ON THE RECONSTITUTION OF THE CRYSTALLINE COMPONENTS OF THE SEA URCHIN FERTILIZATION MEMBRANE

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ABSTRACT

The characteristics of the reconstitution of a crystalline component of the sea urchin fertilization membrane are presented. The reassembly of large aggregates of cylindrical or tubular components is effected by the addition of calcium or other divalent cations. The reassembly requires a slightly alkaline pH and is little affected by increasing ionic strength. Reassembly is strongly inhibited by treatment with reducing agents such as dithiothreitol. The role of this protein in the formation of the fertilization membrane and its possible relation to the calcium-insoluble proteins of the mitotic apparatus are discussed.

INTRODUCTION

A previous report (2) has described the isolation of a crystalline or paracrystalline material released from the cortical granules of the sea urchin *Strongylocentrotus purpuratus* at fertilization. This material was shown to consist of sheets of cylinders or tubules approximately 180 A in diameter. These arrays are identical, ultrastructurally, with the crystalline arrays found by Inoué et al. (8) and Humphreys and Kreutziger (7) on the surfaces of the fertilization membrane by means of freeze-etching. Ito et al. (10) have also described tubular elements in sections of acrolein-fixed fertilization membranes from *Arbacia*. These tubular elements represent two of the structural components of the fertilization membrane which is a tripartite structure (see Austin [1]). The third central component is an amorphous substance, presumably derived from the vitelline coat.

The present work demonstrates that the crystalline material, like the hyaline substance, is precipitable by calcium and other divalent cations. Under the proper conditions, this aggregation results in the reassembly of the tubular elements and their association into large sheets.

MATERIALS AND METHODS

The methods for isolation and partial purification of the crystalline material from the sea urchin *Strongylocentrotus purpuratus* have been described previously (2). The purified material was dissolved by dialysis for 12–24 hr against 10 mM Tris-HCl, pH 7.6, at 4°C. These solutions were then centrifuged at 100,000 *g* for 60–120 min to remove any large aggregated material. Solutions were stored frozen at −20°C until used. No apparent decrease in calcium precipitability has been observed over periods of several months in these preparations.

The extent of precipitation was quantitated be removing the aggregated material by centrifugation (5000 *g* for 10 min or 100,000 *g* for 60 min) and determining the protein concentrations of the resulting supernatants. Protein determinations were done by the method of Lowry et al. (14) with bovine plasma albumin as a standard. Determinations were done on aliquots of the supernatants taken up in 1 N NaOH or on 10% PCA precipitates redissolved in 1 N NaOH.

Reassembly was effected either by addition of small volumes of concentrated calcium chloride solutions or by dialysis against solutions of the specified

calcium concentration; pH experiments were done by dialyzing samples against buffered solutions adjusted to the desired pH and calcium concentration. The buffers used were Tris (Tris[hydroxymethyl]-aminomethane, pH 7–9, Mann Research Labs, Inc., New York) and TES (N-tris-[hydroxymethyl]-methyl-2-aminoethane-sulfonic acid, pH 6–8, Sigma Chemical Co., St. Louis, Mo.). Both buffers were assumed to have negligible calcium binding constants (5). Where necessary, Δ pH-, Δ temperature corrections were applied with use of the values given by Good et al. (5).

A modified Leitz ortholux microscope with rectified-strain-free optics was used to make the polarization observations.

RESULTS

In the ultracentrifuge, the solutions of crystalline material dissolved by dialysis against low salt solutions are polydisperse. At least three major components are present, with uncorrected sedimentation rates of 6, 8.5, and 13S (2). Preliminary evidence from gel electrophoresis of the protein in reducing and denaturing solvents suggests that these components are aggregates of several polypeptide chains. The least molecular weight of this material has been determined in reducing and denaturing solvents and is approximately 33,000–35,000. This value is in agreement with values calculated from amino acid data. A more extensive study of the physical and chemical characteristics of this protein is in progress and will be the subject of a separate report. For the present experiments, the polydisperse solutions have been used in the reconstitution experiments without further fractionation.

