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Plasma membrane regionalization and compaction of mouse cleaving embryos: Effect of microtubule and microfilament inhibitors

Regionalización de la membrana plasmática y compactación en embriones de ratón en segmentación: Efecto de inhibidores de microtúbulos y microfilamentos

L. IZQUIERDO[1], T. LOPEZ[1] and A. PANUNCIO[2]

[1] Departamento de Biología, Universidad de Chile
Casilla 653, Santiago, Chile
[2] Departamento de Histología y Embriología,
Montevideo, Uruguay

Two-cell embryos were incubated during 44 h in media containing either microtubule or microfilament inhibitors. The lowest doses that inhibit cleavage were found to be 0.5 μg/ml for colchicine and colcemid, 5 μg/ml for cytochalasin B and 0.5 μg/ml for cytochalasin D. After incubation with the minimal doses of these drugs, embryos were either fixed immediately or transferred to fresh media without drugs and cultured for different times before fixation. Then, embryos were processed for scanning electron microscopy. Following incubation with microtubule inhibitors, less than 10% of the embryos were compacted and about 20% became so in fresh medium. Regionalization was revealed by the cytochemical demonstration of alkaline phosphatase or 5'-nucleotidase on the apposing surfaces of blastomeres in 50% of the embryos, including all those that were compacted. By scanning microscopy microvilli were seen evenly distributed on the embryo surface, as they are observed in normal 2-cell embryos. Following incubation with microfilament inhibitors, few embryos were compacted and about 90% compacted within 4 h in fresh medium. Alkaline phosphatase activity was detected between blastomeres in all compacted embryos and in one half of those still uncompacted. Scanning microscopy showed patches of microvilli on the outer surface which had become otherwise smooth. During incubation in fresh medium, microvilli concentrated in two large patches placed at the antipodes of the embryo. From these results we conclude that cytokineses is not required for cell membrane regionalization, that blastomeres regionalize before they compact, that microtubule and microfilament inhibitors affect differently the array of microvilli on the external surface of arrested embryos.

As soon as 8-cell mouse embryos compact different plasma membrane regions, or domains, become evident: apposed cell surfaces turn smooth while the free surface of the embryo remains microvillous, cytoplasmic droplets localize under the apposed surfaces and then, when apical tight junctions seal the clefts between peripheral blastomeres, the droplets diminish as the blastocoel cavity appears and the blastocyst forms (7, 8, 10, 39). It has been proposed that spatial signals are encoded during early cleavage in the form of cell membrane regions (17) and we have reported a regionalization of mouse blastomeres which is revealed by the cytochemical demonstration of alkaline phosphatase and 5'-nucleotidase (19, 20). The activity of both enzymes is detected prior to compaction, from the late 4-cell stage onwards, on plasma membrane regions localized on the apposing surfaces of adjoining blastomeres. At the morula stage, all apposed cell surface are labeled by the reaction products of these enzyme markers whereas the outer surface of the embryo remains unlabeled. Around the peripheral blastomeres, the transition from the labeled to the unlabeled region is abrupt and coincides with the site where zonular tight junctions will become established and seal the interblastomeric spaces. This coincidence suggests that the regionalization of the plasma membrane may provide a spatial reference for the local assembly of proteins involved in tight junction formation (15).

Our published data also suggest that the regionalization of the plasma membrane during cleavage is probably not a direct consequence of cytokinesis since it appears during the 4-cell stage interphase; this possibility, however, is tested here by arresting cleavage *in vitro* with different drugs.

Regionalization of the plasma membrane normally precedes compaction of blastomeres, which occurs at the 8-cell stage (8), and compaction seems to be necessary for the maintenance of regionalization since the alkaline phosphatase labels on a blastomere surface soon disappear when this is dislodged from the morula (unpublished observations). The relationship between regionalization and compaction is analyzed here by means of drugs that either interfere with microtubules (colchicine and colcemid) or with microfilaments (cytochalasine B and D). The effect of these inhibitors on the plasma membrane regionalization of mammalian blastomeres has not been studied but their effect on compaction has been reported by several authors (9, 24, 30, 31, 34, 38). These results, however, are not readily summarized because the methodology differs in the developmental stages considered, the concentration of drugs, the duration of treatments and the observational criteria. We incubated 2-cell embryos for 44 h at the lowest concentration of inhibitors that arrest cell divisions and after different times in fresh culture media without inhibitors, embryos were either processed for alkaline phosphatase or 5'-nucleotidase demonstration with the light and electron microscope or examined by scanning electron microscopy so as to describe the cell surface and cell associations.

