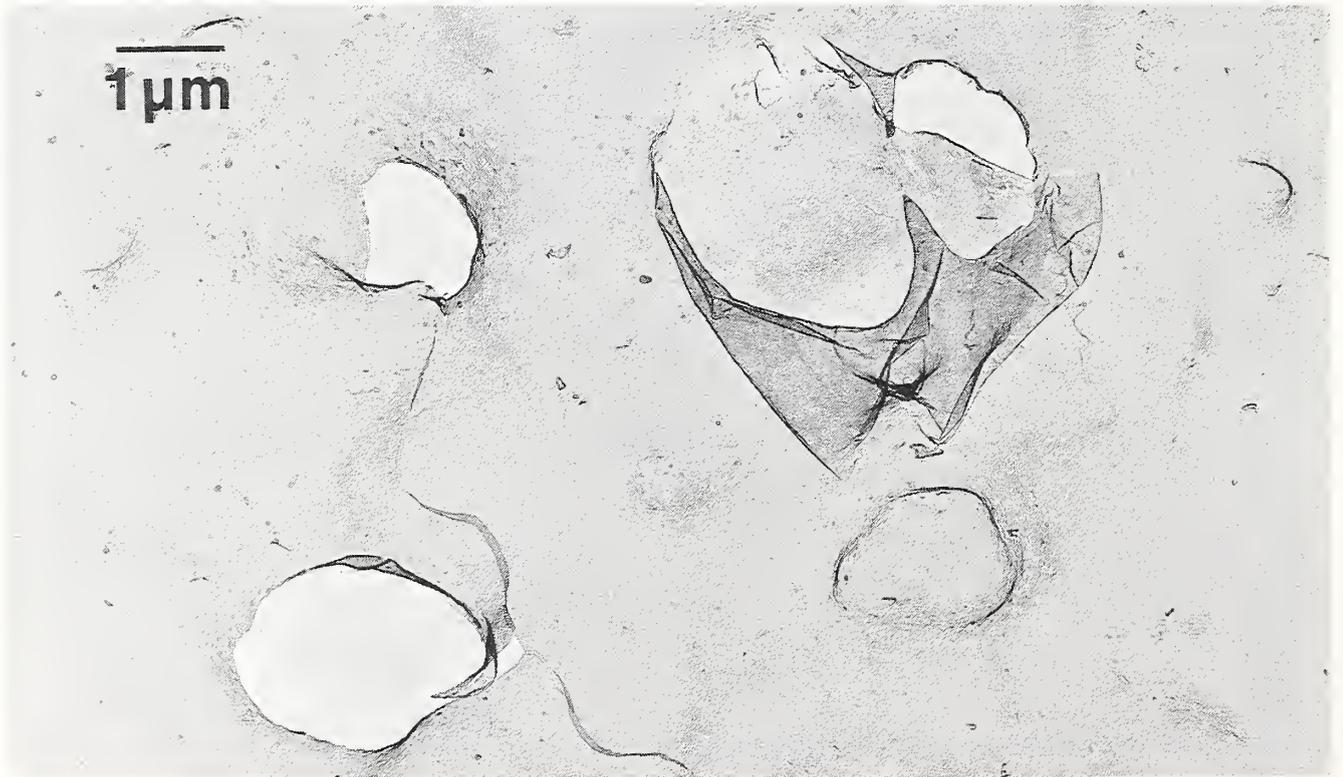


Figure 5. (top) Replica of an MCE filter showing convoluted topography.
Figure 6. (bottom) Craters and holes in an MCE replica.

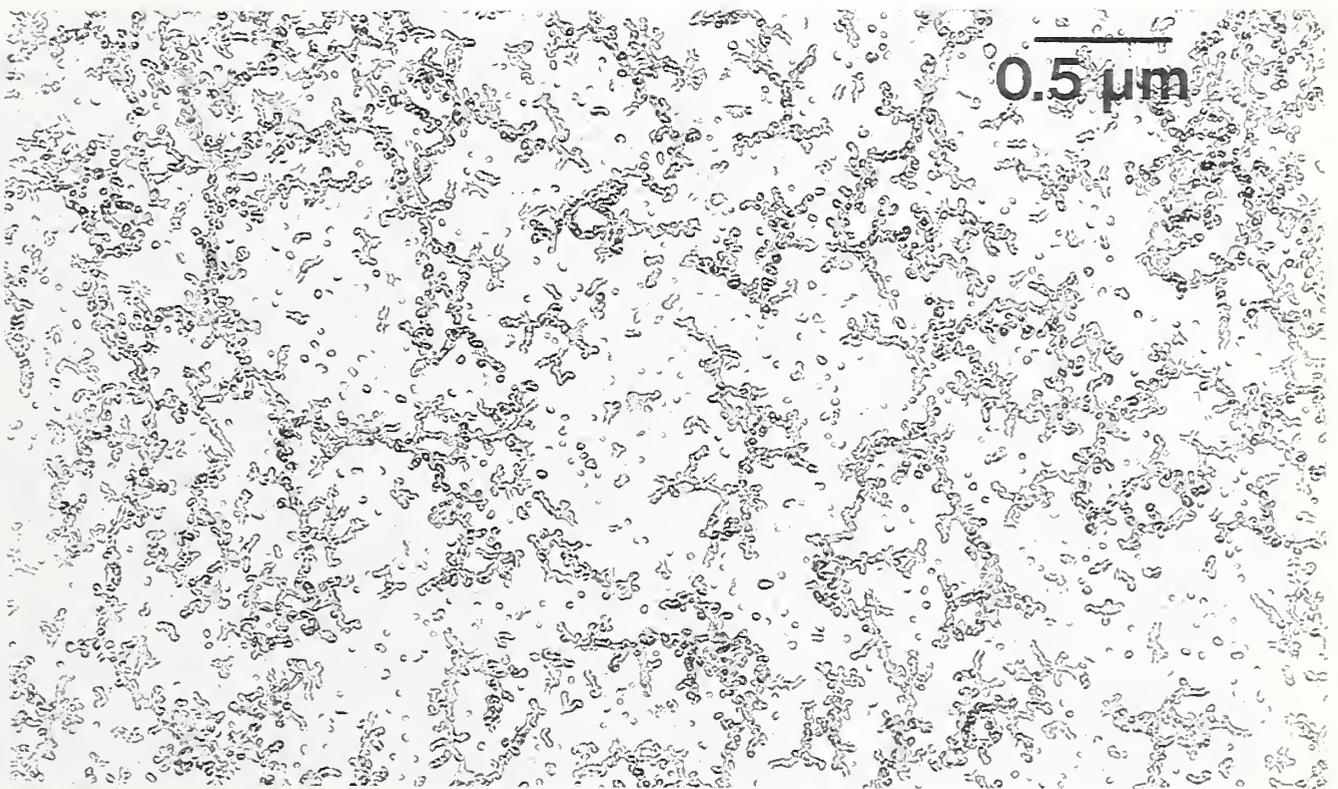
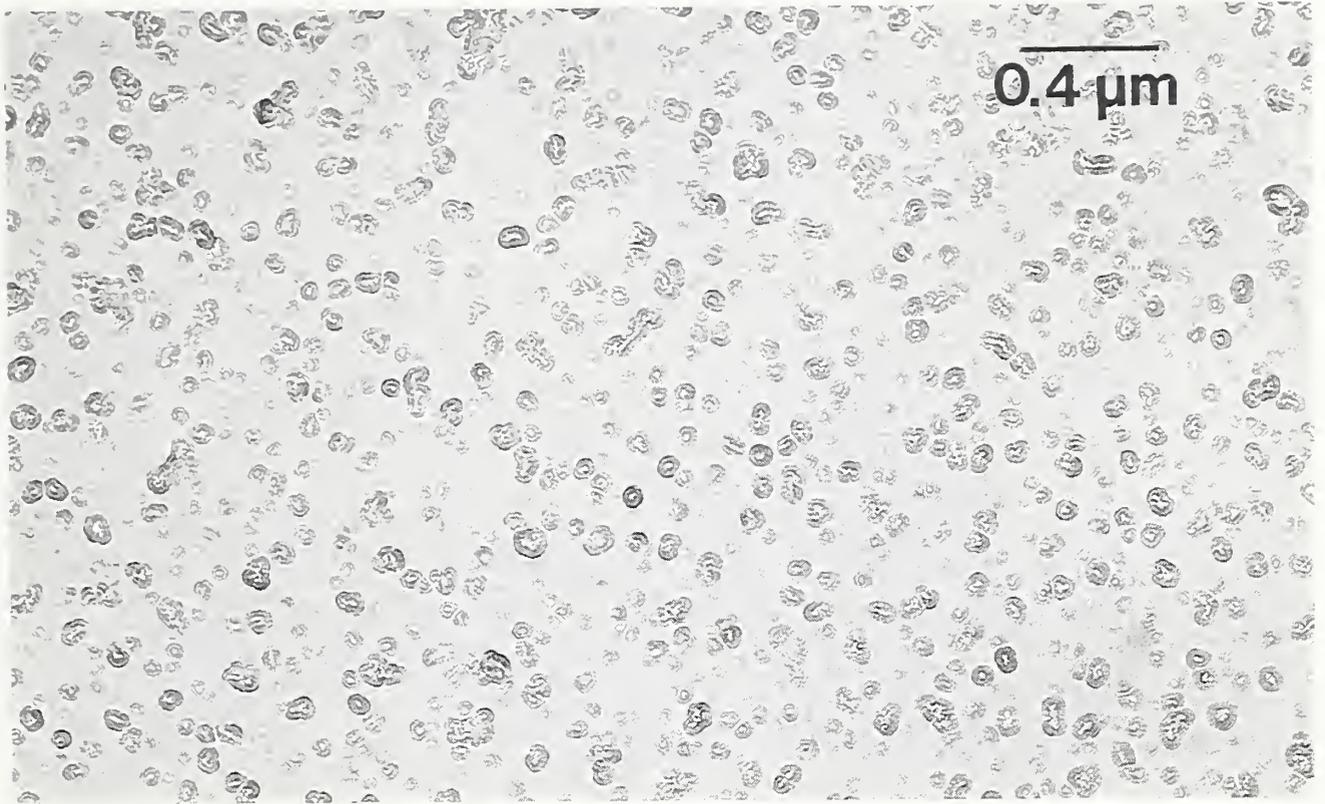


Figure 7a. (top) Type 1 texture on an MCE replica.
Figure 7b. (bottom) Type 2 texture on an MCE replica.

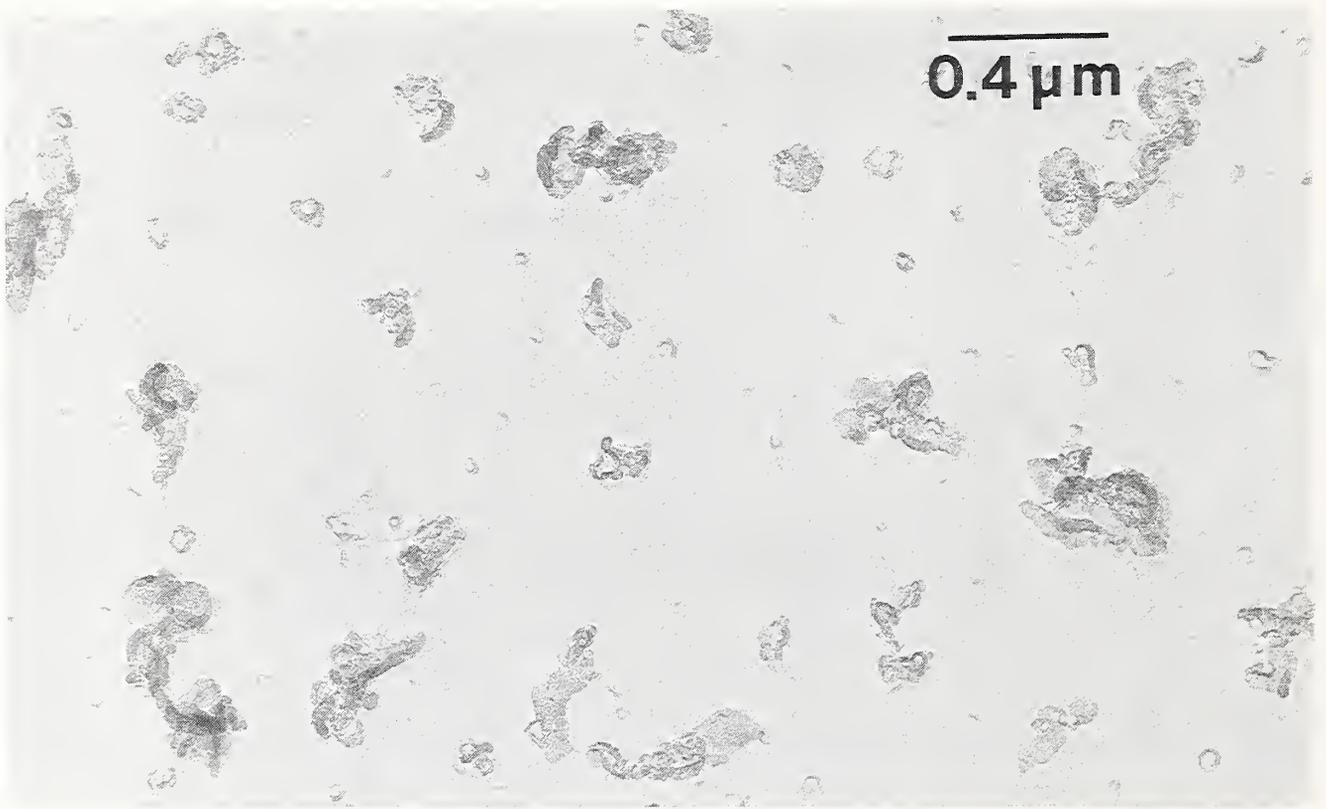


Figure 7c. (top) Type 3 texture on an MCE replica.
Figure 8a. (bottom) Clouded layer on a PC replica near a grid bar.

