The Contribution of Lower Oxides of Osmium to the Density of Biological Specimens in Electron Microscopy*

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ABSTRACT

Centrifugally stratified eggs of the sand-dollar, *Dendraster excentricus*, have cytoplasmic structures segregated into distinct layers. Fat droplets, yolk granules, and mitochondria are separated by "hyaline" layers of protoplasm. Aggregations of particles of 150 to 200 A diameter ("heavy bodies") are found near the mitochondrial layer and a concentration gradient of free 150 to 200 A particles corresponds to a similar gradient of basophilia in thick sections. Eggs fixed in buffered osmium-tetroxide at pH 7.4 and embedded in methacrylate were sectioned and floated on a "bleaching" solution of acidified hydrogen peroxide. "Bleached" sections showed a considerable loss in general density and especially a loss in the sharp images of cellular membranes. It was shown that such loss is not due to sublimation of structure in the electron beam. Assuming that the "bleach" acts principally to reoxidize lower oxides of osmium, it was concluded that reduced and bound lower oxides of osmium play a major role in creation of the electron micrograph image, especially in the delineation of phospholipide components of cellular membranes. Particles of 150 to 200 A diameter showed little or no loss in density, but rather a high intrinsic electron density. Refractometric data were presented to substantiate the tentative conclusions.

The interpretation of electron micrographs of biological specimens fixed by osmium tetroxide is compromised by uncertainty about the contribution to image contrast of reduced and bound osmium (4, 10–12, 19). Are differences in density in a thin section due to good preservation of local variations in concentration of intrinsic solids or are they due to local accumulations of bound osmium compounds in various states of oxidation?

"Bleaching" of osmium-fixed tissues has been known to cytologists for a good many years (13). Such treatment with an oxidizing agent essentially reoxidizes deposits of reduced osmium in the tissue, making soluble as tetroxides (18) the black or brown lower oxides so that they may be removed by washing. The question to be examined here is what this treatment does to *electron densities* in thin sections.

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Materials

The egg of the sand-dollar, *Dendraster excentricus*, whose particulate inclusions have been stratified by centrifugal force (Fig. 5) seemed well adapted to this study. Oil droplets of the centripetal end may be considered as mainly lipide on the basis of straining properties and density. Yolk platelets, which are present in *Dendraster* oocytes and in many other species, contain both protein and lipide (9). In amphibian eggs about one-half of egg lipide is found in the yolk (5). "Heavy bodies" (2) which lie between the yolk and mitochondrial layers in the sand-dollar egg contain granules 150 to 250 A in diameter which are strongly basophilic. They probably correspond to the RNA-rich particles of Palade (14) of more familiar vertebrate cells. Mitochondria are layered near the centrifugal pole. These show a dense matrix and typical membranes with internal cristae. The extreme centrifugal tip shows numerous free 150 to 250 A particles which grade off in concentration up through the yolk layer. A similar gradient of basophilia can be noted in adjacent thick sections.

Methods

Eggs of *Dendraster*, shed into sea water, were centrifugally stratified and fixed at 0°C. in 1 per cent osmium tetroxide in veronal buffer of pH 7.4 to which sucrose had been added to a concentration of 0.23 M. The eggs were then dehydrated in alcohols, embedded in methacrylate, sectioned at 200 to 500 A and mounted on grids with methacrylate in place. Observations were made with an RCA EMU 3C with compensated pole piece and an objective aperature of 50 microns. Kodak contrast lantern slides were used.

Osmium was removed from sections by floating them on the surface of a solution containing 15 per cent hydrogen peroxide and 0.6 N HCl for 10 minutes at room temperature. From the bleaching bath the sections were transferred to a distilled water wash, a dilute solution of oxalic acid to remove residual oxidant, and a final water wash before being picked up on grids for observation. For purposes of comparison, adjacent sections were examined without treatment.

