NUCLEAR MEMBRANE FUSION IN FERTILIZED *LYTECHINUS VARIEGATUS* EGGS

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ABSTRACT

Fusion of apposed nuclear envelopes is frequently seen at telophase during postmitotic reorganization of the nucleus, but only rarely at other times in the cell cycle. We attempted to define an experimental system for studying changes in the nuclear envelope related to the cell cycle by varying the time of pronuclear apposition in fertilized *Lytechinus variegatus* eggs. This approach was based on the assumption that the period from fertilization to metaphase of the first cleavage division corresponds to the period from telophase to metaphase in the generalized cell cycle. The experimental approach used was to block the movement of the pronuclei with Colcemid and then to release this block at varying times after insemination by photochemically inactivating the Colcemid. The results show that apposed pronuclear envelopes can fuse from soon after insemination until the anticipated time of prometaphase. Fusion occurred in about 3 min as scored by light microscopy and this time did not vary significantly with the time after insemination. The potential for nuclear fusion is not restricted to pronuclei alone since diploid nuclei in binucleate cells could be fused using centrifugation in solutions of Colcemid to bring the nuclei into apposition. It is suggested that the potential for nuclear fusion is not necessarily related to the cell cycle and that modification of the nuclear envelope, possibly by association with chromatin or other fibrous material restricts nuclear fusion in most multinucleated cells.

INTRODUCTION

Nuclei do not usually fuse even when in apposition for prolonged times. This has been demonstrated in a wide variety of experimentally induced and naturally occurring multinucleated cells (1). When nuclear fusion does occur it can usually be attributed to events occurring during mitosis, involving either the formation of a common spindle (2, 3) or the fusion of karyomeres (chromosomes bounded by nuclear envelopes) during late anaphase or telophase. Since fusion of nuclear envelopes is common during postmitotic reorganization of the nucleus (4, 5, 6), the potential for this fusion appears to vary during the course of the cell cycle.

A biologically important instance of nuclear envelope fusion occurs during syngamy, i.e., the fusion of haploid nuclei (pronuclei) after fertilization. In some organisms, including the sea urchin, syngamy occurs soon after fertilization and well before prometaphase; in others, pronuclei come into apposition but do not appear to fuse until metaphase (7, 8). The potential for fusion of pronuclei envelopes in the sea urchin might be related to the cell cycle in the period preceding the first division, since pronuclear fusion normally occurs in this organism at a time when the cytoplasm and nuclei act as though in telophase. This

proposition is based on the observation of Hinegardner, Rao, and Feldman (9, 10) that DNA synthesis begins at the usual time of pronuclear fusion in preparation for the first cleavage and at telophase in subsequent cleavages. The monaster which forms after fertilization also seems comparable to the coarse telophase aster both in appearance and with respect to subsequent behavior (7). The view that a delay in pronuclear apposition may inhibit fusion of the pronuclei (reference 6, p. 354; 11) has been stated concisely by Allen, based on a study of fertilization of *Psammechinus miliaris* eggs in capillary tubes. He says (reference 11, p. 410), "One striking effect of lengthening the copulation path is to delay the meeting of the nuclei and thus prevent their fusion to form the synkarion. The egg has apparently lost its competence for this process soon after it would normally occur in the spherical egg."

These considerations suggested that changes in the state of the nuclear envelope occur after telophase and that these changes might be accessible to analysis in the period between fertilization and nuclear envelope breakdown at prometaphase. We examined this possibility using light to reverse photochemically (12) the Colcemid inhibition of pronuclear movement (13), thereby varying the time of nuclear apposition.

MATERIALS AND METHODS

Lytechinus variegatus were obtained from Jack Rudloe, Panacea, Fla. and maintained in Instant Ocean (Aquarium Systems Inc., Eastlake, Ohio 44094) for several months before use. Spawning was induced with 0.5 M KCl and the eggs were then fertilized and allowed to develop in artificial seawater prepared according to the Woods Hole MBL formulation. All experiments were carried out in Philadelphia at room temperature (~22°C).

