Electron Micrographs of Myelin Figures of Phospholipide Simulating Intracellular Membranes.*

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In an earlier study (3) it was observed that if cells isolated from guinea pig testis were allowed to stand in Tyrode solution, the endoplasmic reticulum gave rise to concentric systems of membranes (Fig. 1) strongly reminiscent of the myelin figures studied in great detail by Nageotte (6). These findings suggested that an electron microscope study of phospholipide films formed *in vitro* might shed some light on the behavior of cytoplasmic membranes observed *in vivo*, and might also provide information of value in interpreting the chemical nature of the membrane components responsible for the dense lines seen in electron micrographs of cells. In thin sections of osmium-fixed tissue, the individual membranes (~90 A) exhibit a characteristic fine structure consisting of two dense layers (~30 A) separated by a region of lower density (~30 A). Owing to our poor understanding of the mechanism of fixation and staining with osmium tetroxide, this double-contoured appearance has been variously interpreted. Some investigators, adhering to the traditional view that osmium reacts primarily with the double bonds of unsaturated fatty acids, have considered the dense components of the membranes to be lipide. Other workers, taking cognizance of recent studies (1) showing that osmium also reacts with certain amino acids, have interpreted the two dark lines as representing mainly protein, while the light interspace is believed to consist of a bimolecular leaflet of lipide (8–10). The lack of staining of the latter with osmium has usually been attributed to a high degree of saturation of the hydrocarbon chains or to extraction of lipide in the course of specimen preparation. The model systems of membranes described in this preliminary report were produced *in vitro* by hydrating phospholipides which, although inhomogeneous in lipide content, were essentially free of protein and other high molecular weight compounds. Thus, in electron micrographs of these artificial membranes any density can be interpreted as lipide or as a product of the reaction of lipide with the fixatives used.

The phospholipides, consisting mainly of lecithins were prepared by acetone precipitation of a total lipide extract of eggs, and were used fresh or after short periods of storage. Small amounts were hydrated in distilled water for 1 to 2 hours at 1–4°C. The resulting myelin figures took various forms, the commonest being tubes with thick walls consisting of multiple layers of closely spaced membranes. Of less frequent occurrence, but of greater interest for the problem at hand, were hollow spheres or membrane-limited vesicles of graded size, arranged one within the other (Figs. 2 and 3). These concentric lamellar systems of phospholipide were fixed for 1 hour in potassium permanganate (5) or in Dalton's chrome-osmium fixative (2), slowly dehydrated in ethanol, and embedded in 90 per cent *n*-butyl methacrylate/10 per cent methyl methacrylate (7) or in araldite (4). The latter embedding medium was found to be more suitable for studying thin films in cross-section because of its greater stability under the electron beam. Methacrylate often sublimed in the beam and allowed the sections of membrane to fall over so that they were seen on the flat instead of in edge-on view. Electron micrographs were made at original magnifications of 1500 to 30,000 with an RCA model EMU-3B microscope or with a Siemens Elmiskop II.

The form and arrangement of membranes as seen under the phase contrast microscope (Figs. 2 and 3) are preserved by both chrome-osmium and permanganate fixatives and withstand the dehydration, embedding, and cutting procedures with relatively little distortion (Figs. 4 and 5). At low magnification, the phospholipide membranes appear as thin black lines, where seen on edge, or as less dense narrow ribbons where sublimation of the methacrylate has permitted the 300 A sections of membrane to fall over so that they are seen in oblique or surface view (Fig. 9). The tubular myelin figures, in transverse sections, consist of 5 to 50 or more closely approximated concentric circles. The vesicular figures are made up of 2 to

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30 circles or ovals one within the other. These are often eccentrically arranged so that all the membranes are closely approximated at one pole of the figure and more widely separated at the other (Fig. 5). In some instances, the membranes in such concentric systems have a marked tendency to be associated in pairs 200 to 1000 A apart (Figs. 7 and 8) which, in some respects, resemble the paired membranes that bound the cisternae of the endoplasmic reticulum in secretory cells (Fig. 6). In other instances, circumferentially oriented membranes are arranged around the periphery of an amorphous central mass. Such appearances probably represent membranes delaminating from the surface of an unhydrated mass of phospholipide.

At higher magnification there is a distinct difference in the appearance of permanganate-fixed and chrome-osmium–fixed membranes. In surface view those fixed in permanganate have a fine granular texture of a rather uniform density. Chrome-osmium–fixed membranes also show a fine uniform granularity of appreciable density, but superimposed on this homogeneous background is a random stippling of very dense granules 30 to 50 A in diameter that appear to vary in number and size, depending upon local conditions. Their chemical nature is unknown.