Calcium

Addition of calcium to these solutions at protein concentrations of 1–2 mg/ml results in the formation of a precipitate. Within 5 min (with this protein concentration at 0–5°C) the solutions begin to resemble suspensions undergoing a phase separation. The suspended material or precipitating phase takes on a distinct micellar or coacervate-like appearance (Fig. 1 *a*). This material is termed the "early Ca precipitate." (Precipitate is used here to mean something which is large enough to be observed in the light microscope; no inference

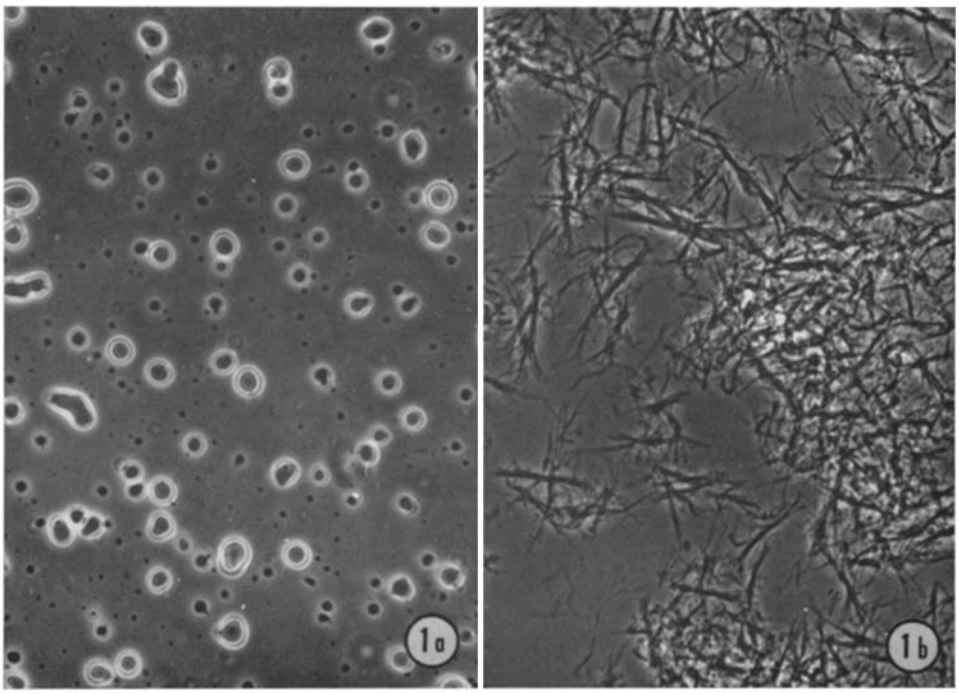

FIGURE 1 Fig. 1 *a* is an early precipitate, approximately 5 min after addition of calcium ($CaCl_2$ to 20 mM. Fig. 1 *b* is the same solution after 60 min. Protein concentration = 1–2 mg/ml, 10 mM Tris-HCl, pH 7.6, 0°C. Phase × 600.

as to structure is intended). Within 60 min (1–2 mg/ml, 0–5°C, pH 7.6, 20 mM $CaCl_2$) this early Ca precipitate has undergone a profound transition to a fibrous form (Fig. 1 *b*). In polarized light the fibers show a strong positive birefringence (Fig. 2). In the electron microscope, negatively stained preparations of this reconstituted material are essentially identical with those of material polymerized in vivo (Fig. 3 *a*). Some difficulty arises with the negative staining of these structures because most of the common negative stains (phosphotungstic acid, molybdate, etc.) have large calcium-binding constants. This leads to the breakdown or partial disassembly of the sheets into individual tubules and eventually to complete dissolution (Fig. 3 *b*).

