# Studies on the Mitotic Apparatus of the Sea Urchin by Means of Antigen-Antibody Reactions in Agar

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(Received for publication, August 3, 1959)

ABSTRACT

The primary purpose of the experiments reported in this paper was to gain information on the molecular origin of the mitotic apparatus. Antisera were prepared against unfertilized sea urchin (*Strongylocentrotus purpuratus*) egg antigens and mitotic apparatus antigens. These were permitted to react with various antigen solutions in Ouchterlony agar gel diffusion plates, and the resultant precipitation patterns analysed. The results revealed that the mitotic apparatus contains probably no more than two antigens (precursor-1 component and precursor-2 component) and that these are shared by the unfertilized egg. Absorption and fractionation techniques indicated that in the unfertilized egg the precursor-1 component is present both as a "soluble" protein and as an insoluble form tenaciously associated with intracellular structural elements. A survey of dividing and non-dividing tissues for the precursor-1 component revealed that it was restricted to tissues in which mitotic activity could be detected microscopically.

No immunochemical relationship could be detected between the mitotic apparatus and proteins extracted, by various methods, from the lantern muscle.

## INTRODUCTION

Mazia and Dan (1952) reported the first successful mass isolation of the mitotic apparatus from sea urchin eggs entering the first cleavage division. The term, the mitotic apparatus, which they applied to the division figure as it appeared in isolation, includes the asters and centrioles, the spindle, and the chromosomes. The existence of a method for isolating intact mitotic apparatus from cells permitted a direct experimental study of the molecular origin of this structure (for preliminary report see Went, 1959).

Two basically different modes of origin of the mitotic apparatus were considered. The first of these is the *de novo* synthesis of the mitotic apparatus from very small non-specific units, such as amino acids or small polypeptides. The second envisages its formation as the result of the assembly of preexisting molecular subunits (precursor molecules) present in the unfertilized egg which undergo little or no modification in structure during their incorporation into the definitive mitotic apparatus.

In its broadest sense the precursor concept of the origin of the mitotic apparatus is not new, having been postulated with a variety of ramifications by some of the early cytologists. In connection with the origin of the achromatic figure Boveri (1887) felt that it remained only to correlate temporally the established phases of nuclear transformations with the individual stages of archoplasmic transformation. The archoplasm is a fibrillar component of the cytoplasm. By means of the picric-acetic acid reaction he could detect a difference in composition between the archoplasm and other cell substances. He was of the opinion that the entire achromatic figure originated in the cytoplasm (*Zellsubstanz*). Wilson (1925) pointed out that the amphiaster was considered by Klein (1878) and van Beneden (1883) to arise from a radial regrouping of preexisting protoplasmic reticulum about two "centers of attraction." Boveri modified this hypothesis by saying that the formation of the amphiaster was not merely a regrouping of the reticulum, but a new formation arising from a specific granular substance which

collected around central structures and then differentiated into astral rays and spindle fibers.

The existence of a mitotic apparatus precursor at the molecular level is implied in more recent work. Pease (1941) has been able to cause the disappearance of the spindle under the influence of high hydrostatic pressure, and Anderson (1956) points out that molecules held together by ionic forces tend to be dissociated by high pressure. Heilbrunn and Wilson (1955) indicated that different agents (isotonic KCl, heat, cold, acid, alkali, ether, and UV), which can bring about the initiation of cell division in *Chaetopterus* eggs, all caused an increase in viscosity of the interior protoplasm.

Swann (1958) presents an interesting discussion on the possible relationships between the mitotic apparatus protein and division.

The most fruitful experimental approach appeared to be that of identifying the protein of isolated mitotic apparatus with a corresponding protein in cells that had not yet entered division. Specifically, the Ouchterlony gel diffusion method (Ouchterlony, 1949; Oudin, 1952; Ouchterlony, 1958) turned out to be the most powerful tool used to gain information about the molecular origin of the mitotic apparatus.

## *Materials and Methods*

*Obtaining Gametes.*—Most of the material used in the investigation was obtained from the eggs of the sea urchin *Strongylocentrotus purpuratus.* The urchins were induced to shed their gametes by injecting 2 to 3 ml. of 0.5 M KCl into the perivisceral coelom (Tyler, 1949). The gametes were collected at 6°C. The eggs were washed before use, but the sperm could be used directly to make the suspensions suitable for inseminating egg suspensions.

*Ethanol Preservation of Material.*—Unfertilized eggs and embryos were routinely preserved in about 30 per cent ethanol (*v/v*) at −10°C. The washed eggs or embryos to be preserved were concentrated by gentle centrifugation and the supernatant sea water decanted, leaving just enough in the centrifuge tube to resuspend the material. Into this thick suspension was rapidly poured a 10 to 20 volume excess of 30 per cent ethanol prechilled to −10°C. The material could then be stored at −10°C. until needed.

Other *S. purpuratus* tissue, *S. franciscanus* eggs, and *Lytechinus* egg were similarly preserved in cold ethanol. The larger pieces of tissue were cut into small fragments before mixing with the cold ethanol.

*Isolation of Mitotic Apparatus from S. purpuratus Material:*

*1. From Ethanol-Preserved Material.*—In general, the procedure described by Mazia and Roslansky (1956) and Rustad (1959) was followed.

*2. Directly from Living Material.*—A more recent technique developed by Mazia and collaborators permits the isolation of mitotic apparatus directly from living material. Its success depends upon the action of the S-S compound dithiodiglycol (DTDG) to stabilize the division figure (Mazia, 1958, 1959). Only the basic principles of the procedure will be presented here. The fertilization membranes were removed from the fertilized eggs by a combination of chemical and physical treatments after which they were incubated in calcium-free sea water. At the desired stage of the first cleavage division the embryos were concentrated and shaken briefly in hypotonic medium containing DTDG and versene (EDTA). This exploded the cells, uniformly dispersing the cytoplasm and liberating intact mitotic apparatus. The medium was then made isotonic by adding an equal volume of approximately 2 M dextrose containing DTDG and versene in the same concentration as the hypotonic medium. A fine mesh sieve was used to separate the mitotic apparatus from the unwanted cytoplasmic particles.

*Dissolving the Isolated Mitotic Apparatus.*—Solutions of mitotic apparatus dissolved by three different techniques have been used in various experiments. (1) Digitonin-isolated mitotic apparatus suspended in distilled water have been dissolved by adding dilute NaOH until pH 10.5 was reached, at which point no mitotic apparatus were detectable by phase contrast microscopy. The solution was then centrifuged in a Spinco model L ultracentrifuge at 100,000 *g* for 5 to 7 minutes to remove undissolved cytoplasmic debris. The supernatant was dialysed against distilled water at pH 7.5 and represented the dissolved mitotic apparatus used in the experiments. (2) Digitonin-isolated mitotic apparatus have also been dissolved in 0.05 M mersalyl at pH 9 according to a procedure developed by Zimmerman (1958). (3) Mitotic apparatus isolated directly from living material have been dissolved in water at pH 8.5–9.3.