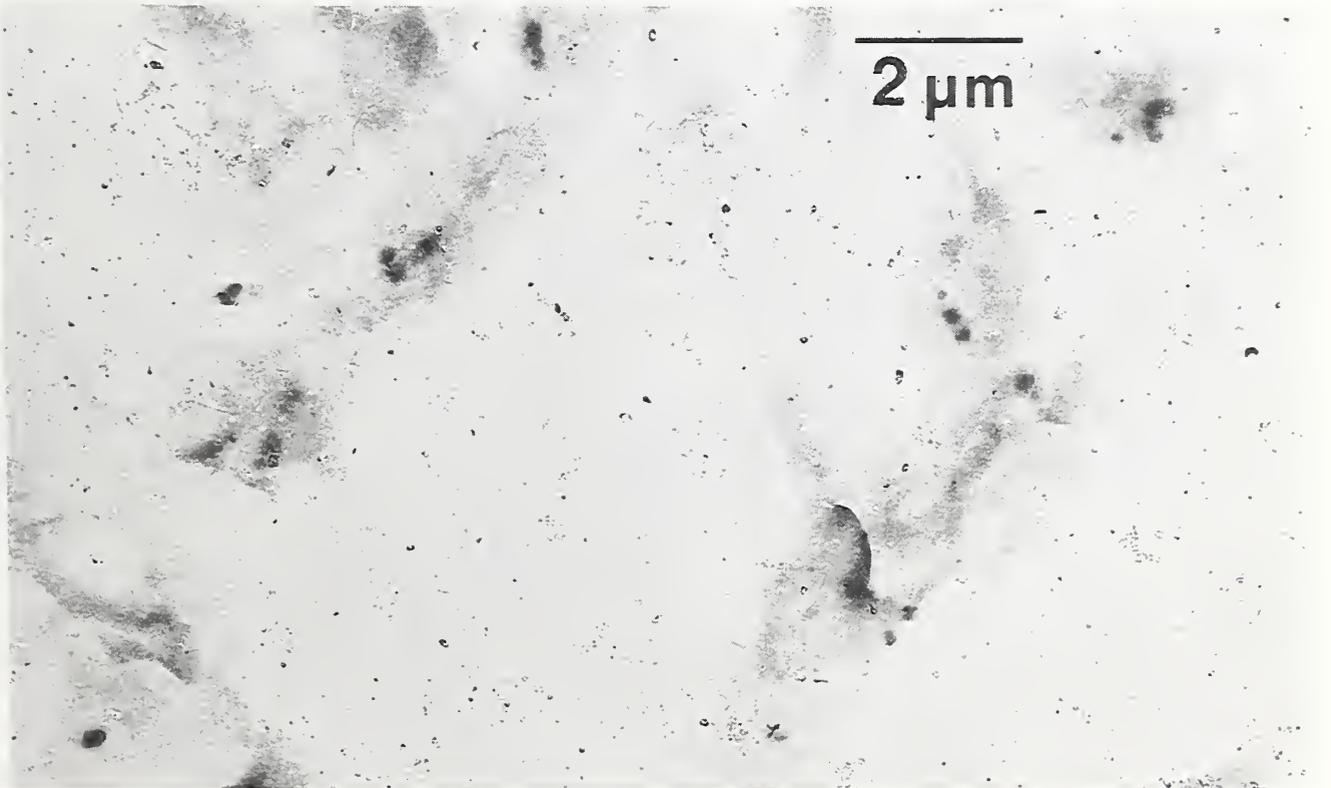
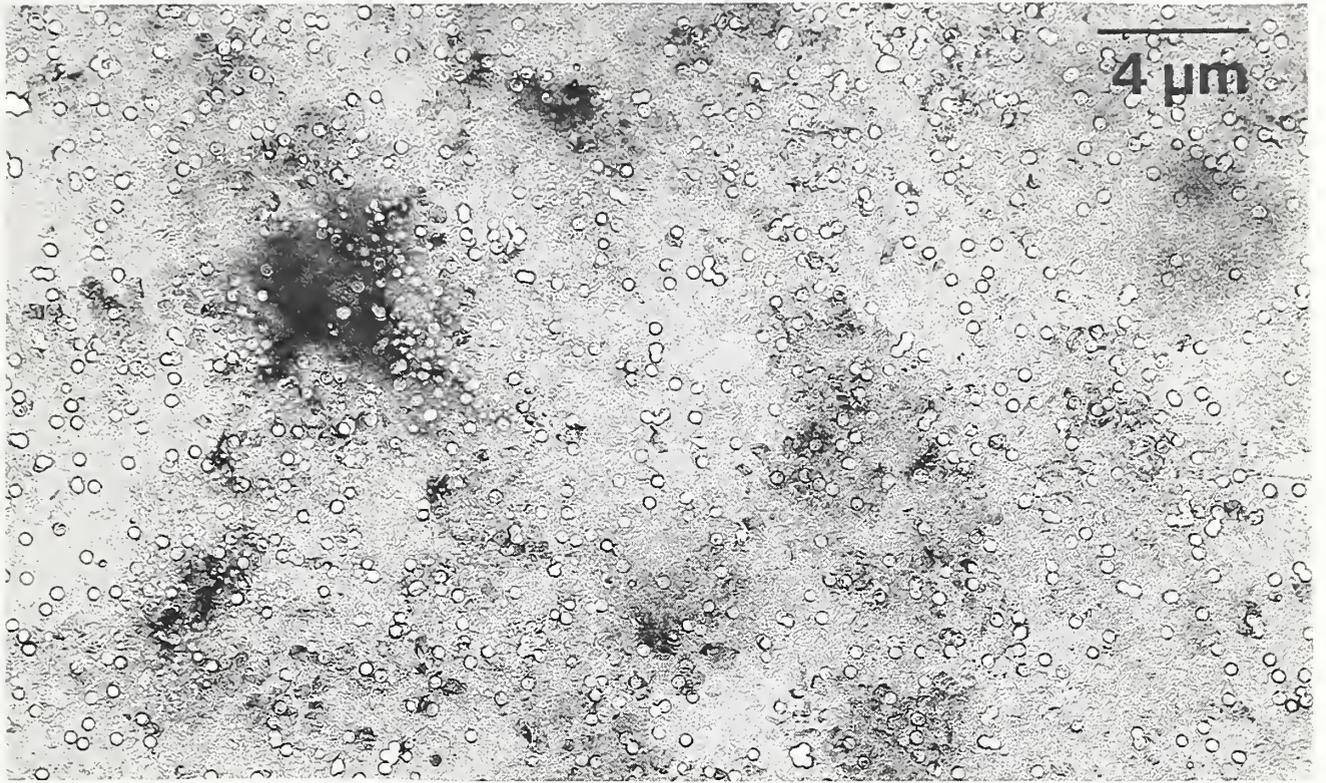


Figure 8b. (top) Patches of clouded material on a PC replica.
Figure 8c. (bottom) Patches of clouded material on an MCE replica.

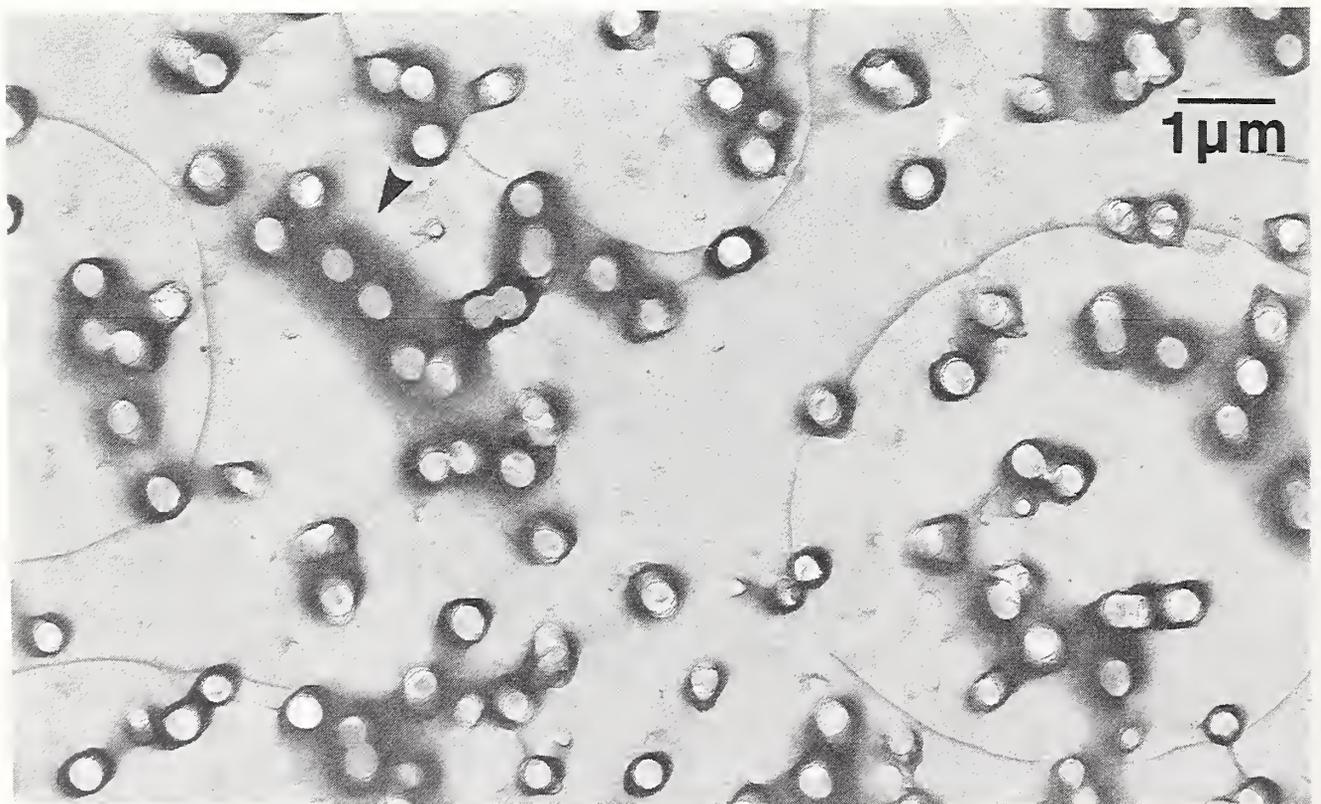
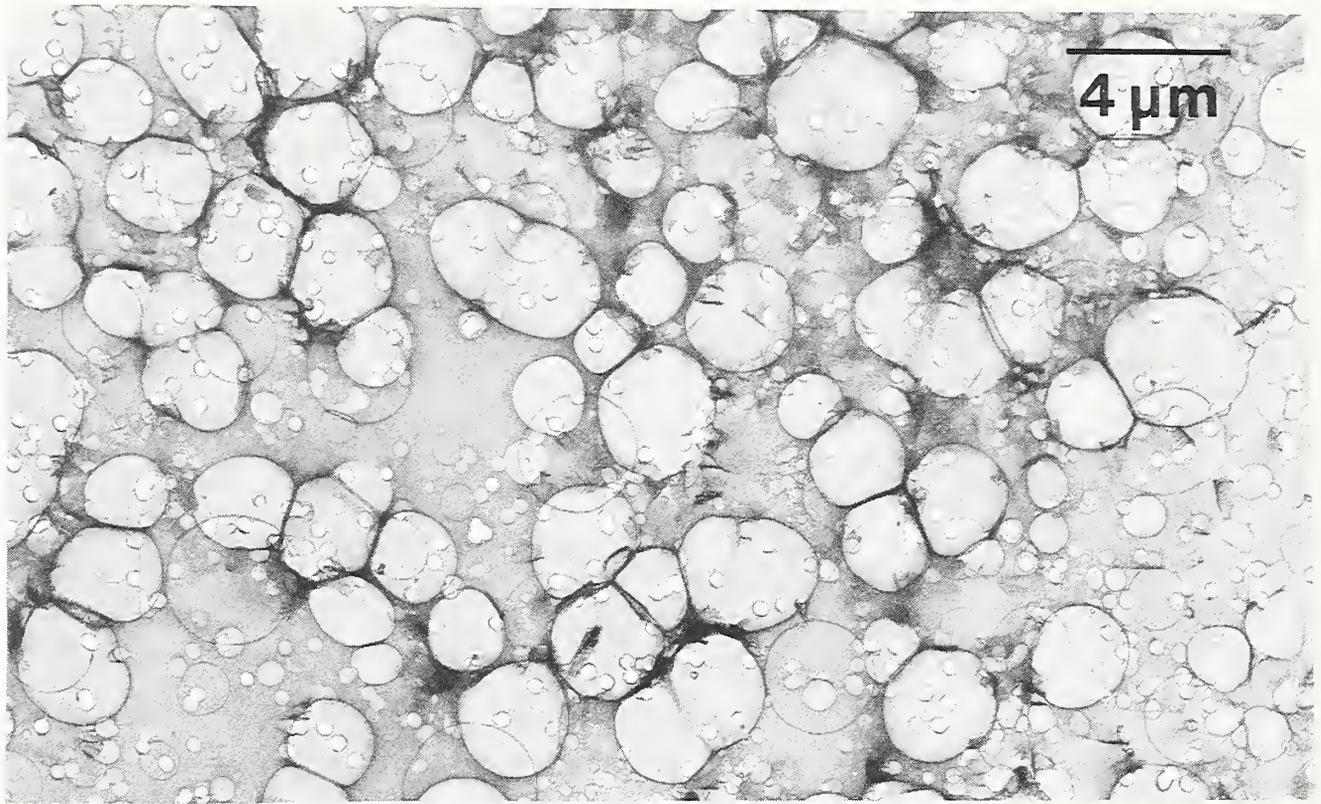


Figure 9. (top) Bubble-like mesh (or overlying mesh) on a PC replica
Figure 10. (bottom) Highlighted pore (white arrow),
interconnected pores (black arrow) on a PC replica

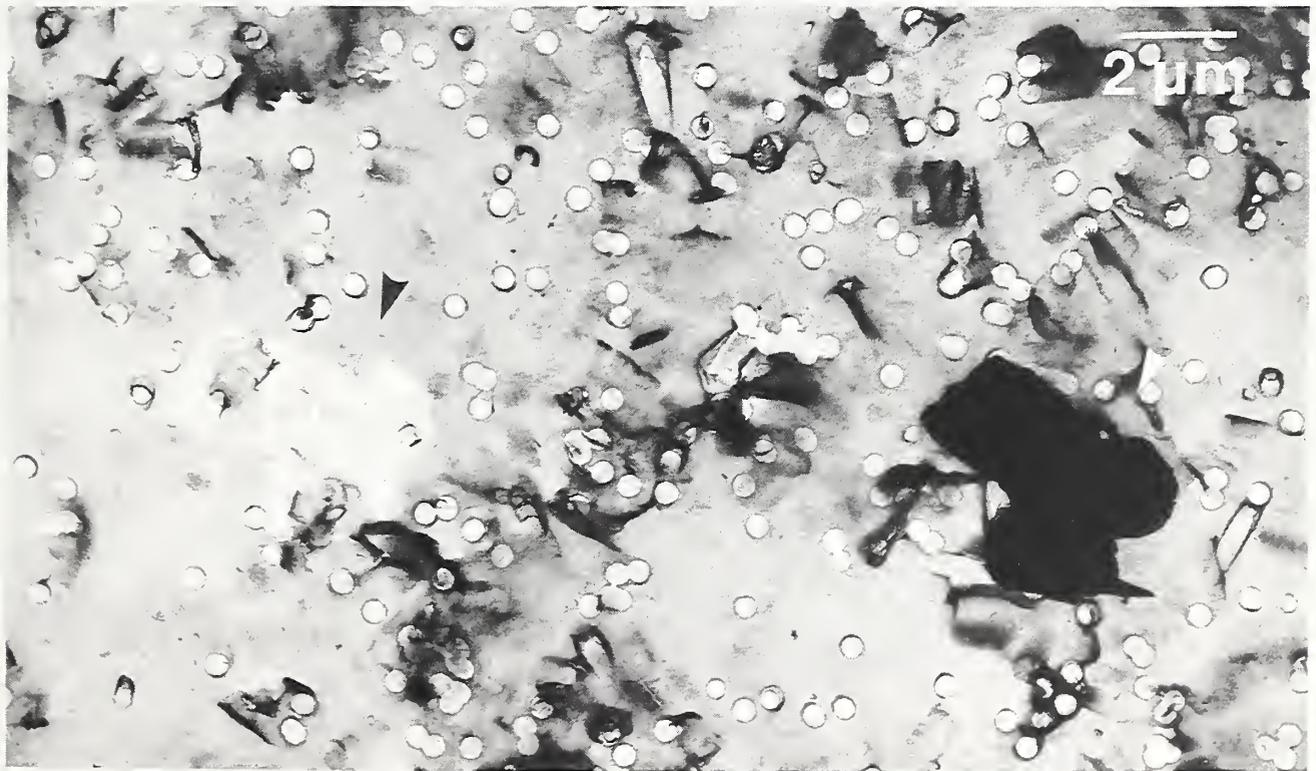


Figure 11a. (top) Displaced particle recognized by the size and shape of hole in MCE replica
Figure 11b. (bottom) Displaced particle recognized by change in texture of PC replica
(original location of particle indicated by black arrow; particle indicated by white arrow)

