Hokkaido University

Causal Analytic Study of Surface Contractile Activity in *Tubifex* Egg: Mechanism of Polar Body Extrusion and Deformation Movement

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CONTENTS

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INTRODUCTION

In addition to activating the egg and providing an opportunity for amphimixis, the penetration of the spermatozoon into the egg causes in most, if not all, animals far-reaching displacements of the cytoplasmic constituents of the egg (Davidson, 1976). Therefore, the distribution of various cytoplasmic inclusions in the egg at the beginning of cleavage may become considerably different from that in the unfertilized egg, and even qualitatively new areas may sometimes appear. These changes in the organization of the egg at the time of fertilization may be of a most profound importance for further development of the fertilized egg (Gurdon, 1974).

Displacements of the cytoplasmic constituents have been observed best in those eggs where easily distinguishable kinds of cytoplasm are evolved during the growth and maturation of the oocytes; for example, the formation of a pole plasm in *Lymnaea* eggs (Raven, 1970), the strictly defined distribution of several kinds of pigment granule-bearing cytoplasms in *Styela* eggs (Conklin, 1905), the supraequatorial distribution of ascorbic acid-containing granules in *Aplysia* eggs (Peltrera, 1940), the appearance of grey crescent in amphibian eggs (Pasteels, 1964), and so on. Although displacements of materials are not microscopically detectable in *Cerebratulus* eggs, factors

specifying the apical tuft and gut seem to be accumulated in defined sites of the egg following fertilization (Yatsu, 1904; Freeman, 1978).

A conspicuous segregation of ooplasm also occurs after fertilization in the freshwater oligochaete, *Tubifex*; the animal and vegetal pole plasms become apparent before the beginning of cleavage (Penners, 1922). These plasms are enormously rich in mitochondria but devoid of yolk granules (Lehmann, 1956; Weber, 1958). The segregation becomes apparent immediately after extrusion of the second polar body. When *Tubifex* eggs are centrifuged during metaphase of the second meiotic division, contents of the eggs are dislocated to different parts in individual eggs and are separated into different layers. In the eggs left to themselves after centrifugation, the pole plasm material starts flowing and is rearranged in the interior of the egg, thus redistributed in the animal and vegetal poles. The appearance of pole plasms in these centrifuged eggs is synchronous with that in intact (not centrifuged) eggs. When the centrifugal force is applied to eggs which have already completed the ooplasmic segregation, however, the pole plasms are hardly displaced (Lehmann, 1938). Since the cortical layer of the egg cannot be displaced by the centrifugal force, Lehmann (1940, 1956) supposed that the accumulation of ooplasm is accomplished through an "intracellular affinity" (or interaction) between the pole plasm material and the egg cortex at

the animal and vegetal poles. The importance of the cortex in the ooplasmic segregation is also suggested in *Lymnaea*, *Aplysia* and *Styela* eggs (Raven, 1964; Peltrera, 1940; Conklin, 1931).

The cortical layer of the egg is a thin layer of ooplasm and including the plasma membrane. It is hardly displaced by the centrifugal force. As for the structure of the cortical layer, there are several reports in sea urchin and amphibian eggs. Marsland (1956) suggested that the cortex of *Arbacia* eggs is a gelated layer. However, in the electron microscopic study of the same material, Mercer and Wolpert (1962) argued against such a gelated cortex. From the inside view of the surface layer of sea urchin eggs, Schatten and Mazia (1976) demonstrated scanning electron microscopically that the cortex is composed of a uniformly arranged network of fibrous bundles. A fibrous structure of the cortical layer has been also reported in eggs of various amphibian species (Franke *et al.*, 1976). Although these findings are important for understanding the immovable property of the surface layer at centrifugation of the egg, it is still unknown whether, and if it is the case, how the cortical layer determines the site of accumulation and segregation of specific cytoplasmic materials that are important for the embryonic development. There must be some local difference in the cortical layer of the egg.

An example of local peculiarity in property of the cortical layer may be found in the case of the extrusion of the polar body, which is an event usually found after fertilization in various animals except coelenterates and sea urchins (see Austin, 1965). According to Chambers (1917), a local relaxation of the egg surface occurs at the animal pole and the extrusion of the polar body follows there. Since the meiotic apparatus has been intimately associated with the egg surface at the animal pole (Conklin, 1917), it seems reasonable to suppose that the apparatus exerts some influences on the egg surface causing the relaxation of the cortical layer at the animal pole (Chambers, 1917). When the meiotic apparatus is displaced inward by the centrifugal force, the meiotic division results in the extrusion of an unusually large body from the egg which is often called "giant polar body" (Conklin, 1917; Clement, 1938; Yamamoto, 1971). Therefore, the association of the meiotic apparatus to the surface of the animal pole may confine the area participating in the formation of polar body to be narrow; thus, the extruded polar body is expected to be small in size (see Lehmann and Huber, 1944).

Besides the importance of the cortical layer in the ooplasmic segregation, the egg surface of *Tubifex* shows dynamic contractile activities at the time of extrusion of the polar body, *i.e.*, immediately before the ooplasmic segregation. Meridionally

running grooves are formed on the surface of the animal hemisphere and the equatorial region of the egg. Since the *Tubifex* egg extrudes the first and second polar bodies after fertilization, the contractile activity takes place twice before the beginning of cleavage. As the result of these surface contractile activities, conspicuous changes in egg shape are observed, and a series of the shape-change in such eggs has been called "deformation movement" (Matsumoto and Kusa, 1966). Penners (1922) has stressed the importance of deformation movement in the ooplasmic segregation.

A similar change in egg shape occurs in the case of development in *Ilyanassa* and *Dentalium*; in these animals, the cytoplasm at the vegetal pole of the egg is pushed out in the form of a polar lobe. Although the egg shape of *Tubifex* at the deformed state is different from that of the molluscs, it is probable that the deformation movement is caused, in principle, by the same mechanism as the polar lobe formation (see Lehmann, 1956; Conrad, 1973). Informations for the polar lobe formation are imprinted in the cortical layer of the egg, since the site of polar lobe formation on the egg surface is not changed after centrifugation (Verdonk, 1968; Clement, 1968). In *Tubifex*, however, the running pattern of grooves on the egg surface is easily altered by the centrifugal force (Lehmann, 1946), suggesting that it is determined by factors that are movable

through centrifugation. According to Lehmann (1946), information from the meiotic apparatus is one of these factors. As stated already, the meiotic apparatus of *Tubifex* eggs is intimately associated with the cortical layer at the animal pole. A "special anchorage device" appears to be developed for the association. Therefore, it would be desirable to know the fine structure of the cortical layer at the aminal pole. Such a study might disclose relations of the ooplasmic segregation, the deformation movement, the meiotic apparatus and the cortical layer.

The deformation movement takes place at the time of extrusion of the first and second polar bodies and immediately before the furrowing at the egg cell divisions. It would be worthwhile to study why the contraction is seen on the egg surface at the specific periods. Is there any structural change in the cortical layer of *Tubifex* eggs during the deforming and non-deforming phases ? If any, what factors control such changes ? In order to know the relation between the deformation movement and the ooplasmic segregation, an analytical study of the contractile activity of egg surface was performed.

In the first part of the paper, the fine structure of cortical layer at the animal pole and in the equatorial region observed by the transmission and scanning electron microscopes was described. Changes in the structure of these regions were followed up to the time of completion of the second meiotic

division (immediately before the appearance of pole plasms). In the next part, the contractile activity of the surface was observed after treatment of eggs with cytochalasin B which is known to exert effects on the function and structure of microfilaments. Furthermore, by the use of calcium ionophore, the underlying mechanism of periodic appearance of the activity was studied in relation to the intracellular Ca^{++} level. In the last part, factors which determine the running pattern of grooves on the egg surface were analyzed by a series of centrifugation experiments. Involvement of the meiotic apparatus in the surface contractile activity was also examined.

MATERIALS AND METHODS

Animals: Materials used in the present study were a freshwater oligochaete, *Tubifex hattai*, collected from the stream running through the campus of Hokkaido University. This species is one of the most common freshwater oligochaetes in Japan and inhabits also in Europe and Canada. In natural circumstances, the worms usually form clumps either with the same or different oligochaete species (*Limnodrilus* sp., *Branchiura* sp., *Rhizodrilus* sp., *etc.*) on the soft muddy bottom of stream.

The distinction and separation of *Tubifex hattai* from other oligochaete species could be easily accomplished. By a gentle stirring of environmental water, *Tubifex* and *Limnodrilus* could be separated from other species, since these worms were very contractile worms and showed a particular reaction to the agitation of environmental water (Yamaguchi, 1965). Furthermore, *Tubifex* was distinguished from *Limnodrilus* having an enveloping muddy tubes. These muddy tubes were not removed off from the body surface of *Limnodrilus* by washing in running water.

The breeding season of *Tubifex hattai* appears to be unlimited (Hirao, 1964), since the worms possessing oocytes in the ovisac are found at any season of the year in natural as well as in laboratory conditions. It was found, however, that the number of mature worms decreased slightly in winter (from November to February) even in laboratory conditions (rearing temperature, 12°±1°C).

For rearing, collected worms were placed in vats containing well water. The bottom of the vats was covered with 30-40 mm thick soft mud. The rearing vats were cooled by constantly running water at 12°±1°C. The worms were nourished with yeasts, boiled squashed potatoes and corn-flour every two weeks. One day before use, clumps of worms were taken out and washed several times in running water for removing soft mud. They were

transferred into new vats without mud.

Eggs: To obtain newly laid eggs, mature worms possessing large oocytes in the ovisac were placed in finger bowls containing well water and a 15 mm thick layer of sterilized sand (Hirao, 1965). The bowls were placed in a dark room at 20°-25°C. In this system, starved worms deposited cocoons within 1-4 days. By the inspection of contents of the bowl at 3-4 hours intervals, cocoons deposited were collected in a petri dish containing a culture medium described below and kept at 18°-20°C. Worms which had already deposited cocoons were returned to vats with soft muddy bottom.

Synchronously developing eggs were freed from a single cocoon with the aid of a pair of watchmaker's forceps in the culture medium. By the observation of egg shape and the polar body in the perivitelline space, developmental stages of these eggs were determined under the dissecting microscope. Eggs were allowed to develop in the culture medium until they attained to desired stages.

Composition of culture medium: For the culture of developing eggs dissected out of cocoons, Inase's culture medium (1960b) was used. It was composed of 5 mM NaCl, 1.4 mM KCl, 8.1 mM $CaCl_2$ and 2 mM $MgSO_4$ and adjusted to pH 7.1 with $NaHCO_3$. In some experiments, a Ca^{++}- and Mg^{++}-free culture medium was used. It had the same osmotic pressure as Inase's medium and contained 5 mM EDTA.

Light microscopy: Besides the observation of live eggs under the dissecting microscope, the internal structure of eggs were studied in sections. For this purpose, eggs were fixed in Zenker's fluid for 6 hours. They were washed in running water overnight and dehydrated through a graded series of ethanol. After an overnight immersion in methyl benzoate, eggs were embedded in paraffin. Serial sections were cut at 8 μm thick and adhered on glass slides with albumen-water method. Deparaffinized sections were stained with Delafield's hematoxylin and eosin.

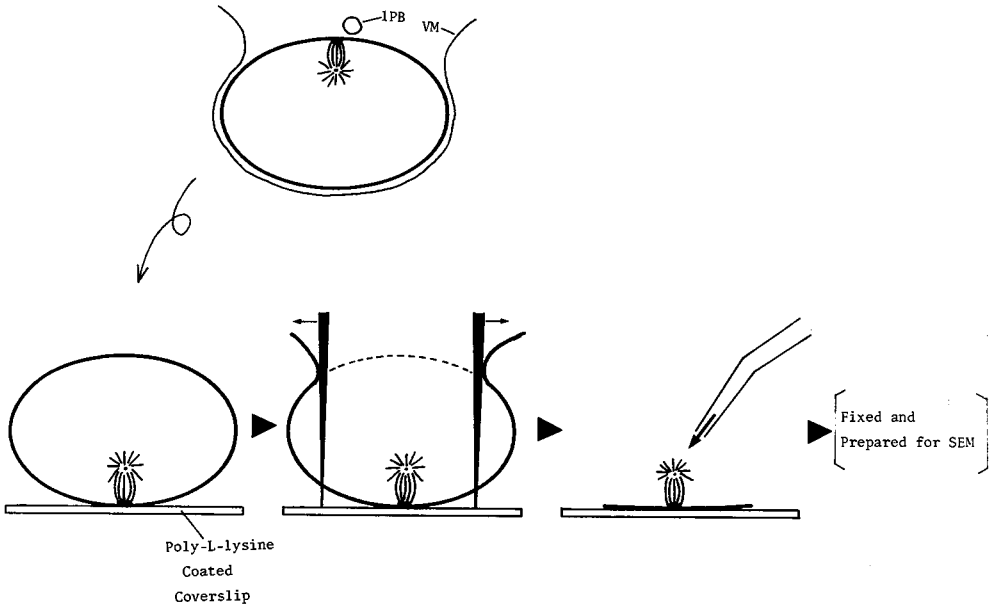
Transmission electron microscopy: Eggs were fixed in 5% glutaraldehyde for 6-12 hours and rinsed overnight in 6 changes of 0.1 M sucrose. After each egg was halved or quartered with a sharp razor, pieces of eggs were postfixed in 1% osmium tetroxide for 2 hours. The fixatives and 0.1 M sucrose were made with 0.1 M sodium cacodylate-HCl buffer solution (pH 7.4) and all the procedures were performed at 0°-4°C. After dehydration with ethanol, pieces of eggs were embedded in Epon 812 according to the method of Luft (1961). Sections were cut with glass knives on a Porter-Blum ultramicrotome MT-1. Thin sections in silver to gold ranges were mounted on copper grids. They were observed in a Hitachi HS-7 or JEOL JEM-100S electron microscope after successive staining in half saturated aqueous solution of uranyl acetate (for 10 minutes) and in Reynolds' lead citrate solution (for 1 minute). In order to know the gross structure of pieces, 1 μm thick

sections were observed under the light microscope after staining in 1% toluidine blue.

Scanning electron microscopy: After fixation in glutaraldehyde, whole eggs were postfixed in osmium tetroxide and dehydrated in ethanol. The removal of the vitelline membrane was carefully performed with a pair of watchmaker's forceps. These eggs were dried in a Hitachi critical point dryer (model HCP-1) by the technique using CO_2 with amyl acetate as the transition fluid (Anderson, 1951) and coated with a thin layer of gold. They were observed in a Hitachi-Akashi MSM-2 or JEOL JSM-T20 scanning electron microscope.

Isolation of egg cortex: A clean square coverslip of 5x5 mm in size was immersed in a 0.1% aqueous solution of poly-L-lysine-HCl (Protein Res. Fund., Osaka); the coverslip was washed with deionized water (Mazia *et al.*, 1975). The polylysine-coated coverslip thus prepared was placed in a petri dish containing 5 ml of the following "cortex isolating solution" modified from Forer and Zimmerman (1975): 5% glycerol, 1% dimethyl sulfoxide, 0.5 M $MgCl_2$, 5 mM phosphate buffer and 0.01 mM EGTA [ethylene-glycol-bis(β-amino-ethylether)- N, N'-tetra acetic acid] (pH 7.0).

The vitelline membrane of eggs immediately after extrusion of the first polar body or some time later was carefully removed with watchmaker's forceps. Two or three of these eggs were placed onto a polylysine-coated coverslip with the animal pole



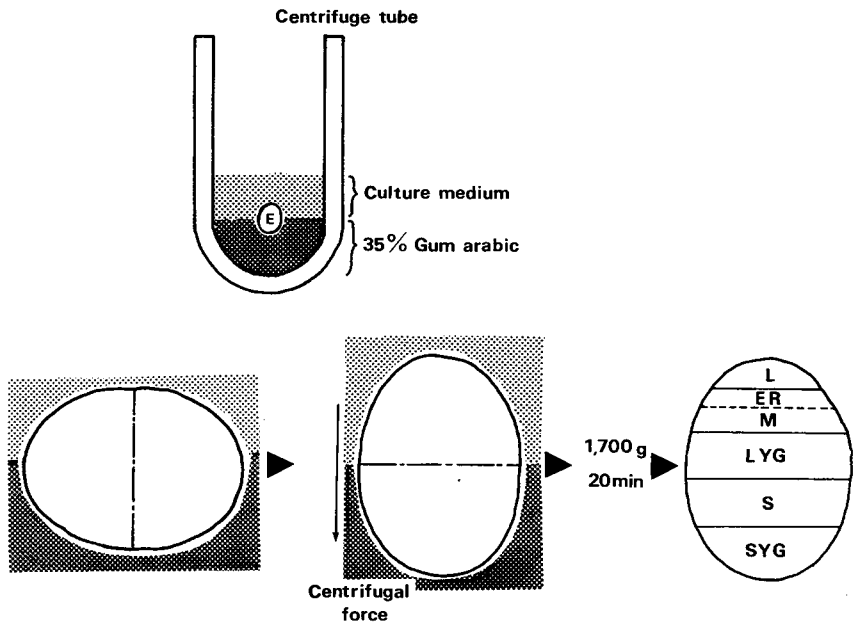
Text-fig. 1. The procedure for isolation of the cortical layer of the animal pole. In the "cortex isolating solution", the vitelline membrane is removed from the egg at early I-3 stage. The eggs are placed on the polylysine-coated coverslip with their animal pole downward. The upper part and sides of the egg which are not in contact with the coverslip are removed. The remaining cytoplasm is flowed away by a jet stream of the "cortex isolating solution". 1PB, first polar body.

downward (Text-fig. 1). The eggs adhered electrostatically to the positively charged polylysine-coated surface of the coverslip. The upper half (vegetal hemisphere) and the sides of the eggs which are not in contact with the coverslip were carefully removed with a pair of watchmaker's forceps; by this procedure, the animal hemisphere of the egg was obtained to be attaching to the coverslip. The endoplasm remaining in the dissected animal hemisphere was flowed away by a jet stream of "cortex isolating solution". The stream was applied to the hemisphere by hand from a pipette with a tip of 200-300 μm diameter at

an angle of about 45° degree (Text-fig. 1). The flow rate of the solution was 3-5 ml/min. The isolation of the egg cortex was finished within 1 minute and was carried out at room temperature (about 20°C).

Immediately after the isolation, 5 ml of 10% glutaraldehyde (in 0.1 M cacodylate-HCl buffer, pH 7.4) were applied to the egg cortex by means of a pipette with a tip of 5 mm diameter. The solution in the petri dish was gently mixed with and replaced by freshly prepared 5% glutaraldehyde (in 0.05 M cacodylate buffer, pH 7.4). The renewal of solution was repeated at least 3 times. Care was taken to keep the coverslip under the surface of the solution in the renewal of the solution, because the surface tension of the fixative could easily disperse and destroy fragile structures such as a meiotic apparatus associated with the egg cortex. After immersion in 5% glutaraldehyde for 30 minutes at room temperature, the fixed specimens on the coverslip were rinsed in 0.1 M sucrose for 60 minutes and postfixed in 1% osmium tetroxide (in 0.05 M cacodylate buffer, pH 7.4) for 60 minutes at 0°-4°C.

Centrifugation of eggs: In order to apply the centrifugal force perpendicularly to the egg axis, a system described by Lehmann (1948) was used. Inase's culture medium was gently poured to the above of 35% gum arabic solution (in deionized water) contained in a glass centrifuge tube; the contents of

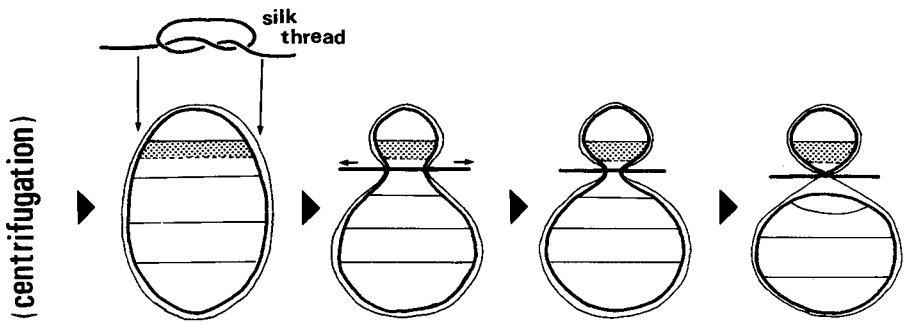


Text-fig. 2. The apparatus for the centrifugation of eggs and the stratification of the egg interior. The egg (E) is placed in the culture medium-gum arabic system prepared in a glass centrifuge tube. It floats at the boundary between the culture medium and gum arabic. When the centrifugal force is applied, the egg axis (dashed line) is perpendicular to the centrifugal axis. The egg interior is stratified into 5-6 layers: L (lipid droplet), ER (endoplasmic reticula), M (mitochondria), LYG (large yolk granule), S (opaque plasma), and SYG (small yolk granule) layers.

the tube were separated each other and the boundary was rather clear (Text-fig. 2). When eggs were put into the tube, they were floated at the boundary; the egg axis was placed parallel to this boundary when the centrifugal force was applied. Eggs were centrifuged in this system for 20 minutes at 4000 r.p.m. (1700*g*). The developmental stage of eggs at which the centrifugation was performed ranged from immediately after extrusion

of the first polar body to the extrusion of the second polar body. Immediately after the end of centrifugation, they were washed several times in Inase's culture medium at 18°-20°C. In order to know what change in the centrifuged eggs corresponds to an event in the normal development, intact (not centrifuged) eggs from the same cocoon served as the control.

Production of egg fragments: Immediately after the extrusion of the first polar body, eggs were centrifuged. A loop of silk thread of a suitable size was hung on and gradually constricted the centrifuged egg with the vitelline membrane (Text-fig. 3). The operation was performed in Inase's culture medium



Text-fig. 3. The procedure to obtain egg fragments. The eggs are centrifuged as shown in Text-figure 2. A looped silk-thread is hung on and the egg is constricted with a pair of watchmaker's forceps. Small nucleated and large non-nucleated egg fragments are produced. The stippled area represents stratified endoplasmic reticular zone.

at 18°-20°C. These ligatured eggs were then washed with the culture medium thoroughly to remove gum arabic. Within about 10 minutes, each egg was separated into halves at the ligaturing point and egg fragments were obtained (Text-fig. 3).

Chemicals and procedures of treatment: Cytochalasin B (Sigma or Nakarai Chem.) and ionophore A23187 (a gift from Dr. R. Hamill of Eli Lilly Co., Indianapolis, Indiana, USA) were dissolved in dimethyl sulfoxide at a concentration of 10 mg/ml and 5 mg/ml respectively. They were stored at -20°C. Aqueous stock solutions of colchicine (10 mg/ml) and 2,4-dinitrophenol (80 μg/ml) were made in deionized water and stored in a refrigerator. Immediately before use, small volumes of these stock solutions were diluted with Inase's or Ca^{++}-and Mg^{++}-free culture medium.

One or two eggs from every cocoon used in experiments served as the control and the remaining 4-7 eggs were treated with drugs. In each experiment, 50-60 eggs from 10-15 cocoons were used. Usually, eggs were treated for a definite period of time with drugs (pulse treatment) and returned to the medium without drugs. In several cases, a continuous treatment with drugs was also performed. All treatments were performed at room temperature (18°-20°C).

Historical Note on Experimental Studies of Early Development

In 1922, Andreas Penners first observed the cleavage of eggs in a European species of freshwater oligochaete, *Tubifex rivulorum* and showed that the first cleavage gives rise to blastomeres of unequal size: small AB and Large CD cells. The latter cell contains clear animal and vegetal pole plasms. Furthermore, the same author (1922, 1923) followed the prospective fate of these blastomeres during embryogenesis and suggested that the pole plasms segregated in 2*d* and 4*d* cells of late cleavage stages play important roles in embryonic differentiation. In order to verify the suggestion, Penners (1924a, b, 1926, 1934, 1937, 1938) performed a series of experiments in the same material: He made the first cleavage to produce two blastomeres of equal size by heat-treatment. Furthermore, Penners killed a certain blastomere of normally cleaving eggs by the ultraviolet irradiation. In these experiments, eggs whose first cleavage was induced to be equal developed to "double embryo". Furthermore, he convincingly demonstrated 1) that, while an aggregation of *A*, *B* and *C* cells without *D* cell develops to only a formless mass of ectoderm and endoderm, isolated *D* cell can produce a small perfectly proportioned animal, 2) that, the nervous system and the circular muscle arise from the ectoderm germ band derived from 2*d* cell, and 3) that, the longitudinal muscle and

primordial kidneys are produced by the mesodermal germ band derived from *4d* cell. These results strongly support the suggested importance of the pole plasm in embryonic differentiation of *Tubifex*. Inase (1960a, 1967, 1968) also worked on the cell lineage during the embryonic development in a Japanese species, *Tubifex hattai*, and came to the same conclusion.

From the late 1930s on, Lehmann and his coworkers of the University of Bern investigated the development of *Tubifex* eggs focussing on the segregation of the pole plasms. Lehmann (1938, 1940; 1941b, 1948) centrifuged these eggs and found that the sites of the segregation of pole plasms are not altered after centrifugation. From these results, he postulated the importance of egg cortex, which may be immovable after centrifugation, in the determination of the site of the pole plasm segregation. Furthermore, Lehmann (1941a, 1948) performed the histochemical study on the pole plasms. He demonstrated a high activity of a mitochondrial enzyme, indophenol oxidase, in them. Subsequently, Carrano and Palazzo (1955) also showed high activities of other mitochondrial enzymes in the pole plasms. These data strongly suggested that the pole plasms are rich in mitochondria. In fact, after introduction of the electron microscope, the abundance of mitochondria in the pole plasms was clearly demonstrated (Lehmann and Wahli, 1954; Lehmann and Mancuso, 1957, 1958; Lehmann and Henzen, 1963; Henzen, 1966; Weber, 1956,1958, 1962).

Another conspicuous phenomenon in the development of *Tubifex* is that the egg shows a series of the shape-change (Penners, 1922; Woker, 1944). As stated already, this is called "deformation movement" (Matsumoto and Kusa, 1966) and is a manifestation of the surface contractile activity of the egg. The movement is regularly observed at the time of the meiotic divisions and cleavage (Huber, 1946). Matsumoto and Kusa (1966) performed a cinematographic analysis of the movement in eggs of Japanese *Tubifex*. Lehmann (1946), observing deformation movement in eggs subjected to the centrifugation, suggested that the site of the meiotic apparatus has relations to shape-change of the egg. According to Woker (1943, 1944) and Rötheli (1949, 1950), however, antimitotic reagents exerted no effects on the deformation movement. Thus, the significance of the meiotic apparatus in the process of the deformation movement still remains to be clarified in future.

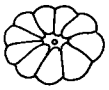
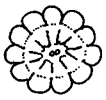
RESULTS

[I]

Description of the Early Stages of Development —from Metaphase of the First Meiotic division to the First Cleavage

The meiotic division in *Tubifex* eggs is initiated during the time when it is present in the ovisac of mature worms (Hirao, 1964): the germinal vesicle breaks down and the first meiotic apparatus is formed in the subcortical region of the animal pole. At oviposition during which fertilization occurs, the egg nucleus is still at metaphase of the first meiotic division (Hirao, 1968).