*Preparing Solutions for Injection.*—Three principal protein solutions were used for the immunization of the rabbits. One was digitonin-isolated mitotic apparatus dissolved at pH 10.5 which gave rise to the anti-DMA serum.

The second represented soluble proteins in a 0.1 M KCl extract of ethanol-preserved unfertilized eggs. The eggs were centrifuged at −10°C. to remove the ethanol and extracted in 5 to 10 volumes of 0.1 M KCl at 0–6°C. for several hours. No buffer was added and the final pH was usually about 6.7. The suspension was then centrifuged at about 140,000 *g* for 35 minutes and the supernatant represented the 0.1 M KCl extract of unfertilized eggs. When $CaCl_2$ to a final concentration of 0.05 M was added to the supernatant, there appeared a fibrous-protein precipitate termed the calcium-

insoluble fraction. This protein, whose properties were described by Kane and Hersh (1959), was put into solution by dialysis against distilled water. With rigorous "purification" it was possible to prepare a solution of this protein which was immunochemically homogeneous. In practice, however, the solutions of calcium-insoluble fraction injected were not immunochemically homogeneous and the crude preparations proved to be convenient carriers for the major antigens of interest. For this reason the antiserum prepared against solutions of the fraction will be referred to as the "antiserum to unfertilized egg antigens."

The third protein solution used was prepared from mitotic apparatus isolated directly from living material. The homologous antiserum will be called the anti-FMA serum.

*Extraction Method.*—Extracts were prepared by exposing gently homogenized, ethanol-preserved cells and tissues to the desired extraction medium (*i.e.*, 0.1 M KCl, 0.6 M KCl, distilled water, etc.) for several hours and centrifuging at 140,000 *g* for 35 minutes. The pellet was discarded and the supernatant retained for immunochemical analyses. Unless otherwise stated, the protein concentration of the various solutions was not known.

*Immunochemical Procedures:*

*Obtaining Antiserum.*—Rabbits were used as the antibody formers and were immunized by three intravenous injections at 1 week intervals. The antigen-protein concentration varied from 5 to 15 mg. per injection (1 to 2 ml.). The rabbits were bled 5 days to 1 week after the final injection. No trial bleedings were performed. The antiserum was separated from the clot, passed through a membrane filter (millipore; HA) into sterile serum bottles, and stored at −10°C. The antisera were used undiluted.

*Preparing Ouchterlony Plates.*—The Ouchterlony method used was basically that described by Oudin (1952) and Ouchterlony (1958). The Ouchterlony plates were prepared on lantern slide cover glasses and the agar was about 4 to 5 mm. thick. After the agar had solidified, it was covered with the lid of a 2 inch petri dish and placed in the refrigerator. The best results were obtained with plates which had been left uncovered in the refrigerator for several hours prior to use. The reactants were introduced into the wells with medicine droppers, the petri dish cover sealed into position with vaseline and incubated for the desired length of time (15 to 72 hours) at room temperature (21–26°C.) in an insulated box. The wells were usually "topped off" 1 to 4 hours after the initial filling.

The concentration of the reactants was usually not known, but was usually in the range from 10 to 20 mg. protein per ml. except for solutions of mitotic apparatus which were never more concentrated than 5 mg. protein per ml.

When nothing was known about the immunochemical behavior of a particular antigen solution, it was tested at three different concentrations (undiluted, 1:5 dilution, and 1:10 dilution) to reduce the possibility of not detecting some antigenic component in the solution.

The precipitation band patterns were reproduced either by placing the plate in an Omega enlarger at the level where the negative is normally placed and projecting the image on high contrast paper, or by photographing the plate from above.

*Absorption Procedures.*—Experiments were also performed which necessitated absorbing antiserum with the particulate fraction from sea urchin eggs and tissues. The particulate fraction is here defined arbitrarily as everything in a homogenate that could be sedimented in 35 minutes at 140,000 *g*. Before this fraction was used to absorb the antiserum, it was exposed to various extraction media. In general, this was performed as follows: Ethanol-preserved material was homogenized in a glass piston-type homogenizer (with a loosely fitting piston) in about 10 volumes of the desired extraction medium. After extraction in the cold, the suspension was centrifuged for 30 to 35 minutes at 140,000 *g* and the supernatant was discarded. The particulate fraction was reextracted and centrifuged as before. Next it was washed three times in cold saline, centrifuging at 140,000 *g* for 30 to 35 minutes after each wash. The particulate fraction was then used for absorbing the antiserum. The latter was tested for the presence or absence of specific antibodies by the Ouchterlony gel diffusion method.

*Effect of pH upon the Precursor-1 Component and the Precursor-2 Component.*—Samples of 0.1 M KCl extracts of unfertilized eggs (pH 7.3) were each initially adjusted to a different pH (ranging from 8.4–10.7) with NaOH. About 15 to 20 minutes later the pH of each solution was readjusted. This was necessary because the pH of those solutions initially adjusted to the highest pH values had dropped by 0.5 to 0.8 pH units during this time. They were next dialyzed for several hours against saline containing phosphate buffer at pH 6.5. The final pH of the dialysate was 6.7. The solutions were then made to react with anti-DMA and anti-FMA sera in Ouchterlony gel diffusion plates (Fig. 3).

## EXPERIMENTAL RESULTS AND DISCUSSION

### *Evidence for the Presence of a "Precursor" Component:*

*Results.*—In Fig. 1 can be seen three Ouchterlony plates that summarize the results of an immunochemical comparison between dissolved mitotic apparatus solutions and antigens obtained from unfertilized eggs. Antiserum prepared against unfertilized egg antigens (*ANTI EGG*) was placed in the top well of each plate. In Fig. 1 *A* the other two wells were filled with a care-

fully purified preparation of the calcium-insoluble fraction (*CIF*) and the supernatant to this fraction (*SUP*). These represent proteins from unfertilized eggs. There are three bands (the P, I, and C bands) assignable to the supernatant, and only one (the C band) to the calcium-insoluble fraction. The C bands were seen to fuse (interfere) with each other in the original Ouchterlony plate, but this relationship has become lost in subsequent photographic procedures. The I band is actually a composite of two or three separate bands, none of which was seen to fuse with the C band. In Fig. 1 *B* a solution of digitonin-isolated mitotic apparatus dissolved at pH 10.5 (*DMA*) was compared with the supernatant (*SUP*) from the calcium-insoluble fraction. The dissolved mitotic apparatus gave rise to only one band which clearly fuses with the P band assignable to the superantant fraction. The "halo" around the well containing dissolved mitotic apparatus appears to be an infrequent non-specific reaction in the agar. Fig. 1 *C* compares a solution of digitonin-isolated mitotic apparatus (*DMA*) with a solution of dissolved mitotic apparatus isolated directly from living material (*FMA*). Each solution gave rise to only the single band, the P band, and these can be seen to fuse. The antigen responsible for the P band has been termed the precursor-1 component of the mitotic apparatus. When reacted with antiserum to unfertilized egg antigens and anti-DMA serum, digitonin-isolated mitotic apparatus dissolved in mersalyl resulted in a solution immunochemically indistinguishable from the other mitotic apparatus solutions.

