Zeitschrift für Zellforschung 69, 418—427 (1966)

Anatomisches Institut der Universität Zürich, Abteilung für Elektronenmikroskopie

# A STEREOLOGIC ELECTRON MICROSCOPE STUDY OF "TUBULAR MYELIN FIGURES" IN ALVEOLAR FLUIDS OF RAT LUNGS* **

EWALD R. WEIBEL, GONZAGUE S. KISTLER and GIAN TÖNDURY

With 10 Figures in the Text

*(Received June 3, 1965)*

**Summary.** The three-dimensional structure of a composite material found in alveolar exudate of oxygen poisoned lungs but also present in normal lungs is stereologically analysed. It is composed of tubules of 450 Å diameter which are tightly packed in a quadratic lattice. The wall of the tubule is formed by "four-winged" osmiophilic filaments which are located in the corners of the quadratic lattice; their interior is made up of a hydrophilic substance which contains either a tubule or a filament of moderate electron density. The osmiophilic substance of the walls is continuous with associated myelin figures which can be resolved into lamellae with a periodicity of 42 Å and can thus be considered to be water crystals of phospholipids. The nature of the content of the tubules, which presumably exerts the formative force on the phospholipid lamellae to form tubules, remains undetermined.

In an electron microscope study on the pulmonary pathology of oxygen toxicity in the rat (KISTLER, CALDWELL and WEIBEL 1965) we observed masses of a material which aggregated in a peculiar and very regular lattice in exudate formed within the alveoli. CAMPICHE (1960) had found similar structures to occur in necrotic alveolar epithelial cells of young rats who had been breathing in an oxygen enriched atmosphere for the first 6 days of life; he described them as "fingerprint-like myelin figures". POLICARD et al. (1957) found similar myelin figures in the lung in inflammatory processes caused by experimental application of fine-particulate silicate. This might indicate that these layered masses are produced as an effect of various noxae on lung tissue.

However, checking through our extensive material of normal rat lungs from various sources we observed that identical layered material could be found on the surface of the alveolar epithelium in almost any normal lung, though in very small amounts. In the following we shall attempt to analyze the three-dimensional configuration of these peculiar structures.

## Material and Methods

The material used in this study consisted of rat lung tissue fixed *in situ* by instillation of 1% $OsO_4$ (phosphate buffered at pH 7.4) into the airways of animals anesthetized by an intraperitoneal injection of Pentobarbital. After fixation, the tissue blocks were dehydrated in

* Dedicated to Prof. W. BARGMANN in honor of his 60th birthday.

** The research reported here has been sponsored by the Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung (Nr. 2569); by the Stiftung für wissenschaftliche Forschung an der Universität Zürich; by the National Institutes of Health, USPHS, through grant RF-57; and the 6570th Aerospace Medical Research Laboratories under contract AF 61(052)-784 through the European Office of Aerospace Research (OAR), United States Air Force.

graded ethanol and embedded in Epon 812 (LUFT 1961). Sections were cut on an LKB-Ultrotome I using a Du Pont diamond knife; our routine sections show a silver interference color and are therefore 600—900 Å thick. Section contrast was enhanced by staining with lead salts either by REYNOLD's (1963) or by KARNOVSKY's (1961) method. Micrographs were obtained in a Philips EM 200 electron microscope operating at 60 or 80 kV.

The rats used in this study had been derived from various sources; their lungs had been collected over a number of years for different projects. The three-dimensional analysis was mainly done on large aggregates of this layered material found in alveolar exudate formed in rat lungs after exposure to pure oxygen for up to 3 days (KISTLER, CALDWELL and WEIBEL 1965).

## Results

### *1. Description of the appearance of sectioned material on electron micrographs*

Fig. 1 shows a large mass of the material to be described, which is filling a niche in the alveolar surface. It is external to the alveolar epithelium. At this low magnification one can well observe an over-all pattern reminiscent of finger-prints. For a more refined description of the pattern we have to distinguish between four regions which are labelled A, B, C and D on Fig. 1 and are further illustrated in Figs. 2—4.

In *region A* (Fig. 2) parallel bands of uniform spacing are outlined by an intensely osmiophilic line; the repeating period is about 450 Å. The space between these lines appears filled with a less dense material which forms two gray lines separated from each other and from the osmiophilic line by fine gaps which are lighter in appearance. The thickness of both types of lines and the width of the gaps are of approximately the same order of magnitude of 75 Å.

