THE ISOLATION OF NUCLEI FROM EGGS AND EMBRYOS OF THE SEA URCHIN

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ABSTRACT

Nuclei can be isolated from the eggs and embryos of the sea urchin by a four step method: (1) washing with dextrose solution until the cells become weak, (2) cytolysis in 0.002 M Mg^{++} solution, (3) homogenization of the cytolyzed cells by agitation, and (4) separation of the nuclei from the rest of the homogenate by centrifugation on a layered sucrose gradient. Contamination of the isolated nuclei is less than 1 per cent. The yield varies between 20 per cent and 80 per cent depending on the developmental stage used.

In a previous paper a method was described for the isolation of nuclei from eggs of the sea urchin *Echinometra mathaei* and several other species (1). The method, however, could not be successfully applied to the eggs of *Strongylocentrotus purpuratus*, a common urchin of the west coast of North America, nor could it be used for isolating nuclei from embryos. The present paper will describe a new method which permits the isolation of nuclei from the unfertilized eggs and developmental stages up to gastrula of *S. purpuratus* and probably most other species. Nuclei isolated by this method contain at least two enzymes, a DNA polymerase and a deoxyribonucleotide kinase (2).

MATERIALS AND METHODS

Eggs are obtained by induced shedding using 0.5 M KCl. After collection, the eggs are washed in a large volume of sea water, filtered through nylon mesh, and settled. The excess sea water is drawn off and the eggs are centrifuged at 500 *g* (1500 RPM) for 2 minutes. (All accelerations are calculated from the bottom of the centrifuge tubes.) An International PR-2 refrigerated centrifuge and 250 ml centrifuge bottles are used. After centrifugation the excess sea water is removed and the volume of packed eggs approximated. This volume is considered to be 1 volume for the purpose of this description, and usually amounted to about 100 ml for unfertilized eggs. Smaller volumes are equally usable. Unless otherwise stated the preparation is kept at 4°C or less. If this precaution is not observed, clumping may occur.

It is important that embryos do not have fertilization membranes. These can be removed by the method of Mazia *et al.* (3). If the membranes are not removed they will contaminate the final nuclear preparation. Membrane removal is, of course, not necessary if hatched embryos are to be used.

Washing of Unfertilized Eggs

The eggs are suspended in 3 volumes of 1.5 M dextrose in distilled water, then centrifuged at 500 *g* for 5 minutes, and the supernatant is discarded. Generally this step is repeated 4 or 5 times. Each time the eggs are resuspended in the 1.5 M dextrose by gentle swirling. During this process of washing, the appearance of the egg sediment changes. After the first wash the eggs become more buoyant than normal, the apparent volume of the packed eggs increases, and, unless the centrifuge is decelerated gradually, the eggs tend to mix with the supernatant. Usually by the fourth washing a major portion of the eggs will again pack well. At this time the supernatant becomes cloudy, indicating that some of the eggs are cytolyzing. If a significant number of the eggs still settle poorly, this fraction is poured off and washed with 2 volumes of 1.5 M dextrose until the eggs pack well. Finally the packed eggs are pooled and washed once more with 1 volume of dextrose solution, then centrifuged, and the cloudy superna-

tant is discarded along with any remaining buoyant eggs.

Washing of Embryos

Embryos do not have to be washed so thoroughly as eggs. Washing is complete for *S. purpuratus* embryos when a significant number of cells begin to break apart. This usually occurs after the fourth wash. Even if the cells do not fall apart, four washes are adequate.

Homogenization of Unfertilized Eggs

The washed eggs are next swirled just enough to break up any clumps and transferred to an Erlenmeyer flask about 20 times larger than the original volume of eggs. To this is added 5 volumes of 0.002 M $MgCl_2$ or $MgSO_4$. The flask is swirled just enough to keep the eggs suspended and is not cooled. Under these conditions the eggs cytolyze but do not disperse because of the presence of the Mg^{++} ions. The temperature of the Mg^{++} solution determines the time required for cytolysis. Temperatures close to 0°C prolong this stage, and many of the eggs do not cytolyze even after 15 minutes. At about 10°C, which is the temperature used, the eggs cytolyze in a few minutes. This process is followed by taking samples for microscopic examination or, with experience, by examining the appearance of the eggs as they drain down the inside of the flask. When most of the eggs have cytolyzed, 5 volumes of ice cold 2 M dextrose containing 0.006 M Mg^{++} are added and the suspension is shaken just enough to break up the cytolyzed eggs.