*Discussion.*—These data are in agreement with the concept that the mitotic apparatus can arise through the assembly of preexisting molecular subunits. They reveal that there occurs in the unfertilized sea urchin egg an antigen (the precursor-1 component) immunochemically identical to an antigen invariably present in all solutions of dissolved mitotic apparatus. It is important to stress that the antiserum used to establish this identity was prepared against unfertilized egg antigens.

The reasons for using the calcium-insoluble fraction for immunization, rather than isolated mitotic apparatus in some form, should be mentioned. Physical characterization of this fraction indicated a strong similarity to the mitotic apparatus protein (Kane and Hersh, 1959). Thus, it was speculated that the structural involvement of the calcium-insoluble fraction in the mitotic apparatus was possible (Mazia, 1957). With very rigorous purification it had been possible to obtain solutions of the calcium-insoluble fraction that yielded only a single band in gel diffusion studies (Fig. 1 *A*). In practice, however, the solutions used in the immunization of the rabbits were known to contain other unfertilized egg antigens. This can account for the spectrum of antibodies in the homologous antiserum (antiserum to unfertilized egg antigens). The heterogeneous nature of this antiserum disclosed that the calcium-insoluble fraction bore no simple structural relationship to the mitotic apparatus, while revealing the presence of the precursor-1 component in the unfertilized egg.

*Experiments with Antiserum to Dissolved Digitonin-Isolated Mitotic Apparatus (Anti-DMA Serum):*

*Results.*—Fig. 2 summarizes in one Ouchterlony plate the evidence for the existence of the precursor-1 component in unfertilized eggs. Antiserum to unfertilized egg antigens (*ANTI EGG*) reacted with dissolved mitotic apparatus (*DMA*) to yield one band which fused with one of the numerous bands that resulted when it reacted with a solution of unfertilized egg antigens. The reaction of the anti-DMA serum (*ANTI DMA*)with dissolved mitotic apparatus also yielded only one band which fused with one of two formed in its reaction with unfertilized egg antigens. The anti-DMA serum reacted with unfertilized egg antigens to yield two bands, but only one resulted from the reaction with dissolved mitotic apparatus. This is interpreted as indicating that the immunizing solutions of dissolved mitotic apparatus contained the second antigen in quantities too small to be detected by gel diffusion methods, but large enough to elicit the formation of antibodies.

*Discussion.*—The anti-DMA serum has shown that the mitotic apparatus may be considered to be antigenically a relatively simple system which appears to contain no antigens not shared by the unfertilized egg. The precursor-1 component is definitely established as an important structural entity of the mitotic apparatus. The anti-DMA serum reacts with some other unfertilized egg antigen whose presence in the immunizing solutions is clearly indicated by this behavior.

Although only a single anti-mitotic-apparatus serum (anti-DMA) had been used in the experiments reported so far, eight other antisera were developed against antigens obtained from unfertilized eggs. These antisera all reacted with the precursor-1 component to form the P band, but some qualitative differences in behavior toward other antigens were observed. The band patterns were highly reproducible and predictable when known antigen and antiserum systems were used.

The P band was the first to appear during the incubation period and was invariably the closest band to the well containing the antiserum. These

facts made it possible to identify with great accuracy the P band, without the aid of reference solutions. In all cases of slightest doubt, the experiment was repeated using a reference solution of dissolved mitotic apparatus.

*Evidence for a Second Component Associated with Dissolved Mitotic Apparatus:*

*Results.*—After the existence of the precursor-1 component had been clearly established, a technique for isolating the mitotic apparatus directly from living material was developed by Mazia and collaborators. When solutions of mitotic apparatus, isolated by this procedure, were made to react with antiserum to unfertilized egg antigens (*ANTI EGG*) and anti-DMA serum, a second antigenic component was consistently observed in addition to the precursor-1 component (see Fig. 8, arrow 2). This second component was shared by the unfertilized egg and will be referred to as the precursor-2 component. It was infrequently detected in solutions of mitotic apparatus isolated by the digitonin method. However, it could always be detected in solutions of mitotic apparatus isolated directly from living material. On the other hand, when anti-FMA serum (antiserum against mitotic apparatus isolated directly from living eggs) was made to react with solutions of dissolved mitotic apparatus and unfertilized egg antigens, a single band was observed in each case, but it was not the P band. The band observed was identified with the precursor-2 component. So, although the immunizing solutions were known to contain the precursor-1 component in addition to the precursor-2 component, the rabbit had directed all its antibody-forming capacity towards the latter component. What had happened to the precursor-2 component in solutions of digitonin-isolated mitotic apparatus in which it could not be detected? A readily testable hypothesis was that its reactive properties had been modified by the elevated pH (pH 10.5) at which digitonin-isolated mitotic apparatus are put into solution, while they are relatively unaffected at pH 8.9–9.5, which is the range used for dissolving mitotic apparatus isolated from living material. Accordingly, aliquots of 0.1 M KCl extracts of unfertilized eggs were adjusted briefly to pH 8.4, 9.1, 10.0, 10.5, and 10.7, returned approximately to neutrality, and made to react with anti-DMA (Fig. 3 *A*) and anti-FMA (Fig. 3 *B*) sera. We see in Fig. 3 *A* a P band associated with each well indicating that the combining effectiveness of the precursor-1 component is not greatly affected by the pH values tested; however, the antigen (the precursor-2 component) responsible for the reaction with the anti-FMA serum (Fig. 3 *B*) was very sensitive to elevated pH. The ability of the precursor-2 component to react with the antiserum could be destroyed by exposure to elevated pH in the range between 9.1 and 10.0. The digitonin-isolated mitotic apparatus can be demonstrated to contain the precursor-2 component.

*Discussion.*—The difference in behavior between the anti-DMA serum and the anti-FMA serum can be explained on the basis of the experiment just described. It shows that the combining effectiveness of the precursor-2 component can be nearly completely destroyed by the conditions used to put digitonin-isolated mitotic apparatus into solution, while it is unaffected by the procedure employed for dissolving mitotic apparatus isolated from living material.

On the basis of the limited experimental evidence, the precursor-2 component should be considered as an important structural unit of the mitotic apparatus. No modification of the precursor concept is required, for the precursor-2 component is shared by the unfertilized egg.

