Published April 1, 1969

A COMPARATIVE STUDY OF THE ISOLATION OF THE CORTEX AND THE ROLE OF THE CALCIUM-INSOLUBLE PROTEIN IN SEVERAL SPECIES OF SEA URCHIN EGG

R. E. KANE and R. E. STEPHENS

From the Pacific Biomedical Research Center, University of Hawaii, Honolulu, Hawaii 96822, and the Department of Biology, Brandeis University, Waltham, Massachusetts 02154

ABSTRACT

A comparative study was made of the isolation of the cortex in the eggs of several sea urchin species. Since the isolation method developed by Sakai depends on the presence of magnesium in the medium, the protein composition of the cortex was investigated to determine whether the protein component of the egg described by Kane and Hersh which is gelled by divalent ions, is present in these cortices. Isolation of the cortex was found to require the same divalent ions at the same concentrations as protein gelation, and in the eggs of some species much of the gel protein of the cell was found in the isolated cortical material. In the eggs of other species a smaller fraction of this protein was found in the isolated cortex, although it was more concentrated there than in the endoplasm, and in one species this protein appeared to be uniformly distributed throughout the cell. These results indicate that this protein is localized in the cortical region of the eggs of some species of sea urchin, possibly in the cortical granules, but also point up the fact that results from one species cannot be uncritically extrapolated to others.

INTRODUCTION

The existence of a differentiated cortex at the periphery of the sea urchin egg has been hypothesized for many years, based on a number of experimental observations. The first evidence for the presence of a differentiated cortical region in the sea urchin egg came from the microdissection studies of Chambers (1) in 1917, who observed that the surface of egg cells was denser than the interior and suggested the presence of an extensible gel at the surface. In later experiments based on the coalescence of oil droplets with eggs, Chambers (2) noted that these drops formed a dumbbell shape when flowing back out of the egg, which indicated a rigid cortex. More recently, by microscopic studies on whole and sectioned urchin eggs which had been stratified by centrifugation, Mitchison (3) has estimated the thickness of this cortex to be 1–2 μ. After this, the studies of Hiramoto (4) on several species of Japanese sea urchins indicated a thickness of 3–4 μ. These values were obtained by inserting a microneedle through the egg and measuring the thickness of the cortex pushed out on the opposite side, with corrections for optical effects at the egg surface.

A somewhat more indirect line of evidence for the presence of a rigid cortex at the egg surface comes from studies of the behavior of cytoplasmic particulates under centrifugal force. Brown (5)

in 1924 followed the movement of the red pigment granules of the *Arbacia* egg under centrifugal force and hydrostatic pressure, and postulated the existence of a cortical region having a higher viscosity than the interior of the cell, whose properties changed during division. These results were confirmed and extended by Marsland (6) and Marsland and Landau (7) who estimated the thickness of the cortex of *Arbacia lixula* to be 6 μ. In addition to studies on the pigment granules, the behavior of the cortical granules of the egg has been used to investigate the nature of the cortex. Harvey (8) first noted the presence of granules at the surface of the sea urchin egg which were immobile under centrifugation; they were later studied extensively by Moser (9, 10). In eggs which have been stratified by centrifugal force so as to form a clear zone, the granules are seen to retain their original distribution at the periphery of the egg; this leads to the assumption that they are held in position by a gel cortex. This work has been reviewed and discussed by Heilbrunn (11), who considered calcium to be critical in controlling the properties of this cortex, its rigidity increasing in excess calcium and decreasing in oxalate or other calcium-binding agents.

The electron microscopic studies of Mercer and Wolpert (12) have failed to confirm the existence of such a cortex. These investigators found no evidence of a differentiated layer at the surface of the egg of *Psammechinus miliaris*, the cytoplasmic particulates penetrating between the cortical granules. Their observations also indicated that the membrane of the cortical granules was fused at some point with the plasma membrane of the egg, thus explaining how the material of the cortical granules passes "through" the intact plasma membrane at fertilization. More important for the question discussed here, this might also explain why these granules remain immobile under centrifugal force, for, if their membranes are joined to the plasma membrane, the granules will be held in place without the necessity for a gelled cortex.

However, this lack of evidence for the cortex at the ultrastructural level has not prevented the isolation of apparent "cortical" material from the eggs of a number of species of sea urchins. Motomura (13) found that the cytolysis of eggs in acidified distilled water allowed the isolation of a cortical material apparently containing the cortical and pigment granules. At about the same time, Allen (14) found fragments of cortex adhering to a glass surface after jellyless eggs had been allowed to adhere and were removed, and he later (15) was able to separate some cortical material from the eggs by centrifugation. The first practical method for preparing large quantities of isolated cortex was that of Sakai (16). After removal of the jelly (and the fertilization membrane in the case of fertilized eggs) the eggs were washed and crushed in 0.1 M $MgCl_2$, and the resulting cortical material was centrifuged and washed in the same medium. This procedure yields an intact cortical hull containing on the order of 5% of the cell protein. Sakai measured the bound —SH groups of such cortical preparations and found changes in the ratio of SH/protein-N over the first cleavage. In a later experiment, Sakai (17) separated the cortex proteins into water-soluble, 0.6 M KCl-soluble, and KCl-insoluble fractions and found the fluctuation of bound —SH in the cortex to result from changes in the 0.6 M KCl-soluble fraction. After further studies on the behavior of the sulfhydryl groups of cortices of eggs blocked in ether (18) and on the behavior of these groups in protein extracts of whole cells (19, 20), Sakai returned to the study of cortical proteins and claimed (21) that under appropriate experimental conditions the 0.6 M KCl-soluble proteins of the cortex underwent an electron-transfer reaction with the calcium-insoluble protein, which was first isolated from the sea urchin egg by Kane and Hersh (22). Later a calcium-insoluble protein was prepared from isolated mitotic apparatuses (MA) and reacted with the 0.6 M KCl-soluble protein of the cortex (23).

