ISOLATION OF SPINDLES FROM THE SURF CLAM *SPISULA SOLIDISSIMA*

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INTRODUCTION

The introduction by Mazia and Dan (1952) of a successful technique for the isolation of the mitotic apparatus (MA) ushered in a new period in the study of mitotic mechanisms. The first isolation techniques used alcohol-fixed cells and various agents, such as digitonin, ATP, etc., to disperse the cytoplasm. In 1961, Mazia *et al.* developed a technique for direct isolation of the MA from living eggs with no prior fixation. The essential element in the isolating medium was dithiodiglycol. Kane (1962 *a*) showed that the dithiodiglycol could be replaced by hexanediol, a 6-carbon chain glycol not containing sulfur. A reliable technique was obtained using only buffered hexanediol solutions. Although spindles had been isolated from organisms other than sea urchins (including *Spisula*, Dan *et al.*, 1952), with the alcohol-digitonin method, essentially all work to date has been done on sea urchin spindles. The following is a description of a mass isolation technique developed for eggs of the surf clam *Spisula solidissima*. It follows Kane's (1962 *a*) method closely, with special modifications necessary for adaptation to molluscan eggs.

METHODS AND MATERIALS

The essential part of the technique is removal of the vitelline membrane (VM) (Rebhun 1962 *a* and *b*), since presence of the membrane prevents dispersal of the cytoplasm and thus liberation of the MA. In early experiments 1 M urea solutions at about pH 7.50 to 8.00 were used to disperse the VM. Although urea will work, the eggs become very fragile and much breakdown occurs. The following technique has been found reliable and has been performed at least thirty times.

Eggs of *Spisula solidissima* are obtained, as described by Allen (1953), and fertilized or parthenogenetically activated with KCl-sea water, Allen (1953). At the desired stage (see below) they are spun out of sea water (or sea water plus KCl, in the case of activation) and resuspended in 1 M glycerol, held at pH 8.0 by 0.02 M KH_2PO_4 buffer, for 2 to 3 minutes. The effect of the glycerol is similar to that of the isotonic NaCl previously used for membrane dispersal in *Spisula* (Rebhun, 1962 *b*); *i.e.*, it causes a swelling of the fibrous layers of the membrane, a pinching off of the microvilli at their bases, and fragmentation and dissolution of the pieces of membrane. However, glycerol works with 100 per cent efficiency on both fertilized and unfertilized eggs, whereas NaCl is highly variable from batch to batch and works very slowly if at all on the unfertilized eggs.

Demembranated eggs are spun out of glycerol (with a hand centrifuge) and resuspended in 10 volumes of 1 M hexylene glycol (HG) (recommended to us by Kane; it is a 6-carbon branched chain glycol which can be used instead of hexanediol) buffered to pH 6.0 to 6.5 with 0.01 to 0.02 M phosphate buffer. After 2 to 3 minutes in HG the cells are broken, with a vortex mixer, and the MA are liberated. The cytoplasm tends to clump at pH 6.0, and many experiments were done at pH 6.5. However, the spindles begin to dissolve at this pH and one must work rapidly, cooling the suspension of MA as soon as they are obtained, centrifuging them out of pH 6.5 HG, and resuspending them in pH 6.0 to 6.1 HG solutions, in which they appear to be stable for several days.

With this technique, spindles may be obtained from eggs at several stages in development. *Spisula* eggs are obtained in the germinal vesicle stage and only undergo further development upon fertilization or parthenogenetic stimulation (Allen, 1953; Rebhun, 1959). The present technique allows 1st and 2nd polar body spindles and first cleavage spindles to be obtained from fertilized eggs, and 1st and 2nd polar body spindles and large monasters to be obtained from parthenogenetically activated eggs (which do not cleave in *Spisula* but do form monasters). Preparations for obtaining spindles are made 5 to 6 minutes before the stage at which spindles are desired. Thus, metaphase spindles at first cleavage are obtained if cells are suspended in glycerol at about the time the zygote nuclei have broken down and the 1st cleavage spindles are forming (Rebhun, 1959). Cleavage continues in glycerol and possibly in the early stages of HG penetration.