To quantitate the Ca reconstitution, the precipitates were removed by centrifugation and the protein concentration was determined in the supernatant. Fig. 4 illustrates the results of such an experiment at an initial protein concentration of 1.3 mg/ml. Curve *b* is the extent of formation of large aggregates; the criterion for "solubility" is a 5000 *g* for 10 min centrifugation. Curve *a* is a similar experiment in which a more stringent "solubility" criterion, 100,000 *g* for 60 min, was used to show the formation of smaller aggregates at lower Ca concentrations. In this series of experiments, both curves appear to level off at 80–85%. Redissolution of these precipitates and subsequent reconstitution at the *same* initial protein concentrations gave similar curves and a similar "plateau" value. These reconstitution and redissolution experiments have been carried through four cycles with little observed difference in the extent of precipitation. This would appear to rule out the possibility that the precipitation and reconstitution are actually "purifying" the crystalline components.

The "plateau" value of the per cent precipitation curves could be significantly varied by changing the initial protein concentration; in general, the higher the initial protein concentration, the higher the "plateau" value and the lower the Ca concentration necessary to effect the transition. Regardless of the initial protein concentration, the concentration of soluble protein at a given Ca concentration is a constant value for any given set of conditions (pH, temperature, etc.). This type of relationship is similar to that observed in the solubility of simple inorganic compounds.

Other Divalent Cations

The ability of other alkaline earth metals to precipitate this protein was tested by dialysis of the protein against the given salt followed by a 100,000 *g* for 30 min centrifugation. The results are given in Table I. A constant salt concentration of 20 mM

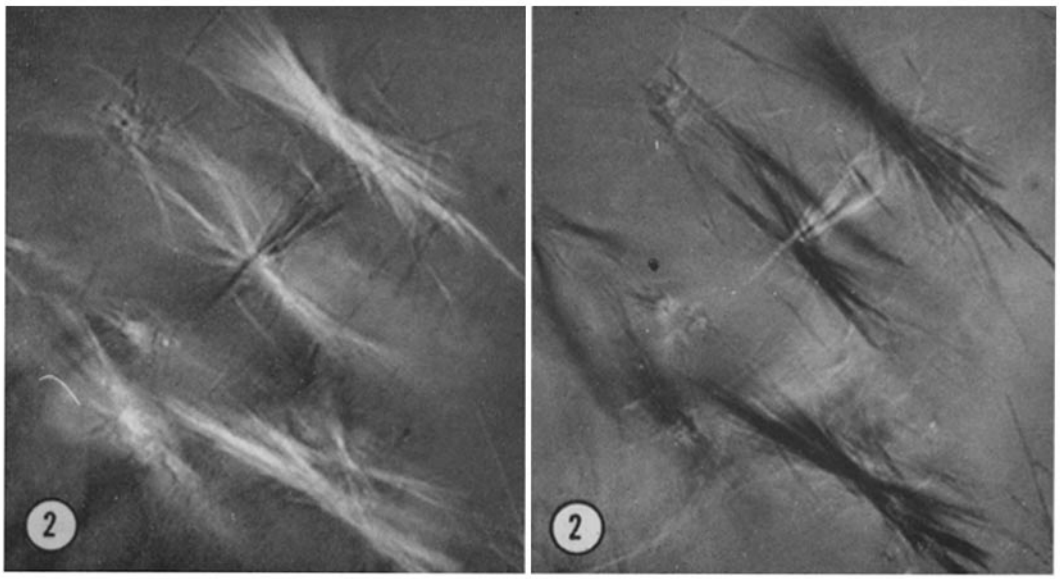

FIGURE 2 Polarization micrographs of reconstituted material at different compensator settings. × 1000.

