THE EFFECTS OF CHRYSOTILE ASBESTOS DUST ON LUNG MACROPHAGES MAINTAINED IN ORGAN CULTURE

AN ELECTRON-MICROSCOPE STUDY

J. M. G. DAVIS

From the Department of Pathology, University of Cambridge

Received for publication February 15, 1967

In a previous study (Davis, 1967) a technique of organ culture was reported which allowed lung macrophages to be maintained for at least 9–10 days. This technique was developed in order to study in detail the uptake of asbestos dust by these macrophages, a thing that had been difficult to accomplish *in vivo*. Experiments with guinea-pigs (Davis, 1963*a*, *b*; Holt, Mills and Young, 1964) had, however, shown that very large amounts of dust could be taken up by individual lung macrophages which were able to combine to produce multinucleate giant cells in only a few days. Later many macrophages became converted to fibroblasts, the dust still remaining in the fibroblast cytoplasm. It was of great interest, therefore, to find that in the organ culture normal undusted macrophages could undergo conversion to fibroblasts. The system was obviously well suited to the study of this process in dusted cells.

MATERIALS AND METHODS

The apparatus and methods of culture used previously to study normal lung tissue (Davis, 1967) were also used in this study of dusted macrophages. It was decided to use chrysotile asbestos dust as this was known to cause few problems of embedding and sectioning for electron-microscope examination, and a sample of finely milled dust was obtained from Dr. P. F. Holt of Reading University. This dust was similar to that used in previous experiments (Holt *et al.*, 1964) and consisted mainly of fibres 5 μ or less in length. Initially it was found difficult to introduce the dust to the cultures in a satisfactory manner. If the dust was simply added to the culture medium none of it penetrated into the lung at all. Even simple injection of a suspension of dust in culture fluid was unsatisfactory as most of the dust was very quickly squeezed out of the cut surface of the lung. Finally it was found necessary to inject the dust into the tip of a small lung lobe and then tie a suture at the injection site to retain the dust. The final method adopted was as follows. Chrysotile asbestos was suspended in physiological saline to give a concentration of approximately 5 mg. per ml. This suspension was sterilised by being brought quickly to the boil and was then allowed to cool during which time the dust sediments to the bottom of the container. This allows 75 per cent of the saline to be drawn off and this is replaced by an equal volume of sterile culture fluid. The mixture which is kept at 37° is injected into the fine points of small lung lobes so as to distend the lung slightly at the injection site. A suture is then passed round the tip of the lobe and tightened, after which the sutured bag of tissue is severed from the rest of the lung and placed in a culture chamber (Fig. 1). At the end of each experiment the lung tissue was divided into two lobes. One was fixed in formol saline for light microscopic study, while the other was fixed in buffered osmium tetroxide for electron-microscope examination. This tissue was embedded in Araldite by the method of Davis (1959) except that total embedding time was reduced to 3 days before the tissue was dispensed into gelatin capsules to harden. After sectioning the tissue was stained on the grids with lead citrate (Reynold, 1963) before being examined in the electron microscope.

RESULTS

The appearance of guinea-pig lung containing asbestos dust differed from normal lung slices maintained in organ culture in two respects. In the first place it was found impossible to inject dust without distending the lung slightly and this made it difficult to keep the diameter of the cultures down to the 2 mm. through which oxygen can adequately diffuse. As a result central necrosis was often found in the dusted cultures with a band of living tissue about 1 mm. thick on the outside. In addition to this injection of dust containing fluid into the alveoli caused them to collapse rather more quickly than a normal lung and in some

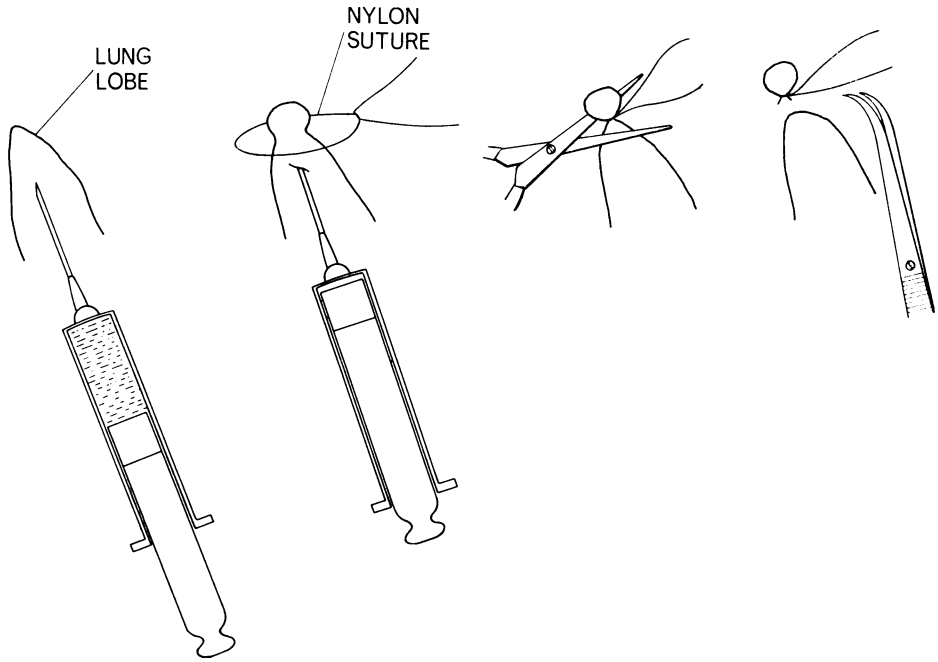


FIG. 1.—A diagrammatic representation of the method used to inject asbestos dust into the lung pieces to be maintained in organ culture.

cases the original outline of the alveoli was difficult to follow after as little as 3 days of culture. Apart from this, however, the lung tissue as a whole seemed unaffected by the presence of the asbestos dust and could be kept alive for at least 9 days. During this time the alveolar epithelial cells and the cells forming blood vessels and bronchioles showed exactly the same structures as the corresponding cells in undusted cultures when examined by both light and electron microscopy. In fact the only difference to be seen between dusted and undusted cultures by light microscope methods was the presence of foamy macrophages in the tissue spaces of the former.