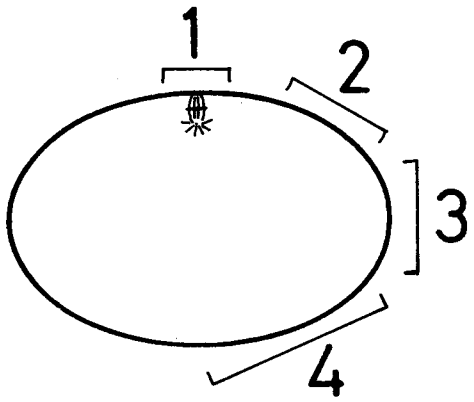
Fertilized eggs require about 10 hours to divide into two cells. For convenience of the description, the developmental period between oviposition and the completion of the first cleavage is divided into 6 stages, I-1 to I-6 (Text-fig. 4).

STAGE	I-1 (60)	I-2 (60)	I-3 (90)	I-4 (120)	I-5 (180)	I-6 (90)
EGG SHAPE						
NUCLEAR EVENT	1st Meiotic Metaphase	1st Polar Body Extrusion	2nd Meiotic Metaphase	2nd Polar Body Extrusion	Karyogamy	1st Cleavage

Text-fig. 4. The time course of *Tubifex* egg between oviposition and first cleavage at 20°C. Numerals in parentheses are the average duration (minutes) of each stage. A typical egg shape and the nuclear events are presented for each stage.

I-1 stage (metaphase of the first meiotic division; spherical egg stage): Immeidately after oviposition, eggs are flaccid in the living state. In sectioned materials, the metaphase spindle of the first meiotic division is seen apart from the egg surface. After 30-40 minutes, the egg becomes spherical in shape (Fig. 1a). Concomitantly with this change of the egg shape, the meiotic apparatus moves toward the egg surface and finally the outer pole of the spindle anchors in the cortical layer of the animal pole (Fig. 1b). The axis of metaphase spindle is perpendicular to the surface. In living eggs, the animal pole is discernible by a yolk-free, clear spot where the first meiotic spindle is located.

I-2 stage (extrusion of the first polar body; the first deformation movement): About 60 minutes after oviposition, the surface of the animal hemisphere and equatorial region of eggs becomes wrinkled (for the terminology of egg regions, see Text-fig. 5). This is the initiation of the first deformation movement. With the lapse of time, many protuberances resulted from the grooving activity of the surface appear around the equatorial region and on the animal hemisphere. In this way, the deformation movement attains the climax (Fig. 2a). The shape-change of eggs lasts about 60 minutes; the protuberances disappear and the egg surface becomes smooth, thus the egg regaining the spherical shape. During the movement, the first polar body is



Text-fig.5. Terminology of egg regions used. 1, animal pole; 2, animal hemisphere; 3, equatorial region; 4, vegetal hemisphere.

extruded from the egg (Fig. 2b).

I-3 stage (metaphase of the second meiotic division; spherical stage between the first and second deformation movement): After completion of the first deformation movement, the smoothness of the egg surface is maintained for about 90 minutes (Fig. 3a). During this period, the second meiotic apparatus is formed at the animal pole (Fig. 3b). The axis of spindle is perpendicular to the egg surface and the outer pole of the spindle anchors in the cortical layer of the animal pole (Fig. 3b).

I-4 stage (extrusion of the second polar body; the second deformation movement): About 3.5 hours after oviposition, the egg surface again becomes wrinkled on the animal hemisphere and equatorial region. This is the initiation of the second deformation movement, which lasts about 2 hours, and conspicuous protuberances are formed on the equatorial surface of the egg at the

climax of the movement (Fig. 4a). The egg at the climax is apparently different in shape from that at the first movement (compare Fig. 4a with Fig. 2a). After cessation of the movement, it returns to the spherical shape. The second polar body is extruded from the egg at the climax of the movement. Chromosomes remaining in the zygote are transformed into karyomeres during the late phase of the movement (Fig. 4b).

I-5 stage (spherical egg stage immediately before the first cleavage; pole plasm segregation): After cessation of the second deformation movement, the smoothness of the egg surface is maintained for about 3 hours. During this period, clear ooplasm devoid of yolk granules and lipid droplets accumulates at the animal and vegetal poles of the egg (segregation of pole plasm) (Fig. 5a). Concomitantly with the pole plasm segregation, karyogamy occurs near the center of the egg (Fig. 5b). At about 1 hour after the onset of stage I-5, the egg forms the first cleavage spindle, whose axis is perpendicular to the animal-vegetal axis of the egg. The asters at both poles of the spindle are different in size.

I-6 stage (first cleavage; the third deformation movement): At about 8.5 hours after oviposition, some grooves appear on the equatorial surface of the egg. This is the initiation of the third deformation movement. All grooves are the same in appearance (Fig. 6a). Meanwhile, two of these grooves becoming

cleavage furrow divide the egg into two cells at 10 hours after oviposition. As the furrowing proceeds, the remaining grooves of the third movement disappear from the equatorial surface of the egg. Since the cleavage asters are unequal in size, small (*AB*) and large (*CD*) blastomeres are resulted from the division (Fig. 6b). The pole plasms are found only in *CD* cell.

[II]

Structural Changes of the Cortical Layer and the Surface Grooving Activity of Eggs

As described in the preceding chapter, the grooving activity is observed on the surfaces of the animal hemisphere and the equatorial region but not on the vegetal hemisphere. In this chapter, the fine structure of the cortical layer is described in relation to the process of the second deformation movement. The term "cortical layer" denotes the peripheral layer of egg cytoplasm including the plasma membrane devoid of membraneous organelles.

(1) *Fine structure of the animal pole*

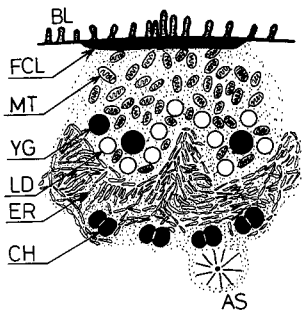
The important karyological events occurring before and during the second deformation movement are: (i) the formation of meiotic apparatus and (ii) the extrusion of the second polar body. In the following description, these events will be treated separately though they are a continuous process.

(i) *Formation of the second meiotic apparatus*

The metaphase spindle of the second meiotic division is found in eggs about 30 minutes after extrusion of the first polar body which occurs at I-2 stage. Previous to the formation of the spindle, microfilaments are detected in the cortical layer of the proximal part of the cytoplasmic bulge for the first polar body which is formed on the surface of the animal pole (mid I-2 stage) (Fig. 7). The individual filament measures 5-7 nm in diameter. Concurrently with the constriction of the proximal part of the bulge, mitochondria and lipid droplets are accumulated near the constriction in the egg proper (Fig. 7).

I-3 stage, 0 minute: At the beginnig of I-3 stage, no trace of the meiotic apparatus is detected at the animal pole. Beneath the cortical layer of the animal pole, there is a large aggregation comprising mitochondria, lipid droplets and some yolk granules where the first meiotic apparatus had been

previously located (Fig. 10). This aggregation appears to be intimately associated with the inner aspect of the cortical layer. Furthermore, there is an accumulation of endoplasmic reticula with some microtubules around the aggregation (Fig. 10). The accumulation takes an appearance as if it is extruded from the center of the aggregation (Text-fig. 6). However, these masses of cytoplasmic inclusions do not seem to be closely related with each other. The degrees of associations between the cortical layer and the aggregated mitochondria, lipid droplets and yolk granules and between this aggregation and the accumulation of endoplasmic reticula and microtubules are studied in the following way. The animal pole of the egg is fixed on a polylysine-coated coverslip; after removal of the vegetal hemisphere by fine forceps, the ooplasm remaining in the animal hemisphere is washed away by the "cortex isolating solution". Figure 11 shows



Text-fig. 6. Diagrammatic illustration of the fine structure of animal pole at I-3 stage, 0 min. AS, aster; BL, blebs; CH, chromosome; ER, endoplasmic reticula; FCL, filamentous cortical layer; LD, lipid droplet; MT, mitochondria; YG, yolk granule.

the inside view of the animal pole of the egg thus prepared. The aggregation is found in association with the cortical layer. However, the accumulation of endoplasmic reticula and microtubules around the aggregation mostly disappears from these preparation; only a portion connected with the central portion of the aggregation can be seen (Fig. 11, asterisk).

The cortical layer is electron-dense, 0.2-0.3 μm thick at the limited area of the animal pole, but is so thin in some other places adjoining this area that it appears as if no cortical layer is present in these places (Fig. 10). The cortical layer is rich in microfilaments which seem to be a part of those observed at the time of the extrusion of the first polar body (Fig. 13; also see Fig. 7). Each of these filaments measures 5-7 nm in diameter. A number of vesicles separate the cortical layer from the subcortical region of the egg (Fig. 13). A relatively clear cortical layer is obtained only when a rather strong jet of the "cortex isolating solution" is applied to the inner surface of the animal hemisphere dissected out from the egg in the way described above. The aggregation comprising mitochondria, lipid droplets and yolk granules located just beneath the cortical layer of the animal pole disappears in such preparation. However, some vesicles are seen embedded in the isolated cortical layer (Fig. 12). As seen in Figure 12a, the cortical layer is thickest at the animal pole and becomes

thinner toward the periphery of the pole, but it is not tapering uniformly in all directions so that the thick cortical layer at the animal pole looks as if it protrudes pseudopodial processes toward the periphery of the pole (Fig. 12). In the scanning electron micrograph of the outer surface of the egg, a considerable number of blebs are seen in the area at the animal pole. The blebs are particularly abundant on the limited area of the pole, but on the region around this limited area they decrease in number. Furthermore, their distribution is not homogeneous (Fig. 8): In the isolated cortical layer, the distribution of blebs coincides with the tapering route of the thick cortical layer tentatively named pseudopodial processes (compare Fig. 8 with Fig. 12).

I-3 stage, 15 minutes: The cytoplasmic organelles are neither aggregated nor accumulated at the animal pole. In the subcortical region of the animal pole, formation of the second meiotic apparatus is in progress. However, the apparatus is structurally not the same with that of the fully formed one; although the inner aster looks fully developed, the peripheral aster and the spindle are rather small in size. Careful observations reveal that microtubules constituting the peripheral aster are apparently smaller in number than those constituting the inner one. A centriolar structure is detected in the center of the inner aster (see Fig. 23) but not in the peripheral aster.

The length of spindle fibers is nearly the same with that of fully second meiotic spindle. The chromosomes do not possess kinetochore microtubules on their surface (Fig. 15) and are not regularly arranged on the metaphase plate of the spindle; they are found in a region near the outer (peripheral) pole, *i.e.*, the boundary region between the peripheral aster and the spindle. A number of mitochondria and lipid droplets are found in this particular region, while these inclusions are never detected in the region exclusively occupied by peripheral aster and spindle microtubules (Fig. 14).

As in the preceding phase, the cortical layer of the animal pole is still electron-dense and rich in filamentous materials. The cortical layer is now invaded by microtubules of the peripheral aster of the second meiotic apparatus (Fig. 16). So far as the present observation is concerned, it is not clear whether these microtubules terminate on the plasma membrane or not. As seen in Figures 14 and 16, the surface of the animal pole possesses a number of villous projections. Since these projections contain vesicles and some microtubules of the peripheral aster, they are different in structure from microvilli containing highly organized microfilaments. Blebs observed in the scanning electron microscopy on the surface of the animal pole are no other than these projections. Thus, villous projections of the cytoplasm on the surface of the animal pole will be referred to as

blebs in the present study. These blebs are now distributed in groups on the narrow area of the animal pole and take an appearance of a bouquet placed on the surface (Fig. 9).

Although the inner aster of the developing meiotic apparatus is always lost, the bulk of the apparatus can be isolated from the animal hemisphere of the egg with the overlying cortical layer (Fig. 17). This fact indicates that the associations of the cortical layer, peripheral aster and the spindle are intimate but the inner aster is not closely connected with the spindle. Figure 18 clearly shows the intimate association of the peripheral aster with the cortical layer.

I-3 stage, 30 minutes: At the animal pole, the fully developed meiotic apparatus can be found, in which the number of microtubules of the peripheral aster and the spindle is evidently larger than that of the preceding phase (Figs. 19 and 20). Some mitochondria and lipid droplets are still detected in the boundary region between the peripheral aster and the spindle (Fig. 19). Chromosomes with fully elongated kinetochore microtubules (see Fig. 22) are regularly arranged on the metaphase plate of the spindle (Fig. 19).

The fine structure of the cortical layer is the same with that in the preceding phase (Fig. 20). Microtubules from the peripheral aster are found to penetrate into the electron-dense,

filamentous cortical layer (Fig. 20). Some of them extend into blebs on the animal pole. The animal pole of eggs is externally recognized by a bouquet of these blebs. The intimate association of the meiotic apparatus with the cortical layer is also seen in these eggs. Unlike the preceding phase, the meiotic apparatus isolated from eggs usually rises perpendicularly to the polylysine-coated coverslip (compare Fig. 21 with Fig. 17). This may be due to the increased degree of association of the apparatus with the cortical layer. The inner aster is, however, lost from the apparatus during the isolation; thus, the association of the inner aster with the spindle seems to be less intimate.

(ii) *Extrusion of the second polar body*

After the second meiotic apparatus is fully developed, eggs remain at metaphase for about 60 minutes (I-3 stage). As the meiosis proceeds further, some changes on the outer surface of the animal pole become apparent through the scanning electron microscopy (Fig. 28, compare with Fig. 9). At late metaphase, the outer surface of the pole is classified into two areas, undulating area and smooth area (Fig. 28). The undulating area bears a number of blebs. These blebs are smaller in size than those found in the preceding phase and arranged to form a bouquet at the animal pole. Although the undulating area is a circular zone of the animal pole, the bouquet always takes an eccentric

position in the zone (Fig. 28). In the cytoplasm just beneath the undulating area, the meiotic apparatus is found in sections. The diameter of the circular undulating area is comparable to the width of the spindle. The smooth area surrounds the circular undulating area.

The fine structure of the cortical layer is different each other in these areas. In the smooth area, the layer is electron-dense and contains microfilaments and microtubules of the peripheral aster. The diameter of each filament is 5-7 nm. Although some filaments and microtubules are found in the portion having blebs on its outer surface, the cortical layer of the undulating area is usually very thin and devoid of microfilaments. Careful observations of microfilaments and microtubules reveal a different pattern of distribution in the cortical layers of the smooth area and of the undulating area with surface blebs: microfilaments and microtubules run parallel to the surface in the smooth area, though they are arranged perpendicularly or obliquely in the region near the undulating area (Fig. 30). In the undulating area having surface blebs, a few number of microfilaments run parallel to the surface but microtubules show a random arrangement (Fig. 30). Blebs on the surface of the animal pole contain a certain number of microtubules and vesicles but no microfilaments (Fig. 29).

As the *anaphase* movement of chromosomes is initiated, the egg surface swells out just over the meiotic apparatus and the formation of a cytoplasmic bulge occurs. At the same time, the asters located at both poles of the apparatus become indistinct. The outer pole of the spindle is now found in the cytoplasmic bulge of the surface. The distal cytoplasm of the bulge is rich in mitochondria and lipid droplets (Fig. 32). The diameter of of the bulge is almost comparable to the width of the metaphase spindle (Fig. 32). When the egg surface is observed in the scanning electron microscope, the distal portion of the cytoplasmic bulge has rugged surface, whereas the other regions of the bulge possess the relatively smooth surface (Fig. 31). Judging from the external structure and the dimension, it may be thought that the surface of the distal portion of the bulge corresponds to the undulating area of the preceding phase. Although some blebs are still found on the surface of the distal portion of the bulge, the clear bouquet of blebs that had been present previously is not seen at this phase. Instead, a number of rod-shaped processes are found on the other regions of the surface of the cytoplasmic bulge (Fig. 31).

Except in the top and proximal regions of the bulge, the cortical layer is thin and does not contain filamentous materials in the cytoplasmic bulge; vesicles in the subcortical region appear as if they are directly exposed to the surface, but

their actual fusion with the plasma membrane does not take place. In the top and the proximal region of the bulge, the cortical layer is thick and contains filamentous materials (Figs. 33 and 34). In particular, the cortical layer of the proximal region is considerably thick and contains a number of microfilaments. These filaments run perpendicularly or obliquely to the surface (Fig. 34). The cortical layer of the egg proper which is connected to that of the proximal region of the bulge is also thick and contains microfilaments. They run parallel to the surface. Microtubules are few in number in the cortical and subcortical regions of these eggs and actually no microtubules are detectable in the distal region of the bulge (Fig. 34).

At *telophase*, the cytoplasmic bulge is constricted from the egg proper (Fig. 36), but no remarkable changes in the structure of the surface are seen (Fig. 35). Except the constricted region, the cortical layer of the bulge is very thin and contains no microfilaments (Fig. 36). Mitochondria and vesicles are always found near the surface of the bulge (Fig. 39). In the constricted region connecting the bulge with the egg proper, however, the cortical layer is thick and contains microfilaments (Figs. 37 and 38). These microfilaments are arranged perpendicularly or obliquely to the surface. In this phase, the chromosome decondensation begins in the egg proper (Fig. 39, inset) but not in the constricted bulge (Figs. 36 and 39).

(2) *Fine structure of the equatorial region*

Up to the time of the first cleavage, the cortical layer of the equatorial region shows periodic alterations in structures in relation to the deformation movement. At non-deforming I-1, I-3 and I-5 stages, the layer is very thin and membraneous organelles of the ooplasm lie near the plasma surface of the egg, whereas in eggs undergoing the deformation movement (I-2 and I-4 stages) the cortical layer is thick and rich in filamentous materials.

(i) *Chronological description of the second deformation movement —— observations in living eggs*

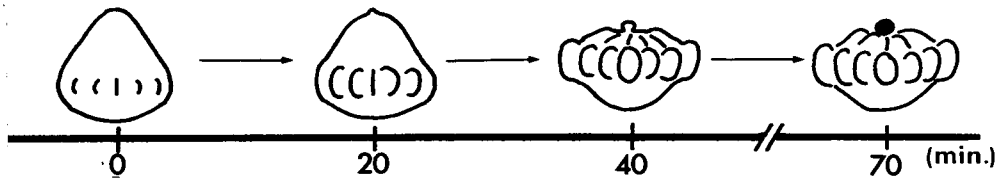
At about 3.5 hours after oviposition (beginning of I-4 stage), the initiation of the second deformation movement is noticed by the appearance of dimples on the smooth egg surface. The dimples are formed at intervals on the equatorial surface and soon develop into shallow grooves (*shallow groove phase*) (Fig. 44a). Within 30-40 minutes, grooves increase in depth and the deformation movement attains its climax, so that the egg looks as if it possesses many protuberances on the equatorial surface. In the *climax phase*, each protuberance is constricted around its proximal part (Fig. 45a). After persistence of the climax phase that lasts for 30-40 minutes, the first symptom of relaxation appears: the proximal constrictions of protuberances relax

(*relaxing phase*) (Fig. 46a). Equatorial grooves gradually disappear within 20-30 minutes and the egg surface becomes smooth, which results in the rounding up of the egg (Figs. 47a and 48a). The second polar body is extruded from eggs during the movement. The time-course of the second deformation movement is diagrammatically illustrated in Text-figure 7.

(ii) *Organization of microfilaments in the cortical layer*

The fine structure of the equatorial cortical layer was observed in eggs at different phases of the deformation movement.

The cortical layer at the bottom of shallow grooves is 0.2 μm thick and contains a small number of microfilaments (about 15 filaments per 1 μm long section of the cortical layer) (Fig. 44b). These filaments are relatively short in length and arranged perpendicularly or obliquely to the surface (Fig. 44c). In the climax phase, the cortical layer is considerably thick at the bottom of the groove, where microfilaments are longer than the preceding phase and increase in number (about 100 filaments per 1 μm long section of the cortical layer). The arrangement of microfilaments in the cortical layer of the groove bottom is the same with that in the preceding phase (Fig. 45b). The surface of grooves is highly indented. Figure 52b shows a scanning electron micrograph of the infinitestimally indented surface of the bottom. This fact may suggest that microfilaments in the cortical layer are the structural components



Text-fig. 7. The time course of the second deformation movement between the shallow groove phase and the climax phase.

responsible for the grooving activity of *Tubifex* eggs; the contraction of filaments arranged perpendicularly to the surface in the cortical layer may pull the plasma membrane inward, thus forming the grooves. If so, the outer end of perpendicularly arranged filaments must have some connection with the plasma membrane and the inner end anchors on the surface of membraneous organelles in the subcortical layer of the egg.

The number of microfilaments in the cortical layer of the groove bottom apparently decreases in the mid-relaxing phase. At the same time, they show a random arrangement in the cortical layer. In the late relaxing phase, the cortical layer at the bottom contains only a small number of microfilaments (about 7 filaments per 1 μm long section of the cortical layer) (Fig. 47b, c). Each filament also decreases in length. Shortly after completion of the deformation movement, a few number of

filaments are still seen in the cortical layer of the equatotial region of spherical eggs (Fig. 48b, c).

As mentioned already, the increase in depth of grooves during the deformation movement results in the formation of protuberances on the equatorial surface. The cortical layer of protuberances is very thin in the distal part; membraneous organelles such as mitochondria and endoplasmic reticula are found near the surface. Throughout the course of the deformation movement, microfilaments are not seen in the distal part of protuberances. On the other hand, the cortical layer of the proximal part of protuberances adjoining the bottom of grooves is relatively thick and contains microfilaments arranged obliquely to the surface.

(3) *Evidence for the involvement of microfilaments in the surface grooving activity — Effects of cytochalasin B*

In the preceding sections, it was noticed that a number of microfilaments are detected invariably at certain sites in the cortical layer exhibiting an active contractile activity; for example, the cortical layer at the proximal constriction of the cytoplasmic bulge during the polar body formation and at the bottom of grooves during the deformation movement. The

cortical layer of the animal pole was rich in filamentous materials after completion of the first meiotic division (I-2 stage). It may be possible to suppose that microfilaments in the animal pole of the egg at metaphase of the second meiotic division (I-3 stage) originate, at least in part, from those found in the furrowing site of the first meiotic division. As stated already, the intimate association exists between the second meiotic apparatus and the cortical layer at I-3 stage. It is unknown whether microfilaments in the animal pole participate in this association or not.

In order to verify the active participation of microfilaments in the polar body extrusion and the deformation movement, the following experiments were performed. For this purpose, cytochalasin B is a useful tool, since it is well known to inhibit such contractile events as cell division and cell movement which are microfilament-dependent processes (Wessells *et al.*, 1971; Copeland, 1974).

In a preliminary experiment, 0.5% dimethyl sulfoxide (DMSO), which was used to dissolve cytochalasin B, was found not to exert any detectable effect on the process of the polar body extrusion and the deformation movement. Furthermore, no appreciable difference in the effectiveness of cytochalasin B was recognized whether the vitelline membrane of eggs was removed or not. Therefore, the following experiments were performed using eggs with the vitelline membrane.

(i) *Effects on the animal pole*

Eggs at 20 minutes before the beginning of I-3 stage (undergoing the first deformation movement, I-2 stage) were immersed in the solution of 50 μg/ml cytochalasin B for 40-60 minutes and fixed immediately after the treatment. During the treatment, the first polar body is extruded from the egg and the second meiotic apparatus is formed. In the intact control eggs, the electron-dense cortical layer of the animal pole is 0.2-0.3 μm thick and is rich in filamentous materials: The peripheral and inner asters of the second meiotic apparatus have been well developed. Chromosomes on the metaphase plate possess well developed kinetochores (Fig. 27). The outer surface of the animal pole possesses a bouquet of blebs. In cytochalasin B-treated eggs, following facts are noticed: (a) The electron-dense cortical layer of the animal pole is thinner than the intact control eggs. The former eggs contain only a few filamentous materials (Fig. 25). (b) The inner aster has been fully developed (Fig. 24) but microtubules that form the peripheral aster are small in number. (c) The regions as usual occupied by the peripheral aster and the spindle in intact eggs are largely filled with endoplasmic reticula (compare Figs. 25 and 26 with Figs. 19 and 20). A number of mitochondria are present in the region of the peripheral aster (Fig. 25). (d) Blebs on the

outer surface of the animal pole are small in number. These facts suggest that the formation of microtubules has been inhibited in these eggs. Therefore, cytochalasin-sensitive structure(s) responsible for the microtubule assembly of the peripheral aster and the spindle may exist in *Tubifex* eggs. In all of cytochalasin-treated eggs, the extrusion of the second polar body is invariably inhibited, though the anaphase movement of chromosomes proceeds normally.

In order to know the exact mechanism of the failure in the extrusion of the second polar body, the process of the meiotic division was observed in detail in eggs treated with cytochalasin B for 60 minutes at the beginning of I-3 stage (after completion of the first deformation movement). In these eggs, the cytoplasmic bulge for the polar body is once formed on the surface of the animal pole (Fig. 40). The outer surface of the top and the periphery of the bulge is densely covered with blebs, whose number per unit area is much larger than that in the intact control egg (compare Fig. 41 with Fig. 31). When the animal pole of experimental eggs is observed in sections, the egg surface is indistinct owing to the presence of a number of blebs (Fig. 42). In some places, bundles of microfilaments running parallel to the surface can be seen in the cortical layer (Fig. 43).

With the lapse of time, the cytoplasmic bulge is absorbed into the egg proper without attaining the maximal height observed in the intact egg (compare Fig. 41 with Figs. 31 and 35). As a result, the egg surface of the animal pole becomes flat. In these eggs, the external structure of the animal pole is similar to that of the egg with the low cytoplasmic bulge: together with a considerable number of blebs, the egg surface is very indistinct. The cortical layer of the pole never possesses microfilaments but contains fine granules. Since the similar fine granules are not observed in the intact eggs, these granules may represent residues of microfilaments disrupted by cytochalasin B. Therefore, the failure of the polar body extrusion may be resulted from the disruption of, if not all, microfilaments in the cortical layer of the animal pole.

(ii) *Effects on the equatorial region*

When eggs at early I-4 stage (shallow groove phase of the second deformation movement) are immersed in 50 μg/ml cytochalasin B, the disturbance in continuation of the movement is apparently observed 40 minutes later on the equatorial surface of the egg. During this period, the deformation movement in the intact control eggs advances nearly to the climax phase which persists for a further 30-40 minutes. In the experimental eggs, however, the movement is already at the relaxing phase:

the eggs round up during 10-15 minutes without showing distinct features of the climax phase.

Cytochalasin B-treated eggs were fixed shortly before and after appearance of the disturbance of the movement (30 and 45 minutes after immersion in cytochalasin). In eggs immersed for 30 minutes, no difference in the organization of microfilaments in the cortical layer is detected between the experimental and control eggs: the filaments lie in the bottom of grooves and are arranged perpendicularly or obliquely to the surface. No filaments are observed in the distal portion of protuberances. In eggs treated with cytochalasin for 45 minutes, however, the cortical layer is thin compared with that of the control eggs and contains no distinct microfilaments (Fig. 49).