While these experiments present a strong case for the precursor origin of the mitotic apparatus, independent evidence must be forthcoming before the existence of a molecular "precursor" can be placed on a firmer basis. It remains to be shown that the two precursor components of the unfertilized egg are actually incorporated into the definitive mitotic apparatus with little or no modification in configuration. This will have to await elucidation of the metabolism of these components.

*Intracellular Distribution of the Precursor-1 Component:*

*Results.*—The unfertilized egg antigens studied thus far were present in the soluble fraction of a 0.1 M KCl extract of ethanol-preserved material. This gave no basis for any conclusions concerning the intracellular distribution of the precursor-1 component. It was known that cytoplasmic particles of sea urchin eggs behave as osmometers (Harris, 1943) and the possibility that the precursor-1 component may be wholly confined within them was investigated. Living eggs were gently homogenized and extracted in isotonic electrolyte and non-electrolyte. Ethanol-preserved eggs extracted in an identical manner provided control solutions. The soluble fractions of each extract were made to react with antiserum to unfertilized egg antigens. The resultant gel diffusion patterns can be seen in Fig. 4. The wells of both *A* and *B* were filled as follows: isotonic sucrose extract of living eggs (well *1*); isotonic KCl extract of living eggs (well *2*); isotonic sucrose extract of preserved eggs (well *3*); and isotonic KCl extract of preserved eggs (well *4*). The antiserum was placed in the center well. The antigen concentration in each well of Fig. 4 *A* was one-fifth that of the corre-

sponding well in Fig. 4 *B*. The photograph in Fig. 4 *A* was taken after 18 hours incubation, while the plate in Fig. 4 *B* was incubated for 42 hours. This was necessary in order to show the P band to best advantage, since its appearance is more sensitive to variation in antigen concentration and duration of incubation than any of the other bands. In Fig. 4 *A* there is a P band associated with every well, indicating the presence of the precursor-1 component in each extract. Fig. 4 *B* shows the appearance of the other bands. All that remains of the P bands in the latter is the diffuse horseshoe-shaped pattern around the center well, although at a much earlier stage of incubation they were as distinct and well defined as in Fig. 4 *A*. The band pattern in Fig. 4 *B* is clearly influenced by the source of the extracts and the extraction medium. The C bands, clearly visible in association with wells *3* and *4*, appear to be lacking in the patterns assignable to wells *1* and *2*. However, subsequent experiments unambiguously established the presence of the calcium-insoluble fraction, the antigen responsible for the C band, in the extracts of living eggs.

It is evident from the above that the precursor-1 component may be present in a "soluble" form in the unfertilized sea urchin egg, but the data permit no decision about the possibility that it may exist in an insoluble form associated with the particulate fraction (defined earlier). Samples of the particulate fraction were each subjected to a different extraction medium and each used to absorb an aliquot of antiserum containing antibodies to the precursor-1 component. Fig. 5 summarizes the absorption data. The samples of particulate fraction were extracted with unbuffered KCl solutions of different concentrations—0.1 M (well *1*), 0.5 M (well *2*), 1 M (well *3*), and 2 M (well *4*)—and then used to absorb aliquots of antiserum prepared against unfertilized egg antigens. In Fig. 5 *A* the center well was filled with the soluble fraction of a 0.1 M KCl extract of unfertilized eggs while that of Fig. 5 *B* was filled with digitonin-isolated mitotic apparatus dissolved in dilute alkali. Well *5* contained unabsorbed antiserum. It should be emphasized that the antisera occupied the peripheral wells in Figs. 5 *A* and *B* instead of their usual position in the center wells. We will first consider the situation with the unabsorbed antiserum. In Fig. 5 *A* the P band is no longer visible owing to the length of the incubation (45 hours); however, it was clearly visible earlier. Two other bands are distinctly visible. In Fig. 5 *B* the P band is readily visible. In sharp contrast to these results, in neither plate at any time during the period of observation was there any trace of a P band in association with wells containing absorbed antiserum.

*Discussion.*—The data presented above demonstrate the presence of an insoluble precursor-1 component tenaciously associated with the particulate fraction of the unfertilized egg and a "soluble" form always present in the supernatant of a 0.1 M KCl extract of the material. The insoluble form was detected by the ability of the particulate fraction, when incubated with antiserum, to remove from the antiserum the antibodies that combine with the precursor-1 component to form the P band. This is a specific immunological manifestation and not the result of non-specific absorption of the antibodies to the particulate fraction. The evidence for this was found in another absorption experiment (not described) in which one of the samples of antiserum to unfertilized egg antigens, that had been absorbed with an aliquot of extracted particulate fraction, could still react with the precursor-1 component while the other absorbed antisera could not.

By extracting living, unfertilized eggs briefly (for 1 hour) with isotonic sucrose (0.95 M) and isotonic KCl (0.5 M) solutions and testing the extracts for the presence of the precursor-1 component by gel diffusion methods, it was learned that the soluble form was not restricted in distribution to cytoplasmic particles.

The following experimentally established facts emerge in connection with the qualitative intracellular distribution of the precursor-1 component: (1) it is found as an insoluble antigen rather firmly attached to the particulate fraction; (2) it occurs in a "soluble" form in unfertilized eggs; and (3) it is invariably observed in solutions of dissolved mitotic apparatus. It is tempting to speculate on the possible interrelationships of the three "states" of the precursor-1 to each other and their over-all significance to the assembly of the definitive mitotic apparatus. Ultracentrifugal experiments (Kane and Hersh, 1959) on the soluble fraction of developing *Arbacia* eggs disclosed the diminution and ultimate disappearance, at metaphase of the first cleavage division, of a specific component. It reappeared after cleavage. The tentative interpretation was that it represented the structural protein incorporated into the mitotic apparatus (Mazia, 1957). The same experiment performed on *S. purpuratus* failed to yield a similar relationship. In their experiments, Kane and Hersh took no precautions to prevent lysis of the yolk particles, for which reason the results cannot be compared directly to the immunochemical data. However, their results are sufficiently consistent within themselves to indicate that the observed differences between the two species may be real. It can be imagined that the soluble precursor-1 component may be

in equilibrium with the insoluble pool, on the one hand, and the developing mitotic apparatus, on the other. In this manner, the insoluble pool would represent the main reservoir of the precursor-1 component, being converted into the soluble form as an intermediate step before its assembly into the mitotic apparatus. Consequently, the level of the soluble pool may remain relatively constant during the formation of the mitotic apparatus and may or may not be exhausted at the time the mitotic apparatus is fully formed. It has been shown experimentally that there exists at metaphase of the first cleavage division the soluble form of the precursor-1 component at a level approximating that observed in unfertilized eggs.