The recent work of Yazaki (24) has considerably clarified the problem of the sulfhydryl reactions of the calcium-precipitable proteins of the sea urchin egg. She has demonstrated that the precipitate formed on the addition of calcium to a water extract of eggs (which she terms Ca-ppt 1) can be divided into two fractions by dialysis against 0.01 M Tris, pH 7.0. The fraction that dissolves on dialysis, and which can be purified by reprecipitation and redialysis, is termed the HyS and is identical to the Kane-Hersh protein in its gelation behavior and ultracentrifugal properties. The insoluble fraction that remains after dialysis is termed Ca-ppt 2 and is a different protein, which exists as a contaminant in unpurified calcium-insoluble protein preparations. It is this component that undergoes cyclical changes in the oxidation state of its —SH groups, while the HyS fraction

shows no such activity. Yazaki convincingly demonstrates that it is this Ca-ppt 2 component of Sakai's calcium-insoluble protein (21) that is involved in the sulfhydryl interaction with the 0.6 M KCl-soluble protein of the cortex and, by implication, would also be responsible for the —SH activity of the calcium-insoluble protein of the mitotic apparatus (23). This latter conclusion is strengthened by the fact that in Sakai's experiments (23) the calcium-insoluble fraction from the MA is redissolved by EDTA and not by dialysis and does not show the characteristic hypersharp ultracentrifugal peak of the Kane-Hersh protein. Thus, whatever the significance of the —SH interaction of the Ca-ppt 2 with the 0.6 M KCl-soluble fraction, the Kane-Hersh protein is obviously not involved in this reaction, and it is this protein with which we are concerned here.

One of the most striking features of the Sakai procedure (16) for the isolation of the cortex is that the conditions used for isolation are precisely those required for the gelation of the Kane-Hersh protein. This protein is obtained by extracting urchin eggs in solutions of low ionic strength. It exists in such solutions as an extremely asymmetric molecule, which forms a transparent gel on the addition of a number of divalent cations, each at a different concentration. One of the ions effective in gelation is magnesium at 0.1 M; this suggests the possibility that this protein may be one of the components of the isolated cortex; a preliminary report on this question has appeared (26). A second result of Yazaki's investigation (24) is pertinent here. She found that a fluorescent antibody for the HyS (Kane-Hersh) protein stained the periphery of the unfertilized egg and the hyaline layer of the fertilized egg. She suggested that this protein might be located in the cortical granules of the egg and extruded at fertilization to form part of the hyaline layer. Earlier electron microscopic studies of Endo (25) had indicated that these granules contributed to both the fertilization membrane and the hyaline layer. On these grounds also, the Kane-Hersh protein would be found in the cortical region, and this provides an additional motive for the reexamination of the cortex isolation procedure. In the present experiments, the conditions for cortex isolation have been studied in detail, the protein components of the cortex identified and measured and these results related to quantitative measurements of soluble, calcium-precipitable, and total protein of the egg. To obtain results of the most convincing generality, these experiments were carried out on a number of different sea urchin species, and major differences in the behavior and quantity of the isolated cortex were found among these species.

MATERIALS AND METHODS

The eggs of the Hawaiian sea urchins *Colobocentrotus atratus*, *Echinometra mathei*, *Tripneustes gratilla*, and *Pseudoboletia indiana* were used for these experiments, and eggs of the Woods Hole species *Arbacia punctulata* were utilized for some comparative studies. The eggs were obtained by the injection of isotonic KCl into the body cavity or by electrical shock. After shedding, the eggs were collected by hand centrifugation and washed several times in seawater, and the jelly was removed by brief exposure to seawater adjusted to pH 5. Egg proteins were separated into soluble and insoluble fractions by homogenizing the eggs in 10 times their volume of solution in a Teflon-glass-homogenizer and centrifuging at 25,000 *g* for 20 min at 4°C. Dialysis was carried out for 24–48 hr at 4°C with continuous stirring. Sodium and potassium chloride gave identical results; sodium was used in most cases to eliminate interference in the Lowry procedure.

Protein determinations were made by the method of Lowry et al. (27), with a serum albumin standard. Total egg protein was determined by taking up the cells in 1 M NaOH. Protein measurements were made on aliquots of the soluble fractions of eggs and cortex, and determinations of gel protein were made on gel samples taken up in NaOH or on aliquots after redissolving by dialysis. The protein of eggs and cortices insoluble in low salt was determined by taking up this material in 1 M NaOH. All protein measurements are given in terms of milligrams of protein per milliliter of packed, jellyless eggs. Sedimentation studies were performed in a Beckman Spinco Model E analytical ultracentrifuge (Spinco Div., Beckman Instruments, Inc., Fullerton, Calif.), equipped with Schlieren optics, phase plate, and rotor temperature control. All ultracentrifuge runs were made at 20 ± 1°C.