Most of the work during the development of the method was done with parthenogenetically KCl-activated eggs at first polar body stages, for the following reasons. First, at 22° to 23° C, the first polar body spindle forms at about 15 minutes after fertilization and remains in metaphase for about 10 minutes

before the polar body is formed. 1st cleavage metaphase lasts for only 2 to 4 minutes. Thus, asynchrony in development is much less apparent in isolated spindles of 1st polar body stage (more cells "pile up" in metaphase than in 1st cleavage spindles). Second, activation is generally 100 per cent efficient and can be performed in egg concentrations up to 20 per cent (volume eggs to total volume egg suspension). *Spisula* eggs are not fertilizable by any technique we have found, in concentrations over 1 per cent, although once fertilized in low concentration they will develop at high concentration. The percentage of fertilization is rarely 100 per cent in concentrations over ½ per cent, and this means that eggs must be settled from relatively large volumes of sea water if any reasonable quantity of eggs (5 to 10 cc) are used. Finally, with the first polar body spindle, the whole period from fertilization to spindle isolation takes about ½ hour, compared with about 1¼ hours for 1st cleavage spindles. Spindles from *Arbacia* were obtained by Kane's (1962) method using HG at about pH 6.1 to 6.2.

Microphotographs were taken with a Wild phase contrast microscope on high contrast copy, Kodak film.

OBSERVATIONS AND DISCUSSION

Spindles from *Spisula* at all stages are much closer in refractive index to the HG-egg homogenate than spindles from *Arbacia* and, indeed, *Spisula* spindles are difficult to recognize in the homogenate without some experience. After spinning them at 500 to 1000 *g* for 2 to 3 minutes and resuspending in pH 6.0 to 6.1 HG solutions, the spindles are clearly seen and further centrifugation clears up most of the suspended small particles although particles still adhere to the isolated spindles after several cycles of centrifugation, as can be seen in Figs. 1 and 2. When *Spisula* and *Arbacia* spindles are compared the degree of contamination does not appear to be greater. However, the considerably greater transparency of *Spisula* spindles and higher optical density of the adhering particles make the contamination more apparent.

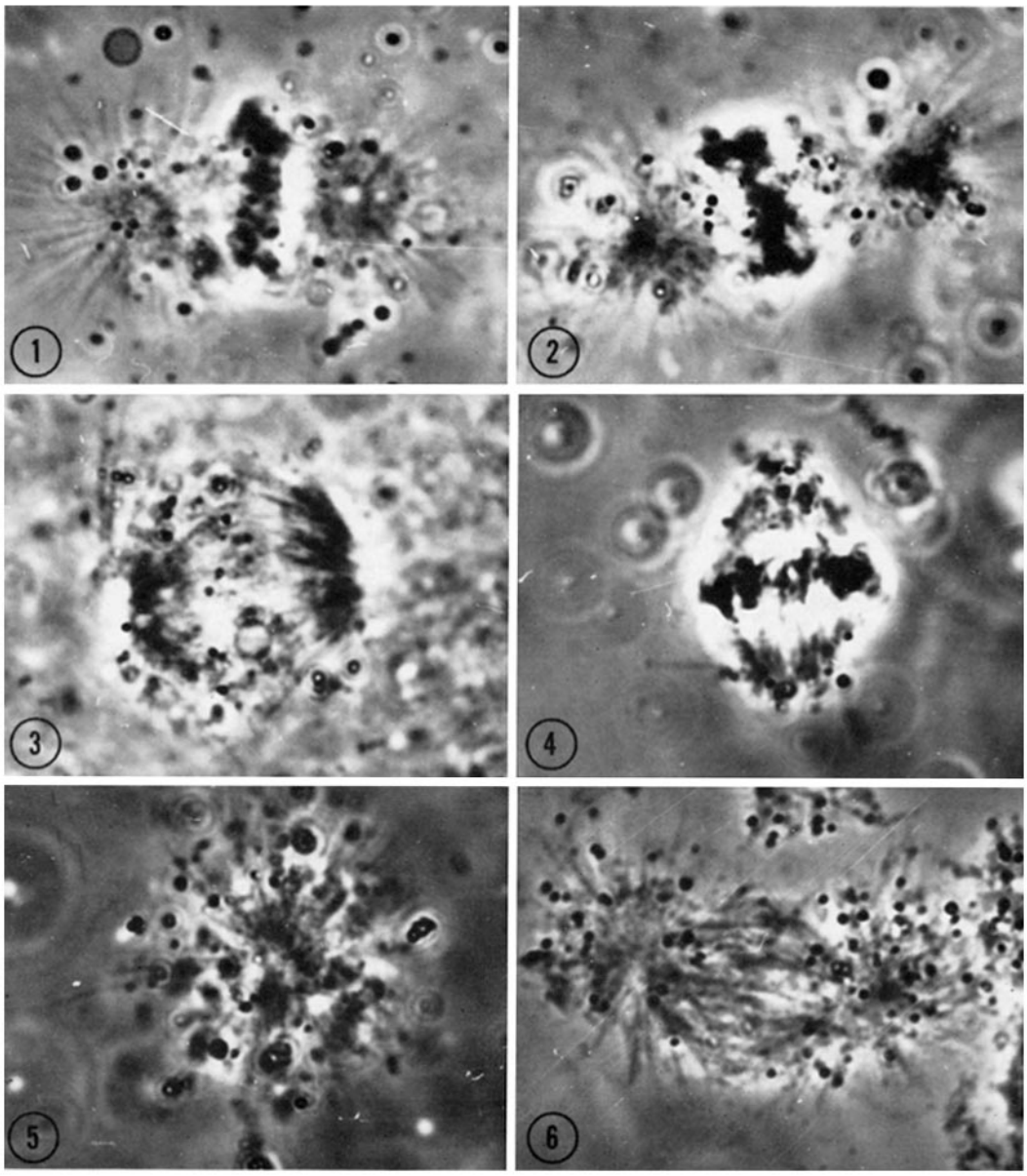
In both *Spisula* and *Arbacia* spindles, asters tend to break free after several cycles of centrifugation and resuspension. This is more frequent in *Spisula* than *Arbacia*. In Fig. 3, a late anaphase spindle, and in Fig. 4, a metaphase spindle without asters may be seen. In Fig. 5 an isolated aster is shown.

