THE ISOLATION OF A MAJOR STRUCTURAL ELEMENT OF THE SEA URCHIN FERTILIZATION MEMBRANE

JOSEPH BRYAN

From the Department of Zoology, University of California, Berkeley, California 94720

ABSTRACT

Procedures for isolating the contents of the cortical granules from the ova of the sea urchin, *Strongylocentrotus purpuratus*, are reported. Dithiothreitol is used to remove the vitelline coat; the "demembranated" eggs are then subsequently activated with butyric acid. By means of these procedures, the hyaline protein and crystalline or paracrystalline material have been isolated from the cortical granules. The crystalline material consists of sheets of cylinders or tubules 150–200 A in diameter. This material is believed to be a major structural element of the fertilization membrane which, in the absence of the vitelline coat, does not form.

INTRODUCTION

The first visible changes in an inseminated echinoderm ovum are the structural changes in the egg cortex which precede the formation of the fertilization membrane. These involve the initial elevation of the vitelline coat, the breakdown of the cortical granules, and the concomitant release of material into the perivitelline space (for reviews see 3, 26, 27). This complex series of events results in the formation of the fertilization membrane and the deposition of the hyaline layer. The work of Wolpert and Mercer (30) and of Endo (5) has demonstrated that the membrane is derived in part from the vitelline substance (or vitelline coat) which combines with material released from the cortical granules. These two "substances" unite and subsequently undergo a progressive structuralization which ultimately results in a trilaminar "membrane" (2, 3, 5). In a brief note, Ito et al. (19) have pointed out that in favorable cross-sections of acrolein-fixed material the membrane appears to be composed of two layers of tubules, running antiparallel, which sandwich a third amorphous region (3). These authors indicate that the tubules are approximately 150 A in diameter and that the amorphous region is approximately 200 A thick, for a total membrane thickness of 500 A. Using a freeze-etching technique, Inoué et al. (15) report finding crystalline arrays on the surface of isolated, dried fertilization membranes. These arrays are described as a gridlike network with repeat dimensions of 110 and 155 A with an angle of 74° between the axes of the grid. More recently Humphreys and Kreutziger (12), again using freeze etching, have described a similar crystalline array with slightly different dimensions. These arrays are composed of 197 A strands or cylinders. Regularly repeating units 108 A apart run diagonally across the cylinder at an angle of 72°. Each diagonal unit appears to consist of four or five subunits. These authors suggest that the cylinders are formed from subunits 40–45 A in diameter assembled into a helix with a pitch of about 72°.

A number of authors (2, 5, 27) have reported the presence of laminar or rodlike structures of approximately the same size in the perivitelline space. The presence of these rodlike structures has

also been observed in the sea water around fertilized eggs which have previously been treated with proteolytic enzymes to remove or alter the vitelline coat and prevent the formation of an intact membrane (29). No attempt to recover these structures appears to have been reported.

The present work details a method for collecting the entire contents of the cortical granules. Particular emphasis has been placed on recovering the hyaline substance and the laminar structures or crystalline arrays usually found on the membrane. The technique of isolation is based in part on the observation by Epel and Mazia (6) that dithiothreitol (DTT) can remove or alter the vitelline coat and thus prevent elevation of the fertilization membrane.

MATERIALS AND METHODS

The eggs of the west coast urchin *Strongylocentrotus purpuratus* were used for the present experiments. Gametes were obtained by pouring 0.5 M KCl into the opened body cavity. The eggs were collected by hand centrifugation and washed several times in filtered sea water. The jelly coat was removed by a brief exposure to sea water adjusted to pH 5.0.

Protein concentrations were estimated by the method of Lowry et al. (23). Bovine plasma albumin was used as a standard. Determinations were run on material taken up in 1 N NaOH.

Sedimentation experiments were done with a Beckman Spinco Model E analytical ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.).

For electron microscopy, one drop of material in sea water was transferred to a carbon-stabilized, parlodion-coated copper grid; excess fluid was removed by blotting on filter paper and replaced with a drop of stain, which was immediately removed by blotting. 1% phosphotungstic acid (pH 6.8) or 1–2% ammonium molybdate (pH 7.4) were used as negative stains. Specimens were examined in a Siemens Elmiskop I electron microscope.

Polarization micrographs were taken with a modified Leitz Ortholux microscope equipped with strain-free rectified optics (American Optical Company, Southbridge, Mass.).