Electron-microscope examination of the dusted tissue showed, however, that the dust is quite well distributed from the original injection site, and is very quickly taken up by the lung macrophages. During this study no evidence was seen that

the asbestos dust was taken up by any cells apart from the macrophages or that the dust was able to penetrate the tissues by mechanical action. Dust was present within the macrophages within as little as 30 min. and almost all macrophages contained dust by 2 hr. after the culture was set up (Fig. 2). The macrophages in a dusted area develop elongated phagocytic processes on their surface membranes within a few min. and if these contact a dust particle they surround it and fold back onto the cell surface to enclose it in a phagocytic vacuole or phagosome (Fig. 3). The phagocytic processes have a fairly constant thickness of about 1000 Å but they vary considerably in length and some can be as long as 10 μ. Initially phagosomes are dilated spherical structures which, in these experiments at least, contain culture fluid in which the dust particles float freely (Fig. 4). The longest period that dust particles can remain in phagosomes is impossible to determine as fresh phagocytosis occurs to some extent throughout the experiment, but within 2 hr. of the start of the experiment some asbestos dust has escaped from the phagosome and can be found free in the cytoplasm (Fig. 11). It is not possible to be certain of the reason for phagosome rupture in all cases. Sometimes it may be due to chemical damage to the phagocytic membrane, but in certain cases it is obvious that long dust fibres have punctured the phagosome wall by purely mechanical action. Whatever the cause of the membrane rupture, however, cytoplasmic material flows into a punctured phagosome, the membrane of which remains intact for much of its circumference (Fig. 5). This membrane, however, eventually collapses (Fig. 6) and contracts enclosing any remaining dust to form a dense structure that in the past would have been called a cytosome, but whose present terminology is rather confused. This point is dealt with in the discussion. Asbestos containing " cytosomes " are shown in Figs. 7, 8, 9 and 10. It can be seen that in addition to the dust, these bodies contain a considerable amount of debris including portions of membranes that often have been folded and packed to produce laminated or " myelin " structures. Dust containing " cytosome " structures were absent from macrophages during the first few days of culture and were most common after 3 or 4 days indicating that it takes some time for the phagosome to contract fully. During the process of dust phagocytosis it was noticeable that the number of primary lysosomes in the cells increased considerably. These structures are spherical granular masses about 0·3 μ in diameter and surrounded by a single membrane (Fig. 15). They are believed to contain katabolic enzymes which can be liberated into the phagosomes to undertake digestion. A few of these structures are present in normal lung macrophages, but it was found in this study that they become much more common from about 48 hr. onwards. This probably means that new ones are produced if the macrophage enters a prolonged period of phagocytic activity.

The fate of asbestos dust in macrophages that has just been described, where the dust is either finally liberated into the cytoplasm or walled up in " residual bodies ", is by far the most usual one. In some cells, however, the phagosome seems neither to rupture or contract and as new phagosomes are formed the cell takes on the highly vacuolated appearance of a " foamy macrophage ". In these cells most of the dust is retained in phagosomes for several days and very little of it is ever found free in the cytoplasm. How these cells differ from normal macrophages is uncertain but most of the cell structures including mitochondria are quite normal so that it does not seem likely that a foaming macrophage is simply a dying cell.

In the previous paper on the structure of normal lung maintained in organ culture it was reported that the lung macrophages were actively phagocytic but they did not combine to form giant cells. In dusted cultures, however, dust containing macrophages quickly combine and quite large giant cells can be formed within 24 hr. The process of giant cell formation in culture is exactly similar to that described by Davis (1963*b*) for giant cell formation in live guinea-pig lungs. The macrophage phagocytic processes become entangled and contract to bind the cells together (Fig. 12). Eventually the dividing cell walls between the individual macrophages break down and a truly multinucleate cell is produced. This process can occur very quickly and some such cells were found after as little as 24 hr. of

EXPLANATION OF PLATES

FIG. 2.—An alveolar macrophage from guinea-pig lung maintained in organ culture for 2 hr. In this short time the cell has phagocytosed a considerable amount of asbestos dust much of which is still contained in the phagosomes but some of which (arrowed) is already lying free in the cell cytoplasm. ×22,200.

FIG. 3.—A phagocytic process from a guinea-pig lung macrophage engulfing crystals of chrysotile asbestos dust. This cell had been maintained in organ culture for 2 hr. ×60,000.

FIG. 4.—A bundle of chrysotile crystals contained in a phagosome from a guinea-pig lung macrophage. This cell had been maintained in organ culture for 1 hr.

FIG. 5.—A phagosome from a guinea-pig lung macrophage in the process of rupturing. The particle of asbestos dust (arrowed) appears to be escaping into the cell cytoplasm. At the same time cytoplasmic material can be seen flowing into the phagosome. This cell had been maintained in organ culture for 24 hr. ×42,000.

FIG. 6.—A punctured phagosome from a guinea-pig lung macrophage. Much of the contained chrysotile asbestos dust has remained within the phagosome and the phagosome membrane is collapsing and contracting around it. This macrophage was maintained in organ culture for 48 hr. ×53,000.

FIGS. 7, 8, 9 and 10.—These 4 figs. show stages in the contraction of phagosomes containing asbestos dust. The photographs were taken from guinea-pig lung macrophages maintained in organ culture for 3 days. Most of these contracting phagosomes contain cell debris, especially membranes, in addition to the asbestos dust. ×54,000 in each case.

FIG. 11.—An area of cytoplasm from a guinea-pig lung macrophage maintained in organ culture for 3 days. Crystals of chrysotile asbestos are shown lying completely free in the cell cytoplasm. ×54,000.

FIG. 12.—Guinea-pig lung macrophages combining in the early stages of giant cell formation. The phagocytic processes of these cells have interdigitated and the cells are effectively tied together. These macrophages had been maintained in organ culture for 24 hr. ×18,000.