Eggs were fixed for 30 min in 2% glutaraldehyde in 90% seawater, pH 7.5–8.0, postfixed for 30 min in 1% OsO_4 in seawater, and embedded in Araldite by the method of Luft (28). The sections were stained in lead citrate (29) and uranyl acetate in methanol (30) and examined in a Philips 300 electron microscope.

RESULTS

Cortex Isolation in the Eggs of Colobocentrotus atratus and Echinometra mathei

The first aim of the present experiment was to duplicate the Sakai isolation technique (16) on the

eggs of several Hawaiian sea urchins and, if successful, to determine whether the Kane-Hersh gel protein could be identified as a component of the resulting cortices. The eggs of the urchin *Colobocentrotus atratus* were used for most of these investigations, since they are available through most of the year. Comparative experiments, to be described shortly, later showed these eggs to be unusually favorable material for these experiments. The method of Sakai was duplicated by transferring washed jellyless eggs of *C. atratus* to a solution of 0.1 M $MgCl_2$ and crushing them gently in a homogenizer. After several washings in the same solution an orange-yellow pellet was obtained, containing recognizable cortex pieces, but of a variety of sizes, and with some contamination by cytoplasmic material. If the induction or retention of gelation of the Kane-Hersh gel protein is involved, isolation should also be possible with other divalent ions at concentrations which gel this protein. The use of 0.02 M $CaCl_2$ proved to be more successful than $MgCl_2$ with the eggs of the species: the cortices appeared to have more mechanical resistance, because they usually remained as intact cortical hulls, and total yields were larger. Further improvement was obtained by buffering the solution sufficiently to overcome the acid reaction which accompanies cytolysis and thus increase the ease of dispersing the cytoplasm, an effect which has been observed previously (31). The final procedure adopted consists of the transfer of jellyless eggs to 0.02 M $CaCl_2$, buffered with 0.02 M Tris at pH 7.5, and allowing them to cytolyze at room temperature. A clear "cortical" region is formed at the periphery as the wave of lysis passes over the cell; this cortical material is then freed of cytoplasm by gentle homogenization, separated by centrifugation at 400–500 *g*, and washed repeatedly in the same solution at 0°C until no further particulate matter is removed. This usually requires 10 or more washings. The cortices become increasingly adherent to each other as they are washed, and the final product is a white pellet composed entirely of intact cortical hulls (Fig. 1). To demonstrate that other ions which cause gelation of the Kane-Hersh protein allow isolation of the cortex, we performed a few experiments in 0.05 M $MnCl_2$, with results similar to those in magnesium.

Isolated cortices washed several times in low salt solution swell and begin to disintegrate; this indicates that the gel protein may be responsible for their physical integrity. To insure a sufficient reduction of the calcium concentration to bring about the complete solubilization of the protein, the cortices were dialyzed for 24 hr or more against a large volume of low salt solution, usually 0.05 M NaCl buffered at pH 7.5 with 0.01 M Tris. The bulk of the material present in the isolated cortex goes into solution under these conditions, and the remaining insoluble material is removed by centrifugation at 25,000 *g* after dialysis. Analytical ultracentrifugation of the soluble extract (Fig. 2) showed a protein having the highly concentration-dependent sedimentation rate and characteristic hypersharp peak of the Kane-Hersh protein to be the major component present. Relative amounts cannot be estimated in such runs, because the extreme sharpness of this peak causes it to exceed the limits of the optical system at concentrations much above 1 mg/ml, and minor components in the solution are usually invisible at the low over-all concentration required. Quantitative determinations of the gel protein were made by adding 0.02 M $CaCl_2$ to the soluble fraction, allowing it to stand for 10–15 min at 0°C with gentle agitation, separating the resultant gel by centrifuging at 4,000 *g* for two min, and washing several times in 0.02 M $CaCl_2$ to remove any trapped soluble material. Under the

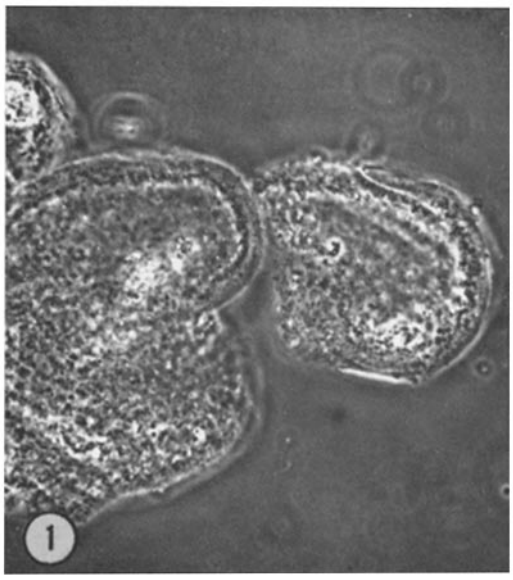

FIGURE 1 Cortices of *C. atratus* eggs isolated in 0.02 M $CaCl_2$-Tris. Phase contrast. × 270.

Published April 1, 1969

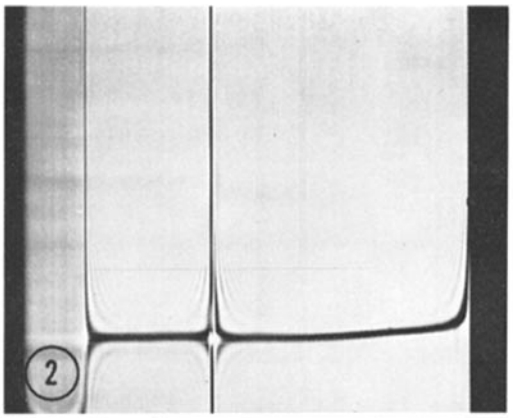

FIGURE 2 Soluble fraction of calcium-isolated *C. atratus* cortices, extracted in 0.05 M NaCl, 0.01 M Tris, pH 7.5. 72 min after reaching speed of 56,000 rpm, bar angle 60°. Uncorrected sedimentation coefficient 4.1S.