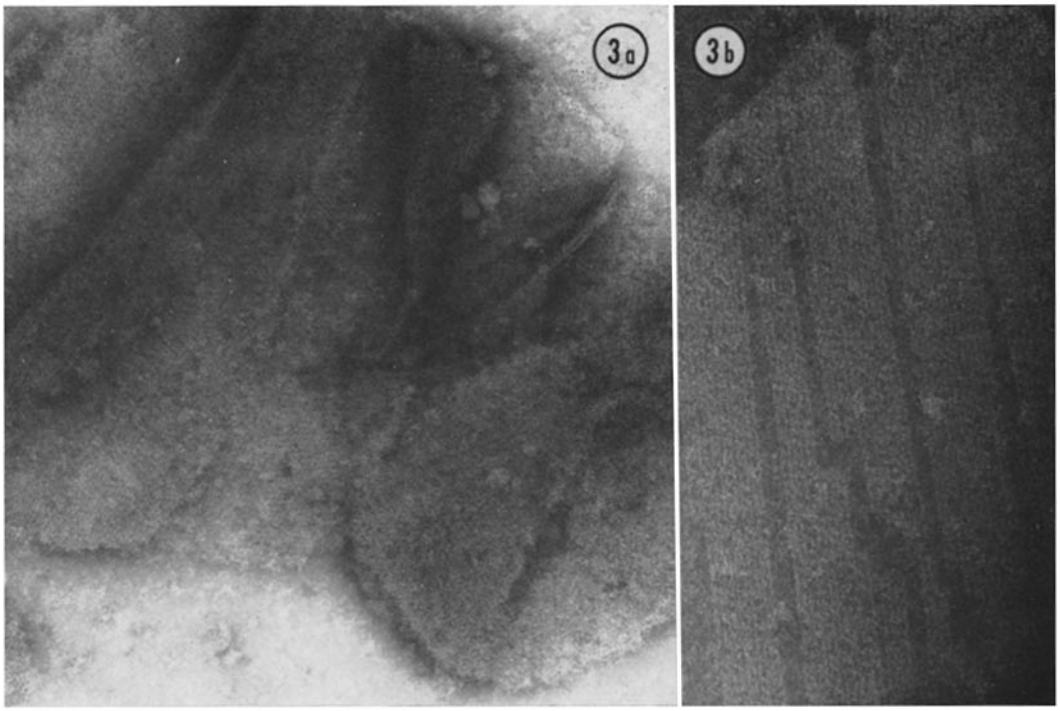

FIGURE 3 Electron micrographs of reconstituted material. Negatively stained with 1% ammonium molybdate, *a*, × 72,000; *b*, × 120,000.

was used throughout. In the case of Ca and Mg, this concentration was in the "plateau" region for these salts at the concentration of protein used. Complete solubility curves for strontium (Sr) and barium (Ba) have not yet been determined.

pH

The effect of pH on the reconstitution of these structures was examined by dialyzing protein samples against solutions with constant Ca concentration at various pH's. The results of these experiments are shown in Fig. 5. The solubility criterion for this experiment was a 5000 *g* for 10 min centrifugation (20 mM Ca—20 mM buffer). Similar results were obtained when the 100,000 *g* for 60 min centrifugation was used. This curve represents, in effect, a phase diagram for the crystalline material. To the left of the dotted zones, the material exists as disordered, amorphous aggregates (Fig. 6 *a*). In the limited region within the dotted zone, the material becomes more structured and rodlike elements are sometimes visible (Fig. 6 *b*). To the right of this region, essentially all the material exists in the fibrous form (Fig. 6 *c*). This entire transition occurs with a pH change of less than 0.3 pH unit. The transition is accompanied by a relatively small increase (15–20%) in precipitability. With increasing pH, particularly above 8.5, there is a pronounced decrease in the aggregate size. Above pH 9, the aggregates become difficult to resolve with the light microscope.

Ionic Strength

The effect of increasing salt concentrations is shown in Fig. 7. With increasing ionic strength there appears to be a very slight decrease in the precipitability of the crystalline material. Once formed in the presence of calcium, the crystalline structures are stable in the presence of high salt (0.5 M KCl), even in the absence of added calcium.