In other experiments, non-deforming spherical eggs at I-3 stage were treated with cytochalasin of the same concentration for 40 or 60 minutes and were transferred into the normal culture medium without the drug (pulse treatment). Immediately after treatment, the control and experimental eggs were still spherical in shape (I-3 stage). When the intact control eggs initiate the second deformation movement, the inhibition of the movement becomes obvious in cytochalasin-treated eggs. At least, in eggs subjected to the pulse treatment for 60 minutes, no sign of the deformation movement is detected on the

Table 1

Effects of immersion in 50 μg/ml cytochalasin B on the second deformation movement

Stage of immersion-onset	Length of immersion (min.)					
	5	10	20	30	40	60
Early I-2	++	++	++	+	+	−
Early I-3	++	++	++	++	+/−	−
Mid I-3	++	++	++	++	+/−	−
Early I-4	++	++	++	++	(+)*	(+)*

++, normal deformation movement; +, regressed deformation movement and shortening of its duration; -, no grooving; (+)*, deformation movement which entered the relaxing phase without climax phase.

egg surface (Table 1). These eggs do not form grooves on the equatorial surface until the cleavage stage of the control eggs. If the pulse treatment is performed for 40 minutes, the inhibition of the deformation movement is incomplete; some eggs form grooves but remainings do not. Grooves are, even if they are formed, shallower and smaller in number than those of the intact control eggs. At the climax phase of the control eggs, protuberances formed on the equatorial surface of experimental eggs are not conspicuous (Fig. 51a). No constriction appears around the proximal portion of protuberances, thus the inhibited movement of experimental eggs does not attain the typical climax phase. Within 50-60 minutes all grooves disappear from the equatorial surface of these eggs but are observable for about 100 minutes in the control eggs.

If the pulse treatment for 30 minutes is performed in the early phase of the first deformation movement (I-2 stage), the first movement itself is not influenced by cytochalasin B but the incomplete second movement occurs (Table 1).

In order to know why the second deformation movement is entirely abrogated in the above experiments, the fine structure of the cortical layer of the equatorial region was observed in sections. As stated already, no microfilaments are detected in the cortical layer of eggs treated for 60 minutes at I-3 stage and completely inhibited the movement. In eggs treated for 30 minutes at I-2 stage or for 40 minutes at I-3 stage and showed the incomplete second deformation movement, however, some microfilaments are found in the cortical layer of grooves formed. Unlike the perpendicular arrangement in the control eggs, these microfilaments run parallel to the surface of grooves (Fig. 50). When these grooves are observed with the scanning electron microscope, folds observed in experimental eggs are much smaller in number and shallower than those observed in the intact control eggs (compare Fig. 51 with Fig. 52).

The experimental results described in this section show that the deformation movement is initiated with the appearance of microfilaments in the cortical layer. The disruption of microfilaments results in the inhibition of the movement. Therefore, it is concluded that the deformation movement of *Tubifex* egg is a microfilament-dependent process.

(4) *Regulation of periodic changes in the structure of the cortical layer*

What factor(s) regulats the appearance and disappearance of microfilaments in the cortical layer ? Recent investigations on non-muscle cells have demonstrated the importance of Ca^{++} in regulating the microfilament-dependent contractile activity (Schroeder, 1975). Conrad and Williams (1974b), immersing *Ilyanassa* eggs in isotonic solution of $CaCl_2$, succeeded to induce the formation of a constriction on the egg surface which resembles the polar lobe formation. Since the cortical layer in the constricted region contains microfilaments, they suggested that the organization of microfilaments is due to the increased level of Ca^{++} in these eggs. Because the formation of grooves is a manifestation of the microfilament-dependent contractile activity of the egg surface, it is interesting to study in *Tubifex* eggs whether the rise of the intracellular Ca^{++} level induces the organization of microfilaments in the cortical layer and elicits the surface contractile activity or not. For inducing the rise of the intracellular Ca^{++} level, ionophore A23187 was used in the present study (see Steinhardt and Epel, 1974). Eggs were continuously treated with this drug.

When spherical eggs at I-3 and I-5 stages are immersed in 5 and 10 μg/ml ionophore, a very slight distortion of the egg

surface occurs within 4-5 minutes. The formation of relatively clear grooves ensues only in eggs which had been immersed in early phase of I-3 or I-5 stage. These grooves are exclusively localized on the equatorial surface but not on the animal hemisphere. They are apparently shallower than those formed in the normally deforming eggs (Figs. 53 and 54a). Ultrastructural examinations of ionophore-treated eggs reveal the presence of highly organized microfilaments in the cortical layer at the groove bottom (Fig. 54b, c); no microfilaments are detected in the corresponding regions of the spherical control eggs kept in 0.1% dimethyl sulfoxide. Afterwards, the surface of experimental eggs becomes again smooth without showing typical features of the deformation movement and eggs regain the spherical shape. The normally scheduled second deformation movement is not disturbed even after the "additional grooving activity" is induced by the treatment with ionophore at I-3 stage.

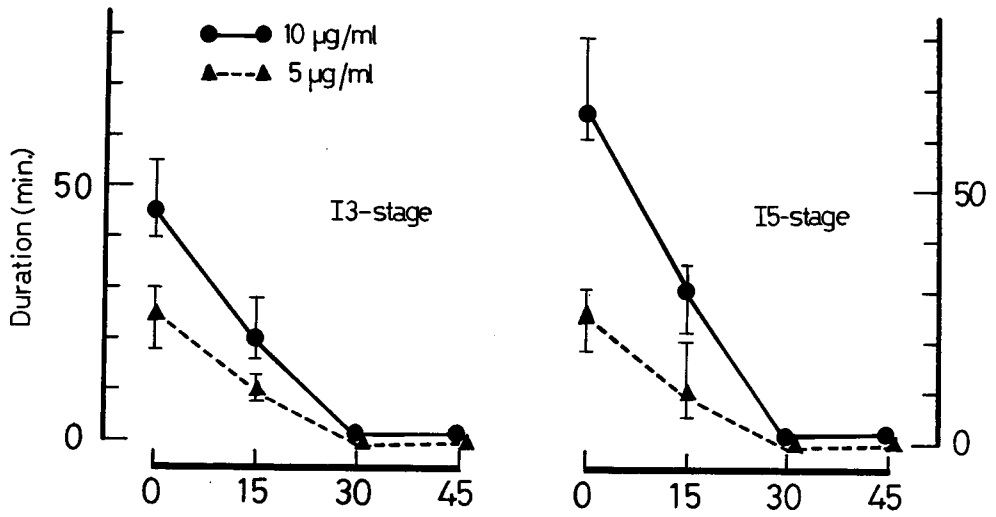
The induced groove-forming activity depends solely on the presence of ionophore but does not require either Ca^{++} or Mg^{++} in the external medium (Table 2). The length of the duration showing the grooving activity on the surface is closely related to the developmental phase of the beginning of the treatment and the concentration of ionophore. No sign of the groove-forming activity is detected when eggs at 30 minutes or more after beginning of I-3 or I-5 stage are immersed in ionophore

Table 2

Induced deformation movement in the *Tubifex* egg

Solution*	Initiation of treatment**							
	Stage I-3				Stage I-5			
	0	15	30	45	0	15	30	45
NCM	–	–	–	–	–	–	–	–
NCM+A23187	++	+	–	–	++	+	–	–
NCM+DMSO	–	–	–	–	–	–	–	–
CMF	–	–	–	–	–	–	–	–
CMF+A23187	++	+	–	–	++	+	–	–
CMF+DMSO	–	–	–	–	–	–	–	–

* A23187=10 μg/ml; DMSO (dimethyl sulfoxide)=0.1%. ** Time-lapse (min.) after beginning of each stage, *i.e.*, after comlete disappearnce of grooves from the preceding deformation movement at stage I-2 or I-4. NCM, normal culture medium; CMF, Ca^{++}-and Mg^{++}-free medium.
++, deep groove (see Fig. 54a); +, shallow groove (see Fig. 53); -, no grooving (no deformation movement).



Text-fig.8. Duration of ionophore-induced grooving activity as a function of the time of beginning of treatment. Ionophore was dissolved in Ca^{++}-and Mg^{++}-free medium. Each value represents the mean of 40-50 eggs. Abscissa: time-lapse after the beginning of each stage.

(Text-fig. 8). If 30 minute-old eggs of I-3 stage are centrifuged at 1700*g* for 20 minutes and treated with 10 μg/ml ionophore in Ca^{++}- and Mg^{++}-free culture medium, however, a deep constriction is formed on the surface of the protoplasmic layer stratified and disappears within 60-70 minutes (Fig. 55; for details of stratification after centrifugation, see Text-fig. 2 in page 15). The cortical layer of these eggs contains microfilaments at the site of the constriction. Therefore, it should be concluded that non-deforming eggs (I-3 and I-5 stages) possess functional contractile elements or their precursors in the protoplasm and the intracellular Ca^{++} level plays an important role in the organization of microfilaments.

The normal deformation movement (I-2 and I-4 stages) proceeds in Ca^{++}- and Mg^{++}-free culture medium without any disturbance. When the treatment with ionophore (10 μg/ml in the normal or Ca^{++}- and Mg^{++}-free culture medium) is begun in either the shallow groove or the climax phase of the first or the second movement, ionophore does not exert any effect. However, ionophore of the same concentration exerts some effcts on the process of the relaxation: if the immersion is begun in the late relaxing phase, remnants of grooves on the equatorial surface increase their depth again. Sometimes, several new grooves appear on the surface, so that the duration of the second deformation movement extends for 40-50 minutes longer

than in the control eggs.

The meiotic events, pole plasm segregation and the first cleavage proceed normally under the presence of ionophore in the normal or Ca^{++}- and Mg^{++}-free culture medium. However, all eggs undergo cytolysis within 24 hours even in the Ca^{++} bearing culture medium.

[III]

Regional Characteristics of the Egg Surface in Relation to the Grooving Activity

In the preceding chapter, it was noticed that microfilaments occurred in the cortical layer are the contractile machinery for the formation of grooves in *Tubifex* eggs. The organization of microfilaments in the cortical layer seems to be regulated by the intracellular Ca^{++} level. As stated already, the surface of *Tubifex* eggs undergoing the deformation movement shows the grooving activity not only on the equatorial region but also on the animal hemisphere. When eggs are treated with ionophore and are induced to rise their intracellular Ca^{++} level, however, the resulted grooving is found exclusively on the equatorial surface and none on the animal hemisphere. This fact implies that the mechanisms of groove formation on the surface of these

two regions are different each other.

Since the extrusion of polar body occurs at the climax phase of the deformation movement, the grooving on the egg surface or the organization of microfilaments in the cortical layer might be not only controlled by the intracellular Ca^{++} level but also influenced with information from the meiotic apparatus in the animal pole (Lehmann, 1946). To clarify the requirement of nuclear information for the formation of surface grooves, experiments were performed using an inhibitor of oxidative phosphorylation, dinitrophenol, and an inhibitor of microtubule, colchicine.

(1) *Chronological relation of the deformation movement to the polar body extrusion*

In order to know the relation between the surface grooving activity and the meiotic processes, the extrusion of polar body was observed in the scanning electron microscope.

(i) *The first polar body extrusion*

The formation of shallow grooves is first recognizable on the equatorial surface of the egg. At this time, the cytoplasmic bulge forming the first polar body attains the maximal height at the animal pole (late anaphase; Fig. 56a); no apparent constriction around the proximal part of the bulge is observed

(Fig. 56b). After 5-10 minutes, concurrently with the appearance of the proximal constriction of the bulge (Fig. 57b), shallow grooves also appear on the surface of the animal hemisphere, leaving a narrow circular zone around the cytoplasmic bulge (Fig. 57a). Afterwards these grooves are individually confluent with the previously established equatorial ones and the deformation movement attains the climax phase. Along with the extrusion of the first polar body, radial folds appear on the narrow circular zone around the connecting site between the polar body and the egg proper (Fig. 58). After extrusion of the polar body, the movement enters the relaxing phase. Finally the grooves and folds disappear and the egg surface becomes smooth.

(ii) *The second polar body extrusion*

The initiation of the second deformation movement is detected by the appearance of shallow grooves on the equatorial surface of the egg. Unlike the first movement, however, these grooves occurs long before the formation of the cytoplasmic bulge forming the second polar body (Fig. 59). At this time, the egg nucleus is still at late metaphase. In the following 15-20 minutes, formation of the bulge is initiated at the animal pole (Fig. 60). The maximal bulge with its proximal constriction is clearly observed after 20 minutes; at this time,

grooves appear for the first time on the surface of the animal hemisphere of the egg (Fig. 61). From this phase of the movement on, a circular zone devoid of grooves is observed on the surface around the bulge. Radial folds are, however, also formed on this zone at the climax phase of the movement (Fig. 61b). After extrusion of the polar body, the retraction of grooves occurs. Finally the grooves and folds disappear, thus the egg surface becomes smooth.

The following conclusions are drawn from the above observation: Although the chronological relations of the meiotic process to the deformation movement are different between the first and the second polar body extrusions, the onset of the movement is invariably characterized by the appearance of equatorial grooves. The participation of the egg surface of the animal hemisphere in grooving is the second step of the movement. In both movements, this phase coincides with the time of furrowing for the polar body extrusion. Grooves are never formed on the vegetal hemisphere.

(2) *Effects of dinitrophenol (DNP) and colchicine on the deformation movement and polar body extrusion*

Using dinitrophenol (DNP) or colchicine, or both, four series of experiments for the first deformation movement and

five series for the second deformation movement were carried out. The experimental scheme and results are summarized in Text-figures 9 and 10.

At the concentration employed, DNP-treatment for 20-40 minutes shortly before and after the onset of the movement does not affect polar body extrusion. On the other hand, colchicine-treatment completely destroyed the meiotic apparatus and other microtubular system (Fig. 65b, c).

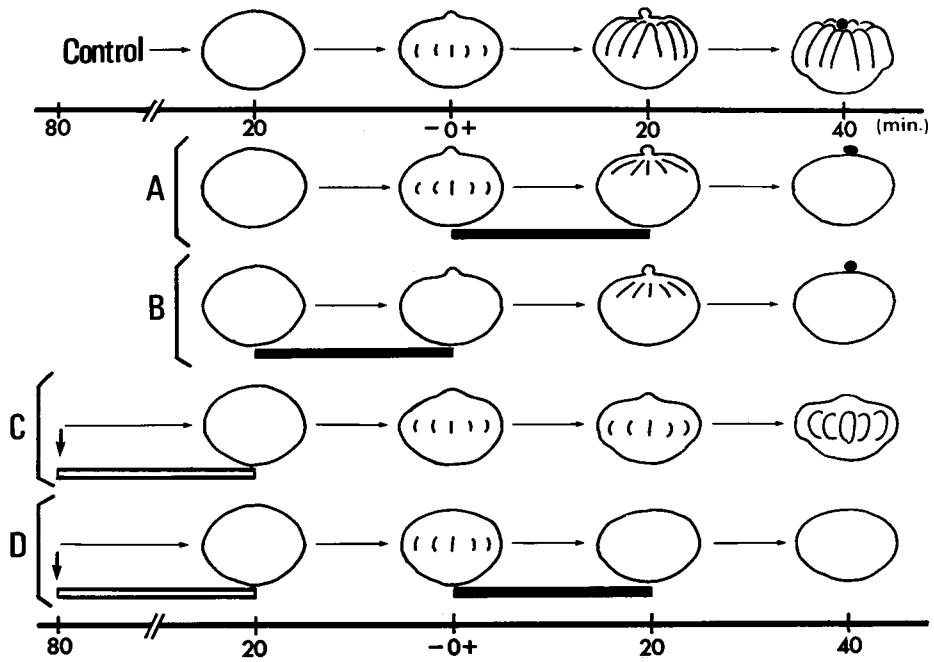
(i) *On the equatorial grooving*

When eggs in the shallow groove phase of the movement (early I-2 or I-4 stage) are exposed to DNP, active deepening of the equatorial grooves ensues for the first 10 minutes (Text-figs. 9A and 10A, B). However, most of these grooves disappear within 20 minutes. The appearance of equatorial grooves can be prevented if the eggs are treated with DNP for 20 minutes preceding to the onset of the movement (late I-1 or I-3 stage) (Text-fig. 9B).

When eggs at an early phase of the movement are treated with DNP for 20 minutes and transferred to the normal culture medium (pulse treatment), grooves already formed disappear from the equatorial surface at the end of DNP-treatment; in the normal culture medium, equatorial grooves do not reappear in the case of the first deformation movement (Text-fig. 9A, B)

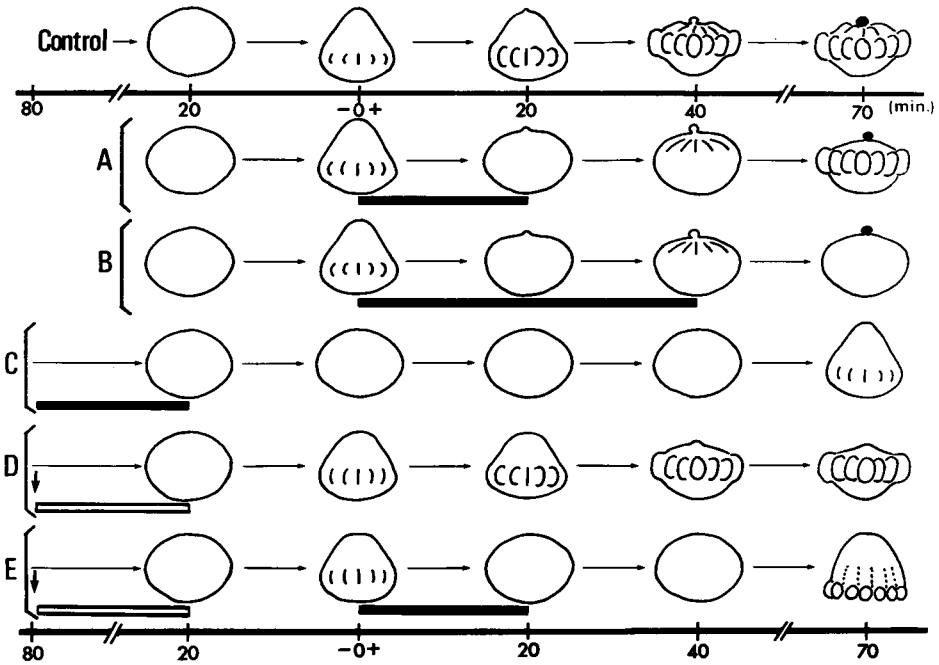
but are clearly re-formed at the second movement (Text-fig. 10A; Fig. 64a). If the duration of DNP-treatment is prolonged up to 30-40 minutes, however, the re-formation of equatorial grooves is manifestly prevented even in the case of the second deformation movement (Text-fig. 10B).

When eggs at the early metaphase of the second meiotic division (early I-3 stage), immediately after the termination



Text-fig. 9. Schematic illustration of the manner and effect of treatments with dinitrophenol and colchicine. The first deformation movement. Solid bars indicate the exposure-time of eggs to dinitrophenol (20 minutes); open bars with vertical arrows are for colchicine-treatment (60 minutes). The illustrated eggs are all viewed from the side, and the black dots on the animal pole are the first polar bodies.

of the first deformation movement, are exposed to DNP for 60 minutes (Text-fig. 10C), the second movement begins in the culture medium after a delay of 60-90 minutes. Since shallow grooves first appear on the equatorial surface, the sequence of this retarded movement seems to be normal in the initial phase. The later deepening process of the equatorial grooves, however, proceeds very slowly; therefore, grooves on the animal hemisphere



Text-fig. 10. Schematic illustration of the manner and effect of treatments with dinitrophenol and colchicine. The second deformation movement. The length of the dinitrophenol-treatment is 20, 40 or 60 minutes and is indicated by solid bars. The exposure of the eggs to colchicine is indicated by open bars with vertical arrows (60 minutes) and is performed between the first and the second deformation movements. The illustrated eggs are all viewed from the side, and the black dots on the animal pole are the second polar bodies.

appear while the equatorial grooves are relatively shallow (Fig. 67). A similar retarding effect of DNP on the second deformation movement is also observed when eggs are pretreated during the first movement (I-2 stage) for 20 minutes (Text-fig. 9A, B, D).

At the concentration used, colchicine never disturbs the groove-forming activity of the equatorial surface (Text-figs. 9C and 10D): In the climax phase of the movement, the morphological features of this region are similar to those of the control eggs (Fig. 65a).

When eggs at early I-2 or I-4 stage are treated with colchicine (60 minutes) at late I-1 or I-3 stage and are further subjected to pulse-treatment of DNP (20 minutes), shallow grooves for the second deformation movement are barely re-formed on the equatorial surface but not at all in the first movement (Text-figs. 9D and 10E). In spite of a weak groove-forming activity of the equatorial surface, unusually deep grooves appear in the second deformation movement on the vegetal hemisphere after termination of the treatment (Text-fig. 10E; Fig. 66).

(ii) *On the animal hemisphere grooving*

In contrast to the high susceptibility of the equatorial surface to DNP, the groove-forming activity of the animal

hemisphere is not disturbed by the pulse-treatment with DNP (Text-figs. 9A, B and 10A, B). The scanning electron microscopic study on eggs treated with DNP for 20 minutes reveals that the animal hemisphere grooving occurs concurrently with the appearance of the proximal constriction of the cytoplasmic bulge forming the polar body (Fig. 62). These grooves deepen to some extent (Fig. 63), but their persistence is limited to about 20 minutes, which is far shorter than that in the control eggs (40-50 minutes). Along with the retraction of the grooves on the animal hemisphere, equatorial grooves are re-formed in the case of the second deformation movement (Text-fig. 10A; Fig. 64), but not in the case of the first movement (Text-fig. 9A, B).

In contrast to the equatorial surface, the groove-forming activity of the animal hemisphere is completely prevented by treatment with colchicine (Text-figs. 9C, D and 10D, E). Figure 65 explicitly shows that colchicine-treatment does not inhibit the equatorial grooving but suppresses the animal hemisphere grooving (compare with Fig. 63).

(iii) *Effects of DNP-treatment on the number of microfilaments*

It was concluded from the preceding experiments that the equatorial surface of the egg is highly susceptible to DNP compared with the animal hemisphere. Since microfilaments undoubtedly participate in the deformation movement of the *Tubifex* egg,

Table 3

Relative densities of microfilaments in cortical region before and after DNP-treatment

	Time (min) after the onset of DNP-treatment			
	0	20	40	60
Animal pole	100	193 (330)	360 (402)	423 (387)
Animal hemisphere	100	46 (141)	79 (215)	107 (375)
Equatorial region	100	0 (461)	0 (518)	195 (560)

Eggs in the very early phase of the second deformation movement were treated with dinitrophenol for 20 minutes and fixed at 20-minute intervals. The measurements were carried out at the animal pole, animal hemisphere and equatorial regions. For the counting of the number of microfilaments at each period and each region, 25 transmission electron micrographs of high magnification (x60000) from five eggs were prepared. Five-micrometer long sections of the cortical layer of the animal pole and animal hemisphere were divided into 25 equal parts; the two parts that contained the largest number of microfilaments were selected and used for further analysis. In the equatorial region, the number of microfilaments in a 1-μm long sample of the cortical layer, at the bottom of an equatorial groove was counted. For the size of divided cortical layer, see Figure 68. Values from 50 measurements were averaged; each numeral presented expresses the percentage of the mean number of microfilaments at the onset of dinitrophenol-treatment (shallow groove phase of the second deformation movement). Numerals in parentheses are of control eggs. The mean number of microfilaments ± standard deviation in the shallow groove phase of the second deformation movement was 1.5±0.4 (animal pole), 3.1±0.5 (animal hemisphere) and 6.7±0.3 (equatorial region).

it is interesting to count the number of microfilaments per unit area of the cortical layer in DNP-treated eggs. For this determination, three regions of the egg surface were chosen: the animal pole, animal hemisphere and equatorial region.

Eggs in the shallow groove phase of the second deformation movement were pulse-treated with DNP for 20 minutes (Text-fig. 10A) and were fixed at 20-minute intervals. Meridional sections were prepared, passing through the egg axis for examination of the animal pole and animal hemisphere, and sections through the equatorial plane for the equatorial region. These sections gave longitudinal features of microfilaments, making it easier to count them.

When the number of microfilaments in each region is compared with that of the control eggs, it is always small in DNP-treated eggs (Table 3). At the end of the treatment, some filaments can still be observed in the animal pole and animal hemisphere, but never detected in the equatorial region (Fig. 68). After washing the treated eggs in the culture medium without DNP, a period of 30-40 minutes is required for the reappearance of microfilaments in the equatorial region (Table 3).

The number of microfilaments gradually increases at the animal pole after DNP-treatment, and this incremental pattern seems to resemble that of the normal eggs. In the animal hemisphere, however, the increase in the number of microfilaments seems to be repressed after DNP-treatment (Table 3).

(3) *Is the grooving pattern of the egg surface changed after centrifugation ?*

The results obtained from the previous experiments suggest the following possibility: The groove-forming activity is closely related to the meiotic events on the animal hemisphere but *not* on the equatorial region. In order to know relations between the groove-forming activity and the cytoplasmic organelles other than microfilaments, the pattern of grooving was observed in centrifuged eggs.

(i) *Stratification of egg components after centrifugation*

When spherical eggs (I-3 or early I-4 stage) are centrifuged at 1700*g* for 20 minutes, they slightly elongate in the centrifugal direction (Fig. 71), but restore the original shape within 10 minutes after the end of centrifugation (Fig. 69). The author failed to observe whether the cortical layer is displaced by the centrifugal force or not. In these eggs, the stratification of the egg interior occurs and is observable in the living state. The interior consists of following five layers (Figs. 70 and 71): (1) centripetal lipid layer, (2) protoplasmic layer with the nucleus, endoplasmic reticula, Golgi complexes and mitochondria, (3) large yolk granule layer, (4) "opaque plasma" layer (Weber, 1958), and (5) centrifugal small yolk granule layer. Careful examinations of the components in each

layer reveal that the separations of lipid droplets and yolk granules from other components are relatively clear. Mitochondria and endoplasmic reticula are however not sufficiently separated from other components of the egg and contaminate the layers other than the protoplasmic layer (Figs. 73 and 74). In the protoplasmic layer, endoplasmic reticula are found in a centripetal part and mitochondria occupy its centrifugal part. The egg nucleus is found in the centripetal part of the protoplasmic layer, thus being embedded in endoplasmic reticula (Figs. 71 and 72). Although the author failed to actually observe the sperm nucleus in the present study, it may be also contained in the protoplasmic layer. The "opaque plasma" layer is mainly composed of amorphous materials of low electron density.

When eggs are left to themselves after centrifugation at I-3 stage, the stratified components do not start flowing and rearranging themselves in the egg interior until the onset of the grooving activity (60 minutes after centrifugation) (compare Fig. 72 with Fig. 71). If the centrifugation is carried out at early I-4 stage, however, flowing of mitochondria and endoplasmic reticula begins immediately after the end of centrifugation, though lipid droplets and yolk granules do not initiate the rearrangement. Later, all of these centrifuged eggs divide into two cells of unequal size.