METHODS

Two-cell embryos were collected from spontaneously ovulating mice of the Swiss-Rockefeller strain around 30 h after ovulation and cultured in polystyrene Petri dishes (Corning) in microdrops of Bigger's medium (2) supplemented with 4 mg/ml of Bovine Serum Albumin (Calbiochem) under mineral oil in an atmosphere of 5% (v/v) CO_2 in humid air at 37°C.

The culture media contained one of the following drugs or a combination of them: colchicine (0.25 – 4 μg/ml), colcemid (0.25 – 4 μg/ml), cytochalasin B (2.5 – 10 μg/ml), cytochalasin D (0.25 – 2 μg/ml). All drugs were purchased from Sigma, except for cytochalasin D which was kindly provided by Dr. M. Flashner from the Chemistry Department of State University of New York at Syracuse. The drugs used in cultures were diluted from stock solutions containing 1 mg colcemid, or 5 mg cytochalasin B, or 1 mg cytochalasin D, per 1 ml dimethyl sulfoxide (DMSO); colchicine was dissolved in distilled water. DMSO in Bigger's medium, at a concentration which is more than twice the highest used in any of our experimental series, did not prevent. development of 2-cell embryos to normally looking blastocysts and therefore, control cultures were usually run in media without DMSO.

After incubation for 44 h, the embryos were either fixed immediately or rinsed several times in Bigger's medium containing no drugs and cultured in it for 0 to 4 h; in some cases though, the embryos were cultered overnight before fixation. In order to enhance cytoskeletal components under observation with the electron microscope, embryos were fixed for 60 min in 3% glutaraldehyde in phosphate buffer 0.1 M pH 7.4 containing 1 or 2% tannic acid and postfixed for 60 min in 2% OsO_4 in phosphate buffer (37). The material was dehydrated in graded acetones, embedded in Spurr's low viscosity resin (Polyscience), and thin section were stained with uranyl acetate and lead citrate.

The methods used for the cytochemical demonstration of alkaline phosphatase and 5'-nucleotidase with the light and electron microscope have been discussed elsewhere (19, 20).

For scanning electron microscopy embryos were fixed for 30 min at room temperature in 3% glutaraldehyde in 0.05 M cacodylate buffer pH 7.4, rinsed and postfixed in 2% OsO_4 during 90 min. The zona pellucida was removed, prior to or after fixation, in 0.5% Pronase (Calbiochem). In order to observe the apposing surfaces, blastomeres from fixed embryos were drawn apart with strong jets of fluid. The material was then mounted on metal plaquettes covered with 1% gelatine, dehydrated through a graded acetone series and critical point dried from liquid CO_2. The samples were coated with 30 nm thick layer of gold in a Polaron E5000 sputtering device and examined with the scanning attachment of a Philips M-300 electron microscope.

Two kinds of control embryos were studied: normal 2-cell embryos similar to those beginning treatment with drugs and late morulae or early blastocysts which had been cultured during 44 h from the 2-cell stage without drugs in the medium.

RESULTS

Definitions: The following description refers to regionalization, compaction and blastulation in arrested 2-cell embryos that differ in many features from the embryos in which these events normally occur; therefore, it seems convenient to define these terms operationally. Plasma membrane regionalization was recognized by the cytochemical demonstration of alkaline phosphatase or 5'-nucleotidase. In normal embryos as well as in arrested embryos,

regionalization was detected exclusively on the apposed surfaces of blastomeres. Compaction was recognized under the light microscope by the flattening of blastomeres against each other and under the transmission or scanning electron microscope, by the dissappearance of microvilli on the apposed cell surfaces, which become smooth. Blastulation, or rather, incipient blastulation, was recognized in arrested 2-cell embryos by the enlargement of the space between the blastomeres.

Experiments with microtubule inhibitors

Doses. Two-cell embryos were incubated during 44 h in media containing either colchicine or colcemid at concentrations ranging from 0.25 to 4 μg/ml, so as to determine the lowest dose that arrest cleavage completely. After running experiments with 277 embryos, the lowest dose was found to be 0.5 μg/ml for both drugs, which is 10 times higher than the dose determined by Siracusa *et al.* (34). The role of microtubules on compaction has been analysed by Ducibella (9) with colchicine, at a concentration two times higher than the dose used here and by Pratt *et al.* (31) with colcemid, at the same concentration used by us.

Light and electron microscope observations. After incubation of 2-cell embryos with colchicine or colcemid during 44 h, less than 10% of them were in different stages of compaction. When culture continued for 4 h in fresh medium without drugs, the number of compacting or compacted embryos increased to 20%; culture overnight did not change this percentage significantly. Observations by light and electron microscopy were performed on 216 embryos, excluding controls, the great majority of them treated with colcemid.