OBSERVATIONS

In the untreated sections density was high in cellular membranes of all kinds (Figs. 1, 3, and 7), in yolk platelets (Fig. 1), and in fat droplets. Sections floated only on 0.6 N HCl appeared the same as untreated sections. It was apparent immediately, however, that bleached sections had suffered a marked loss of density. Sections were difficult to find at all in the microscope. Contrast improved slightly as the beam intensity increased and, visually, the sections showed no unusual deterioration in the electron beam. Sharply defined membranes, with densities equivalent to those in unbleached sections were now apparently absent (Figs. 2, 4, 8, 9), fat droplets were empty areas, and yolk platelets had lost the definition of constituent yolk granules, appearing now as diffusely arranged material (compare Figs. 1 and 2). Mitochondrial membranes had virtually disappeared but close examination showed that although membranes were no longer sharply defined, they could still be made out at least in some places (Fig. 4 at arrows) as denser boundaries in a comparatively dense mitochondrial mass.

Because of the many factors influencing contrast on the negative of an electron micrograph, it is difficult to make reliable estimates of absolute contrast and its variation from negative to negative. We can speak with greater confidence, however, about relative densities in the same micrograph. As expected, yolk and fat droplets show the greatest relative loss of density, changing from greater to less density than the surrounding material.

"Hyaline" protoplasm between the oil cap and yolk layers is characterized by small membrane-enclosed vesicles with diffusely scattered material between them (Fig. 7). The density of these structures in a centrifugal field is even less than the protein-lipide complexes of the yolk platelets. The nucleus usually lies in this layer and stands out with lower general density. Bleaching removes most of the density of the vesicular membranes and ground material without having much effect on the contents of the nucleus. This leaves the nucleus with greater density than the structures of the surrounding "hyaline" layer (Fig. 8).

The "heavy bodies" and free particles of the centrifugal pole, which presumably are nucleoprotein in nature, show little or no loss in density after bleaching. The non-particulate substances of the heavy end, lose enough density to make the "heavy bodies" and 150 A particles stand out in greater contrast after bleaching (Fig. 9) than before. It is interesting to note in passing that the diffuse material which is seen in so many kinds of nuclei after osmium fixation reacts to bleaching in much the same way as do the cytoplasmic structures of nucleoprotein composition. Note especially the aggregates of dense material along the nuclear membrane in Fig. 8 even though the membrane itself is invisible (compare pictures in references 1, 16, 17).

The refractive index of fixed and dehydrated cellular solids may be considered an average of the refractive indices of the constituent substances times their partial volumes. The refractive index of frozen-dried cellular solids, in general, varies between 1.530 and 1.546 (unpublished observations) and binding of osmium would be expected to raise the refractive index. Stratified eggs, fixed and embedded in methacrylate as usual, were sectioned at 1½ microns and mounted on a glass slide. The methacrylate was removed in xylene and the eggs photographed in phase contrast in an oil of refractive index 1.460 (Fig. 5). The various layers can be easily seen in the two adjacent cells. Refractive index measurements were made on these cells with a phase microscope during successive mountings in oils of graded indices of refraction. Because the sections showed a barely perceptible brownish tint, measurements were made with white light and with light of about 5300 A (green) and 6000 A (red). The results with all were the same. Measurement uncertainty was about ±0.004. The same sections were then treated 15 minutes with the bleaching solution, washed, dehydrated, and mounted again in oil of refractive

TABLE I

Refractive Index of Dendraster Egg Sections after Osmium Fixation and Subsequent Bleaching

Layer	Refractive index		
	Before bleach	After bleach	Difference
Oil droplets	1.630	1.566	0.064
Upper hyaline	1.574	1.566	0.008
Yolk platelet	1.592	1.562	0.030
Lower hyaline, mitochondria and heavy tip	1.568	1.564	0.004

index 1.460. Fig. 6 shows the same sections photographed with the identical phase set-up. Table I lists the refractive index measurements of the sections both before bleaching and after.

The differences in refractive index due to bleaching agree well with the subjective impression of losses of density in the various layers as observed in the electron microscope. In addition, it should be noted that the refractive indices of the various layers of the *bleached* egg although now quite similar, still are considerably higher than those of frozen-dried materials. This would suggest that organically bound osmium is still present despite losses of reduced osmium compounds.

Although the image of the bleached sections were observed to become *more* distinct as low beam intensity was increased for normal focusing, the possibility still remained that the beam had initially caused a sublimation of solid structures. The question is whether the diffuse image obtained after bleaching is due to removal of osmium oxides from structures or whether removal of osmium has simply removed the stability of the structures in the electron beam. Thus, it has previously been shown (7) that formaldehyde fixation can give a fair image if the beam is kept low enough to prevent sublimation of the actual structures.