PROCEDURES AND RESULTS

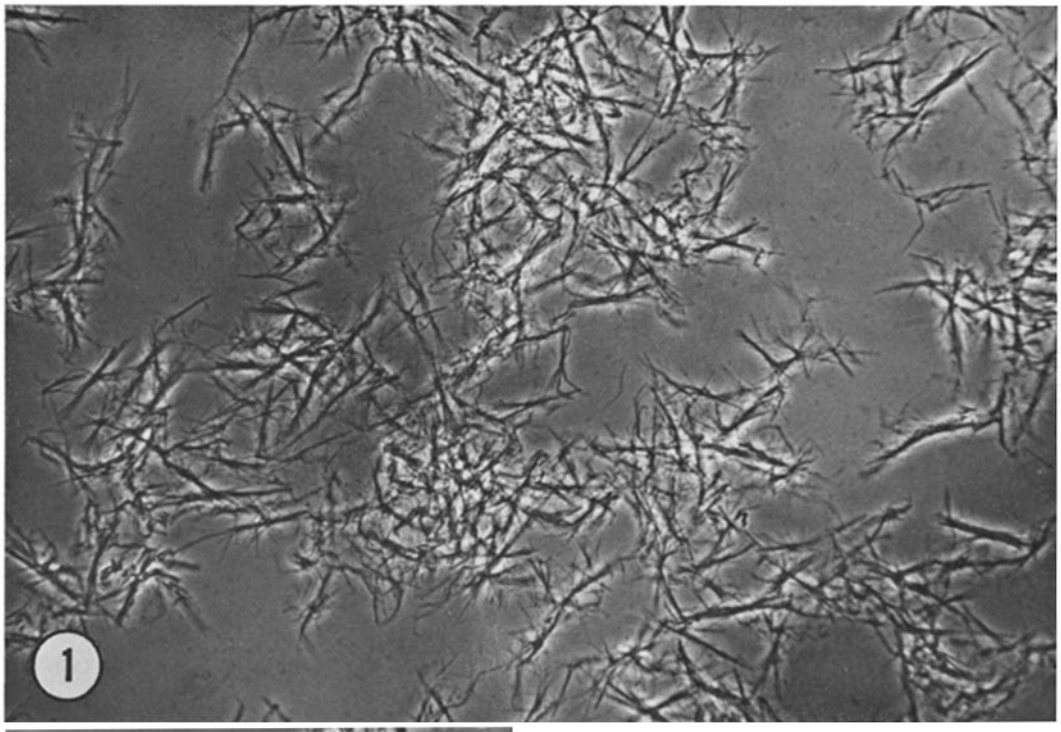
Removal of the Vitelline Coat

The vitelline coat was removed or inactivated by a method originated by Epel and Mazia (6). A loose pellet of dejellied eggs was suspended in an equal volume of 20 mM dithiothreitol (DTT) in filtered sea water adjusted to pH 9.4 with 1 N NaOH. The eggs were stirred gently for 3 min, then collected by hand centrifugation, and washed three times in 10–20 volumes of filtered sea water. After this treatment the eggs will fertilize, but will not raise fertilization membranes.

Parthenogenetic Activation of Eggs

The eggs were activated by means of a variation of the butyric acid (BA) method originally described by Loeb (22). In the present application, the BA treatment has only been used to produce a cortical response; however, cleavage and abnormal development can be induced (22). A dense suspension (15–30%) of eggs was stirred gently at 2–4°C. The pH was measured continuously. 0.2 N butyric acid was added until the pH reached 4.7–4.5. The eggs were then stirred for 2 min, at which time the pH was adjusted to the desired value with 1 N NaOH.

Upon neutralization (pH 5.0–5.5) of the butyric acid, the contents of the cortical granules were released into the surrounding sea water. Since there appeared to be a differential either in the rate of "release" of the crystalline substance versus the hyaline substance or in the rate of their solubilization after the cortical granules open to the exterior, several variations in the experiment were possible at this stage. These variations suggest that the crystalline substance is always released rapidly and presumably completely whereas the hyaline substance tends to leave the eggs or solubilize more slowly, with complete release requiring extended periods of time. The variations were mainly concerned with the pH, which was observed to play a critical role in the gelation or polymerization of both the hyaline and crystalline substances. In the range between 5.5 and 6.5, release of these substances occurs rapidly after BA treatment, but polymerization seldom occurred. In this pH range the supernatants above the eggs had a "granular" appearance, suggesting that particulate material had been released intact from the cortical granules. In favorable preparations the release of material could often be observed. The egg surface begins to bleb and bubble, and a large number of small particles (0.4–0.8 μ) are released into the sea water. These often aggregated to form larger particles. It was convenient to work in this pH range, because polymerization could be delayed until the eggs had been completely removed. One disadvantage of this procedure, for some purposes, is that



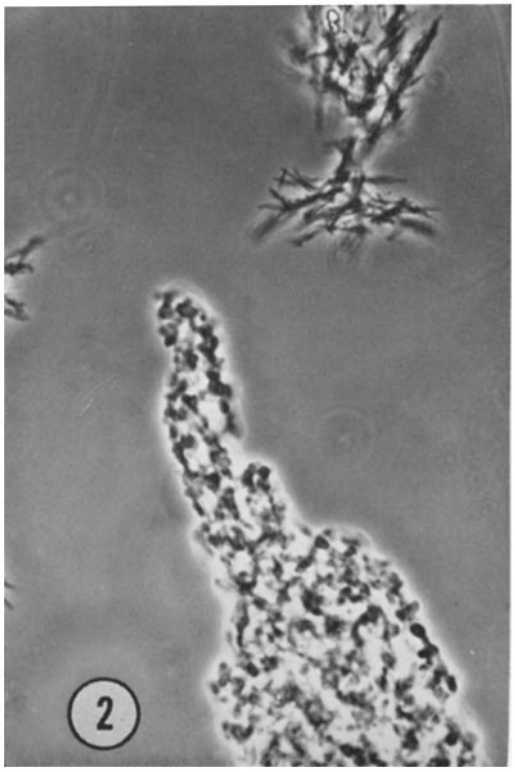


FIGURE 1 Crystalline material in sea water adjusted to pH 7.6. Phase, × 700.