*The Precursor-1 Component in Embryonic and Adult Tissues:*

*Results.*—Tenth molar KCl extracts of blastulae and prism larvae were compared to a similar extract of unfertilized eggs. These extracts were adjusted to the same protein concentration of about 1 mg./ml. (as determined by the method of Lowry *et al.* (1951)) and reacted in agar with antiserum to unfertilized egg antigens. The antiserum was placed in the center well of Fig. 6 and the peripheral wells were filled as follows: unfertilized egg extract (*UNF. EGG*), blastula extract (*BLAS.*), and prism larva extract (*PRISM*). The wells labelled with 1:5 contained a 1:5 dilution of the extract used in the wells not so labelled. The P band is associated with each extract, indicating the presence of the precursor-1 component in the three stages tested. The relative displacement of the bands from the center well suggests no more than a threefold difference between highest and lowest concentrations of precursor-1 component in the extracts. The incubation time of 20 hours was too short to reveal the presence of any other antigens.

The situation with ethanol-preserved tissues from adult sea urchins can be seen in Fig. 7. The tissues (ovary, testes, and gut) were extracted in 0.1 M KCl and adjusted to the same protein concentration. Mitotic apparatus isolated directly from living material and dissolved at pH 9 served as a reference solution (*FMA*), but its concentration was not determined. The antiserum to unfertilized egg antigens (*ANTI EGG*) was placed in the center well, while extracts of ovary (*OVAR.*), testes (*TEST.*), and gut (*GUT*) were placed in peripheral wells. The arrow indicates the position of the P band, assignable to the well containing the dissolved mitotic apparatus (*FMA*), which clearly fuses with a band associated with the ovary extract. The testes and gut, on the other hand, appear to be entirely devoid of the precursor-1 component. Indeed, the gut extract failed to give rise to any bands of precipitate.

*Discussion.*—The embryonic tissue contains more precursor-1 component than would be expected if a direct quantitative relationship to the number of visible mitoses existed. Phase contrast observations on freeze-substituted squashes of blastulae revealed very few division figures—less than 10 per cent. Such behavior is consistent with the precursor concept, since one of its provisions was that the precursor-1 component must be present in cells *preparing* for division.

The results with the extract of ovary and testes are of special interest. Feulgen-stained sections of gonads from the same animals that furnished the material for the extracts were prepared. In the ovary there were a few mature ova to be seen, but no division figures were observed. The precursor-1 component detected in the extract of ovary could represent that which was stored in the mature ova in anticipation of the first cleavage division. When the Feulgen-stained sections of testes were examined, no meiotic or mitotic divisions could be seen, while gel diffusion techniques failed to reveal the presence of the precursor-1 component.

The lack of any detectable precursor-1 component in gut tissue was a little surprising, since one might expect some mitotic activity to be taking place there at all times. Smears examined under phase contrast did not appear to contain any dividing cells. No sections were made.

It appears that tissues in active mitosis and cells preparing for division contain appreciable quantities of the antigen termed the precursor-1 component. The data suggest that it would be possible to identify cells preparing for division by the presence of the precursor-1 component, provided that cells not anticipating division lack this antigen—not a proven inference.

There is an alternative interpretation. The precursor-1 component has been found in embryonic tissues, but not in any of the adult tissues tested (for this consideration the ovary cannot be included). This could be accounted for by assuming that different antigens are involved in the mitotic apparatus of adult and embryonic tissue. Unfortunately, this interpretation cannot be tested for lack of suitable adult sea urchin tissue from which mitotic apparatus can be isolated.

*Interspecies Distribution of the Precursor-1 Component:*

*Results.*—Unfertilized eggs of *S. franciscanus*, which occurs locally predominantly in the subtidal regions,

and a species of *Lytechinus* (not positively identified), collected off the Coronados Islands of Baja California, were extracted in 0.1 M KCl and compared immunologically to unfertilized egg extracts of *S. purpuratus* by means of antiserum to the unfertilized egg antigens of the last-named species. These results are summarized in Fig. 8. The antiserum was placed in the center well. The solution of mitotic apparatus isolated directly from living material gave rise to two bands, arrow 1 indicating the P band. The precursor-1 component is clearly present in the extract of *S. franciscanus*; indeed the over-all pattern is very similar to that observed for *S. purpuratus*. The *Lytechinus* extract, on the other hand, gave no indication of containing any precursor-1 component. No bands assignable to the *Lytechinus* egg extract fused with the P band associated with the solution of mitotic apparatus and the end of the P band proximal to the *Lytechinus* extract was not deflected, as would likely have been the case had there been even small amounts of precursor-1 component present in the *Lytechinus* eggs.

*Discussion.*—It has been thought that proteins of similar function may exhibit similar antigenic properties, irrespective of phylogenetic affiliations (Boyd, 1951). This is not a new or original concept and there has been little general experimental support for it (Boyd, 1956). However, owing to the universally unique function of the mitotic apparatus, it seemed worthwhile to look for the precursor-1 component in species other than *S. purpuratus*. The investigation was restricted to the soluble fraction of the 0.1 M KCl extracts. The results show that the precursor-1 component is present in the two species of *Strongylocentrotus* and absent from the species of *Lytechinus* tested. If a common mechanism of mitosis exists, apparently immunochemically unrelated molecules are involved.

*The Precursor-1 Component and Muscle Proteins:*

*Results.*—Bands of muscle were removed from the Aristotle's lantern of numerous urchins, preserved in cold ethanol, and after homogenization (Went, 1958) were extracted in 0.1 M KCl, 0.6 M KCl (both at pH 7.5–8.0), and distilled water at pH 10.5. The extracts were adjusted to the same protein concentration of about 7 mg./ml. (Lowry *et al.*, 1951) and reacted with antiserum to unfertilized egg antigens. At no time during the incubation period were there any bands associated with these extracts of muscle.

*Discussion.*—There is no demonstrable evidence for an immunochemical relationship between the precursor-1 component and the soluble proteins from adult sea urchin muscle. Data in support of the above are to be found in the experiments of Holtzer *et al.* (1959). They observed that fluorescein isocyanate-labelled antibodies against chick myosin did not react with the mitotic apparatus of chick fibroblast cells.

## GENERAL DISCUSSION

These experiments have shown that it is possible to think of the formation of the mitotic apparatus in terms of the spatial rearrangement of preexisting molecules present in the unfertilized egg. There are no antigens foreign to the unfertilized egg detectable in the mitotic apparatus. Before the precursor concept can be considered "proved," it must be demonstrated that the individual molecules comprising the mitotic apparatus are, indeed, the same ones that occur in the egg. Currently, this cannot be investigated for lack of information regarding the metabolism of the two precursor components.