(ii) *Formation of grooves on the surface of centrifuged eggs*

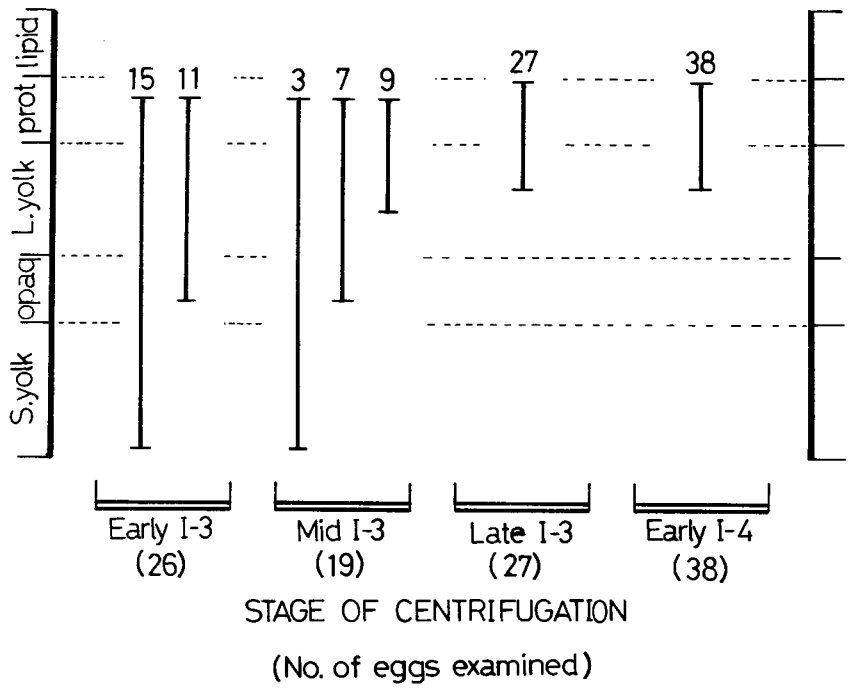
The sequence and pattern of grooving occurred on the surface were followed in detail in the living eggs centrifuged at I-3 and I-4 stages.

Eggs at I-3 stage: When eggs are centrifuged at early or mid I-3 stage, they begin to form grooves on the surface after about 50 minutes. The formation is precisely synchronous with the onset of the second deformation movement in the intact eggs. Grooves appear first on the surfaces of the large and small yolk granule layer, and run parallel to the centrifugal axis (Fig. 75, arrows). About 30 minutes later, a shallow constriction sometimes appears around the surface of the protoplasmic layer, which indicates that the layer also possesses the grooving activity. The surface of these centrifuged eggs continues to show the grooving activity for 20-40 minutes longer than the intact eggs and finally becomes smooth. Although these centrifuged eggs do not extrude the second polar body, the first cleavage in normal pattern occurs synchronously with the control eggs.

Immediately after centrifugation at late I-3 stage, the intact eggs are in a very early phase of the second deformation movement. These centrifuged eggs form constriction on the surface of the protoplasmic layer indicating that the

layer possesses the groove-forming activity (Fig. 77a). As a result of the appearance of constriction, the centripetal lipid layer is sometimes separated from others (Fig. 77b). The nucleus is not contained in the separated lipid layer. Probably owing to the constriction on the egg surface, mitochondria and endoplasmic reticula in the protoplasmic layer start flowing and rearranging themselves in the egg interior (Fig. 78). Thirty or forty minutes later, grooves running parallel to the centrifugal axis appear also on the surface of the large yolk granule layer (Fig. 77c, d). No eggs extrude the second polar body.

The time after onset of I-3 stage, at which the centrifugation is performed, affects not only the sequence of grooving on the surface but also the dimension of the egg surface involved in the groove-formation: In eggs centrifuged at early I-3 stage, about two thirds of the surface of centrifuged eggs (ranging from the protoplasmic layer to the small yolk granule layer) show the grooving activity. When the centrifugation is performed at late I-3 stage, however, the egg surface involved in the grooving is apparently small in dimension: the groove-forming activity is observed on the surface of the protoplasmic and the large yolk granule layers but not on the small yolk granule layer (Text-fig. 11). If the centrifugation is at mid I-3 stage, the groove-forming activity of the surface of the small yolk granule layer is detected in only 3 out of 19 eggs.



Text-fig. 11. Areas showing surface contractile activity in eggs centrifuged at various stages. Eggs were centrifuged at 1700*g* for 20 minutes and were left to themselves. The range of the regions showing the activity is expressed by the length of vertical lines. Numerals on the top of each line shows the number of eggs. Ordinate indicates stratified layers in the egg.

These results indicate that the dimension of the egg surface involved in the formation of grooves is closely related to the length of the duration between the centrifugation and the onset of grooving.

Eggs at I-4 stage: When the centrifugation is performed at the onset of I-4 stage (immediately after the appearance of

dimples on the equatorial surface), the sequence of grooving on the surface is the same with that observed in eggs centrifuged at late I-3 stage. The separation of the centripetal lipid layer from others occurs frequently.

If eggs at 20 minutes following the onset of I-4 stage are centrifuged, grooves previously formed disappear during the centrifugation; in the intact eggs, the equatorial surface has deep grooves. The centrifuged eggs never re-form grooves. Synchronously with the intact eggs, the centrifuged eggs are divided into two cells without showing further surface grooving activity comparable to the second deformation movement.

(iii) *Microfilaments in centrifuged eggs*

As stated already, the author failed to decide whether the cortical layer of the egg is displaced after centrifugation or not. When the cortical layer of eggs centrifuged at early I-3 stage is examined with the electron microscope, it is very thin immediately after centrifugation but becomes thick in the grooving sites within 50 minutes. Although microfilaments are not found in the thin cortical layer, they appear with the onset of the grooving activity and are arranged perpendicularly or obliquely to the surface in the thick cortical layer (compare Fig. 76 with Fig. 74). Since the author did not observe the

cortical layer of eggs centrifuged at I-4 stage, it is unknown why grooves previously formed disappear during the centrifugation.

(4) *To what degree is the surface grooving independent of the meiotic events ?*

In the preceding chapter, it was noticed that the surface grooving activity is closely related to the meiotic events on the animal hemisphere but not on the equatorial region. However, the activity was observed in the centrifuged eggs where the extrusion of the polar body was failed; therefore, the grooving activity on the animal hemisphere does not appear to be resulted from the extrusion of the polar body. Is the equatorial grooving entirely independent of the nucleus and induced by an innate activity of the egg surface ? To answer this question, experiments were performed in non-nucleated and nucleated fragments of eggs.

Eggs at early I-3 stage were centrifuged and stratified previously. The stratified eggs were ligated with a silk thread around the protoplasmic layer and separated into small centripetal and large centrifugal fragments (Text-fig. 3 in page 16). The centrifugal fragment mainly comprises the "opaque plasma" and yolk granule layers, and includes a part of the protoplasmic layer. It does not contain the nucleus (non-nucleated fragment). The lipid layer and the bulk of protoplasmic layer

constitute the centripetal fragment which invariably contains the nucleus (nucleated fragment).

Synchronously with the onset of the second deformation movement and with the furrowing of the first and second cleavages in whole eggs (not centrifuged, intact), the surface of non-nucleated fragments shows a grooving activity (Figs. 79-84). Grooves appear on the surfaces of the large yolk granule layer and the protoplasmic layer; the activity is observed for about 60 minutes and is shorter in duration than that observed in the intact eggs. At the cleavage stage of the intact eggs, no division occurs in these fragments. The groove-forming activity of the surface is completely diminished by 50 μg/ml cytochalasin B but not by 2 mg/ml colchicine. Furthermore, microfilaments are actually observed in the cortical layer of grooving sites (Fig.88). These facts indicate that the formation of grooves on the surface of non-nucleated fragments is due to the contraction of microfilaments in the cortical layer and that the activity corresponds to that observed on the equatorial surface of the intact eggs.

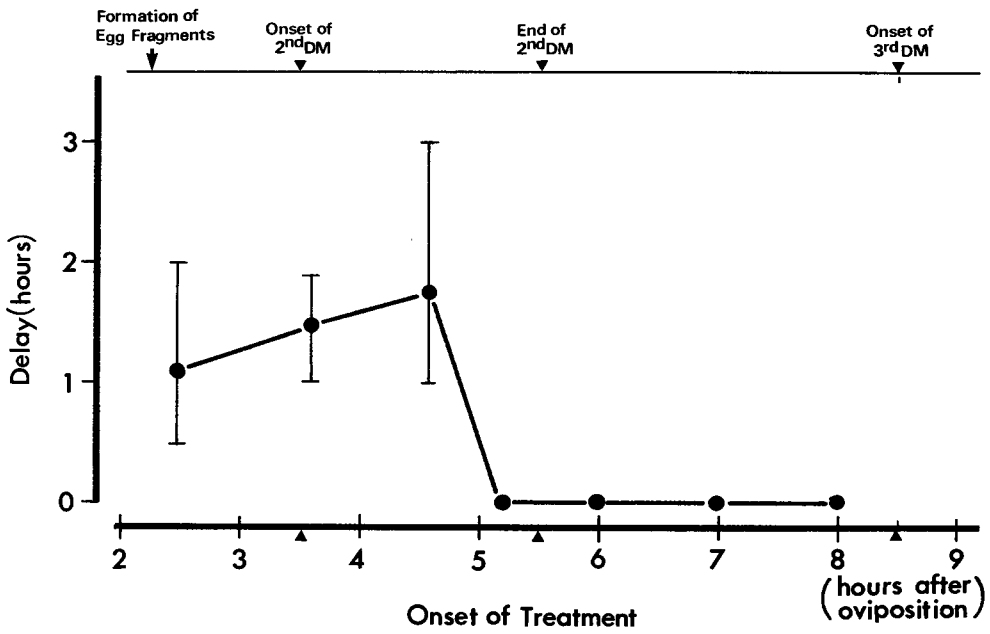
In nucleated fragments, the grooving activity is not clear on the surface. However, these fragments obviously form grooves on the entire surface after removal of the vitelline membrane (Fig. 85). To induce the grooving activity in these fragments,

therefore, it is obvious that the surface should be widely separated from its overlying membrane. The nucleated fragments do not extrude the polar body. However, they are equally divided at the first cleavage of the intact eggs (Fig. 86) and repeats further divisions (Fig. 87). It is not certained in the present study whether the mitotic spindle involved in this division is the meiotic spindle itself which serves the extrusion of the polar body.

(5) *Protein synthesis and periodic appearance of grooves on the surface of non-nucleated fragments*

As described in the preceding section, non-nucleated fragments of *Tubifex* eggs periodically show the grooving activity. This fact suggests the presence of cytoplasmic factors which regulates cycles of the surface contractile activity. It is interesting to know whether the protein synthesis, one of biosynthetic processes, is involved in this regulation. For this purpose, the formation of surface grooves was investigated in non-nucleated fragments after inhibition of the protein synthesis. An inhibitor of the protein synthesis, cycloheximide, was used.

When fragments are treated with 20 μg/ml cycloheximide for 20 minutes at I-3 or mid I-4 stage of the whole eggs (not centrifuged, intact) and transferred into the culture medium



Text-fig. 12. Cycloheximide-induced delay of the appearance of the grooving activity in non-nucleated fragments as a function of the onset time of treatment. Delay of the beginning of the grooving activity corresponding to the third deformation movement (3^{rd}DM) in intact eggs is presented. The ends of the second and the third deformation movement in the intact eggs are indicated upper part of the figure. Egg fragments are treated with 20 μg/ml cycloheximide for 20 minutes (pulse-treatment). Each plotted point represents the mean value from 35-40 egg fragments.

(pulse treatment), grooves are formed without any disturbance on the surface synchronously with the deformation movement in the intact eggs. However, the grooving activity of fragments which will be observed at the time of the furrowing of the first cleavage in the intact eggs (the third deformation movement) is detected with delay of 1-2 hours (Text-fig. 12). The pattern

and the duration of the grooving are not affected by the cycloheximide-treatment. The grooving activity comparable to that observed at the time of the second cleavage of the intact eggs (the fourth deformation movement) is initiated without delay in these fragments. As stated already, no fragments divide.

If fragments are subjected to the same treatment at late I-4 or I-5 stage of the intact eggs, the formation of grooves is initiated precisely synchronously with the third deformation movement of the intact eggs. The onset of grooving which will be observed at the fourth deformation movement of the intact eggs is however delayed by about 1.5 hours. Therefore, the stage showing the response to cycloheximide in these fragments apparently differs from that observed in the previous experiment (treated at I-3 or mid I-4 stage of the intact eggs). This fact indicates the presence of specific time to be influenced by cycloheximide in relation to the groove-formation.

The results of the above experiments again indicate that the surface of non-nucleated fragments can produce grooves. Since the inhibition of the protein synthesis in fragments results in the delay of the onset of grooving activity, it is supposed that the onset of groove-forming activity in *Tubifex* eggs is regulated through a control of biosynthetic processes, such as protein synthesis.

DISCUSSION

It has been known in a wide variety of animal species that the eggs show regular changes of shape in early stages of embryonic development. The extent of the shape-change is however different among species. The followings are the typical examples of such shape-change: polar lobe formation in molluscan and polychaetous annelid eggs (Wilson, 1925; Raven, 1966; Conrad, 1973), peristaltic constrictions of barnacle and ascidian eggs (Reverberi, 1971; Lewis, 1977), surface constriction spreading in a wave-like fashion after fertilization and at cleavage of amphibian eggs (Hara, 1971; Sawai and Yoneda, 1974; Hara *et al.*, 1977).

The deformation movement of *Tubifex* eggs is a typical example of dynamic egg-shape change occurring in the early embryonic development. Since eggs of terrestrial oligochaetes, *Eisenia* and *Lumbricus*, do not show any comparable shape-change in early stages of the development (see Wilson, 1889; Devries, 1973), the deformation movement in *Tubifex* eggs seems to be unique for the early development of oligochaete eggs.

Since the observations by Penners (1922), the deformation movement of developing *Tubifex* eggs which occurs prior to cleavage attracted attentions of several investigators. In early ultrastructural analysis of *Tubifex* eggs, however, Hess (1959),

Lehmann and Henzen (1963) and Henzen (1966) failed to demonstrate cytoplasmic structures responsible for the deformation movement. These authors merely reported the increase in amount of cortical cytoplasm in the vicinity of grooves which appeared during the movement. In 1966, Cloney fairly demonstrated the contractile role of caudal epitheliar microfilaments during tail retraction in ascidian larvae. Since that time, the importance of microfilaments in cellular locomotion have been reported in a considerable number of non-muscle cells of various organisms (for review, see Wessells *et al.*, 1971; Schroeder, 1973, 1975; Pollard, 1975).

It has been known that a variety of types of cellular processes are inhibited in many organisms by the treatment with cytochalasin B; *e.g.*, polar lobe formation in eggs of *Ilyanassa* (Conrad and Williams, 1974a) and *Sabellaria* (Peaucellier *et al.*, 1974), and peristaltic constrictions in fertilized eggs of the goose-neck barnacle (Lewis, 1977). Cytochalasin B is also known as an inhibitor of cytokinesis (Arnold, 1976). A high susceptibility to this drug of the deformation movement in *Tubifex* eggs is also shown in the present study. Several investigators have noticed that the disruption of microfilaments takes place concomitantly with the cytochalasin B-inhibition of cellular processes (Wessells *et al.*, 1971; Schroeder, 1970, 1972; Conrad and Williams, 1974a; Luchtel *et al.*, 1976;

Lewis, 1977). The present electron microscopic observations demonstrated that the periodic appearance of microfilaments in the cortical layer occurs concurrently with the formation of grooves on the egg surface. In view of these facts, it is safe to say that the deformation movement in *Tubifex* eggs is a microfilament-dependent process. Furthermore, the present observations disclosed the importance of the pattern of microfilament-arrangement in the cortical layer; when eggs are treated with cytochalasin B at I-2 or I-3 stage, grooves formed were much shallower and smaller in number than those in the intact eggs. These eggs were relaxed without attaining the climax phase. Microfilaments were considerably small in number after treatment with cytochalasin and ran parallel to the surface in the cortical layer of shallow grooves, whereas they were in the intact eggs arranged perpendicularly or obliquely to the surface at the bottom of grooves. The incompleteness of the deformation movement in cytochalasin B-treated eggs is therefore attributed to the decreased number of microfilaments as well as the inadequate pattern of their arrangement in the cortical layer.

Subcellular mechanism of the formation of surface grooves

As for the primary site of biological action of cytochalasin B (see Copeland, 1974), the continuity between the

plasma membrane and microfilaments has been suggested to be affected with this drug (Luchtel *et al.*, 1976). Recently Lin and Lin (1979) showed that the surface membrane of human erythrocytes possesses cytochalasin-binding complexes: The complexes seem to participate in the formation of microfilaments as well as in the establishment of the continuity between the plasm membrane and filaments. From these facts, these authors supposed that cytochalasin B binds these complexes and inhibits the formation of microfilaments. Since the deformation movement of *Tubifex* eggs requires considerable increase in length and number of microfilaments, the insufficiency of the surface grooving activity in cytochalasin B-treated eggs may be due to the inhibition of the formation of microfilaments in the cortical layer. Furthermore, the disturbance of the continuity between the plasma membrane and microfilaments produced by cytochalasin B-treatment might change the arrangement of filaments.

An inhibitor of oxidative phosphorylation, dinitrophenol, also prevents the equatorial grooving of *Tubifex* eggs. Preliminary experiments showed that a similar inhibition of the grooving activity on the egg surface is obtained by inhibitors of cytochrome oxidase cycle, antimycin A ($3x10^{-4}$M), KCN (10^{-3}M) and NaN_3 ($7.5x10^{-4}$M). Although some side effects of dinitrophenol have been reported by Slater (1967), the equatorial grooving in *Tubifex* eggs might be prevented by dinitrophenol

through the inhibition of ATP production. As for the role of ATP in microfilament-dependent cellular activities, it is a well known energy source for the contraction process of cells (Taylor *et al.*, 1973; Izzard and Izzard, 1975; Rodewald *et al.*, 1976). Furthermore, Hatano and Oosawa (1966) and Puszkin and Berl (1972) demonstrated the importance of ATP in the polymerization of microfilaments; it binds to monomer of microfilaments and is involved in their polymerization. Therefore, it is possible to suppose that the absence of microfilaments in the cortical layer after treatment with dinitrophenol is resulted from the disturbance of polymerization of filaments caused by the inhibition of ATP production. Since the animal hemisphere grooving occurred normally even in eggs treated with dinitrophenol, the cytoplasmic ATP level should be considered to be enough for the contraction of microfilaments to occur. The polymerization of microfilaments in the equatorial region appears to require a high ATP level than the animal hemisphere.

The number and length of microfilaments considerably increased during the first half of the deformation movement. These filaments in the cortical layer were arranged perpendicularly or obliquely to the surface in the equatorial region; the outer end of each filament appeared to be associated with the plasma membrane. Except for microfilaments, no granules and organelles were detected in the cortical layer. The

arrangement of microfilaments in the cortical layer strongly suggests that the polymerization or formation of microfilaments is initiated on the inner aspect of the plasma membrane and proceeds inward. As a result, cytoplasmic granules and organelles may be expelled from the cortical layer (Schroeder, 1972, 1973, 1975). As stated already, materials which may participate in the formation of microfilament and in the establishment of the continuity between the plasma membrane and the filaments have been isolated from the plasma membrane fractions of human erythrocytes and of microvilli of the intestinal brush border (Lin and Lin, 1979; Tilney, 1975); the materials from erythrocytes are composed of actin, spectrin and other two peptides, and those from microvilli are identified to be homologous to α-actinin constituting Z-line of striated muscles. Although no reliable evidence for the mode of association of microfilaments with the plasma membrane was obtained in the present study, the above mentioned facts implies that the site of polymerization or formation of microfilaments in *Tubifex* eggs is the inner aspects of the plasma membrane.

The heavy meromyosin binding ability of cytoplasmic filaments has recently been studied in many types of cells. According to these studies, actin filaments appear to exist in bundles of microfilaments such as contractile ring in cytokinesis (Perry *et al.*, 1971), contractile bands in cells undergoing

morphogenetic shape changes (Spooner *et al.*, 1973) and acrosomal filaments of arthropod and molluscan sperm (Tilney, 1975; for review,also see Pollard, 1975). It has been believed today that contractile proteins, actin, myosin, tropomyosin and α-actinin, are widely found in eukaryotic non-muscle cells (Cohen and Cohen, 1972; Fine *et al.*, 1973; Pollard and Weihing, 1974; Pollard, 1975; Lazarides, 1975; Fujiwara and Pollard, 1976). From these facts, the sliding filament model of muscle contraction (when activated by Ca^{++}, bipolar myosin filaments pulling on a bipolar array of actin filaments using energy from ATP) is generally accepted to be reasonable starting point for thinking about the organization of all actomyosin-based non-muscle movement (Durham, 1974). Unlike skeletal muscles, the contractile apparatus in non-muscle cells is said to be extremely labile; it repeats a rapid formation and breakdown in living cells. In *Tubifex* eggs, microfilaments appeared transitorily in the cortical layer but soon disappeared in the early embryonic development. Although no data are available on the biochemical nature of these filaments, this might be a reflection of the labile property of contractile apparatus in *Tubifex* eggs.

To obtain knowledge on the subcellular mechanism of the deformation movement in *Tubifex* eggs, factors dominating the periodic appearance of microfilaments in the cortical layer

should be analyzed. It was found that ionophore A23187 induced the formation of grooves on the equatorial surface, Since this drug has a function to transport Ca^{++} across the biological membranes (Reed and Lardy. 1972), the present result indicates that the polymerization or formation of microfilaments in the cortical layer is regulated, at least in part, by the intracellular Ca^{++} level. In eggs and embryos of frogs and squid, ionophore A23187 also elicits microfilament-dependent contractile responses (Schroeder and Strickland, 1974; Arnold, 1975; Moran and Rice, 1976). Conrad and Williams (1974b) immersed *Ilyanassa* eggs in isotonic solution of $CaCl_2$ and succeeded to form rings composed of microfilaments at the bases of induced polar lobe-like protuberances. The sufficiency of Ca^{++} to act as an activator of the cortical contraction has been directly demonstrated by intracellular microinjections of Ca^{++} into eggs of some animal species (Gingel, 1970; Baker and Warner, 1972; Hollinger and Schuetz, 1976; Conrad and Davis, 1977). These facts may indicate that Ca^{++} specifically activates the polymerization of microfilaments in invertebrate and vertebrate eggs. Although the involvement of Ca^{++} in the *in vitro* polymerization of actin filaments has been demonstrated by Miki-Noumura and Kondo (1970) in sea urchins, little is known about the mode of *in vivo* participation of Ca^{++} in the formation of microfilaments.

Since the ionophore-induced deformation movement does not require external Ca^{++}, it may be supposed that the rise in the intracellular concentration of Ca^{++} for eliciting the contractile activity on the surface of *Tubifex* eggs is induced by the release of Ca^{++} from intracellular stores. The absence of microfilaments in spherical I-3 and I-5 stages may be attributed, in part, to the low availability of intracellular Ca^{++}. If the above supposition is correct, then it is possible to expect that the periodic appearance of microfilaments in the cortical layer of *Tubifex* eggs is regulated by oscillatory behavior of intracellular Ca^{++}: When the intracellular Ca^{++} concentration is above threshold, microfilaments are polymerized; if Ca^{++} is again bound to some membraneous organelles, the degradation of filaments occurs. It is still remained unknown however what factor(s) triggers the rise or decline of the intracellular Ca^{++} concentration.

The subcellular mechanism of the deformation movement is considered as follows. When the intracellular concentration of Ca^{++} once rises, the polymerization of microfilaments occurs on the inner aspects of the plasma membrane and proceeds inward with an expense of ATP as the energy source. The site of polymerization possesses a cytochalasin B-binding property, and the formed filaments are actin in nature. During the

polymerization and elongation of microfilaments, the intracellular Ca^{++} level is maintained above a threshold and the constant production of ATP has occurred. When the filaments contract with the use of energy from ATP, mechanical force pulls the plasma membrane which results in the formation of grooves on the egg surface. When the intracellular Ca^{++} is resequestrated by membraneous organelles of the eggs, the degradation of microfilaments begins and mechanical force for pulling the plasma membrnane diminishes. With a gradual disappearance of filaments, the retraction of grooves occurs and finally the egg regains the smooth surface.

Dependence of surface grooving activity on the meiotic process

Since Morgan (1935b) demonstrated the occurrence of the polar lobe formation in non-nucleated fragments of *Ilyanassa* eggs, the "autonomous" aspect has been particularly stressed for the egg-shape change in the early embryonic development (Verdonk *et al.*, 1971; Conrad, 1973). Although the deformation movement of *Tubifex* eggs occurs concurrently with the extrusion of the polar bodies, the surface grooving activity on the equatorial region seems to be different in property from that on the animal hemisphere: The activity of the equatorial surface seems to be independent of the progress

of meiotic division while that of the animal hemisphere appears to be closely related to the nuclear events.

The meiosis-independent property of the activity on the equatorial surface was ascertained in colchicine-treated eggs where the meiotic apparatus was disrupted to inhibit the formation of the polar body. The occurrence of surface grooving activity in non-nucleated fragments gives further evidence for the presence of nucleus-independent activity in *Tubifex* eggs. On the surface of the animal hemisphere, however, an invariable parallelism was detected between the onset of the groove-forming activity and the progress of meiotic division in normal and dinitrophenol-treated eggs: Grooving of the animal hemisphere always appear with the furrowing for the extrusion of polar body (early telophase of meiotic division). When the meiotic apparatus was disrupted by colchicine, not only the polar body extrusion but also the animal hemisphere grooving were inhibited. These facts suggest that functions of the meiotic apparatus are requisite for the surface grooving activity of the animal hemisphere, as they are for the polar body extrusion.

In eggs of spherical I-3 and I-5 stages, the author succeeded in eliciting the groove-forming activity with calcium ionophore A23187. In this case, the activity was always detected on the equatorial surface, but not on the animal hemisphere.

Accordingly, the rise in intracellular Ca^{++} level is closely related with the initiation of the equatorial grooving but is not enough to induce the groove-formation on the animal hemisphere; perhaps another factor or factors are necessary for the initiation of grooving on the animal hemisphere. The presence of time lag between the onset of the equatorial and the animal hemisphere grooving also implies a difference in triggering mechanism of the surface contractile activity of these two regions.

As in the case of the furrowing for the polar body extrusion (Longo, 1972), the surface grooving of *Tubifex* eggs is a manifestation of the force-generating function of microfilaments. The furrowing is known to be triggered by "cleavage stimuli" from the meiotic apparatus (Rapperport, 1971). Therefore the meiosis-dependent activity on the animal hemisphere, which appear about 100 μm away from the meiotic apparatus, may possibly be elicited by similar "stimuli" that might be communicated by the microtubular or endoplasmic reticular system. These organelles are found, though few in number, in the subcortical region of *Tubifex* eggs. Although it remains to be elucidated whether the "stimuli" reach as far as the equatorial region, it is certain that the equatorial grooving does not require the stimuli which might emanate from the meiotic apparatus.

What factors determine the orientation of running of grooves on the egg surface ?

From the results of centrifugation experiments, Morgan (1935a) and Clement (1968) postulated that information for the polar lobe formation of *Ilyanassa* eggs is imprinted in the cortex. Therefore it might be possible to expect that the cortical layer foreshadows in some way the pattern of egg-shape changes in *Tubifex*.