Under the electron microscope, blastomeres presented 1 nucleus of irregular shape containing 2 or more nucleoli that had scarcely any nucleolonema around their amorphous core (Fig. 2) and thus resembled the nucleoli observed in 2-cell control embryos (7). In the cytoplasm, instead, most organelles and inclusions were typical of control morulae except for mitochondria, which were dense, roundish and carried few cristae (Figs. 2, 3) as they are usually observed in 2-cell embryos. Lattice-like material, crystalloid bodies, multivesicular and multimembranous bodies were abundant (Figs. 3, 4), as well as droplets of the kind involved in blastocoel formation (7, 39). In embryos fixed with tannic acid, microfilaments could be observed while microtubules were absent, but after 18 h in fresh medium these reappeared in small numbers near to blastomere contacts.

Demonstration of alkaline phosphatase and 5'–nucleotidase. Following a 44 h incubation with colchicine or colcemid, 92 out of 184 embryos (50%) showed alkaline phosphatase activity and 52 out of 95 (55%) showed 5'–nucleotidase activity. In additional experiments we observed that the percentage of embryos showing alkaline phosphatase activity was maintained when colcemid concentration was increased 8 fold and also when incubation begun 4 h earlier or 4 later than the standard time; that is, colcemid concentration and age of the embryos did not affect membrane regionalization, within certain limits.

Observations with the light microscope were somewhat imprecise as to the localization of the enzyme reaction products due to light refraction at the edge of blastomeres (Fig. 1) and therefore, results were confirmed by electron microscopy. Alkaline phosphatase activity was demonstrated only on cell membrane regions facing the adjoining blastomere (Figs. 1, 5). The enzyme activity was detected in 50% of the embryos, including all those that were at any stage of compaction. In advanced stages of compaction the intercellular space was wider and the enzyme reaction product lining the cell surface became discontinuous or disappeared (Fig. 5) as in normal nascent blastocysts (20). Alkaline phosphatase activity was not detected on crystalloid bodies (Fig. 5) as it is often the case with normal morulae (20). We did not study 5'–nucleotidase activity by electron microscopy.

Scanning electron microscopy. Most embryos that have been incubated during 44 h with colcemid showed approximately the same even distribution, height and density of microvilli that could be observed on the surface of 2-cell controls. In the few embryos that were in the process of compaction (Fig. 6), disaggregated blastomeres showed reduced microvilli on the contact surface (Fig. 7). We studied by scanning microscopy in this experimental series 149 embryos, all of them incubated with colcemid.

Experiments with microfilament inhibitors

Doses. Two-cell embryos were incubated for 44 h in media with different concentrations of cytochalasin B (2.5 to 10 μg/ml) or cytochalasin D (0.25 to 2 μg/ml). Experiments on 146 embryos showed that the lowest doses that inhibit cleavage were 5 μg/ml for cytochalasin B and 0.5 μg/ml for cytochalasin D. These concentrations are similar to those established by Siracusa *et al.* (34); moreover, the dose of cytochalasin D corresponds to that used by Pratt *et al.* (30) and to the lowest dose used by Kimber and Surani (24).

Light and electron microscopy. Following the incubation of 178 embryos with cytochalasins B or D, compaction was seldom observed but it begun as soon as embryos were transferred to fresh medium without the inhibitors; after 4 h, 90% of the embryos were compacted. Under the electron microscope, blastomeres were seen tightly pressed against each other and in some embryos an incipient blastulation could be recognized. Occasionally compacted embryos showed one blastomere partially engulfed by the other, as formerly reported by Kimber and Surani (24). Blastomeres presented 1 to 3 nuclei containing several nucleoli each. Nucleoli had a prominent nucleolonema (Figs. 8, 9) as is usually seen in normal morulae (7). Chromosomes and spindles were found in very few embryos. As to cytoplasm components, including mitochondria, they were typical of late morulae though lattice-like material and particulary crystalloid bodies, seemed to be more abundant (29). In embryos fixed with tannic acid immediatelly following incubation with cytochalasin D, microvilli showed microfilaments that seemed to be less precisely aligned than in controls.

Demonstration of alkaline phosphatase. In embryos fixed following incubation with cytochalasins, alkaline phosphatase was detected on the apposing cell surfaces of about 45% of the embryos, including the few which were compacting or compacted (Fig. 8). During culture in fresh medium this percentage increased, surpassing 90% after 4 h. The events followed a strict sequence: apposed cell surfaces regionalized, their microvilli disappeared, the interblastomeric space narrowed (Figs. 10, 11) and later became wider, thus suggesting a nascent blastocoelic cavity. Alkaline phosphatase activity has been detected in the cytoplasm on some crystalloid bodies, as in normal embryos (20). In these experiments, 648 embryos, including controls, were used, and 84 of them were examined with the electron microscope.

