

The Effect of Partial Protein Extraction on the Structure of the Eggs of the Sea Urchin, *Arbacia punctulata**†

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PLATES 1 TO 4

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ABSTRACT

The structure of the eggs of the sea urchin, *Arbacia punctulata*, has been investigated after the removal of one-half of the cellular protein. The procedure involves treatment of the eggs with 30 per cent ethanol at -10°C . followed by extraction of the soluble proteins with water. The eggs remain intact, although all of the cytoplasmic matrix is removed. Most cell structures can still be identified, although only the membranes of most remain. The mitochondria lose all of their matrix but retain the inner membranes or cristae.

The annulate lamellae appear unaffected by this extraction procedure, remaining intact and apparently undamaged. The nuclear envelope is also retained, although it often undergoes a curious disorganization, apparently as the result of the separation of its two layers. The significance of these observations with respect to the structure of the envelope is discussed.

INTRODUCTION

In a recent study (1) the major soluble proteins of the sea urchin egg were characterized by physical methods. The extraction procedure used in these investigations was an adaptation of a method originally developed by Mazia (2) in connection with the isolation of the mitotic apparatus from dividing cells, and involves treatment of the eggs in 30 per cent ethanol at -10°C . for a minimum of 24 hours. The eggs remain intact in this solution and when transferred to water or salt solution the soluble proteins can be extracted without breaking the cells. Approximately 15 per cent of the total cell protein is removed by the alcohol and an additional 30 per cent is extracted by the water, leaving a "skeleton" which resembles the normal cell but contains only one-half of its original protein. The present work is an electron microscopic investigation into the structure of these extracted eggs.

The water extract, contrary to what one might expect, contains only two major ultracentrifugal components. The heavier fraction consists of ribosomes (also referred to as RNP particles), rich in nucleic acid, with a sedimentation coefficient of 20 Svedberg units (S). The other component is a protein having a sedimentation coefficient of 7S, which viscosimetric evidence shows to be quite asymmetric. No other component is present in sufficient quantity to be identifiable by the methods used. Manipulation of the ethanol extract presents some technical difficulties and its composition has not been investigated in detail.

The structure of the extracted cell is of interest from several points of view. The extraction must remove more material than has been identified, since the material extracted by the ethanol is lost and other components may be present in the water extract in too small a concentration to be visible in the ultracentrifuge. The examination of sections of these cells should make clear the extent of the extraction in a way not possible through the study of extracts alone. More important, the study of extracted cells is an effective way to gain information about the insoluble components.

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One would like to know which cellular structures remain and what modifications, if any, have been induced by the extraction procedure. The reaction of the insoluble structures to the extraction must be a reflection of their composition and such information thus has a direct bearing on more general problems of cellular organization.

Thin sections of the eggs of *Arbacia punctulata* were made quite early in the history of the technique by McCulloch (3, 4), who observed many of the major structural features. More recently, Gross *et al.* (5) have reported on the structure of the mitotic apparatus in these cells. In this work the eggs were given an ethanol treatment similar to that used here, but OsO_4 fixation was performed in the subzero ethanol, resulting in the coagulation of the cytoplasmic proteins rather than their extraction. In a series of papers, Afzelius (6-9) has reported in considerable detail on the ultrastructure of the eggs of a number of European species of sea urchin. Recently, Pasteels *et al.* (10) have observed the structure of stratified eggs of the urchin *Paracentrotus lividus*. In addition to a study of the mitotic figure by Kurosumi (11), Kurosumi *et al.* (12) have investigated the cytoplasmic RNA of the eggs of the urchin *Heliocidaris crassispina*. Among related forms, the oocytes of the sand dollar *Dendraster excentricus* have been the subject of a study by Merriam (13). This investigation is particularly relevant here, since it is concerned with those cytoplasmic inclusions that have been termed "annulate lamellae" by Swift (14). These units, which are similar in structure to the nuclear membrane, are the most notable feature of the cytoplasm of the extracted eggs studied in the present investigations. They were first observed in the cytoplasm of the sea urchin egg by Afzelius (6), who considered them to be fragments of the nuclear membrane remaining from previous maturation divisions. Since that time, they have been observed in many other forms (14) and seem widespread in occurrence.