FIGURE 2 Hyaline material (bottom) polymerized in sea water at pH 7.6. Crystalline material included for comparison. Phase, × 900.

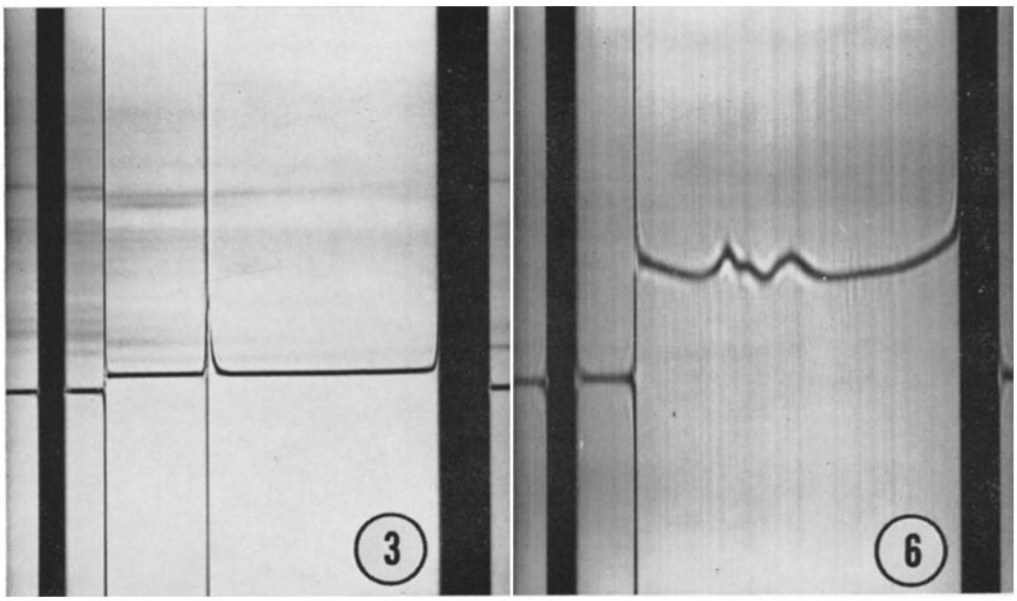


FIGURE 3 Sedimentation pattern of hyaline protein in 10 mM Tris-HCl pH 7.5. Concentration, 3 mg/ml. 128 min after reaching a speed of 47,660 rpm. Bar angle = 80°.

FIGURE 6 Sedimentation pattern of crystalline protein dissolved by dialysis against 10 mM Tris-HCl, pH 7.5. Concentration, 3 mg/ml. 64 min after reaching a speed of 59,780 rpm. Bar angle = 60°.

increasing amounts of hyaline are released on continued stirring at pH 6.

An alternative to keeping the eggs at an intermediate pH was to adjust immediately to pH 8. In this condition the hyaline tended to gel normally and stay with the eggs. However, some care had to be exercised in using lower egg concentrations and generally working faster, otherwise the oocytes tended to become entangled in the polymerizing fibrous masses of crystalline material. Under this condition it was difficult or impossible to obtain clean separations. It was also necessary to avoid adding excess base which resulted in the formation of inorganic precipitates and concomitant loss of protein.

To obtain both the hyaline and crystalline materials for further analysis, eggs were treated with butyric acid as described above, then adjusted to pH 6.0–6.2, and allowed to stir for 10 min. The long stirring time resulted in the release of increased quantities of hyaline substance. After 10 min the eggs were removed by hand centrifugation and discarded. The supernatant was then adjusted to pH 7.5–8.0 with 1 N NaOH and stirred on ice for 30–60 min. After this final pH adjustment, within 1–3 min, the supernatant became highly turbid as the crystalline material and the hyaline substance polymerized.

Fig. 1 is a low-power phase micrograph of the material which forms in these supernates after raising the pH. The major part of the material was composed of long fibrous elements. The hyaline substance appeared to polymerize independently of this fibrous material and formed an amorphous gel (Fig. 2). When these suspensions were centrifuged at 100–200 *g* for 10 min in conical centrifuge tubes, the hyaline substance formed a tight cohesive pellet at the tip of the tube; the crystalline substance formed a fluffy white layer over this pellet. Initial purification was effected by resuspending the crystalline material and removing the hyaline pellet. This procedure was repeated through two washes with filtered sea water. The separated hyaline and crystalline materials were then dissolved by dialysis against 10 mM Tris-HCl (pH 7.5) for 12–24 hr. In the ultracentrifuge, the dissolved hyaline substance exhibits the characteristic hypersharp sedimentation boundary of the Kane-Hersh (20) protein (Fig. 3). This material has a marked concentration dependence as shown