Fig. 10 is a thin section of an unbleached egg which had been exposed only to a low electron beam during a 6 minute photographic exposure. The beam was then raised to high intensity and a two second exposure made of the same field (Fig. 11). Similar treatment was given a bleached section and the paired micrographs are shown in Figs. 12 and 13. It can readily be seen that essentially the same image appears in the low beam as in the high. The greater distinctness and contrast of the high beam pictures is taken to indicate that embedding methacrylate is lost (7). Such evidence suggests that loss of contrast and sharp outlines due to bleaching is not simply a result of structural instability in the beam.

DISCUSSION

The increased density of oil droplets and yolk granules after osmium fixation and the loss of most of their density in bleaching is expected because of their content of lipides and the known ability of unsaturated bonds of fatty acids to reduce osmium-tetroxide (3). Perhaps oxidative removal of density from the background substances of the upper hyaline layer can also be explained by the presence of lipides.

Proteins relatively uncomplexed with lipides might be expected in the matrix of mitochondria as well as in the background substances, especially of the heavier layers of the stratified egg. Bleaching causes little or no loss of electron density in mitochondrial matrix and only a small loss in the background substances of the heavy end. Nucleoprotein structures of the cytoplasm and the wispy material within the nucleus show little or no loss in density with bleaching but retain a high intrinsic density. As mentioned earlier, the most striking change produced by bleaching is the almost complete loss of contrast in all membranes of the cytoplasm as well as the nuclear membrane.

Loss of contrast and a considerable degree of total density should not be taken to mean, however, that bleaching removes *all* electron density. Study of Figs. 2, 4, 8, 9, and 13 show that appreciable image-forming density remains. It seems possible that bleached images presented in this study may have as much actual density as images of formalin-fixed material (7). If a difference in image quality exists between osmium-fixed and bleached material and formalin-fixed material, perhaps this is due to the fact that the bleaching process involves an oxidation step which might produce some structural damage. Structural damage, if present, is not felt to be severe enough to alter the conclusions of this study.

In an attempt to interpret these observations let us make the following assumptions: (*a*) The bleaching treatment used in these studies acts principally to reoxidize and remove deposits of lower oxides of osmium. (*b*) Oil droplets and yolk contain the most significant amounts of lipides. (*c*) Mitochondrial matrix and background substances at the heavy end of centrifuged cell are principally protein in nature.

To the extent that these assumptions are valid, it seems reasonable to conclude that osmium-tetroxide fixation does contribute to specimen density in electron micrographs. The most marked density contrasts would be due to lower oxides of osmium, although stabilization and a small degree of density may be contributed by organically bound but unreduced osmium. The presence of such unreduced osmium is suggested in this study by the relatively high residual index of refraction after bleaching. Further, it would seem that the ability to reduce osmium is greatest in the lipides. Proteins have a comparatively modest ability to reduce osmium and hence show little or no loss in density with bleaching. However, they probably bind enough osmium to raise their index of refraction and increase their stability in the electron beam. Nucleic acids do not reduce osmium to any appreciable extent but have a high intrinsic density. These conclusions are supported by test tube experiments on the reactivity of osmium with organic substances (3, 15, 18).

If this reasoning is correct then it follows as highly likely that membranes, as seen in electron micrographs, represent deposits of lower oxides of osmium as well as a lower intrinsic density. Of the known major constituents of membranes, namely proteins and phospholipides, the latter would be the most likely to reduce the osmium, especially in light of the fact that structural phospholipides in animal tissues have an appreciable content of unsaturated fatty acids (6). Reversal of the sign of birefringence of membrane systems by osmium-tetroxide fixation (8) can also be interpreted to mean that osmium is deposited in the negatively birefringent, or phospholipide, component of the membranes.