Scanning electron microscope observations. Following incubation with cytochalasin, discrete patches of microvilli were revealed on the external surface of the embryos which was otherwise smooth (Fig. 12). After 4 h culture in fresh medium the microvilli appeared concentrated in two large patches placed at the antipodes of the embryo (Figs. 13, 14, 15). These patches, which occupied about 1/3 of the free surface of the embryo, were circumscribed by larger microvilli (Fig. 16) that resembled those closing the gap between compacted blastomeres in control morulae. In disaggregated blastomeres, a wide concavity was observed when they had partially engulfed their partner (Fig. 17). Our observations comprised 184 embryos, all of them treated with cytochalasin D.

Experiments with microtubule and microfilament inhibitors.

Following incubation during 44 h of 2-cell embryos in a medium containing colcemid

(0.5 μg/ml) plus cytochalasin D (0.5 μg/ml), cell membrane regionalization and compaction were seldom evident. After 4 h in fresh medium without the drugs no progress was observed.

Under scanning electron microscopy most embryos exhibited a typical colcemid effect (see above) which was not modified in fresh medium. Few embryos showed long and sparse microvilli, while others presented patches of microvilli, thus suggesting the cytochalasin effect described above. Occasionally, embryos were seen in which one blastomere had many more microvilli than the other.

Experiments on the simultaneous effect of colcemid and cytochalasin D were performed on 166 embryos.

DISCUSSION

Plasma membrane regionalization does not require cytokinesis

Our results show that the arrest of cytokinesis does not prevent regionalization of the plasma membrane; therefore, a model based exclusively on "new" membrane formation at the advancing edge of the cleavage furrow (17) would not account for regionalization. Now, regionalization without cleavage demands that the marker enzymes, once inserted in the plasma membrane, somehow form a cluster facing a similar one on the adjoining blastomere. We had formerly suggested that the enzyme molecules might be put and held in place through their attachment to the cytoskeleton (20), but that conjecture is not fully supported by our present results since drugs that interfere with microtubule or microfilament integrity do not impede regionalization necessarily. However, the arrest of cytokinesis does not necessarily imply a complete disruption of the cytoskeleton. Microtubules seem to disappear in embryos incubated with colcemid, but a similar or higher dose only reduces, without suppressing, the microtubule network of mammalian 3T3 cells (6). As to microfilaments, we could observe them in microvilli after incubation with cytochalasin and a similar finding has been reported by Pratt *et al.* (31). Anyhow, a role of actin in regionalization should not be dismissed since only 50% of the embryos were regionalized after cytochalasin treatment while most of them became so when transferred to fresh medium.

If the embryo cytoskeleton endures the drug treatments, albeit only partially, one may still ascribe to it a role in preventing the marker enzymes from diffusing in the plane of the plasma membrane and also a role in establishing and keeping the regions of adjoining blastomeres in precise register. An interesting review by Tucker (36) on cytoskeletal coordination and intercellular signalling adds substance to these conjetures. Now, assuming that the cytoskeleton depolymerizes entirely as a consequence of these drug treatments, one might suggest that the marker enzymes form clusters on the cell surface through ligands that cross-link them and that the same or similar ligands link the enzyme clusters on apposing cell surfaces. Of course, positioning of surface molecules by attachment to the cytoskeleton or by cross-linking are not mutually exclusive alternatives.

Cell membrane regionalization precedes compaction.

Following treatment with cytoskeletal inhibitors all embryos at any stage of compaction were regionalized while uncompacted embryos were not always so. A constant temporal precedence of regionalization over compaction suggests a causal relationship but does not prove it, since both phenomena may well be independent manifestations of a basal morphogenetic programme. An analogous case, the temporal precedence of regionalization over organelle polarization in the rat embryo, has been discussed elsewhere (27). If the relationship is not causal, that is, if the development of one defined state is not a consequence of another state that is previous to it, one would expect that their temporal relationship is coordinated by a developmental clock.

Among the different mechanisms that have been proposed for time-keeping during embryonic development, the most

elaborated are based on reckoning of cell cycles and, particularly, on DNA-replication cycles (16). However, this is not necessarily the kind of clock that signals for regionalization, compaction and blastulation since these events, as observed in our experiments, occur often timely and always sequentially even when cytokineses is arrested and nuclear divisions are retarded by comparison with the controls. DNA replication, of course, might have continued keeping time and we cannot at present discard this possibility.