Materials and Methods

Ripe eggs of *Arbacia punctulata* were obtained by the application of 10 volts AC across the test (15). The eggs were washed several times in sea water, spun down, and the sea water decanted. A large volume of 30 per cent ethanol at -10°C . was added rapidly and the resulting suspension stored at -10°C . After a minimum of 24 hours the eggs were spun out at -10°C . in a refrigerated centrifuge, the alcohol

decanted, and 10 times the volume of the egg sediment of distilled water at 0°C . added. The eggs were extracted with this solution for 24 hours and then fixed in 1 per cent OsO_4 in sea water for 1 hour at 0°C . After dehydration in a graded ethanol series, the eggs were embedded in a mixture of 80 per cent butyl and 20 per cent methyl methacrylate, using 0.2 per cent benzoyl peroxide as a catalyst and a polymerization temperature of 60°C . Thin sections were cut on a Sjöstrand ultramicrotome, flattened with xylene vapors (16), and examined in an RCA EMU-3C electron microscope with a 50 or a 30 μ objective aperture.

OBSERVATIONS

For comparative purposes the appearance of the cytoplasm of a normal, unfertilized, osmium-fixed egg is shown in Fig. 1. Several large yolk platelets are visible in the lower section of this figure. A number of mitochondria can be identified by means of their internal organization and are found clustered about the oil droplets. The cytoplasm is somewhat vesicular and is filled with dense, 150 A granules. These particles are randomly distributed in the cytoplasm and do not appear to be attached to membrane systems.

The appearance of the egg cytoplasm after ethanol and water extraction is shown in Fig. 2. The most obvious result of the extraction is a great reduction in density, due to the removal of both the background material and the contents of most of the structural components. Swelling during embedding makes no appreciable contribution to this emptiness of the cytoplasm, as the volume of the extracted cells in methacrylate is the same as untreated cells. It is of interest to note that the extracted cells are translucent by transmitted light, as compared to the opaque normal cells. The loss of background density in Fig. 2 is clearly the result of the total removal of the 150 A particles which make up a large part of the cytoplasmic matrix of the normal cell. The oil droplets are present in the extracted cells in the same numbers as in normal cells. This might be expected, since the 30 per cent ethanol would be a poor solvent for such large lipid masses, particularly at the low temperatures involved. The yolk platelets have lost most of their contents and remain only as large membrane-bound vesicles, often containing a few yolk remnants. Clumps of material which appear to be liberated yolk particles are also found scattered in the cytoplasm. The mitochondria are still recognizable by means of their inner membranes or cristae, although the

interior is empty and of the same density as the background. There is often evidence of a break in the outer membrane, which is presumably the mechanism of escape of the contents. The mitochondrial membrane is quite dense and clearly double in some cases, while in others it is reduced to a lightly staining ribbon with no apparent structure. This variation can sometimes be seen even within a single mitochondrion. The characteristic grouping of the mitochondria around the oil droplets persists, implying that these units are joined with sufficient strength to resist the effects of the extraction.

The general appearance of these cells gives the impression of considerable disorganization and it is surprising to find that the annulate lamellae survive. Some examples of these structures in an unextracted egg are given in Fig. 3, and their appearance after extraction is shown in Figs. 4 and 5. The lamellae are scattered throughout the cytoplasm in both normal and extracted cells and are not organized in any regular pattern. Morphologically they do not appear to be damaged in any way and their dimensions are similar to those of annulate lamellae observed in other egg cells (6, 17). They are present in all stages of the first division cycle examined. These units consist of two parallel membranes, approximately 90 Å thick, separated by a space of 200 Å. The lamellae are interrupted at regular intervals by heavy-walled annuli, which have an over-all diameter of 1100 Å and a wall thickness of 250 Å. The lamellae are very clearly resolved and the walls of the annuli somewhat less so, although this is also true in the illustrations of Afzelius (6) and appears to be typical. The two lamellae usually form a vesicle at the ends of these units, as noted by Rebhun (17), although these are often difficult to observe. A particularly clear case is shown in Fig. 5. It appears that the space within the vesicle is continuous with the space between the membranes.

Since the annulate lamellae survive the extraction without damage and are similar in structure to the nuclear envelope, one might predict that this latter structure would also survive. That such is the case is demonstrated in Fig. 7, which illustrates a section of nuclear envelope in an extracted cell, which can be compared to the normal nuclear envelope in Fig. 6. The most unusual feature of the nuclear envelope in extracted cells is the presence of large blisters. These swellings appear randomly along the membrane and have no apparent regularity. In Figs.

8 and 9 it can be seen that these blisters result from the separation of the two layers of the envelope, the space within the swelling being continuous with the interlamellar space. The areas between the swellings appear undamaged, and the annuli are often more clearly visible than in normal cells. The membranes over the swelled area are considerably modified in appearance. They have a very low density and have lost the complex annular structures. Careful examination of the long region of disorganized envelope in Fig. 9 also fails to reveal any evidence of holes or pores in the residual membranes. It is apparent from the figures that these ribbons are considerably expanded over the normal state, and the twisting which they undergo indicates that they are quite flexible.