In the course of the deformation movement, grooves were formed on the animal hemisphere and the equatorial region but not on the vegetal hemisphere. Furthermore, these grooves always ran meridionally on the egg surface. The orientation of running of grooves was however easily altered after centrifugation. Therefore the orientation is determined in relation to ooplasmic components which can be displaced by the centrifugation. It might be possible to suppose that the spatial organization of microtubular or endoplasmic reticular system is responsible for determining the orientation of grooves. If this supposition is valid, the running pattern of grooves on the animal hemisphere may be predetermined by the position and the axial orientation of the meiotic apparatus.

In *Tubifex* eggs centrifuged at I-3 stage, the dimension of egg surface involved in the grooving was closely related to the length of duration between the centrifugation and the

onset of grooving: The longer the eggs were left after centrifugation, the larger the dimension was resulted. If the centrifugation is performed at late I-3 or early I-4 stage, grooves formed first on the surface of the protoplasmic layer and the grooving activity on the surface of large yolk granule layer appeared with the delay of about 40 minutes. When grooves appeared on the surface of yolk granule layer, the nucleus of the intact eggs was at meiotic telophase: The grooving activity of the yolk granule layer was never detected at the meiotic metaphase of the intact eggs. Therefore the grooving activity of the yolk granule layer resembles that on the animal hemisphere of the normally deforming intact eggs and may be meiosis-dependent. When equatorial grooves appear in the intact eggs, the grooving activity (formation of a constriction) of centrifuged eggs was found exclusively on the protoplasmic layer. In eggs treated with ionophore A23187 immediately after centrifugation at early I-3 stage, grooves were also restricted to only the protoplasmic layer. Thus, it may be supposed that the grooving activity of the protoplasmic layer is meiosis-independent.

The surface contractile activity of the protoplasmic layer has been also observed in centrifuged eggs of *Urechis* (Morgan and Tyler, 1935), *Ilyanassa* (Clement, 1938) and *Arbacia* (Marsland *et al.*, 1960). Tilney and Marsland (1968) observed the fine

structure of the cortical layer in centrifuged *Arbacia* eggs and demonstrated the occurrence of microfilaments in the grooving site of the protoplasmic layer: these authors supposed that the contractile activity of the protoplasmic layer is independent of nuclear events and that Ca^{++} is released in the layer. These facts suggest that microfilaments or their precursors are accumulated in the protoplasmic layer after centrifugation.

Grooves formed on the surface of centrifuged eggs always ran parallel to the centrifugal axis. Thus, some ooplasmic factors determining exact sites of grooving and displaced after centrifugation should show, if they exist, a particular distribution in the centrifuged eggs. Since ooplasmic inclusions such as mitochondria, endoplasmic reticula, lipid droplets and yolk granules were stratified after centrifugation and did not show any change in the pattern of separation in the egg interior even when the grooving was initiated, it is probable that one of the factors determining the sites of grooving is the distribution of precursors or monomers of microfilaments in the egg; these precursors may not be observable in the electron microscopy and are displaced after centrifugation. Probably these invisible precursors diffuse from the accumulated site of the protoplasmic layer after the end of centrifugation. The diffusion may occur along the centrifugal axis, thus the surface

of yolk granule layer shows the grooving activity. The above mentioned consideration allows us to speculate that, in the normal deformation movement, protoplasmic streaming translocates contractile elements in the endoplasm and determines the running pattern of grooves on the surface. During the course of the polar body extrusion of starfish, a similar endoplasmic streaming has been demonstrated (Hamaguchi and Hiramoto, 1978). Freeman (1978) has already pointed out the importance of astral microtubules of the meiotic apparatus in translocation of factors specifying apical tuft and gut formation in *Cerebratulus* eggs. In *Tubifex* eggs, however, asters of the meiotic apparatus appear to play no roles in the translocation of contractile elements, because the normal equatorial grooves running parallel to the egg axis were observed after destruction of the meiotic apparatus by colchicine.

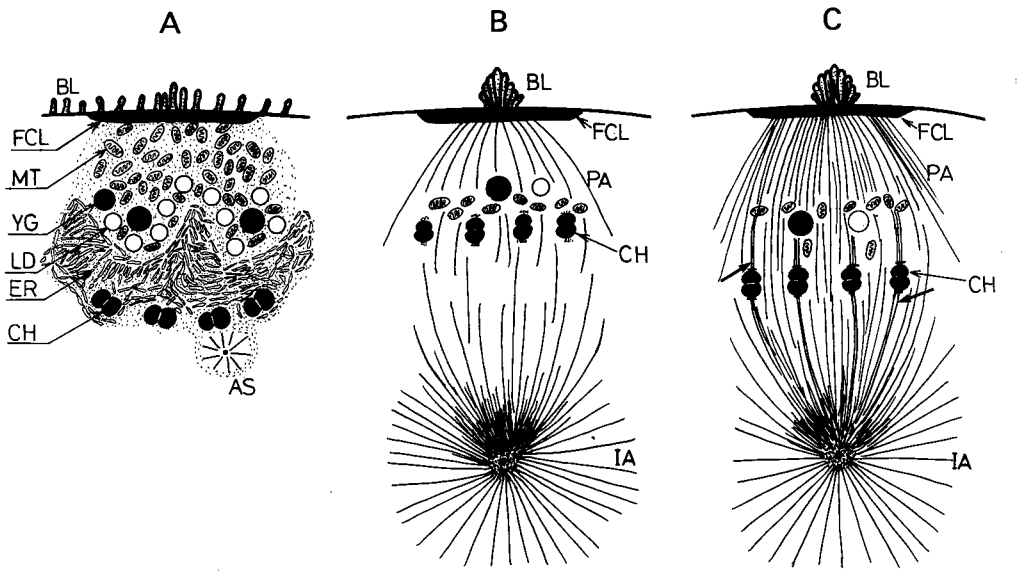
How is the meiotic apparatus fixed at the animal pole ?

As is well known, the process of polar body extrusion from the egg is, in principle, the same with that of the cytoplasmic division in somatic cells. According to Rappaport (1971), the polar body extrusion requires "stimuli" from the meiotic apparatus: microfilaments are organized, under the administration of the meiotic apparatus, in the cortical layer of the furrowing site

and contract to form furrows (Wolpert, 1960; Rappaport, 1971; Schroeder, 1975). In the polar body extrusion, however, the size of daughter cells is extremely different each other. The unequal division is caused by the anchorage of the peripheral pole of the meiotic apparatus in the cortical or subcortical layer of the animal pole. Therefore, the intimate connection of the peripheral pole of the meiotic apparatus with the egg surface may be of importance for the unequal division of the meiosis. Conklin (1917) demonstrated by the centrifugation of *Crepidula* eggs that the first meiotic apparatus at metaphase is firmly attached to the surface; the association is, however, rather loose at the second meiotic metaphase and becomes firm again at anaphase. In *Cerebratulus* eggs, the meiotic apparatus is streched with a fine needle but cannot be dislodged without causing disorganization (Chambers, 1917). These classical works also suggest strongly the presence of "special anchorage device" in the cortical layer which is responsible for the connection of the meiotic apparatus with the egg surface.

The animal pole of *Tubifex* eggs at I-3 stage was externally characterized by the presence of blebs. Some microtubules constituting the peripheral aster were extended into blebs. These blebs may be formed and persisted by a contractile function of filamentous materials in the cortical layer, because

their distribution on the surface of animal pole was considerably modified after treatment with cytochalasin B. When the endoplasm of eggs is washed away from the animal hemisphere isolated, the second meiotic apparatus at metaphase was found still attached to the cortical layer. Microtubules constituting the peripheral aster of the meiotic apparatus invaded the cortical layer. Although no conclusive evidence was obtained from the present observation, the firm attachment of the meiotic apparatus to the egg surface is possibly established by an intimate association of these microtubules with the cortical layer. The structure of the cortical layer of the animal pole at I-3 stage resembled that observed in the furrowing site for the first polar body extrusion. This suggests that filamentous materials observed in the layer at I-3 stage are microfilaments or their derivatives. These materials became hardly detectable when eggs were treated with cytochalasin B at early phases of the formation of meiotic apparatus (early I-3 stage). After completion of the meiotic apparatus formation, however, filamentous materials in the cortical layer became less susceptible to cytochalasin B. This fact implies that physico-chemical properties of microfilaments undergo some changes during the process of meiotic apparatus formation. As the formation of apparatus goes on, microtubules increased in number and



Text-fig. 13. Diagrammatic representation of the formation of the second meiotic apparatus. The animal pole at 0 (A), 15 (B) and 30 (C) minutes after the beginning of I-3 stage. AS, aster; BL, blebs; CH, chromosome; ER, endoplasmic reticula; FCL, filamentous cortical layer; IA, inner aster; LD, lipid droplet; MT, mitochondria; PA, peripheral aster; YG, yolk granule. Big arrows in (C), kinetochore microtubules.

invaded the cortical layer (Text-fig. 13). Therefore, it appears that microfilaments become less susceptible to cytochalasin B concurrently with the establishment of the intimate association between the peripheral pole of the meiotic apparatus and the egg surface. Accordingly microfilaments having less susceptible property to cytochalasin seem to play some roles in fixing the meiotic apparatus at the animal pole.

Little is known about the mechanism of attachment of the meiotic apparatus to the egg surface in other animals. Although

the cortical cytoplasm overlying the second meiotic apparatus contains microfilamentous materials in mouse eggs, the role of these materials in fixing the apparatus at the animal pole is obscure (Nicosia *et al.*, 1977). The cortical layer overlying the meiotic apparatus of *Mytilus* and *Spisula* eggs does not contain filamentous materials (Longo and Anderson, 1969, 1970). Thus, the attachment of the peripheral pole of the meiotic apparatus to the egg surface in these eggs does not require the participation of filamentous materials.

How are microtubules of the peripheral aster formed ?

McIntosh *et al.* (1969) outlined the process of mitotic apparatus formation as follows: At the onset of the formation, microtubules appear radially around the centriolar region and astral rays are formed. Concurrently with the formation of asters, microtubules appear on the kinetochore of chromosomes. They increase in length and form a continuous spindle; the shape of spindle is determined by lateral interactions between microtubules from the peripheral and inner asters. Chromosomes are arranged on the metaphase plate of the spindle, and the mitotic apparatus is fully formed. There are a number of observations indicating that both the pericentriolar region and the kinetochore of chromosomes have an ability to organize microtubules

(Fawcett, 1966; Rhodin, 1974; Telzer *et al.*, 1975; McGill and Brinkley, 1975; Gould and Borisy, 1977; Telzer and Rosenbaum, 1979). Therefore, these loci have been called microtubule organizing centers (see Pikett-Heaps, 1969). Considering the structural homology between the *meiotic* apparatus and *mitotic* apparatus, it is reasonable to suppose that microtubules forming the meiotic apparatus are also produced by the functions of pericentriolar materials and the kinetochore of chromosomes. In fact, Weisenberg and Rosenfeld (1975) demonstrated that pericentriolar materials of *Spisula* eggs are capable of acting as a site for the initiation of microtubule assembly. Therefore, in *Tubifex* eggs, the kinetochore of chromosomes and regions of the peripheral and inner asters are expected to be the microtubule organizing centers. The structure of peripheral aster is however undoubtedly different from that of the inner aster: Although a centriolar structure is detected in the center of inner aster, it is not found in the peripheral aster. These facts suggest that the mechanism for the microtubule formation in the peripheral pole of meiotic apparatus is different from that in the inner pole.

Since the peripheral pole of the meiotic apparatus fully developed was embedded in the cortical or subcortical layer of the egg, it is probable that the formation of peripheral aster microtubules is influenced by materials in the cortical

layer. Observations on the cortical layer isolated from the animal pole revealed close connections of peripheral aster microtubules with the filamentous cortical layer. When the cortical layer became very thin and lost its filamentous inclusions after treatment of eggs with cytochalasin B, microtubules of the peripheral aster and the spindle also decreased in number. Since microtubules of the inner aster and kinetochore appeared to be normal in number, cytochalasin B itself may not inhibit directly the microtubule formation. In the intact eggs, mitochondria and lipid droplets located near the egg surface at the initial phase of the formation of meiotic apparatus were all expelled from sites concurrently with the appearance of peripheral aster microtubules. In cytochalasin-treated eggs, however, these organelles and inclusions remained near the egg surface. These facts may indicate (1) that the microtubule formation for the peripheral aster requires the presence of cytochalasin-sensitive factors, and (2) that the formation seems to be initiated in the cortical layer and proceeds toward the subcortical region of the egg. The filamentous cortical layer at the animal pole is assumed to be a site of the formation of microtubules (microtubule organizing center), though the fine structure of the cortical layer was different from that of the reported microtubule organizing center; the pericentriolar region and the kinetochores of chromosomes are granular in appearance

(Telzer *et al.*, 1975; Weisenberg and Rosenfeld, 1975) but the cortical layer of *Tubifex* eggs does not contain granular materials. The meiotic spindle was formed just beneath the filamentous cortical layer. Since some microtubules of the peripheral aster participate in the formation of spindle, the decrease in number of microtubules forming the spindle fibers after cytochalasin B-treatment may be ascribed to an unsuccessful assembly of peripheral aster microtubules.

How is the cytoplasmic bulge formed on the egg surface before the polar body extrusion ?

Previous to the extrusion of polar body, a cytoplasmic bulge is formed on the surface of the animal pole. Chambers (1917) postulated that the formation of bulge is resulted from weakening or relaxing of "rigidity" of the egg surface induced by the influence of the peripheral aster. Wolpert (1960) supposed that "local differentiation" of the egg cortex at the animal pole and an increase in the internal pressure of the egg are important for the formation of cytoplasmic bulge. Recent experiments in starfish clearly showed that the increased internal pressure of the egg causes a protoplasmic streaming toward the animal pole (Hamaguchi and Hiramoto, 1978).

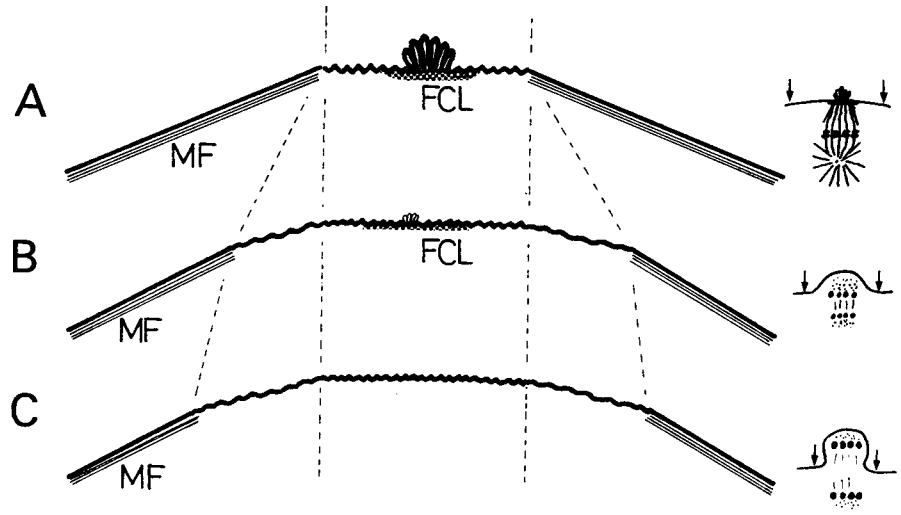
In *Tubifex* eggs, "local differentiation" of the surface of animal pole was detected first at late metaphase of the

meiotic division: The surface overlying the peripheral aster showed an undulation, but the neighboring area was relatively smooth. During the period up to early anaphase of the meiosis, the egg surface expanded in the undulating area to form a cytoplasmic bulge. With the enlargement of the bulge, the relatively smooth surface neighboring the expanded region also showed an undulation; it formed the side of the bulge. In sections, no filamentous materials were detected in the cortical layer beneath the undulated surface. When the bulge attained full size, the materials were observed only in the proximal periphery of the bulge which had the undulated surface. It may be supposed therefore, that the disappearance of filamentous materials from the cortical layer has some relation to the surface undulation. Since the filamentous materials in the cortical layer are contractile elements of *Tubifex* eggs, the surface undulation following the disappearance of these materials may be a manifestation of the weakened contractile activity of the cortical layer. The cortical layer beneath the relatively smooth surface was rich in the filamentous materials. Therefore, it is assumed that microfilaments endow a "rigidity" of the cortical cytoplasm and maintain the egg surface smooth. If this assumption is correct, a local decrease in "rigidity" of the cortical layer is induced by the disappearance of microfilaments from the site and is one of factors responsible for the formation

of a cytoplasmic bulge on the egg surface.

As stated already, microfilaments were observed in the cortical layer beneath the smooth surface of the animal pole. Therefore, the physiological state of this region is assumed to be favorable for the organization and function of microfilaments. Concurrently with the occurrence of surface undulation, microfilaments gradually disappeared from the cortical layer of the animal pole. At the same time, the inner aster of the meiotic apparatus decreased in size. The disappearance of microfilaments from the microtubule-organizing cortical layer of the animal pole might give rise to disassembly of microtubules. However, the present observation could not answer to the question why microfilaments disappear from the cortical layer of animal pole. At any rate, the disappearance of microfilaments may weaken "rigidity" of the cortical layer and allow the expansion of egg surface for the formation of cytoplasmic bulge.

As the cytoplasmic bulge increases in its surface area, the cortical layer became very thin and lost microfilaments (Text-fig. 14). How did the bulge increase its surface area ? In cases of cleavage of various animal eggs, new plasma membranes originated from membraneous organelles of the egg protoplasm have been reported to be inserted to old membranes and provide for the increased cell surface (Arnold, 1976). However, this seems



Text-fig. 14. Distribution of microfilaments (MF) in the cortical layer at various phases of the second meiotic division. A, late metaphase; B, mid anaphase; C, early telophase. The areas presented correspond to those indicated by two arrows in the right row. In order to be proportioned with A and for convenience of the comparison, the contour in B and C is simplified by eliminating the shape of the cytoplasmic bulge. FCL, filamentous cortical layer.

not the case of the polar body extrusion in *Tubifex* eggs; no fusion of the plasma membrane with membraneous organelles can find in any phase of the enlargement of bulge.

When the meiotic division reached telophase, filaments located in the proximal periphery of the cytoplasmic bulge show contraction, so that the base of the bulge is constricted to extrude the polar body from the egg. According to Rappaport (1971), the constricting region of dividing egg is determined by asters of the mitotic apparatus. Since the size of cytoplasmic

bulge at the animal pole of *Tubifex* eggs was nearly the same with that of the meiotic spindle, the egg surface which is involved in the polar body formation and contains no microfilaments in the undulating cortical cytoplasm may be determined by aster of the meiotic apparatus.

Why microfilaments disappear from a specific part of the cortical layer ? Since the plasma membrane seems to contain particles linking to microfilaments (intramembrane particles) (Bluemink and Tertoolen, 1978), the translocation of these particles toward the proximal periphery of the bulge might occur during the polar body extrusion. Longo (1979) succeeded to inhibit the formation of a cytoplasmic bulge as well as the polar body extrusion by treatment of *Spisula* eggs with concanavalin A; in this case, the organization of microfilaments in the cortical layer has been failed. He supposed that some cross-linkage of components in the egg surface is produced after treatment with this lectin and inhibits the process of the polar body extrusion. If his assumption is correct, a dynamic alteration in the surface architecture should occur at the time of the meiotic division.

The enlargement of the cytoplasmic bulge was not only due to the increase in diameter of the bulge at the proximal level but also to the increase in its height. Probably microfilaments located in the proximal periphery of the bulge

prevent an unlimited increase in diameter of the bulge, which results in a considerable increase in its height; concurrently with a slight movement of the egg surface toward the animal pole which is resulted from the enlargement of the cytoplasmic bulge, perpendicularly arranged microfilaments at the base of the bulge may pull the egg surface inward and prevent the increase in diameter of the bulge. It is expected from these considerations that, by the disruption of microfilaments, the cytoplasmic bulge decreases in its height but increases in diameter. In fact, the height of a bulge formed in eggs treated with cytochalasin B was low compared with that in the intact eggs. The same results have been obtained by Longo (1972) in *Spisula* eggs treated with this drug. Therefore, it can be said that microfilaments in the proximal periphery of the cytoplasmic bulge are important not only for the extrusion of the polar body but also for the formation of the cytoplasmic bulge at the animal pole.

Significance of the deformation movement in the ooplasmic segregation

As for the biological significance of the deformation movement, Penners (1922) supposed that it is of importance for the accumulation of pole plasm in *Tubifex* eggs. In the present study, the ooplasmic segregation became apparent immediately

after the end of the second movement. Concurrently with the formation of surface grooves, a marked displacement of the ooplasmic inclusions was noticed. It is highly probable that changes in the organization of the egg cytoplasm is caused, in part, by the formation of equatorial grooves. The accumulations of plasm are, however, observed at the animal and vegetal poles, even when the movement was inhibited after treatment of eggs with cytochalasin B; in this case, the amount of plasm accumulated appeared to be slightly reduced compared to the intact eggs. However, there is a possibility that the quality or composition of plasms accumulated at both poles of the cytochalasin B-treated eggs might differ from that observed in the intact eggs. Since no further development was observed in these treated eggs, it is still remained unknown whether the accumulated pole plasms in the cytochalasin B-treated eggs are qualitatively the same or not with those of the normally developing eggs in respect of the behavior in the embryonic differentiation. Even when spherical eggs were treated with ionophore A23187 to elicit an unusual groove-forming activity, no precocious accumulation of pole plasms was not induced. Therefore, it is unlikely that the deformation movement or the surface contractile activity of *Tubifex* eggs plays a definitive role in the ooplasmic segregation. Since the displacement of ooplasmic substances occurs concurrently with the formation

of surface grooves during the deformation movement, the deformation movement might accelerate the process of the ooplasmic segregation in *Tubifex* eggs.

SUMMARY

(1) The underlying mechanism of early development was analyzed in a freshwater oligochaete, *Tubifex hattai* NOMURA. For this purpose, living eggs as well as fixed and sectioned specimens processed for the scanning and transmission electron microscopy were used.

(2) The early developmental period was divided into the following six stages: I-1 stage (metaphase of the first meiotic division), I-2 stage (extrusion of the first polar body), I-3 stage (metaphase of the second meiotic division), I-4 stage (extrusion of the second polar body), I-5 stage (karyogamy), and I-6 stage (the first and second cleavage).

(3) Shortly after extrusion of the first polar body at I-2 stage, no trace of the meiotic apparatus was detected in the animal pole; instead, a large aggregation of membraneous

organelles and filamentous materials were seen at the pole. Within 15 minutes, the second meiotic apparatus was formed; its inner aster appeared to be fully developed, whereas the microtubules constituting the peripheral aster and the spindle were small in number. After another 15 minutes, the apparatus was fully formed (I-3 stage). A centriolar structure was detected in the inner aster but not in the peripheral aster.

(4) At the time of the extrusion of polar bodies (I-2 and I-4 stages), the egg surface at the equatorial region and the animal hemisphere showed a contractile activity. As a result, deep grooves were formed on the egg surface and the egg shape was considerably changed. The change of the egg shape is here designated as the "deformation movement". At other developmental stages, however, the egg surface was smooth and did not show any groove-forming activity.

(5) The peripheral cytoplasmic layer, or the cortical layer, of the egg was devoid of membraneous organelles such as mitochondria and endoplasmic reticula. At the animal pole, it contained filamentous materials and was electron-dense. As the development of the meiotic apparatus proceeded, the cortical layer was invaded by microtubules from the peripheral aster. The surface of the animal pole was markedly indented and formed "blebs". Some microtubules from the aster extended

into these blebs.

(6) In the scanning electron microscopy, the surface of the animal pole at late metaphase of the second meiotic division was classified into two areas, the undulating area and the smooth area. The surface blebs were arranged in a bouquet and placed on the undulating area. In sectioned materials, the cortical layer contained filamentous materials beneath the smooth area but not the undulating area. When a cytoplasmic bulge took place forming the second polar body, filamentous materials were found exclusively at the proximal periphery of the bulge but not at its distal and side portions.

(7) The inner surface of the cortical layer was isolated from the animal pole of the egg immediately after extrusion of the first polar body and was observed in the scanning electron microscope. A thick central portion of the layer looked as if it protruded pseudopodial processes toward the periphery. When the meiotic apparatus was formed, the cortical layer with the apparatus could be isolated: In this preparation, however, the inner aster was invariably lost. The association of the cortical layer with the meiotic apparatus was accomplished by means of the microtubules of the peripheral aster that penetrated into the layer.

(8) After treatment of eggs with cytochalasin B, filamentous materials in the cortical layer disappeared, indicating that they were microfilaments.

(9) When the eggs were treated with cytochalasin B at late I-2 stage, the microtubules of the peripheral aster and the spindle at I-3 stage (the second meiotic metaphase) were small in number, whereas those of the inner aster and kinetochore appeared to be normal in these eggs. Although a cytoplasmic bulge took place forming the polar body after treatment with cytochalasin B, its height was low compared to that in intact eggs. No extrusion of the polar body was seen in such eggs.

(10) The cortical layer of the equatorial region was found to contain microfilaments during the deformation movement (I-2 and I-4 stages) but not at other (non-deforming I-1, I-3 and I-5) stages. Microfilaments were organized in the equatorial region at the time of onset of the movement and arranged perpendicularly or obliquely to the egg surface. The number of microfilaments in the cortical layer increased toward the climax phase of the movement but gradually decreased as the surface grooving activity was diminished.

(11) The groove-forming activity of the equatorial surface was inhibited by the treatment of eggs with cytochalasin B.

The degree of inhibition depended on the time length of the treatment and the developmental stages at which the eggs were exposed to cytochalasin B. In the cortical layer of these treated eggs, microfilaments did not come out or at least their arrangement was disrupted.

(12) A groove-forming activity was elicited on the equatorial surface of the spherical eggs by ionophore A23187. It was found that the activity was independent of either external Ca^{++} or Mg^{++} and accompanied the organization of microfilaments in the cortical layer. The response to ionophore was most distinct in round eggs at the beginning of I-3 and I-5 stages (immediately after the disappearance of surface grooves formed during the deformation movement). The susceptibility of the egg to this drug gradually decreased with the lapse of time after the end of the preceding movement.

(13) Dinitrophenol inhibited the equatorial grooving but not the animal hemisphere grooving in the concentration which did not disturb the meiotic events. The disruption of filaments by dinitrophenol took place more easily in the equatorial region than the animal hemisphere. Colchicine, in the concentration that destroyed the meiotic apparatus, completely inhibited the surface grooving activity on the animal hemisphere but not

on the equatorial region. From these facts, it was assumed that the contractile activity of the egg surface on the animal hemisphere is different in property from that seen at the equatorial region.

(14) When the centrifugal force was applied to eggs at round I-3 and deforming early I-4 stages, the interior of the egg was stratified into 5 layers: centripetal lipid layer, protoplasmic layer, large yolk granule layer, "opaque plasma" layer, and centrifugal small yolk granule layer.

(15) The centrifuged eggs showed the same surface grooving activity at the time of the deformation movement seen in the intact eggs. Grooves appeared in these eggs running in parallel to the centrifugal axis. The dimension of egg surface involved in grooving was found closely related to the time length between the centrifugation and the onset of the grooving. Microfilaments were seen in the grooving sites.