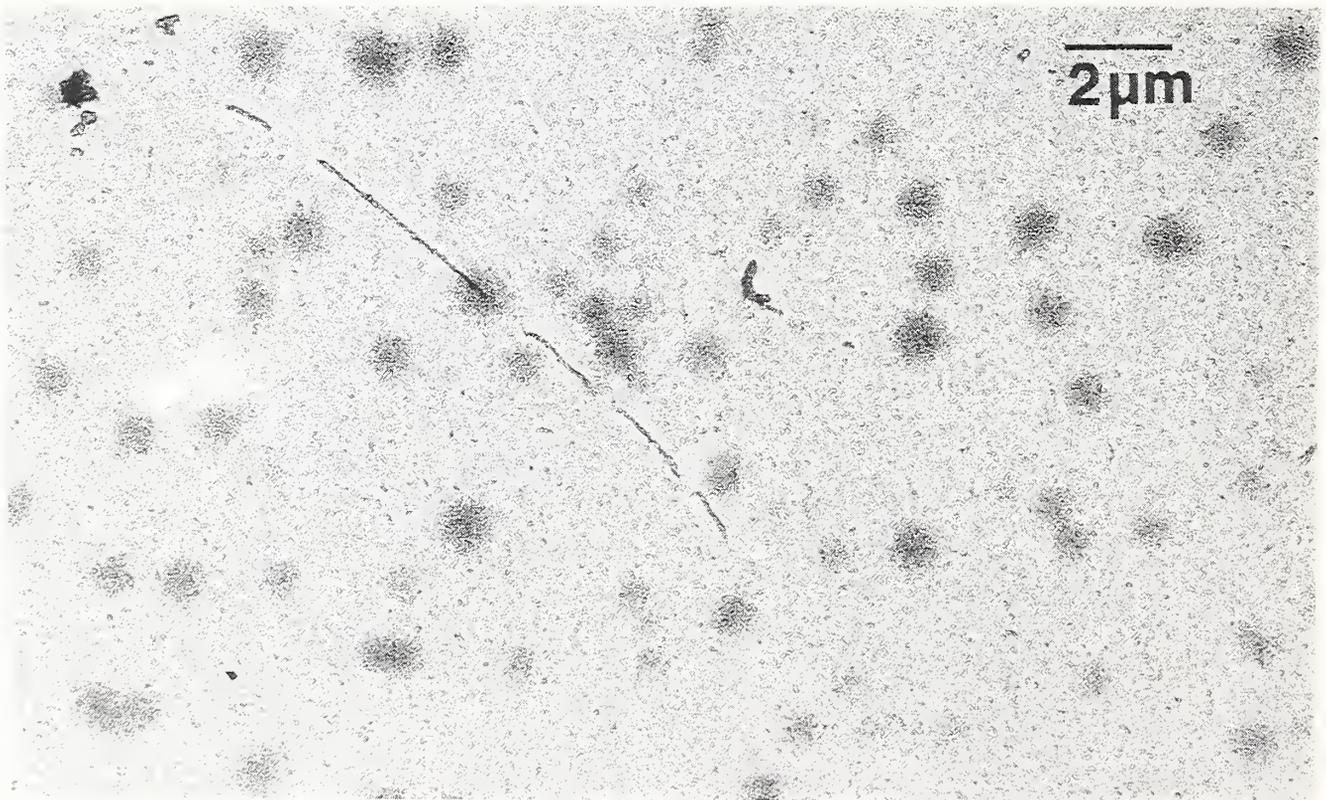
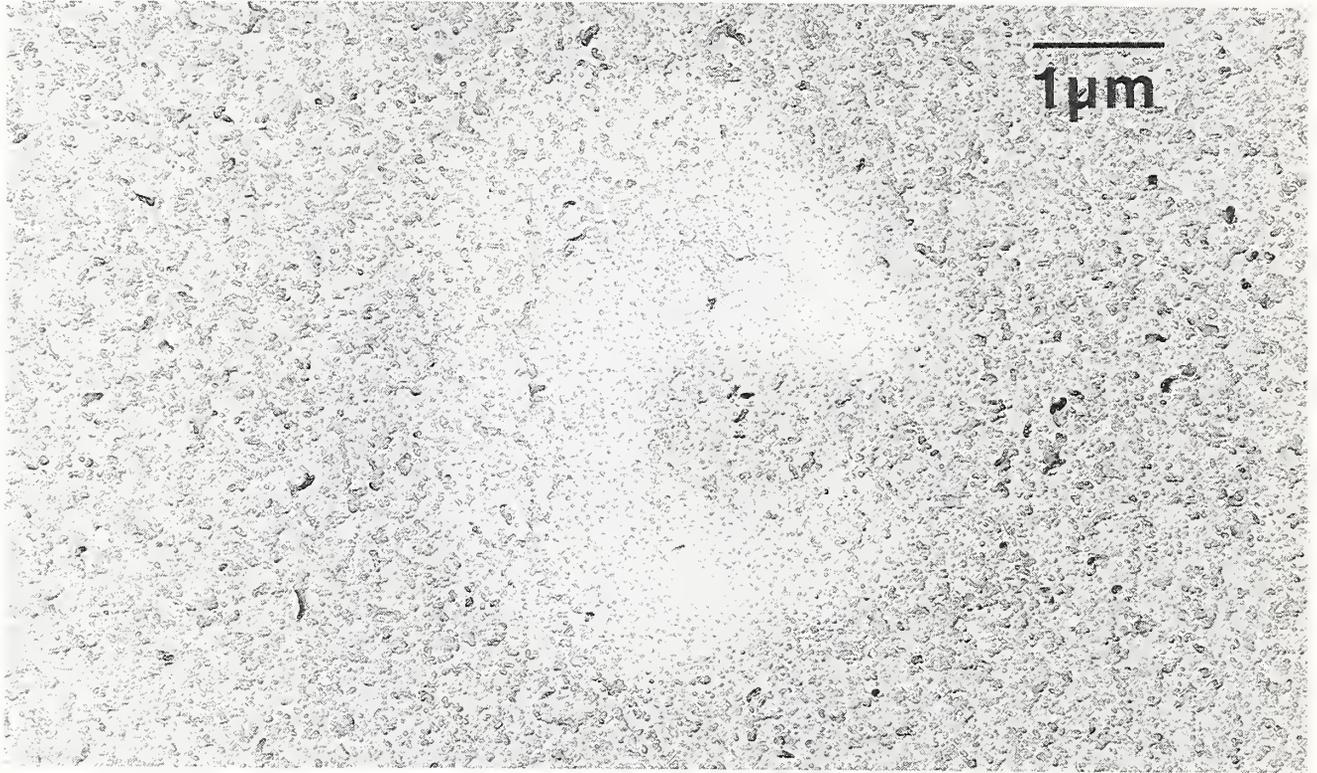


Figure 11c. (top) Missing particle recognized by change in texture of MCE replica.
Figure 12. (bottom) Round spots in an MCE replica.



0.4 μm

Figure 13. Carbon tube on an MCE replica

III. Determining the Acceptability of Replicas for Analysis

The goal of evaluating replicas for problems and artifacts is to judge if a grid square or grid preparation is or is not acceptable for analysis. This involves recognition of problems and artifacts, quantification of their effect on analyses, and comparison to established criteria for acceptance and rejection. The previous section gave a qualitative description of sample preparation problems and artifacts and their possible effects on analyses. In this section, criteria for acceptance and rejection of replicas and methods for quantification of the effect of sample preparation problems and artifacts on analyses are discussed.

General discussion of quantification

Quantifying the effect of sample preparation problems and artifacts on TEM analysis of asbestos can be a challenging problem. This is partially because the analyses are not automated and the TEM analyst is the detector. An analysis can then be affected by people-related factors such as the amount of long-term attention required by the analyst to distinguish asbestos structures from background features or by the ability of the analyst to distinguish between various gray levels. At present, the judgement of the effect of such features on an analysis is subjective. In the list below, the various effects and aspects and limitations of their quantification are discussed.

Features that obscure asbestos structures: These features affect analyses by reducing or eliminating the visibility of structures or by degrading the quality of electron diffraction or chemical information obtained from structures. The effect can be either total so that an asbestos structure is completely obscured or it can be partial. A total obscuring effect is obtained from thick particles through which electrons are not transmitted. A partially obscuring effect is obtained from a thin clouded layer through which structures are visible and analyzable.

If features are totally obscuring, a method for quantification is to determine the projected area of the feature and to compare it to the total area of a grid square or grid. If features are partially obscuring, a judgement must be made as to the usability of the area associated with the feature. This judgement can be affected by the setup of the microscope - for example, apertures in the column or beam current. At present, the judgement of the effect of partially obscuring features is subjective. When a judgement has been made that a replica associated with such features is not usable for analysis, the feature can be quantified by determining the projected area of the feature and comparing it to the total area of a grid square or grid.

Features that provide excessive detail or that mimic asbestos structures: These features require increased attention by the analyst. This increased attention will possibly slow an analysis or tire an analyst if sustained over a long time and could lead to structures being missed by operators. Ideally, to quantify the effect of certain features, numerical values could be assigned to represent the degree of detail present (e.g., determining a fractal dimension for the features) or to represent the degree certain features mimic asbestos fibers or structures. Studies would then be conducted to establish the correlation between these values and the number of false negatives obtained by analyst. Such studies have not been done. The practical quantification of these features is limited to the determination of their projected area as discussed above for obscuring features.

Features that are inhomogeneously distributed over a grid or grid square: A nonrandom distribution of features over a replica may affect the statistical validity of an analysis. The presence of the feature can bias and limit the choice of grid squares for analysis. Furthermore, it is conceivable that a nonrandom distribution of a feature reflects an inhomogeneous distribution of structures. For example, the presence of a nonrandomly distributed split replica could relate to a nonrandom distribution of asbestos structures if the particles are causing the splitting. Ideally, homogeneity would be quantified by determining the projected area of randomly-chosen sections of a grid or grid square. A numerical value would be determined reflecting the degree of inhomogeneity. At present only subjective, qualitative determinations of the inhomogeneity of a feature are made.

Features that indicate physical loss or gain of particles: Such features include those for which an area measurement can be used for quantification including overlapping replica, splitting of replica, missing replica and craters and holes. Features that cannot be quantified in this manner are missing or displaced particles indicated by holes or changes in texture. For this case, an estimate can be made of the percentage of particles on the grid or grid square that is missing or displaced.

Features that modify how structures are counted: An additional effect of sample preparation features on the analysis is the possibility of modifying structure counts. The AHERA method contains a specific protocol (or counting rules) for determining the number of structures in a particle. As discussed for split replica, in Part II of the handbook (Figure 3b), a split can separate a fiber arrangement into two structures. The structure count is then increased over what it would have been without this feature. Another modification of structure counts may occur if there are difficulties in distinguishing sample preparation features from matrix materials. For example, if an asbestos fiber is associated with a sample preparation feature and does not protrude 0.5 μm from the feature, it would not be counted as a structure. The same fiber on a different replica preparation free of sample preparation features would be counted as a structure. Quantification of this effect is only done indirectly by projected area estimates of the feature.

Determination of projected area of a feature

From the discussion in the previous section, it is clear that determining projected area is an important means of quantifying the effect of a feature. The projected area of a feature can be quantified by either image analysis or by calibrated visual estimates. Image analysis requires specialized hardware and software. The number of pixels in a feature is determined and ratioed to the number of pixels in the total available replica of a grid square or grid. Calibrated visual estimates require use of reference images for which the projected area of a feature has been determined. The area of a feature is determined by comparison to the areas in the calibrated visual images.

A series of reference images for calibrated visual estimates are provided in Figures 14-21^{*}. Images were analyzed using the public domain software program "Image 4.2"^{6,7}. Figures 15 a-d show the particle loading for four different grid squares. The top images of Figures 16-21 are digitized images of a replica. The bottom images are binary images in which the area affected by the sample preparation problem is highlighted in black. The areal percentage of the sample preparation problem is determined by dividing the number of pixels in the highlighted area by the number of pixels in the entire image. These figures can be used as standards for estimating the areal extent of a sample preparation problem or artifact.

^{*} Note: it is suggested that the reader estimate the areal percentages of the features in the figures before looking at the figure caption.

Criteria for judging the acceptability of replicas

For consistency in evaluation between analysts and laboratories, it is necessary to set up criteria for the acceptability of replicas. Previous guidelines for establishing criteria were given in the AHERA method², in the National Voluntary Laboratory Accreditation Program (NVLAP) Handbook for Airborne Asbestos Analysis⁸ and in the Environmental Protection Agency Quality Assurance Guidelines⁹. In this section, the criteria for analysis by the AHERA method are reviewed and expanded and revised criteria for the features discussed in this handbook are given.

AHERA method

The criteria given by the AHERA method for rejection of grid squares or grids are as follows:

- 1) Grid openings with greater than 5 percent openings or holes.
- 2) Grid openings with greater than 25 percent particulate matter.
- 3) Grid openings with nonuniform loading of particulate matter.
- 4) Grids for which less than 50 percent of the grid openings covered by replica are intact.
- 5) Grids for which the replica is too dark because of incomplete dissolution of the filter.

Expanded and revised criteria

The criteria listed by the AHERA method were developed before recognition of the range of possible sample preparation problems. The following list contains an expanded set of criteria for rejection of replicas.

- 1) Grid squares with obscuring features, excessive detail, features that mimic asbestos, missing replica (holes) or splits that individually or in sum cover greater than 5 percent of the area.
- 2) Grid squares for which greater than 5 percent of the particles are displaced or missing.
- 3) Grid squares with greater than 10 percent particulate matter.
- 4) Grids with a significantly nonrandom loading of particulate matter or distribution of sample preparation features (as discussed on p. 21).
- 5) Grids for which less than 50 percent of the available grid squares are covered by the replica section.
- 6) Grids for which more than 50 percent of the replica is unacceptable.
- 7) Grids with a convoluted replica (MCE filters) or a thick replica (greater than ~50 nm).