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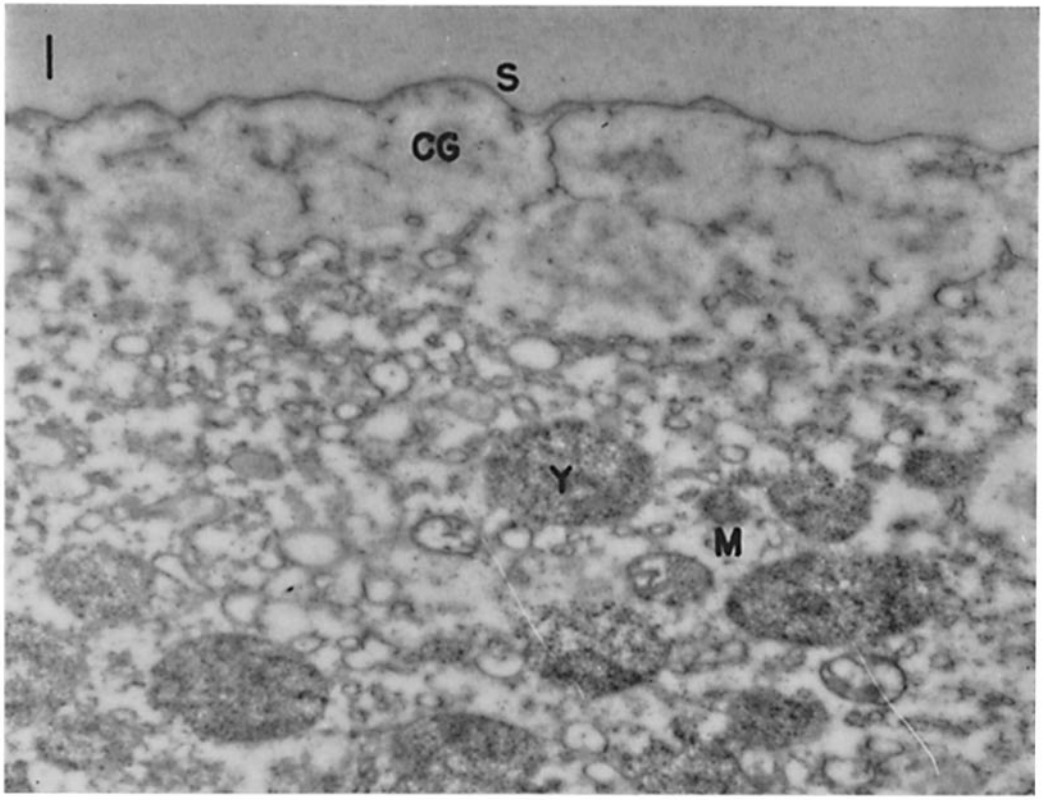
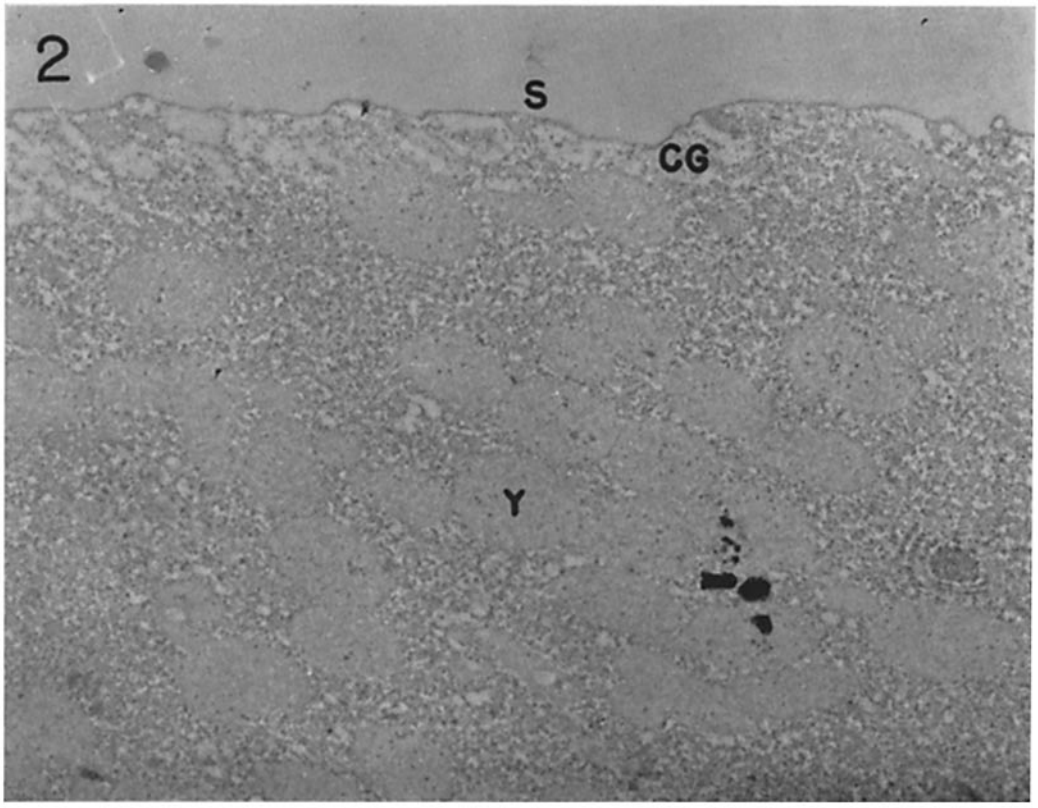
Explanation of Plates