In *region B* (Fig. 3) the parallel banding is replaced by a group of dark dots arranged in a regular square lattice. The spacing of the dots in both directions is again about 450 Å. At closer look these dots appear more like four-spiked stars with the tapering spikes lying in the square lattice. The square area between four dots contains a somewhat circular profile formed by a gray line. Its interior space as well as the gap separating the circle from the components of the osmiophilic square lattice are lighter in contrast.

In *region C* (Fig. 4) a parallel banding is formed by alternating gray and light lines. The repeating period is about 320 Å. The darker lines of this pattern show less contrast than those in region A. Both gray and light bands are of about the same width ($\sim$160 Å); they are not sharply delineated.

In addition to these three regions exhibiting a distinct pattern we find areas such as D in Figs. 1 and 4 which appear gray with an indication of faint fuzzy streaking of no apparent regularity.

### *2. Stereologic analysis of the three-dimensional structure of this material*

The four patterns described were found on random sections of the material. They were formed by projection of the section content onto the photographic plate. The question must therefore be asked, whether these distinct patterns can have arisen from one and the same three-dimensional lattice which may have been sectioned in different directions.

Of several possible hypothetical models only one appeared to satisfy all conditions; it is illustrated diagrammatically in Fig. 5. It consists of prismatic

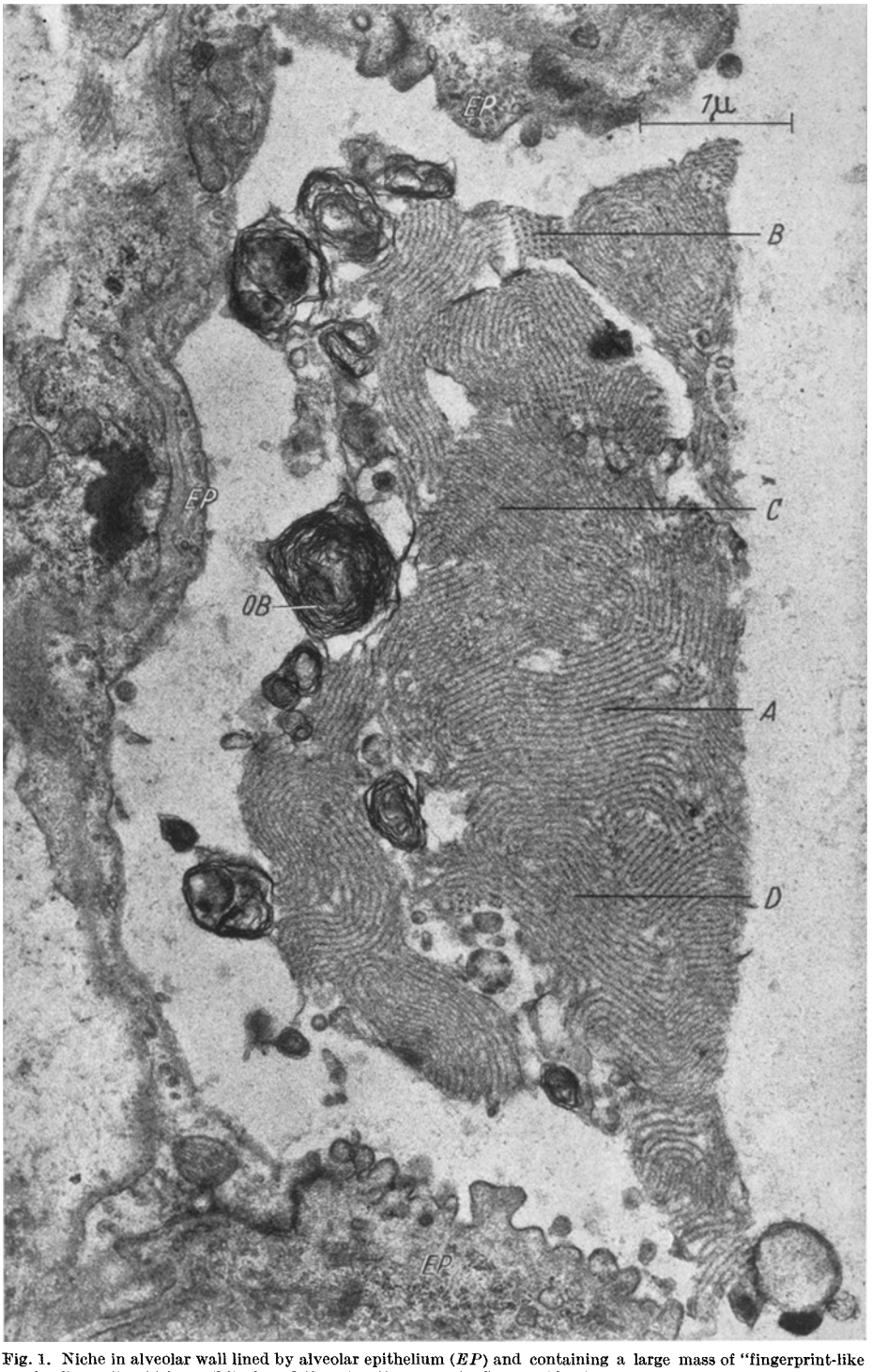


Fig. 1. Niche in alveolar wall lined by alveolar epithelium (*EP*) and containing a large mass of "fingerprint-like myelin figures", which exhibits four different patterns *A*—*D*. Spherical bodies composed of osmiophilic lamellae (*OB*) are associated with this material. Rat lung after 72 hours exposure to pure $O_2$. × 21,000

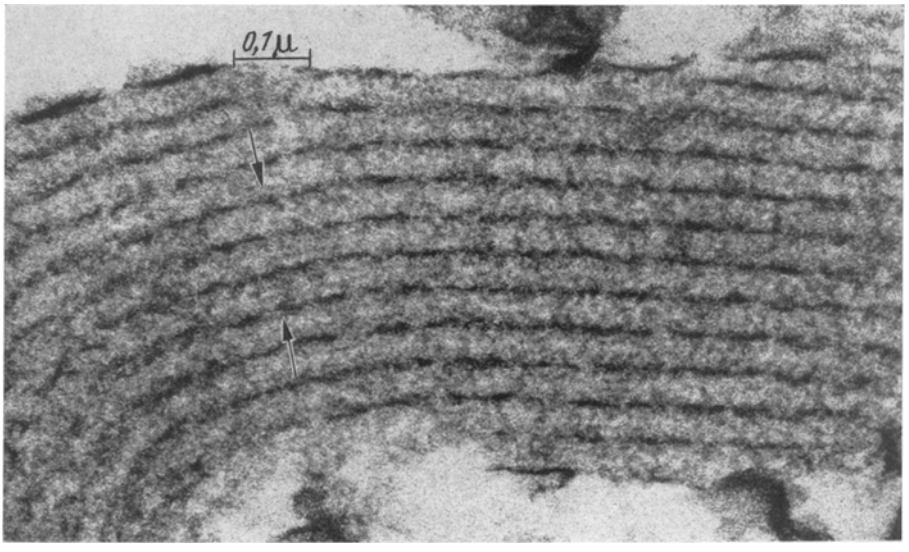


Fig. 2. Pattern *A* of 450 Å spacing of osmiophilic lines with two finer gray lines in between. Arrows mark course of photometer tracing of Fig. 7. × 100,000