Homogenization of Embryos

The homogenization of embryos is essentially the same as for unfertilized eggs except that 0.002 M Mg^{++} is used in the 2 M dextrose solution. Embryos past the 4 cell stage cytolyze more readily in the Mg^{++} solution than unfertilized eggs, and this step can be carried out at temperatures close to 0°C. Cells of embryos later than the 8 cell stage often do not readily break up after cytolysis even with strong shaking. A more effective method is to force the suspension through a number 18 hypodermic needle at the rate of about 2 ml per second. Embryos with more than 200 cells can also be homogenized in a Waring Blendor at slow speed (30 volts).

Isolation

The homogenate is centrifuged at 1000 *g* (2200 RPM) for 30 minutes. The supernatant, containing most of the yolk and small particles, is discarded. The sediment contains large debris and nuclei.

The nuclei are separated from the rest of this sediment by high speed gradient centrifugation on a layered sucrose gradient using a Spinco SW 25 swinging bucket head and model L ultracentrifuge. The gradient is formed in 34 ml cellulose nitrate tubes (1 × 3 inches) and consists of 3.5 ml layers of 2.5 M sucrose solution diluted with water to contain 95, 80, 70, 60, and 50 per cent 2.5 M sucrose. This produces layers with densities of 1.301, 1.255, 1.224, 1.192, and 1.160, respectively. The crude nuclear sediment is layered over the gradient and the tubes are spun at 56,000 *g* (20,000 RPM) for 45 minutes. The nuclei come to rest between the 95 per cent and 80 per cent layers (Fig. 1). All material above the

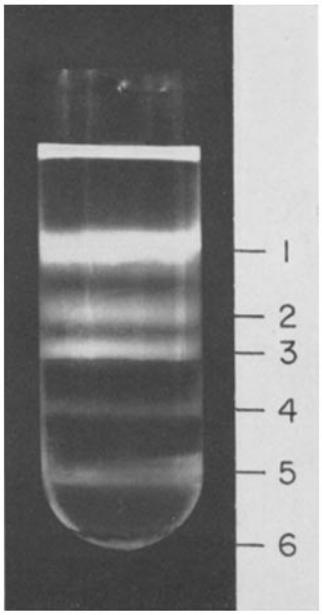


FIGURE 1

Sucrose gradient after centrifugation. 1, whole eggs and yolk; 2, broken eggs; 3, small debris; 4, broken and contaminated nuclei; 5, nuclei; 6, dirt.

nuclei is drawn off and the nuclear layer removed with a pipette and diluted with an equal volume of 0.002 M Mg^{++}. This leaves the nuclei in about 1.2 M sucrose and 0.001 M Mg^{++}, and at an average concentration of about 2×10^6 nuclei per ml for unfertilized eggs and up to 2×10^8 per ml for embryos past the 400 cell stage. The nuclei can be concentrated by centrifugation at 300 *g* for 10 minutes. Because of their fragile nature some damage usually results if they are then resuspended.

DISCUSSION

Fig. 2 illustrates the appearance of nuclei in a good preparation from unfertilized eggs. At this stage it is most difficult to isolate nuclei cleanly because of

the very unfavorable nucleo-cytoplasmic ratio, the nucleus occupying no more than 0.2 per cent of the egg. However, if the isolation is carried out with care, clean nuclei can be consistently prepared. Contamination is usually a fraction of 1 per cent and can be approximated by determining the total volume of non-nuclear material (assuming it to be spherical) visible under the phase contrast microscope as compared with the volume of nuclei in the same field. Almost all visible contamination consists of pieces of fertilization membrane; mitochondria and pieces of cytoplasm are normally not present. Free microsomes or other small cytoplasmic components would not sediment under the conditions of isolation.

Fig. 3. is an electron micrograph of a nucleus from a 200 cell embryo and demonstrates the lack of contaminating cytoplasmic material. The torn nuclear membrane is the result of the method of preparation, which required centrifuging the nuclei into a pellet and then breaking the pellet into small pieces to allow adequate fixation. This same tearing is also seen under the phase contrast microscope but is seldom seen in nuclei removed directly from the sucrose gradient. Fig. 4 is a section through a 200 cell embryo, and comparison with Fig. 3 illustrates the morphological changes that occur during isolation.

