Concentrations and dimensions of coated and uncoated asbestos fibres in the human lung

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ABSTRACT   Concentrations and length distributions of uncoated and coated amphibole-type fibres in samples of human lung taken at necropsy were measured by optical microscopy using the membrane filter technique that enables fibres with diameters down to about 0·2 μm to be detected. The subjects included 10 who died with mesothelial tumours, three with lung cancer, and eight of other causes. Measurements of fibre concentrations are compared with those of other workers. It can be deduced from the length distributions that fibres less than 5 μm long are cleared from the lung more efficiently than longer ones. The clearance of short fibres appears to be inhibited in subjects with asbestosis, however. The length distributions of uncoated and coated fibres were dissimilar. In general, few fibres < 10 μm in length were coated and few > 40 μm in length were uncoated. The probability of a fibre of given length, however, becoming coated varied considerably from subject to subject. Possible reasons for this are discussed.

The inhalation of fibrous materials may cause lung fibrosis or tumours, which can be either bronchial or mesothelial in origin. Most studies with experimental animals have indicated that fibrogenicity is dependent on fibre length.[1-3] Fibres are probably only actively fibrogenic if they exceed a critical length, which is probably about 10-20 μm. Studies on the carcinogenic effects of fibres have also emphasised the significance of length.[4-7] Long fibres are thought to be more carcinogenic than short and long thin fibres may be particularly active in this respect.

Most current studies of asbestos fibres in human lung are concerned either with estimating the concentration of uncoated fibres present[8] and their topographic distribution[9] or with the identification of the fibre types.[10] As many fibres in human lung are too thin to be detected with the optical microscope, transmission or scanning electron microscopes, often with analytical attachments, are generally used for such investigations. With the electron microscope, however, it is difficult to measure the length of long uncoated and coated fibres (which may exceed 300 μm in length) and therefore to obtain accurate fibre length distributions.

There is a consensus among workers measuring asbestos fibre concentrations in air (using membrane

filters and phase contrast optical microscopy) that fibres with diameters equal to or greater than about 0·2 μm can be detected. Pooley[11] has reported mean values for the diameters of various types of fibre isolated from human lung tissue. The values for crocidolite and amosite were 0·14 and 0·24 μm respectively, which suggest that it should be possible to detect a reasonable fraction of amphibole fibres with the optical microscope. The corresponding value for chrysotile was 0·11 μm. Ashcroft and Heppleston,[12] who counted fibres in a Fuchs-Rosenthal counting chamber, claimed to have detected between 12% and 30% of the total fibres present in human lung. It would be expected that this could be improved on using the membrane filter technique. A preliminary comparison of fibre densities on membrane filters by electron and optical microscopy indicates that it is possible to detect about 30% of crocidolite fibres and at least 50% of other amphiboles by optical microscopy.

In digesting lung tissue for the determination of either fibre concentration or dimensions, any process that may damage fibres either by shortening or by longitudinal splitting must be avoided. Ashcroft and Heppleston[12] pointed out that drying tissue before maceration leads to fracture of the longer uncoated and coated fibres and that fibre counts may be exaggerated as a result. The rapid dimensional changes in and local overheating of

Table 1 *Details of subjects from whom lung tissue was obtained at necropsy*

Ref	Sex	Age at death	Cause of death	Asbestosis (A) or plaques (P)	Occupational exposure
M1	F	60	Mesothelioma	P	Gas mask factory 1940-5 and 1955-9
M2	M	56	Mesothelioma	A	Cleaner in shipyard
M3	M	70	Mesothelioma	P	Asbestos factory most of working life
M4	M	47	Mesothelioma	P	General labourer at glass factory before 1965
M5	M	81	Mesothelioma	P	Boiler maker 1913-62
M6	F	72	Spontaneous pleural mesothelioma	—	Tobacco factory all her life
M7	M	80	Mesothelioma	P	Shipyard worker from 1922 to retirement in 1962
M8	M	64	Mesothelioma	NA	Shipyard worker (driller) 1927-77
M9	M	75	Mesothelioma	—	Shipyard apprentice at 17. Joined LNER at 21. From 1945 to 1967 worked for BR engineering
M10	M	76	Mesothelioma	—	Chemical industry from 1931 until retirement in 1964
C1	M	56	Lung cancer	NA	Pipe lagger for eight years before death
C2	M	60	Undifferentiated lung cancer	P	Worked in shipyard for unspecified period
C3	M	70	Adenocarcinoma of lung	NA	Probably coalworker but worked in asbestos factory for one year in 1946
O1	M	56	Diffuse reticulosarcoma	—	Docker
O2	M	71	Cirrhosis of the liver	P	Mixed paint containing asbestos up to 1958. No exposure since
O3	M	88	Cardiac failure	—	Retired bricklayer
O4	F	87	Haemopericardium	—	NA
O5	M	72	Myocardial fibrosis	—	NA
O6	F	77	Pulmonary embolism	—	NA
O7	M	19	Multiple injuries	—	NA
O8	F	60	Cerebral haemorrhage	—	Housewife