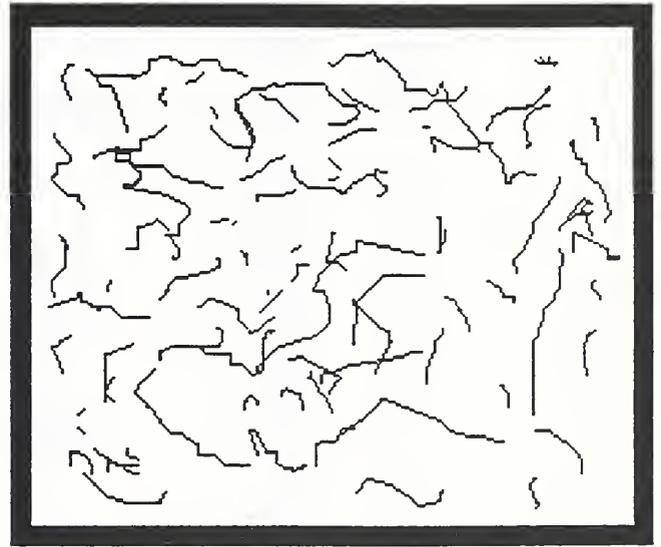
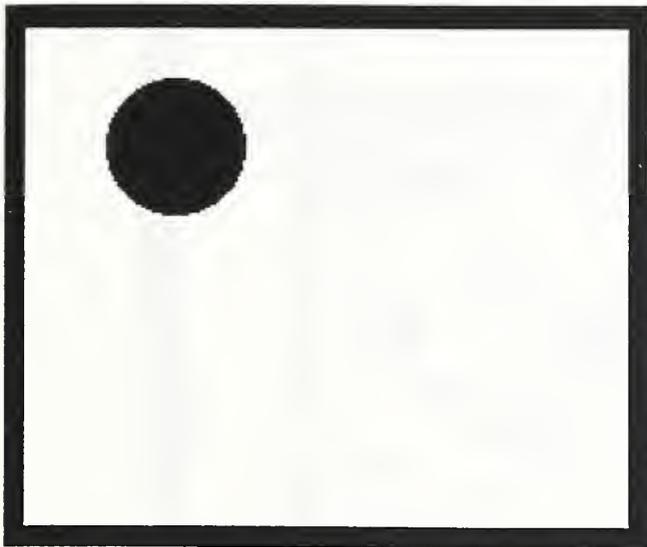
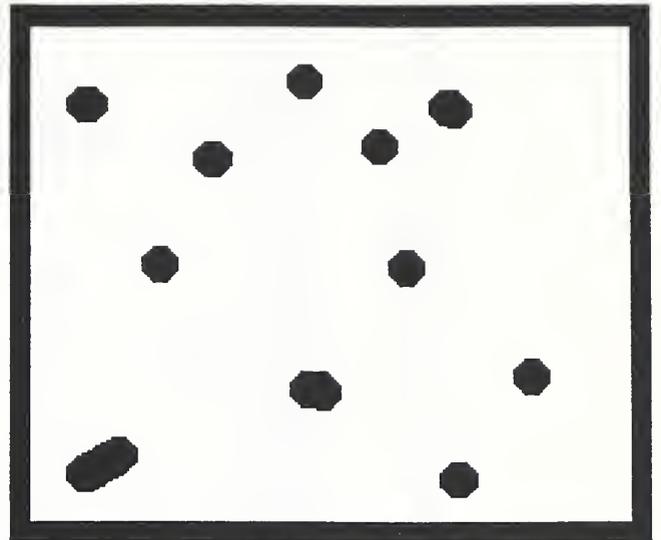
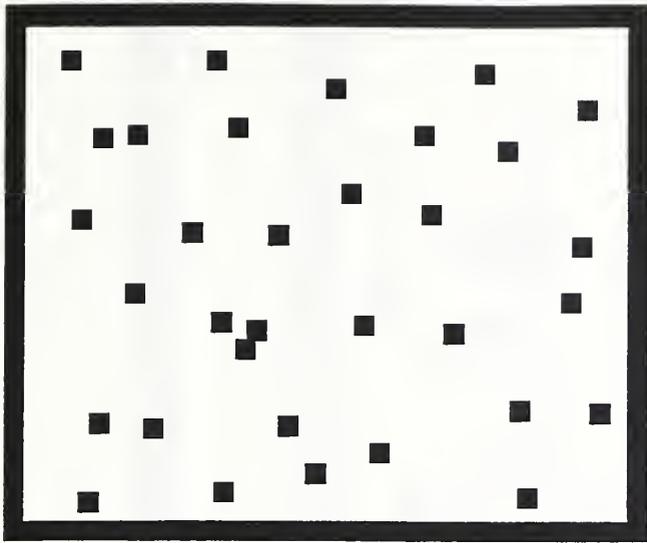


Figure 14 a-d. Examples of particles comprising 5 percent of the area within a grid square.



Figure 15 a-d. Particle loadings on grid squares (interior of grid squares is $\sim 90 \mu\text{m}$ in width); (a) (top) particles comprise 3 percent of grid square area, (b) (bottom) particles comprise 10 percent of grid square area (continued next page).

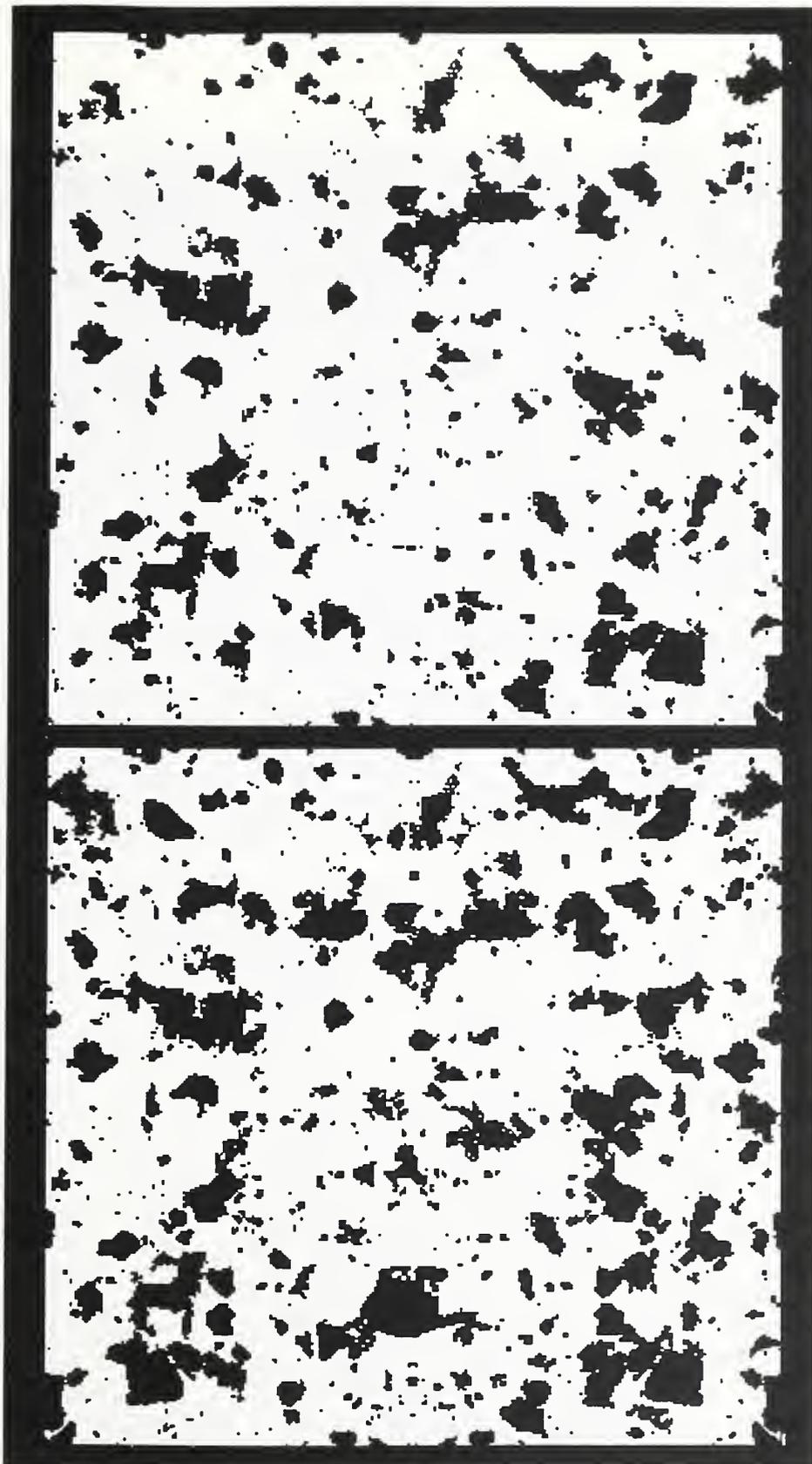


Figure 15. Particle loadings on grid squares (continued); (c) (top) particles comprise 17 percent of grid square area, (d) (bottom) particles comprise 25 percent of grid square area.

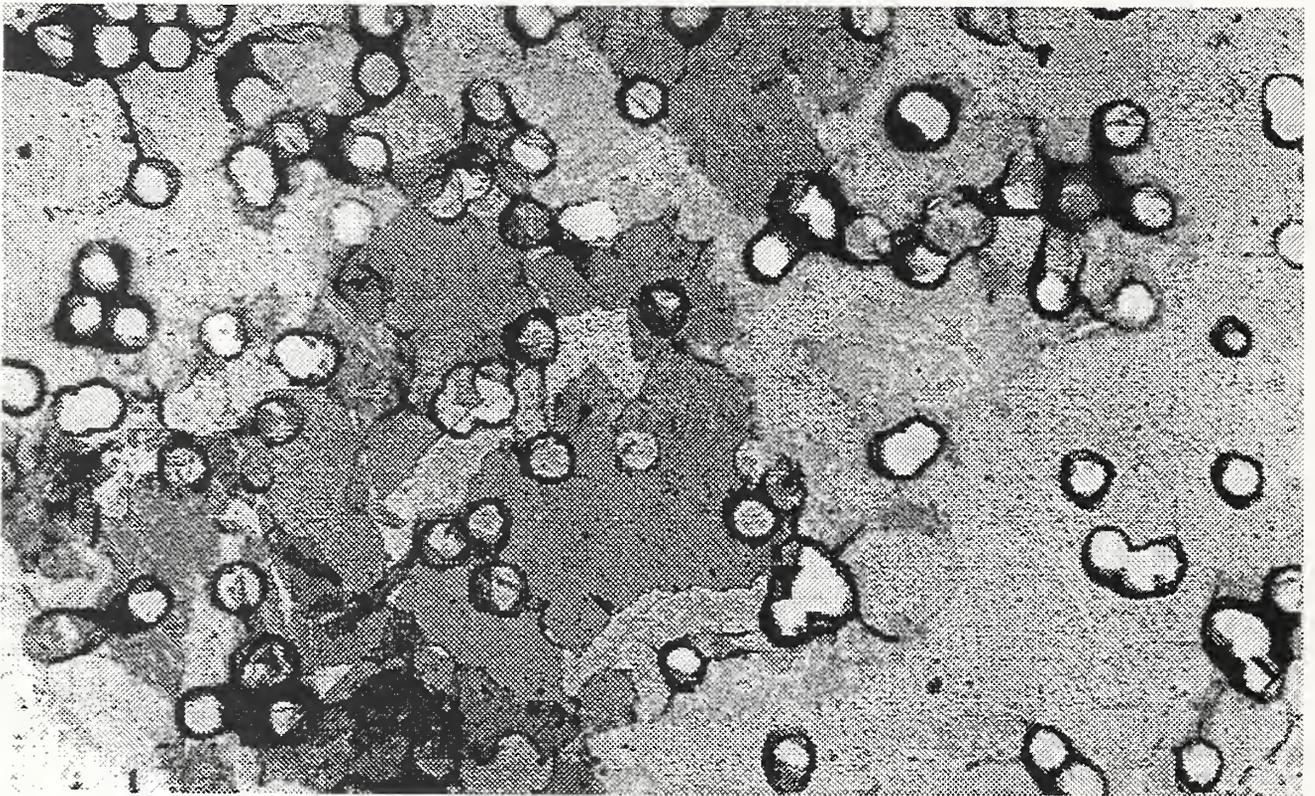


Figure 16. Image of overlapping replica on a PC filter replica (top). The overlapping replica covers 32 percent of the total image (black areas in bottom image).

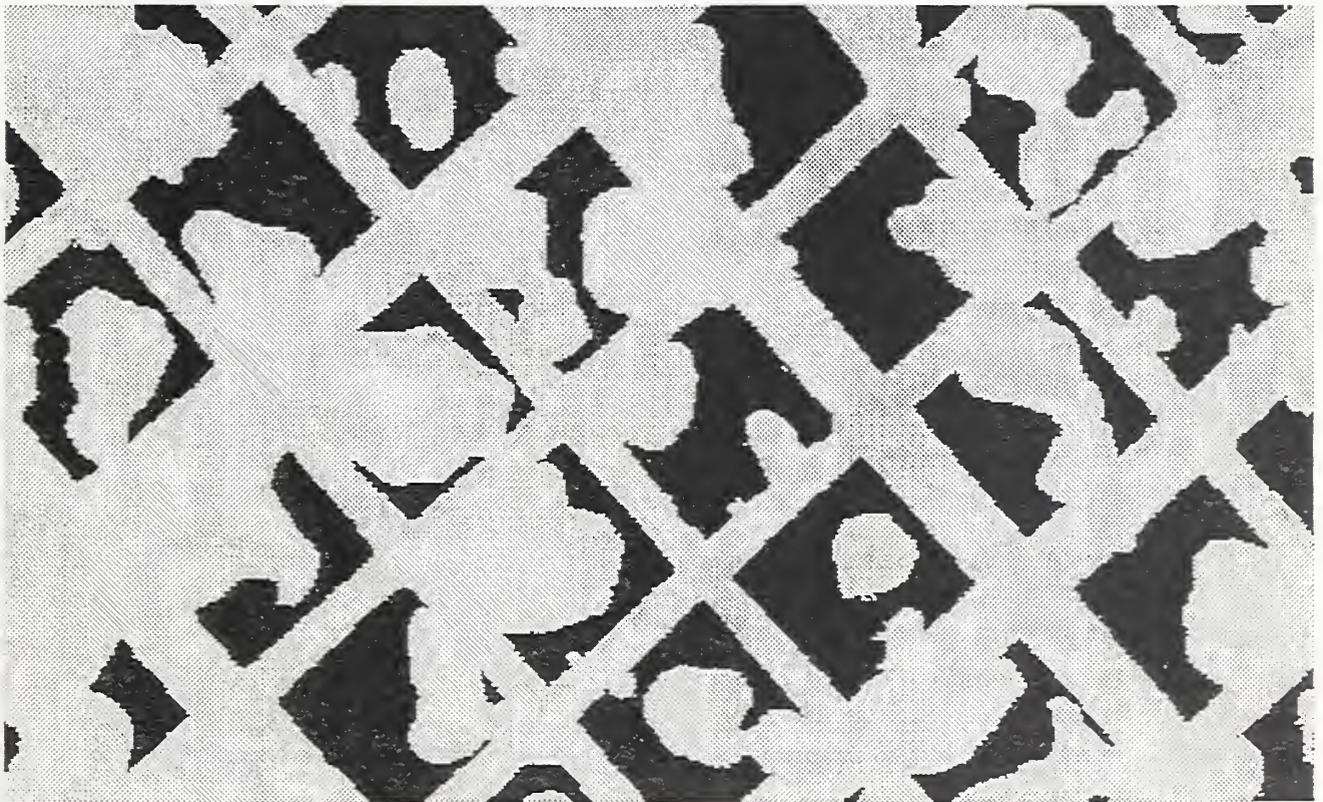
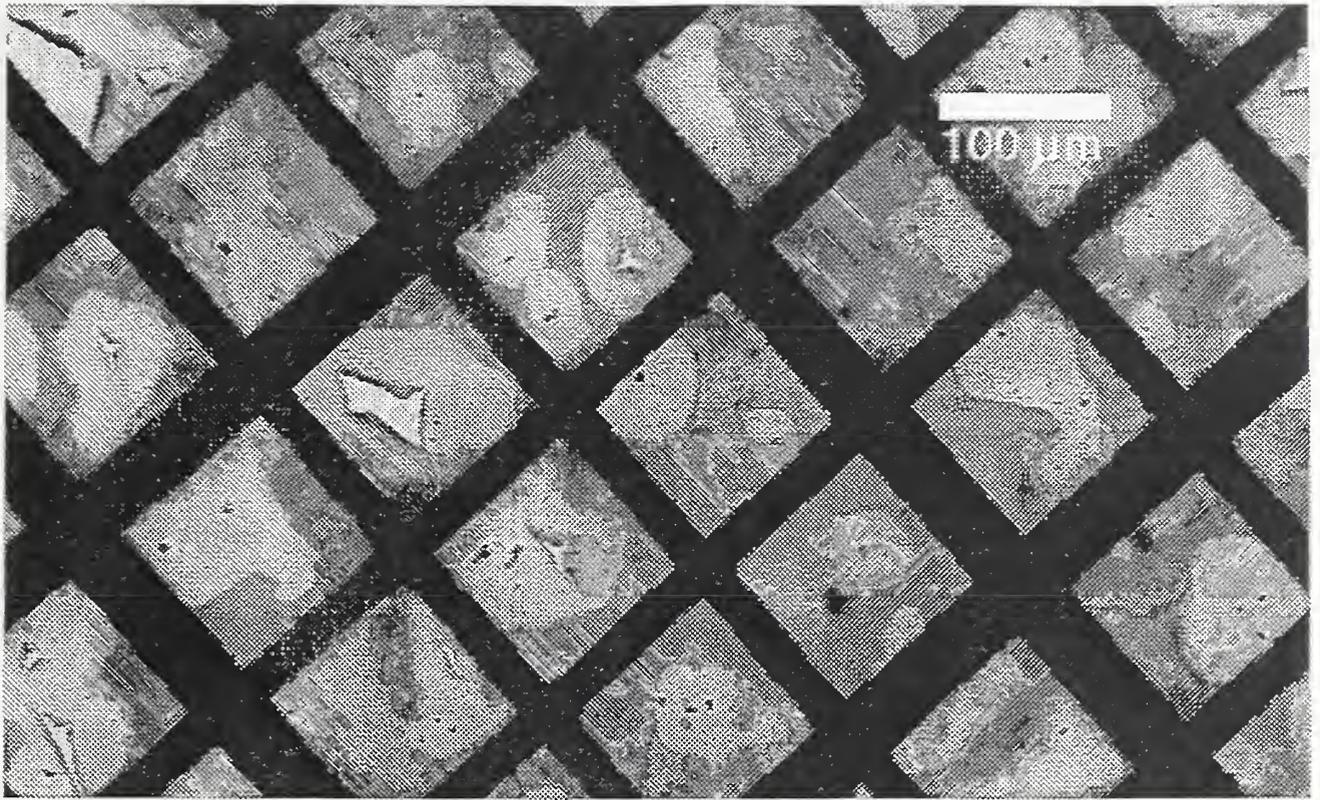


Figure 17a. Image of a clouded layer and split replica on an MCE replica (top). The clouded areas cover 52 percent of the total image (black areas in bottom image; grid bars not included in area determination).