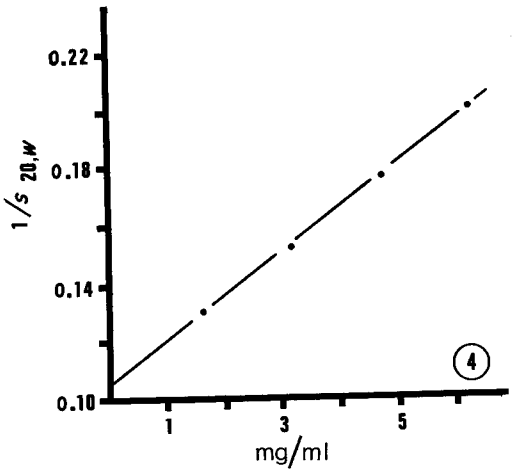


FIGURE 4 Concentration dependence of hyaline protein sedimentation in 10 mM Tris-HCl, 50 mM NaCl, pH 7.8. $s^{\circ}_{20,w} = 9.5$.

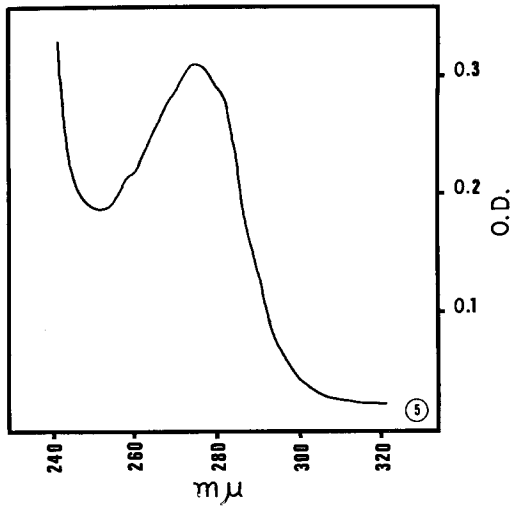


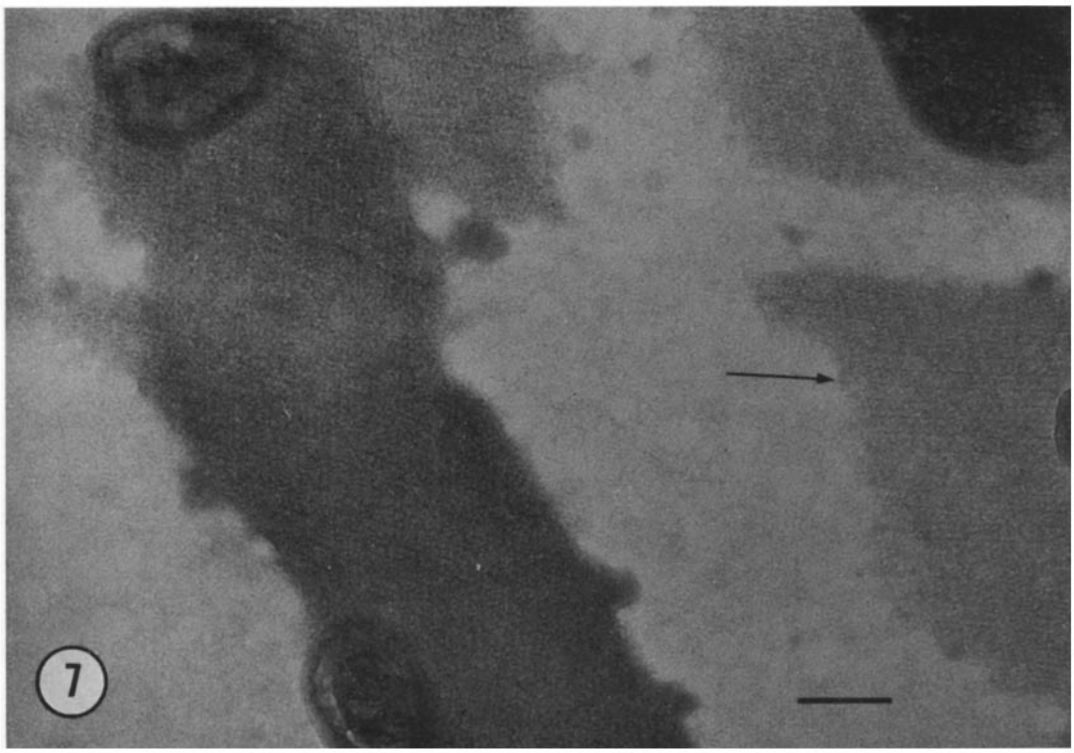
FIGURE 5 UV spectrum of dissolved crystalline protein in 10 mM Tris-HCl, pH 7.5.

in Fig. 4. The plot of $1/s_{20,w}$ vs. concentration extrapolates to $s^{\circ}_{20,w} = 9.5$. The addition of divalent cations (Ca, Mg, etc.) causes solutions of this protein to gel. Its behavior is identical with the Ca-precipitable material studied by Kane and Hersh and shown by Yazaki (31) to originate from the hyaline layer.