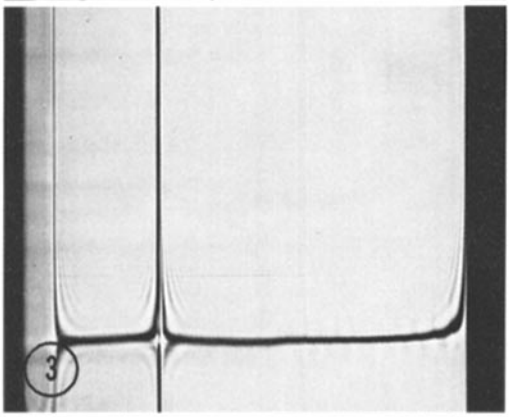

FIGURE 3 Soluble fraction of magnesium-isolated *C. atratus* cortices, extracted in 0.05 M NaCl, 0.01 M Tris, pH 7.5. 72 min after reaching speed of 56,000 rpm, bar angle 60°. Uncorrected sedimentation coefficient 3.4S.

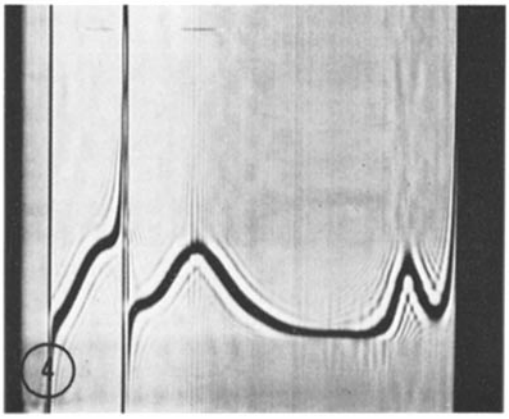

FIGURE 4 Soluble fraction of *C. atratus* eggs, extracted in 0.05 M NaCl, 0.01 M Tris, pH 7.5. 40 min after reaching speed of 56,000 rpm, bar angle 60°.

conditions of the experiment, the cortices isolated from 1 ml of packed *C. atratus* eggs contain 12 mg of soluble protein, of which 10 mg (or more than 80%) is gelled by calcium. About 6 mg of protein is present in the insoluble material remaining after extraction.

Quantitative measurements were made on the original cortex isolation procedure of Sakai (16) in the light of this information. Confirming the visual observations, isolation in 0.01 M $MgCl_2$ solution was found to reduce the total yield of material, but not to change the relative composition of the fractions obtained. The soluble extract is similar to that of cortices isolated in $CaCl_2$ (Fig. 3), but the yields of protein are halved, 6 mg of soluble protein being obtained, of which 5 mg is

gel protein. The gel protein thus makes up the same proportion of the soluble protein in both calcium- and magnesium-isolated cortices, but the over-all yield is twice as great in calcium.

Extraction of the low salt–insoluble cortex residue with 0.6 M salt to prepare the "contractile" protein of Sakai (17, 21, 23) yielded only very small amounts of protein, on the order of 1 mg per ml of packed cells. This value is much lower than the values reported by Sakai (17), who found about one-third of the total protein-N of the cortex of *Hemicentrotus pulcherrimus* in this fraction. Since the total insoluble protein of the *C. atratus* cortex is only one-third of the protein present, this may indicate a species difference (the cortices of *T. gratilla*, to be described shortly, contain much more insoluble protein) or may be due to incomplete extraction of the water-soluble components. The gel protein is partially soluble in high salt, and will appear in this fraction of not completely removed by water extraction.

The values for the protein composition of the isolated cortical material can then be related to the protein content of the whole egg. 1 ml of packed *C. atratus* eggs contains 163 mg of total protein, so the 18 mg of cortical protein represents about 11% of the cell protein. The distribution of the egg protein into soluble and insoluble fractions depends on the extraction medium used. The maximum amount of calcium-insoluble protein is extracted by homogenization of the eggs in very low ionic strength, such as 0.01 M Tris buffer. However, a fraction of this is heterogeneous material which precipitates rather than gels with calcium and which has physical properties different from those of the gel protein. This material appears equivalent to the Ca-ppt 2 fraction of Yazaki (24) and can be eliminated by dialysis and regelation. However, the precipitation of this material from 0.01 M Tris extracts is repressed by the addition of 0.05 M NaCl, and, if the eggs are homogenized directly in 0.05 M NaCl, only the Kane-Hersh gel protein is extracted. In the experiments reported here, extracts of *C. atratus* eggs and cortices were usually made in 0.05 M NaCl, 0.01 M Tris, pH 7.5; and 0.05 M NaCl was added to Tris extracts before gelling with calcium. Analytical ultracentrifugation of the soluble fraction of the egg (Fig. 4) shows a variety of components, as would be expected, but the sharp peak of the gel protein is easily identified. Under these conditions the soluble components averaged 126 mg of protein per ml of cells, of which 15 mg was gelled by calcium. Of this 15 mg of gel protein, 10 mg or two-thirds of the total is present in the isolated cortex. Thus, although the isolated cortex contains only a relatively small fraction of the total cell protein, it contains two-thirds of the total gel protein present in the cell.