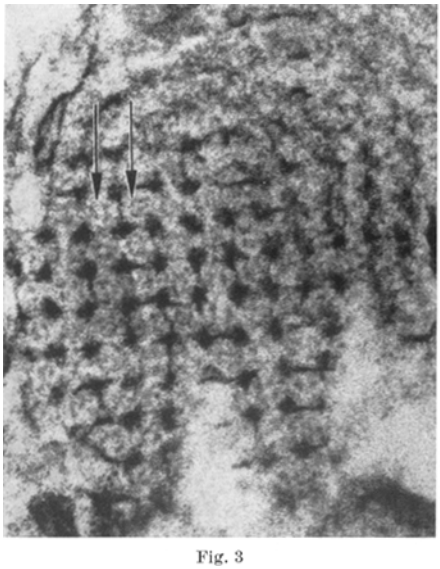

Fig. 3

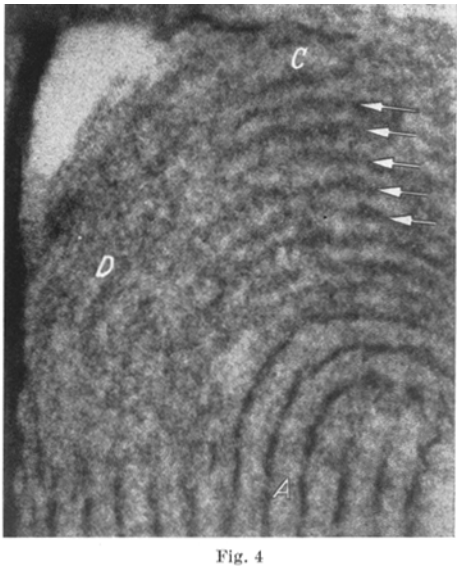


Fig. 4

Fig. 3. Pattern *B* of four-spiked stars in square lattice of 450 Å spacing. Arrows indicate circular profiles in squares. × 100,000

Fig. 4. Patterns *A*, *C* and *D*. Arrows indicate 320 Å spacing of gray bands in pattern *C*. × 100,000

tubules of about 450 Å diameter, which are formed by a strongly osmiophilic material and are packed in a square lattice. In the corners of the square lattice the osmiophilic material forms "four-winged filaments" whose "wings" are connected by fine membranes. The corners of the prismatic tubules are thus rounded off. A second smaller tubule lies inside the principal tubule; it is formed by a less osmiophilic substance. The diameter of this inner tubule is of the order of 250 Å.

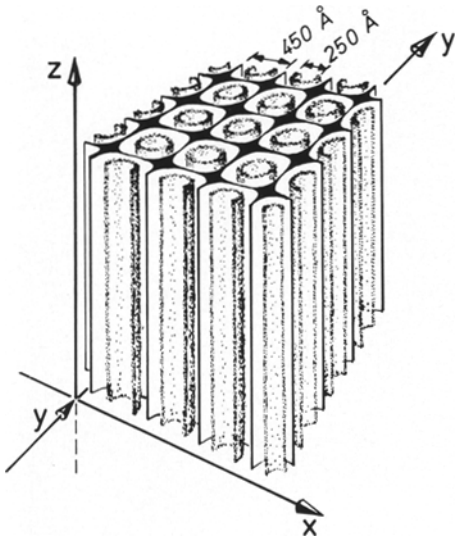


Fig. 5. Model of three-dimensional structure of "tubular myelin figure"

Fig. 6 shows the different patterns obtained on electron micrographs if this hypothetical model lattice is sectioned at different angles with a section thickness of 900 Å.

If the lattice is cut parallel to the $(x, y)$ plane (Fig. 5) the pattern of fourspiked stars is obtained with a 450 Å spacing in both lattice directions. The central tubule appears as circular profile. This corresponds to the pattern observed in region B (Fig. 3).

If the model is sectioned parallel to the $(x, z)$ or to the $(y, z)$ plane a pattern of parallel banding with a repeating period of 450 Å results. Considerations on the density of the different components revealed that a line of maximal contrast would occur at the axis of the osmiophilic "filaments", since the "wings" perpendicular to the section plane would be viewed on edge and scatter most electrons. The less dense inner tubule would be recognized as two faint lines separated by a lighter gap. This pattern was observed in region A