The crystalline material also dissolves under the same low ionic strength conditions. The UV spectrum of the dissolved, nondialysable material (Fig. 5) indicates the presence of protein. In the ultracentrifuge, these solutions are polydisperse, with at least three major components with uncorrected sedimentation rates of 6.0, 8.5, and 13 s (Fig. 6). Work is in progress to determine if these components represent separate proteins or aggregates of a single protein.

When negatively stained preparations are examined in the electron microscope, the fibrous elements observed in the phase microscope now appear to be composed of sheets of material rolled into rodlike structures of highly variable diameter and length, often running for many microns. The sheets which form the rodlike structures are composed of strands or cylinders which are approximately 150–180 A in diameter, with an average of 165 A. The strands themselves often appear to be right-handed helices with a somewhat variable pitch (Fig. 7). The subunits, although difficult to discern, are approximately 40–50 A. In some preparations or after very brief sonication, single tubules or fragments of tubules are often visible. A helix with a pitch angle of 40–45° is occasionally observed in these tubules (Fig. 8). This variation in pitch angle and the inability to clearly see the 72° pitch observed in freeze-etched specimens are believed to result from the fact that the tubules are somewhat transparent. The observed images are therefore a superposition of all the subunits in the tubule. To test this possibility, negatively stained specimens were observed in the EM and intentionally photographed when the plane of focus was above the specimen plane. While this procedure does not produce the most esthetically pleasing micrographs, it does tend to reduce or eliminate the contributions from the back subunits of the tubules. In this case a shallow pitch angle of 68–72° is clearly demonstrated. In addition the surface subunits now appear somewhat clustered and an approximately 110 A periodicity is discernible on the cylinders (Fig. 9). The isolated elements are therefore quite similar to if not identical with the crystalline structures seen in the intact fertilization membrane.

The highly organized nature of the crystalline material is also reflected in their birefringence. Fig. 10 is a polarization micrograph of the crystalline material at different compensator settings. The material exhibits a strong positive birefringence. No attempt has yet been made to determine the coefficient of birefringence or to decide whether the birefringence is primarily intrinsic or form birefringence.



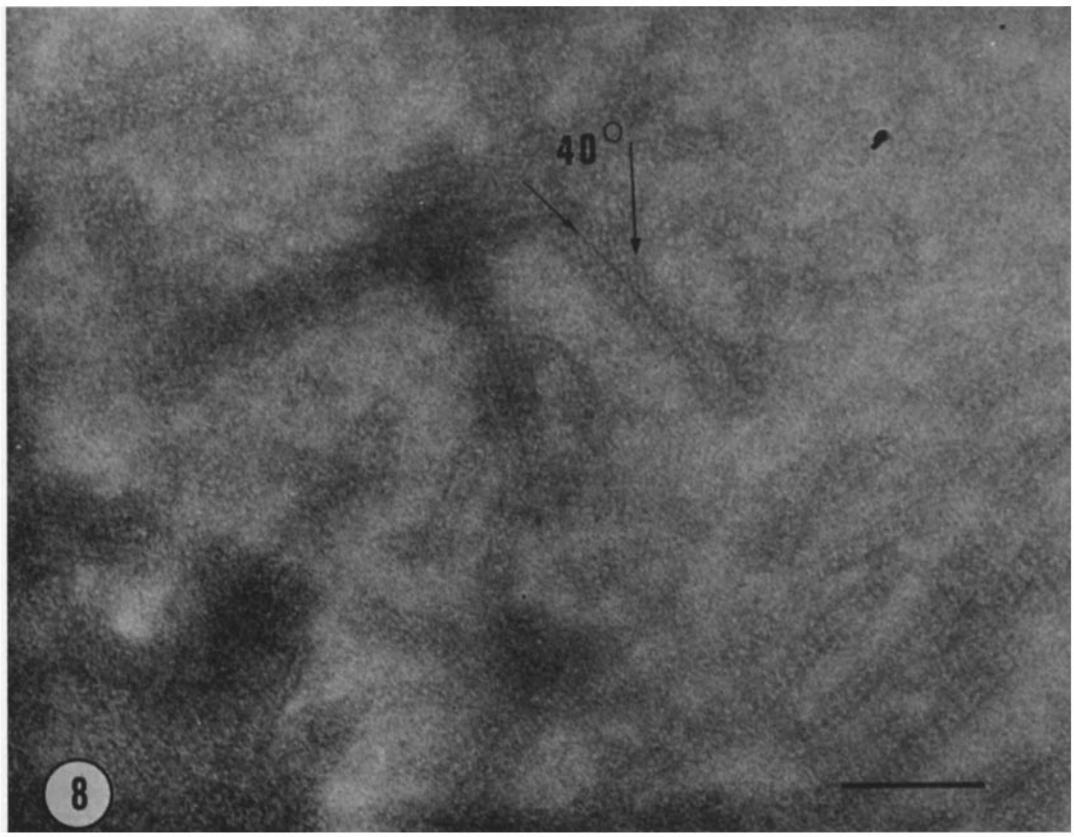


FIGURE 7 Crystalline material spread in sea water, negatively stained with 1% phosphotungstic acid (PTA). Arrow indicates 170 A periodicity × 128,000. Scale = 0.1 μ.