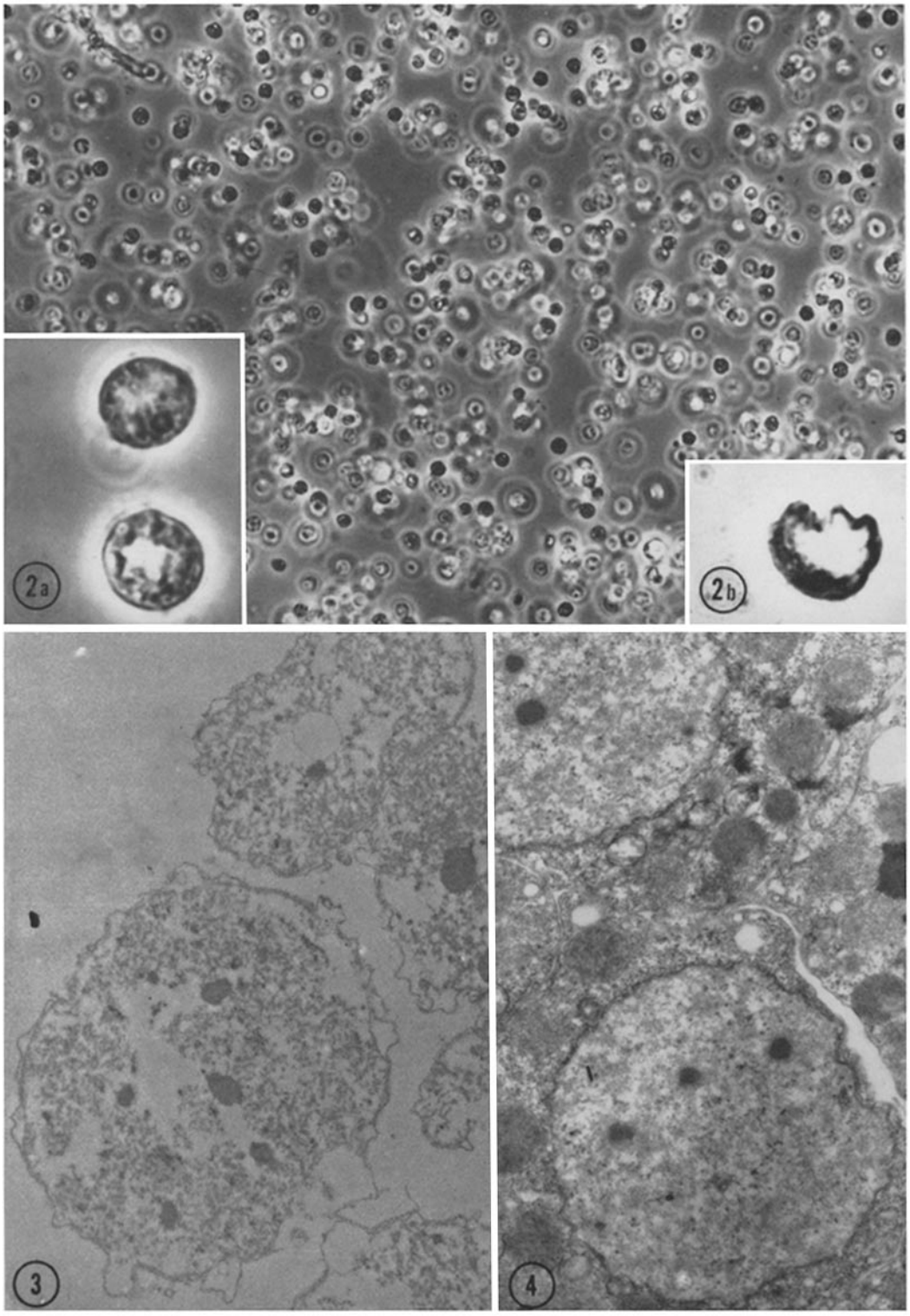
Yield, measured by determining the number of cells serving as raw material and the final number of isolated nuclei, varies between 20 per cent and 80 per cent, depending upon the developmental stage used. Unfertilized eggs and stages up to about 64 cells approach the lower figure, owing to the weakness of the nuclei and/or the fact that a large portion of the cells are in mitosis. The highest yields come from 300 to 500 cell embryos.

The above method has also been used to isolate nuclei from eggs and embryos of *S. franciscanus*. These eggs have a volume three times that of *S. purpuratus* and therefore the homogenization step is less effective and the yield is lower.

Fig. 2, *a* illustrates the appearance of nuclei from unfertilized eggs at high magnification. It is easily seen that the nuclear contents are clumped. This occurs at the time the water and divalent ion reach the nuclei and is not the normal condition in the nucleus. If the divalent ion is not present this clumping does not occur and the nuclei are very fragile and cannot be isolated. Nucleoplasmic clumping is not so apparent in later developmental stages. For example, in the 200 cell embryo (Fig. 3) the distribution of nuclear material is not greatly altered.