Earlier experiments of ours on the morula-blastocyst transition in half and double embryos showed that blastulation occurs simultaneously in all experimental conditions, eventhough the number of cells at the transition is significantly less than half or twice the number in control embryos (12); and in experiments on the effect of Li^+ on early embryos we observed that cleavage was delayed while blastulation was not and therefore, the number of cells in nascent blastocysts decreased as Li^+ concentration increased (18). Cell differentiation, as judged by electron microscopy, is not altered in half or double embryos nor does it seem to be so in embryos treated with Li^+ prior to or after blastulation (1, 13). Therefore, our present and former results probably conform to Satoh's hypothesis of a cytoplasmic clock for morphogenetic events though they may not conform to his complementary hypothesis about a DNA-replicating clock for cell differentiation (33).

Our observations on the timing of development remind those on the ctenophore *Mnemiopsis* in which the reversible inhibition of cleavage by cytochalasin B does not retard development (review in 14) and they also recall observations on the maintenance of cyclic cortical activity in anucleated fragments of sea urchin and amphibian embryos (25, 40).

The cytoskeleton in early morphogenesis.

Our results show that only few colcemid-arrested or cytochalasin-arrested embryos are able to regionalize and compact; however, once in fresh medium most of the latter succeed in doing so while the former are considerably less successful. A more remarkable difference between the effect of the two drugs in observed on the outer surface of the embryo: on those treated with colcemid or colcemid plus cytochalasin, the surface resembles that of 2-cell controls while cytochalasin-treated embryos show discrete patches of microvilli on an otherwise smooth surface. In fresh medium, these patches coalesce and come to lie at the antipodes of the embryo. The cortical phenomenon described suggests the morphogenesis of an abortive morula that exposes on the outside its smooth interblastomeric surfaces. As this process is not observed in colcemid-arrested-embryos, it seems that it requires the maintenance of the microtubule network; however, microfilaments must also be involved since an overall rearrangement of microvilli occurs when embryos are transferred from cytochalasin-containing media to fresh media.

A recent paper by Sutherland and Calarco-Gillam (35) shows that when compacted mouse morulae are treated with cytochalasin for 4 to 24 h, most microvilli disappear and those remaining are frequently found in randomly distributed clumps; then, after 20 min in fresh medium, a more even distribution of microvilli is observed. Their results and ours resemble, except for the changes of microvilli distribution in fresh medium; however, since the methods differ in developmental stage, lenghts of incubation and recovery, we may be describing the same phenomenon. A reorganization of microvilli is also detected in normally cleaving embryos. Johnson and his associates (review in 21) have recognized two cell subpopulations in 16-cell morulae and more advanced stages: inner apolar cells and outer cells with a clump of microvilli at their apical pole. The polarization in intact embryos may correspond to the patches of microvilli we described in cytochalasin-treated embryos and if this is correct, one would conclude that cell cleavage is not necessary for the development of polarity provided that blastomeres have the proper developmental age and a focal cell contact (22, 23).

The cytoplasmic rearrangement of

Chaetopterus eggs activated by KCl represent the classic case of "differentiation" without cleavage (3, 26). It has been recently shown that this process requires DNA synthesis (4, 5) and that the effect of KCl is also observed when eggs are activated with cytochalasin whereas colcemid inhibits the process entirely (11). The latter result parallels the observations reported here but we cannot assume that early morphogenesis is generally dependent on transformations of a microtubule scaffold since in other cases, as in axis fixation of *Fucus* eggs, the morphogenetic process is inhibited by cytochalasin B and not by colchicine (32). Anyhow, morphogenesis without cleavage and the effect thereon of drugs that interfere with microtubules or microfilaments, suggest the operation in early development of a transcellular cytoskeleton. As to how a spatial differentiation might occur in the absence of cellularization, interesting possibilities arise from the evidence on the role of the cytoskeleton in protein synthesis (review in 28). However, it is still unclear whether the spatial configuration of the cytoskeleton might specify which proteins are synthesized and where.