When individual phospholipide membranes are viewed in cross-section at high magnifications, it is noticed that each membrane has a structure consisting of two dense lines, 25 to 30 A wide, separated by a clear space of the same width. This bilaminar structure can be made out in material fixed in osmium tetroxide, but it is often obscured by the presence of the dense particles adhering to the membranes. It is most clearly seen in permanganate-fixed material (Fig. 10).

The observations presented in this preliminary note demonstrate that the phospholipides are not extracted to any great extent by the methods of fixation and dehydration commonly used in the preparation of tissues for electron microscopy. Electron micrographs of phospholipide membranes in thin sections are strikingly similar in appearance to cellular membranes prepared in the same way. In the case of the artificial membranes described here, the presence of protein cannot be invoked to account for the double contour of the membranes seen in sections at high magnification. It is possible therefore, that the bilaminar structure apparent in permanganate-fixed cell membranes is largely due to their phospholipide component. It cannot be stated at present with any degree of assurance whether it is the hydrophilic or hydrophobic component of the bimolecular leaflets of phospholipide which is responsible for the two dense lines observed.

In an effort to adduce further evidence on this point, studies are in progress on the mode of fixation and staining of membranes made from carefully purified and well characterized phospholipides. Attention is also being directed to the effect of various environmental factors (protein, ions, pH, etc.) on the behavior of phospholipide membranes *in vitro*.

Bibliography

1. Bahr, G. F., Continued studies about the fixation with osmium tetroxide, *Exp. Cell Research*, 1955, **9**, 277.
2. Dalton, A. J., A chrome-osmium fixative for electron microscopy, *Anat. Rec.*, 1955, **121**, 281.
3. Fawcett, D. W., and Ito, S., Observations on the cytoplasmic membranes of testicular cells examined by phase contrast and electron microscopy, *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 135.
4. Glauert, A. M., and Glauert, R. H., Araldite as an embedding medium for electron microscopy, *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 191.
5. Luft, J. H., Permanganate—a new fixative for electron microscopy, *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, 799.
6. Nageotte, J. Morphologie des gels lipoïdes, Actualités Scientifiques et Industrielles, Paris, Herrmann et Cie., 1936.
7. Newman, S. B., Borysko, E., and Swerdlow, M., New sectioning techniques for light and electron microscopy, *Science*, 1949, **110**, 66.
8. Robertson, J. David, Some aspects of the ultrastructure of double membranes, Ultrastructure and Cellular Chemistry of Neural Tissue, (H. Waelsch, editor), New York, Paul Hacker, Inc., 1957.
9. Schmitt, F. O., and Geschwind, N., The axon surface, Progress in Biophysics and Biophysical Chemistry, (J. A. Butter and B. Katz, editors), New York, Pergamon Press, **8**, 1957.
10. Sjöstrand, F. S., The ultrastructure of cells as revealed by the electron microscope, International Reviews of Cytology, (G. H. Bourne and J. F. Danielli, editors), New York, Academic Press, Inc., **6**, 1956.

Explanation of Plates

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FIG. 1. A phase contrast photomicrograph of two anuclear portions of Sertoli cells isolated from guinea pig germinal epithelium and allowed to stand in Tyrode solution for 1 hour. The cytoplasmic membranes have become reorganized into concentric lamellar systems (*cf.* reference 3). × 2200.

FIG. 2. A phase contrast photomicrograph of concentric arrays of membranes formed *in vitro* by hydration of a preparation of phospholipide. These myelin figures bear a striking resemblance to the concentric intracellular membranes depicted in Fig. 1. An electron micrograph of an area similar to that enclosed by the rectangle is shown in Fig. 7. × 2500.

FIG. 3. A photomicrograph of a larger mass of concentric membranes formed by hydration of phospholipide in distilled water. × 2500.

FIG. 4. A low power electron micrograph of a thin section of a myelin figure that was fixed in permanganate and embedded in araldite. The wrinkled, irregular contour of the membranes, which is probably artefactual, is more marked in this specimen than in material fixed in chrome-osmium and embedded in methacrylate. × 7000.

FIG. 5. An electron micrograph of a myelin figure consisting of several membranous hollow spheres of graded size one within the other. These are often eccentric in position, with the limiting membranes closely approximated on one side and more widely spaced on the opposite side. Several membranes from an area such as that enclosed by the rectangle are seen at higher magnification in Fig. 9. Osmium fixation, methacrylate embedding. × 4500.