Reducing Agents

In view of the known effect of a variety of reducing agents on the "hardening" of the fertilization membrane, the effects of mercaptoethanol and dithiothreitol (DTT) on Ca precipitability were tested. Protein was incubated with 20 mM DTT, 20 mM Tris-HCl, pH 7.6, for 60–120 min. The

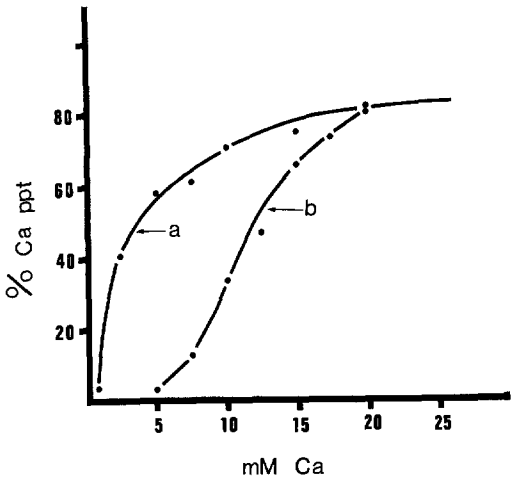

FIGURE 4 Extent of calcium precipitability of the crystalline protein. Curve *a* was obtained from a 10^5 *g* for 60 min centrifugation while curve *b* is from a 5000 *g* for 10 min centrifugation. Initial protein concentration = 1.3 mg/ml, 10 mM Tris-HCl, pH 7.6, 5°C.

DTT was removed by passage through a Sephadex G-100 column (1.5 × 15 cm), and the protein peak was collected and tested for precipitation by addition of calcium. The results are given in Fig. 8. Curve *a* is a control experiment; curve *b* is after preincubation with DTT. Treatment with a sulfhydryl reducing agent profoundly alters the Ca precipitability of the crystalline protein. In 20 mM Ca, a concentration sufficient to polymerize greater than 80% of the untreated material, less than 2% of the reduced protein is affected. At higher Ca concentrations filamentous structures begin to form; the normal crystalline material has not, however, been observed.

Protein Concentrations

Some of the effects of varying protein concentration have been mentioned with regard to solubility; one other effect is quite apparent. At protein concentrations greater than 2–3 mg/ml, the precipitation occurs very rapidly. Under these conditions the formation of micelles or coacervates is quite abbreviated. This leads to the formation of small aggregates instead of the larger crystalline arrays. Similar effects were observed with Ca concentrations greater than 30–35 mM.

DISCUSSION

The first visible events after insemination of an echinoderm ovum are the changes associated with the formation of the fertilization membrane. These changes have been reviewed in detail by several authors (1, 4, 19, 21). In brief they involve the elevation of the vitelline coat, the release of the contents of the cortical granules, and the subsequent formation of the "hardened" fertilization membrane. Epel et al. (4) have recently correlated these events with a decrease in pH and an increase in light scattering in egg suspensions. The decrease in pH has been attributed to a release of acidic polysaccharides by Ishihara (9). Endo (3) and others (21, 24) have demonstrated the incorporation of material from the cortical granules into the fertilization membrane. The evidence identifying the present crystalline material with the structural elements in the fertilization membrane has been presented (2).

TABLE I

Salt*	Ca ppt
	%
$MgCl_2$	63
$CaCl_2$	79
$SrCl_2$	74
$BaCl_2$	70

* 20 mM salt, 10 mM Tris-HCl, pH 7.6, 5°C

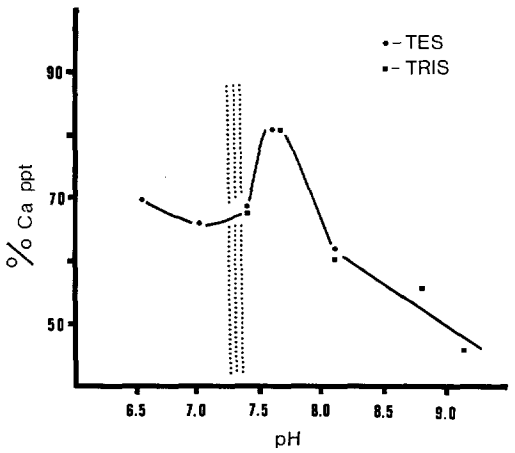

FIGURE 5 Effect of pH on calcium precipitability. The criteria for solubility is a 5000 *g* for 10 min centrifugation, calcium concentration = 20 mM ($CaCl_2$), buffer concentrations = 20 mM, 5°C, initial protein concentration = 1–2 mg/ml. The dotted region is the transition zone between amorphous and crystalline aggregates.