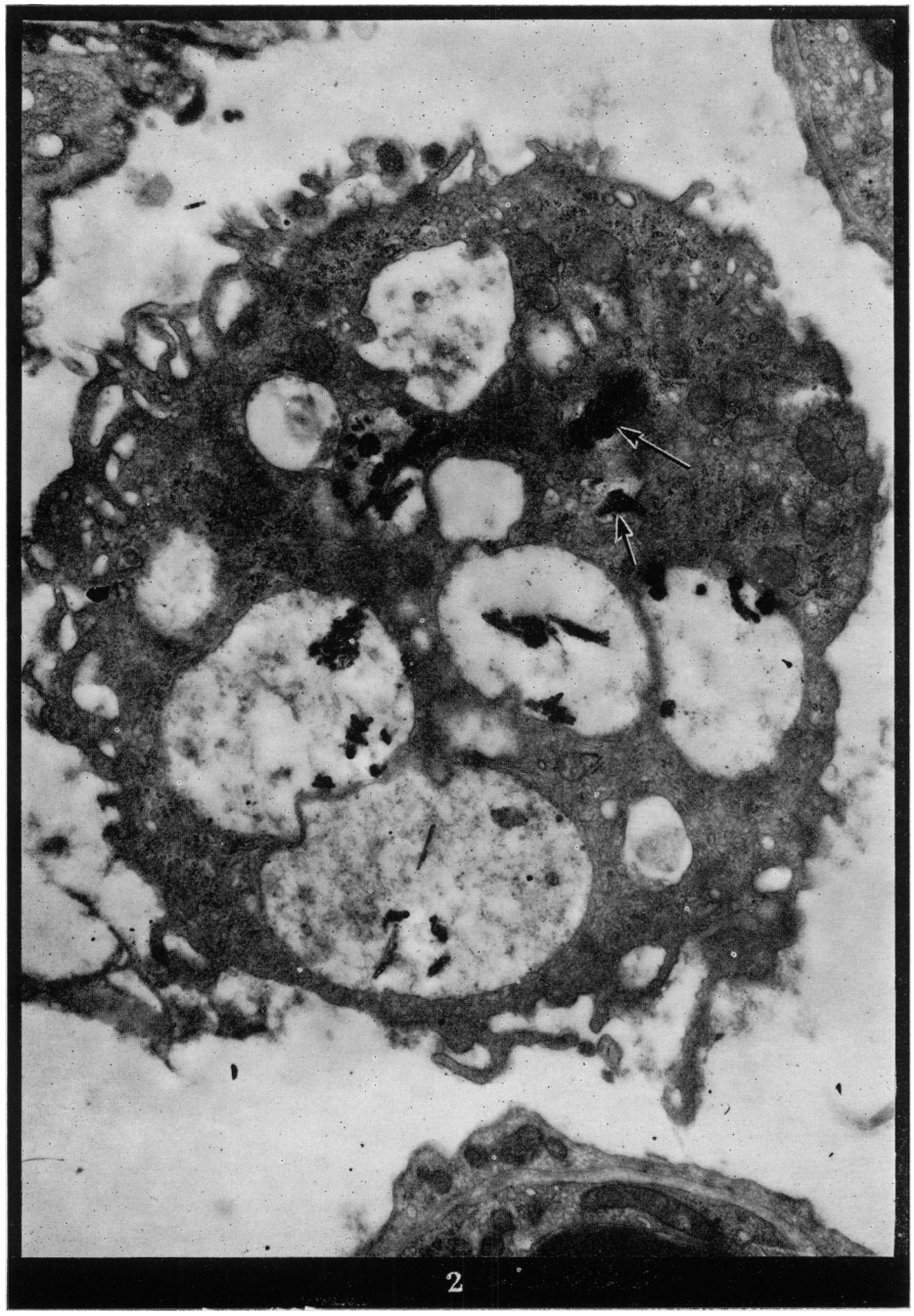
FIG. 13.—A multinucleate giant cell found in guinea-pig lung after only 24 hr. of organ culture. In this short time the junction line between the individual macrophages had disappeared and the cell membranes have fused. Phagocytic processes are, however, still present on the surface of this giant cell (arrowed) and it therefore seems likely that the cell could still pick up asbestos dust. ×6,300.

FIG. 14.—A lung macrophage from guinea-pig lung maintained in organ culture for 24 hr. The cell is still actively phagocytic but considerable numbers of new α-cytomembranes have recently been produced (arrowed).

FIG. 15.—An area of cell cytoplasm from a guinea-pig lung macrophage maintained in organ culture for 4 days. New α-cytomembranes are present as well as crystals of chrysotile asbestos dust lying free in the cytoplasm. This photograph shows 2 typical primary lysosomes which are arrowed. ×42,000.

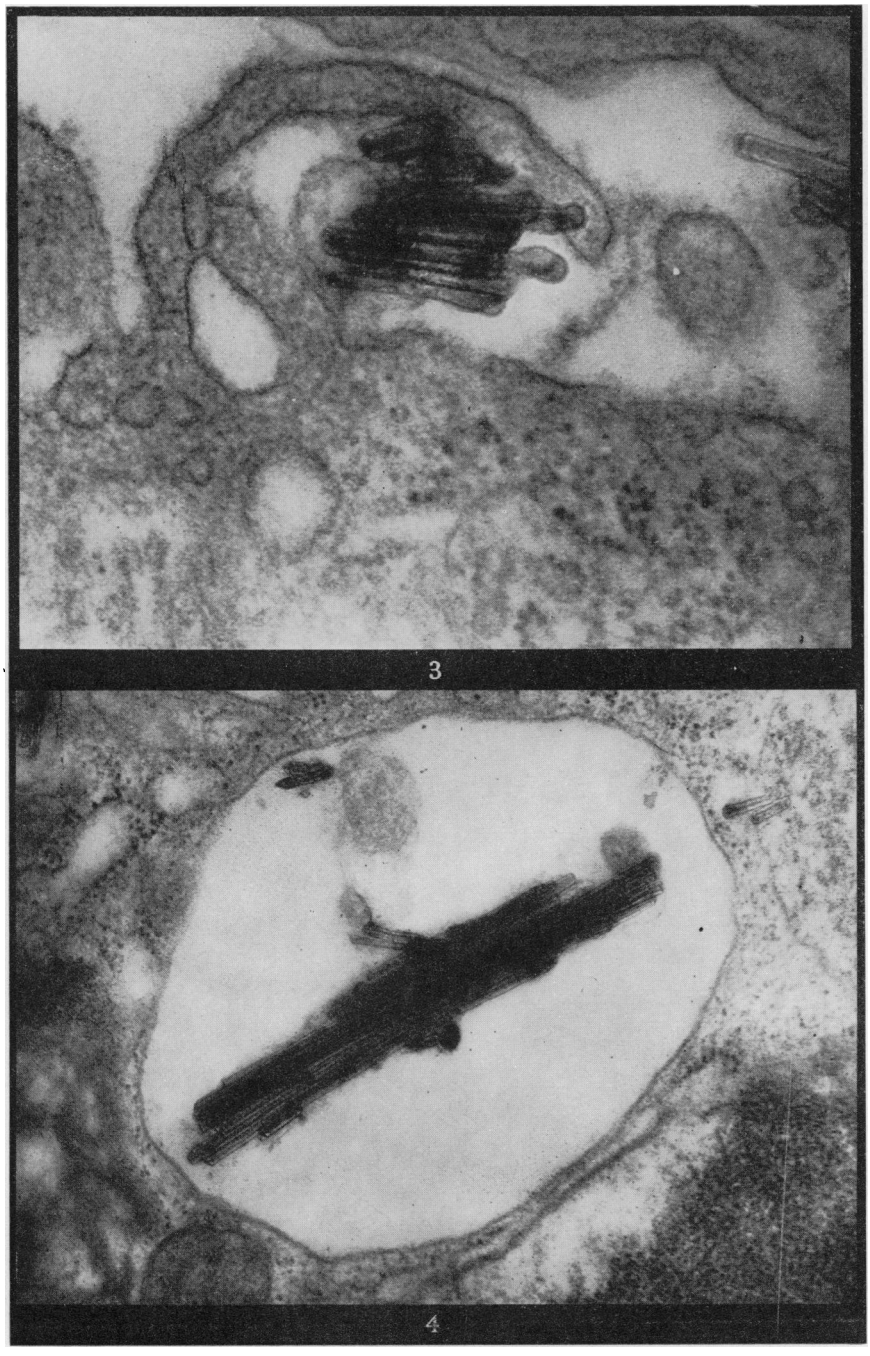
FIG. 16.—A cell from guinea-pig lung maintained in organ culture for 6 days. The cell contains a particle of asbestos dust (arrowed) and numbers of long α-cytomembranes. This cell was found surrounded by collagen or reticulin fibres and had no phagocytic processes on its surface membrane. The α-cytomembranes are not short or dilated enough for the cell to be considered a typical fibroblast and it probably represents an intermediate stage in the conversion of dust containing macrophage to dust containing fibroblast. ×26,000.