DISCUSSION

The appearance of the extracted cell demonstrates that this procedure has removed all of the cytoplasmic matrix and liberated most of the contents of the cell structures, leaving only the membranous component of the cell intact. The loss of the 150 Å granules from the cytoplasm accounts for the appearance of the 20S fraction in the water extract, although it cannot be determined from these experiments whether the extracted particle represents the intact granules or a breakdown product. These particles are very easily extractable even without the use of ethanol (1), presumably due to their free dispersal in the matrix of the cytoplasm. Kurosumi *et al.* (12) have made a point of the existence of many free 150 Å granules in the eggs of *Heliocidaris crassispira*. The 7S component is probably also present in the cytoplasmic matrix, but it is too small to be visualized in these electron micrographs and its localization thus cannot be established by a comparison of normal and extracted cells.

Most of the cytoplasmic structures can still be identified, although in most cases only the membranes remain. The matrix of the mitochondria is completely lost leaving only the empty outer membrane, usually containing a number of recognizable cristae. These structures resemble the "membrane fraction" obtained by Watson and Siekevitz (18, 19) by treatment of mitochondria with sodium deoxycholate. In the present experiments, the boundary of the mitochondria varies from an organized double membrane to an undifferentiated ribbon. The appearance of these structures gives the impression that some material has been

removed from these membranes, but this cannot be verified with the present data. However, it seems reasonable to suppose that these ribbons may be a structural protein backbone which forms the basis of the membrane. Certainly, considerable amounts of protein must be involved in these membranous components to account for the one-half of the cell protein that remains in the extracted cell.

In view of the rather serious disorganization of the mitochondria, the retention of the complex structure of the annulate lamellae is unexpected. The composition of these structures is unknown, although in view of their basophilia it has been suggested (13, 17) that the annuli may contain RNA. The role of these structures in the cytoplasm is also unknown, although they seem to be of general occurrence, having been found in the larval pancreas of *Amblystoma* and in the rat testis by Swift (14) and in a rat mammary carcinoma by Schulz (20). Palade (21) has recently classified them as a form of endoplasmic reticulum. However, the resistance of the annulate lamellae to this drastic extraction procedure certainly sets them apart from the usual types of endoplasmic reticulum, which are quite labile and often change their form during isolation (22, 23).

In view of the preservation of the annulate lamellae, the existence of an identifiable nuclear envelope in these cells might be expected. The blisters which are observed on this structure are of considerable interest. Since such formations have not been observed in the untreated cell, they seem to be a result of the extraction. Although they are thus without physiological significance, they are relevant to the problem of the structure of the membrane.

The existence of "pores" in the nuclear envelope was observed first by Callan and Tomlin (24) and it now seems relatively certain, as illustrated by Watson (25), that they are of general occurrence. However, their function, and even their exact structure, is still in doubt. Afzelius (6) presented a model for the nuclear envelope of the sea urchin egg which consists of a double membrane with discontinuities in the form of "holes" covered by a very thin single membrane. A heavy-walled annulus surrounds these "holes" on both the nuclear and the cytoplasmic sides, but does not pass through the envelope. Wischnitzer (26), using amphibian material, claims that an unbroken tube passes through both layers of the envelope, and does not find evidence of any membrane within

the tube. However, Watson (25) has noted that often there is the appearance of a diaphragm in the "pores."

The effects of extraction on the structure of the nuclear envelope provides some additional information but does not completely clarify the situation. The appearance of the disorganized regions indicates that the two layers of the envelope have separated, accompanied by a considerable reduction in density and the loss of all evidence of annuli. Since these changes appear to be a result of the extraction, it may be that some component (or components) is removed from the envelope, thereby causing these effects. The complete absence of pores or holes in these residual membranes admits of several explanations. If one assumes that these membranes are continuous over the entire nuclear envelope, only the additional assumption that the two membranes were joined in the region of the pores is necessary to account for the thin membrane covering the "holes" noted by Afzelius (6) and the diaphragm across the pores mentioned by Watson (25). These regions might be pulled apart during the formation of a blister, thus giving rise to the continuous double membrane in the disorganized regions. If the annuli in the intact nuclear envelope are assumed to be unbroken tubes extending from the nucleus to the cytoplasm, then a more involved explanation is required to account for the absence of holes in the residual membranes. One must hypothesize that the reorganization associated with blister formation caused the closure of the opening in the envelope through which this tube passed. Since this opening must be of the order of 1000 Å in diameter, its disappearance seems unlikely. With regard to the possibility of a diaphragm across the pore, the fact that the nucleus of these cells has been observed to vary in volume in solutions of varying osmotic pressure (27) implies that the envelope is not freely permeable to large molecules, as it should be if holes of 500 Å were present. If some selectively permeable barrier does exist in the pores it might well be formed by the residual membranes demonstrated here.