(16) Non-nucleated egg fragments also showed a surface grooving activity. The formation of grooves was precisely synchronous with the second deformation movement and early cleavages of the intact eggs, and was similarly inhibited by cytochalasin B.

(17) The onset of the groove-forming activity was delayed in non-nucleated fragments after inhibition of the protein synthesis by cycloheximide. The eggs at certain developmental stages were especially sensitive to this drug.

(18) From the results mentioned above, it was concluded that: (a) the deformation movement of *Tubifex* eggs is a microfilament-dependent process; (b) the microfilaments are important for the assembly of microtubules and for the extrusion of the polar body; (c) the egg surface overlying the meiotic apparatus at animal pole differs in sensitivity to cytochalasin B from that of the equatorial region; (d) the periodic appearance of the surface contractile activity is correlated with the change in the intracellular Ca^{++} level of the egg.

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PLATES

Explanation of Plate 1

Figs. 1-6. Photomicrographs showing shape changes in live eggs and nuclear events in sectioned materials (hematoxylin preparations).

Fig. 1. Shortly after oviposition (I-1 stage). (a) Animal pole view. x70. (b) Metaphase spindle of the first meiotic division. x600.

Fig. 2. At I-2 stage. (a) Animal pole view showing the first deformation movement. x70. (b) Extrusion of the first polar body. An arrow indicates chromosomes. x700.

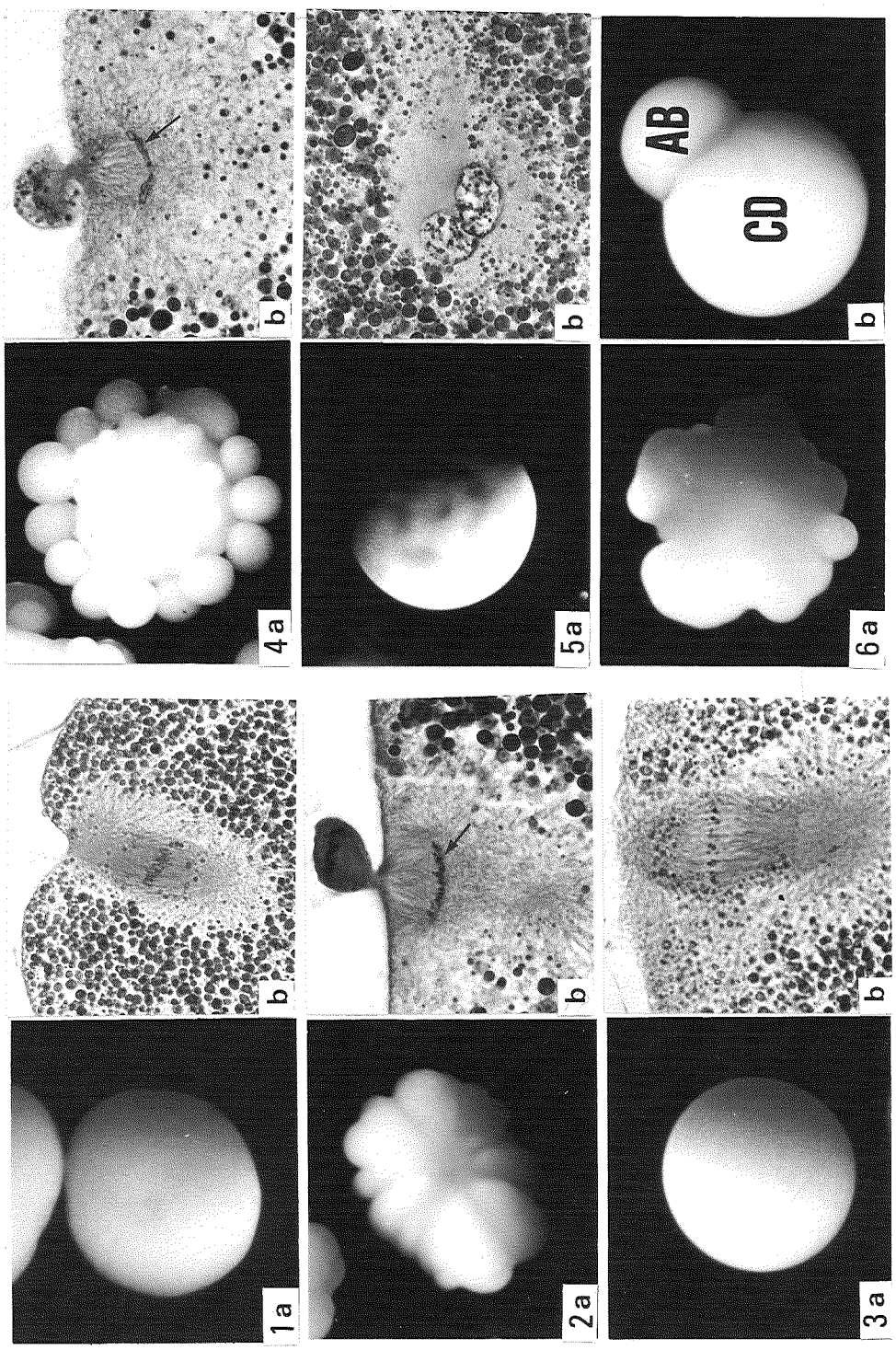
Fig. 3. At I-3 stage. (a) Animal pole view. x70. (b) Metaphase spindle of the second meiotic division. x670.

Fig. 4. At I-4 stage. (a) Animal pole view showing the second deformation movement. Note many protuberances on the equatorial region. x70. (b) Extrusion of the second polar body. An arrow indicates karyomeres. x700.

Fig. 5. At I-5 stage. (a) Animal pole view showing the accumulation of pole plasm. x70. (b) Fusion of pronuclei. x700.

Fig. 6. At I-6 stage. (a) An egg undergoing the first cleavage. Animal pole view. x70. (b) Two-cell stage. Note small *AB* and large *CD* cells. x70.

Plate 1



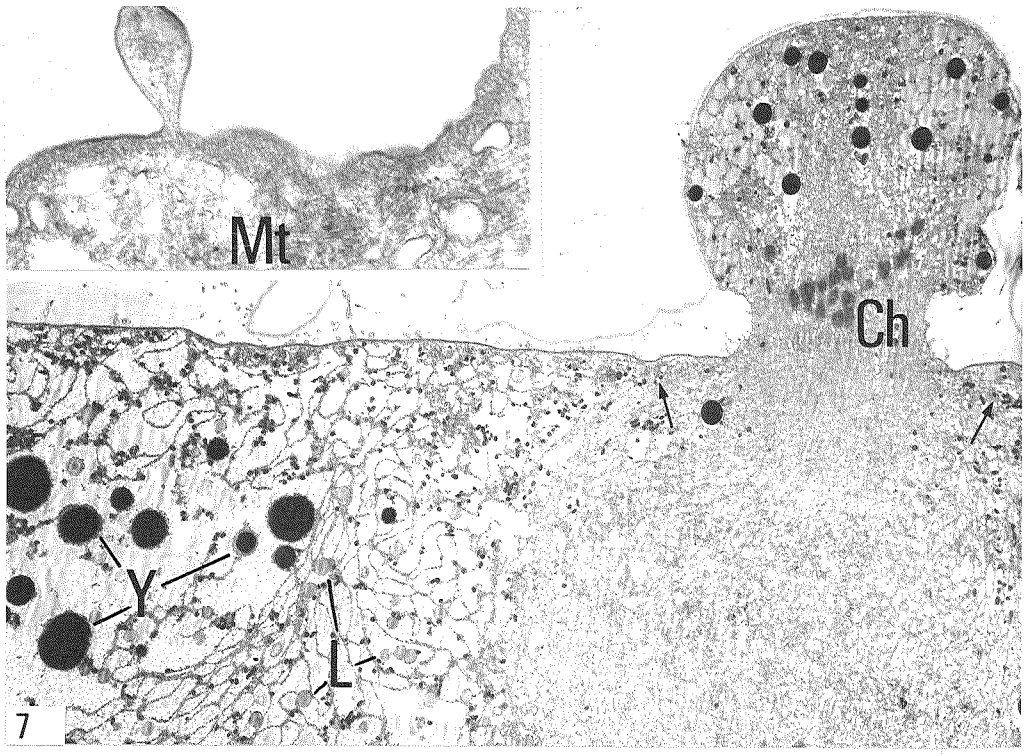
Explanation of Plate 2

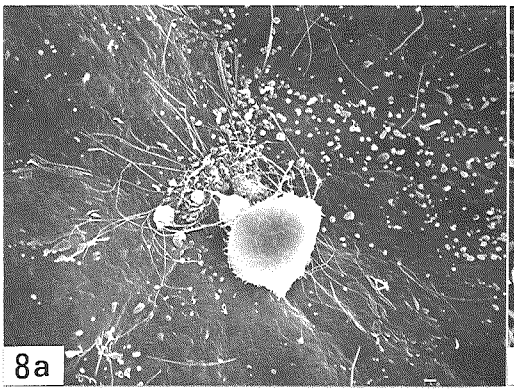
Fig. 7. Transmission electron micrographs of the animal pole of an egg extruding the first polar body. The constriction is evident at the proximal portion of the cytoplasmic bulge for the first polar body. Chromosomes (Ch) are seen in the bulge. Arrows indicate the accumulation of mitochondria toward the constricting region of the bulge. Inset is a higher magnification of the proximal portion of the bulge, showing microfilaments in the cortical layer. L, lipid droplet; Mt, mitochondria; Y, yolk granule. x1500; inset, x30000.

Figs. 8-9. Scanning electron micrographs of the animal pole surface of eggs forming the second meiotic apparatus.

Fig. 8. Immediately after the extrusion of the first polar body (PB). Blebs of various length cover a wide area; they are present in high density at the portion of extrusion of the polar body (Fig. 8b). a, x500; b, x1750.

Fig. 9. Thirty minutes after the extrusion of the first polar body. Most of blebs have been cleared off; they are found on a restricted region in a form of bouquet. The surface appears to be rather smooth (Fig. 9b; compare with Fig. 28). a, x750; b, x2500.


Mt
Ch
Y
L
7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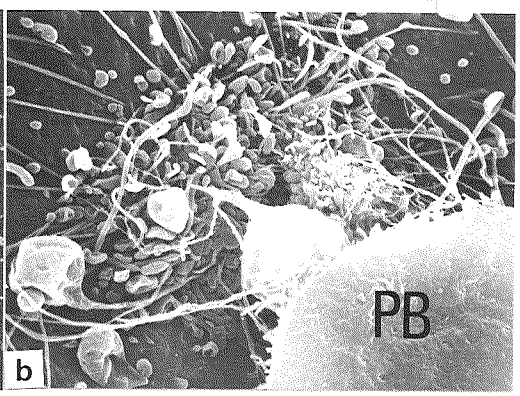
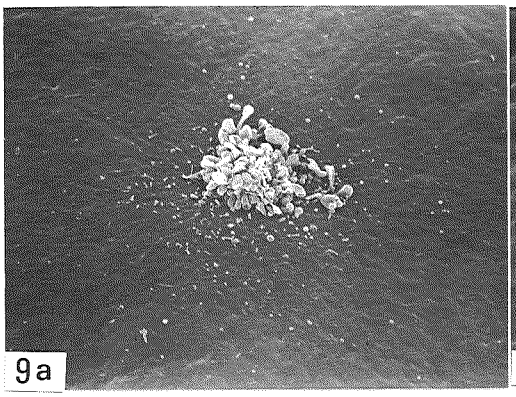

PB
b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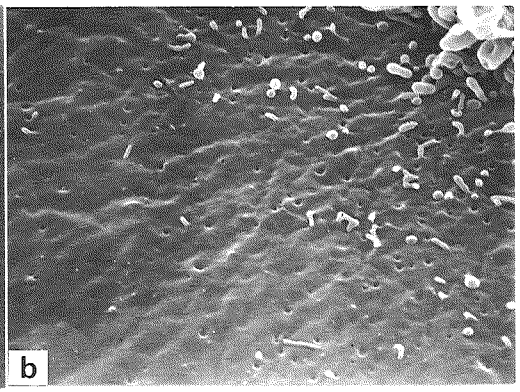
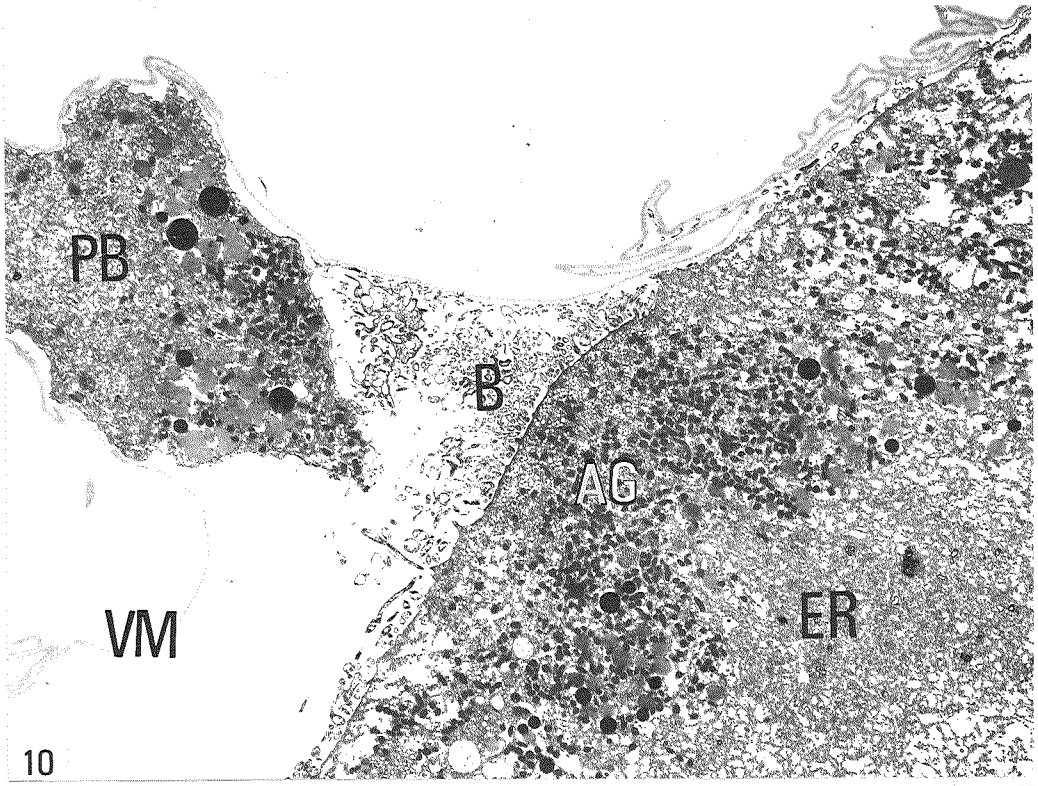
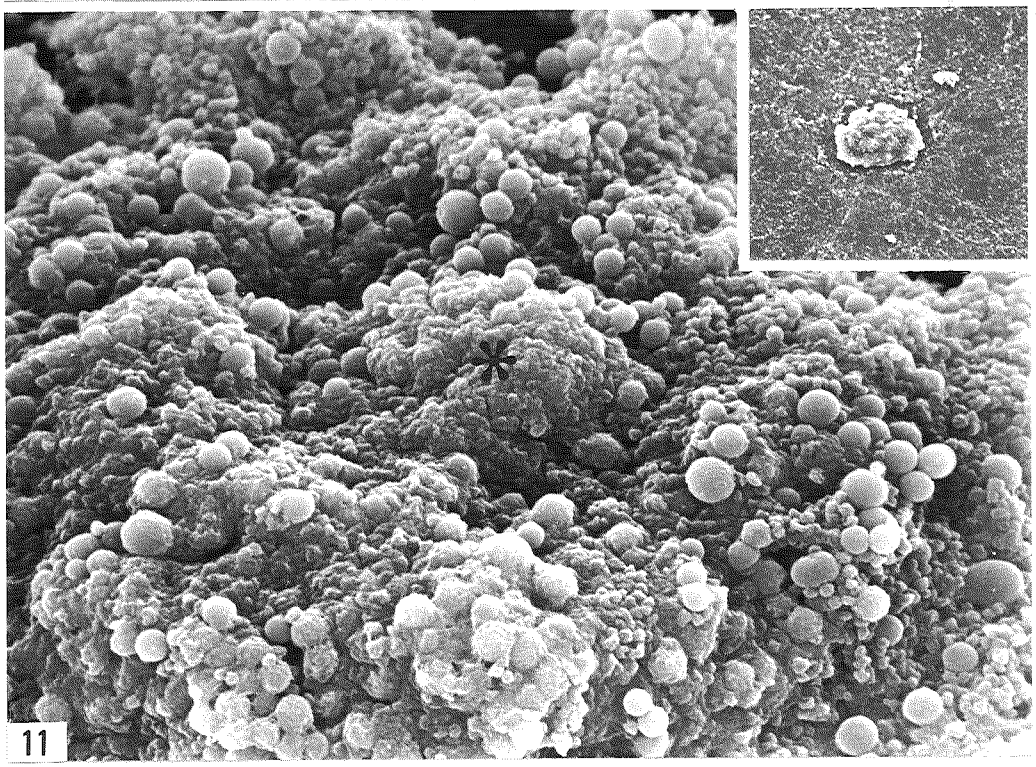

b

Explanation of Plate 3

Fig. 10. Transmission electron micrograph showing the animal pole of an egg shortly after the extrusion of the first polar body (PB). Numerous blebs (B) are present on the surface (see also Fig. 8a). Note aggregation (AG) of mitochondria, lipid droplets and yolk granules, and an accumulation of endoplasmic reticula (ER). VM, vitelline membrane. x2000.

Fig. 11. Scanning electron micrographs of an isolated aggregation of mitochondria, lipid droplets and yolk granules. For isolation procedure, see Text-figure 1. Asterisk indicates endoplasmic reticula remaining in the central part of the aggregation. Inset shows low magnification of the aggregation. x4000; inset, x230.

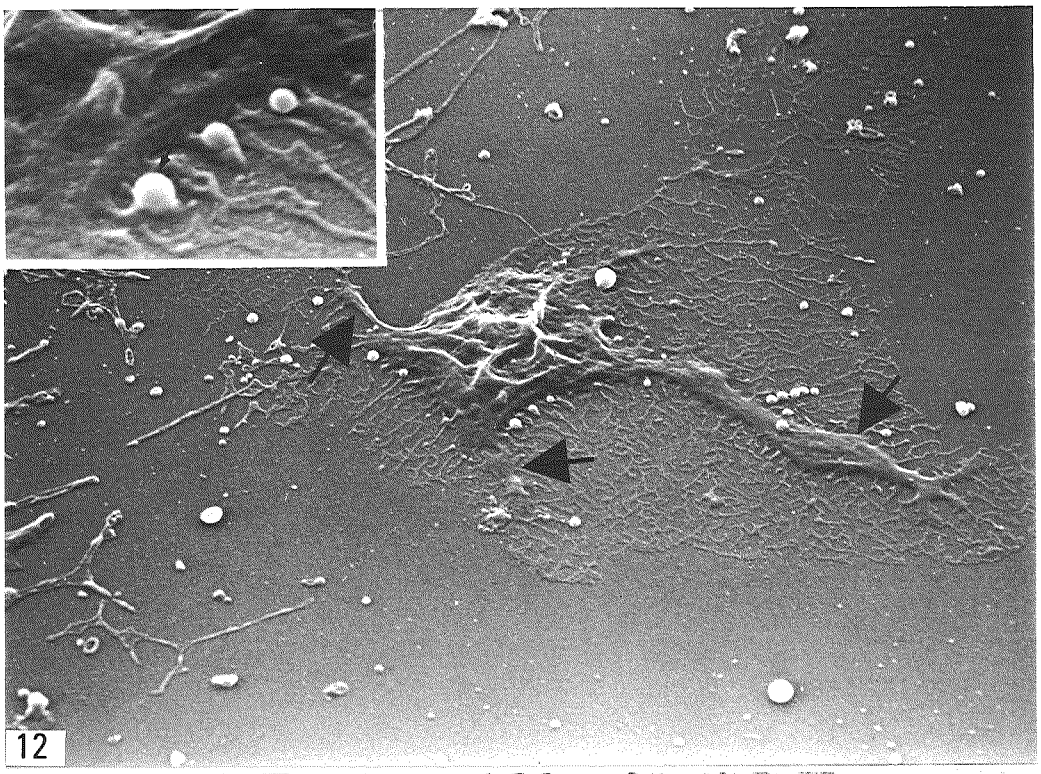

PB
B
AG
VM
ER
10


*
11

Explanation of Plate 4

Fig. 12. Inside view of the isolated cortical layer at the animal pole of an egg shortly after the extrusion of the first polar body. Scanning electron micrograph. After an egg was attached to the polylysine-coated coverslip, egg contents were flowed away (see Text-fig. 1). Note pseudopodial processes (big arrows) of the cortical layer. Vesicles (small arrows in inset) are embedded in the cortical layer. x2300; inset, x8600.

Fig. 13. Transmission electron micrograph of the animal pole of an egg shortly after the extrusion of the first polar body. Note filamentous materials (arrows) in the cortical layer. B, bleb; M, mitochondria. x45000.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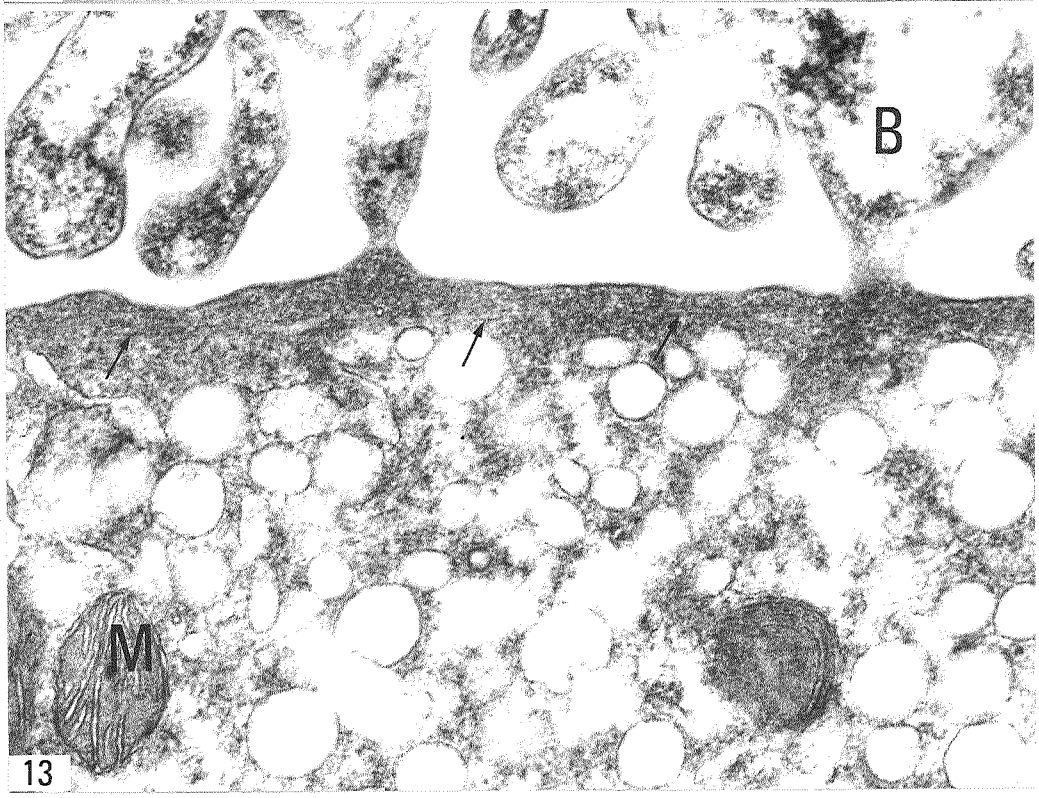

B
M
13

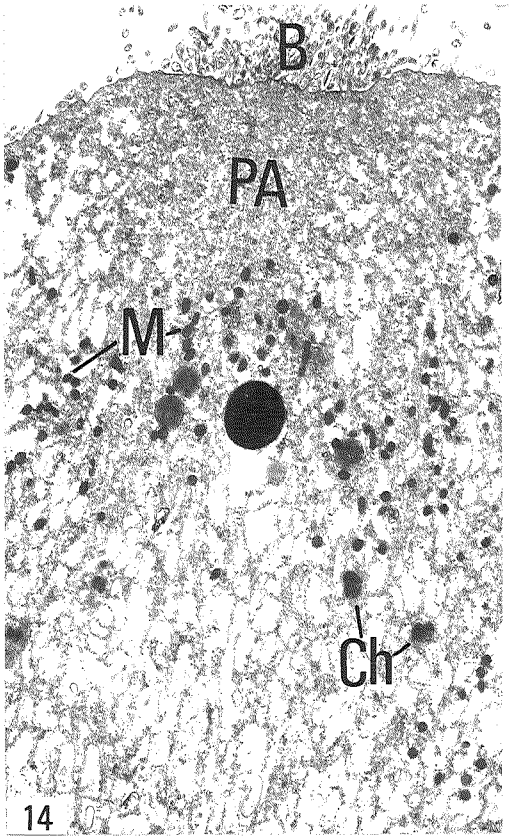
Explanation of Plate 5

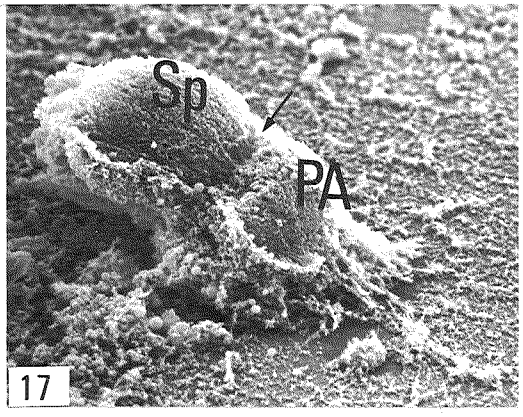
Fig. 14. Transmission electron micrograph showing developing meiotic apparatus in an egg at 15 minutes after the beginning of I-3 stage. At the boundary between the peripheral aster (PA) and the spindle, mitochondria (M) and lipid droplets are located. Chromosomes (Ch) are present near this boundary. B, bleb. x2700.

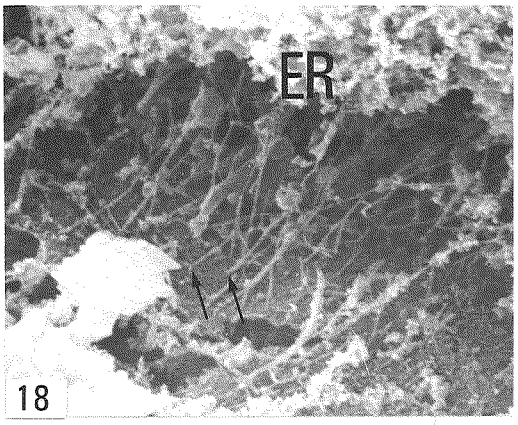
Fig. 15. Higher magnification of a chromosome in Figure 14. Note granular materials (arrow) at the kinetochore region. No kinetochore microtubules are seen. x30000.