Zeiss-Nomarski differential interference optics with objective and condenser numerical apertures of about 0.6 and green light from a mercury arc were used for observation. Using this optical arrangement with slightly flattened sea urchin eggs, we found it possible to detect the nuclear envelope when it was at an angle of 9° or less to the microscope axis. This maximum value was based on the calculated curvature of a spherical nucleus when it was just visible above and below the equator. Correlated light and electron microscope observations on *Haemanthus* endosperm cells by Bajer and Mole-Bajer (14) have previously shown that changes in the nuclear envelope at prometaphase can be detected in living cells with Nomarski optics.

Fertilization-initiated movement of the pronuclei was inhibited when eggs were placed in concentrations of Colcemid greater than 3×10^{-7} M for 30 min before fertilization. This block to pronuclear movement was reversed when Colcemid-pretreated eggs were fertilized, mounted in Colcemid-free seawater, and irradiated so as to photochemically inactivate Colcemid. Irradiation was with 366 nm light (12) using a mercury arc lamp (type 110 Illumination Industries, Inc., Sunnyvale, Calif.) mounted in a Leitz housing equipped with Jena glass UG-1 and BG-38 filters and with several glass heat filters. For the experiments shown in Fig. 2 the time of irradiation was 20 s.

In order to demonstrate fusion of diploid nuclei, binucleate cells were produced by treating cells with Colcemid (3×10^{-7} to 1×10^{-6} M) about 10 min before they would normally cleave or by placing cells which had started to cleave either into cytochalasin B (0.1–0.5 μg/ml) for about 4 min (15) or into seawater at 0°C. The binucleate cells were then treated with Colcemid (1×10^{-6} M) for 10 min or longer and centrifuged in a small air turbine using a maximum force of ~30,000 *g*. A 1–2 mm layer of 1 M sucrose was placed in the bottom of the centrifuge tube so that the cells would elongate in the local density gradient forcing the nuclei into apposition.

RESULTS

Pronuclei moved together and fused within 15 min of insemination in control eggs. In agreement with Zimmerman and Zimmerman (13) we observed that high concentrations of Colcemid applied after fertilization rapidly and completely inhibited pronuclear movements. We also observed that much lower concentrations of Colcemid (5×10^{-7} or greater) when applied for 30 min before fertilization were also effective in inhibiting pronuclear movements.

When cells in which pronuclear movements had been blocked with low concentrations of Colcemid were irradiated with 366 nm light an aster (or asters) formed in association with the male pronucleus. The female pronucleus then moved toward the center of this aster. This movement brought the pronuclei into apposition even though the primary movement of the female pronucleus was not directly towards the male pronucleus. The area of apposition between the two pronuclei increased until it was about half the maximal cross-sectional area of the male pronucleus and the male pronucleus was often seen to bulge into the female pronucleus during this time. After several minutes of contact the strong optical contrast of the apposed nuclear envelopes dropped and a "hole" became apparent. After the hole appeared the