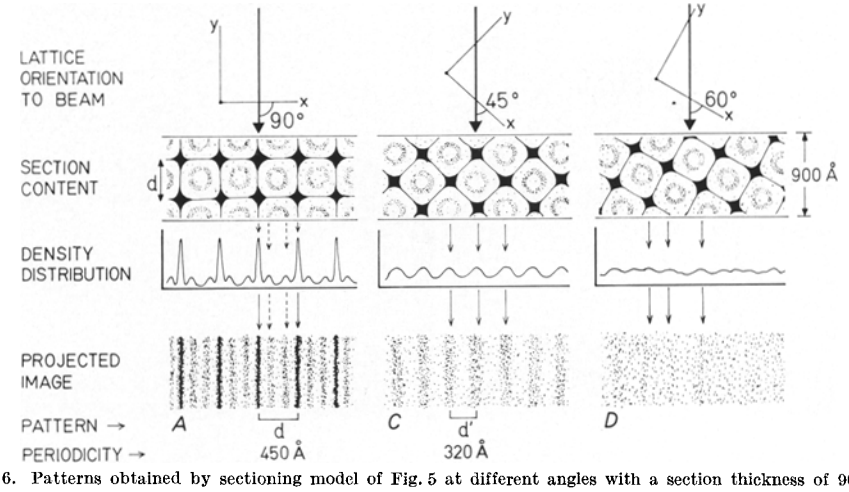


Fig. 6. Patterns obtained by sectioning model of Fig. 5 at different angles with a section thickness of 900 Å

(Fig. 2). A photometer tracing (Fig. 7) of three periods along the axis indicated by arrows in Fig. 2 shows good agreement with the theoretically predicted density distribution shown in Fig. 6 for pattern A.

If the model is sectioned parallel to the $z$-axis but at an angle of $45^0$ to the two other axes the lattice will again be projected as a banded pattern. The dense lines will correspond to the projection of the osmiophilic "filaments"; however, they should be broader, less well defined, and have less contrast than the dark lines

in pattern A since the four "wings" of the filaments will be inclined to the beam axis. The inner tube will not apear as distinct structure on the projected image since it is "behind" the dense filaments. The repeating period of this pattern can be calculated from the repeating period of the square lattice $d$ to be $d' = d \cdot \sqrt{\frac{1}{2}}$. With $d = 450$ Å it should measure about 320 Å. The pattern described for region C (Fig. 4) shows all these characteristics.

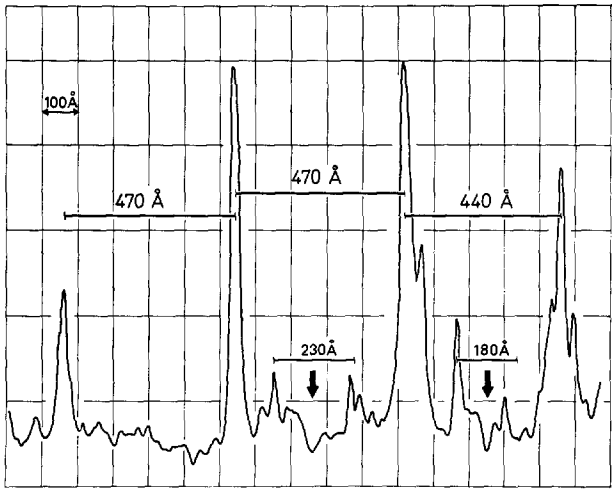


Fig. 7. Densitometer tracing of three periods of pattern *A* (cf. Fig. 2)

Sections of the model which form different angles to the $x$- and $y$-axis will not yield a distinct pattern on projection, except for a faint but irregular streaking. This is found in regions of type D (Figs. 1 and 4). A distinct pattern can be seen on the projected section if the section plane is parallel to the $x$- or $y$-axis but inclined to the z-axis by an angle $\alpha < 30^0$: The pattern will show rectangles whose shorter sides will measure $d = 450$ Å. The length of the longer side will be $d/\cos\alpha$, i.e. it may be up to 20% larger than $d$ and measure 500—540 Å. The contrast of the shorter side should be less than that of the longer side. Such patterns can also be observed.

The qualitative evaluation of the projection patterns obtained by sectioning the hypothetical model of Fig. 5 has revealed that these agree very well with the patterns observed in our electron micrographs. A quantitative check consisted of comparing the repeating periods $d$ and $d'$ of patterns A and C respectively. According to theory we should find $d' = d \cdot \sqrt{\frac{1}{2}}$. From repeated measurements of these periods on different micrographs we find:

$$d = 457 \text{ Å} \quad (\text{S.E. } \pm 6 \text{ Å})$$
$$d' = 325 \text{ Å} \quad (\text{S.E. } \pm 5 \text{ Å})$$
$$d \cdot \sqrt{\tfrac{1}{2}} = 324 \text{ Å}$$

This good agreement between calculated and measured values of $d'$ serves as quantitative proof that the patterns observed on sections are derived from a structure which conforms to the model proposed.