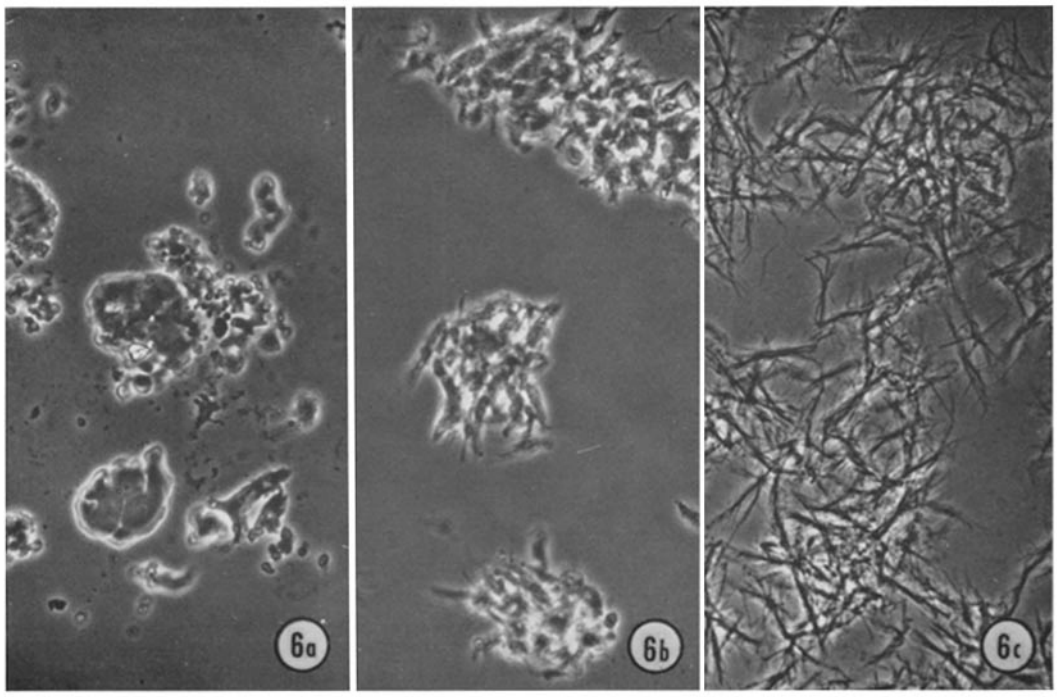

FIGURE 6 *a*, Amorphous material, pH 6.7; *b*, Material in the transition zone, pH 7.3; *c*, Crystalline material, pH 7.5. Phase × 700.

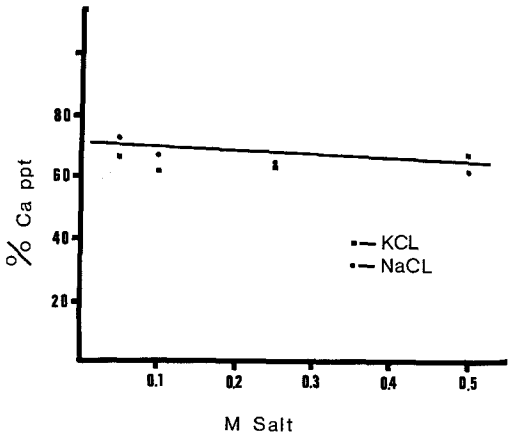

FIGURE 7 Effect of ionic strength on calcium precipitability. The criteria for solubility is a 5000 *g* for 10 min centrifugation. Calcium concentration ($CaCl_2$) = 20 mM, 10 mM Tris-HCl, pH 7.5.