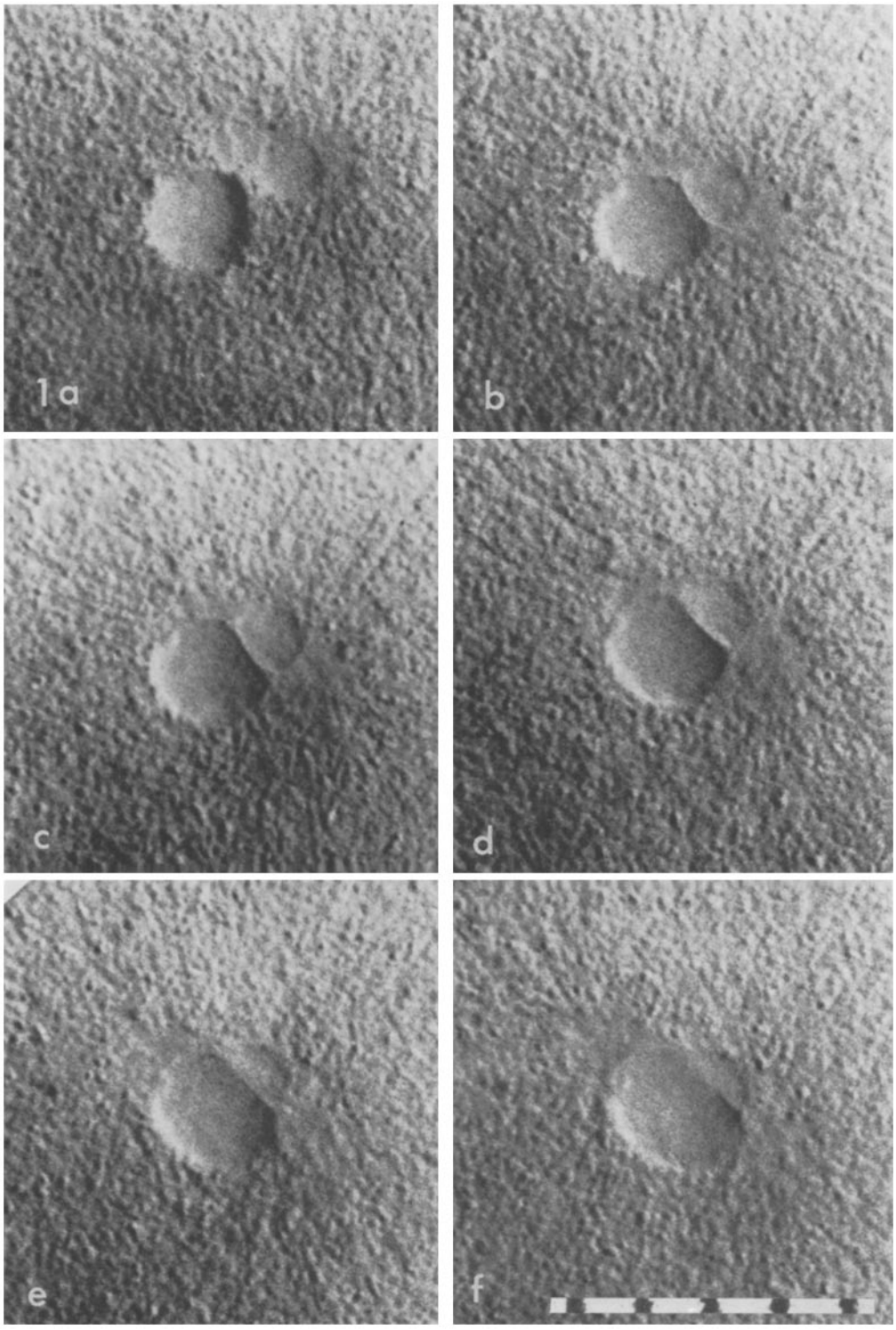

FIGURE 1 Photographs from a movie sequence showing delayed fusion of *L. variegatus* pronuclei. The photographs cover an interval of about 4 min and show an enlarged male pronucleus, a shift in the position of the centers, and possibly some internalization of nuclear envelope as the nucleus rounds up. Nomarski differential interference optics. × 1,000.

membrane indentations at the outer edge of the apposed region opened out and usually became indistinguishable from the rest of the nuclear envelope, although a faint optical boundary was often apparent between the fused pronuclei. An example of the fusion process is shown in Fig. 1.