Fig. 8. Tubular myelin figure of 380 Å spacing. Arrows indicate that the central tubule is replaced by a "filament". × 100,000

As Fig. 1 reveals different patterns can be observed on sections of one and the same aggregate of this tubular material. This is the result of a curved course of bundles of the prismatic tubules, which can be directly observed in regions showing pattern A. Such bundles can split into different branches and also form whorls.

*3. Variations in the pattern and association with other materials*

Among the different specimens studied some degree of variation in the pattern was found. The most significant variation concerned a reduction in the repeating period $d$ to 380 Å while the geometric properties of the observed patterns were unchanged. In these instances it was characteristic that the space between the dense images of the winged osmiophilic filaments contained only one gray line instead of two in pattern A, and a central gray dot instead of a circular profile in pattern B (Fig. 8). Translating these patterns into our model we would find the central tubule of 250 Å diameter replaced by a thinner central filament. This appears to cause a reduction in the diameter of the principal tubules. In the same regions some spaces may widen from 380 Å to 450 Å and then show again a central tubule instead of the filament. The two patterns thus appear to be essentially identical.

Fig. 9 shows a section of a structure exhibiting concentric lines which could often be found associated with the material described. The repeating period was about 270 Å. In structures of this narrow spacing we could never observe other patterns than parallel banding. We can therefore conclude that these structures are formed by concentric osmiophilic shells separated by a less osmiophilic space.

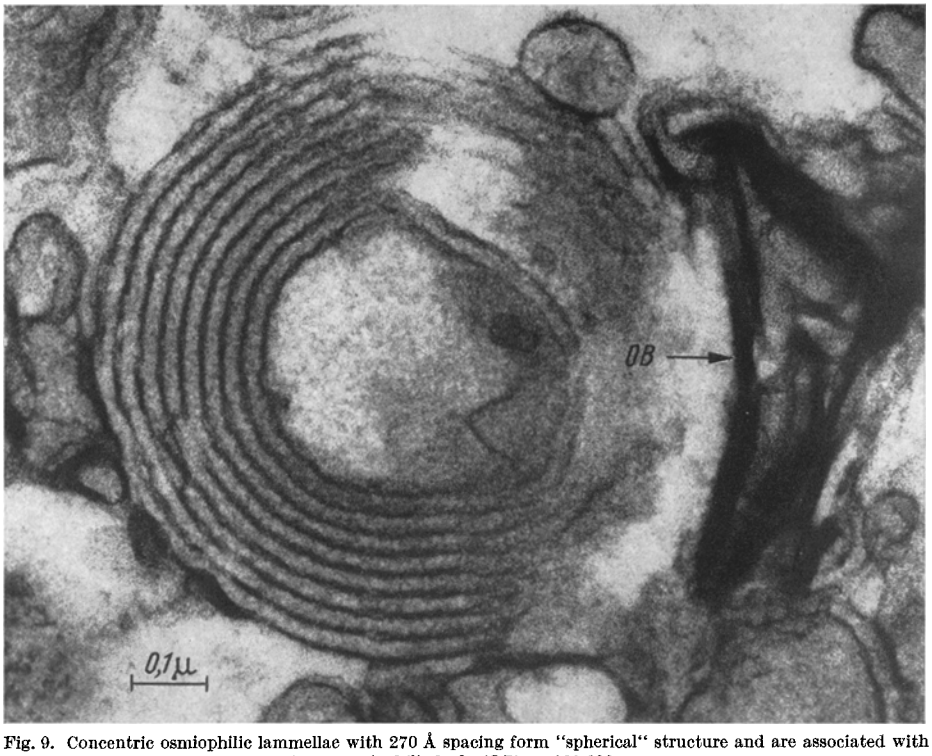


Fig. 9. Concentric osmiophilic lammellae with 270 Å spacing form "spherical" structure and are associated with dense osmiophilic body (*OB*). × 100,000