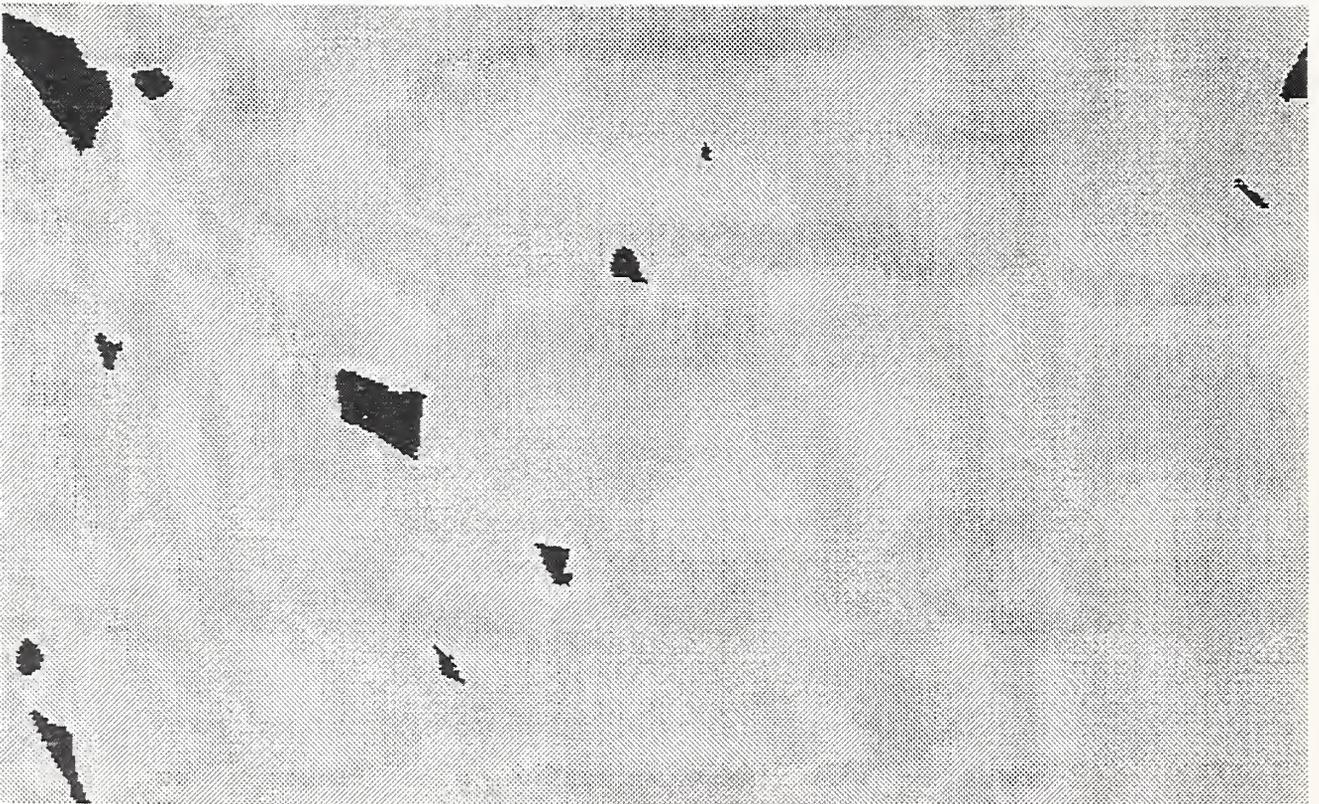
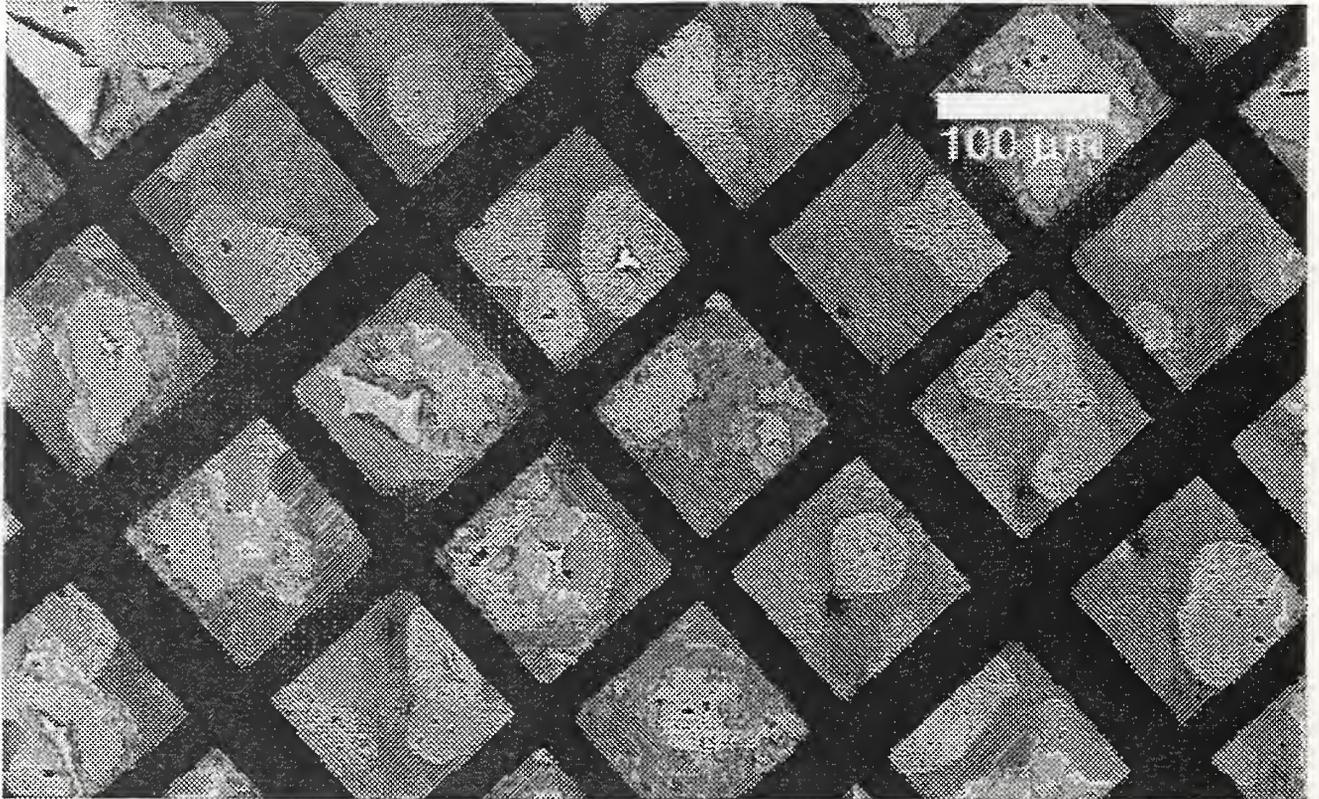


Figure 17b. Image of a clouded layer and split replica on an MCE replica (top). The areas of split replica cover 3 percent of the total image (black areas in bottom image; grid bars not included in area determination).

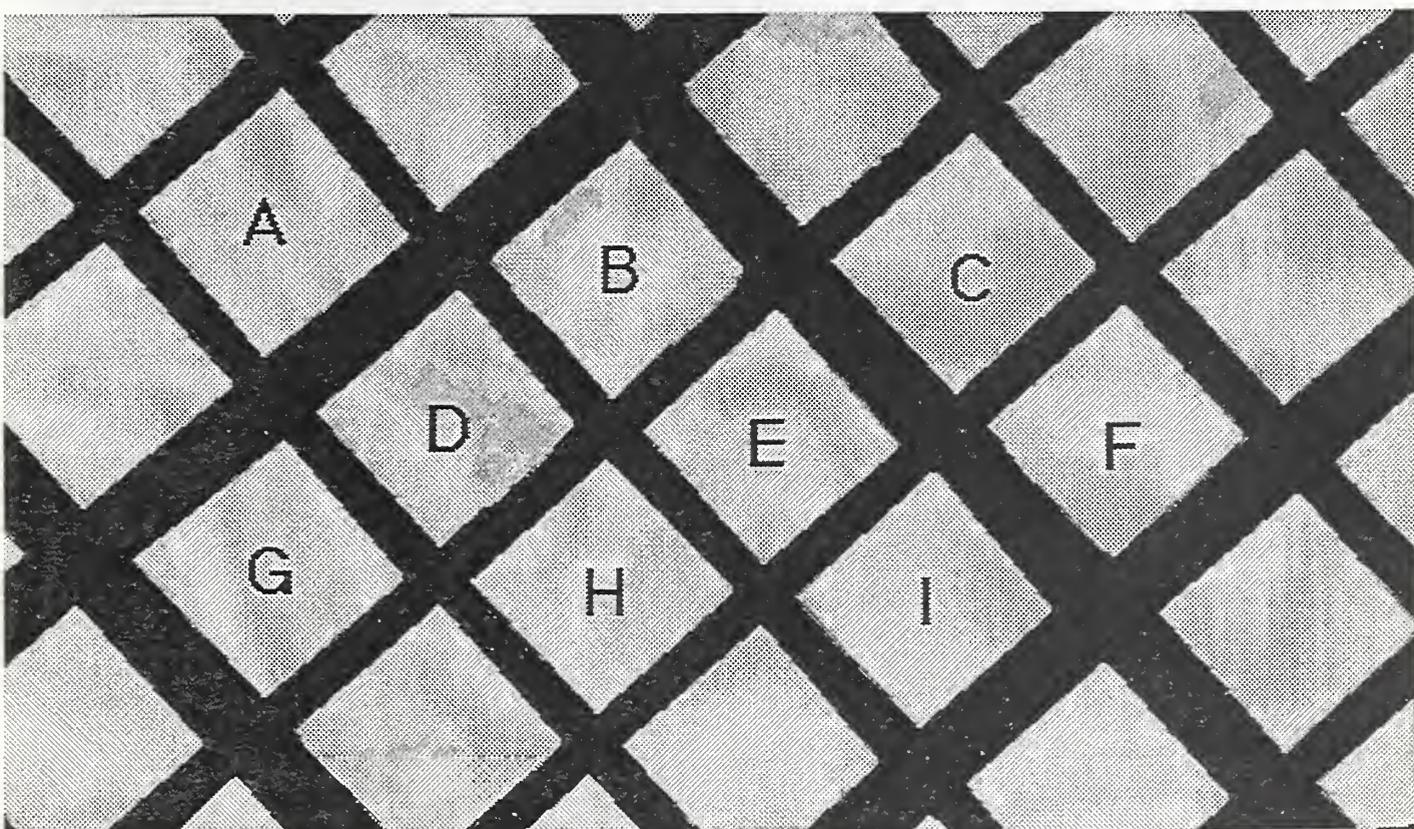
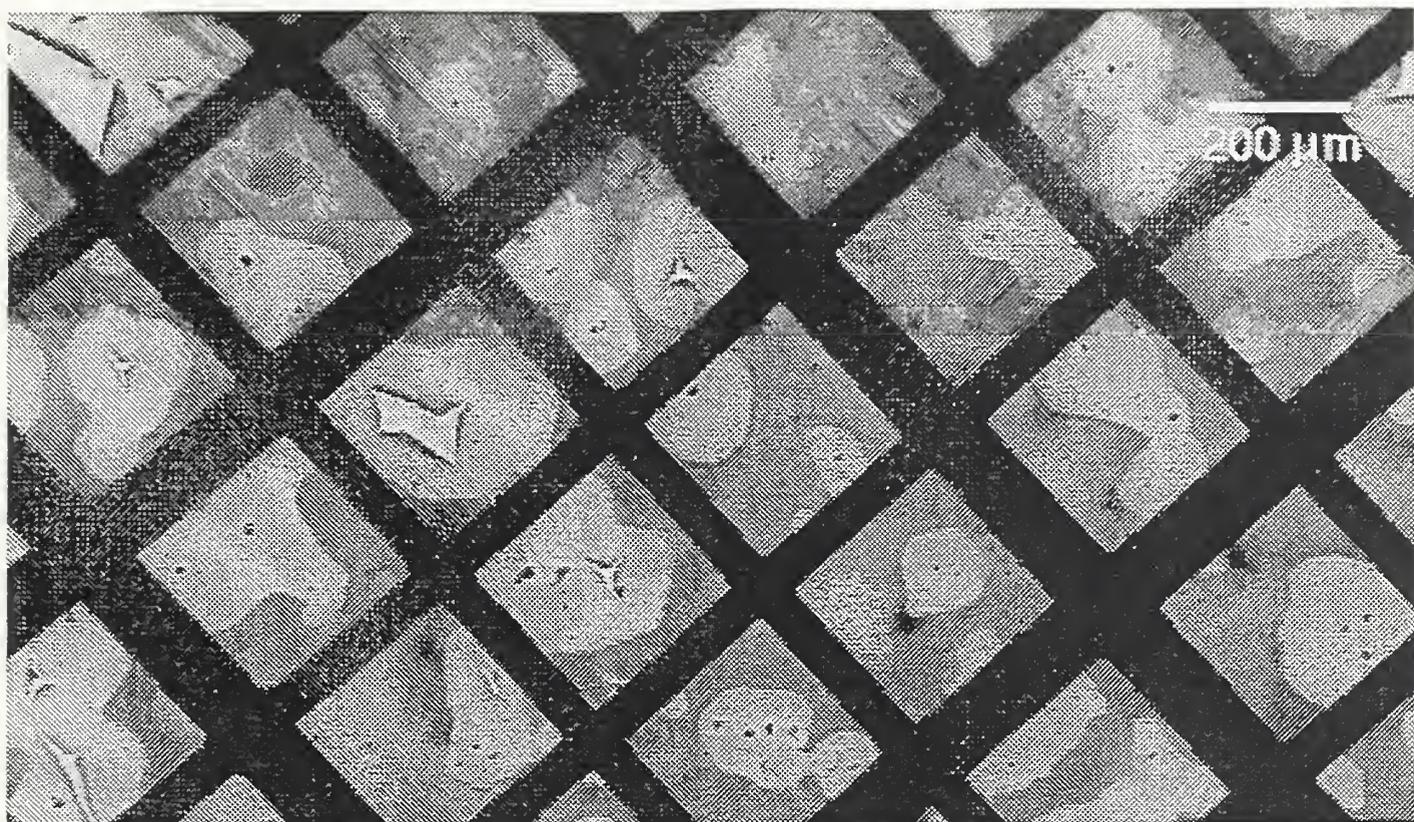


Figure 17c. The percentages of clouded layer on individual grid squares labelled A-I are as follows: A- 64 percent, B- 34 percent, C- 75 percent, D- 31 percent, E- 69 percent, F- 52 percent, G- 41 percent, H- 35 percent and I- 76 percent. The percentages of split replica on three squares are: B- 2.1 percent, D- 12 percent and H- 1.5 percent.