It was observed that an aster could appear after 366 nm irradiation of Colcemid-treated cells at any time after fertilization. It was also observed that this aster would move the female pronucleus towards the center but only when the nuclear envelope boundary looked sharp as it does up to the time of prometaphase. These observations provided an experimental basis for varying the time of pronuclear apposition and determining if there was a restriction on the potential for nuclear envelope fusion. The criterion for fusion was the appearance of a hole followed by opening out of the nuclear envelope. The relationships between the time after fertilization of pronuclear contact and the ability and time to fuse are shown in Fig. 2. Fusion clearly occurred at any time up to the expected time of prometaphase (45 min) as observed in control eggs. Fusion was not observable at later times because the female pronucleus did not move into apposition with the male pronucleus. The time between apparent contact of the pronuclei and fusion was 2–4 min and the time for fusion to occur did not depend significantly on time after fertilization.

When fertilized eggs were placed in high (3×10^{-5}) Colcemid 15 min after fertilization so that pronuclear fusion did occur, dissolution of the nuclear membrane occurred even though there was no sign of a spindle. The time of onset and the speed of dissolution were comparable to nuclear membrane breakdown at prometaphase in non-Colcemid-treated eggs.

When eggs were placed in a low concentration (3×10^{-6} M) of Colcemid 30 or more min before fertilization so that pronuclear fusion was inhibited, nuclear envelope dissolution at prometaphase was not strictly comparable to control eggs. For the female pronucleus, breakdown was delayed usually by 2–10 min and the initial response seemed weaker in that the nuclear envelope crumpled without strong change in optical contrast; the envelope gradually dissolved thereafter. The male pronucleus usually retained its spherical outline of high contrast during this entire period.

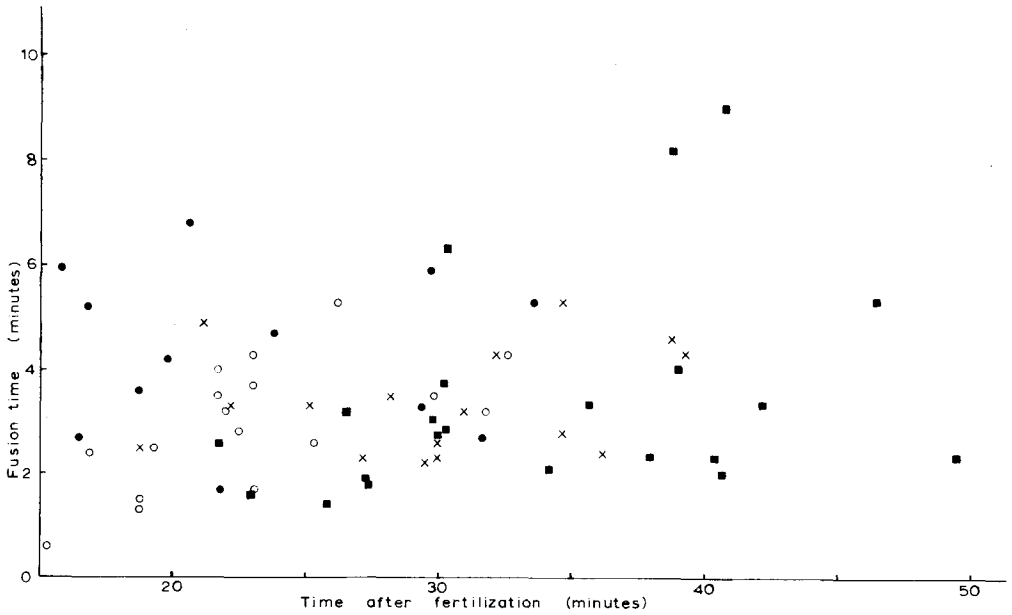

FIGURE 2 Release from Colcemid inhibition was used to vary the time after fertilization when pronuclei came together. This time is shown on the abscissa. The difference between the time when the pronuclei first appeared to touch and when the nucleoplasm first appeared fused was termed the fusion time and is shown on the ordinate. Eggs from four individuals (×, ○, ■, and ●) were used and prometaphase normally occurred about 45 min after insemination in these eggs.

