APPLICATIONS OF FREEZE-SUBSTITUTION TO ELECTRON MICROSCOPE STUDIES OF INVERTEBRATE OOCYTES

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ABSTRACT

A modified freeze-substitution process is described which gives a low percentage (less than 5 per cent) of preparations of invertebrate eggs which appear to be ice crystal-free at the resolution of the electron microscope. The mitochondria show no membranes in these preparations but can be recognized by internal spaces with the size and the distribution of the cristae. The Golgi bodies resemble those seen with diffusion fixatives, but the limiting membranes are here double; that is, they appear to be a triple-layered sandwich with two outer dark approximately 25A layers and an inner light layer of the same thickness. The endoplasmic reticulum is clearly present and resembles that seen with diffusion fixatives. Here again, the limiting membranes are double with the same dimensions as those in the Golgi bodies. The membranes of the Golgi bodies and ER are seen after permanganate but not lead hydroxide staining. The hyaloplasm (or "cell sap") is crowded with 150 to 200A particles and these are also seen lining the ER membranes. In general, the structures as seen with the present technique show considerable similarity to those seen with diffusion fixatives.

INTRODUCTION

Most of the techniques used in the preparation of cells for electron microscopy make use of fixatives such as osmium tetroxide or potassium permanganate which penetrate cells by diffusion. The possibility of disturbing the arrangement of delicately organized components of the cell by diffusion drag forces (Rashevsky, 1948) or by violent dislocations accompanying chemical reaction of the fixative with cell constituents is difficult to eliminate in these procedures. Unfortunately, independent checks of structure on unfixed material, *e.g.* by means of x-ray diffraction, are difficult to carry through except on special material, such as the myelin sheath of nerve (Fernández-Morán and Finean, 1957). Further, the chemical and biochemical properties of cells fixed by osmium tetroxide or permanganate are very different from those of the corresponding living cells.

The use of ultrarapid freezing techniques in electron microscopy would appear to offer advantages both in the immobilization of delicate structures and in the preservation of their chemical and biochemical properties (Pearse, 1960). However, problems associated with ice crystal formation during freezing (Stephenson, 1960) and during dehydration, if the latter is done at too high a temperature ("recrystallization" or "devitrification," Luyet, 1960), must be overcome before these techniques can become standard.

Our own work in the area of freeze-substitution was stimulated by results published by Hancox (1957), Patten and Brown (1958), and Feder and Sidman (1958) for light microscopy. We have attempted to apply the methods to marine invertebrate eggs, whose small size (in our case 60 microns in diameter) offers unique advantages for rapid freezing (see Stephenson, 1960). The results

are encouraging in the sense that a small percentage of eggs are preserved in a way which appears comparable, if not superior, to that obtained with ordinary techniques, but discouraging in the sense that "successful" embeddings never number more than one out of twenty blocks and the number is usually considerably lower than this. However, the goals are worth continued effort for reasons given above and also for reasons emphasized by Fernández-Morán (1959).

MATERIALS AND METHODS

Ripe oocytes of the surf clam, *Spisula solidissima*, were obtained using the methods of Allen (1953). The quenching fluid was prepared essentially according to Feder and Sidman (1958) except that Freon 12, 13, or 14 was used. The liquid Freon was kept in a 250 ml. beaker in a Dewar whose temperature was controlled with liquid N_2. The Freon was stirred with a motor driven glass rod at speeds of approximately 5 to 10 revolutions per second.

Small (approximately 3 to 4 mm. square) pieces of aluminum foil were washed in detergent and then rinsed in distilled water and dried. Such foils probably retain some detergent, since water drops placed on them form flattened films rather than hemispherical drops. Thick suspensions of the previously obtained oocytes were placed on the foil, and excess sea water and eggs were removed until a thin film of water with a single layer of eggs remained. The foils were then plunged into the rotating Freon using fine jeweler's forceps, shaken, and set free. They usually floated on the surface of the liquid although they occasionally stuck to the sides and bottom of the beaker.

The next maneuvers were designed to transfer the frozen cells rapidly into substituting fluid without having to lift the cells out of cold fluid at any time. 5 ml. wide-mouthed serum bottles held in tongs were precooled to liquid N_2 and rapidly transferred into the 250 ml. beaker of Freon containing the floating pieces of foil. The foil was then ladled into the bottles with some liquid Freon. The bottles were rapidly dropped into holes in a precooled Styrofoam block and were then capped with the serum bottle stoppers pierced by two hypodermic needles, a 2 inch 20 to 24 gage needle for introducing and withdrawing fluid, and a ½ inch needle of the same gage which acts as a vent. It is advisable to mark these so that they are readily identifiable, since any fluid that is subsequently introduced into the venting needle by accident may be ejected from the long needle in a thin, high-velocity stream which can be very dangerous to the eyes.

The whole assumbly was then rapidly transferred into a freezer at −80°C. The Freon was removed, either by letting it vaporize or by withdrawing it from the serum bottle with a glass syringe which had been precooled by leaving it in the freezer. Syringes were put in dry and needed no lubrication.

The substituting fluid used most often by us consists of 1 per cent OsO_4 in acetone dried with Linde type 4A, 1/16 inch pellets of molecular sieve and prepared as in Feder and Sidman (1958). Using cooled glass syringes, we introduced 3 to 4 cc. of this fluid or absolute acetone into the 5 cc. bottles containing the tissue and allowed substitution to go on for three days to two weeks. The substituting fluid was then withdrawn and cold acetone without osmium (if osmium had been used) was introduced for at least a day (otherwise the osmium was reduced when the vials were brought to room temperature and the contents of the vial turned black). The vials were brought to room temperature and embedded in either methacrylate or Araldite 502 (the latter, following Finck, 1960). We have had uniformly poor results with methacrylate under many embedding conditions, as compared with the results with the epoxy resin.

Blocks were first screened by observing thick sections (1 to 2 microns) with a phase microscope, and only those blocks showing little obvious ice crystal damage were subsequently thin-sectioned for electron microscopy.