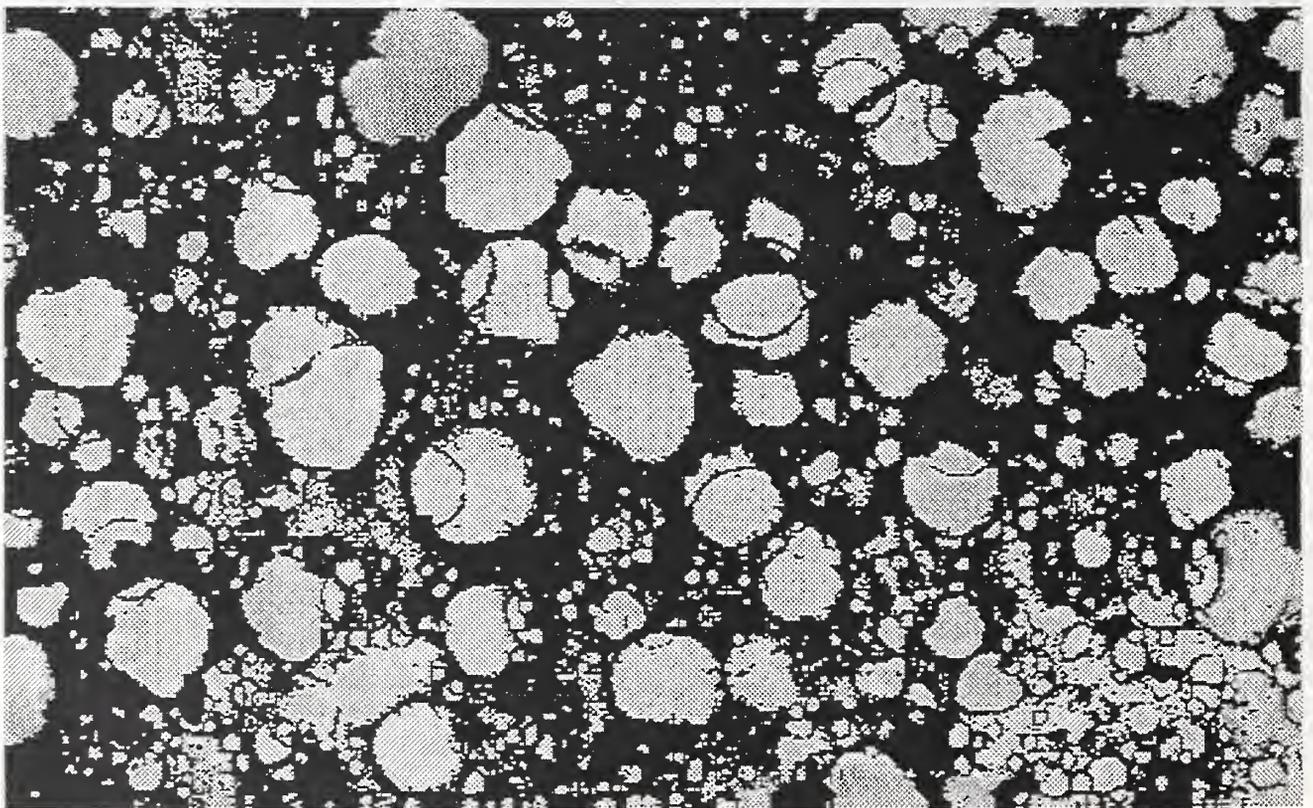
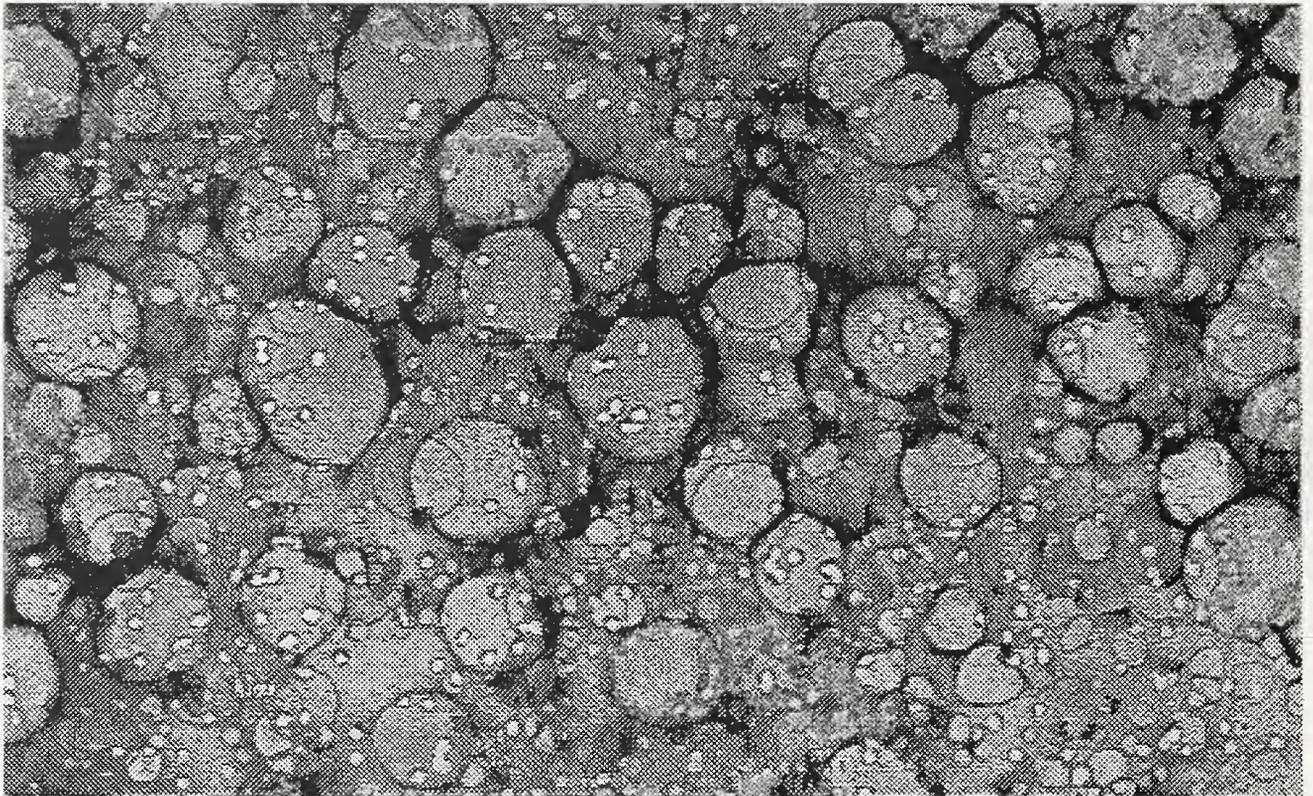


Figure 18. Image of overlying mesh (bubble-like mesh) on a PC filter replica (top).
The overlying mesh covers 59 percent of the total image (black areas in bottom image).

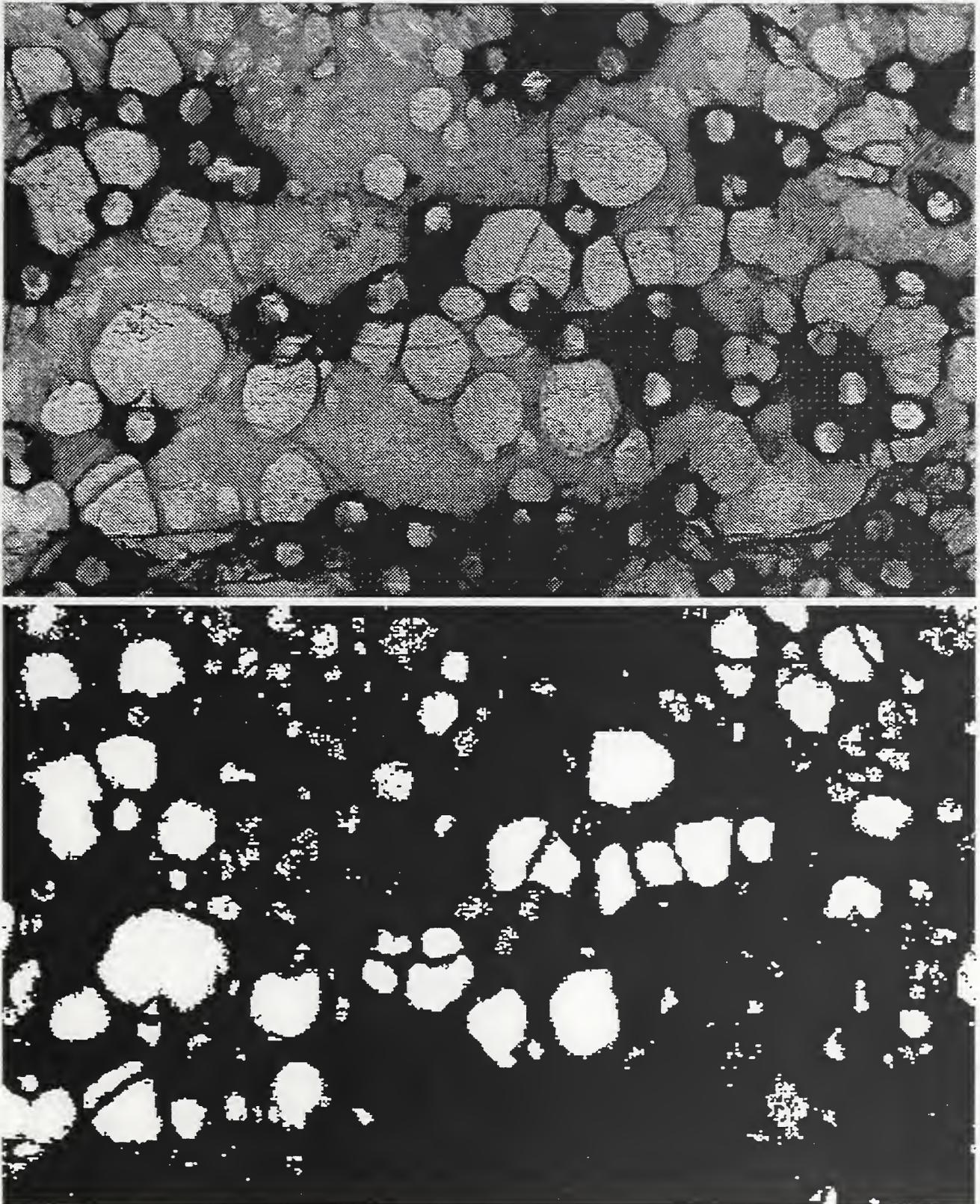


Figure 19. Image of interconnected pores and overlying mesh on a PC replica (top). The interconnected pores and overlying mesh cover 81 percent of the total image (black areas in bottom image).