Occasionally, the nuclear envelope showed some crumpling at about the time the female pronucleus broke down, but this soon reversed. Distinct dissolution of the male pronuclear envelope did not occur until at least 10 min after the female pronucleus had crumpled.

Irradiation of the cell with 366 nm light after dissolution of the male pronucleus frequently caused a male haploid bipolar spindle to form. Similarly, irradiation of the cell just after the female pronucleus broke down did not lead to the recovery of a male haploid spindle but did lead to the formation of a female monopolar spindle in a few instances when the female pronucleus was near a center.

It was possible to demonstrate that nuclear envelopes could fuse at the two-cell stage too. This was done by inhibiting the first cleavage, usually with cold, to give a binucleate cell and then treating this cell with Colcemid to reduce the size of the asters; centrifugation in a sucrose density gradient was then used to bring the nuclei into apposition.

Using this technique, fusion of diploid nuclei was observed in approximately 15 percent of binucleate cells centrifuged in 3×10^{-5} M Colcemid for 15 min at 30,000 *g*. Nuclear fusion was occasionally observed under the microscope after centrifugation was over (Fig. 3).

Orientation of both pronuclei (or nuclei) on a common center was not a necessary condition for fusion. This was apparent experimentally in instances where reversal of the Colcemid block in fertilized eggs gave two centers, only one of which was associated with the male pronucleus. In these situations the female pronucleus moved first to the nearer center and then fused with the male pronucleus at a later time. A related instance is shown in Fig. 4 where two diploid nuclei are fusing without orienting on a common center. Subsequently the fused nuclei in this egg went on to form a functional spindle utilizing one center from each nucleus.

Other evidence suggesting that the center is not directly involved in nuclear envelope fusion comes from similar experiments in which it was possible to obtain pronuclear fusion by centrifugation under conditions where astral motility was strongly inhibited with Colcemid.

DISCUSSION

The present experiments using Colcemid to block the movement of pronuclei have shown that the potential for pronuclei to fuse does not vary from fertilization until prometaphase. It seems unlikely that low concentrations of Colcemid prolonged the period during which fusion can occur: At low concentrations, Colcemid binds fairly strongly with good specificity to microtubule proteins in the sea urchin egg (16); in addition, the cycles of DNA synthesis (13) and of chromatin condensation (17) are not greatly affected by Colcemid or by the failure of pronuclei to fuse. Allen's observation (11) on the restriction of pronuclear fusion in *P. miliaris* is, on the basis of this study, better interpreted in terms of incomplete cytoplasmic activation when eggs are fertilized in capillary tubes, a possibility he recognized (18).

That nuclei in most[1] other multinucleated cells do not fuse when in apposition may reflect differences in nuclear envelope structure or stability related to composition, to association with other structural material, or to less specific environmental effects. Instances of the association of the nuclear envelope with filamentous material (summary in reference 22) or with stage specific proteins (23, 24) have been described, and a particularly clear instance of nuclear envelope differentiation has been described in *Blepharisma* (25) where a new nuclear envelope forms within the remnants of the old. Tooze and Davies (26) observed that sections of nuclear envelope appeared to be stabilized by association with chromatin during early metaphase in frog erythroblasts. This last observation seems particularly pertinent to the present work since a rim of condensed chromatin around the nuclear envelope is not seen in the female pronucleus or in early cleavage of the sea urchin. The apparent absence of chromatin nuclear envelope interaction may be related to the short cell cycle time, the absence of a G_1 period, and the relatively large nuclear volume, all of which are characteristic of early cleavage divisions.

The aster is directly involved in bringing pronuclei into proximity after fertilization but does not appear to be necessary for fusion. Observations of pronuclear fusion in cotton (27) and in an alga, *Bryopsis* (28), suggest that smooth endoplasmic reticulum can act as an intermediate by fusing with both nuclear envelopes and then in some way becoming shorter until the surfaces are

[1] Nuclear fusion has been described in *Oncopeltus* nurse cells (19), in *Rhodnius* fat cells (20) after months of starvation, and in some plant tapetal cells (21), but is apparently a degenerative event.