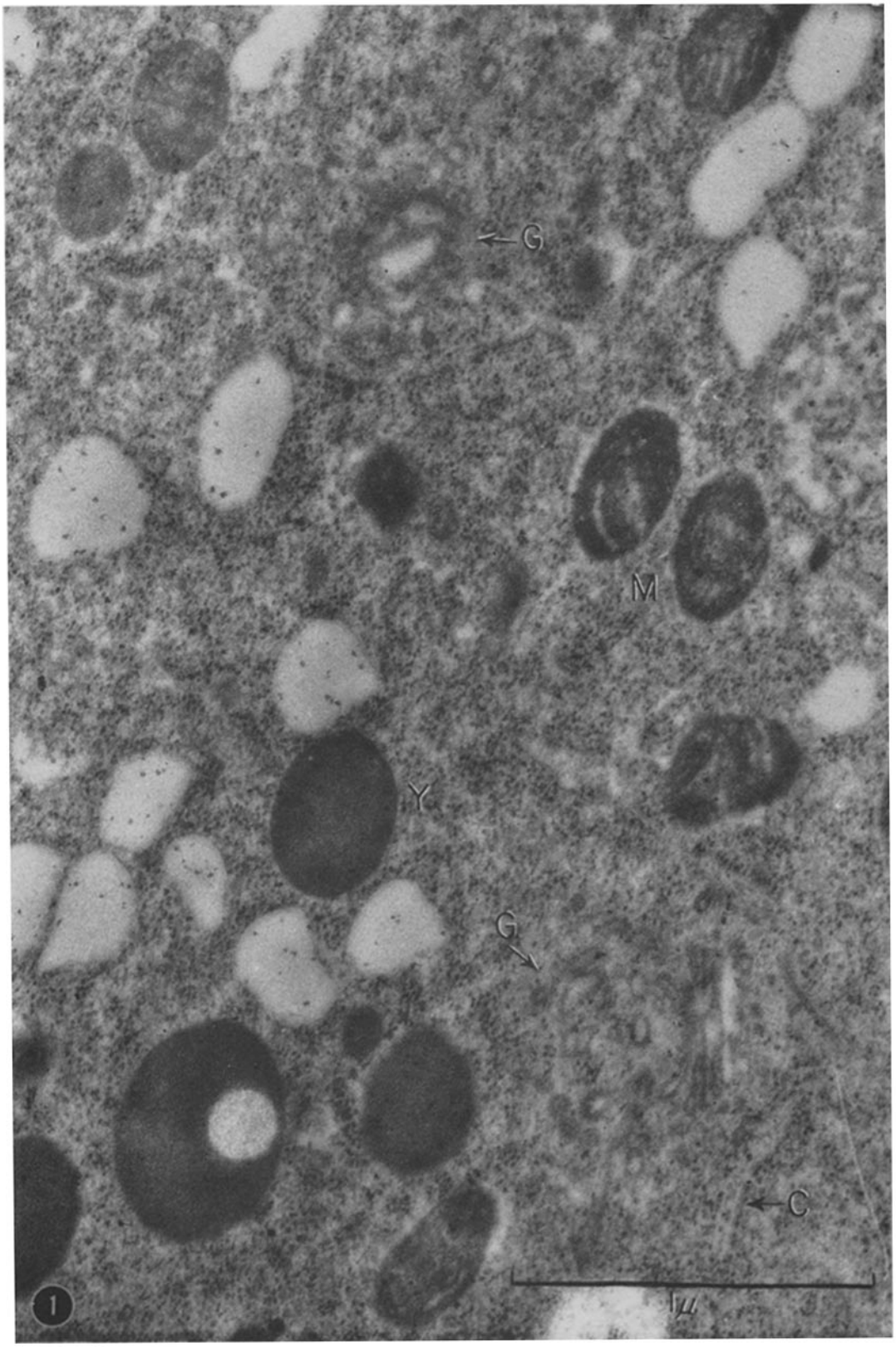
Diffusion-fixed material was prepared as described by Rebhun (1960, 1961).

Sections were cut with a Servall Porter-Blum microtome and mounted on formvar or carbon-formvar grids. The grids were subsequently stained with potassium permanganate following Lawn (1960) or lead hydroxide following Watson (1958) and Peachey (1959).

Observations were made with an RCA EMU-2D microscope.

FIGURE 1

View of the cytoplasm of an oocyte. The mitochondria (*M*) can be recognized by their size and by the "negative" image of the cristae. Individual cisternae (*C*) of the endoplasmic reticulum can be seen. Yolk granules (*Y*) are present. The "hyaloplasm" appears densely packed with small granules which are heavily stained. Two Golgi bodies (*G*) can be seen. In the lower one can be seen some of the elements described in the text; *i.e.*, the elongated, flattened loops and small circular profiles, each with its double limiting membrane. Stained with permanganate. × 57,500.