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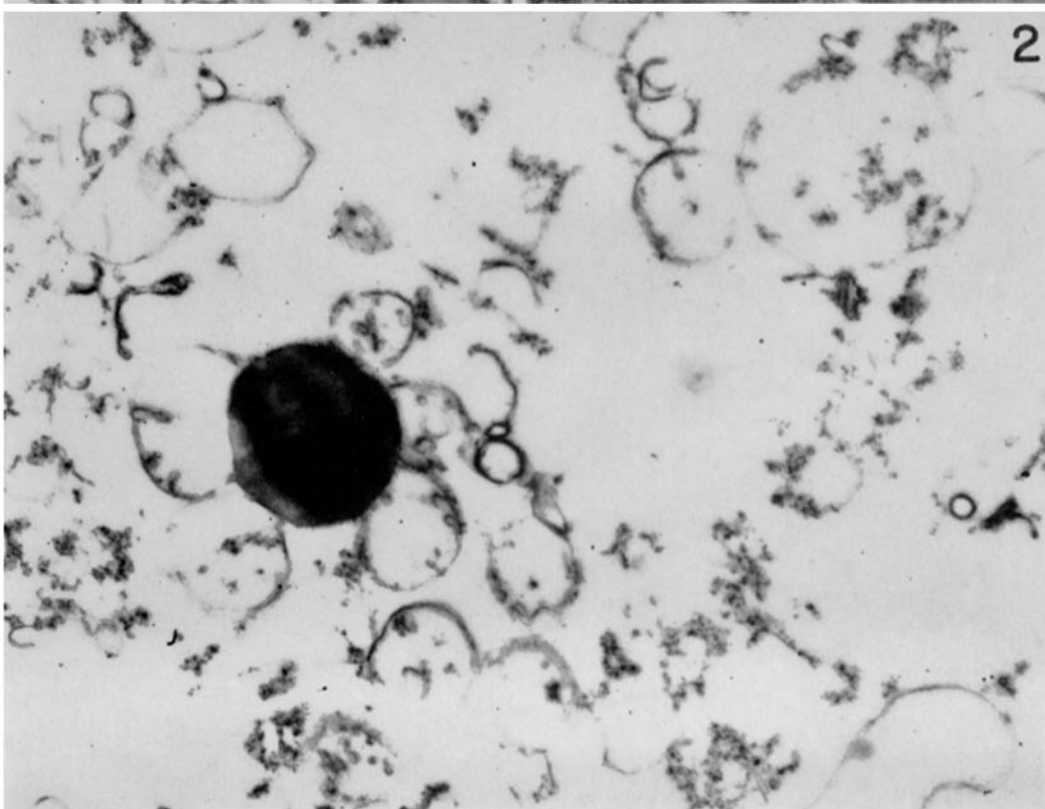
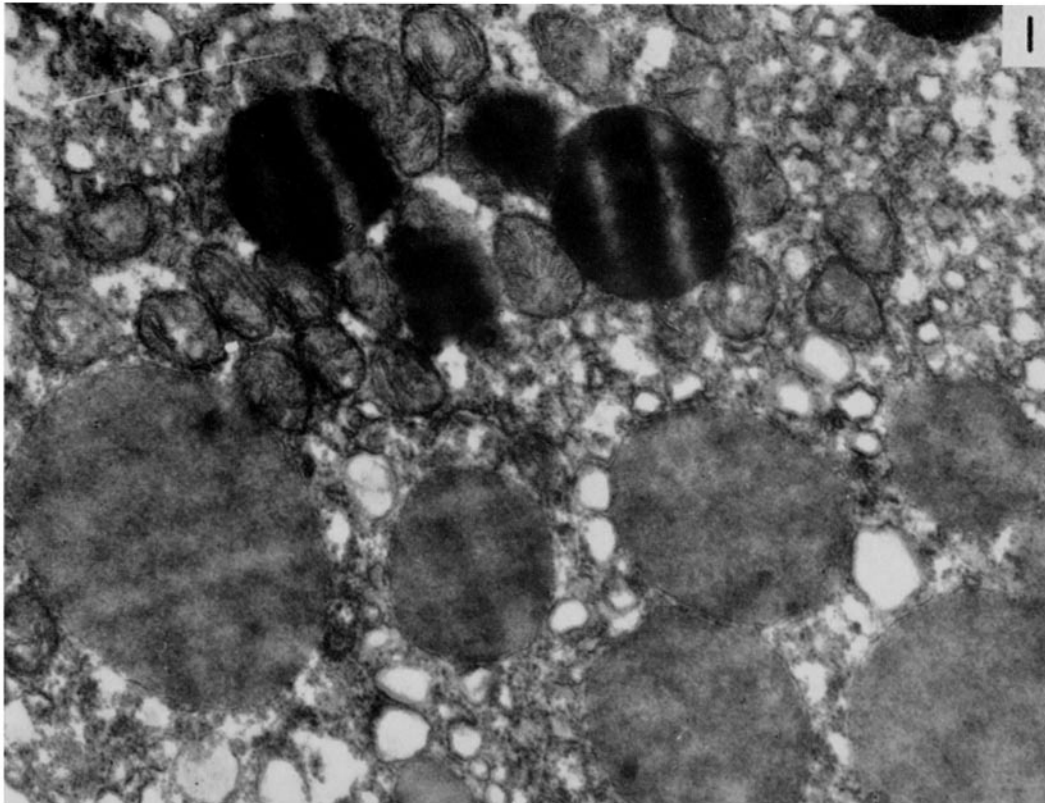
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EXPLANATION OF PLATES