FIG. 17.—A fibroblast from guinea-pig lung maintained in organ culture for 6 days. The cell contains crystals of chrysotile asbestos dust (arrowed) and the α-cytomembranes have become dilated to form sacs which appear to be filled with amorphous material. Banded collagen or reticulin fibres can be seen outside the cell. ×41,600.



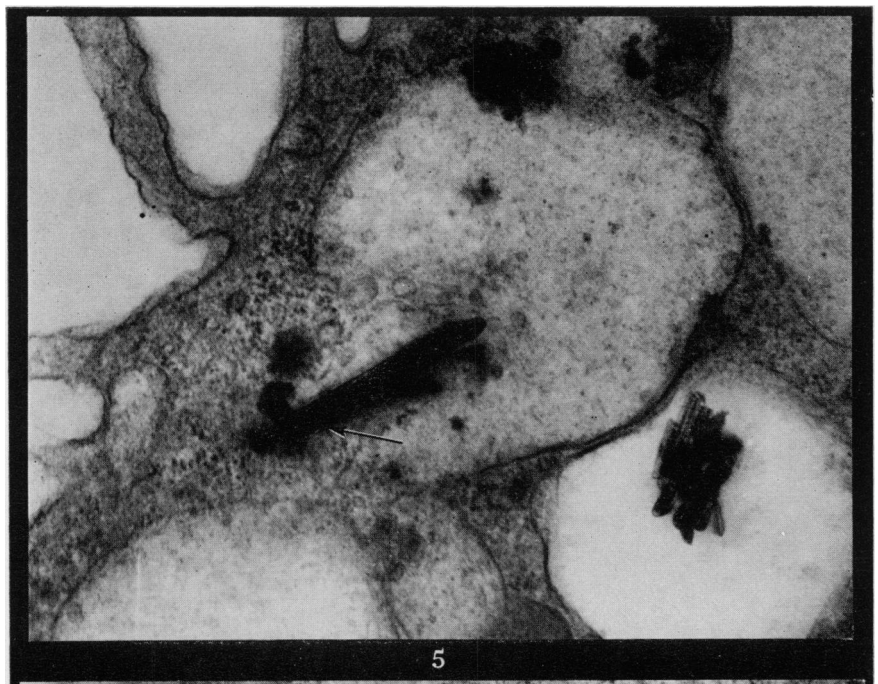
2

Davis.