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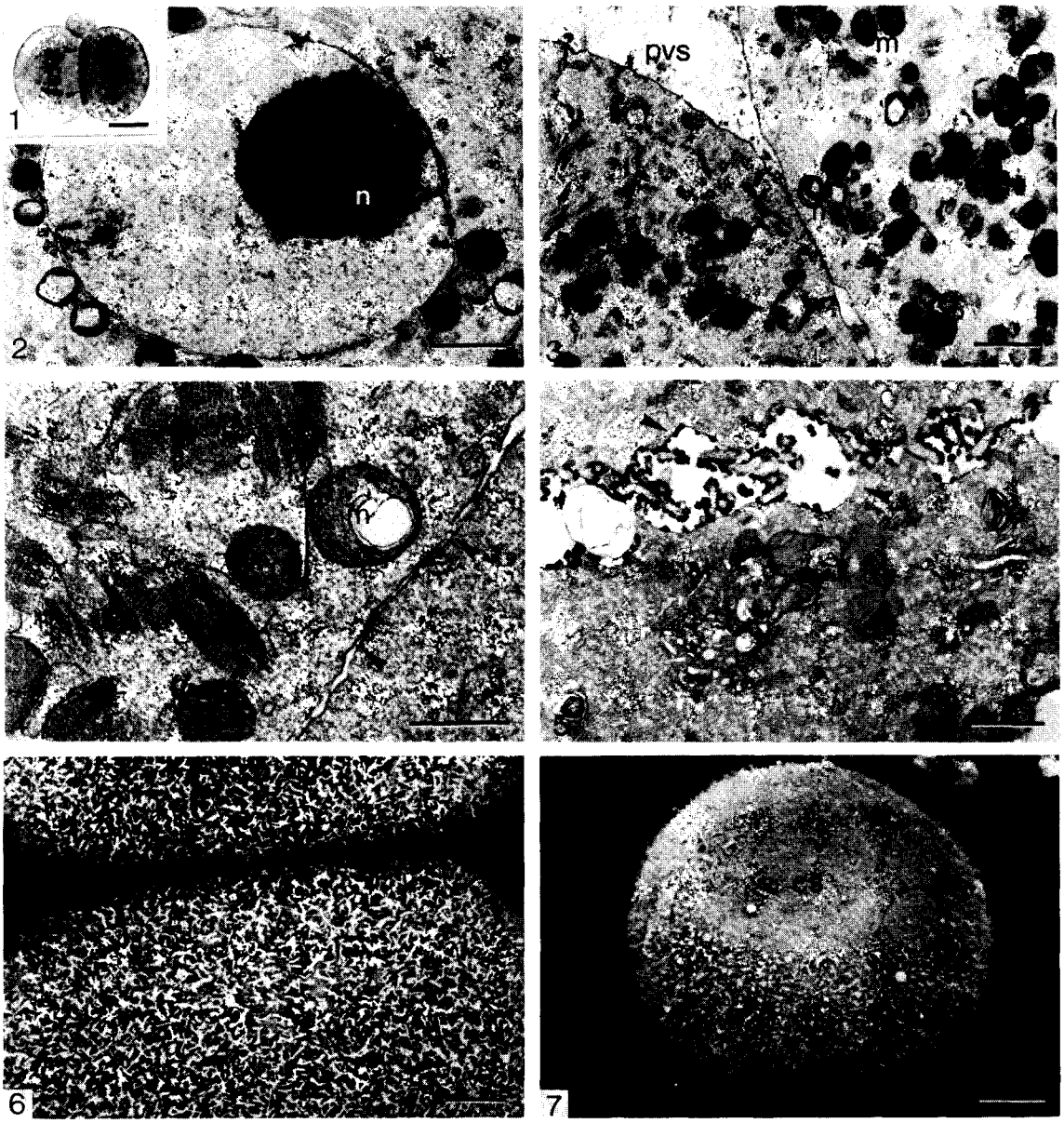


Fig. 1: Alkaline phosphatase demonstration on a whole-mounted colcemid-arrested embryo at the beginning of compaction. Enzyme reaction product between blastomeres (arrow heads). Bar represents 20 μ.

Fig. 2: Embryo fixed with tannic acid following incubation with colcemid. Nucleolus (n) with a prominent amorphous core, dense mitochondria with few christae (m) and lattice-like material (1). Bar represents 1 μ.