Plate 271

Fig. 1. Thin section of mature oocyte. Cell surface (*S*) with underlying cortical granules (*CG*) and showing yolk platelets (*Y*), mitochondria (*M*), and small cytoplasmic vesicles. Fixed in buffered osmium-tetroxide at pH 7.4. Magnification about 21,000.

Fig. 2. Thin section of mature egg. Cell surface (*S*) with cortical granules (*CG*) and yolk platelets (*Y*). Fixed in buffered osmium-tetroxide at pH 7.4 and subsequently "bleached." Magnification about 9,000.



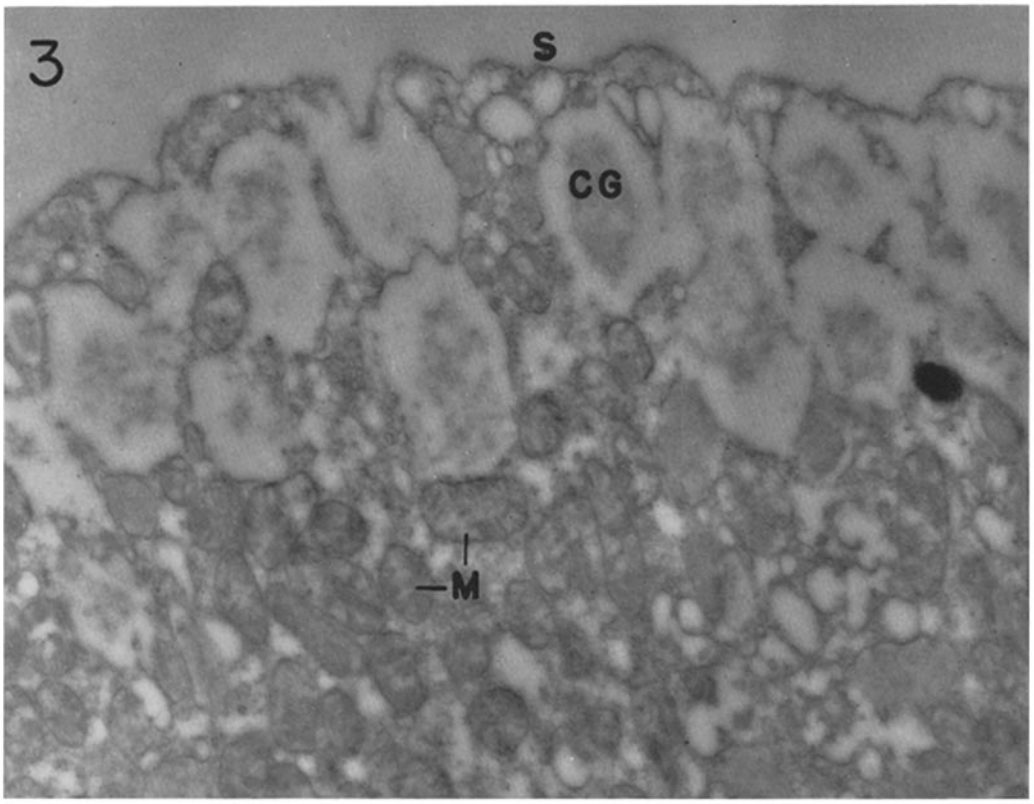


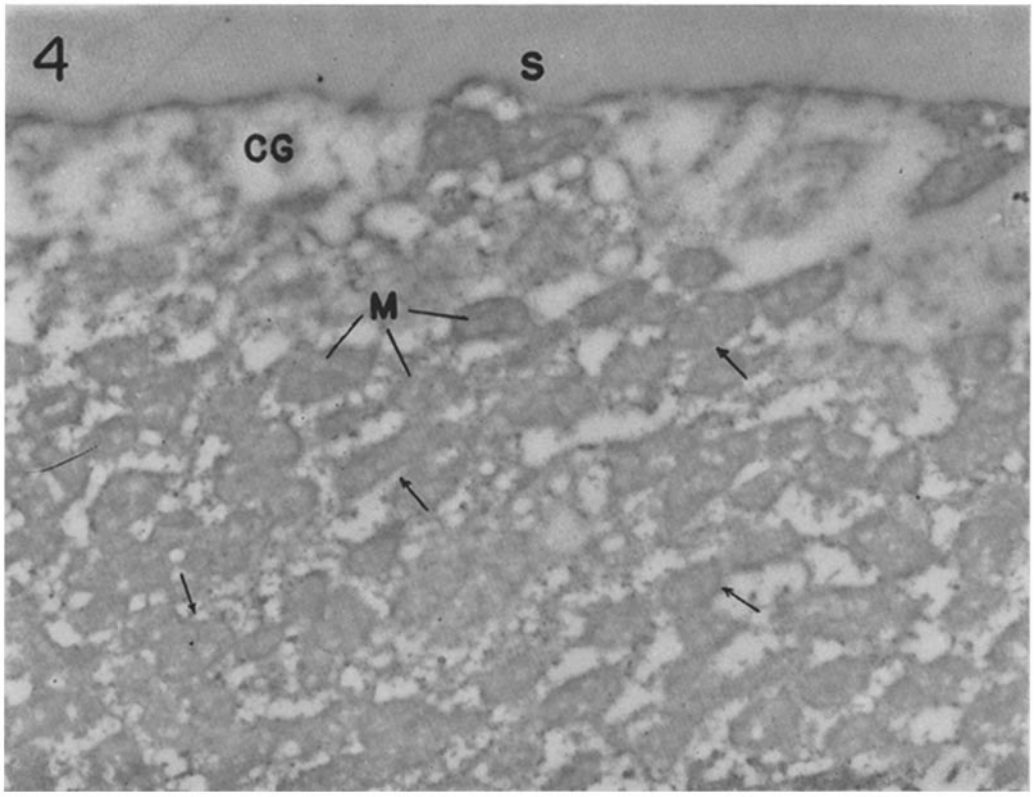
(Merriam: Lower oxides of osmium)

PLATE 272

FIG. 3. Section through mitochondrial layer of centrifugally stratified egg. Cell surface (*S*) with cortical granules (*CG*) and mitochondria (*M*). Fixed in buffered osmium-tetroxide at pH 7.4. Magnification about 23,000.

FIG. 4. Section of mitochondrial layer of stratified egg. Cell surface (*S*), cortical granules (*CG*), and mitochondria (*M*). Note presence of mitochondrial membranes with reduced contrast at arrows. Fixed in osmium-tetroxide but subsequently "bleached." Magnification about 21,000.





(Merriam: Lower oxides of osmium)