NA = Information not available.

tissue during low-temperature ashing make this procedure even more suspect than oven drying. It is well-known that sonication can result in the longitudinal splitting of both amphibole and chrysotile fibres, and must also be avoided. Despite contraindications, drying, low-temperature ashing, and sonication are included in several published procedures for isolating fibres from lung.

In the present investigation the concentrations of amphibole-type fibres were measured in 21 samples of human lung taken at necropsy. The concentrations of uncoated and coated fibres were measured separately; no attempt was made to include chrysotile fibres, most of which would not have been detected. The length of each fibre (both uncoated and coated) was measured and the length distributions determined in each sample for which sufficient data were available.

Methods

MATERIAL
The samples of lung tissue examined were obtained from 10 patients who died with mesothelial tumours, three with other types of lung cancer, and eight who died of other causes. In some cases samples of associated tumour and pleural plaques were also analysed. The mesothelioma lung specimens had been submitted to the Manchester Pneumoconiosis Medical Panel by coroners in the north west of

England in 1977 and 1978. Table 1 gives details of the subjects, together with available information on their occupational histories.

DIGESTION OF LUNG TISSUE
A technique for the digestion of either fresh or formalin-fixed lung tissue using hydrogen peroxide has been described by Morgan *et al*.[13] More recent work has shown that the hypochlorite technique[9] is more convenient. A block (about 0·5 g) is cut from the tissue with a clean scalpel and weighed. A second, adjacent, sample about 1 g in weight is taken to determine the fresh/dry weight ratio. The sample to be analysed is placed in a 50 cm³ plastic screw-top centrifuge tube (Falcon, Cockeysville, USA) and 15 cm³ of Parozone bleach (Jeyes, Thetford, Norfolk) added. The tube is left overnight at room temperature. If digestion is incomplete, a further 5 cm³ of bleach may be added and the tube heated at 50°C. When digestion is complete the tube is cooled and diethyl ether (10 cm³) added. The capped tube is shaken to extract lipids, centrifuged, and the ether removed with a Pasteur pipette. The extraction is repeated with a further 10 cm³ of ether, and residual solvent is removed by heating the tube on a water bath. A few drops of ethanol are added and the walls cleaned with a rubber "policeman" to ensure that all fibres are in suspension. The volume is made up to 25 cm³ with water and an aliquot (generally 2·5 cm³) filtered on a 2·5 cm diam

Millipore GS membrane filter (pore size 0·22 μm). The filter is washed well with distilled water and allowed to dry. Half of the filter is placed on a microscope slide and cleared either with acetone vapour or with a dimethylformamide/acetic acid/ water mixture (Le Guen, personal communication). Finally, the cleared filter is mounted in neutral mounting medium (Gurr) under a coverslip. All reagents used in the digestion procedure should be filtered through membrane filters before use. Even diethyl ether was found to contain fibres. Appropriate blank samples are prepared from time to time to confirm the absence of fibres.

For transmission electron microscopy studies the remainder of the Millipore filter is sputtered with a carbon film. A small disc (3 mm diam) is cut from the filter and placed on an electron microscope grid. The filter is dissolved with acetone vapour, thus depositing the fibres and carbon film on to the grid.

The advantage of this digestion technique is that all operations are carried out in the same tube. Although the sample is centrifuged during the extraction of lipids, only the upper (organic) phase is removed so that losses of fibre, which have been reported by Ashcroft and Heppleston to occur during centrifuging,[12] are avoided. Details and an assessment of the procedure will be published elsewhere.