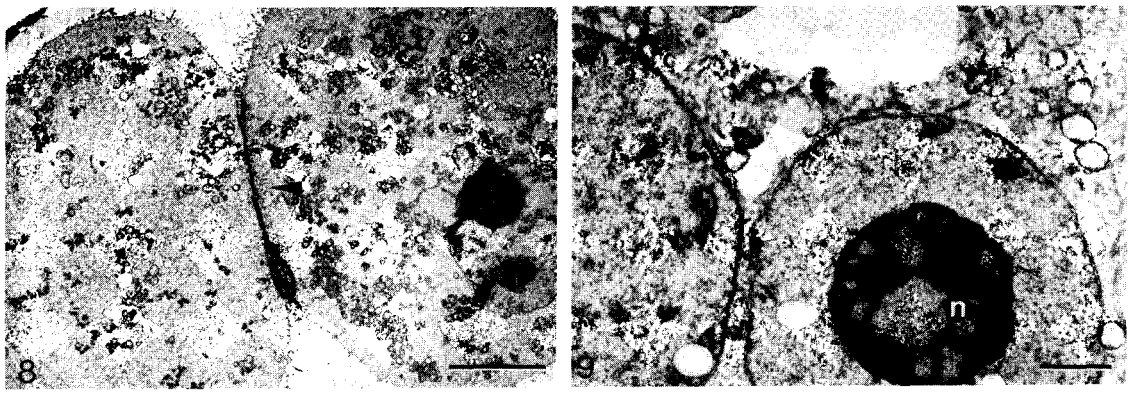
Fig. 3: Colcemid-arrested embryo beginning compaction fixed as in Fig. 2. Perivetelline space (pvs), mitochondria (m), lattice-like material (1), crystalloid bodies (c) and multimembranous bodies (mb). Bar represents 1 μ.

Fig. 4: Compacted colcemid-arrested embryo fixed with tannic acid after 18 h in fresh medium. Blastomere apposition (arrow heads), mitochondria (m) and crystalloid bodies (c). Bar represents 0.5 μ.

Fig. 5: Alkaline phosphatase demonstration on a compacted colcemid-arrested embryo. Diminished enzyme reaction product (arrow heads). Golgi substance (g), crystalloid bodies without enzyme activity (c). Bar represent 1 μ.

Fig. 6: Scanning electron microscopy of a colcemid-arrested embryo beginning compaction. Microvilli resembling those of normal 2-cell embryos. Bar represents 5μ.

Fig. 7: Similar to Fig. 6. A disaggregated blastomere showing reduced microvilli on the contact surface (cs). Bar represents 10 μ.



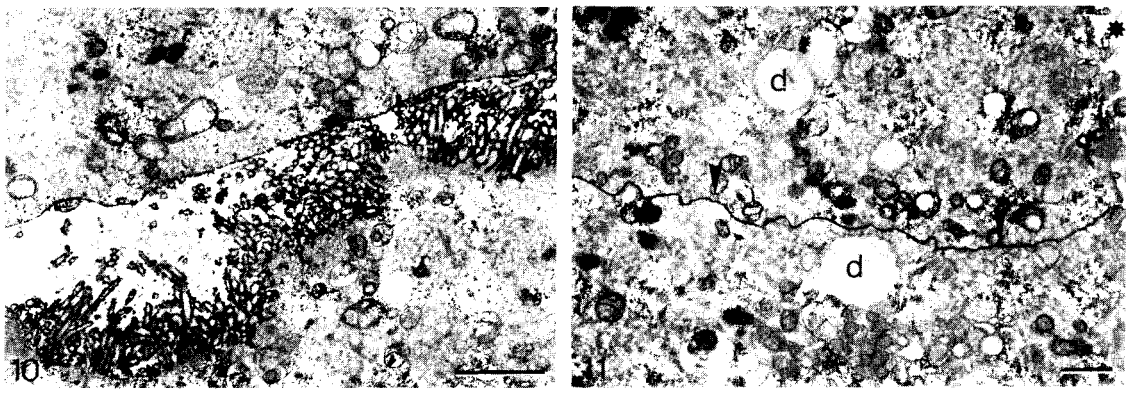


Fig. 8: Akaline phosphatase demonstration on an embryo that begins compaction, arrested by cytochalasin D treatment. Reaction product on apposing cell membranes (arrow head). Bar represents 10 μ

Fig. 9: Cytochalasin D-arrested embryo. Nuclei containing nucleoli (n) with prominent nucleolonema. Bar represents 2 μ

Fig. 10: Alkaline phosphatase demonstration on a cytochalasin D– arrested embryo. The cell surfaces of both blastomeres are labeled by the reaction product. The blastomere below still shows microvilli while the blastomere above has a smooth surface. Bar represents 1 μ

Fig. 11: Alkaline phosphatase demonstration on a cytochalasin D– arrested embryo fixed after 4 h in fresh medium. Apposing cell surfaces of compacted blastomeres labeled by the reaction product (arrow heads). Perivitelline space (asterisk) cytoplasmic droplets (d). Bar represents 1 μ.