PLATE 273

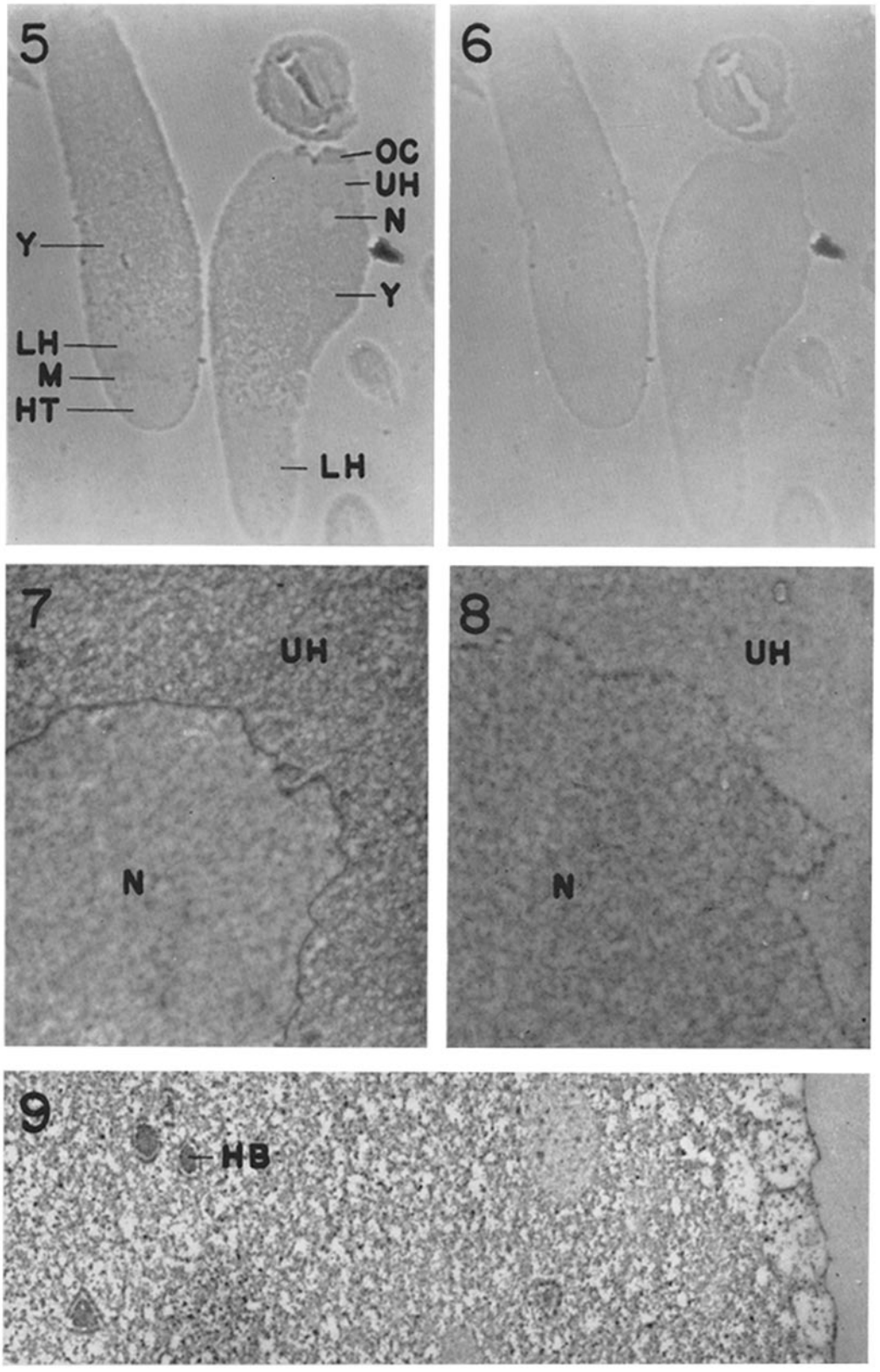
FIG. 5. Phase contrast picture of centrifugally stratified mature oocytes. Sections about 1½ microns thick. The layers from the centripetal pole are: oil cap (*OC*), upper hyaline layer (*UH*) with nucleus (*N*), yolk layer (*Y*), lower hyaline layer (*LH*), mitochondrial layer (*M*), and heavy tip (*HT*). Fixed in buffered osmium tetroxide at pH 7.4. Magnification about 3,000.

FIG. 6. Phase contrast picture of same sections as shown in Fig. 5 but "bleached." Magnification about 3,000.

FIG. 7. Section through upper hyaline layer (*UH*) of a stratified egg showing the nucleus (*N*). Fixed in osmium-tetroxide at pH 7.4. Magnification about 8,000.

FIG. 8. Section through upper hyaline layer (*UH*) of a stratified egg showing the nucleus (*N*). Fixed in osmium-tetroxide but subsequently "bleached." Magnification about 8,000.

FIG. 9. Lower hyaline layer of stratified egg. "Heavy bodies" (*HB*) can be seen in strong contrast to their surroundings. A particulate component of the surrounding substance retains marked density. A few yolk platelets can be seen. Fixed in buffered osmium-tetroxide at pH 7.4 and subsequently "bleached." Magnification about 9,000.