The eggs of the sea urchin *Echinometra mathei* are very similar to those of *C. atratus* with respect to cortex isolation and the quantities of gel protein involved, and were investigated only briefly to confirm the previous results. Cytolysis of jellyless *E. mathei* eggs in 0.02 M $CaCl_2$-Tris solution results in the formation of a clear cortical region, which is sufficiently rigid to be easily separated from the egg by gentle homogenization and washed free of cytoplasmic contamination (Fig. 5). When separation into soluble, calcium-gelable and insoluble fractions was carried out by the same methods used for *C. atratus*, similar results were obtained: 10 mg of soluble protein was present, of which 8 mg (or 80%) was gelled by calcium. 8 mg of insoluble protein remained after extraction.

The protein content of these eggs was also similar to that of *C. atratus*: 165 mg of total protein was measured and 126 mg was soluble in low salt, of which 10 mg was gelled by calcium. Ultracentrifuge runs on these extracts did not differ significantly from those of *C. atratus*. Thus, as in the case

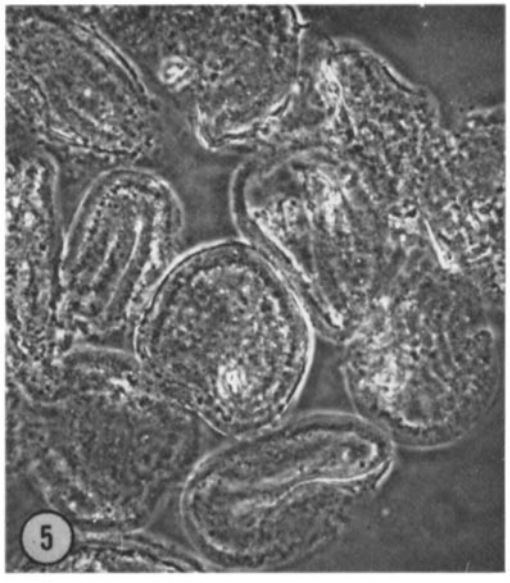

FIGURE 5 Cortices of *E. mathei* eggs isolated in 0.02 M $CaCl_2$-Tris. Phase contrast. × 270.

Published April 1, 1969

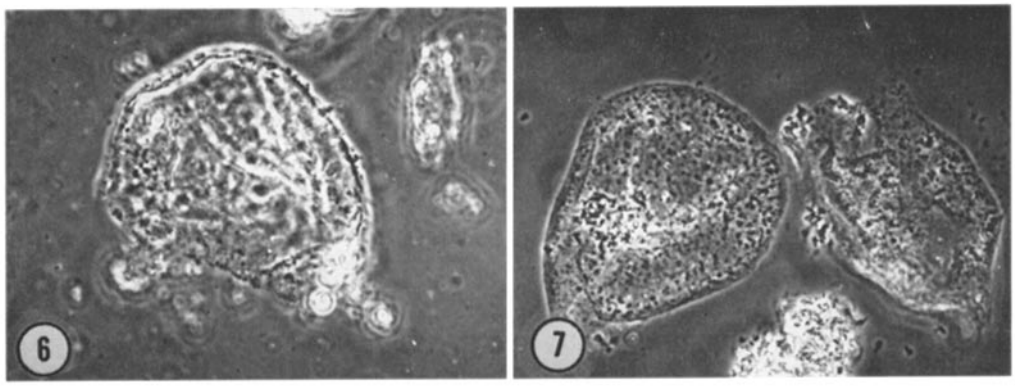

FIGURE 6 Cortex of *T. gratilla* egg isolated in 0.02 M $CaCl_2$-Tris. Phase contrast. × 270.

FIGURE 7 Cortices of *P. indiana* eggs isolated in 0.02 M $CaCl_2$-Tris. Phase contrast. × 270.

of *C. atratus*, the isolated cortex contains most of the gel protein present in the egg. The results of the experiments on *E. mathei* are so similar to those of *C. atratus* that one might be led to what is, in fact, an erroneous generalization: the results with these two species suggest that the calcium-insoluble gel protein is located primarily at the periphery of the urchin egg. However, the experiments with eggs of other species of sea urchins will show that this generalization requires some modification.

Tripneustes gratilla

The method of cortex isolation that was developed with the eggs of *C. atratus* and proved equally successful on the eggs of *E. mathei* gives very different results on the eggs of the urchin *Tripneustes gratilla*. A cortical region is much less evident after lysis of jellyless eggs in 0.02 M $CaCl_2$-Tris solution, and the homogenization and washing procedure used previously yields fewer intact cortices and more fragments of cortical material than with the other two species. These cortices and cortical fragments are thinner and have less of the clear material evident in *C. atratus* and *E. mathei* cortices and contain more dense granular material (Fig. 6). Soluble protein is much lower in these cortices as compared to the two species described above, only 3 mg being obtained per ml of cells, and of this soluble protein 1.6 mg, or about one-half, is gelled by calcium. Insoluble protein is much higher than in *C. atratus* and *E. mathei* cortices, however, and averages 12 mg per ml of cells. These values reflect the microscopic appearance of the cortex, since the high percentage of insoluble material measured may be due to the granular material present and the much smaller gel protein content may be due to the much reduced clear region in these cortices.