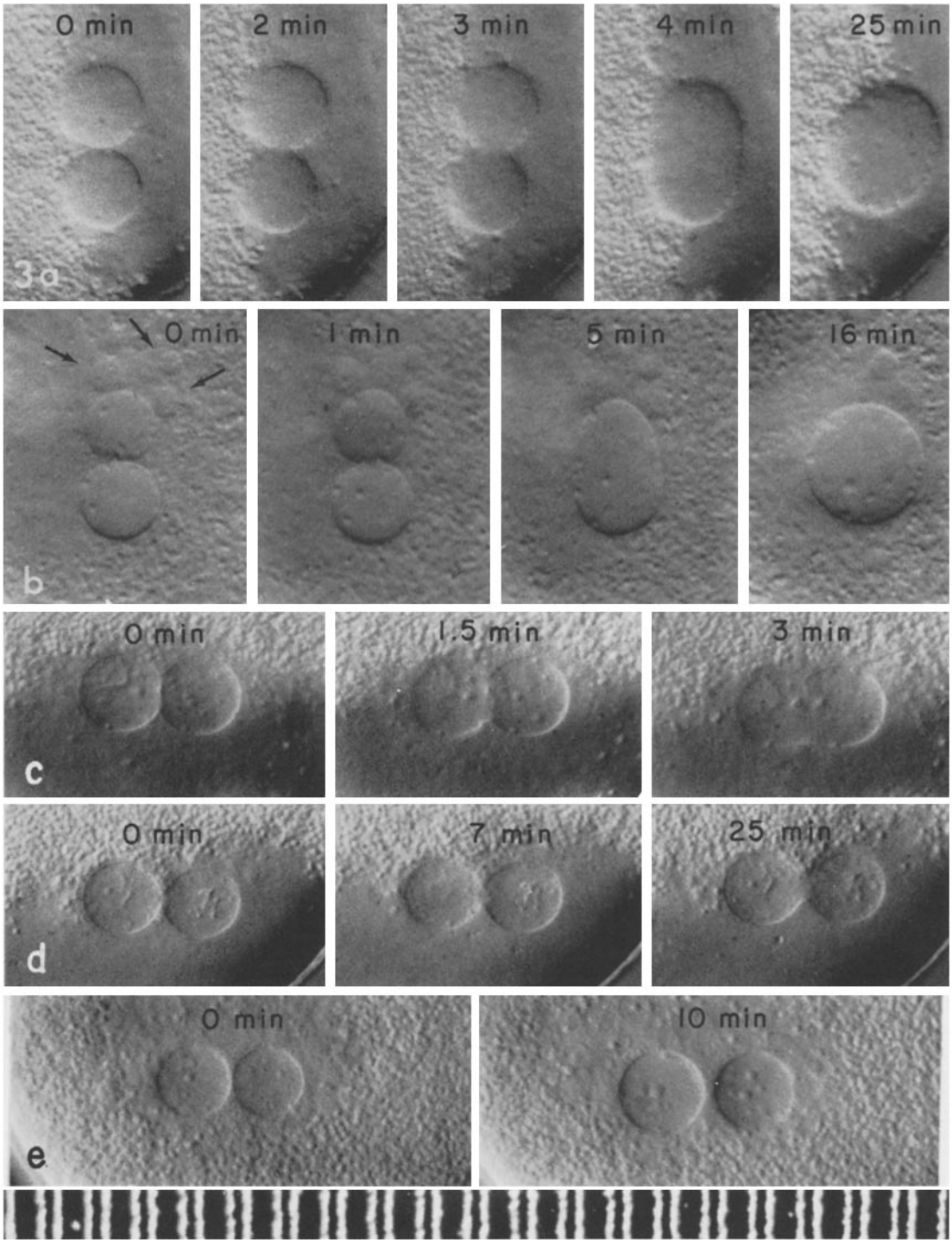

FIGURE 3 Fusion of *diploid* (or near diploid) nuclei after treatment with 1×10^{-6} M Colcemid and centrifugation. The cells shown were made binucleate using cold to inhibit cleavage after the chromosomes had separated at anaphase. (*a*, *b*, *c*) Three instances of nuclear fusion followed by a change in nuclear shape. In *b* several micronuclei (arrows) are visible, suggesting that Colcemid or cold acted in late anaphase. (*d*) Nuclear fusion with little subsequent change in shape. (*e*) Two nuclei which remained in apparent apposition for 10 min before moving apart. Nomarski differential interference microscopy. × 730.