G
M
Y
G
C
1μ
1

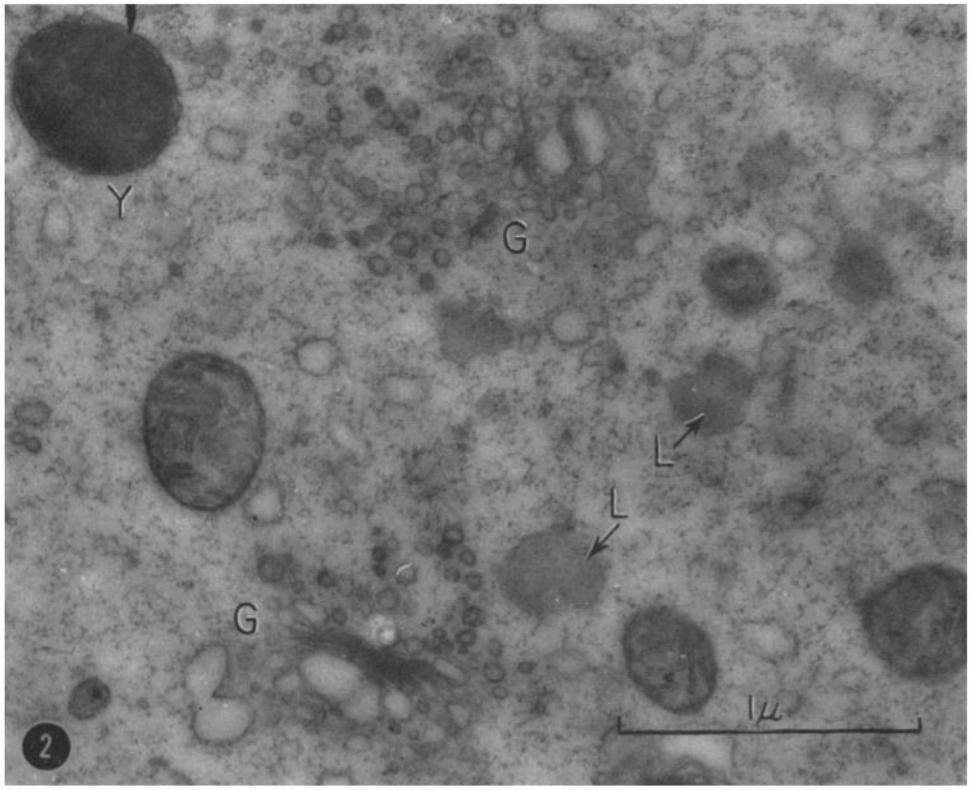


FIGURE 2

Region of an unfertilized oocyte fixed in Palade's fluid and embedded in methacrylate. Two Golgi bodies (*G*) can be seen which consist of flattened membranes parallel to one another and 250 to 500 A vesicles at the ends. Typical mitochondria and yolk (*Y*) and lipid (*L*) granules are also visible. × 40,000.

RESULTS

Quenching Fluid

We have found that, except on a small number of occasions, the only success we have had in avoiding ice crystals visible with the electron microscope has been achieved by use of Freon 12 at temperatures of about −150°C. Although Freons 13 and 14 can be cooled to temperatures from 30°C. to 40°C. lower than those of Freon 12, they have not yielded ice crystal-free preparations, in our hands, to any usable degree at these lower temperatures.

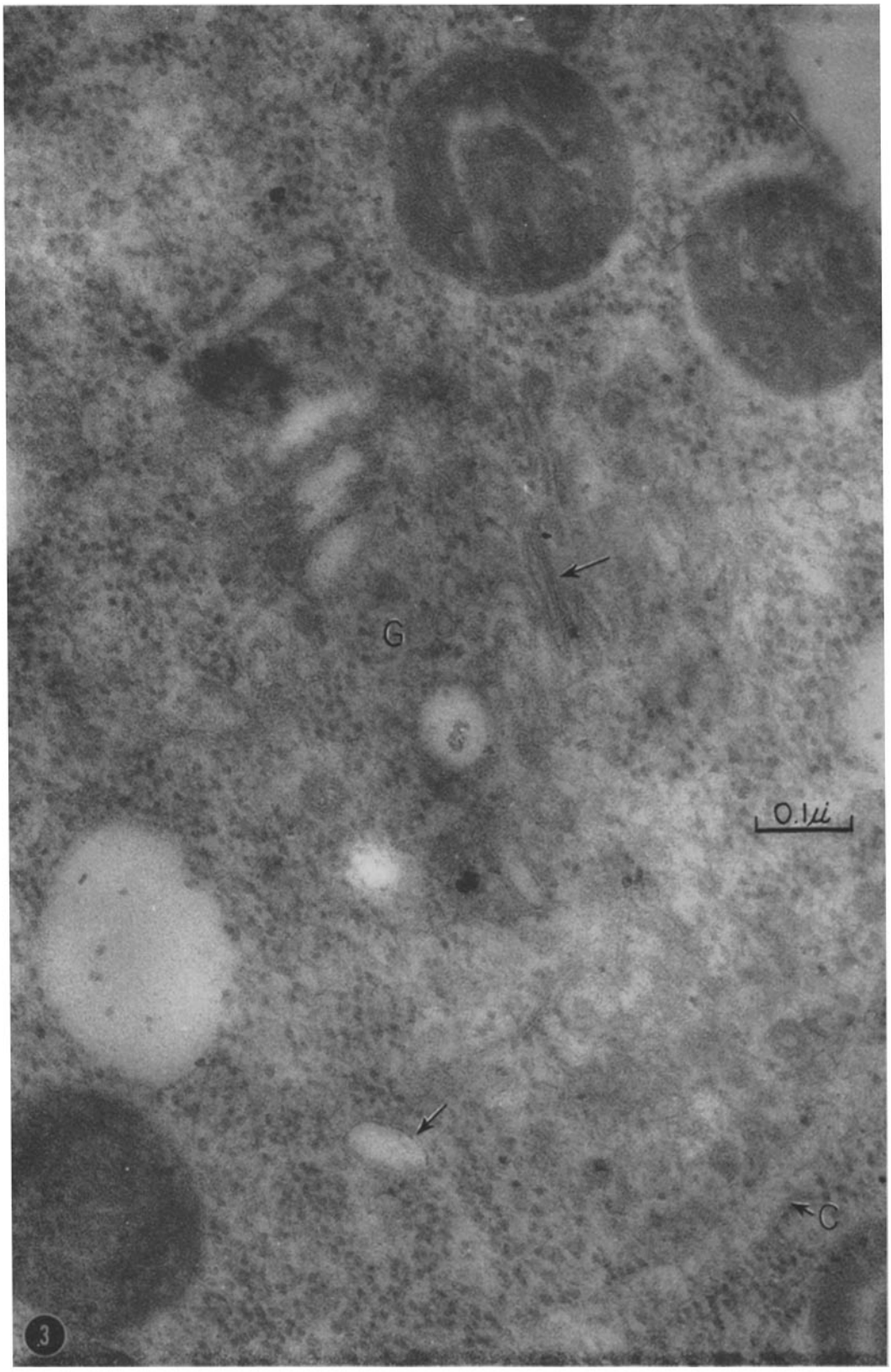
The frequency of successful embeddings which we have obtained in the total project is low. With Freon 12 it varies with the experiment but never exceeds more than one in twenty blocks of embedded material. Within the successful blocks, at most one in two or three eggs is devoid of obvious ice crystals. For eggs prepared by conventional means (Rebhun, 1960, 1961) the percentage of successful embeddings is approximately 5 to 10 per cent and may be less.