MEASUREMENT OF FIBRES
Uncoated fibres greater than about 2·5 μm long were counted and sized under phase contrast with a Zeiss photomicroscope III using a magnification of about ×640 (×10 eyepiece, ×1·6 Optovar, ×40 objective) and Porton-type eyepiece graticule. Coated fibres were counted and sized at a magnification of about ×256 (×16 objective). To measure the density of uncoated fibres continuous scans parallel to the cut edge of the filter were made, until either 200 fibres had been measured or an area of about 2 mm² had been covered. To measure the density of coated fibres it was sometimes necessary to scan the entire half filter. Applying the appropriate corrections, the results were expressed as 10^6 fibres/g dry weight of tissue for uncoated fibres and 10^3 fibres/g dry weight for coated fibres. In terms of wet weight, values were 5 to 10 times lower. The length of each coated or uncoated fibre was measured and classified in the categories given in tables 4 and 5.

Results

Table 2 gives the concentrations of uncoated and coated fibres in lung tissue. The values for uncoated fibres ranged from less than 0·1 to 370 × 10^6/g dry weight. The corresponding values for coated fibres were 0 to 11 500 × 10^3/g dry weight. Included in table 2 are values for the uncoated/coated fibre ratio in cases where the result can be derived with reasonable accuracy. The presence of non-fibrous material, indicative of occupational exposure is also noted. Particles of coal were present in most samples examined. The particles of calcium carbonate in C1 (fig 3) are probably an artifact arising from the use of calcium acetate to neutralise the formalin injected into the lung.

Table 2 *Concentrations of uncoated and coated fibres in lung tissue*

Ref	No of determinations	Uncoated (10^6/g dry wt)	Coated (10^3/g dry wt)	Uncoated/ Coated	Non-fibrous material
M1	2	13	340	38	—
M2	2	370	11500	31	++
M3	1	1·3	5	270	+
M4	3	3·6	86	42	++
M5	4	42	4840	9	++
M6	2	0·6	3·4	200	—
M7	1	0·5	880	0·6	++
M8	1	1·0	140	7	+
M9	3	0·6	35	17	+
M10	2	<0·1	8	10	+
C1	3	0·4	100	4	+++[a]
C2	6	0·1	3·3	40	+
C3	2	<0·1	1·0	—	+++[b]
O1	4	0·9	210	5	++
O2	3	2·1	19	110	+
O3	3	<0·1	1·2	17	++[b]
O4	2	<0·1	0·4	—	+
O5	3	<0·1	0·6	—	+
O6	3	<0·1	Nil	—	—
O7	3	<0·1	<0·1	—	—
O8	3	<0·1	<0·1	—	—

a = Calcium carbonate.
b = Coal.

Table 3 *Concentrations of uncoated fibres in samples of mesothelial tumour or plaque*

Ref	No of determinations	Tumour (T) or plaque (P)	Fibres (10⁶/g dry wt)
M5	1	T	0·03
M7	1	T	0·03
M8	1	T	<0·01
M9	1	T	<0·01
M10	1	T	0·01
O1	1	P	<0·01

Fig 1 *Length distributions of coated (o) and uncoated (●) fibres from subjects M1, M2, and M5.*

Short uncoated fibres were detected in samples of tumour and in plaques and table 3 gives their concentrations. No coated fibres were detected in any of the samples of tumour or plaque examined.

Table 4 gives measurements of the length distributions of uncoated fibres. The results are expressed as the frequency of fibres in six length categories from < 4·9 μm to > 80 μm. The median lengths obtained from cumulative distributions plotted on a logarithmic probability paper (fig 1) are included in table 4. In some cases insufficient fibres were detected to permit analysis of the data in this way. Table 5 gives corresponding values for coated fibres.
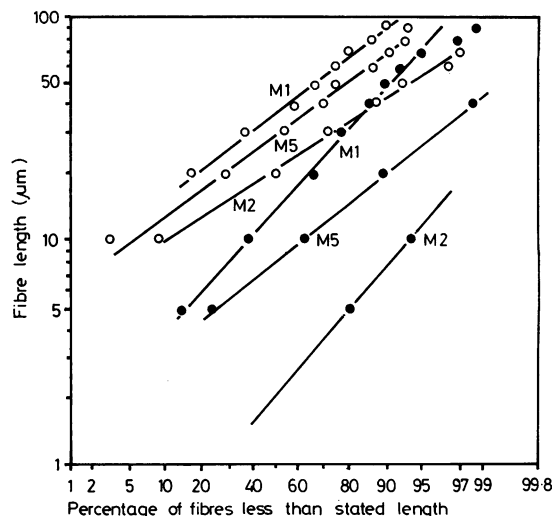
Table 4 *Length distributions of uncoated fibres*