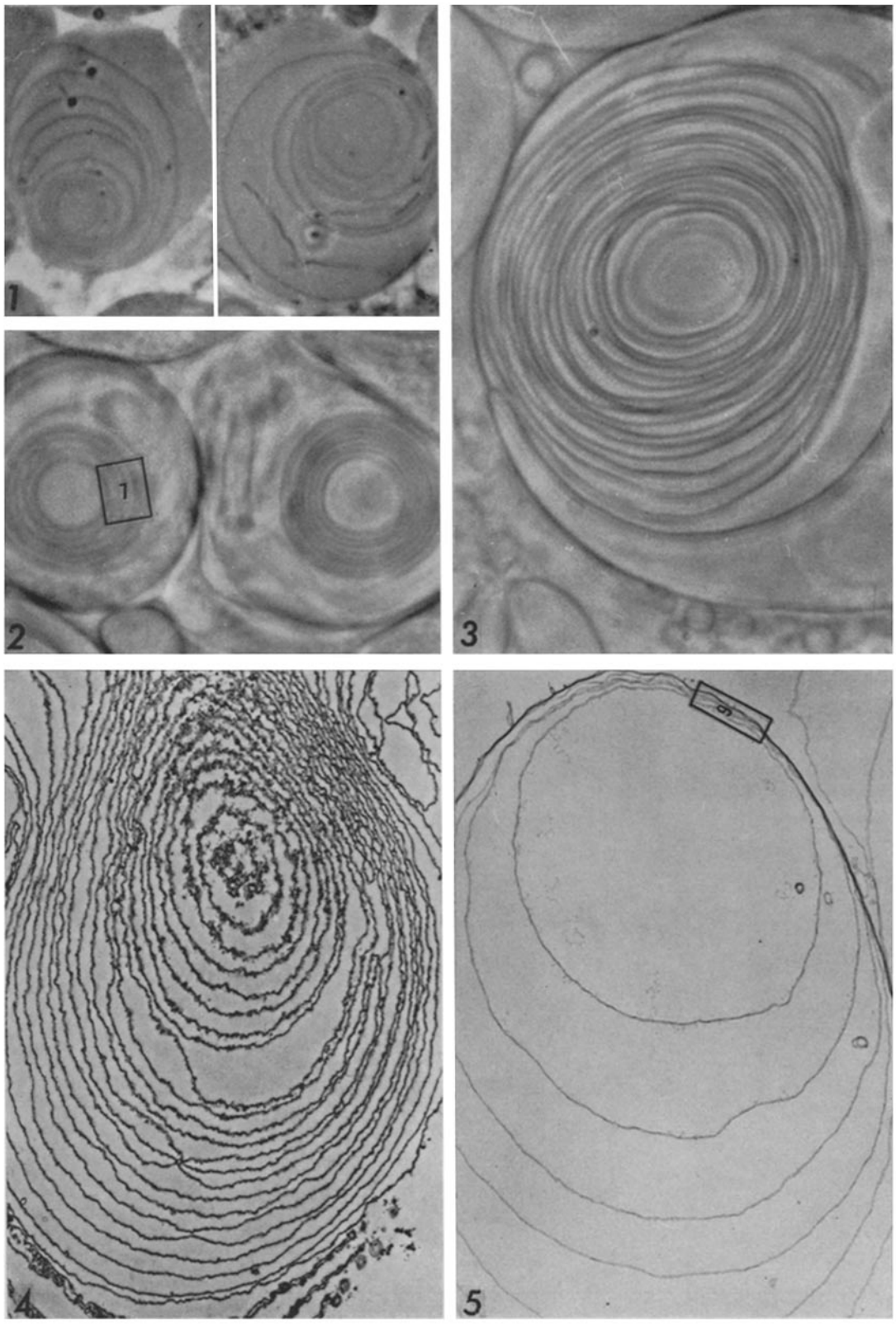

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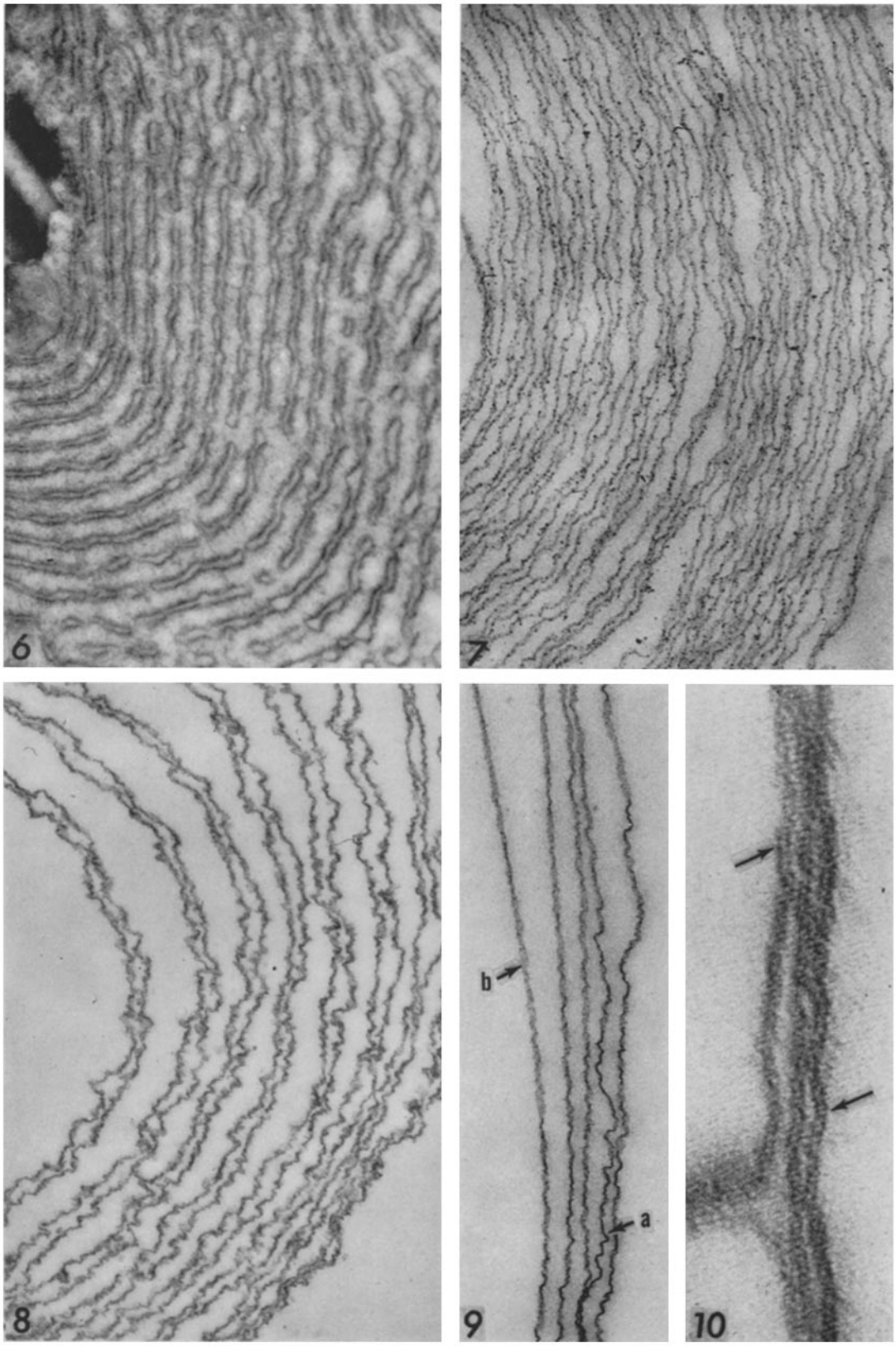
FIG. 6. An area of cytoplasm from a glandular cell showing the familiar parallel disposition of the cisternae of the endoplasmic reticulum. The micrograph is included here for comparison with the concentric membranes in myelin figures (Figs. 7 and 8) which often show a tendency to associate in pairs which bear a superficial resemblance to the membranes that bound the cisternae of the endoplasmic reticulum. × 30,000.

FIG. 7. A transverse section of a myelin figure formed *in vitro* by hydration of phospholipide. With osmium tetroxide fixation, the phospholipide membranes themselves have considerable density and their surface is stippled with minute granules of much greater density. These dense particles appear to be *on*, rather than *in* the membranes. The membranes in this figure show a tendency to associate in pairs. This is seen more clearly in the specimen illustrated in Fig. 8. × 19,000.

FIG. 8. A concentric system of artificial phospholipide membranes fixed in permanganate and embedded in araldite. The wrinkling of the membranes that occurs in the course of this procedure for specimen preparation gives an erroneous impression of their thickness owing to the prevalence of oblique and surface views. The membranes here are clearly associated in pairs separated by 200 to 1000 A, which is of the same order of magnitude as the space between the parallel membranes bounding cisternae of the endoplasmic reticulum in cells. × 23,000.

FIG. 9. Electron micrograph of a small area (*cf.* rectangle, Fig. 5) of an osmium-fixed myelin figure at higher magnification. Where the plane of section is perpendicular to that of the membrane (*e.g.* at *a*), the latter appears as a sharply defined black line. Where the segment of membrane has fallen over as the methacrylate sublimed in the beam (*e.g.* at *b*), it has a gray, ribbon-like appearance in surface view. × 31,000.

FIG. 10. At high magnification the individual lipide membranes are double contoured. The two dense lines separated by a less dense intermediate zone can be seen best at the arrows. The electron micrograph shows a slightly astigmatic image, but the direction of distortion is such as to minimize rather than accentuate the double contour. The two dense components often interpreted as protein in micrographs of cell membranes, cannot be so interpreted in these artificial phospholipide membranes formed *in vitro*. × 230,000.



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