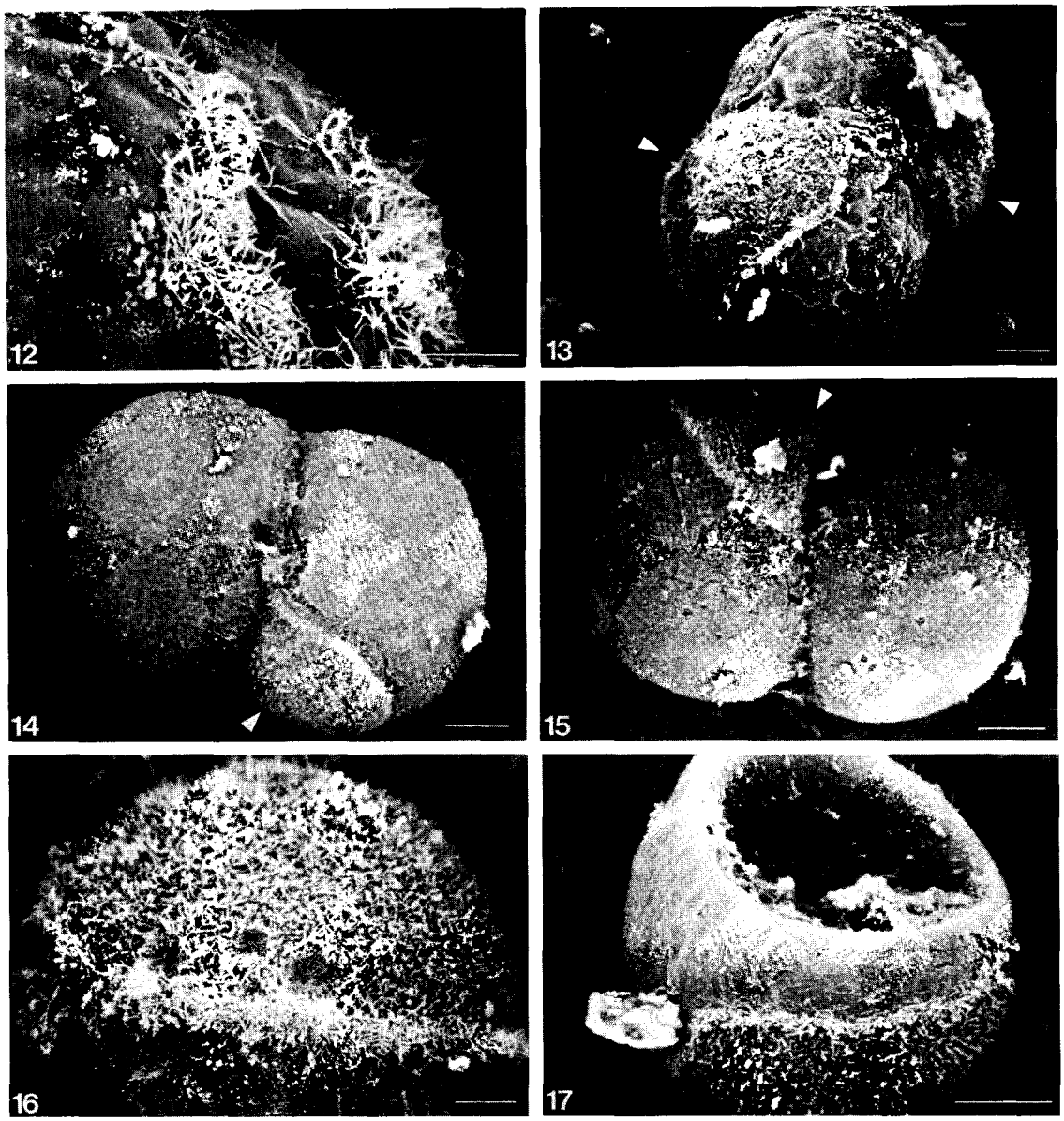


Fig. 12: Scanning electron micrograph of a cytochalasin D – arrested embryo. Patches of microvilli on the smooth external surface. Bar represents 4 μ

Fig. 13: Similar to Fig. 12, but fixed after 4 h in fresh medium. Microvilli concentrate in two patches (arrow heads) at the antipodes of the compacted embryo. Bar represents 10 μ

Figs. 14-15: Similar to Fig. 13. The patches of microvilli (arrow heads) on the two blastomeres of an embryo tilted so as to show both antipodes. Bar represents. 10 μ

Fig. 16: Similar fo Fig. 13, showing an antipode patch circumscribed by larger microvilli. Bar represents 5 μ

Fig. 17: A disaggregated blastomere of an embryo similar to Fig. 13. The concavity corresponds to the engulfment of the adjoining blastomere. Bar represents 4 μ