Fig. 16. Higher magnification of the peripheral region shown in Figure 14. Many blebs (B) are seen on the egg surface. Note that each microtubule (Mt) of the peripheral aster terminates in the electron-dense cortical layer. x40000.

Figs. 17-18. Scanning electron micrographs showing inside view of an egg treated as shown in Text-figure 1. Fifteen minutes after the beginning of I-3 stage. Meiotic apparatus is attached to the egg surface. An arrow in Figure 17 indicates the boundary between the peripheral aster (PA) and the spindle (Sp). Figure 18 is a close-up of the attaching site of the apparatus to the egg surface. Termination of microtubules (arrows) on the surface is evident. Fig. 17, x500; Fig. 18, x7500.


B
PA
M
Ch
14


Sp
PA
17


ER
18


15


B
Mt
16

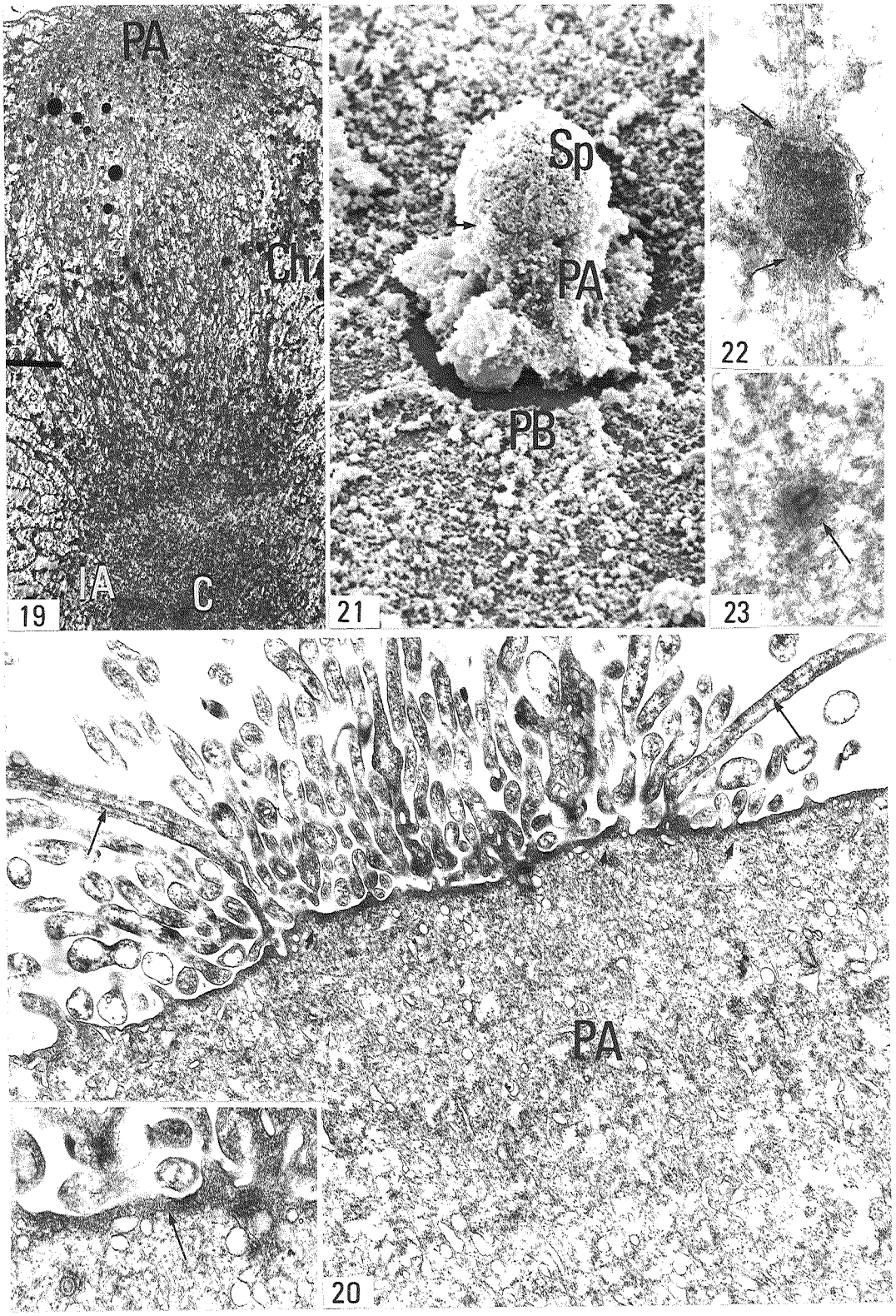
Explanation of Plate 6

Fig. 19. Transmission electron micrograph of the meiotic apparatus (second metaphase) found at 30 minutes after the beginning of I-3 stage. C, centriolar region; Ch, chromosome; IA, inner aster; PA, peripheral aster. x1200.

Fig. 20. Transmission electron micrograph showing the peripheral region of the animal pole. Microtubules (short arrows) from the peripheral aster (PA) terminate in the electron-dense cortical layer. Microtubules (long arrows) are also found in blebs. The cortical layer is consisted of filamentous materials (arrow in inset). x12000; inset, x25000.

Fig. 21. Inside view of an egg at 30 minutes after the beginning of I-3 stage. Scanning electron micrograph. Meiotic apparatus composed of the peripheral aster (PA) and the spindle (Sp) is attached to the egg surface. An arrow points to the boundary of these components of the apparatus. PB, first polar body. x430.

Figs. 22-23. Higher magnification of Figure 19. Figure 22 shows a chromosome with well-developed kinetochore microtubules (arrows). Figure 23 shows the central part of the inner aster; centriole is evident. Note electron-dense pericentriolar materials (arrow in Fig. 23). Fig. 22, x17000; Fig. 23, x10000.


PA
Ch
IA
C
19
Sp
PA
PB
21
22
23
PA
20

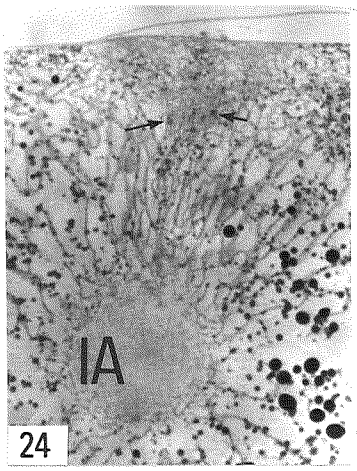
Explanation of Plate 7

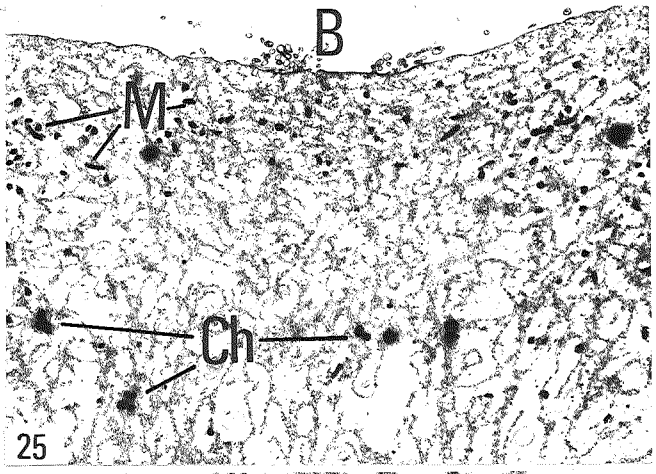
Figs. 24-27. Animal pole of eggs treated with cytochalasin B. Eggs were treated with 50 μg/ml cytochalasin B for 60 minutes at late I-2 stage and fixed at 30 minutes after the beginning of I-3 stage.

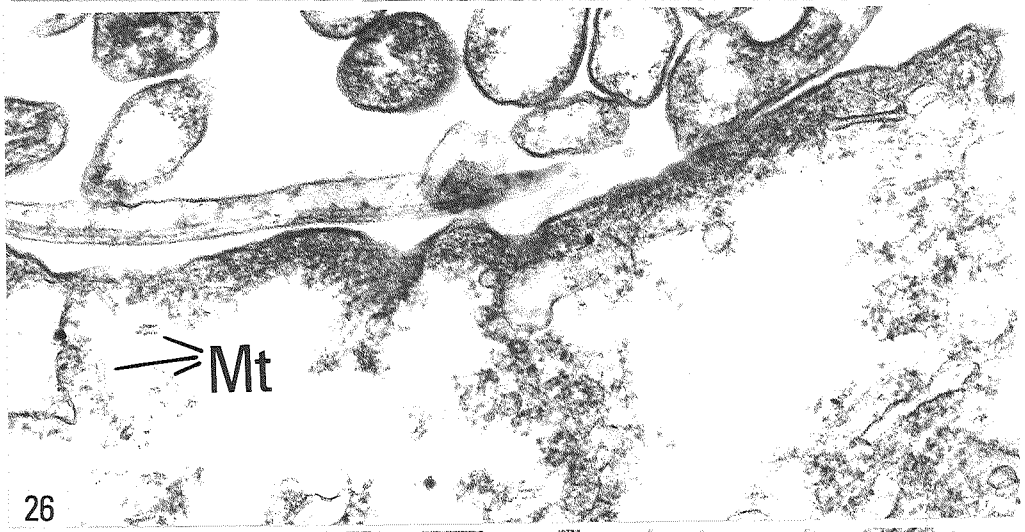
Fig. 24. Photomicrograph of a thick section (toluidin blue preparation). Although the inner aster (IA) has well developed, other parts of the meiotic apparatus show deficient development. Arrows point to chromosomes. x250.

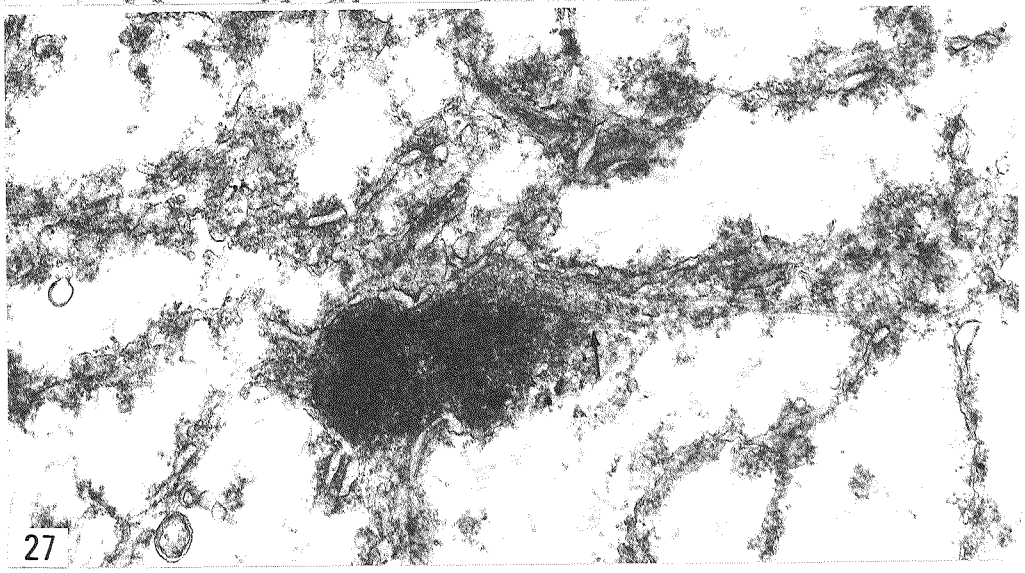
Fig. 25. Transmission electron micrograph showing poorly developed peripheral aster and spindle. Blebs (B) and microtubules are small in number (compare with Figs.14 and 20). Note that mitochondria (M) are present near the surface. x2400.

Figs. 26-27. Higher magnification of Figure 25. Filamentous materials are not found in the cortical layer and the number of microtubules (Mt) is very small (Fig. 26). Kinetochore microtubules (arrow in Fig. 27) are found. Fig. 26, x45000; Fig. 27, x32000.


IA
24

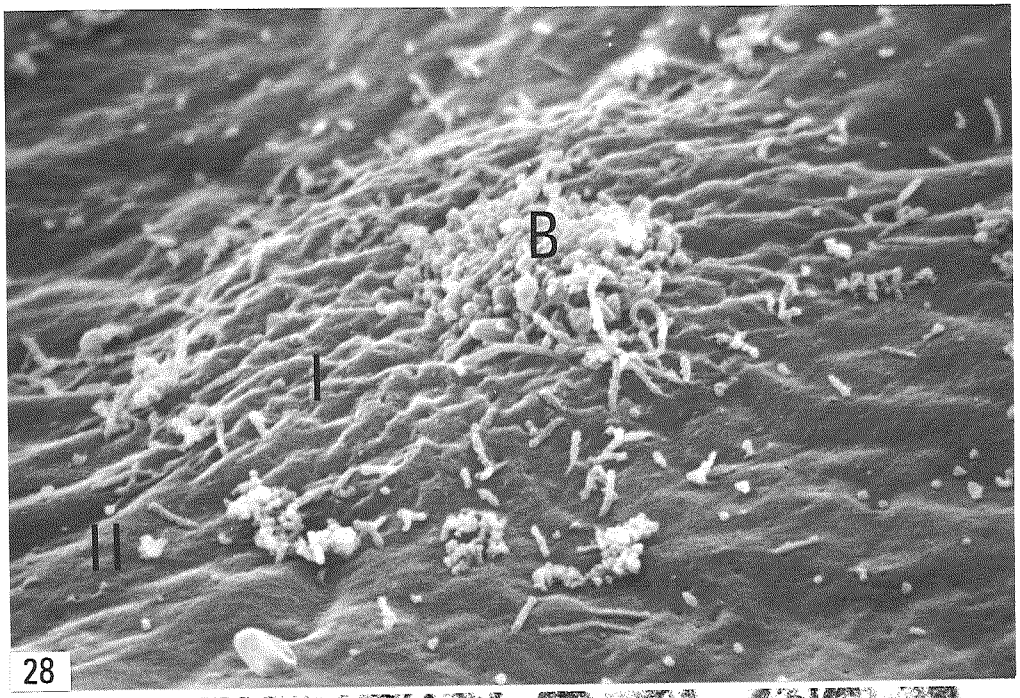

B
M
Ch
25

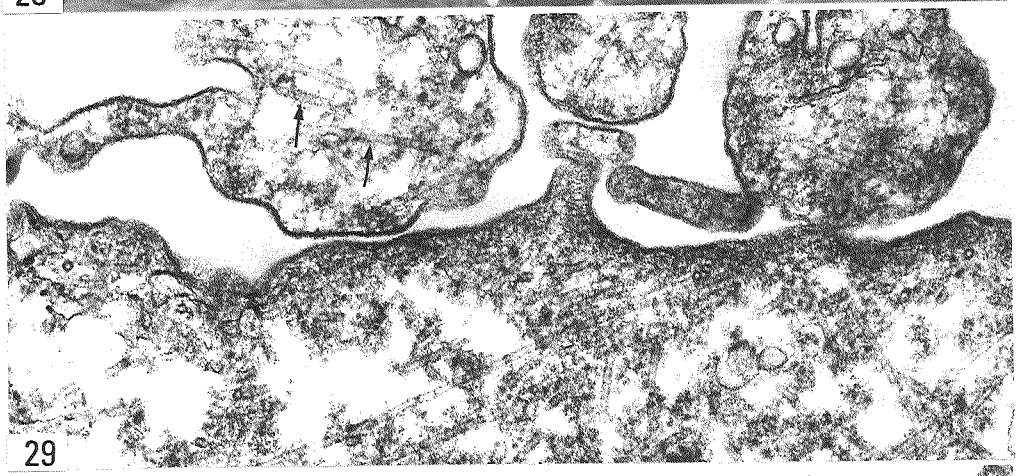

Mt
26


27

Explanation of Plate 8

Figs. 28-30. Scanning (Fig. 28) and transmission (Figs. 29-30) electron micrographs of the animal pole of an egg at late metaphase of the second meiotic division. Two surface areas are discernible (Fig. 28): undulating area (I) and rather smooth area (II). Transmission electron microscopy reveals that the cortical layer of smooth surface is characterized by the presence of microfilaments; at the boundary (big arrow) between undulating (I) and smooth (II) area, microfilaments (small arrow) are arranged perpendicularly to the surface (Fig. 30). A bouquet of blebs (B) is located in the undulating area. The cortical layer of this blebby area contains some filamentous materials (Fig. 29). Some microtubules (arrows in Fig. 29) are seen in blebs. Fig. 28, x3500; Figs. 29-30, x40000.


B
I
II
28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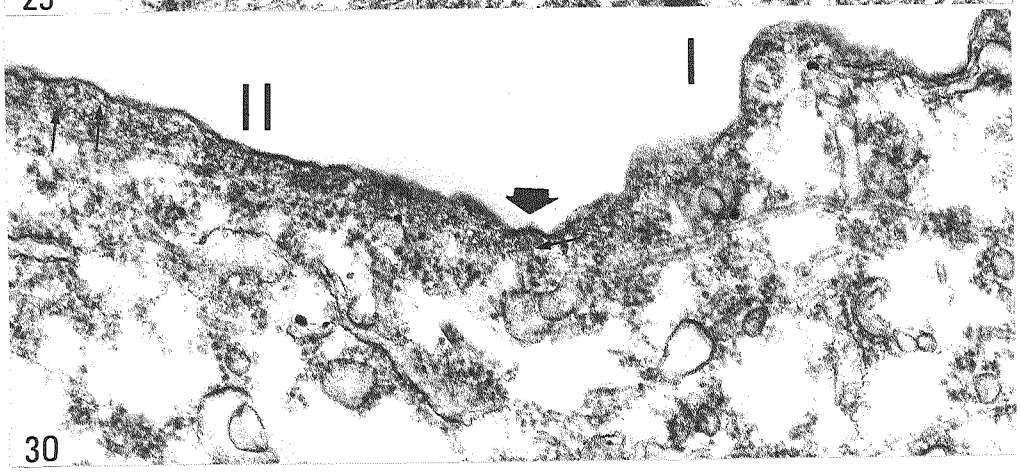

II
I
30

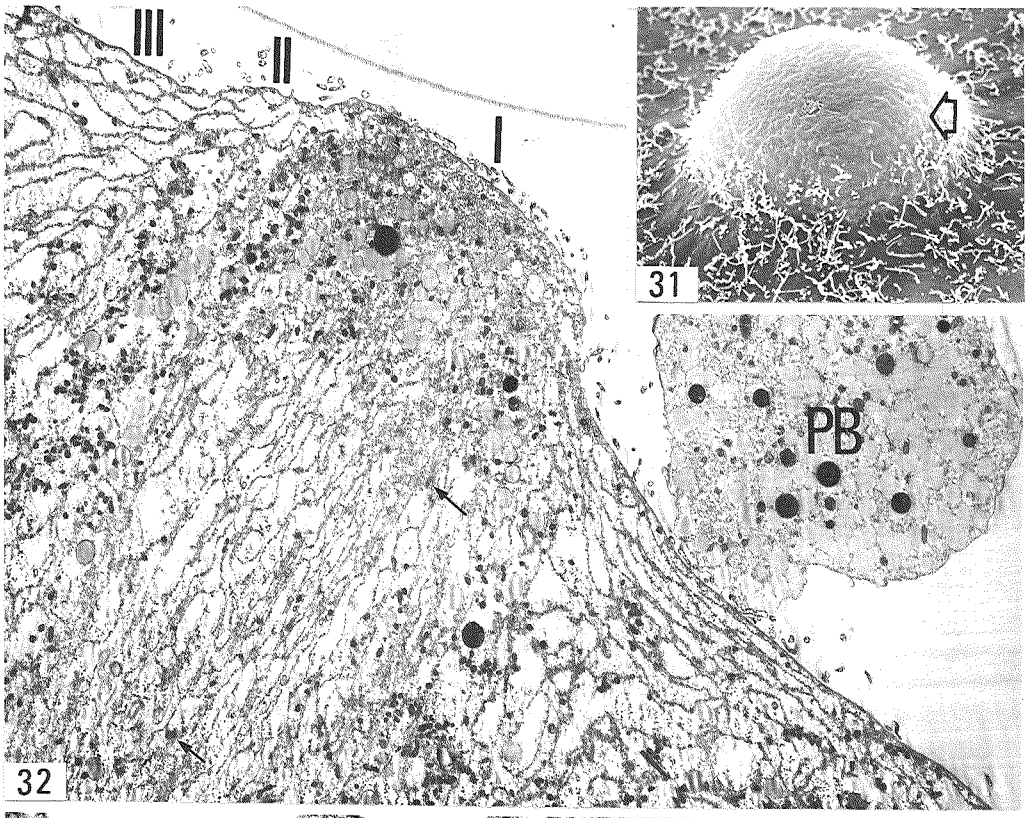
Explanation of Plate 9

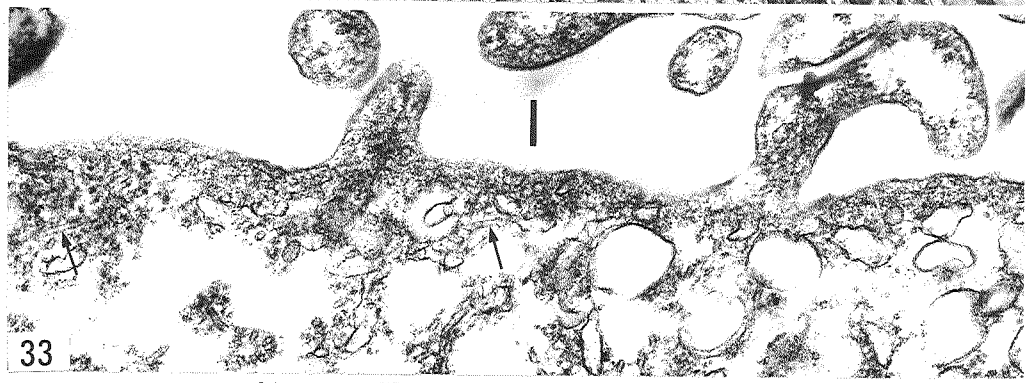
Figs. 31-34. Animal pole of eggs at late anaphase of the second meiotic division.

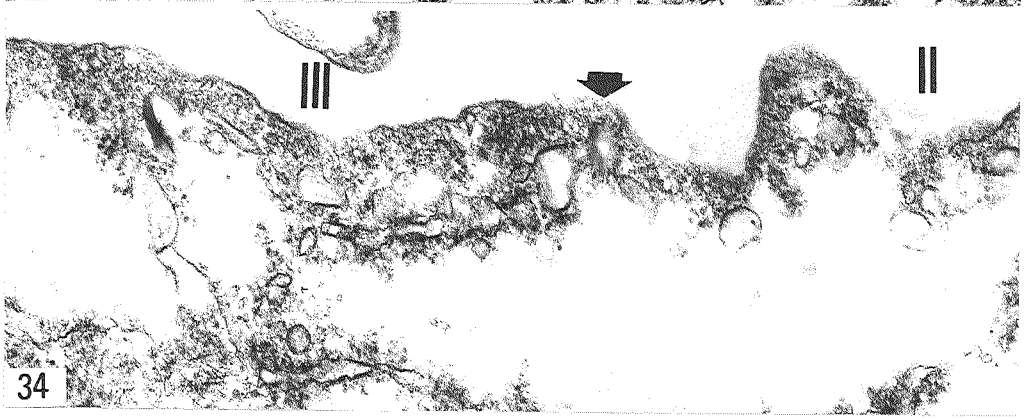
Fig. 31. Scanning electron micrograph showing cytoplasmic bulge. An open arrow points to the boundary between the distal and the side portions of the bulge. Note the difference in the surface morphology of these portions. x1200.

Fig. 32. Transmission electron micrograph showing cytoplasmic bulge and anaphase spindle. Arrows indicate chromosomes. I, distal portion of the bulge; II, bulge side; III, proximal portion of the bulge. PB, first polar body. x2000.

Figs. 33-34. Higher magnification of Figure 32. Portions indicated by Roman numerals (I-III) corresond to those in Figure 32. Figure 33 shows distal portion of the bulge. Some filamentous materials are still found in the cortical layer; microtubules (arrows) are also seen in the subcortical region. Figure 34 shows the boundary (arrow) between the bulge side(II) and the proximal portion (III) of the bulge. Note microfilaments in the cortical layer in the proximal portion. x40000.


III
II
I
PB
31
32


I
33


III
II
34

Explanation of Plate 10

Figs. 35-39. Animal pole of eggs at early telophase of the second meiotic division.

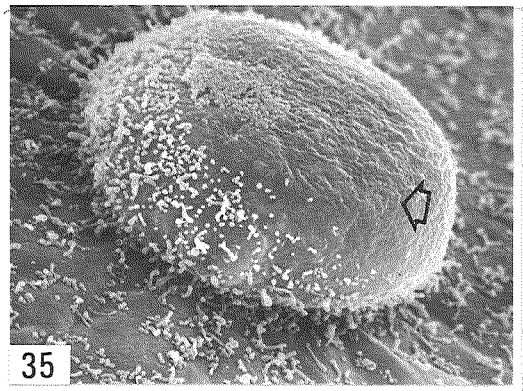
Fig. 35. Scanning electron micrograph of the cytoplasmic bulge with the constriction at its proximal portion. The boundary between the distal portion and the side portion of the bulge is indicated by an open arrow. Note the difference in the surface morphology of these portions of the bulge. x1000.

Fig. 36. Transmission electron micrograph showing the cytoplasmic bulge with the constriction at its proximal portion. Sister chromosomes are separated and found in the bulge and the egg proper. Compared with the chromosomes in the bulge (single arrow), it is clear that those (double arrow) in the egg proper have begun decondensation. VM, vitelline membrane. x1400.

Fig. 37. Cortical layer at the proximal portion of the bulge. Note the cortical microfilaments (arrow) arranged obliquely to the surface. x28000.

Fig. 38. Cortical layer of the egg proper near the cytoplasmic bulge. A bundle of microfilaments (arrow) running parallel to the surface is seen. x28000.