Cytoplasmic Structures

A. *Mitochondria:* Mitochondria can be recognized in sections stained with permanganate or lead

FIGURE 3

The Golgi bodies (*G*) never stain densely with the present technique, but the approximately 75 A double membrane limiting the flattened loops and circular profiles can be seen with reasonable clarity at the arrows. Stained with permanganate. × 115,000.


G
0.1μ
C
3

hydroxide by their size, number, and distribution and by the fact that light spaces in the dense interior correspond in size and distribution to cristae seen in conventional preparations (compare Fig. 1, 3, and 4 with Figs. 2 and 7). In detail, each mitochondrion appears to be surrounded by a space 100 to 200 A thick with no obvious membranes. The interior of the structure is dense except for spaces whose dimensions correspond to those of cristae as seen by ordinary techniques (see figures referred to above). Briefly stated, the mitochondria look as if the usual mitochondrial image had been "inked in" within the inner mitochondrial membrane and outside the cristae, and the membranes removed. This is uniformly seen in all mitochondria in well preserved areas of these eggs. The dense parts of the mitochondrion appear, in these preparations, to consist of closely packed electron-opaque grains of the order of 20 to 30 A in diameter. The latter are possibly artifacts induced by permanganate staining, or possibly represent phase effects due to slight underfocusing (Sjostrand, 1956). However, the possibility cannot be excluded that they represent some components existing in the living cell.

B. *Golgi Apparatus:* In contrast to the mitochondria, which lack obvious membranes, the Golgi bodies can be clearly recognized in permanganate-stained sections by the fact that the membranes correspond in some detail to those seen in sections prepared with ordinary techniques of electron-microscopy (Rebhun, 1960), although their contrast in frozen sections is low; compare Figs. 1 and 3 with Fig. 2. Their profiles consist of loops up to 1 micron in length with a total width of about 250 to 300 A. Several of these are stacked in parallel with spaces of the order of 200 A between, although neighboring loops may occasionally touch. In addition, the region contains spherical profiles 250 to 500 A across, the total profile of loops and circles being of the order of 1 micron in diameter.

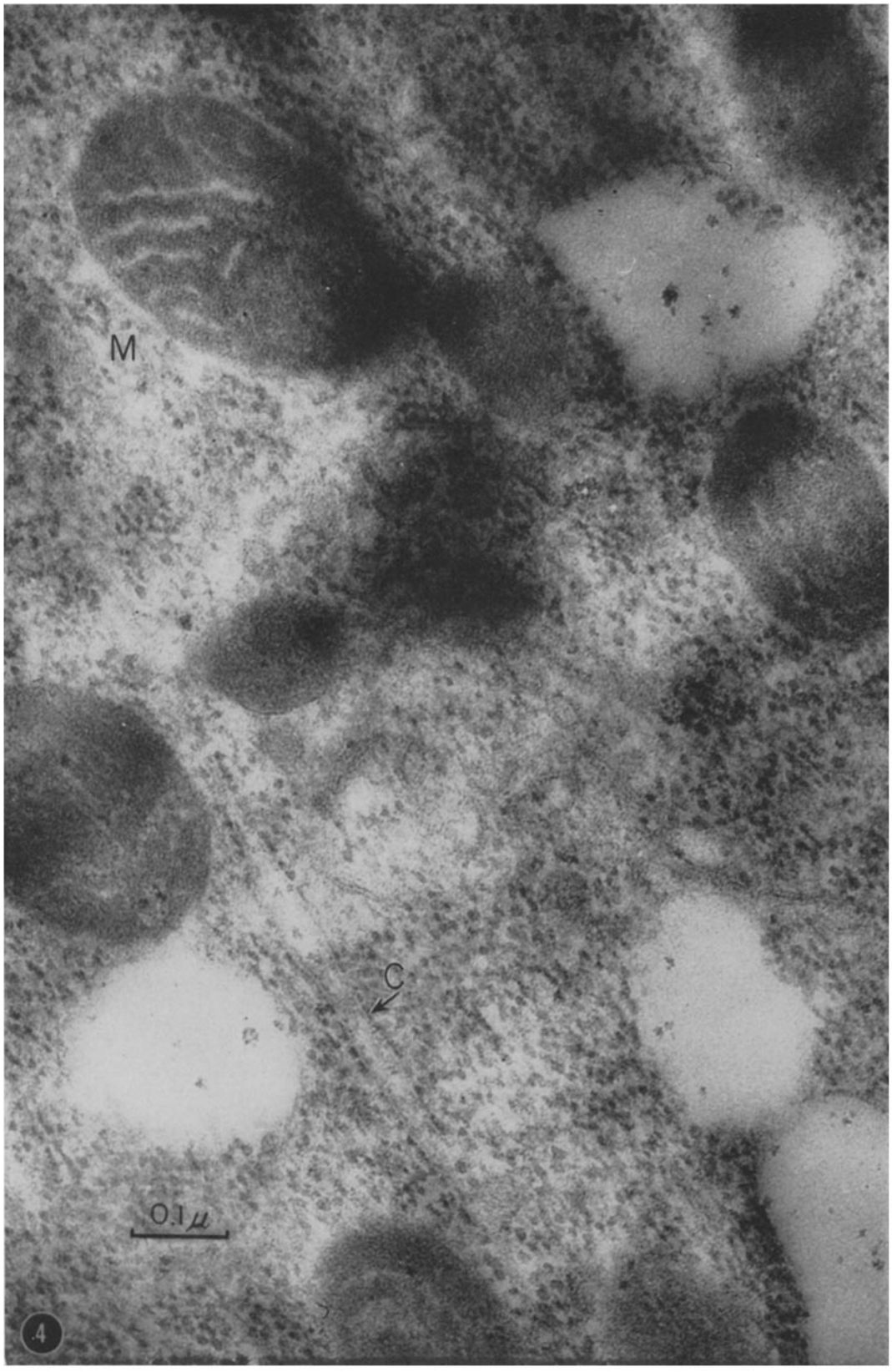
The most striking aspect of these structures is the fact that each loop or circle consists of a "double membrane," that is, a triplet consisting of a dark outer and inner layer, each approximately 25 A in thickness, separated by a space of about the same thickness, the whole complex therefore being about 75 A thick. The dimensions given here are clearly subject to uncertainties of preparation and of optics such as those discussed by Robertson (1960). It should be pointed out that we have, so far, not observed similar regions in sections stained with lead hydroxide, although we think we have seen regions which represent Golgi bodies with the lead technique. The lack of membranes or other clear landmarks, however, makes the identification somewhat uncertain.