Ref	No of fibres	% of fibres of length (μm) 2·5-4·9	5-9·9	10-19·9	20-39·9	40-79·9	>80	Median length (μm)
M1	120	15·0	23·3	28·3	19·2	12·4	1·8	14
M2	217	72·4	19·8	7·8	—	—	—	2
M3	106	36·8	50·0	8·5	4·7	—	—	6
M4	231	7·4	24·2	26·5	27·7	12·6	1·6	15
M5	538	24·9	38·3	26·4	9·1	1·3	—	8
M7	27	11·1	55·6	33·3	—	—	—	9
M8	44	20·5	40·9	25·0	13·7	—	—	8
M9	66	15·2	33·0	37·9	9·1	4·5	—	10
C1	121	11·6	24·8	32·2	26·4	4·9	—	13
C2	88	22·7	38·6	23·9	12·5	2·2	—	9
O1	45	20·0	42·2	17·8	17·8	2·2	—	9
O2	90	25·6	37·8	25·6	10·0	1·1	—	8

Table 5 *Length distributions of coated fibres*

Ref	No of fibres	% of fibres of length (μm) 2·5-4·9	5-9·9	10-19·9	20-39·9	40-79·9	>80	Median length (μm)
M1	112	—	—	17·9	41·0	28·5	12·6	36
M2	118	—	9·3	40·7	36·4	11·8	1·6	21
M3	59	—	1·7	3·4	27·1	45·8	22·1	48
M4	149	—	0·7	6·0	45·6	33·6	14·1	43
M5	274	—	3·3	26·6	40·2	23·2	6·6	29
M7	86	—	—	25·6	44·2	26·8	3·6	29
M8	60	—	—	10·0	40·0	33·3	16·7	41
M9	249	—	0·4	8·8	53·0	30·0	7·6	38
M10	47	—	—	27·7	27·7	34·1	10·7	33
C1	87	—	4·6	27·6	27·5	28·7	11·4	30
C2	110	—	—	18·2	40·0	26·4	15·4	36
O1	105	—	1·9	15·2	45·8	30·6	6·6	34
O2	38	—	—	23·7	44·7	28·9	2·6	30

Discussion

Uncoated fibres were detected in all the samples of lung tissue examined. The concentrations of uncoated fibres in those members of the non-neoplastic "O" series who were unlikely to have been exposed to asbestos occupationally were relatively low ($< 0.1 \times 10^6$/g dry weight of lung). M10 and C3 also fell within the same range. For subjects likely to have been exposed to asbestos occupationally the values ranged from 0.1 to 370×10^6/g dry weight. The only exception to this general rule was M6. Although this subject worked in a tobacco factory all her working life, the concentration of uncoated fibres in her lungs was above the lower limit of the occupationally exposed range.

The best series with which to compare these results is that of Whitwell *et al*[14] who measured the concentration of fibres (uncoated plus coated) in the lungs of patients who had died with mesothelial tumours, lung cancer, and of other causes. These workers used the counting chamber technique and included only those fibres exceeding 6 μm in length as against 2.5 μm in the present study. The effect of this higher cut-off would be to underestimate fibre concentrations, particularly in a case such as M2, where a high proportion of fibres are < 5 μm long, and also in non-occupationally exposed subjects in whose lungs short fibres predominate. In Whitwell's normal control series[14] the fibre concentration was less than 0.1×10^6/g dry weight in all samples, and most were below 0.01×10^6. In his mesothelioma series 80% of the lungs had concentrations exceeding 0.1×10^6/g dry weight as against 90% in the present (smaller) series. In the series published by Ashcroft and Heppleston[12] the concentration of uncoated fibres in subjects likely to have been exposed to asbestos occupationally, but without asbestosis, ranged from 0.1 to 16.4×10^6/g dry weight. In five subjects with moderate asbestosis the mean value was 203×10^6. The present results accord with the findings of the earlier studies.