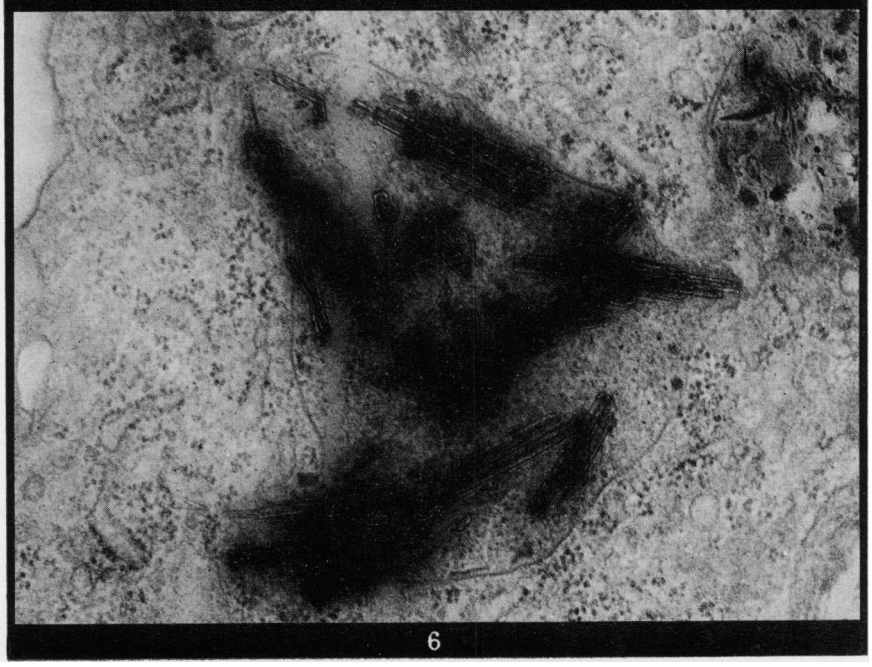


3

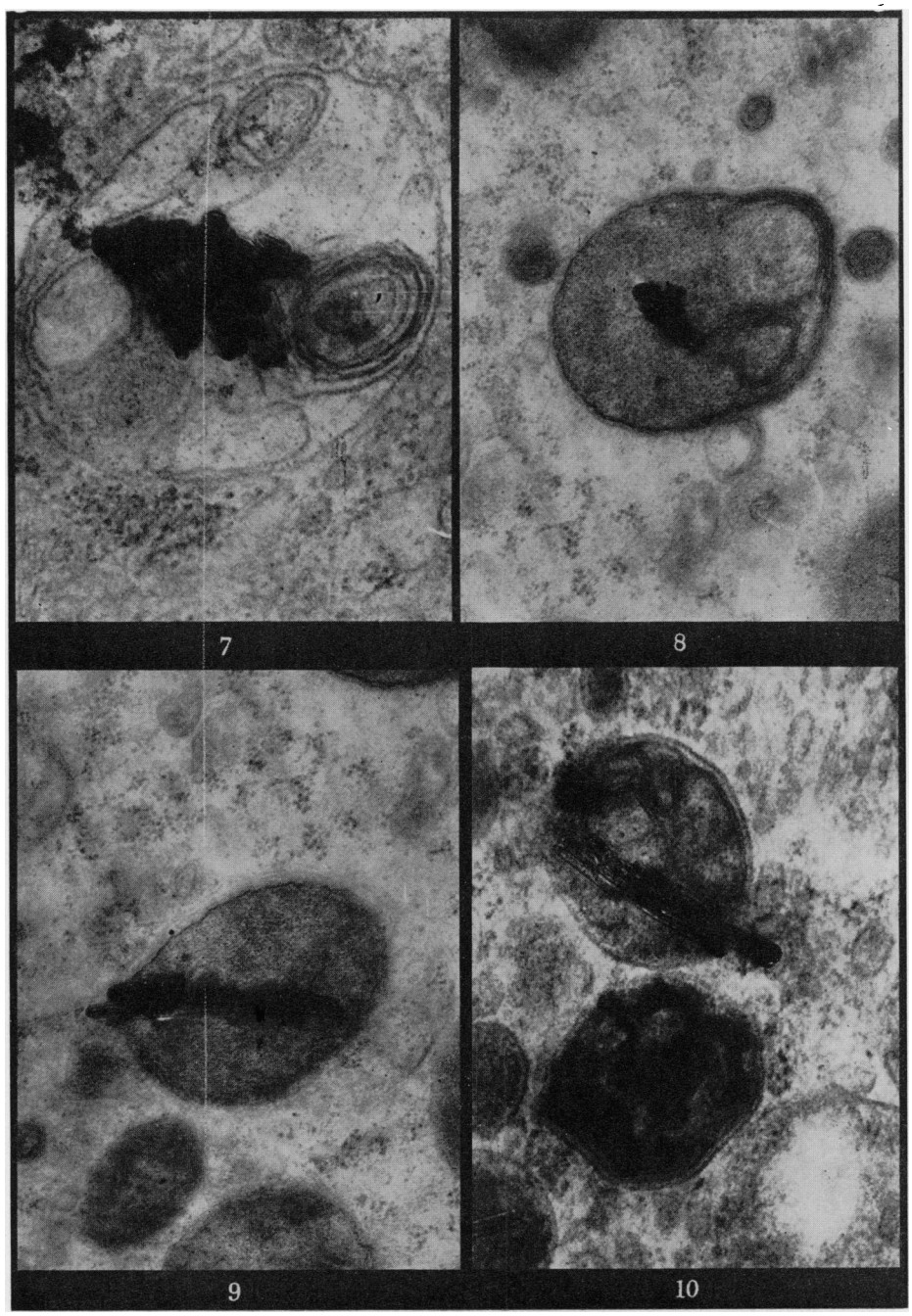
4



5



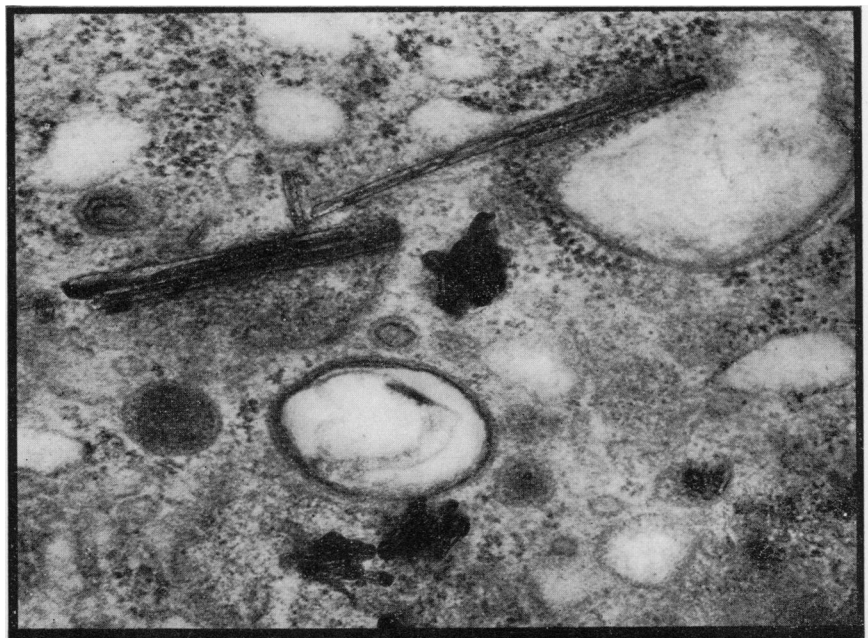