The sea urchin egg represents a cell provisioned and poised for a series of rapid, consecutive mitoses without intervening periods of growth. The penetration of a sperm is its signal to embark upon this predetermined pathway. With the egg being thus endowed with all the requirements to carry it through to a free-living larva, it is not surprising that the levels of the "precursor" component in the later larval stages were not greatly different from that found in the unfertilized egg. If, however, we had been dealing with a cell that engages in an active period of growth between mitotic divisions, the quantitative aspects would be entirely different. Such a cell would have to synthesize as much precursor-1 component and precursor-2 component before the onset of the next mitotic cycle as it contained immediately following the preceding division. In some cells this would represent a considerable amount of the total protein present. When a cell assumes specific specialized functions as a small unit contributing to the integrated activity of the entire organism, it will normally undergo no further mitotic activity. Such a cell could reasonably, but not necessarily, be expected to have lost all the precursor-1 component and precursor-2 component to the mitotic apparatus. This would then make it possible to identify dividing cells and cells anticipating division in a population of non-dividing cells. Using the appearance and disappearance of the two precursor components as a guide would offer a new experimental approach to the problem of the resumption of mitotic activity by otherwise differentiated cells, as seen in regeneration, and the

cessation of mitotic activity prior to differentiation. Inextricably interwoven with this problem is the question of the origin and fate of the precursor-1 component and precursor-2 component.

The author expresses his appreciation and thanks to Professor Daniel Mazia for many stimulating discussions and valuable suggestions. Dr. John Phillips, Jr., is thanked for his advice on immunochemical procedures.

This investigation was performed in the Department of Zoology at the University of California.

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PLATES

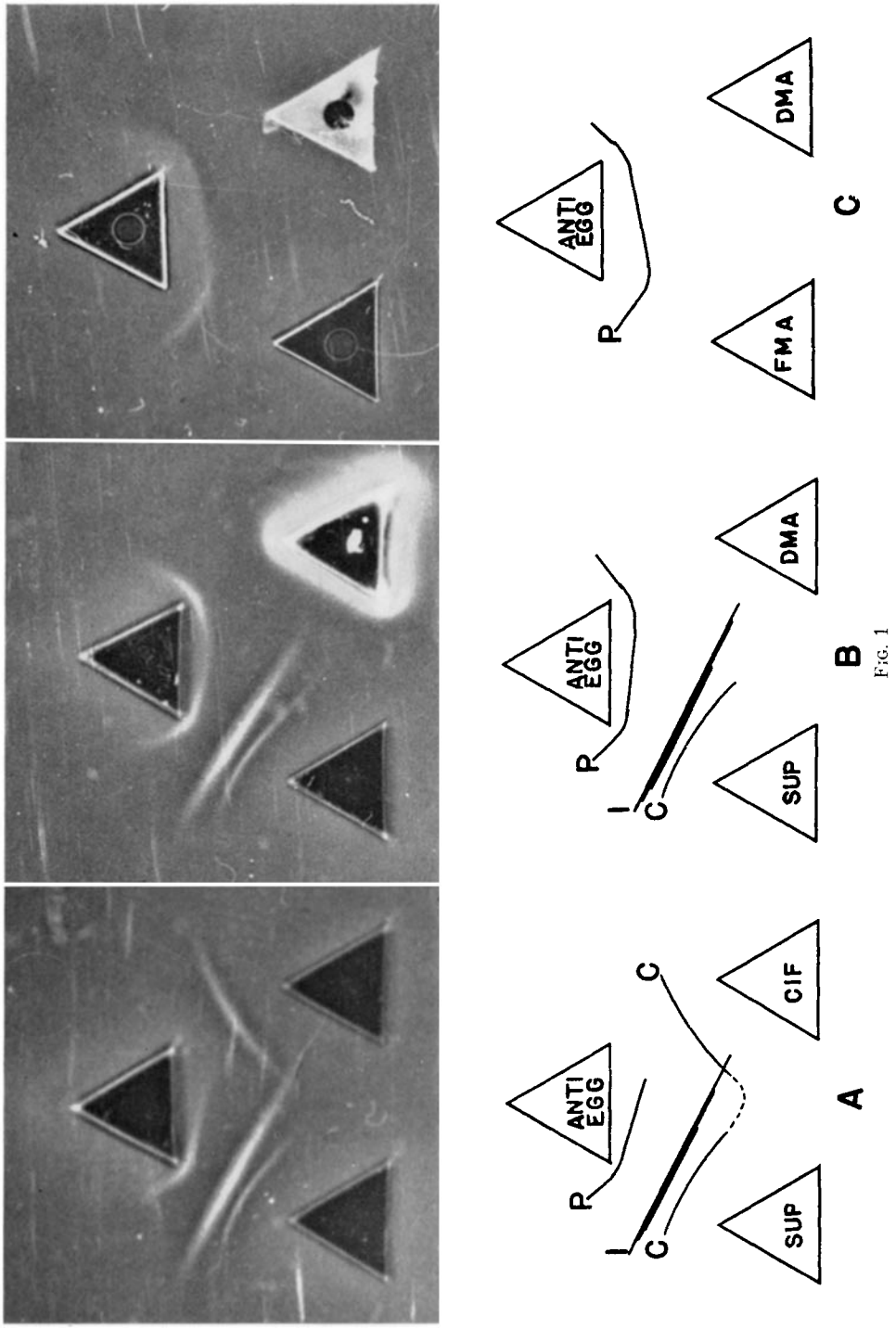


FIG. 1

(Went: Mitotic apparatus of sea urchin)

PLATE 225

FIG. 1. Evidence for the presence of antigens common to dissolved mitotic apparatus and unfertilized eggs. Under each photograph is a corresponding line drawing giving the author's interpretation of the band pattern. The following abbreviations are used: antiserum to unfertilized egg antigens (*ANTI EGG*), calcium-insoluble fraction (*CIF*), supernatant to CIF (*SUP*), dissolved digitonin-isolated mitotic apparatus (*DMA*), and dissolved mitotic apparatus isolated directly from living material (*FMA*). The precursor-1 component is indicated by *P* and the calcium-insoluble fraction band by *C*.

PLATE 226

FIG. 2. Summary of the immunochemical evidence for the existence of a precursor-1 component common to the unfertilized egg and the mitotic apparatus. The arrows indicate the position of the precursor-1 component band (*P* band). The following abbreviations are used: antiserum to unfertilized egg antigens (*ANTI EGG*), dissolved digitonin-isolated mitotic apparatus (*DMA*), antiserum to dissolved digitonin-isolated mitotic apparatus (*ANTI DMA*), unfertilized egg extract (*EGG EXT.*).

FIG. 3. Effect of pH upon the precursor-1 component and the precursor-2 component. The peripheral wells were filled with 0.1 M KCl extracts of unfertilized eggs treated at the pH indicated for each well. Antiserum to mitotic apparatus isolated directly from living material (*ANTI FMA*) and antiserum to digitonin-isolated mitotic apparatus (*ANTI DMA*).