FIGURE 8 Sonicated crystalline material spread in sea water, negatively stained with 1% PTA. Arrow indicates 40° pitch angle. × 182,000. Scale = 0.1 μ.

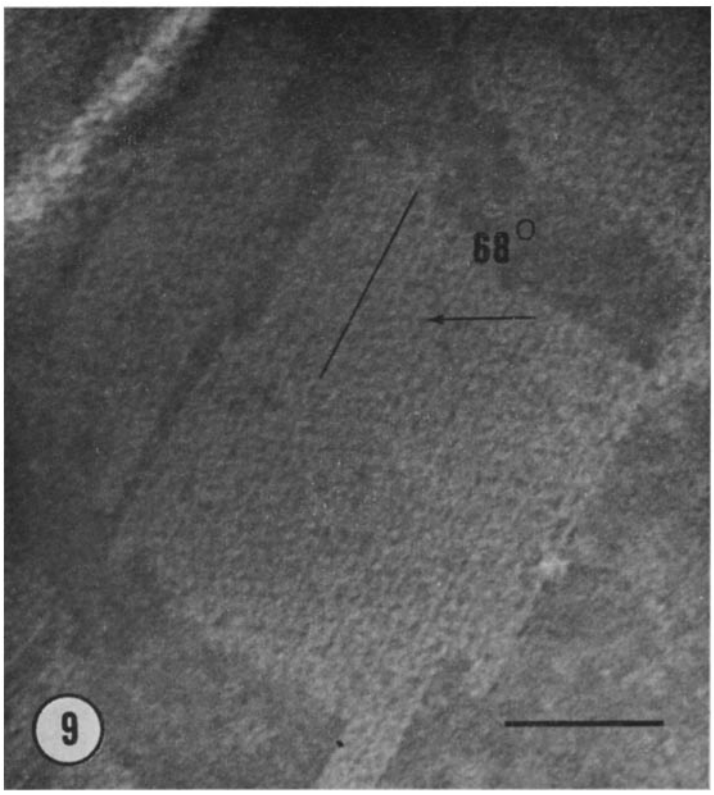


FIGURE 9 Crystalline material spread in 20 mM $CaCl_2$, pH 7.6, stained with 1% ammonium molybdate. Arrow indicates 68° pitch angle. Underfocused specimen. × 218,000. Scale = 0.1 μ.

To attempt to quantitate the release of the crystalline material, eggs were treated with butyric acid at pH 4.5 for 2 min, adjusted to the indicated pH with 1 N NaOH, and then stirred for only a short time (1 min) to minimize the release of the hyaline substance. The eggs were quickly collected by hand centrifugation and the supernatants were adjusted to pH 8, to effect polymerization. Aliquots were removed at each step and protein determinations were done; the results are shown in Table I. The percentage of protein sedimented at 200 *g* is primarily crystalline material, containing less than 10% hyaline substance. There is a great deal of variability in the results obtained with different batches of eggs, and therefore it is difficult to detect any clear pH effect on the release of material. The crystalline material that is released represents approximately 3–5% of the total egg protein under the conditions employed for *Strongylocentrotus purpuratus*. This is a minimal figure since the criterion for solubility is a rather loose one (200 *g*, 10 min spin). The supernatants above the 200 *g* pellet are, however, quite clear. The presence of butyrate does not appear to reduce the polymerization of the crystalline material since dialysis of supernatants against excess filtered sea water does not increase the yield.

DISCUSSION

There have been other reports on the chemical nature of the contents of the cortical granules (27). The work of Immers (13, 14) and of Aketa (1) using $S^{35}O_4$ and various basic dyes, suggests the presence of sulfated acidic polysaccharides. Ishihara (16) has studied the release of acid polysaccharides following fertilization of sea urchin eggs and has proposed that this release accounts for the acid production at the time of fertilization (17) (see also Mehl and Swann (24) and Fujii and Ohnishi (8) for alternative interpretations). Using preparations of dejellied, demembranated eggs