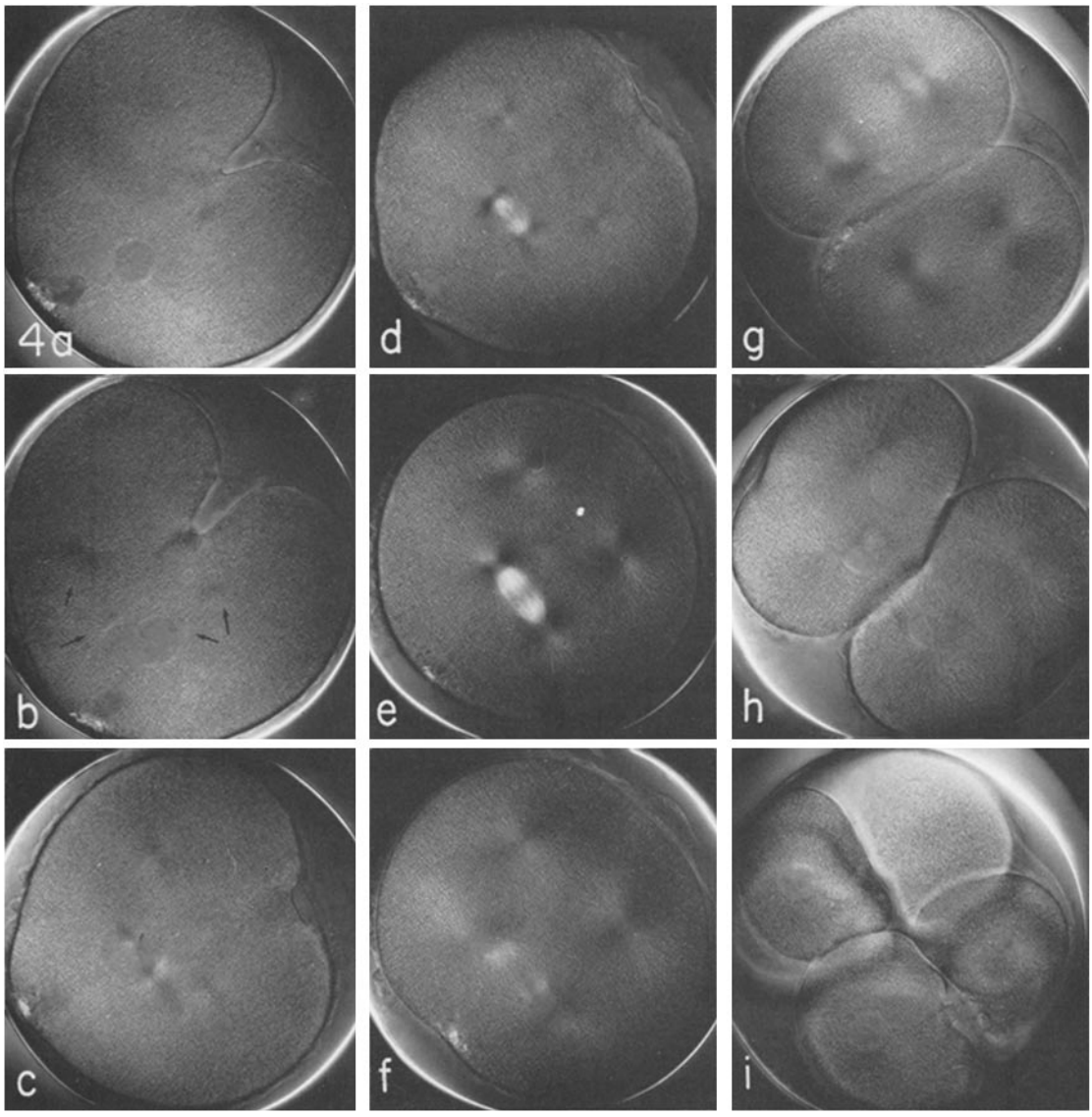

FIGURE 4 Fusion of two diploid nuclei. Cleavage was inhibited with cytochalasin B and the egg was then treated with 1×10^{-6} M Colcemid and centrifuged to bring the nuclei into apposition. In *a*, which was taken 61 min after insemination and 9 min before prometaphase, the nuclei are beginning to fuse. The centers (arrows) are not readily recognized until after 366 nm irradiation, (*b*). Prometaphase is seen in *c*. In *d–g* a functional bipolar spindle leading to cleavage formed between one center from each nucleus. The other two centers become positioned fairly equidistant from all other centers, show an increase in size and birefringence at anaphase and telophase, and determine a weak cleavage axis (*h*) which leads to more extensive (*i*) but still incomplete cleavage at the subsequent division of the nucleated region. Polarized light microscopy. × 350.

brought into contact and fuse. This mechanism could be involved in instances of nuclear fusion of the sort shown in Fig. 4, although it is unlikely that endoplasmic reticulum is involved in normal pronuclear fusion in *Arbacia* (29).

The difference in behavior of unfused male and female pronuclei at the expected time of prometaphase may relate to the effects of delayed duplication and recondensation of the sperm chromatin when the male pronucleus must organize its own

nucleoplasm. An apparently similar effect is seen in frogs (30) and in sea urchins (31) when enucleated eggs which have been fertilized show a delay in the time of cleavage.[2] The basis for the erratic nuclear envelope effects at prometaphase in eggs with unfused pronuclei may be related to experiments of Dettlaff et al. (32). These authors injected nucleoplasm from hormonally activated frog oocytes into nonactivated oocytes and obtained evidence that there is material localized