(Merriam: Lower oxides of osmium)

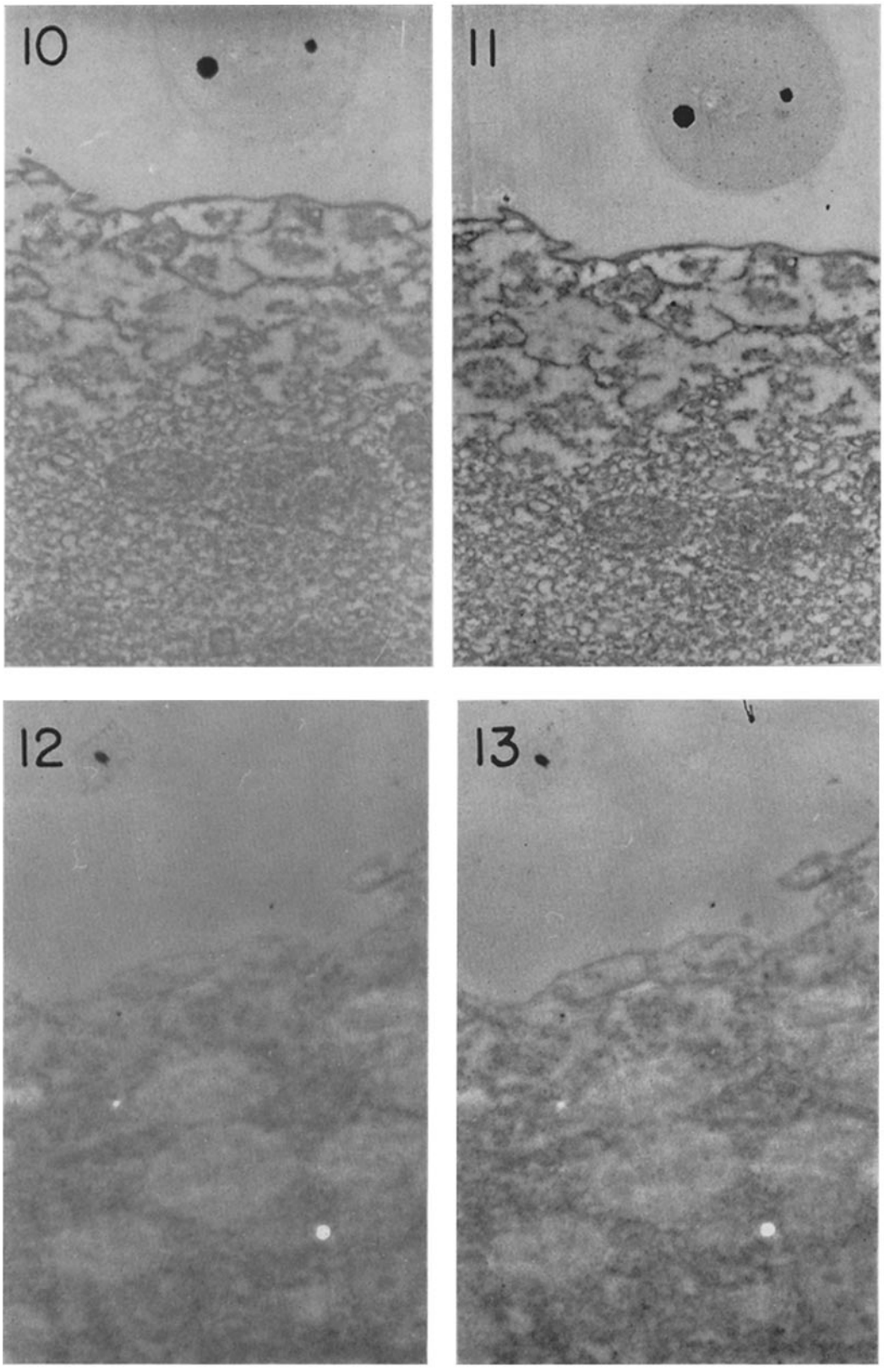
PLATE 274

FIG. 10. Low beam intensity picture of mature egg cytoplasm. Photographic exposure of 6 minutes. Fixed in buffered osmium-tetroxide at pH 7.4. Magnification about 12,000.

FIG. 11. High beam intensity picture of the same field as shown in Fig. 10. Note increased contrast. Magnification about 12,000.

FIG. 12. Low beam intensity picture of mature egg cytoplasm. Photographic exposure of 6 minutes. Fixed in osmium-tetroxide at pH 7.4 and subsequently "bleached." Magnification about 12,000.

FIG. 13. High beam intensity picture of the same field as shown in Fig. 12. Note increased contrast. Magnification about 12,000.



(Merriam: Lower oxides of osmium)