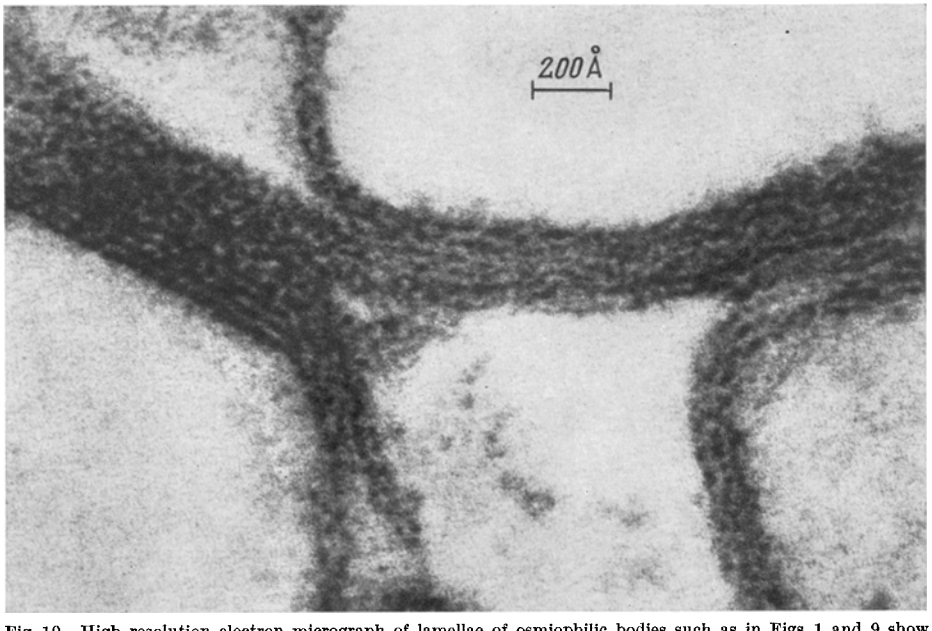


Fig. 10. High resolution electron micrograph of lamellae of osmiophilic bodies such as in Figs. 1 and 9 show 42 Å periodicity. × 500,000

Osmiophilic lamellae of varying thickness were irregularly arranged to form more or less spherical bodies which were always found closely associated with the organized materials described so far (Figs. 1—4, 9). High resolution electron micrographs of such lamellae revealed, that they were composed of alternating light and dark layers with a repeating period of 42 Å on the average (Fig. 10). This spacing has been related to water crystals of phospholipids (STOECKENIUS 1959), so that these lamellae can be regarded as true myelin lamellae. They were frequently found to be continuous with the highly osmiophilic components (lamellae and four-winged filaments) of the described organized structures.

## Discussion

Alveolar exudate formed under the conditions of oxygen poisoning contains large amounts of a peculiar material which could be shown, upon stereologic analysis, to be built of prismatic tubules of about 450 Å diameter which are closely packed in a quadratic lattice. Their wall consists of a highly osmiophilic substance which forms "four-winged filaments" in the corners of the lattice. They contain either a secondary smaller tubule or a filament of less osmiophilic nature.

This material appears to be identical to "finger-print-like" myelin figures described by POLICARD et al. (1957) in inflammatory processes and by CAMPICHE (1960). Both authors have related these figures to cellular degeneration caused by various agents. It may be of particular interest that we have regularly found small amounts of this material in normal rat lungs as well. However, the described masses were always located *outside* of cells; we have never been able to find this conspicuous pattern in intracellular myelin figures, neither in alveolar epithelial cells nor in macrophages. In oxygen-poisoned rat lungs we found "degenerating" alveolar epithelial cells (cf. KISTLER et al. 1965); however, in their osmiophilic granules the spacing of the dense lamellae was irregular and on the average considerably smaller than 450 Å. The same could be said for the lamellated osmiophilic inclusions in the macrophages.

The material described can be considered to represent a particular form of myelin figures. It is frequently found associated with spherical figures composed of concentric osmiophilic lamellae separated by irregular light spaces (Fig. 1). Where these lamellae are oriented normal to the plane of section a fine banding with a repeating period of about 42 Å can be resolved (Fig. 10) which may characterize them as myelin figures formed by repeating bimolecular leaflets of phospholipids in a water phase (STOECKENIUS 1959, 1962). Very frequently a continuity can be observed between these myelin lamellae and the electron dense layers of the two basic lattices described: the 270 Å periodicity of concentric shells (Fig.9), and the osmiophilic "four-winged" filaments forming the walls of the 450 Å tubular lattice. It is therefore suggestive that these osmiophilic components represent phospholipids as well.