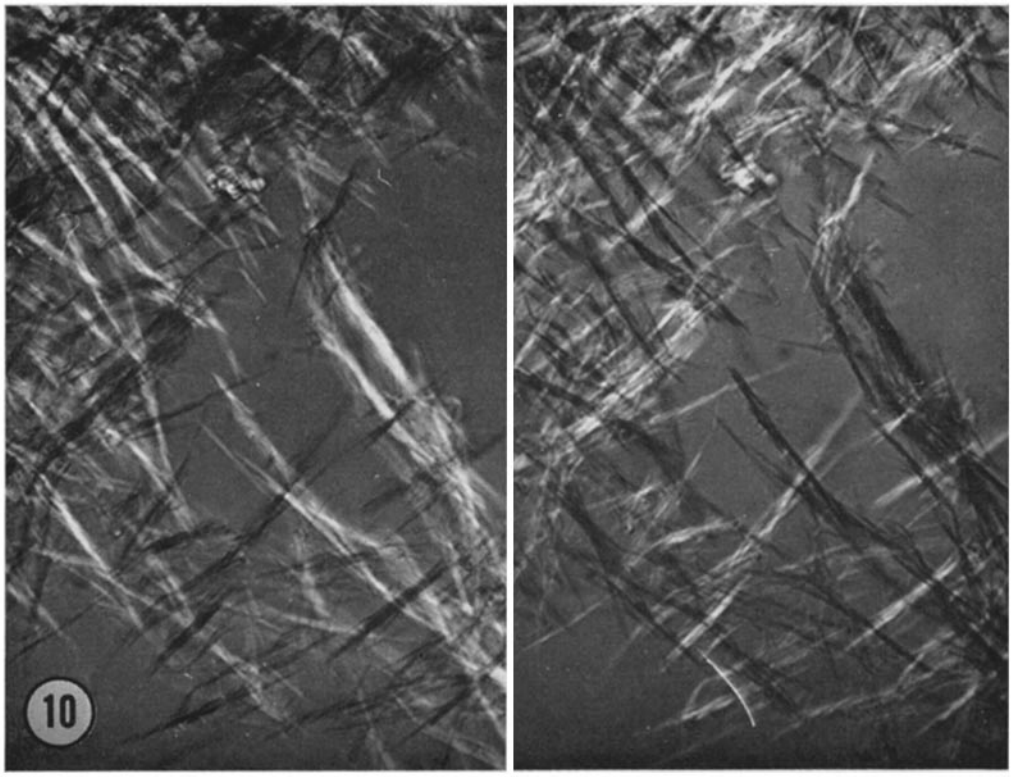


FIGURE 10 Polarization micrographs of crystalline material in sea water (pH 7.6) at different compensator settings. × 1500.

TABLE I

Initial pH*	Total egg protein released	Crystalline‡ protein	Crystalline protein as % total egg protein
	%	%	
5.5	3.0	38	1.1
6.0	9.0	48	4.3
6.5	7.7	45	3.5
8.0	5.7–8.8	44–46	2.5–7.7

* pH after activation with butyric acid at pH 4.5.
‡ Released protein which is sedimentable at 200 *g* in 10 min.

obtained with trypsin, Ishihara (18) has collected material released at fertilization. This "fertilization product" has been characterized, with no attempt at fractionation, although an amino terminal analysis indicated the presence of multiple components. From the chemical analysis, which shows the presence of amino acids, a number of hexoses, sulfate, and hexosamine, Ishihara concludes that the "fertilization product" is an acid glycoprotein. In the absence of any fractionation of the components of the fertilization product, it is difficult to say whether this "acid glycoprotein" is hyaline protein, the crystal protein, or a mixture of these and more.

Metz (25) and Gregg and Metz (10) have also shown that the fertilization product is a complex mixture. At least 11 antigens are released from dejellied eggs and dejellied, demembranated eggs upon activation. At least one of these "cortical response antigens" is a sperm agglutinin which Gregg (9) reports to be functionally indistinguishable from fertilizin, the sperm agglutinin of egg jelly.

Epel et al. (7) have demonstrated the release of a β-1,3, glucanhydrolase (E.C.3.2.1.-39) from the eggs of the sea urchin *Strongylocentrotus purpuratus* into the perivitelline space and the surrounding sea water after insemination. The role of

this enzyme in the cortical response is by no means clear. It is assayed by the release of free glucose from the algal β-1,3-glucan, laminarin, which has led to the idea that this enzyme may function either as a hydrolase by unmasking reactive groups or as a transferase to modify glycoproteins.

The hyaline material has recently been shown by Yazaki (31) to be identical with the Kane-Hersh (20) protein. The material isolated in the present work and referred to as hyaline is identical to the Kane-Hersh protein in sedimentation behavior and calcium precipitability. Kane and Stephens (21) have further localized this material in the cortex before fertilization and have studied its distribution in several species of Hawaiian sea urchins.

The crystalline material is easily distinguishable from the hyaline substance. In the ultracentrifuge, samples dissolved by dialysis against low salt solutions do not behave like the Kane-Hersh protein. Ultrastructurally, the hyaline layer appears to be relatively amorphous while the crystalline material is highly organized. Although both substances aggregate in sea water, they appear to polymerize independently.

The structure of the isolated crystalline material is identical with the structures visualized by Inoué et al. (15) and by Humphreys and Kreutziger (12) on the surface of the fertilization membrane by freeze-etch techniques. These structures are apparently sheets of cylinders or, more specifically, as the work of Ito et al (19) would seem to indicate, sheets of tubules which are approximately 150–200 A in diameter. The variation in the observed dimensions is presumably due to the differences in the methods of preparation and visualization. The individual tubules appear to be wound in a right helical arrangement with a pitch of approximately 70°. Although the subunit structure is difficult to resolve, the subunits appear to be consistent with the 40–50 A value given by Humphreys and Kreutziger (12). These units are "clustered" in some as yet unknown way which produces a 105–110 A periodicity on the surface of the tubules.

The relation between these tubular structures and the more common intracellular microtubular elements is unclear. Other *extracellular* microtubules have been identified with the mastigonemes from the sperm of *Ascophyllum* and *Fucus* (4). These microtubules are composed of globular subunits with a center-to-center distance of about 45 A. In addition they are of smaller diameter (170–190 A) than the cytoplasmic microtubules in these cells and appear to have only approximately 10 subunits per turn rather than the usual 13 subunits. On the basis of the morphological evidence, Bouck (4) concludes that these elements must be considered as an additional class of stable microtubules distinct from the bacterial flagella which they resemble.