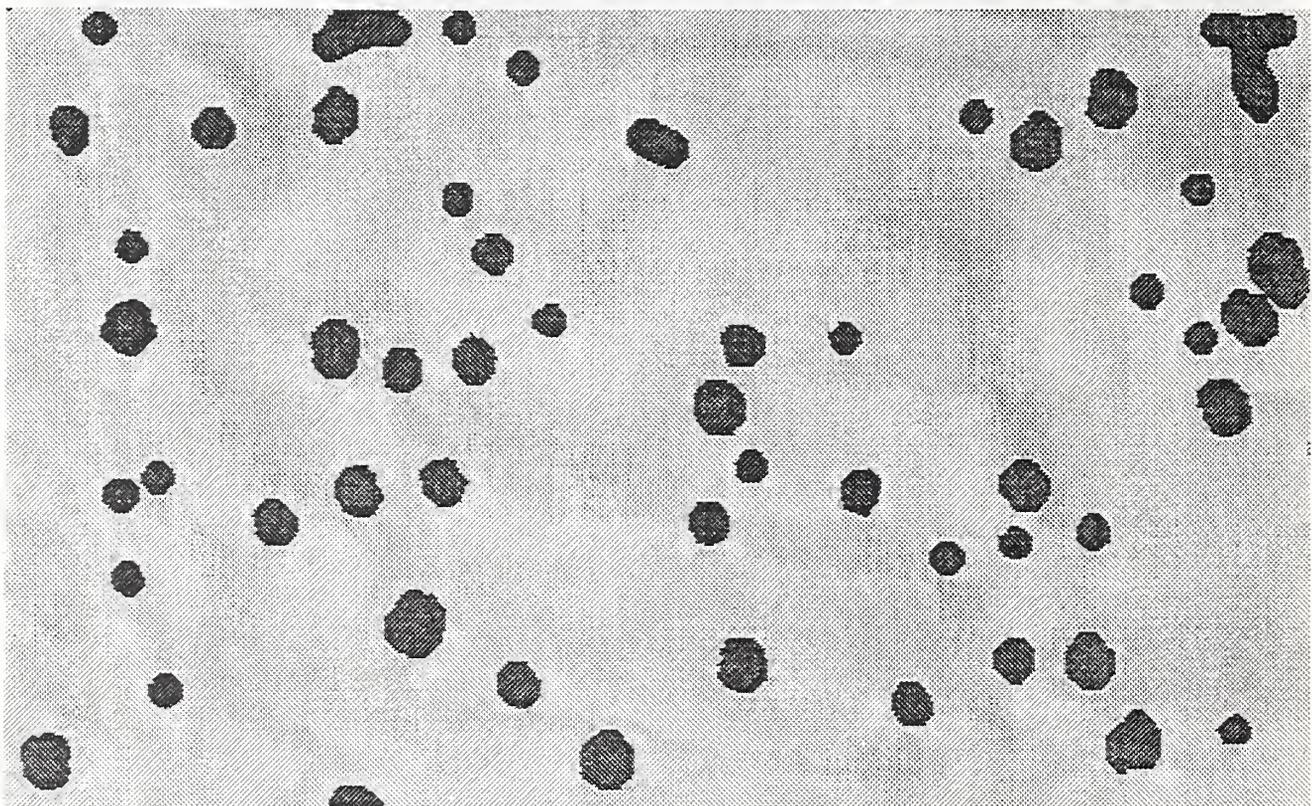
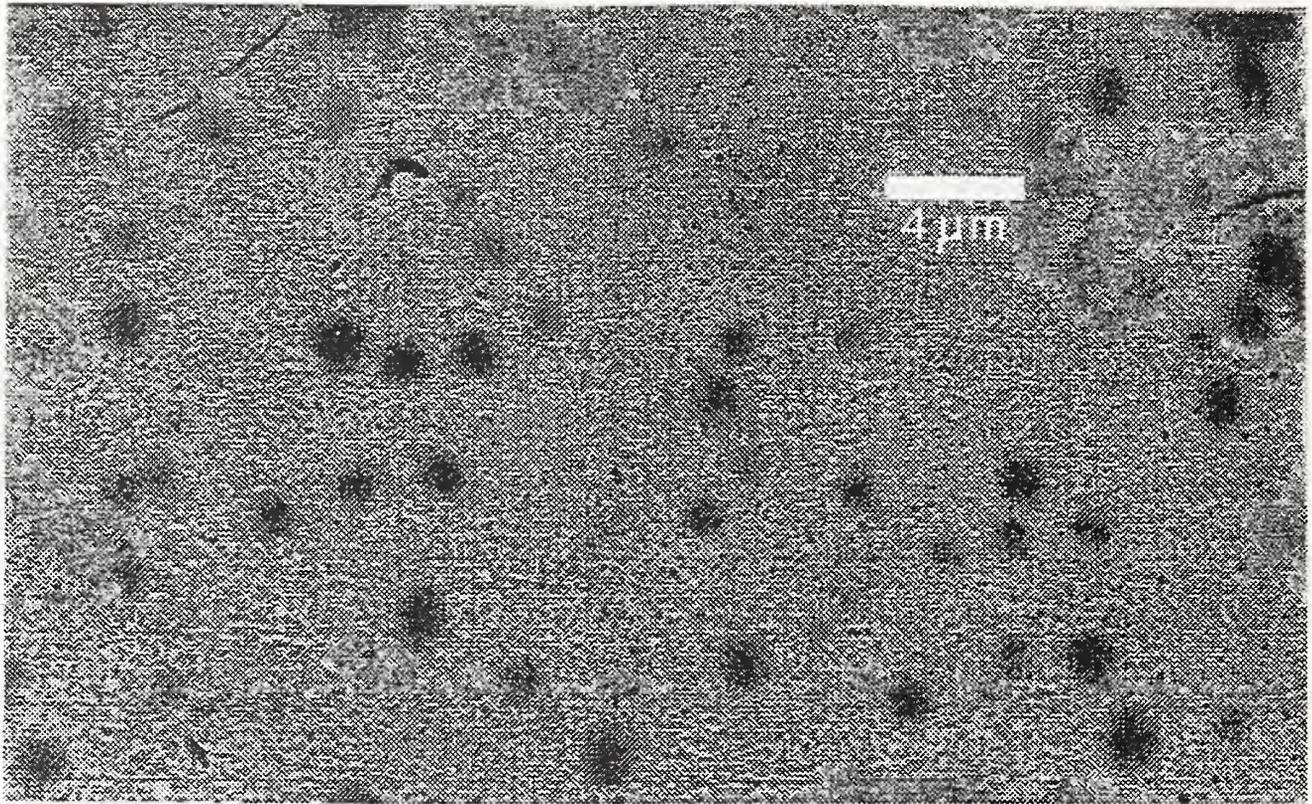


Figure 20. Image of round spots on an MCE replica (top). The round spots cover 9 percent of the total image (black areas in bottom image).

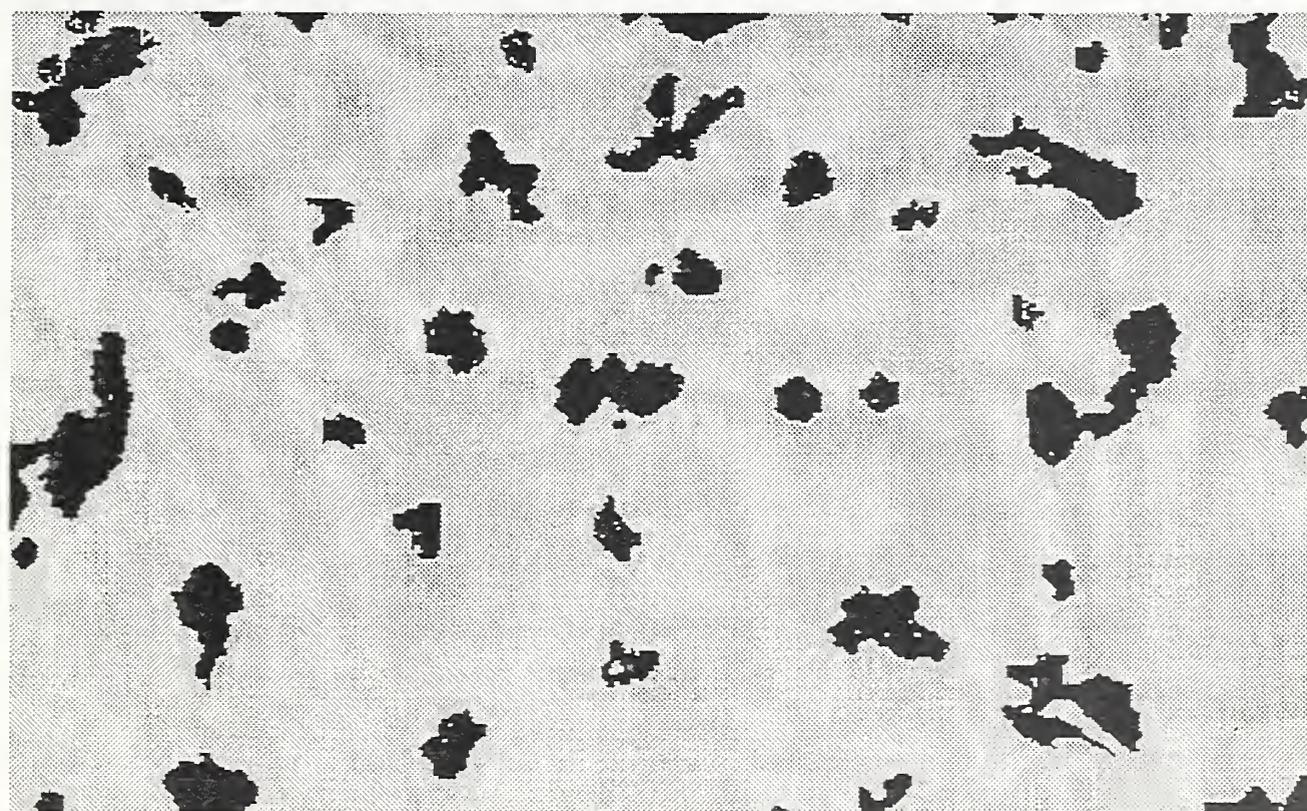
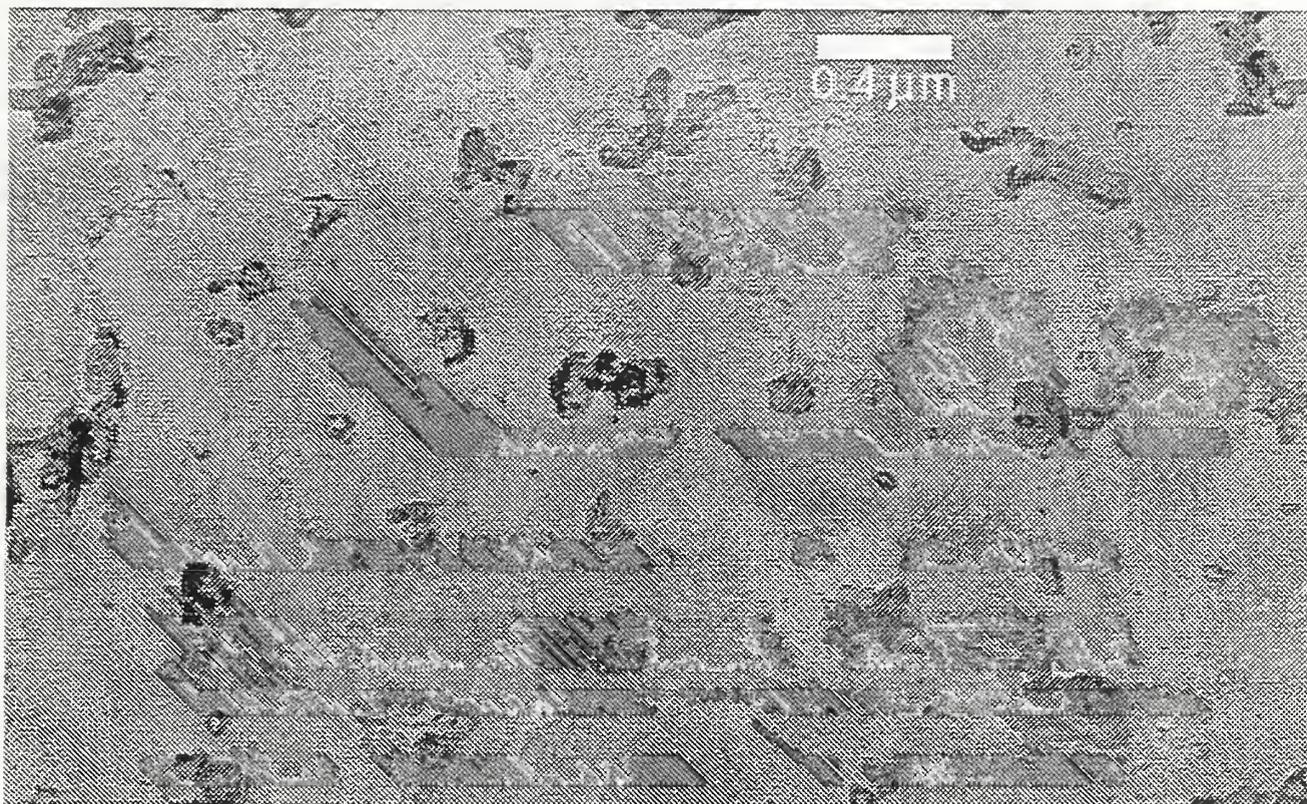


Figure 21. Image of Type 3 texture on an MCE replica (top). The Type 3 texture covers 11 percent of the total image (black areas in bottom image).

IV. Replica Evaluation Procedure and Forms

In this section, methods for evaluating the quality of filter replica preparations are described and forms that can be used with the procedures are given. The section is divided into four parts. Procedures for randomly choosing grid squares for TEM analysis are discussed in Part 1. The evaluation of preparations at low magnification by light microscopy and TEM is discussed in Part 2. A method for the evaluation of preparations by TEM at the analysis magnification is given in Part 3. A worked example of a grid analysis is given in Part 4. Information given in sections II and III of the handbook should be used for definitions of terms and for area estimates of various sample preparation problems.

Part 1. Methods for randomly choosing grid squares for TEM evaluation

Before replica preparations are examined by light microscopy or by TEM, a random ordering of grid squares should be established. The random sequence should not be biased by prior knowledge of the condition of the replica.

To obtain a random ordering of grid squares, the grid squares of a grid must be uniquely labelled. For example, a possible labelling scheme for a grid is shown in Figure 22. Grid squares in each of the nine sections with letters are labelled as shown for the section with the letter E. There are therefore unique labels for each of the 216 grid squares on this grid. Two methods for determining a random ordering of grid squares are described below.

Method 1. Random number tables

A procedure for using random number tables found in statistic books is as follows:

- 1) Obtain a random number table that is for numbers equal to or greater than the number of grid squares on your grid.
- 2) Compile a list of the grid squares for your grid type. In an adjacent column, write the numbers in the random number table that are equal to or less than the total number of grid squares on the grid.
- 3) Examine grid squares in the order indicated by the second column.

Method 2. Computer methods

There are random number generators in many computer programs, such as spreadsheets or databases. The procedure specified by the program should be used to generate a random sequence of grid squares.

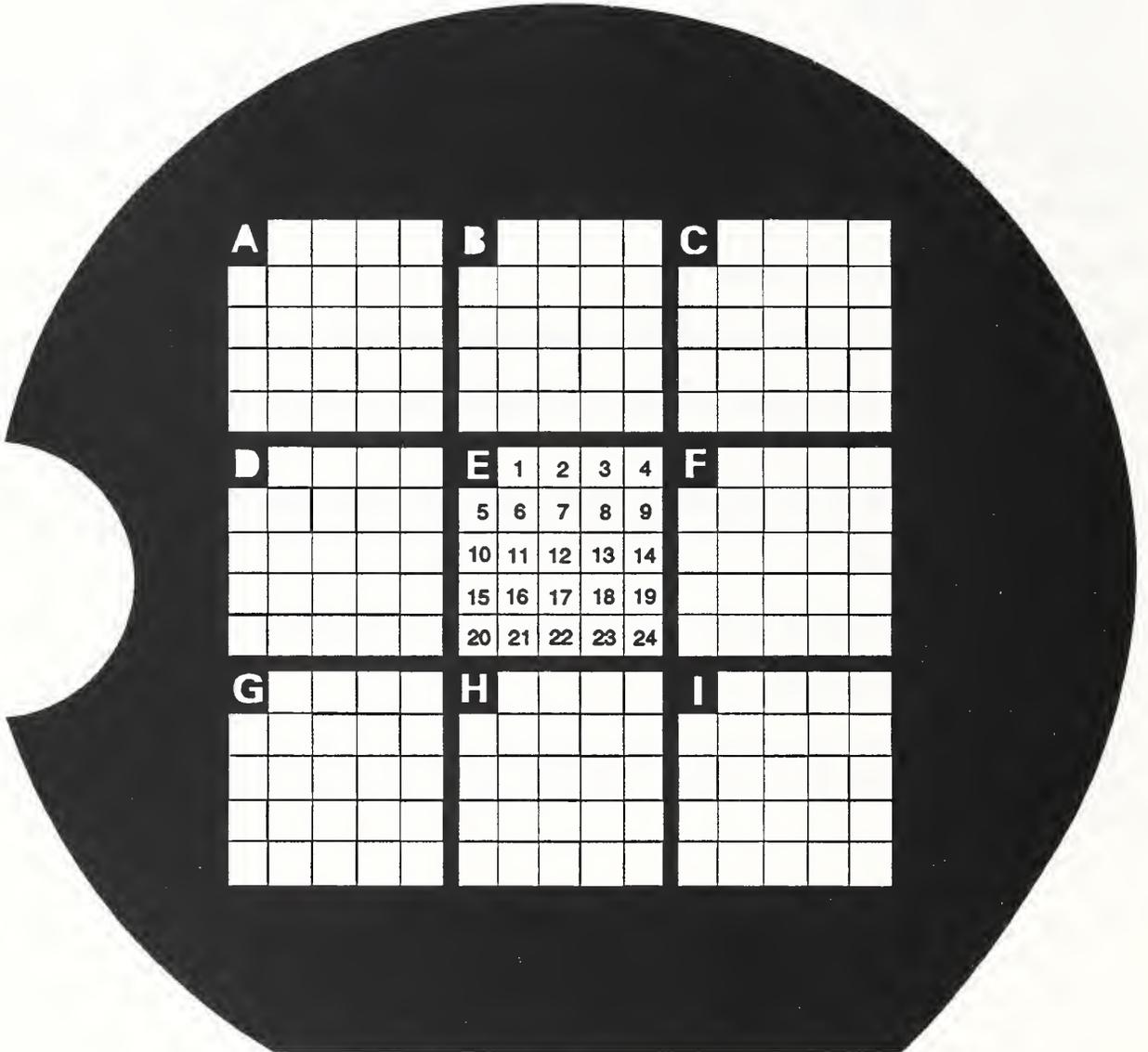


Figure 22. Example labelling scheme for a TEM grid.