Concentrations of uncoated fibres in the samples of tumour and plaque examined were, without exception, relatively low ($< 0.1 \times 10^6$/g dry weight). The concentration of fibre in the tumour samples examined did not appear to be correlated with that in adjacent lung tissue. No coated fibres were detected in any of the samples of tumour or plaque examined.

The concentrations of coated fibres in people likely to have been exposed to fibres occupationally (not necessarily in the asbestos industry itself) ranged from 1 to 11 500 $\times 10^3$/g dry weight of lung. In lung samples from people without evidence of occupational exposure the upper limit appeared to be about 0.5×10^3/g dry weight, and in one subject no coated fibres were detected.

One of the main objectives of this study was to obtain evidence to support the contention that short (< 5 μm) uncoated fibres are cleared from the lung via the airways as has been shown by Morgan *et al*[13] to occur in rats. Gibbs[15] has reported that, of the
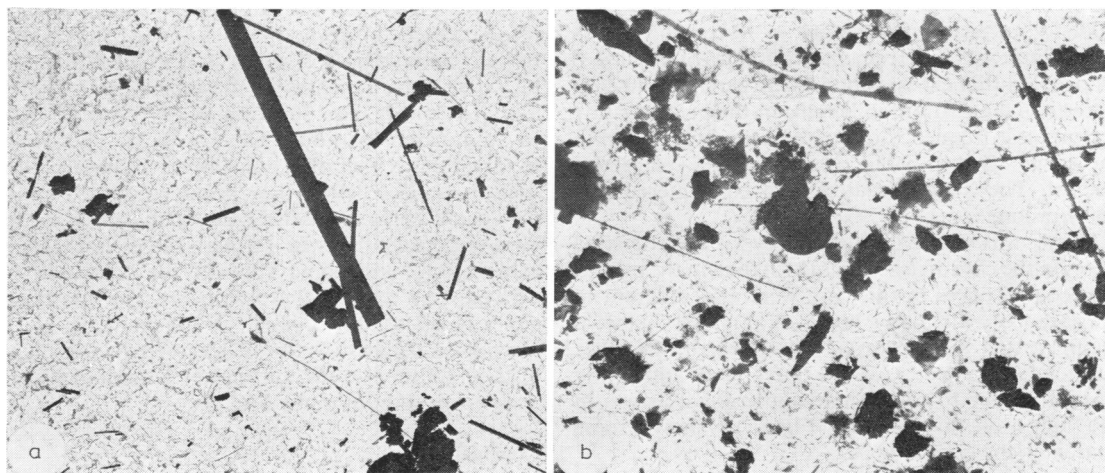

Fig 2 (a) *Electron micrograph of fibres isolated from lungs of subject M2 showing predominance of short (< 5 μm) fibres and wide variation in thickness. Thickest fibre about 1 μm in diameter. (b) Electron micrograph of fibres isolated from lungs of subject M1 showing predominance of relatively long fibres. Thickest fibre about 0.2 μm in diameter (both $\times$ 2400).*

airborne fibres produced in various operations in the asbestos industry, generally over 90% are less than 5 μm in length. The percentage of fibres of this length deposited in the alveolar region of the lung is likely to be at least as high due to the interception of long fibres in the small conducting airways. Table 4 gives length distributions of uncoated fibres for subjects in which more than 25 such fibres were measured. Omitting case M2, for reasons discussed below, the mean frequency of fibres less than 5 μm long was only 19%, which supports the contention that fibres in this range are removed preferentially from the lung, presumably by macrophage-mediated transport to the terminal bronchiole followed by mucociliary clearance. In M2, the only case of asbestosis, the value was 72% and fig 2a illustrates quite clearly the predominance of short fibres in this case. We suggest that the anomalous result from M2 is a direct consequence of the asbestosis and the inability of fibres of any length to be cleared from fibrotic areas of lung. Further work is required to substantiate this conclusion.