6



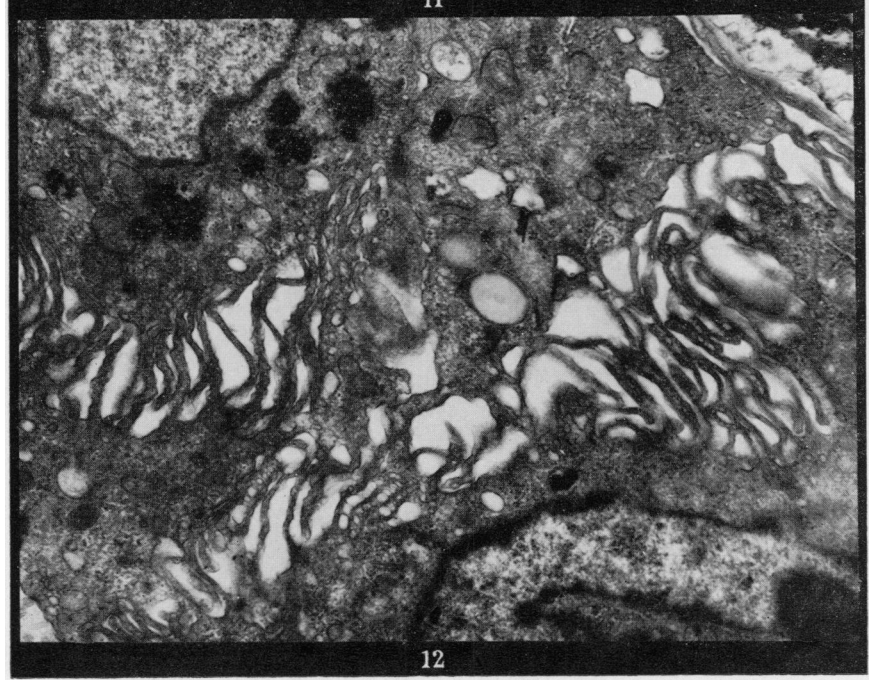
7 8

9 10

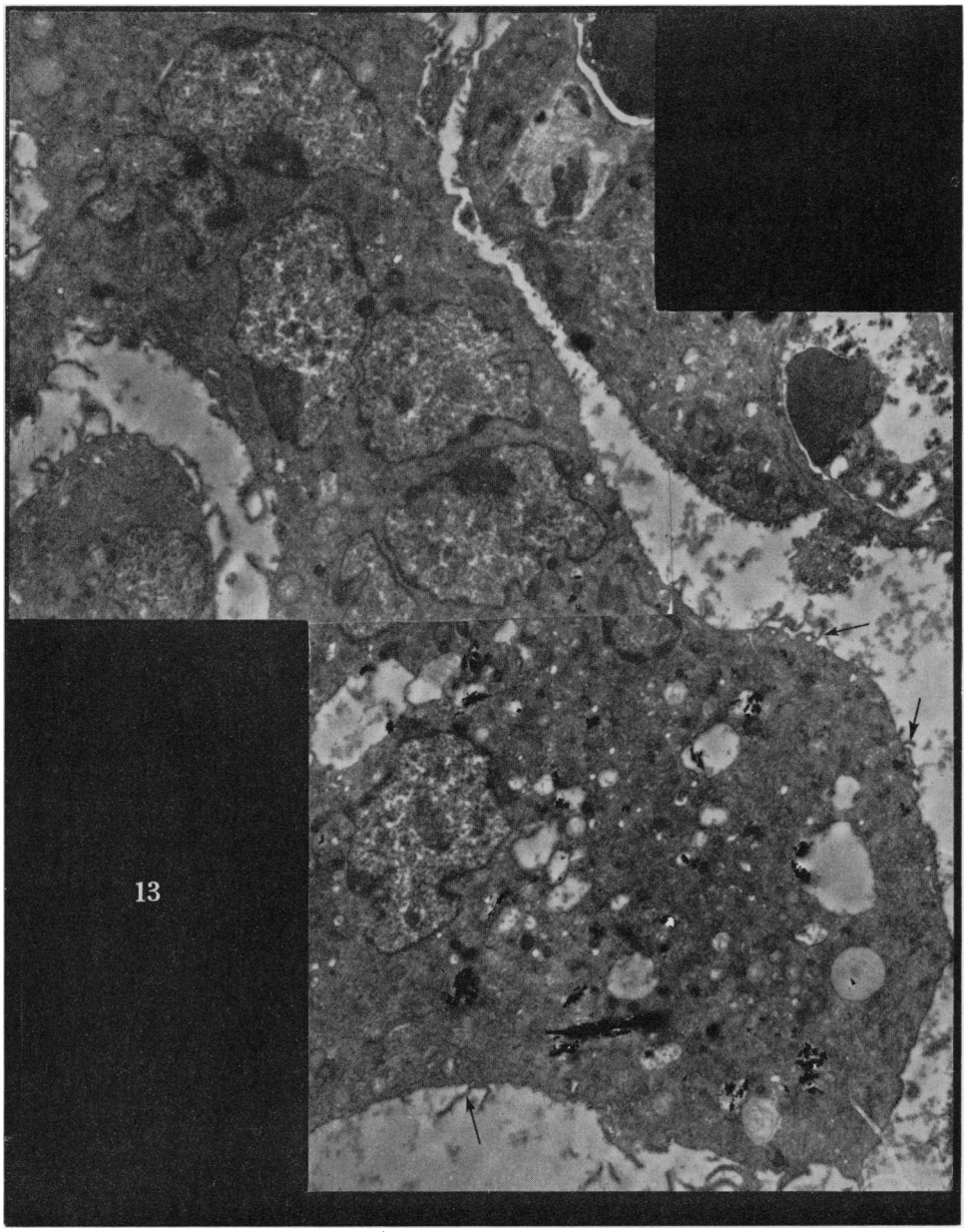
Davis.