C. *Small Granules:* The cytoplasm of the cell, excluding large inclusions (*i.e.*, the "cell sap"), appears to contain great numbers of small granules 150 to 200 A in diameter and approximately spherical in shape, although angular and polygonal-shaped particles are not uncommon. Further, particles lining the endoplasmic reticulum, as discussed below, are almost always flattened on the side facing the membrane part of the ER. Close examination of these granules indicates that some of them may have light interiors (Fig. 4). It seems clear that the granules here described correspond to what have been called "Palade" particles in cells fixed by conventional procedures (compare with Fig. 2 and 7).

D. *Endoplasmic Reticulum:* The ER in these eggs has been the subject of several papers (Rebhun, 1956*a*, 1956*b*, 1961) and consists of annulate lamellae, organized layers of ER resembling that seen *e.g.* in pancreas, and extensive deployments of single cisternae and tubules of various gross dimensions. We shall not discuss the annulate lamellae since their structure is somewhat variable as seen with the present technique, although they are clearly recognizable (see Fig. 5). The gross features of the organized ER (Fig. 8) correspond to those seen with ordinary techniques (Fig. 7) in this material (see also Rebhun, 1961). That is, there are extensive cisternae parallel to one another, the membranes of which are lined by

FIGURE 4

The mitochondrion (*M*) in the upper left shows cristae as well as the 25 to 30 A granularity mentioned in the text. A cisterna (*C*) of the endoplasmic reticulum runs diagonally from middle left to the lower middle of the figure. The approximately 75 A double membrane limiting the ER is clear, as are the flattened 150 to 200 A particles studding the exterior. Notice that some of the small granules appear hollow. Stained with permanganate. × 115,000.


M
C
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4

100 to 200 A particles, somewhat flattened but otherwise resembling those discussed above under C (Fig. 8).

In addition to the masses of organized ER, isolated cisternae and tubules ramify throughout the cytoplasm (Rebhun, 1961). The details of these isolated structures differ in no obvious way from those of the structures which are organized into parallel layers (Fig. 1, 3, and 4).

As in the case of the Golgi apparatus, the most striking feature of these structures is the limiting membrane. This consists of a flattened row of 150 to 200 A particles on a possibly incomplete 25 A membrane (or layer), separated by a space approximately 25 A in width from an inner membrane which is also about 25 A thick (Figs. 4 and 8). It is not clear from these preparations whether there is always an outer 25 A layer to complete the "membrane sandwich," since it may be obscured by the flattened 150 to 200 A particles. In many but not all areas where the particles are somewhat separated, the outer layer does appear to be present. The inner layer is always clear in cross-sections. Thus, except for the possibility that the outer 25 A membrane responds somewhat differently to our procedure than does the inner 25 A membrane, the membranes of the ER appear to be three-layered structures, identical in dimensions to those of the Golgi bodies.

The results with lead hydroxide staining are surprising; no membranes can be seen at all in the ER and, indeed, the latter appears as a set of narrow spaces, with 150 A particles neatly lined up, but no obvious supporting structure (Fig. 6).

E. *Other Inclusions:* Yolk granules and cortical granules may be easily recognized when comparing electron micrographs of permanganate-stained sections with those of sections prepared by ordinary techniques (compare Fig. 1 with Fig. 2). Both types of granules possess surrounding membranes (not easily seen in all cases in frozen material) and both possess dense interiors. The cortical granules are oblong and are ½ to 1 micron wide by 1 to 2 microns long. The yolk bodies are about 1½ to 2 microns in diameter.

The cytoplasm is replete with what appear to be vacuoles about ½ micron in diameter and usually not bounded by an apparent membrane although occasionally one appears to be present. Comparing the electron micrographs of freeze-substituted eggs with those of eggs prepared by ordinary techniques, *e.g.* Figs. 1 and 2, it seems clear that the only structures that these "vacuoles" could represent are lipid granules. This statement is made on the basis of two observations: (*a*) The number of lipid granules in eggs fixed by ordinary means is about the same as the number of "vacuoles" seen in freeze-substituted eggs. (*b*) There are no other structures seen in freeze-substituted eggs numerous enough or of nearly enough the same size as lipid granules to represent them, if the "vacuoles" do not.