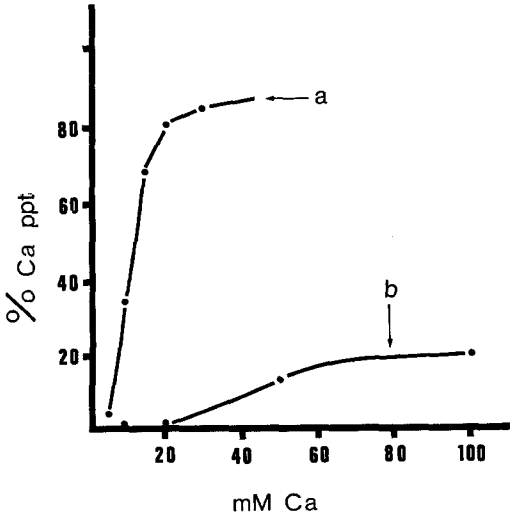

FIGURE 8 Effect of an -SH reducing agent. Curve *a* is a control experiment; curve *b* is after preincubation with 20 mM DTT for 60 min. 20 mM Tris-HCl, pH 7.6. The solubility criterion is a 5000 *g* for 10 min centrifugation.

The reconstitution experiments serve to demonstrate that the requirements for polymerization are very similar to the sea water conditions in which in vivo membrane formation takes place. Sea water contains Ca and Mg at approximately 10 mM and

50 mM, respectively. The in vitro experiments indicate that these conditions would provide a strong driving force for the formation of the crystalline elements of the fertilization membrane, particularly since the local protein concentration within the perivitelline space is likely to be high. Although the polymerization appears to be primarily an ionic effect, the high concentrations of NaCl present in sea water do not appear to interfere with the formation process.

A number of parameters remain to be defined in the in vitro polymerization, particularly those relating to nucleation and the stoichiometry of Ca binding. The question of nucleation has not been directly approached although the requirement for relatively large aggregates is ruled out by the 100,000 *g* precentrifugation. Two possibilities seem likely; either the higher molecular weight components (13S) of the protein solutions serve as nuclei or the initial micelle formation raises the local protein concentration sufficiently so that the system is self nucleating.

A complete study of the Ca-binding properties of this material is in progress, but some preliminary information on the stoichiometry is available from the precipitation and solubility data. The protein which remains soluble at various Ca concentrations appears to follow a solubility product relationship of the form:

$$K_{sp} = (\text{protein} - \text{Ca}_m)\ (\text{Ca})^n$$

where K_{sp} is the solubility product constant, protein $- Ca_m$ is the calcium proteinate in solution which takes part in precipitate formation (i.e. the soluble protein), and n is the additional calcium interaction required for precipitation. The calcium concentration for initial precipitation is determined by both protein $- Ca_m$ and n, while the shape of the solubility curve is determined by n. A preliminary inspection of the low speed data gives a $K_{sp} = 250$ (using molar concentrations, not activities, and assuming a molecular weight of 33,000) and $n = 1$. These values are for 10 mM Tris-HCl, pH 7.6. From the pH experiments, the K_{sp} would be expected to vary with pH and presumably other environmental conditions. The value of $n = 1$ suggests that one additional calcium molecule is necessary for addition of the polymerizing unit (assumed as the 33,000 molecular weight subunit for purpose of calculating K_{sp}) into the crystalline structure. The total number of calcium molecules bound, the possibility of cross-linking, and the exact molecular weight of the polymerizing subunit are currently under study.

The other common calcium-precipitable proteins are difficult to compare directly with the crystalline protein. Casein, which forms micelles rather than "ordered" structures, consists of a number of separable proteins (see McKenzie [17] for a review). The hyaline substance, which is distinct from the crystalline material in the ultracentrifuge, gels into amorphous material. The hyaline substance and the crystalline material do, however, have very similar calcium solubility properties (11, 12). Actin, one of the most throughly studied structural proteins, will polymerize from dilute salt solutions upon addition of Ca or Mg; this polymerization, however, is greatly accentuated by the addition of KCl. Stephens (23) has reported similar findings for the repolymerization of Sarkosyl-dissolved microtubule proteins from the outer fiber doublets of sea urchin sperm.