The eggs of *Tripneustes gratilla* contain only about one-half the total protein found in *C. atratus* and *E. mathei*, the average value being 88 mg per ml of packed cells. The 15 mg of protein in the isolated cortex of this species thus represents 17% of the total cell protein. Of this total cell protein, 57 mg is soluble in low salt, of which only 3 mg is gelled by calcium. This value for calcium-gelable protein was so much lower than the value obtained for *C. atratus* and *E. mathei* that additional extraction procedures were investigated to determine whether it was the maximum obtainable. Since more calcium-insoluble material was obtained from the eggs of the other species by extraction with 0.01 M Tris, this extraction was carried out on *T. gratilla* and some increase was obtained. However, unlike that in the other species, all of this material was Kane-Hersh gel protein or HyS, as characterized by its gelation and sedimentation behavior. Addition of 0.05 M NaCl to this extract, which eliminates the precipitation of the Ca-ppt 2 material in the other species, did not reduce the amount of material insoluble in calcium, and all this material moved as a single hypersharp peak in the ultracentrifuge. Thus, in this case the 0.01 M Tris solution extracts additional gel protein which is not extracted by 0.05 M NaCl. Even under these conditions, however, the maximum amount of gel protein obtained per ml of cells is 5 mg, a value still considerably lower than the values for the pre-

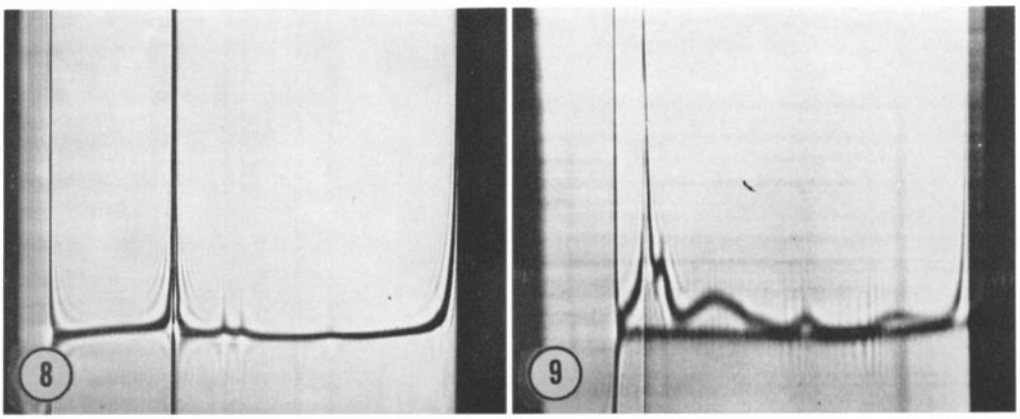

FIGURE 8 Soluble fraction of calcium-isolated *P. indiana* cortices, extracted in 0.05 M NaCl, 0.01 M Tris, pH 7.5. 72 min after reaching speed of 56,000 rpm, bar angle 60°. Uncorrected sedimentation coefficient 3.6S.

FIGURE 9 Soluble fraction of magnesium-isolated *A. punctulata* cortices, extracted in 0.05 M NaCl, 0.01 M Tris, pH 7.5. 16 min after reaching speed of 59,780 rpm, bar angle 60°. Double sector cell. Uncorrected sedimentation coefficient 3.7S.

viously studied species. Of the small amount of gel protein present in these eggs, the fraction present in the isolated cortex is only on the order of one-third, rather than the much larger percentages in *C. atratus* and *E. mathei*.

Pseudoboletia indiana

The eggs of this species differ markedly from those of the species previously investigated with respect to cortex isolation. When prepared by means of the 0.02 M $CaCl_2$-Tris solution used for the other species, the volume of cortex obtained is very small in proportion to the egg volume and consists of cortex pieces of varying sizes. The cortices are extremely thin and fragile and often appear wrinkled (Fig. 7), which is rare in the case of *C. atratus* and *E. mathei* cortices, which seem to be at least semirigid. Soluble protein is lower than in *T. gratilla*, only 1.3 mg being obtained per ml of cells, and 0.6 mg, or one-half of this, is gelled by calcium. With this composition, other components of the soluble extract are just visible in the ultracentrifuge pattern (Fig. 8). Insoluble protein is even more markedly reduced from the values obtained with *T. gratilla*, and averages only 2.6 mg. Total cortical protein is thus only 3.9 mg per ml of cells.

Total protein content in the eggs of this species is 137 mg per ml of cells, a value midway between that for *C. atratus* and that for *T. gratilla*, and the isolated cortex of these eggs thus contains only 3% of the total cell protein. As in the case of *T. gratilla* the extraction of gell protein is quite sensitive to salt, the highest yield being obtained by extraction in 0.01 M Tris, which gives an average value of 3.7 mg of gel protein per ml of cells. The isolated cortical material thus contains only 17% of the total gel protein of the egg, an even lower value than in *T. gratilla*.

Arbacia punctulata

Preliminary experiments on the eggs of this species show yet another pattern of protein distribution. Cortices can be isolated in 0.1 M $MgCl_2$ solution, but the 0.02 M $CaCl_2$-Tris solution which proved more successful with the other species cannot be used, as cytolysis of the egg in this solution causes gelation of the cytoplasm, which does not disperse but breaks up into large clumps on homogenization. Since cortices could not be separated by the usual procedures of differential centrifugation, the use of calcium solutions was abandoned and all isolations were made in 0.1 M $MgCl_2$. In this solution the cells show swelling and lysis accompanied by a wave of pigment granule breakdown. The cortical material is easily freed of cytoplasm by washing, and the pigment is also removed, resulting in a white pellet of cortical pieces which appear quite thin and fragile. Soluble protein in these cortices is 3.2 mg per ml of cells,

TABLE I

Protein Composition of Eggs and Cortices in Various Species of Sea Urchins

Values in mg per ml of packed cells.