The basic isolation procedure can be modified in a number of ways: (*a*) If small amounts of embryos are used the preliminary centrifugation of the homogenate at 1000 *g* can be eliminated. It is only required to concentrate the nuclei into a volume that can be accommodated over the gradient. The entire homogenate can be layered over the gradient if it is no more than 30 or 40 ml. This would contain a relatively small number of nuclei from unfertilized eggs but a large number of nuclei from later stages when the nucleo-cytoplasmic ratio is more favorable. (*b*) The actual volumes of each of the layers of the gradient are not critical and can be varied within limits in order to accommodate different volumes of homogenate. One milliliter in all layers except the 80 per cent layer is adequate if a larger volume of homogenate or sediment is to be centrifuged. The 80 per cent layer must be thick enough to allow for complete removal of the particulate matter on the layer above the nuclei, and for this reason 3.5 ml is about the lower limit. In all cases the tubes should be filled to within a centimeter of the top to prevent collapse during centrifugation. Experience has shown that the number of layers in the gradient cannot be abbreviated. A continuous gradient does not give satisfactory results. (*c*) If the presence of magnesium ions is undesirable, 0.001 M $CaCl_2$ can be used for cytolysis and in the 2 M dextrose. This alters the appearance of the nuclei and moderately increases the amount of contamination. The contents of nuclei from unfertilized eggs do not clump against the membrane but appear as small particles exhibiting brownian motion. These will collect on one side of the membrane during gradient centrifugation. Fig. 2, *b* is a nucleus from an unfertilized egg prepared in this way. Controlled experiments show that the irregular appearance of the membrane is the result of centrifugation and is not caused by the hypertonicity of the sucrose solution. In fact, in all cases the nuclei behave as though they were very porous. This is completely consistent with the electron micrographs of nuclear membranes taken by Afzelius (4). If the 2 M dextrose is made up to contain 0.005 M $CaCl_2$ the nuclear contents clump to the membrane, forming nuclei almost indistinguishable from Mg^{++} nuclei. Usually the contents of embryonic nuclei clump even in 0.001 M $CaCl_2$.

Hypertonic dextrose is used for the wash step



rather than an isotonic solution in order to prevent premature cytolysis of the eggs. At the present time the necessity for this step is not fully understood. It is ineffective when divalent metal ions are present but is not noticeably improved if a chelating agent such as EDTA is added.

One of the main functions is to prevent the formation of fertilization membranes during cytolysis of unfertilized eggs. If eggs are not washed or are washed in dextrose containing as low as 0.001 M monovalent ions, a fertilization membrane will form in the 0.002 M Mg^{++}. If the pH of the dextrose solution is raised above 8, the charged ions have no effect. In this case, or when no ions are present, cytolysis is accompanied by what appears to be bursting of the cortical granules and formation of a layer of gelatinous material around the egg. Because there is no bounding membrane this material is of no consequence in later steps. When a charged ion is present during washing at neutral pH the granules also burst, but in this case the gelatinous material is contained within a membrane. It is believed that the vitelline membrane gives rise to the fertilization membrane (5). Therefore it can be concluded that the wash step affects the vitelline membrane, probably by removing an organic component. Whether other components are affected by this step has not been determined, though it is assumed that the cell membrane and possibly the cytoplasm are also affected. This would explain why embryos, which of course lack a vitelline membrane, also require the wash step if any but an extremely low nuclear yield is desired.

If sea water is gradually added to a suspension of washed unfertilized eggs and these eggs are then fertilized, embryonic development will follow. The only differences from normal embryonic development are that fertilization membranes do not form and simultaneously fertilized eggs do not develop synchronously. It is therefore concluded that the nuclei are under abnormal conditions only after cytolysis has taken place.

Both urea and glycine at concentrations of 1.5 M can substitute for dextrose. However, neither solution shows significant advantages over dextrose and both are potentially more reactive.

Unfortunately this isolation method has one shortcoming. Nuclei cannot be isolated from embryos older than gastrula. When the method is applied to these late stages, nuclei do not separate satisfactorily from their cytoplasmic surroundings and cannot be isolated on the gradient.

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FIGURE 2

Phase contrast photograph of nuclei isolated from unfertilized eggs. For the purpose of photography these nuclei were concentrated by centrifugation at 300 *g*, then resuspended in a small amount of 1.2 M sucrose. × 220.

A. Higher magnification of the same nuclei. × 1500.

B. Nucleus from an unfertilized egg isolated in 0.001 M $CaCl_2$. × 1500.

FIGURE 3

Electron micrograph of a section through a pellet of nuclei isolated from a 200 cell embryo. × 11,000.

FIGURE 4

Electron micrograph of a section through a 200 cell embryo, showing appearance of the nucleus before isolation. × 11,000.

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