Part 2. Low magnification evaluation: light microscopy and TEM

Light microscopy

An optical microscope is used for initial evaluation of a grid preparation. To preclude contamination, this optical microscope should not be used for bulk asbestos analysis. Observe the grid preparation at a magnification where all or most of the grid squares are visible. View the grid and replica for problems related to the grid, replica, presence of filter residue, particle loading, etc. Common-sense can be used at this point to reject a grid if there are obvious major problems. Some examples of severe problems include: 1) a grid that had been manufactured incorrectly so that the grid squares are unusual in appearance (e.g., unusually small squares or missing grid bars), 2) a grid that has been extensively deformed, 3) a replica that is folded in two, 4) filter material that has not been dissolved or 5) a heavy loading of particles. Information concerning the presence of problems should be recorded in the top, left-hand portion of Form 1. The grid should be rejected at this point if it contains a severe problem.

Determine the number of usable grid squares for the grid type used. Do not count squares that have an odd shape or that contain letters or numbers. This number does not have to be determined for each individual grid examined; it should be standard for a given type of grid. Record this value in the bottom table of Form 1. Determine the percentage of available grid squares that are covered by replica (include grid squares containing no replica or split replica if they are within the perimeter of the replica section). Reject the grid if this value is less than 50 percent. Determine the percentage of grid squares covered by replica that is acceptable. Record this information in the bottom table of Form 1. Reject the grid if this value is less than 50 percent.

Low magnification TEM

Scan in at least two perpendicular directions across the grid replica. As done by light microscopy, note any problems related to the grid, coherency of the replica, presence of filter residue, particle loading, etc. Record information related to problems in the top right-hand side of Form 1. In the 'comment' section, the estimated area affected by the problem can be noted. The grid can be rejected at this point if the rejection criteria are met (page 22 of handbook).

Form 1. Low magnification evaluation: light microscopy and TEM

Laboratory:

Sample:

Date:

Analyst:

View grid and replica by light microscopy (LM) and by low magnification TEM. Evaluation can be stopped if rejection criteria are met.

View grid for obvious problems:	LM	Comments	Low Mag. TEM	Comments
- grid problems - deformation of the grid - grid squares of unusual size, etc.				
- replica problems - overlapping replica - splits or holes - thick replica, etc.				
- filter residue problems - clouded features - bubble-like mesh, etc.				
- particle problems - high particle loading (> 10%) - missing or displaced particles, etc.				
- other obvious problems (if yes, describe in comment section)				

Determine the following values (using light microscopy):	Value	Comments
Number of potentially usable grid squares on grid		
Percentage of usable grid squares covered by replica		
Percentage of grid squares covered by replica that are acceptable		

Part 3. Analysis magnification: TEM evaluation

Several grid squares should be examined in the sequence determined by random selection (described in Part 1). The goal is to evaluate each grid square for the presence of artifacts and other features that might affect an analysis for asbestos. A determination is made as to whether a grid square is acceptable or unacceptable for asbestos analysis.

The grid squares should be examined at the magnification used for TEM analysis. The areas next to the grid bars should be examined because some sample preparation problems preferentially occur in this location. At a minimum, two perpendicular traverses should be made across the grid square. The sample preparation problems or artifacts and the estimated percentage of the grid square that is not suited for analysis should be noted on Form 2.

The grid squares should be judged as acceptable, not acceptable or not available for analysis. A grid square is not available for analysis if obscured by the sample holder or if not fully covered by replica because of its occurrence along the perimeter of the filter section. These grid squares do not factor into the analysis of the quality of the filter preparation. If five grid squares are judged acceptable, the replica should be considered acceptable and further evaluation can be stopped. Conversely, if five unacceptable grid squares are found, the replica should be considered unacceptable. Excluding grid squares not available for analysis, a minimum of five and a maximum of nine grid squares are examined (if nine grid squares are evaluated, five have to be either acceptable or unacceptable).

Part 4. Example analysis of a TEM grid

The following is a worked example of how to analyze a prepared TEM grid. Figure 23a shows a schematic of a TEM grid used for this example. The example includes an indexed grid with a carbon film replica that has some labelled preparation artifacts.

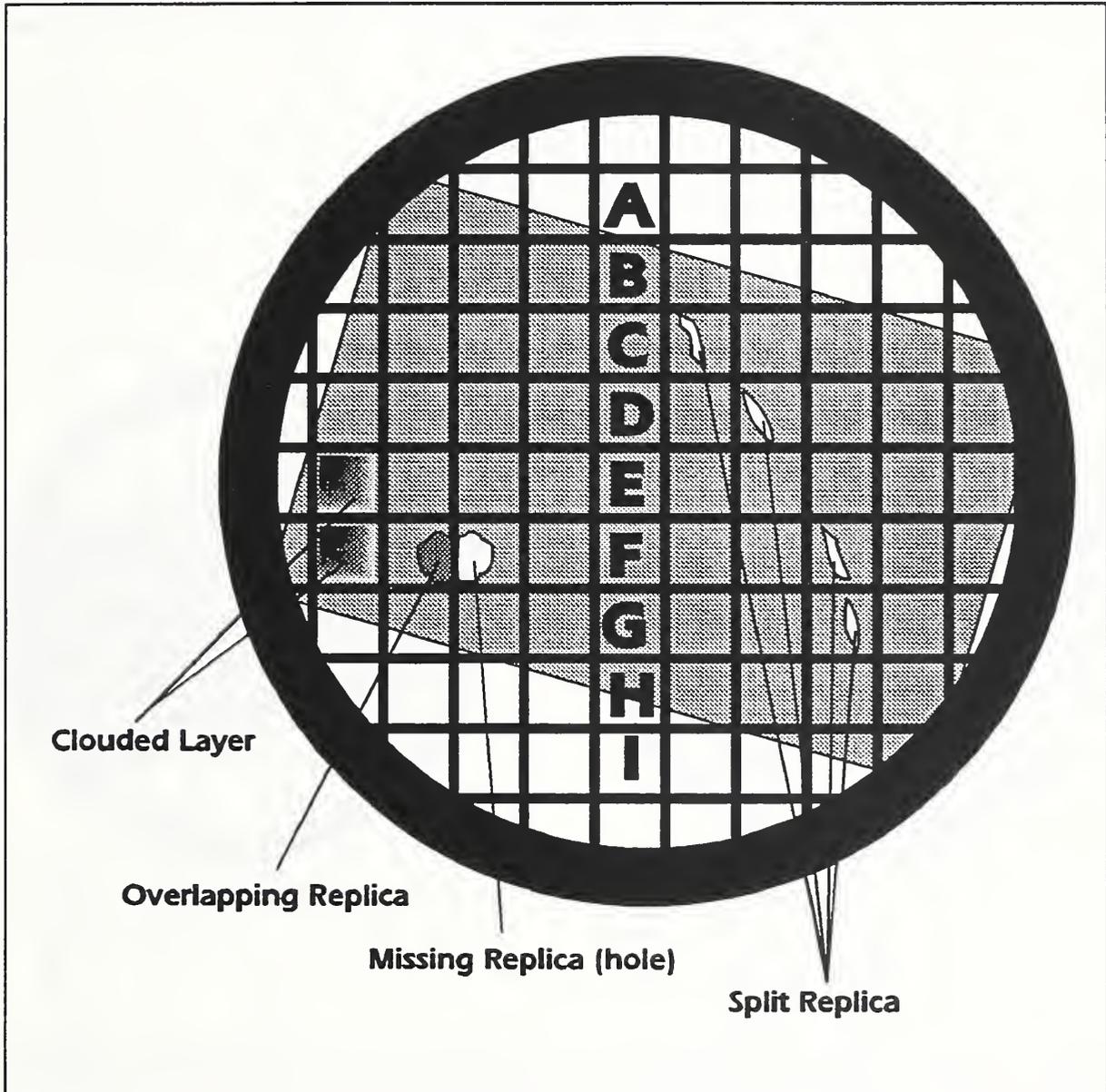


Figure 23a. Schematic of a prepared TEM grid.

Low magnification evaluation

The first step is to view the grid by light microscopy to check for severe problems (top section, Form 1). The grid in Figure 23a passes this portion of the test (Table 2).

In the next portion of the evaluation, the areal coverage of the replica and the percentage of acceptable replica are determined (bottom section, Form 1). First, the number of grid squares available on the grid is determined. From Figure 23b, it is shown that a grid of this type has 60 possible grid squares available for analysis.

For the next step, the number of grid squares enclosed by the perimeter of the replica is determined. Only those grid squares that are completely within the perimeter of the replica are counted. Such grid squares are indicated by stars in Figure 23c. Therefore, there are 39 grid squares within the perimeter of the replica for the 60 available grid squares. Thus the replica covers 65 percent $([39/60]*100)$ of the grid. The grid preparation is considered satisfactory for this portion of the analysis, i.e., the replica covers more than half the grid. The appropriate portion of Form 1 is shown completed for this grid in Table 2.

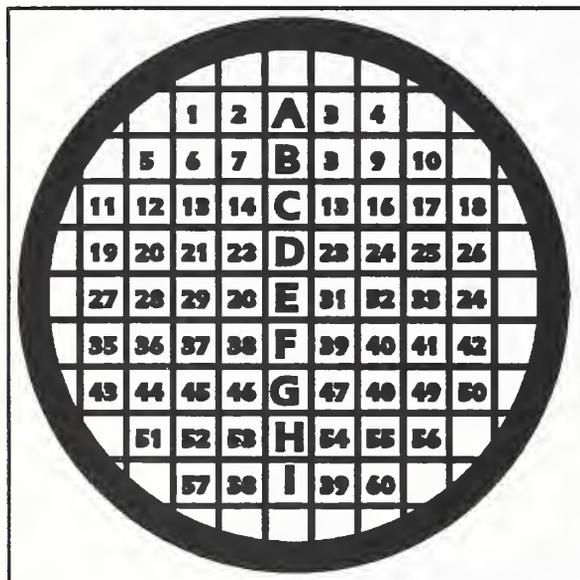


Figure 23b. Grid numbering system allowing identification of each square and showing the total number of available squares.

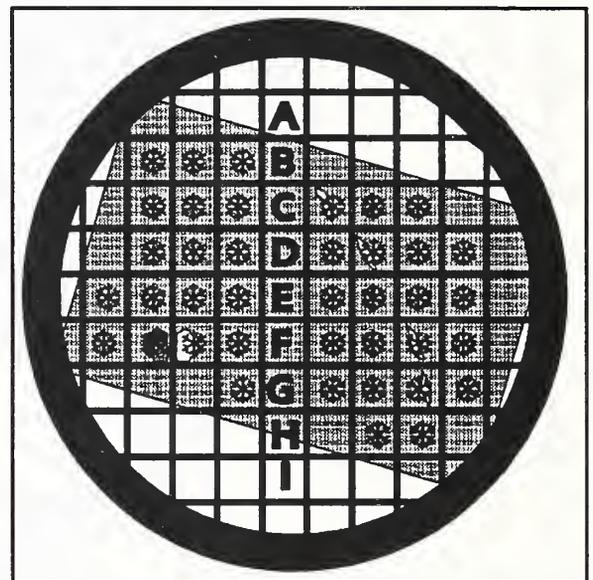


Figure 23c. Schematic showing the squares covered completely by replica (starred).

For the next step in this portion of the test, the number of grid squares that are acceptable is determined. Eight of the 39 grid squares that are fully covered by the replica are unusable due to sample preparation artifacts. Therefore, 31 of 39 squares (79 percent) are deemed adequate by light microscopy. Again, the preparation of the grid is satisfactory because more than half the replica is usable.

For the next step, the grid is observed at low magnification in the TEM. For this grid, no additional problems are noted (Table 2). Note: several preparation artifacts, such as the clouded layers, will be more evident in the TEM than in the light microscope.

Table 2. Example of low magnification evaluation (Form 1) completed for replica preparation shown in Figure 23a.

View grid and replica by light microscopy (LM) and by low magnification TEM. Evaluation can be stopped if rejection criteria are met.

View grid for obvious problems:	LM	Comments	Low Mag. TEM	Comments
- grid problems - deformation of the grid - grid squares of unusual size, etc.	N		N	
- replica problems - overlapping replica - splits or holes - thick replica, etc.	Y	Minor 4 squares - split replica 2 squares - hole, overlapping replica	Y	~ 10% of replica
- filter residue problems - clouded features - bubble-like mesh, etc.	Y	Minor 2 squares - clouded features	Y	~ 5% of replica
- particle problems - high particle loading (> 10%) - missing or displaced particles, etc.	N		N	
- other obvious problems (if yes, describe in comment section)	N		N	

Determine the following values (using light microscopy):	Value	Comments
Number of potentially usable grid squares on grid	60	
Percentage of usable grid squares covered by replica	65%	
Percentage of grid squares covered by replica that are acceptable	79%	

Analysis magnification evaluation

Before examination by TEM, a random set of grid squares should be chosen. A list of random numbers that can be used for choosing the order of analysis of grid squares for four different grids is given in Table 3. For this example, the column marked "grid 2" is used.

Table 3. Example random grid square sequence for four grids in an analysis sequence.

grid 1	grid 2	grid 3	grid 4
27	14	35	46
36	39	47	42
19	8	51	48
12	34	13	6
21	11	33	35
5	31	11	11
50	14	19	45
42	45	43	58
57	33	3	59
58	50	50	43
16	8	21	43
20	60	39	21
22	36	25	1
39	16	17	36
40	22	49	50
48	56	1	32
38	37	2	12

The grid squares are then observed at the magnification used for analysis (Form 2). The first grid square in the list of random numbers corresponds to square number 10. Since this grid square is near the edge of the grid, it is found to be covered by the specimen holder. This grid square is not counted. The next grid opening in Table 3 corresponds to square number 39 which is directly to the right of the letter F. No artifacts are observed and this grid opening is considered available for analysis. Opening 8 is only partially covered by replica so it is disregarded. More grid squares are observed using Table 3 and a summary of the grid square evaluations is given in Table 4.

At this point, the preparation of the grid has been found satisfactory using the TEM examination because 5 out of 6 grid squares were suitable for analysis. The analyst may then proceed with the analysis of the grid for asbestos. If the preparation of the grid was found to be unsatisfactory, then the grid would not be analyzed for asbestos. Instead, a satisfactorily prepared grid would need to be found.

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Appendix A. Definitions of some terms related to asbestos analysis.

General terms:

AHERA: Abbreviation for the Asbestos Hazard Emergency Response Act which was enacted by Congress in 1986. The act mandated that all schools in the United States be evaluated for the presence of asbestos.

AHERA method: Term used in this handbook to refer to the methodology written by the EPA for identification and analysis of asbestos in air-collected samples obtained from schools².

counting rules: Procedure for determining the number of asbestos structures in a particle. A set of counting rules is given in the AHERA method.

Terms related to asbestos particles:

fiber: A structure with greater than or equal to $0.5\ \mu\text{m}$ in length with an aspect ratio (length to width) of 5:1 or greater and having substantially parallel sides.

bundle: A structure composed of three or more fibers in a parallel arrangement.

cluster: A structure with fibers in a random arrangement such that all fibers are intermixed and no single fiber is isolated from the group.

matrix: Fiber or fibers with one end free and the other end embedded in or hidden by a particulate.

structure: A bundle, cluster, fiber or matrix particle that contains asbestos.