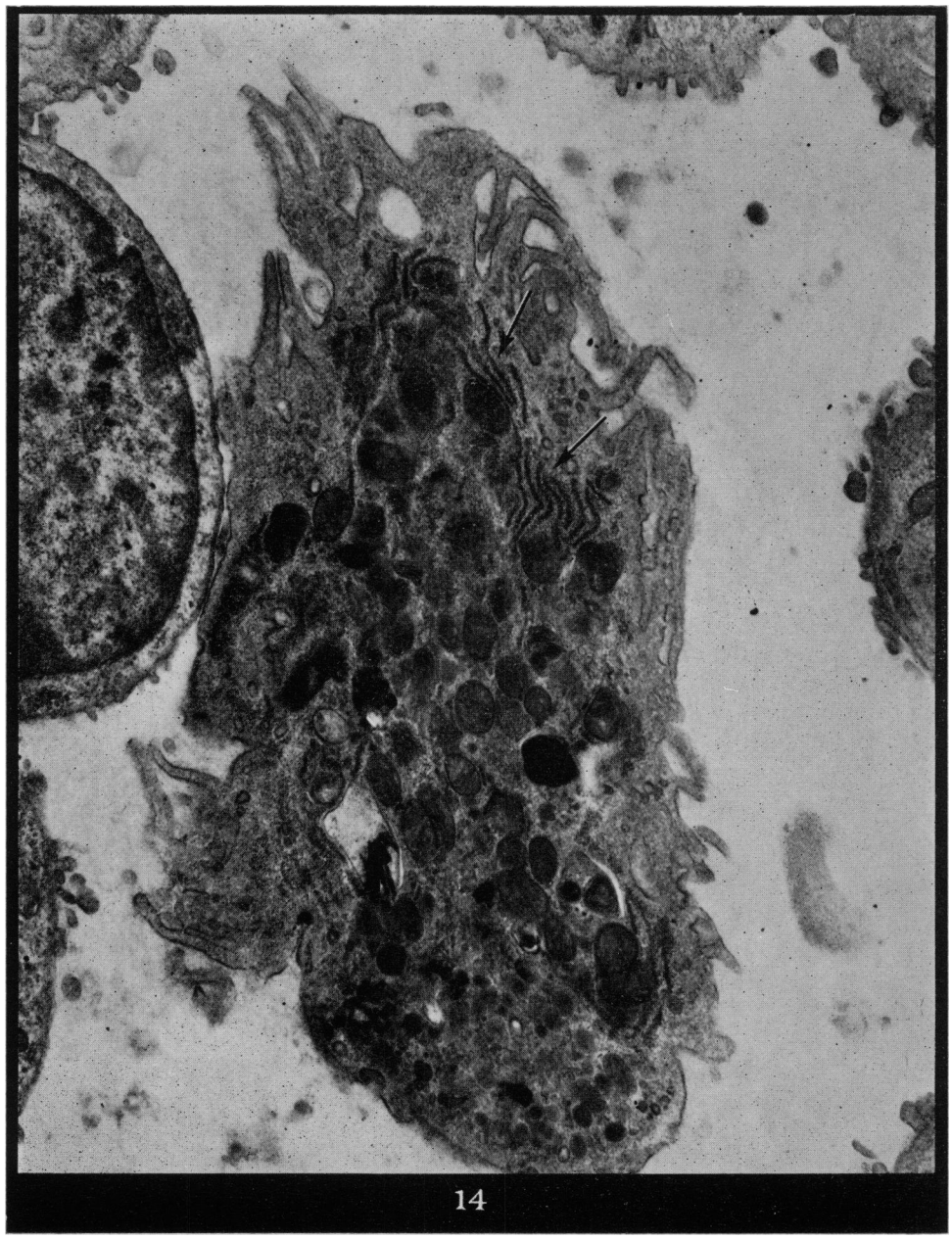


11

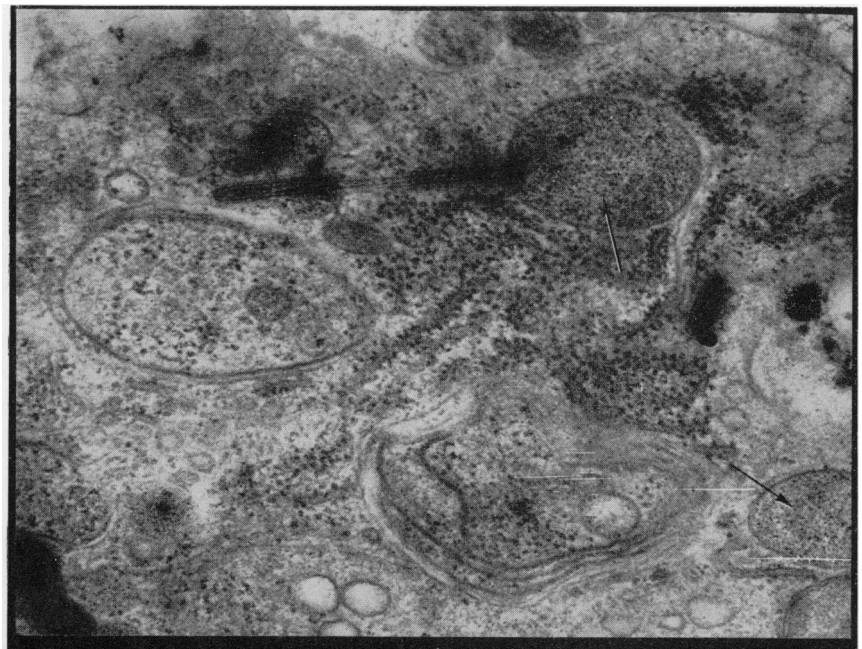


12

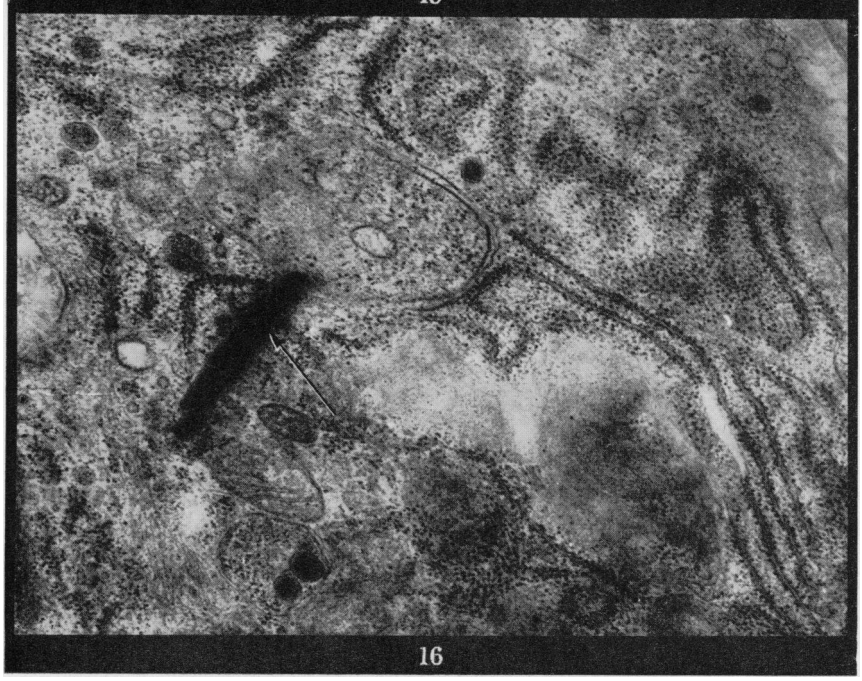

13



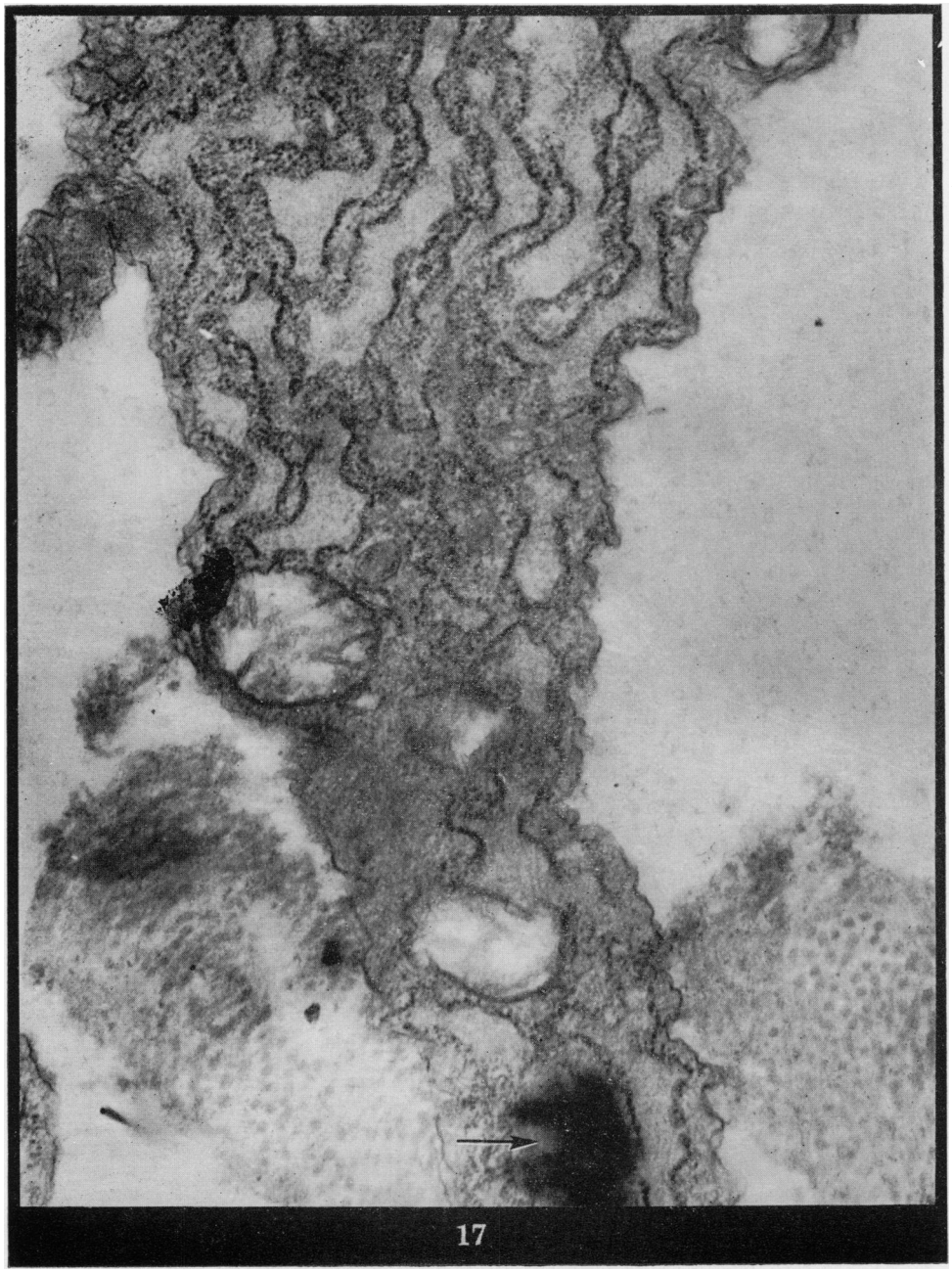
14



15



16



17

Davis.

culture (Fig. 13). Once formed giant cells did not appear to change for the whole 9–10 days of the experiment.