PLATE 1

FIG. 1. General view of the cytoplasm of an unfertilized, osmium-fixed egg. Two dense oil droplets, surrounded by mitochondria, occupy the upper half of the figure. A number of large yolk platelets can be seen below. $\times 35,700$.

FIG. 2. Section of the cytoplasm of an extracted egg. An oil droplet, apparently unchanged by the extraction, is visible. It is surrounded by a group of empty mitochondrial membranes. The large, empty membrane in the upper right corner of the figure is presumably the remnant of a yolk platelet. $\times 35,700$.



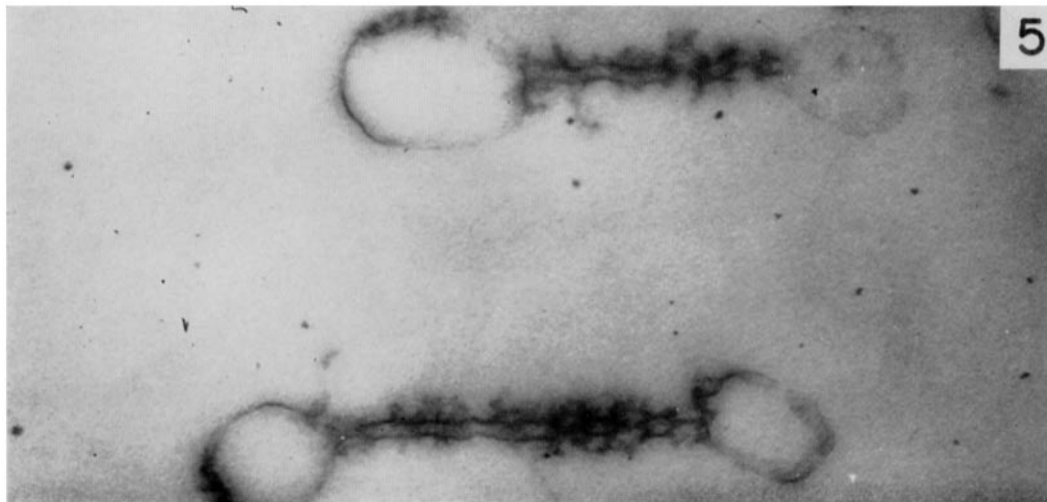
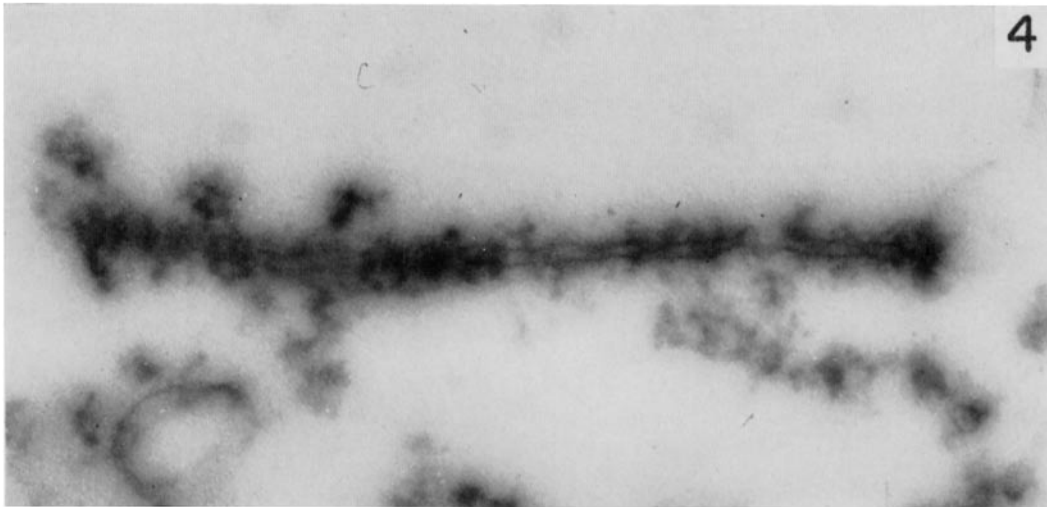
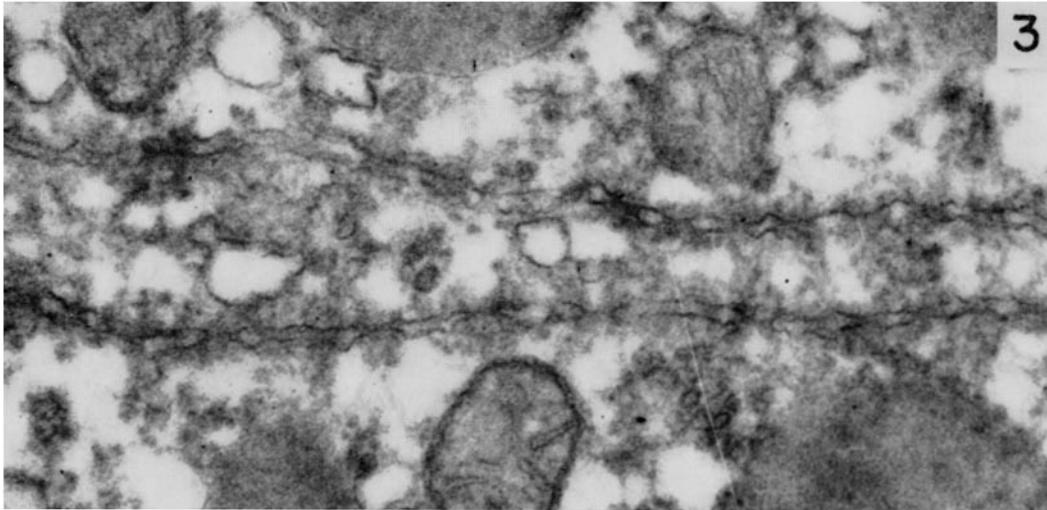
(Kane: Effect of partial protein extraction)

PLATE 2

FIG. 3. Annulate lamellae in the cytoplasm of a normal egg. $\times 57,000$.

FIG. 4. Annulate lamellae in an extracted egg. $\times 57,000$.

FIG. 5. Annulate lamellae in an extracted egg, showing vesicles at ends of lamellae. $\times 57,000$.