Microtubules or microtubule-like elements have also been observed inside the cortical granules of *Mytilus edulis*. Humphreys (11) has described bundles of uniformly spaced microtubules measuring up to 0.8 μ in length. The bundles overlie the granular material found in the cortical granules. These microtubules are about 180 A in diameter, with a wall thickness of 50 A and an electron-lucent center about 80 A in diameter. Each microtubule is separated from its neighbor by about 90 A, or a center-to-center spacing of 270 A. The cortical reaction in *Mytilus* is very attenuated and the fate of these structures has not been examined in detail, although they appear to break down when released into the perivitelline space.

Similar, organized structures (the lamellar structures (2, 27) have been seen in the cortical granules of sea urchin oocytes but have not been clearly demonstrated to be aggregates of tubules. These lamellar structures are transformed into the rodlike objects in the perivitelline space and the crystalline arrays on the fertilization membrane (5, 30). They are the precursors of the paracrystalline material which has been isolated.

In view of the similarity between the present material and other extracellular tubules, it seems reasonable to speculate that this material consists of a complex organization of stable extracellular microtubules. Work is now in progress to define this material chemically and to compare it with intracellular microtubules, particularly those of the mitotic apparatus (28).

It is a pleasure to acknowledge the helpful criticism and stimulus of Dr. Daniel Mazia in whose laboratory this work was done. The author is indebted to Dr. Hidemi Sato for the use of his polarization microscope.

This work was supported by USPHS grant GM-13882 to Dr. Daniel Mazia and by a Helen Hay Whitney Postdoctoral Research Fellowship.

Received for publication 9 September 1969, and in revised form 9 October 1969.

BIBLIOGRAPHY

1. AKETA, K. 1962. *Embryologia.* **7**:223.
2. ANDERSON, E. 1968. *J. Cell Biol.* **37**:514.
3. AUSTIN, C. R. 1968. Ultrastructure of Fertilization. Holt, Rinehart & Winston Inc., New York.
4. BOUCK, G. B. 1969. *J. Cell Biol.* **40**:446.
5. ENDO, Y. 1961. *Exp. Cell Res.* **25**:383.
6. EPEL, D., and D. MAZIA. 1969. In press.
7. EPEL, D., A. M. WEAVER, A. V. MUCHMORE, and R. T. SCHIMKE. 1969. *Science (Washington).* **163**:294.
8. FUJII, T., and T. OHNISHI. 1962. *J. Fac. Sci. Univ. Tokyo Sect. IV Zool.* **9**:333.
9. GREGG, K. W. 1968. *J. Cell Biol.* **39**:56A.
10. GREGG, K. W., and C. B. METZ. 1966. *Bulletin of the Association of Southeastern Biologists.* **13**:34.
11. HUMPHREYS, W. J. 1967. *J. Ultrastruct. Res.* **17**:314.
12. HUMPHREYS, W. J., and G. O. KREUTZIGER. 1968. 26th Annual EMSA Meeting. 136.
13. IMMERS, J. 1961 *a. Ark. Zool.* **13**:299.
14. IMMERS, J. 1961 *b. Exp. Cell Res.* **24**:356.
15. INOUÉ, S., J. P. HARDY, G. H. CONSINEAU, and A. K. BAL. 1967. *Exp. Cell Res.* **48**:248.
16. ISHIHARA, K. 1964. *Exp. Cell Res.* **36**:354.
17. ISHIHARA, K. 1968. *Exp. Cell Res.* **51**:473.
18. ISHIHARA, K. 1968. *Biol. Bull.* **134**:425.
19. ITO, S., J. P. REVEL, and D. A. GOODENOUGH. 1967. *Biol. Bull.* **133**:471.
20. KANE, R. E., and R. T. HERSH. 1959. *Exp. Cell. Res.* **16**:59.
21. KANE, R. E., and R. E. STEPHENS. 1969. *J. Cell Biol.* **41**:133.
22. LOEB, J. 1906. The Dynamics of Living Matter. Columbia University Press, New York.
23. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. *J. Biol. Chem.* **193**: 265.
24. MEHL, J. W., and M. M. SWANN. 1961. *Exp. Cell Res.* **22**:233.
25. METZ, C. B. 1967. Fertilization. C. B. Metz and A. Monroy, editors. Academic Press Inc., New York. **1**:163.
26. MONROY, A. 1965. Chemistry and Physiology of Fertilization. Holt, Rinehart & Winston Inc., New York.
27. RUNNSTROM, J. 1966. *Advan. Morphogenesis* **5**:291.
28. SAKAI, H. 1960. *J. Biochem. Biophys. Cytol.* **8**:603.
29. WICKLUND, E. 1949. *Ark. Zool.* (2) **1**:1.
30. WOLPERT, L., and E. H. MERCER. 1961. *Exp. Cell Res.* **22**:45.
31. YAZAKI, I. 1968. *Embryologia* **10**:131.