In normal guinea-pig lungs the macrophages contain many ribosomes but few if any α-cytomembranes. Within 24 hr. of culturing, however, many macrophages produced sheaves of long α-cytomembranes and this often occurs while the cell is still actively phagocytic (Fig. 14). Eventually this type of macrophage loses its phagocytic processes (Fig. 16) and the long α-cytomembranes break up into short dilated lengths (Fig. 17). The cells finally exhibit the structure that is normally considered typical of fibroblasts, and are usually found surrounded by masses of collagen on reticulin fibres.

From these observations it will be seen that the pathological changes previously reported for dusted guinea-pigs *in vivo* can all be produced in pieces of lung maintained in organ culture. This system should therefore be useful for studying the effect of various chemicals on asbestos uptake and it is hoped in the near future to study the effect of P 204 on cultures dusted with chrysotile.

DISCUSSION

In this study it was reassuring to find that the fate of asbestos dust in organ culture is exactly the same as that previously demonstrated in dusted animals and known to occur in human lungs. Dust is taken up only by the lung macrophages and these may either combine to form giant cells or at a rather later stage become converted into fibroblasts. In this respect the system of organ culture is ideally suited for a cytological study of the changes produced by asbestos. It would be less satisfactory for examining the pathological stimulus of collagen production by asbestos because, as was shown in the preceding paper, fibrosis can occur in the later stages of organ culture of normal lung tissue. This, however, does not invalidate the suggestion that asbestos dust causes fibrosis by direct action on the macrophage cytoplasm, but merely emphasises the fact that fibrosis is a normal pathological process that can be initiated by a number of different stimuli.

This study has confirmed the previous suggestion (Davis, 1963*b*) that giant cell formation does not necessarily result from the presence of foreign material too large to be phagocytosed by a single macrophage. Giant cells formed in the dusted cultures within 24 hr. and none of them was found to contain any dust particles more than 5 μ in length. The reasons for giant cell formation are probably seen by comparing normal and dusted organ cultures of lung tissue. In normal cultures the macrophages are actively phagocytic but remain evenly distributed throughout the tissue and giant cells do not form. It seems likely that in these cultures the stimulus to phagocytose is widespread and the macrophages do not aggregate in any particular area. In dusted cultures, however, the dust fibres tend to occur in clumps and many macrophages become attracted to small areas where they aggregate. When this occurs their phagocytic processes intertwine and giant cells are formed. From this evidence it would appear that giant cell formation results from a localisation of foreign material and the size of this material is not important.

By the use of organ culture it has been possible to follow the uptake of asbestos dust into a phagocytic vacuole or " phagosome " and to study the way in which these vacuoles collapse to form " residual bodies ". This, however, raises the problem of terminology. Originally, all the structures mentioned here in con-

nection with the phagocytosis of asbestos were given the general name of cytosome (Carasso and Favard, 1958; Gieseking, 1958; Karrer, 1958), but now the importance of the different types is recognised. In order to discuss their terminology, however, it will be necessary to summarise the present concepts of the enzymatic digestion of phagocytosed materials and the role of the lysosome. It is now believed that the digestion of phagocytosed materials is carried on inside the phagosome by katabolic enzymes that are secreted into the vacuoles by structures called lysosomes. These lysosomes are dense granular bodies bounded by a single membrane. The enzymes they carry are either inactive or unable to damage the cell because they are separated from the cytoplasmic structures by a membrane. Once in the phagosome the enzymes are activated and digest any organic material present. It is in the subsequent fate of the phagosome and the lysosome enzymes it contains that the confusion arises.

When the phagosome contracts to form a residual body it often contains the remains of membrane structures that become packed together to give a " myelinated " appearance. The residual body is very much smaller than the original phagosome. It contains dense granular material and is surrounded by a single membrane. In fact the residual body is very much like a lysosome except for the debris it contains and some workers have used the word lysosome to describe these structures. Both de Duve (1963) and Goldfischer (Goldfischer, Essner and Novikoff, 1964) consider that the primary lysosome, the phagosome and its residual body should all be included under the general term " lysosome ". As far as the phagosome is concerned this seems undesirable. If the primary lysosome is to be considered as a bag of enzymes ready for use, then the phagosome is an entirely separate structure which is formed from the cell surface during phagocytosis. The fact that the lysosome enzymes may be injected into the phagosome does not alter this fundamental difference. When the phagosome has contracted to form a residual body, however, it does show a similar structure to the primary lysosome apart from containing a certain amount of debris. This fact suggests the idea that the lysosome enzymes may not be used up or inactivated during digestion in the phagosome and are retained in the residual body in a form in which they could be reinjected into another phagosome. If this is the case then the residual body could well be called a " secondary lysosome ", but until this is definitely proved it would be best to retain the term " residual body ". To call such a structure a lysosome unless it can be proved still capable of acting as a source of usable enzymes would obviously be incorrect.

SUMMARY

Chrysotile asbestos dust was injected into small pieces of adult guinea-pig lung maintained in organ culture for 10–14 days. This dust was taken up by the lung macrophages in a matter of hours, but dust was not found in any of the other cells during the course of these experiments. The macrophages in contact with dust produce elongated phagocytic processes on the cell surface and these encircle the dust and fold back onto the cell to form phagocytic vacuoles or phagosomes. The dust may remain in the phagosomes for some time or it can quickly escape into the cytoplasm perhaps by mechanical action. If the dust remains in a phagosome this eventually contracts to form a small dense structure usually called a residual body. In the cultured lung it was found that dust containing macrophages could

quickly combine to form giant cells, and quite large specimens were often found within 24 hr. Some macrophages eventually become converted into fibroblasts with asbestos dust still retained in the fibroblast cytoplasm.

REFERENCES

CARASSO, N. AND FAVARD, P.—(1958) *C.r. Acad. Sci. (Paris)*, **246**, 1594.
DAVIS, J. M. G.—(1959) *Nature, Lond.*, **183**, 200.—(1963*a*) *Br. J. exp. Path.*, **44**, 454.—(1963*b*) *Br. J. exp. Path.*, **44**, 568.—(1967) *Br. J. exp. Path.*, **48**, 371.
DE DUVE, C.—(1963) *Scientific American*, **64**, 208.
GIESEKING, R.—(1958) *Verh. dtsch. path. Ges.*, **42**, 344.
GOLDFISCHER, S., ESSNER, E. AND NOVIKOFF, A. B.—(1964) *J. Histochem. Cytochem.*, **12**, 72.
HOLT, P. F., MILLS, J. M. AND YOUNG, D. K.—(1964) *J. Path. Bact.*, **87**, 15.
KARRER, H. E.—(1958) *J. biophys. biochem. Cytol.*, **4**, 693.
REYNOLDS, S.—(1963) *J. Cell Biol.*, **17**, 208.