It would thus appear that the formative force which causes the formation of these very distinct "water crystals" lies in the substance separating the osmiophilic layers. At this time nothing can be said about the nature of this substance, except that it seems to be a hydrophilic material, possibly protein or mucopolysaccharide (GRONIOWSKI and BICZYSKOWA 1964), since we can observe that the lattice "opens" to the surrounding fluid in the region of these lighter layers (Fig. 1).

Recently, a number of "layered" structures have been described which may, at first sight, bear some similarity to the structures described here. FRIEDMANN et al. (1965) have found laminated cytoplasmic inclusions in degenerating sensory cells of the human inner ear in Ménière's disease; the repeating period of these structures was 1000—1500 Å and thus greatly exceeds that of the structures described here in spite of a certain similarity of the pattern on sections. The laminated material described by MORALES et al. (1964) in the neurons of the lateral geniculate body is of clearly different nature. The periodic structures found by WETZSTEIN et al. (1963) in the subcommissural organ of the rat have been found by stereologic analysis (SCHWINK et al. 1963) to be generated by parallel "in phase" arrangement of tropocollagen molecules; they thus clearly differ in material and structure from the formations described here.

Further experimental analysis will have to elucidate, whether these peculiar formations — which are found abundantly in diseased lungs but are also present in small amounts in normal lungs — derive from destroyed cells or relate to some normal constituents of a lining layer external to alveolar epithelial cells as it was postulated to exist by PATTLE (1958) and others (cf. CLEMENTS and TIERNEY 1965).

## References


CAMPICHE, M.: Les inclusions lamellaires des cellules alvéolaires dans le poumon du raton. J. Ultrastruct. Res. **3**, 302—312 (1960).

CLEMENTS, J. A. and D. F. TIERNEY: Alveolar instability associated with altered surface tension. Handbook of Physiology, Section 3, Volume II, 1565—1583 (1965).

FRIEDMANN, J., T. CAWTHORNE, and E. S. BIRD: The laminated cytoplasmic inclusions in the sensory epithelium of the human macula. J. Ultrastruct. Res. **12**, 92—103 (1965).

GRONIOWSKI, J., and W. BICZYSKOWA: Structure of the alveolar lining film of the lungs. Nature **204**, 745—747 (1964).

KARNOVSKY, M. J.: Simple methods for staining with lead at high pH in electron microscopy. J. biophys. biochem. Cytol. **11**, 729—732 (1961).

KISTLER, G. S., P. R. B. CALDWELL, and E. R. WEIBEL: Pulmonary pathology of oxygen toxicity: an electron microscopic and morphometric study. To be published.

LUFT, J. H.: Improvements in epoxy-resin embedding methods. J. biophys. biochem. Cytol. **9**, 409—414 (1961).

MORALES, R., D. DUNCAN, and R. REHMET: A distinctive laminated cytoplasmic body in the lateral geniculate body neurons of the cat. J. Ultrastruct. Res. **10**, 116—123 (1964).

PATTLE, R. E.: Properties, function, and origin of the alveolar lining layer. Proc. Roy. Soc. B. **148**, 217—240 (1958).

POLICARD, A., A. COLLET et S. PRÉGERMAIN: Etude au microscope électronique des figures myeliniques dans les processus inflammatoires. Bull. Micr. appl. **7**, 49—53 (1957).

REYNOLDS, E. S.: The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. **17**, 208—211 (1963).

SCHWINK, A., P. STANKA u. R. WETZSTEIN: Stereologie periodisch strukturierter Körper in der Umgebung bestimmter Hirngefäße. In: Proceedings of First Internat. Congr. for Stereology, Vienna, 22/1—6 (1963).

STOECKENIUS, W.: An electron microscope study of myelin figures. J. biophys. biochem. Cytol. **5**, 491—500 (1959).

— Structure of the plasma membrane; an electron microscope study. In: Symposium on the Plasma Membrane. Circulation, Suppl. **26**, 1066—1069 (1962).

WETZSTEIN, R., A. SCHWINK u. P. STANKA: Die periodisch strukturierten Körper im Subkommissuralorgan der Ratte. Z. Zellforsch. **61**, 493—523 (1963).



Prof. Dr. E. R. WEIBEL
Anatomisches Institut der Universität
Gloriastraße 19, 8006 Zürich (Switzerland)