In general, the greatest frequency of fibres was found in the 5-10 μm range indicating that fibres in this category are cleared less efficiently than shorter ones. In some subjects no uncoated fibres longer than 40 μm were detected but in others, notably M1 and M4, about 15% of the fibres fell into this category. Pooley[11] reported an upper limit of 40 μm for fibres of crocidolite and amosite in human lung but it is clear from the present work that longer uncoated fibres may be present in many cases.

The length distribution of coated fibres is quite different from that of uncoated. On average, less than 1% of coated fibres were < 10 μm long. This lower limit is in agreement with that of Pooley,[16] who stated that asbestos bodies are rarely formed on fibres less than 10 μm in length. In the present study the greatest frequency of coated fibres was found in the range 20-40 μm but on average 10% exceeded 80 μm. The longest coated fibre encountered was 360 μm long.

There was a greater frequency of short-coated fibres in the case of asbestosis (M2) than in the others. The coated fibres in this case were also different in appearance with many being of dumbell shape, a configuration that occurred only rarely in the other subjects examined. Coated fibres had apparently fused together to form three-dimensional arrays in the lungs of this subject. Figure 3 shows some characteristic coated fibres.

Comparing the length distributions of uncoated and coated fibres shows that the longer the fibre the greater the probability of it becoming coated. The probability of a fibre becoming coated, however, varies considerably from subject to subject. By combining the length distributions of uncoated and coated fibres with their densities (fibres mm^{-2}) on the filter it is possible to calculate, for each subject, the probability of a fibre of given length becoming coated. Taking the 20-40 μm range, for example, the probability of such fibres being coated was 100% in M2. Corresponding values for M1, M4, and M5 were 5%, 13%, and 26% respectively. In view of the significance attached to the presence of long fibres in the lung it seems important to understand the reasons for the observed variation in body formation from subject to subject. It could be due either to variations in the response of individuals to long fibres or the differences in the type and relative quantities of fibres present. Examination of the uncoated fibres in M1 with the electron microscope showed that they were all relatively thin (see fig 2b), and many were only just resolved with the optical microscope. This subject worked in a gas mask factory and was almost certainly exposed only to crocidolite, which suggests that long fibres of intrinsically thin-fibred amphiboles, such as crocidolite, may be coated less readily than thick.

Values for the uncoated/coated ratio vary considerably. As the time between the cessation of exposure and death increases, the value for this ratio will fall due to the removal of short fibres from the lung. In one case (M7) who died at the age of 80 after 16 years in retirement, more coated than uncoated fibres were detected.

At the 1969 Conference on Pneumoconiosis in Johannesburg, Thomson described the asbestos body as the tombstone of the asbestos fibre, implying that it had no pathological significance. If it is accepted, however, that it is the long fibres that initiate pathological changes in the lung then the fact that, in some cases of mesothelioma, virtually all fibres longer than 20 μm have been coated leads to an apparent paradox. This can be resolved only by assuming either that long fibres initiate pathological changes before they become coated or that the coated fibres themselves are capable of doing so. It is suggested that the former is the more likely explanation, and some support for this may be derived from the fact that it appears that thin fibres are not as readily coated as thick. This could provide an explanation for the greater carcinogenicity of crocidolite vis-a-vis the thicker amphiboles such as amosite and anthophyllite.