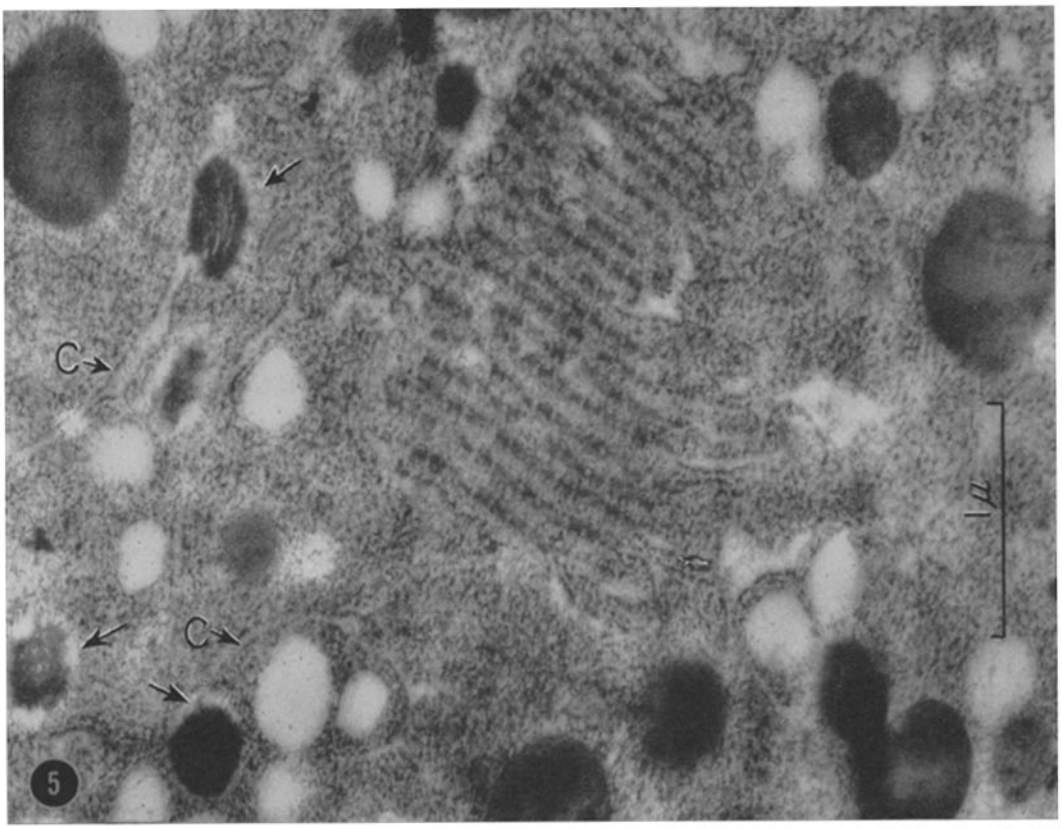
In addition to the above, there are other inclusions and regions of small diameter in the egg which either are too ill defined to allow detailed description at this time, or are difficult to analogize with inclusions known from studies with chemical fixatives.

FIGURE 5

A section of an oocyte which we would interpret as being more poorly frozen than those in the remaining figures. The annulate lamellae are clear but have not been described because our preparations of them, so far, are too variable. Notice that the character of the hyaloplasm appears more reticular than granular, that the cisternae (*C*) are preserved (including the double membrane structure which can be seen in higher power micrographs), that the dark borders lining the cisternae are not clearly granular, and that what we believe are shrinkage spaces can be seen around some mitochondria (arrows). Stained with permanganate. × 31,500.

FIGURE 6

The section was stained with lead hydroxide as described in the text. It should be noted that there is no evidence of a membrane structure in the endoplasmic reticulum, although some scattered, dense material can be seen in the cisternae. The small granules are lined up at the cisterna edges. The picture could be described as yielding a negative image of the ER. × 43,000.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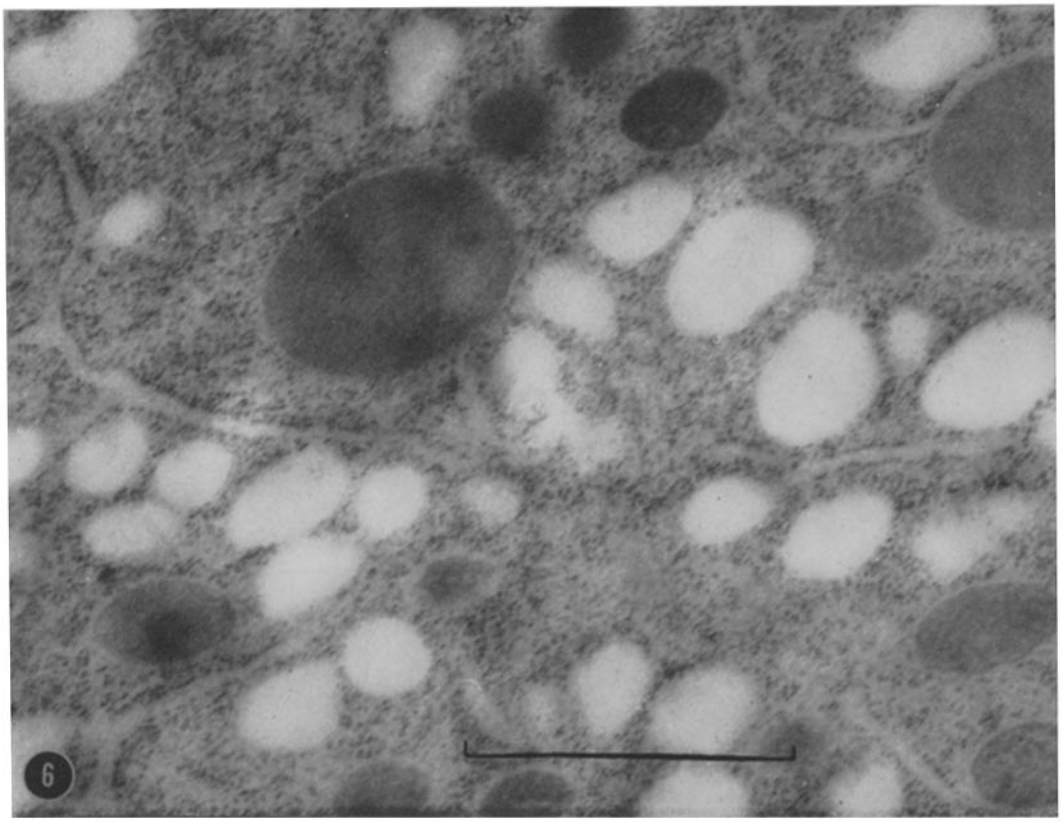
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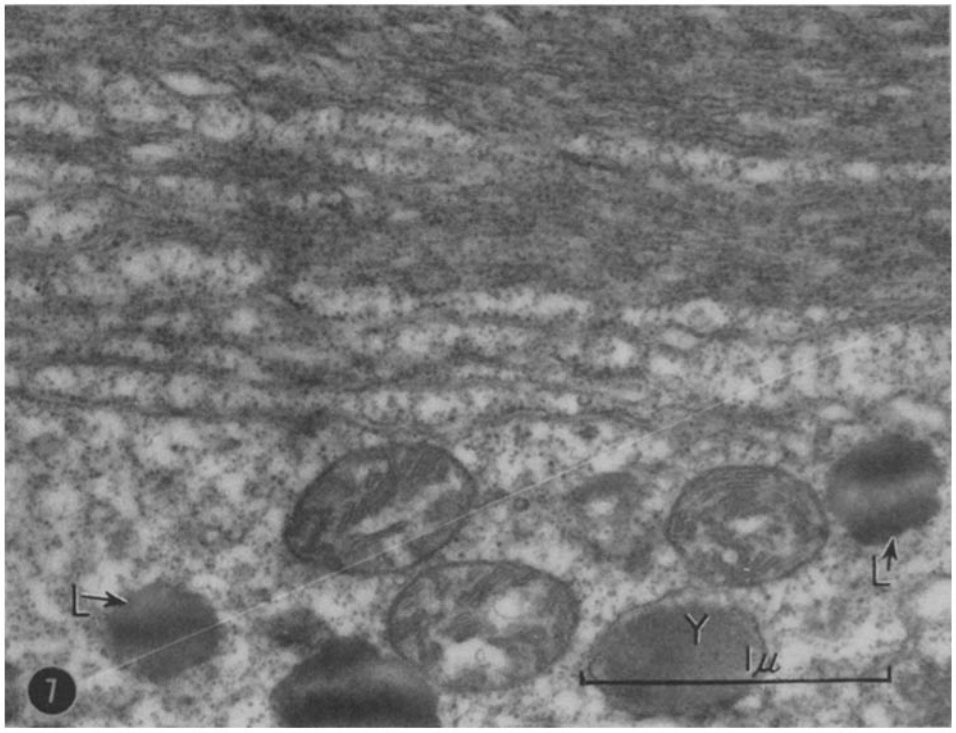