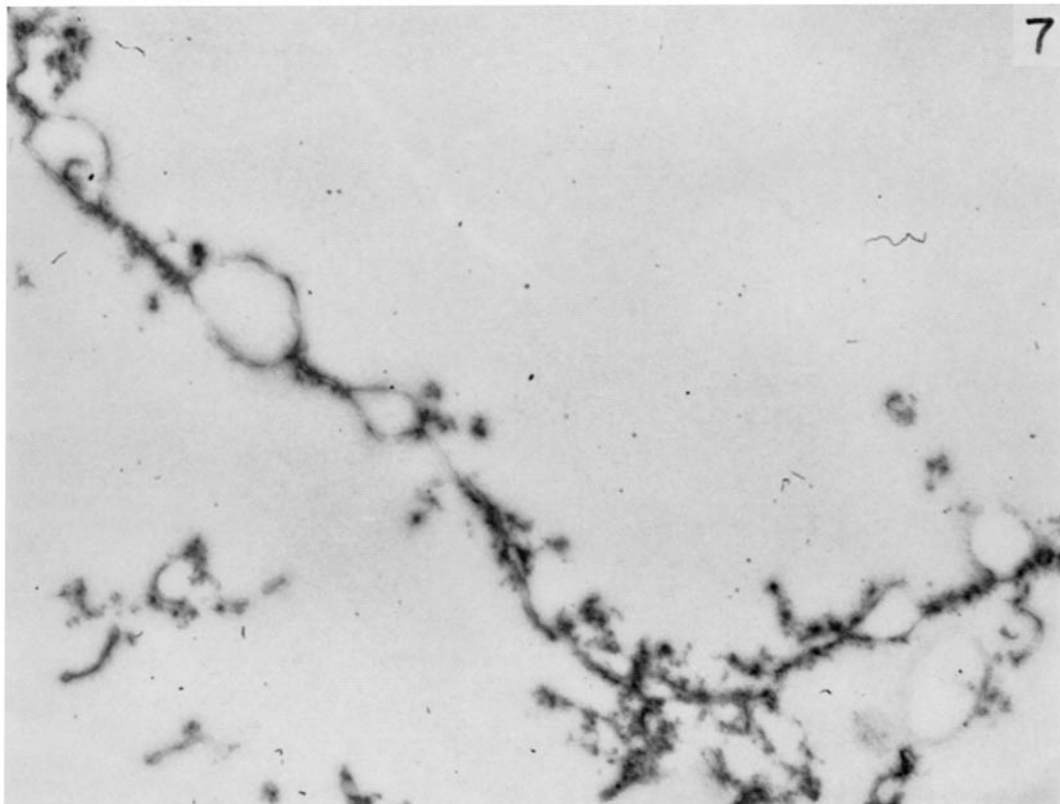
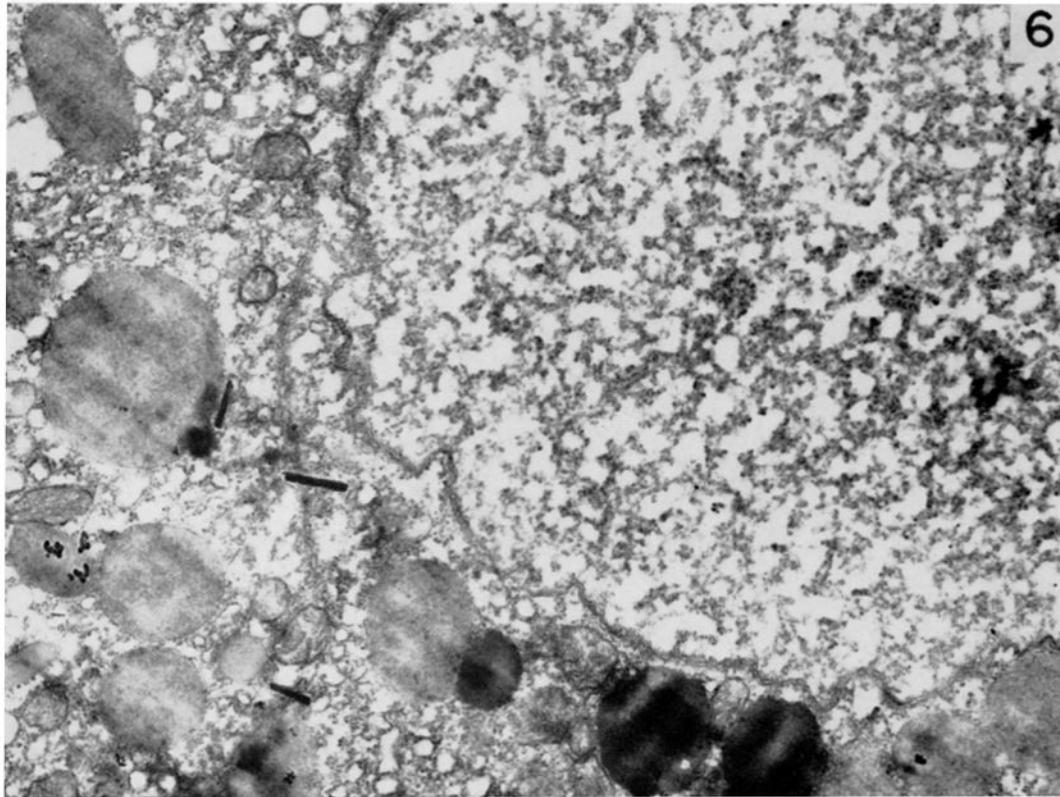


(Kane: Effect of partial protein extraction)

PLATE 3

FIG. 6. Part of the nucleus in normal cell, showing the appearance of the nuclear envelope. An annulate lamella is visible to the left of the nucleus. $\times 22,500$.