Conclusion

We suggest that the measurement of the total number of uncoated fibres in the lung is likely to provide a poor index of pathologically significant dose. This is because the number of very short
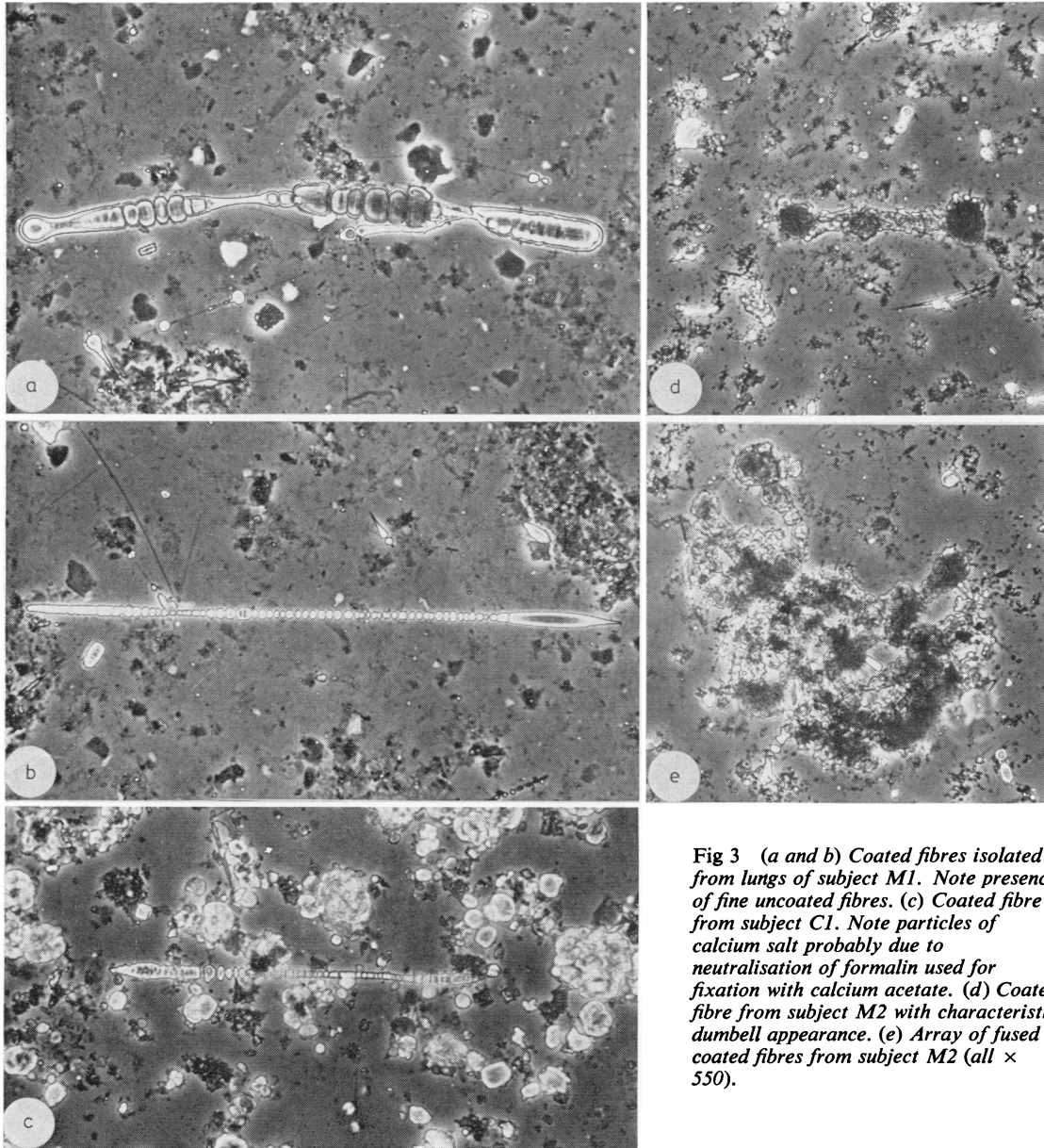
Fig 3 (*a and b*) *Coated fibres isolated from lungs of subject M1. Note presence of fine uncoated fibres.* (*c*) *Coated fibre from subject C1. Note particles of calcium salt probably due to neutralisation of formalin used for fixation with calcium acetate.* (*d*) *Coated fibre from subject M2 with characteristic dumbell appearance.* (*e*) *Array of fused coated fibres from subject M2 (all × 550).*

(and probably pathologically insignificant) fibres is highly variable depending as it does not only on the exposure itself but also on the time available for clearance and whether or not asbestosis is present. If, as suggested by Davis,[17] only fibres greater than 20 µm in length are of pathological significance, then only such fibres should be measured. Many fibres in this category will exist in coated form.

comments. We acknowledge the technical help of Mr R J Talbot and Mr J Pritchard, who obtained the electron micrographs. Samples of lung tissue were provided by Dr Whitwell, Broadgreen Hospital, Liverpool, and Dr Cowdell, Radcliffe Infirmary, Oxford.

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