FIGURE 7

An array of endoplasmic reticulum as seen with conventional techniques (Palade's fixative, embedding in methacrylate). Small granules free or associated with single membranes of the ER, mitochondria with typical structure, and yolk (*Y*) and lipid (*L*) granules are visible. × 41,000.

Unstained Sections

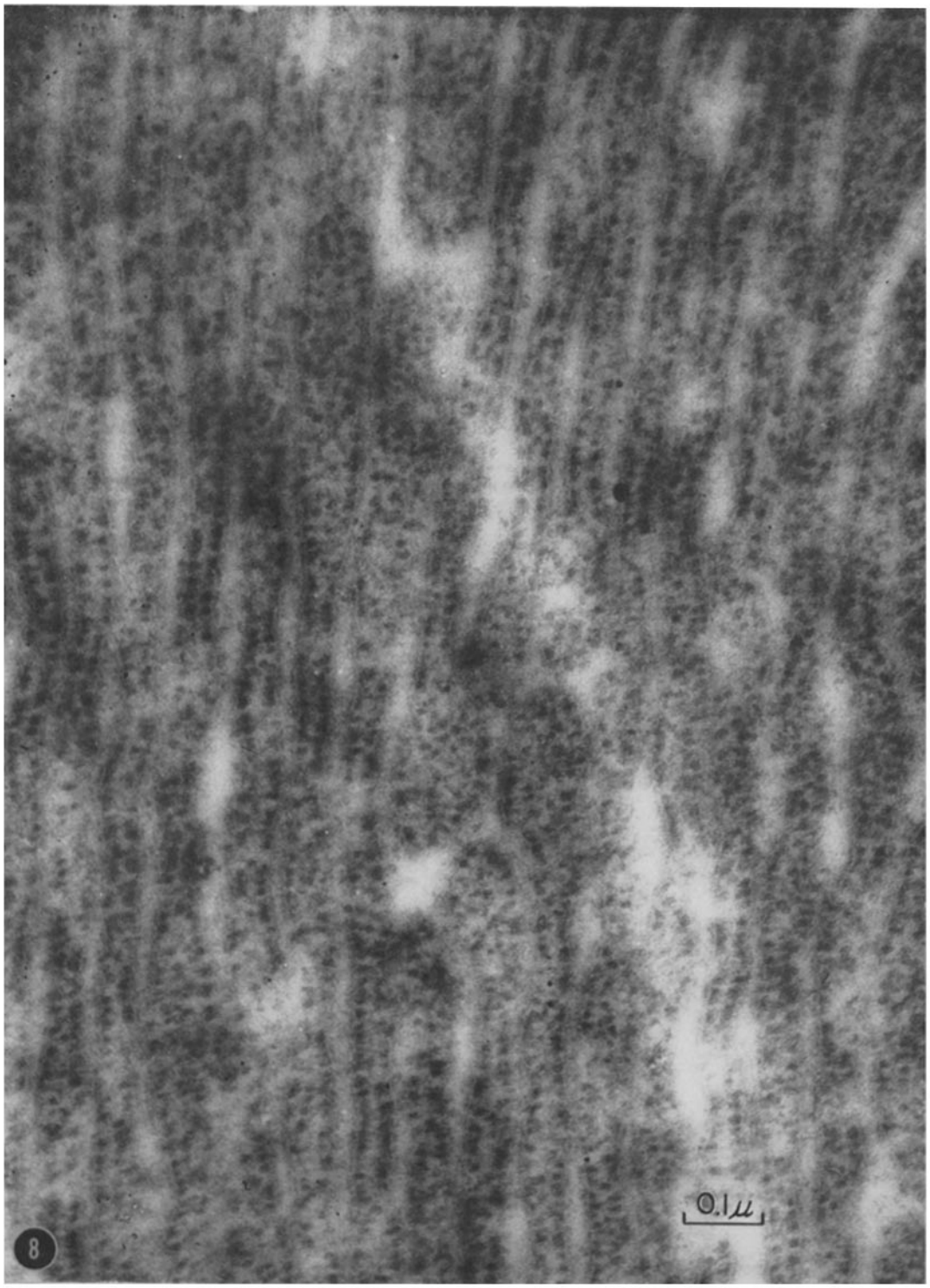
An extensive study of unstained sections was not undertaken because of the very low contrast of the cells after epoxy embedding even with osmium in the substituting fluid. However, the major components of the cell can be grossly seen and the small "Palade" particles are visible.