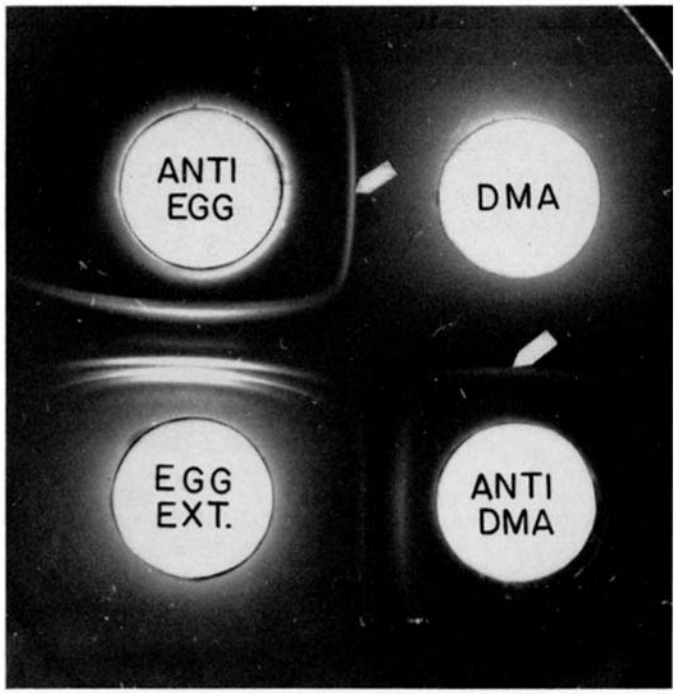


FIG. 2

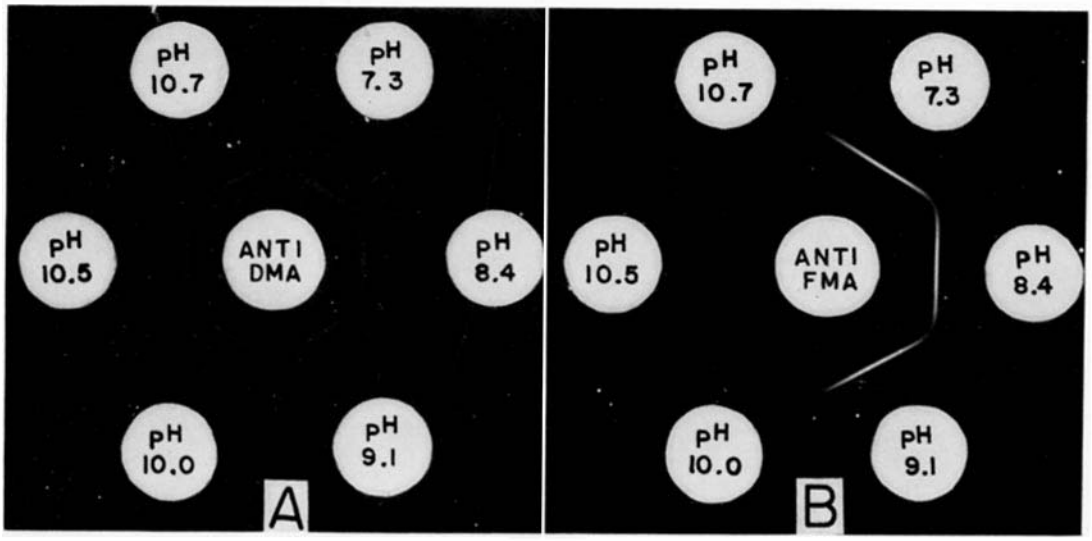


FIG. 3

(Went: Mitotic apparatus of sea urchin)

PLATE 227

FIG. 4. Comparison of isotonic electrolyte and non-electrolyte extracts of living eggs to similar extracts of ethanol-preserved eggs. The peripheral wells were filled as follows: isotonic sucrose extract of living eggs (well *1*), isotonic KCl extract of living eggs (well *2*), isotonic sucrose extract of preserved eggs (well *3*), and isotonic KCl extract of preserved eggs (well *4*). In both *A* and *B* the centerwell was filled with antiserum to unfertilized egg antigens. The plate in Fig. 4 *A* was incubated for 18 hours and the plate in Fig. 4 *B* for 42 hours. The bands are labelled as in Fig. 1.