	C. atratus	*E. mathei*	*T. gratilla*	*P. indiana*
Total egg protein	163	165	88	137
Egg gel protein	15	10	5	3.5
Total cortical protein	18	18	15	3.9
Cortex total as % egg total	11	11	17	3
Cortex gel	10	8	1.6	0.6
Cortex gel as % egg gel	66	80	32	17

whereas only 0.8 mg is gelled by calcium. Insoluble protein remaining after extraction is 4.6 mg. Calcium-insoluble protein, which was approximately 80% of the soluble cortical protein in *C. atratus* and *E. mathei*, and 50% in *T. gratilla* and *P. indiana*, is thus reduced further to 25% in this species. The gel protein thus makes up the smallest fraction of the soluble cortical protein of any species studied, and the ultracentrifuge pattern (Fig. 9) is no longer dominated by the gel protein peak, but approaches a whole egg extract in composition. The total gel protein present in the egg is about 10 mg, so the gel protein isolated in the cortical material represents only 8% of the total. In this species there is little evidence of the concentration of the gel protein at the periphery of the cell, since the gel protein is present there in about the same proportion as in the endoplasm.

For the purposes of discussion, the results from the Hawaiian species are summarized in Table I. Since the significant values are the relative rather than the absolute amounts, the percentages for some values of interest have been computed and are included.

Electron Microscopic Observations

In view of the results of Yazaki (24), which indicate that the gel protein is present in the hyaline layer of the fertilized egg and is localized at the periphery, possibly in the cortical granules, of the unfertilized egg, preliminary electron microscopic observations were made on the eggs of the species studied here. Although marked differences in the amount of gel protein in the isolated cortex were found among the Hawaiian species, no pronounced difference in the number or distribution of the cortical granules was found in the unfertilized eggs of these species. Marked differences are apparent, however, in the cleaving egg. *C. atratus* blastomeres are closely opposed at first cleavage and have a pronounced hyaline layer (Fig. 10*a*); this hyaline layer is evident in electron micrographs as a dense meshwork between the fertilization membrane and the cell surface (Fig. 11*a*). *T. gratilla* blastomeres are somewhat more separated (Fig. 10*b*) and display a thinner and more fragmented hyaline (Fig. 11*b*), whereas the blastomeres of *P. indiana* are much more separated at cleavage (Fig. 10*c*) and have a barely detectable hyaline layer (Fig. 11*c*). This correlation between the gel protein content and hyaline layer structure of the egg supports Yazaki's (24) conclusion that this protein plays a role in the hyaline layer, but the localization of this protein in the cortical granules of the unfertilized egg remains an interesting but as yet unproven possibility.

DISCUSSION

One of the unexpected results of this investigation was the marked differences in composition and experimental behavior found to exist among the eggs of the various species. Only the eggs of *C. atratus* and *E. mathei* are sufficiently alike to be considered similar; the eggs of the other species differ from them in important respects and also differ amongst themselves. With regard to the protein content of the egg, the value for *C. atratus* and *E. mathei* eggs is almost twice that of *T. gratilla*, whereas that for *P. indiana* lies midway between the others. The content of gel protein varies independently of the total protein, and makes up from 4 to 11% of the total cell protein.

Even wider variations are seen in the response of the eggs to the cortex isolation procedure. In the case of *C. atratus* and *E. mathei*, the isolated material contains only 11% of the total cell protein, but has two-thirds or more of the gel protein in the egg, indicating that this component is localized primarily at the periphery. If, as implied in Sakai's experiments (16), this procedure isolates a preexisting differentiated cortical region of the type that has been hypothesized to exist at the egg surface, this protein might provide the physical basis for such a cortex. However, the lack of electron microscopic evidence for such a cortical region in the eggs studied by Mercer and Wolpert (12)