Considerations on Ice Crystals

The technique we have used requires a preliminary scanning of 1 to 2 micron sections with the phase microscope so that the more profitable embeddings may be subsequently sectioned. Much of the material we have prepared has very obvious ice crystal formations in it, using the term ice crystal in the sense in which Gersh and Stephenson (1954) use it. This may leave the cells in a state of almost complete destruction or may yield cells with the barest hint of a background granularity at the light microscope level. From experience, such granularity almost invariably appears as a fine-meshed reticulum in the electron microscope, with mesh size anywhere from 500 to 2000 A in diameter. Even cells whose cytoplasm appears homogeneous in the light microscope may very well possess a distinctly non-homogeneous and reticulum-like structure in the electron microscope (see, *e.g.*, Fig. 5). Some cells show complete destruction of structure at one surface and preservation at the other, equivalent to that in

FIGURE 8

This micrograph is taken from a region of organized endoplasmic reticulum. The small granules and approximately 75 A double limiting membranes are clear and have been described in Fig. 4. Stained with permanganate. × 112,000.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8

Fig. 1. In such sections the sequence of structures seen varies from huge ice crystal spaces through meshes of ever decreasing diameter, through regions such as that in Fig. 5, and finally to more homogeneous regions such as those in Figs. 1, 3, 4, 6, and 8. It should be noted that in regions comparable to that in Fig. 5, there may be no obviously delineated small granules other than what might be taken for nodes at the intersections of a mesh. Membranes of the ER are apparent however (see arrows). Regions such as this usually also show distinct evidences of shrinkage around mitochondria (see Fig. 5), and in general we consider them to be more poorly frozen than regions such as those in the remaining figures. It is primarily on the basis of this continuous distribution of mesh sizes down to that in which the reticulum-like structures disappear that we have chosen preparations such as those in Figs. 1, 3, 4, 6, and 8 as representing the best-frozen preparations, *i.e.*, those most likely to yield interpretable information concerning living ultrastructure.

DISCUSSION

The techniques of cell preparation discussed in this paper have yielded results which are remarkably like those obtained with diffusion fixation techniques although differing from them in certain important details. The most important of these concerns the mitochondria, since with our procedures, membranes are not visualized in these structures. Nevertheless, the mitochondria are clearly recognizable as such since they present an image which is a "negative" of that seen with diffusion fixation; that is, the interior of the mitochondrion, excluding the cristae, appears dense and solidly filled with small 25 to 30 A granules. This would indicate that the usual methods of fixation cause a leaching out of materials which our techniques preserve. The absence of membranes around mitochondria as seen in this work is somewhat remarkable in the light of the fact that in the same sections, after permanganate staining, membranes are quite clearly seen in the Golgi apparatus, in the ER, and also in the cell surface (which we shall discuss at a later date in connection with a study of the vitelline membrane). This may mean either that the membranes are actually preserved by the technique but neither the permanganate nor the lead staining reveals them, or that the technique for some reason does not preserve the mitochondrial membrane. Although no final decision is possible at this moment, we lean toward the interpretation that the mitochondrial membrane is probably preserved but not stained by permanganate or lead.

The presence of "double membranes," or actually triple-layered complexes, bounding the Golgi apparatus and the ER has been reported after permanganate fixation (*e.g.*, Robertson, 1959, 1960). In the present work such membranes are seen after preparation by cryofixation (Fernández-Morán, 1959) and staining with permanganate subsequent to embedding and sectioning. This tends to support the idea that fixation with permanganate is preserving rather than creating the double layers and that these layers do exist in the living cell. The fact that lead hydroxide staining fails to reveal the membranes makes it quite clear that inferences concerning the absence of a particular structure after a given technique of cell preservation depend to a considerable extent on the staining procedure subsequently employed on the material.

With respect to the latter point, we should consider the small 150 A particles which the hyaloplasm contains in great abundance in these preparations. In certain recent work with freezing-drying in the hands of Gersh (Gersh *et al.*, 1957), Finck (1958), Sjöstrand and Baker (1958), and especially Hanzon *et al.* (1959) there have been claims that this small particulate component is not a normal structure in living cells but may be formed upon injury to a delicate hyaloplasmic organization. Hanzon *et al.* (1959) have reported that the particles do appear in pellets derived from homogenates after use of freeze-drying, but that the particles are absent from intact tissues so prepared, unless these have been injured in some way. Our own preparations contain these particles clearly and definitely and in areas in which there is no evidence of ice crystal damage at the resolutions attained and in which the total picture of the preparation appears excellent.

There are two obvious considerations. First, it is by no means impossible that small granules either are destroyed or are actually present but unstained in the tissue preparations of Hanzon *et al.* (1959). The fact that these authors see small granules in pellets from homogenates may be explainable on the basis of the differences in fixation or staining conditions in homogenates and in tissues. Second, there is the possibility that it is our technique, rather than theirs, that has

produced the artifact, *i.e.* has caused small particles to appear in the cells, by processes which are completely unrelated to the initial freezing velocities and ice crystal formation, and which are specific to the substitution as compared with the vacuum dehydration part of the technique. We believe that the totality of evidence, both morphological and biochemical, supporting the existence of Palade granules, together with the fact that we have demonstrated that certain stains (permanganate) *may* reveal structures (membranes) not revealed by other stains (lead), supports the first alternative. That is, the results of Hanzon *et al.* may be due to preparative or staining conditions which fail to reveal small particles after freeze-drying in intact tissues.

It is quite clear, however, that our ignorance concerning the fundamental physical and physicochemical processes occuring during fixation of any sort, and especially fixation by freezing, is such that extreme caution must be exercised in drawing any conclusions about the existence, prior to preparation, of any structure below the limit of resolution of the light microscope. Further work, especially comparison of freeze-dried and freeze-substituted material of the same type, is needed.

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