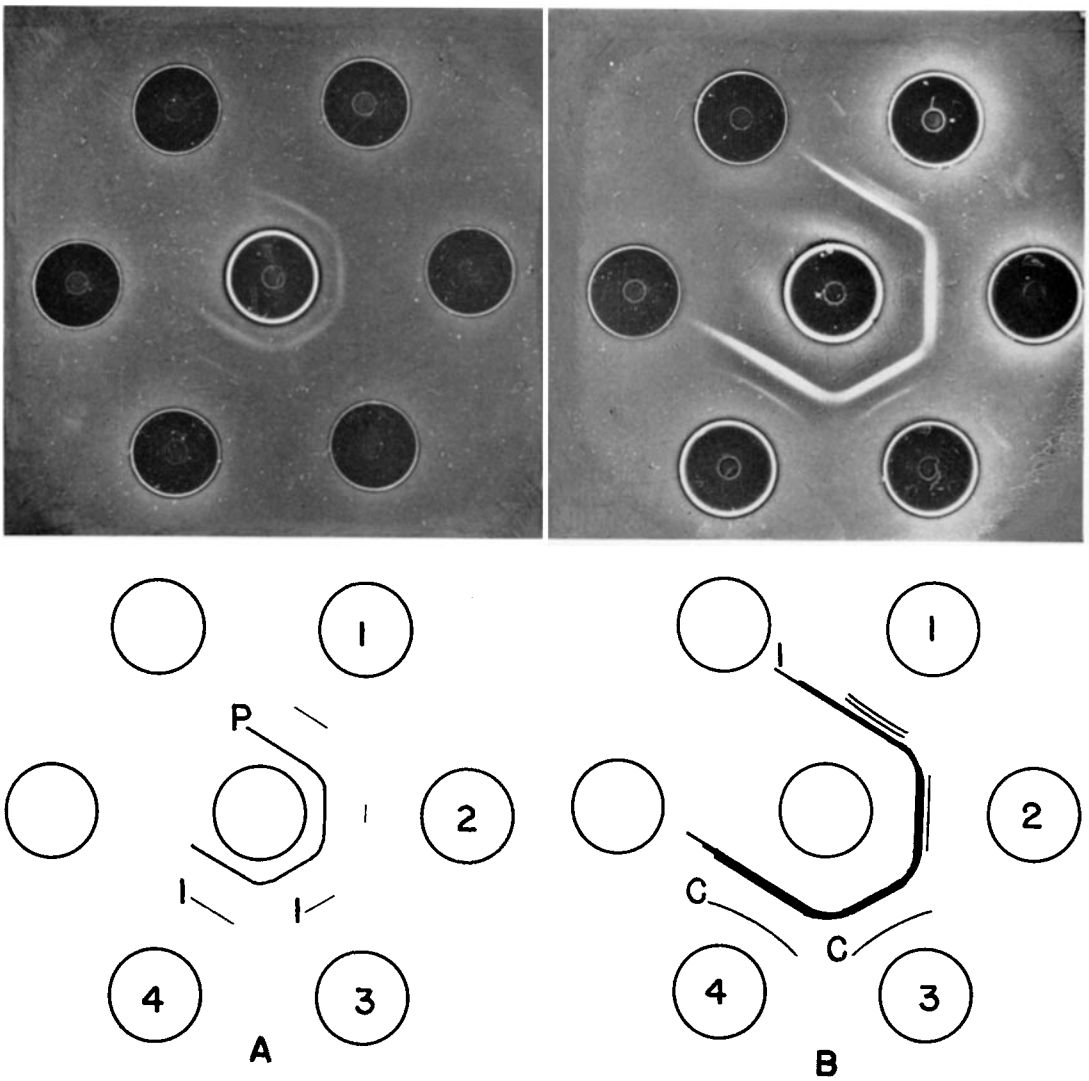


FIG. 4

(Went: Mitotic apparatus of sea urchin)

PLATE 228

FIG. 5. Absorption studies. The peripheral wells were filled with antisera absorbed with unfertilized-egg particulate fraction after the latter had been extracted in 0.1 M KCl (well *1*), 0.5 M KCl (well *2*), 1 M KCl (well *3*), and 2 M KCl (well *4*). Unabsorbed control antiserum was placed in well *5* of each plate. The centerwell of Fig. 5 *A* contained unfertilized egg antigens, while that of 5 *B* was filled with dissolved digitonin-isolated mitotic apparatus.

FIG. 6. Distribution of the precursor-1 component in 0.1 M KCl extracts of embryonic stages. The following abbreviations are used: unfertilized egg extract (*UNFERT. EGG*), blastula extract (*BLAS.*), prism larva extract (*PRISM*). *ANTI EGG* is the same used in Fig. 1.

FIG. 7. Distribution of the precursor-1 component in 0.1 M KCl extracts of adult tissue. The arrow indicates the precursor-1 component band. *FMA*, solution of mitotic apparatus isolated directly from living cells; *OVAR.*, ovarian tissue; *TEST.* testes tissue; *ANTI EGG*, antiserum to unfertilized egg antigens; *GUT*, gut tissue.

FIG. 8. Interspecies distribution of the precursor-1 component. *S. P. EGG*, unfertilized *Strongylocentrotus purpuratus* egg extract; *S. F. EGG*, unfertilized *S. franciscanus* egg extract; *LYT. EGG*, unfertilized *Lytechinus* egg extract. *FMA* and *ANTI EGG* are the same as in Fig. 1. Arrow *1* indicates the precursor-1 band and arrow *2* indicates the precursor-2 band.

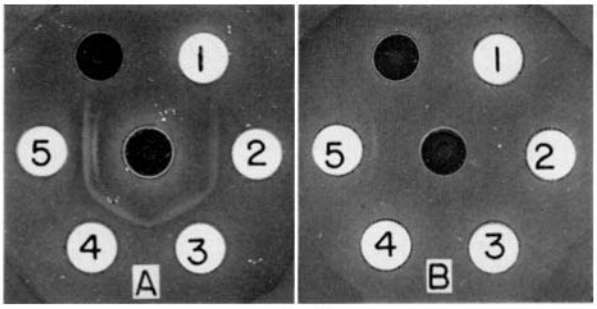


FIG. 5

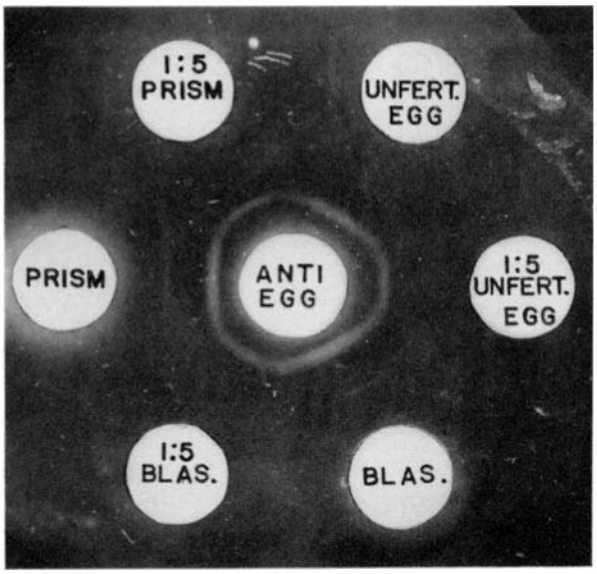


FIG. 6

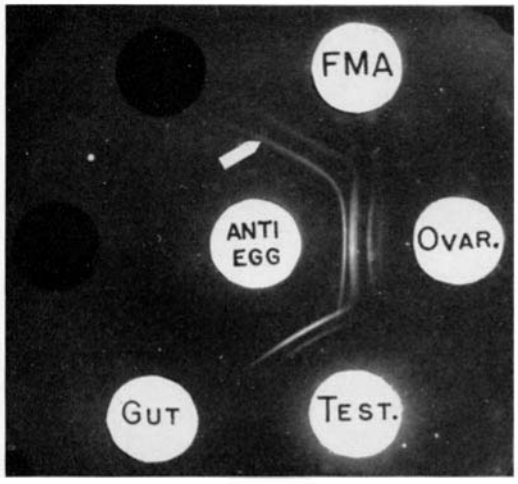


FIG. 7

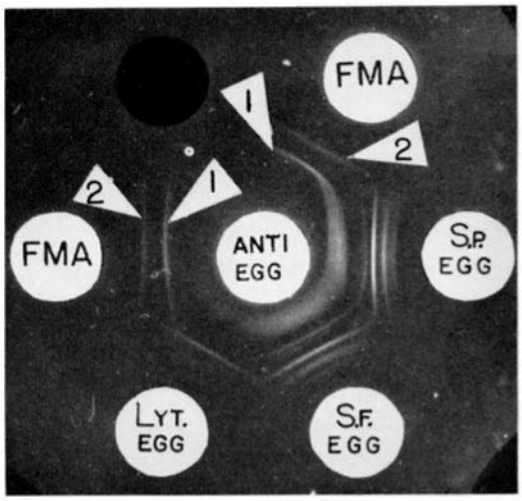


FIG. 8

(Went: Mitotic apparatus of sea urchin)