in the nucleoplasm just before and at the time of nuclear envelope dissolution which can lead to nuclear envelope dissolution in nonactivated oocytes. A similar situation in which "mature" nucleoplasm from the female pronucleus is released into cytoplasm containing an immature male pronucleus may have occurred at prometaphase in our experiments with unfused pronuclei. In this instance the prometaphase response of the female pronucleus as compared to fused pronuclei was clearly reduced, suggesting that the immature male pronucleus exerted a modifying action or that gene dosage within the female pronucleus was restrictive.

The examples of nuclear envelope fusion discussed above involve fusion initiated by apposition of the cytoplasmic faces. But, there is also evidence that the envelope can fuse from the nucleoplasm side. Some protozoa such as *Stentor* pinch their macronucleus in two and experimental work by Chambers (33) and by Marcus and Freiman (34) shows that nuclei in echinoderm eggs and in mouse giant cells can be bisected with a glass needle or with a glass cutter without immediately destroying the nuclei.

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[2] Good synchrony in the replication and condensation of the male and female chromosomes in rapidly cleaving eggs may have been evolutionarily advantageous for spindle function and for egg cleavage. Fusion of the sperm chromatin with the already-accumulated contents, and the established environment of the female pronucleus, could be the basis for improving synchrony in chromosome replication. When fusion of pronuclei was blocked the diameter of the male pronucleus increased from that of the sperm head to ~ 5 μm in 15 min and to ~ 11 μm in 30 min while the female pronucleus had a diameter of ~ 14 μm at the time of fertilization.

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