The present results on the reconstitution of the crystalline protein agree with a number of empirical findings on the nature of the formation of the fertilization membrane. These results come not from studies on the membrane per se, but rather from attempts to obtain demembranated eggs for use in other work, notably the isolation of the mitotic apparatus. Although a great deal of variability exists between species, the use of low ionic strength, calcium-magnesium-free conditions immediately after fertilization usually prevents membrane formation. In some species (*Urechis capo*) the additional use of a chelating agent (EDTA) is required (W. Silva, 1969, personal communication). In others, including *Strongylocentrotus purpuratus*, divalent cations must be removed and a reducing agent added to prevent membrane hardening (16). Monroy (19) has discussed further evidence for the involvement of the oxidation of —SH groups in the membrane hardening process. In general, conditions which will solubilize the crystalline material will prevent membrane formation and subsequent hardening. It should be noted, however, that once the membrane has undergone the hardening process, the use of low ionic strength conditions and reducing agents do not effect dissolution of the formed membrane. Clearly some other processes, besides –SH oxidation and Ca binding, must be involved in membrane hardening.

The packaging of the crystalline material in the cortical granules is of some interest. The pH de-

crease upon the release of material from the cortical granules could be accounted for by at least two mechanisms. One of these is an ion-exchange mechanism, whereby the protein is stored in a protonated form which upon release into the perivitelline space binds calcium with the release of protons. Mehl and Swann (18) have invoked just such an ion-exchange model to explain data obtained by titrating egg suspensions. An alternative model would be to package the protein as a calcium proteinate in an acid environment; upon release into the perivitelline space, the acid is diluted and polymerization could then occur. The present results are consistent with either model but do not differentiate between them.

The relationship of the crystalline "membrane" protein to the other water-soluble, calcium-insoluble proteins which have been isolated from echinoderm eggs remains somewhat obscure. Some information is provided by the work of Yazaki (25). Yazaki has shown that the calcium precipitate (Ca-ppt 1) of a water extract of eggs can be separated into two fractions by dialysis against 0.01 M Tris-HCl, pH 7.0. The fraction which dissolves readily is termed HyS and is identical to the material studied by Kane and Hersh (11). HyS has been shown to be distinct from the crystalline protein (2). The insoluble fraction (Ca-ppt 2) undergoes a cyclical change in the oxidation state of its —SH groups during the cell cycle and is presumed to be the "—SH active" protein(s) in the Ca-precipitable fraction described by Sakai (22). Sakai's protein has been obtained from isolated mitotic apparatus and has been tentatively identified as a microtubule protein (13).

Mazia (15) has proposed that the —SS— dimer of Sakai's protein is the unit which assembles into microtubular structures in the mitotic apparatus. This proposal is in excellent agreement with the present findings which indicate that the oxidized form of the crystalline protein will reassemble, while the reduced form will not. Several pieces of indirect evidence; the Ca insolubility, the reduction by DTT, and a preliminary investigation of the molecular weights point to the conclusion that crystalline material from the fertilization membrane is very similar to the protein isolated by Sakai from the mitotic apparatus. This conclusion is further strengthened by the observations of Harris (6) and Roth and Jenkins (20) that the presence of divalent cations, particularly Ca and Mg, are necessary for the fixation of the microtubules of the spindle fibers at alkaline pH.

Although the low salt solubility properties of the crystalline protein and Ca-ppt 2 appear to be different, it is interesting to note that when Yazaki made fluorescent labeled antibodies against the water-soluble fraction and absorbed with HyS, the remaining antibodies localized on the fertilization membrane. While the water-soluble fraction would contain Ca-ppt 2 in a soluble form, and it is again tempting to speculate on the possible identity of the crystalline material with Ca-ppt 2, clearly other fertilization membrane antigens, including the vitelline substance, could account for this localization. Work is currently in progress to more directly compare the Ca-precipitable proteins from the mitotic apparatus with the crystalline protein.

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