FIG. 7. Length of nuclear envelope in an extracted cell, showing the irregular occurrence of blisters. $\times 22,500$.

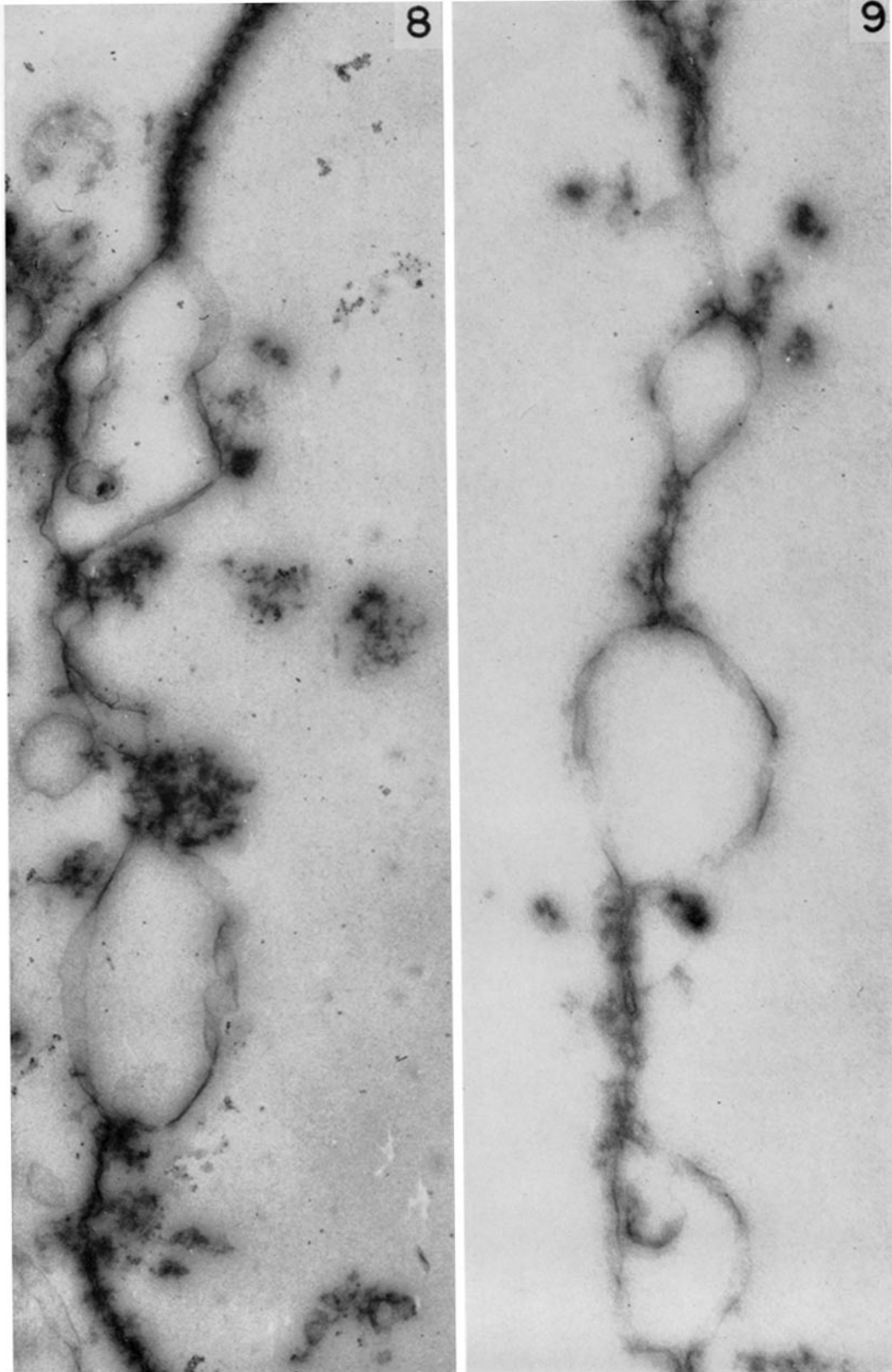


(Kane: Effect of partial protein extraction)

PLATE 4

FIG. 8. Long region of disorganized nuclear envelope in an extracted cell. The envelope has normal structure at top and bottom of the figure, but the entire center section is reduced to an undifferentiated ribbon. $\times 34,000$.

FIG. 9. Higher magnification view of the envelope of an extracted egg, illustrating the formation of blisters by separation of the two layers of the envelope. $\times 57,000$.



(Kane: Effect of partial protein extraction)