Published April 1, 1969

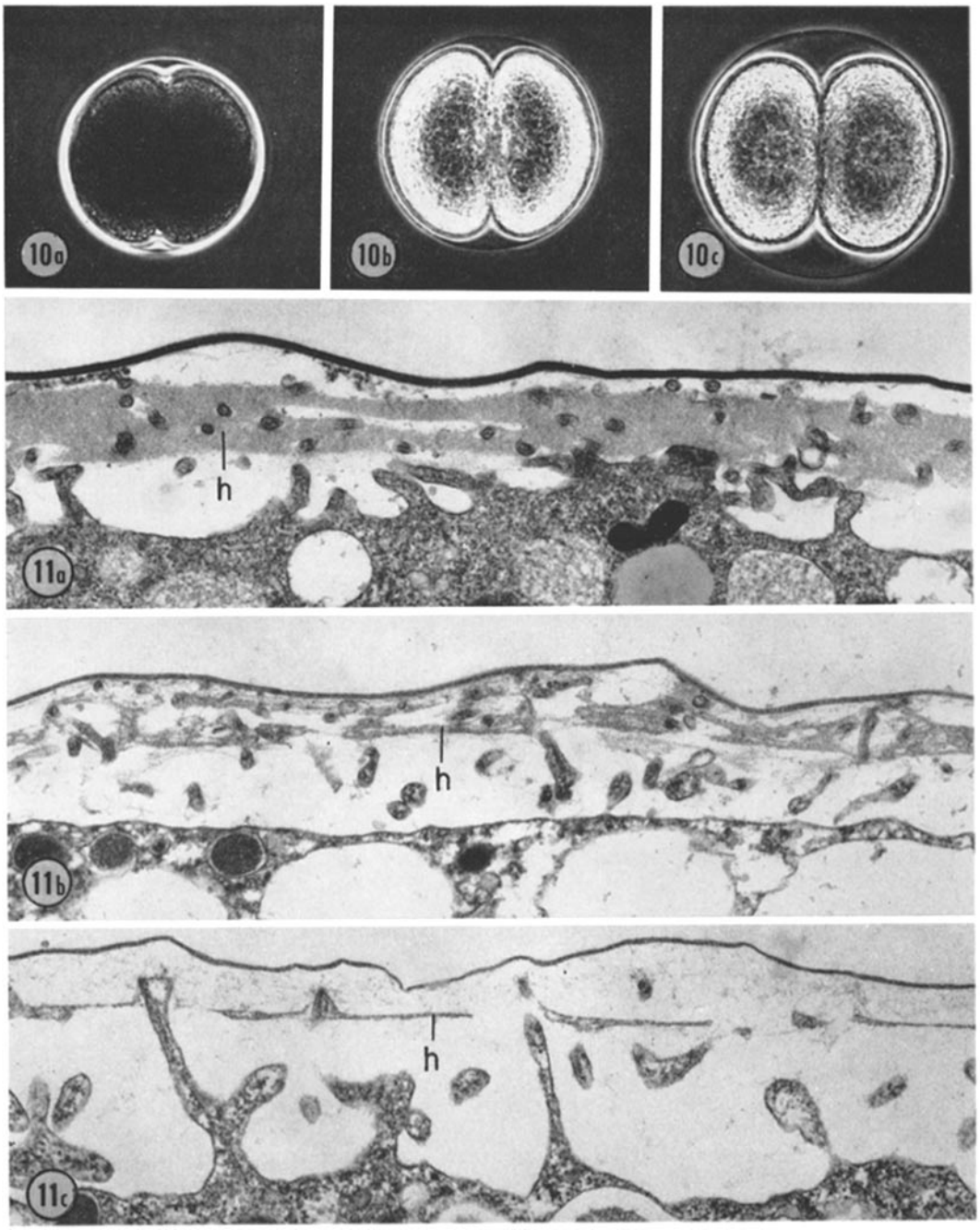

FIGURE 10 First cleavage of (10*a*) *C. atratus*, (10*b*) *T. gratilla*, and (10*c*) *P. indiana*. Phase contrast. × 330.

FIGURE 11 Surface of fertilized eggs of (11*a*) *C. atratus*, (11*b*) *T. gratilla*, and (11*c*) *P. indiana*; *h*, hyaline layer. × 20,000.

and also in the eggs of the Hawaiian species (R. E. Kane, unpublished observations), and the appearance of a clear cortical hull at lysis, supports Yazaki's suggestion (24) that this material may be present in the cortical granules of the unfertilized egg. This material would then be liberated at the time of lysis as a result of cortical granule breakdown, and then gelled by the divalent ions present in the solution. This would agree with Yazaki's demonstration of it in the hyaline layer (24), since this layer apparently originates, at least in part, from these granules (25), but would conflict with Sakai's claim (16) that these granules are present in the isolated cortical material.

The eggs of *T. gratilla* and *P. indiana* respond differently to the isolation procedure, and a thick cortical hull is not seen after lysis. The cortical material from *T. gratilla* contains 17% of the total cell protein, but only 32% of the total gel protein is recovered in this material, which is only about twice as much as would be expected on the basis of a uniform distribution throughout the cell. The gel protein is thus not located primarily in the cortical region and seems not to exist in a concentrated form there. The isolated cortical material of *P. indiana* contains an even smaller fraction of the total gel protein (17%), so this material is clearly not localized there but, as the isolated cortical material contains only 3% of the total cell protein, it is apparent that the gel protein is present in a much more concentrated form near the periphery than in the endoplasm. It can be argued that, as in the case of *C. atratus* and *E. mathei*, the gel protein found in the isolated cortical material originated from the cortical granules, and that this would contribute, in the fertilized egg, to the much reduced hyaline in these species. This can be proven only by an experimental demonstration that the breakdown of the cortical granules at lysis contributes this gel protein to the isolated cortex; such studies are now under way.

The complete localization of this protein in the cortical granules and its subsequent incorporation into the hyaline layer at fertilization seems unlikely in any of these species. It is well known that fertilized eggs stripped of their hyaline by treatment with calcium- and magnesium-free solutions are able to replace at least some of this material, which must have some source within the cell. In addition, there is other evidence, such as the surface precipitation reaction and related phenomena (11), which indicates that a component which gels in the presence of divalent ions is present in the fluid endoplasm of the egg. Further experiments on the partitioning of this protein within the cell are in progress.

The authors wish to express their appreciation to Miss Maudest Milan for her valuable technical assistance.

This investigation was supported by a United States Public Health Service Grant No. GM-14363.

Received for publication 17 July 1968, and in revised form 21 November 1968.

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