Fibrous elements in *Spisula* spindles appear more prominent in the phase microscope than those in *Arbacia* isolated at the same pH, possibly due to a lower refractive index of unoriented material in *Spisula* spindles. Fibers appear to extend into the surrounding medium from the asters and appear relatively rigid though able to bend, (Figs. 1 and 5). What appear to be large swollen vesicles (Fig. 3) may be seen in many *Spisula* spindles. Smaller ones may be seen with greater difficulty in *Arbacia* spindles. These are undoubtedly merely larger elements of vesicular form seen in electron micrographs of *Arbacia* spindles (Kane, 1962 *b*) and may possibly be swollen elements of the endoplasmic reticulum in the spindle (spindle lamellae).

Chromosomes are much more easily dislodged from *Spisula* spindles than from *Arbacia* spindles and tend to be uniformly absent after several centrifugations and resuspensions (see Fig. 6). Chromosomes on 1st polar body spindles are more prominent than those on 1st cleavage spindles, presumably because they are bivalents of the 1st meiotic division (compare Figs. 1 and 2 with 3 and 4). 1st polar body spindles are also shorter and more transparent than 1st cleavage spindles.

Spindles from *Spisula* have two major sources of contamination (disregarding the adherent particles). The first consists of undissolved pieces of the vitelline membrane. Generally, the amount of this contaminant is greater at lower pH's of glycerol and if times shorter than 3 minutes for membrane dissolution are used. The second major class of contaminants are the germinal vesicles which are liberated from unfertilized eggs by the spindle isolation technique and may actually be obtained *en masse* from unfertilized eggs. Another reason for concentrating on parthenogenetically activated eggs, other than those given above, is that little if any contamination from germinal vesicles occurs if activated eggs are used.

The isolation technique described above will thus allow spindles at different stages to be obtained from an organism with determinative cleavage and should offer the possibility of interesting comparative studies with sea urchins, which possess regulative cleavage. In addition, it will be of considerable interest to study relations of spindle protein from polar body and 1st cleavage spindles with regard to the question of whether the 1st (and 2nd) polar body spindle material is disassembled and used as such in the 1st cleavage spindle. Finally, the fact that asters break away from the MA may allow the two parts of the MA, aster, and spindle, to be isolated and studied separately.



FIGURES 1 AND 2 *Spisula* spindles isolated at metaphase of 1st polar body formation. Note relative transparency of the spindle, prominent chromosomes, adherent dark particles, and prominent straight fibers radiating from the asters. × 1500.

FIGURE 3 A spindle isolated at anaphase of 1st cleavage. Asters have come off during preparation of the spindles. Note the large vesicle in interzonal region. Vesicles of this size as well as smaller ones are not uncommon. × 1500.

FIGURE 4 A 1st polar body metaphase with asters missing, presumably mechanically removed during spindle isolation. × 1500.

FIGURE 5 An aster broken from a spindle during spindle isolation at first cleavage. × 1500.

FIGURE 6 A first cleavage spindle showing loss of chromosomes, prominent fibrous structure, dark adherent particles, etc. × 1500.

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