Fig. 39. Distal portion of the cytoplasmic bulge shown in Figure 36. Inset shows decondensing chromosome found in the egg proper. Ch, chromosome. x7000; inset, x20000.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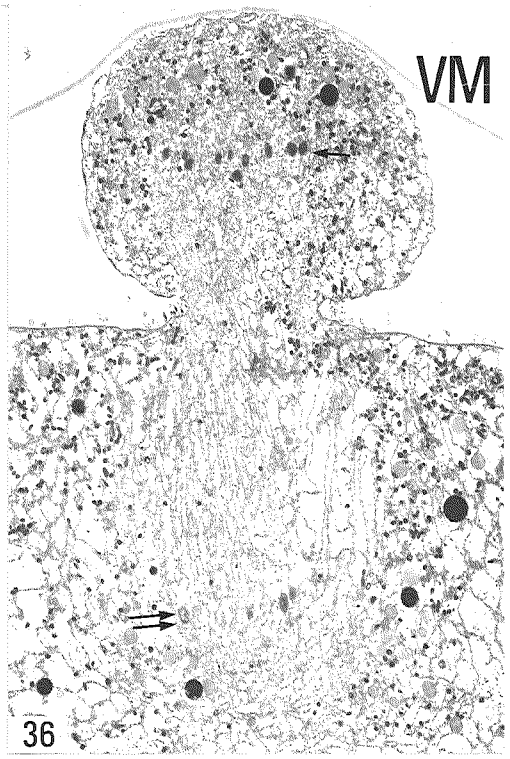
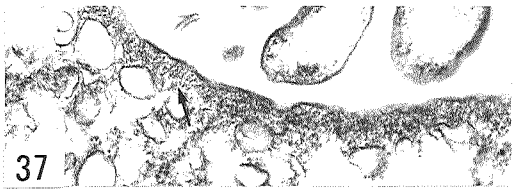

VM
36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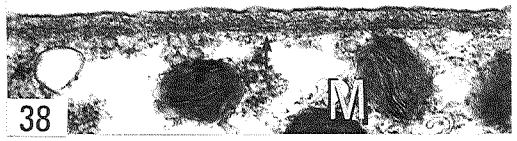
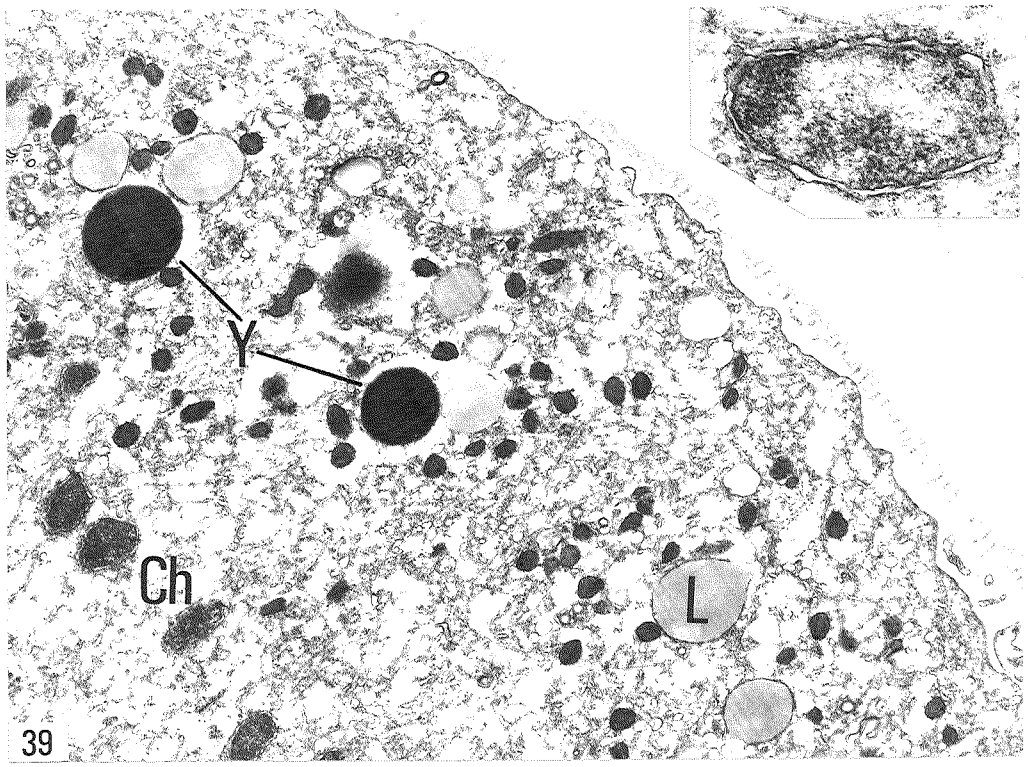

38
M


Y
Ch
L
39

Explanation of Plate 11

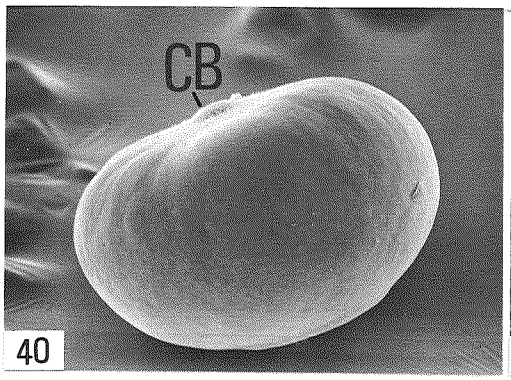
Figs. 40-43. Scanning (Figs. 40-41) and transmission (Figs. 42-43) electron micrographs showing cytoplasmic bulge formation in cytochalasin-treated eggs. Eggs were treated with 50 μg/ml cytochalasin B for 60 minutes at I-3 stage and fixed 60 minutes later.

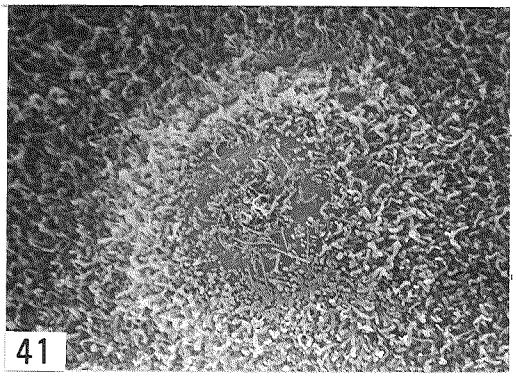
Fig. 40. Side view of an egg. Protrusion of the cytoplasmic bulge (CB) is evident at the animal pole. x100.

Fig. 41. Higher magnification of the animal pole of the egg in Figure 40. Note numerous blebs on the surface around the cytoplasmic bulge. x750.

Fig. 42. Section of a portion of the animal pole. Numerous blebs (B) cover the surface. Note intense indentation of the surface. L, lipid droplet; M, mitochondria. x6000.

Fig. 43. Higher magnification of Figure 42. Bundles of microfilaments (arrow) are seen in the cortical layer. M, mitochondria. x45000.


CB
40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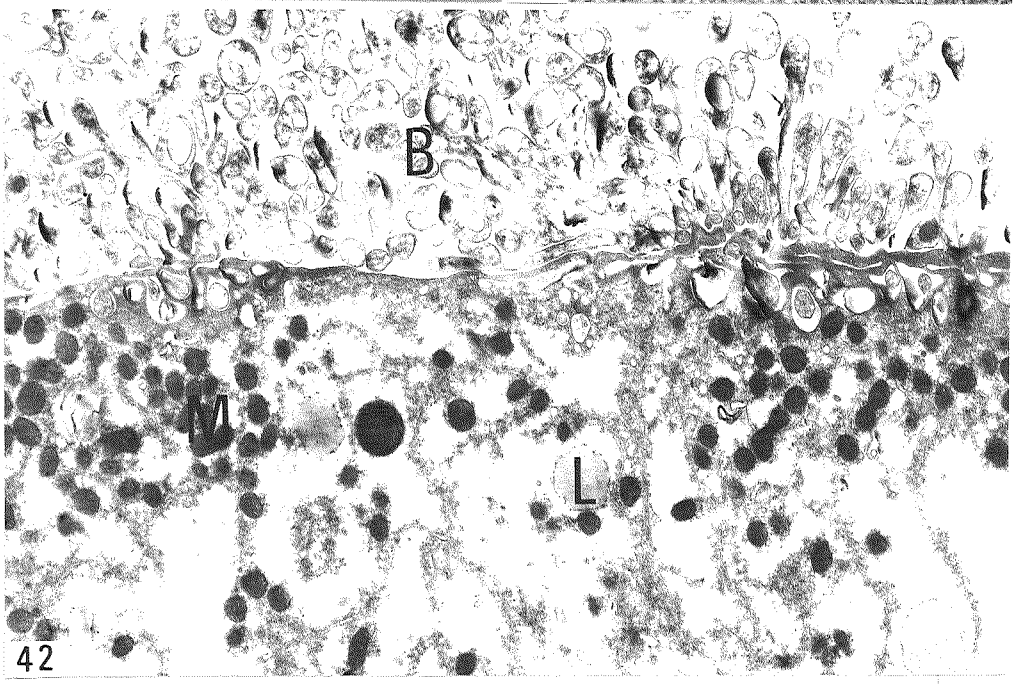
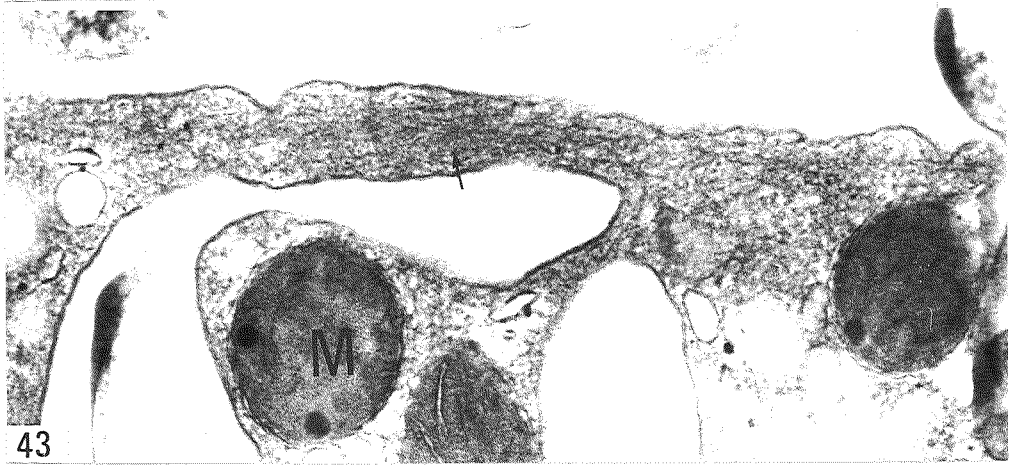
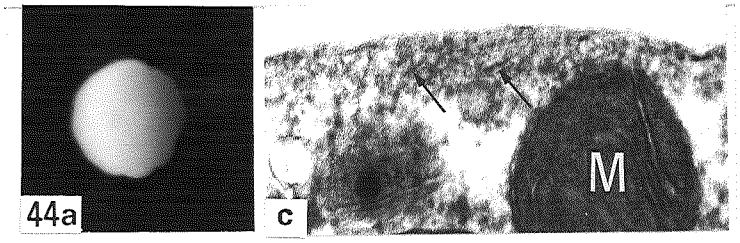

B
M
L
42

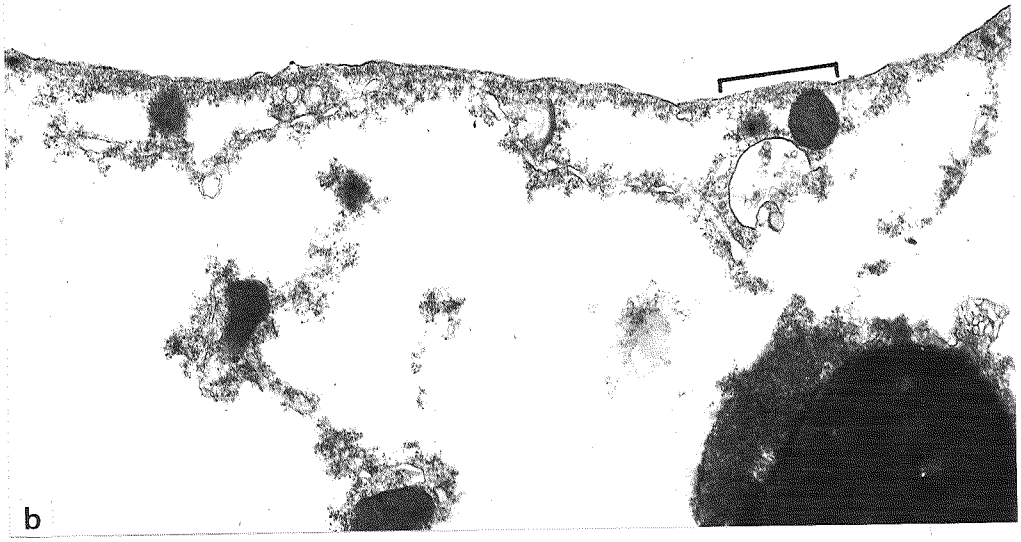

M
43

Explanation of Plate 12

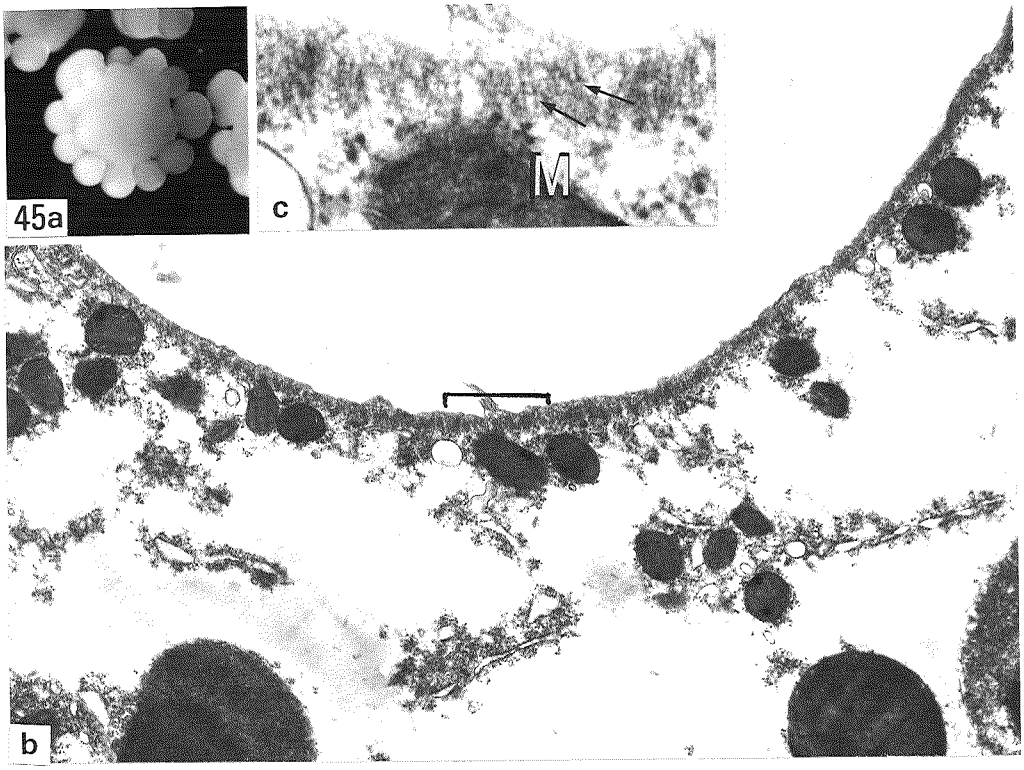
Fig. 44. Shallow groove phase of the normal second deformation movement. (a) Live egg viewed from the animal pole. x35. (b) Transmission electron micrograph of the peripheral region at the bottom of the shallow groove. x16500. (c) Higher magnification of the area indicated in Figure 44b. Microfilaments (arrows) are seen in the cortical layer. x66000.

Fig. 45. Climax phase of the normal second deformation movement. (a) Live egg viewed from the animal pole. Many protuberances are found on the equatorial surface of the egg. Note the constrictions at their proximal parts. x35. (b) Transmission electron micrograph showing the peripheral region at the bottom of the groove. The cortical layer is occupied by microfilaments. x13800. (c) Higher magnification of the area indicated in Figure 45b. Some filaments are indicated by arrows. x69000.


44a
c
M


b

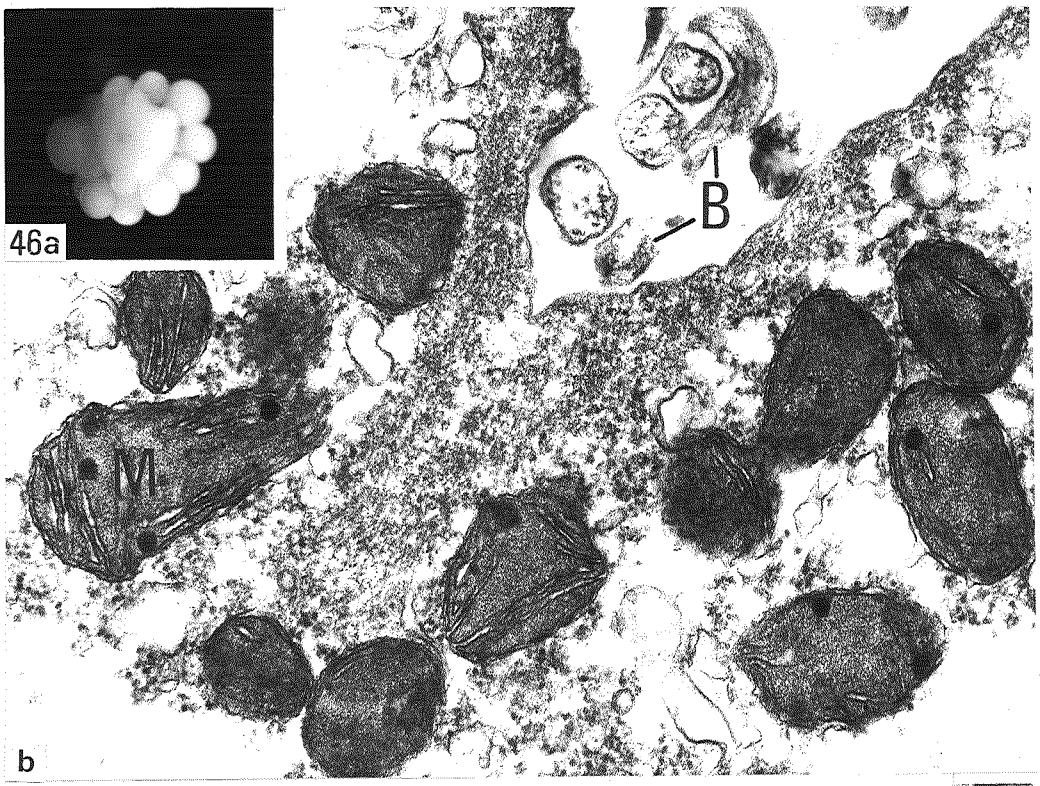
45a
c
M


b

Explanation of Plate 13

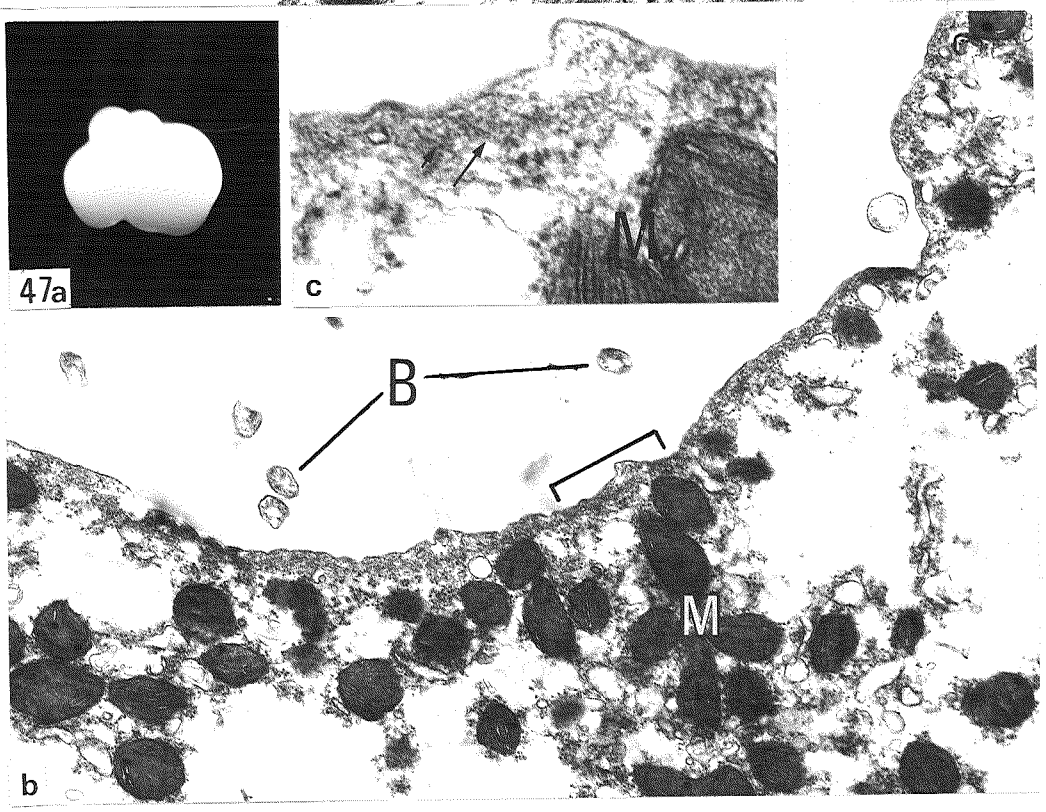
Fig. 46. Early relaxing phase of the normal deformation movement. (a) Live egg viewed from the animal pole. Note the absence of constrictions at proximal parts of the protuberances. x35. (b) Transmission electron micrograph of the bottom of the groove. A large number of microfilaments are seen with regularity in their arrangement. Note blebs (B) over the surface of the bottom. x45000.

Fig. 47. Late relaxing phase of the normal deformation movement. (a) Live egg viewed from the animal pole. x35. (b) Transmission electron micrograph of the peripheral region at the bottom of the retracting groove. B, bleb. x16000. (c) Higher magnification of the area indicated in Figure 47b. Note loosely packed microfilaments (arrows). x60000.


46a
B
M
b

47a

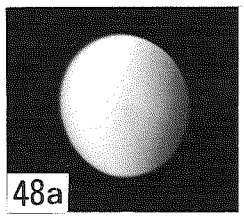
M
c

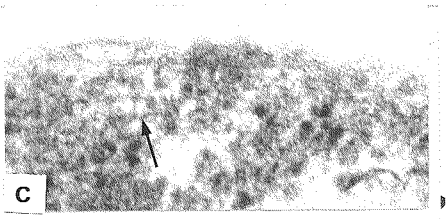

B
M
b

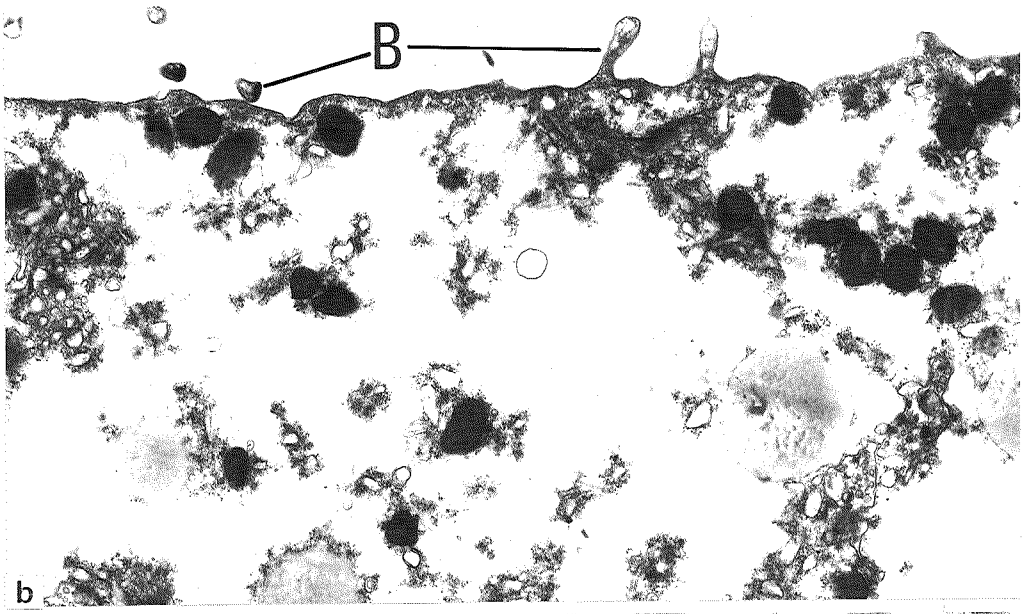
Explanation of Plate 14

Fig. 48. Normal egg shortly after the completion of the second deformation movement. (a) Live egg viewed from the animal pole. x35. (b) Transmission electron micrograph showing a part of equatorial region of the egg with blebs (B) on and over its surface. x12000. (c) Electron-dense cytoplasmic mass containing a few filaments (arrow) found in the peripheral region beneath the equatorial surface. x80000.

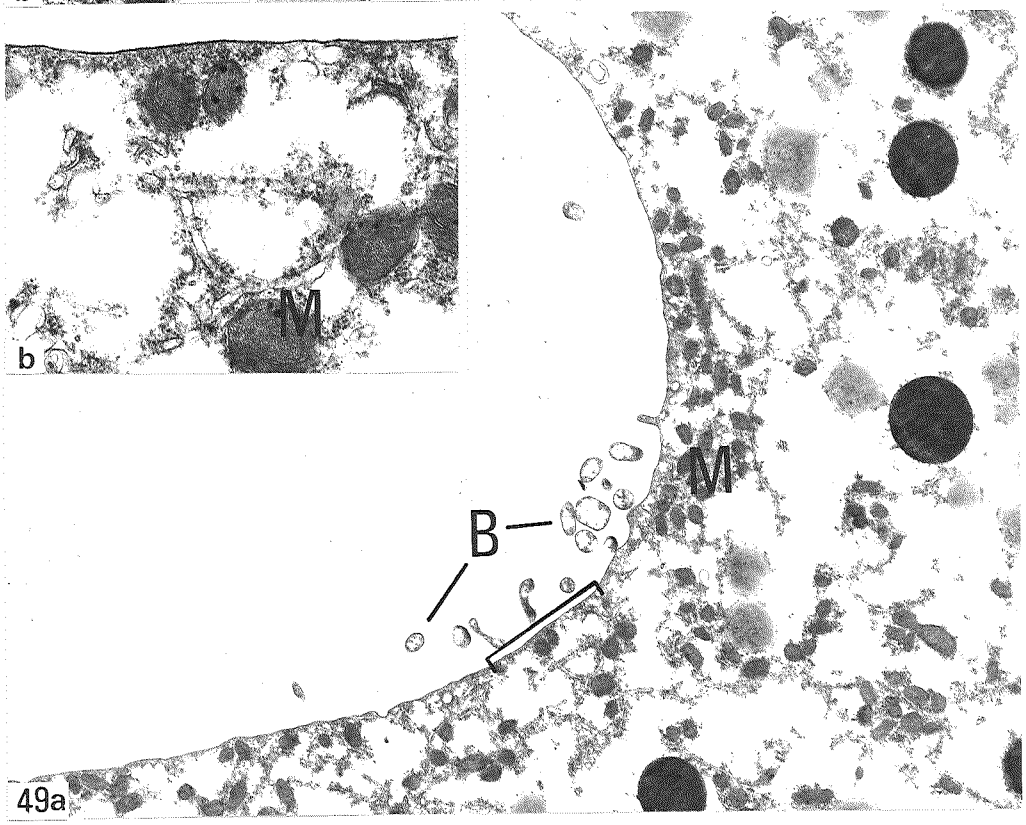
Fig. 49. Transmission electron micrographs of cytochalasin-treated egg. Forty-minute immersion in 50 μg/ml cytochalasin B at early I-4 stage. The egg was fixed at 5 minutes after immersion and was in the state of early relaxing phase. Peripheral region at the bottom of a groove is shown. Note the presence of blebs (B) on the surface. Inset shows a higher magnification of the peripheral region indicated. The cortical layer contains no microfilaments but possesses some vesicles. x6000; inset, x22500.


48a


c


B
b

M
b


M
B
49a

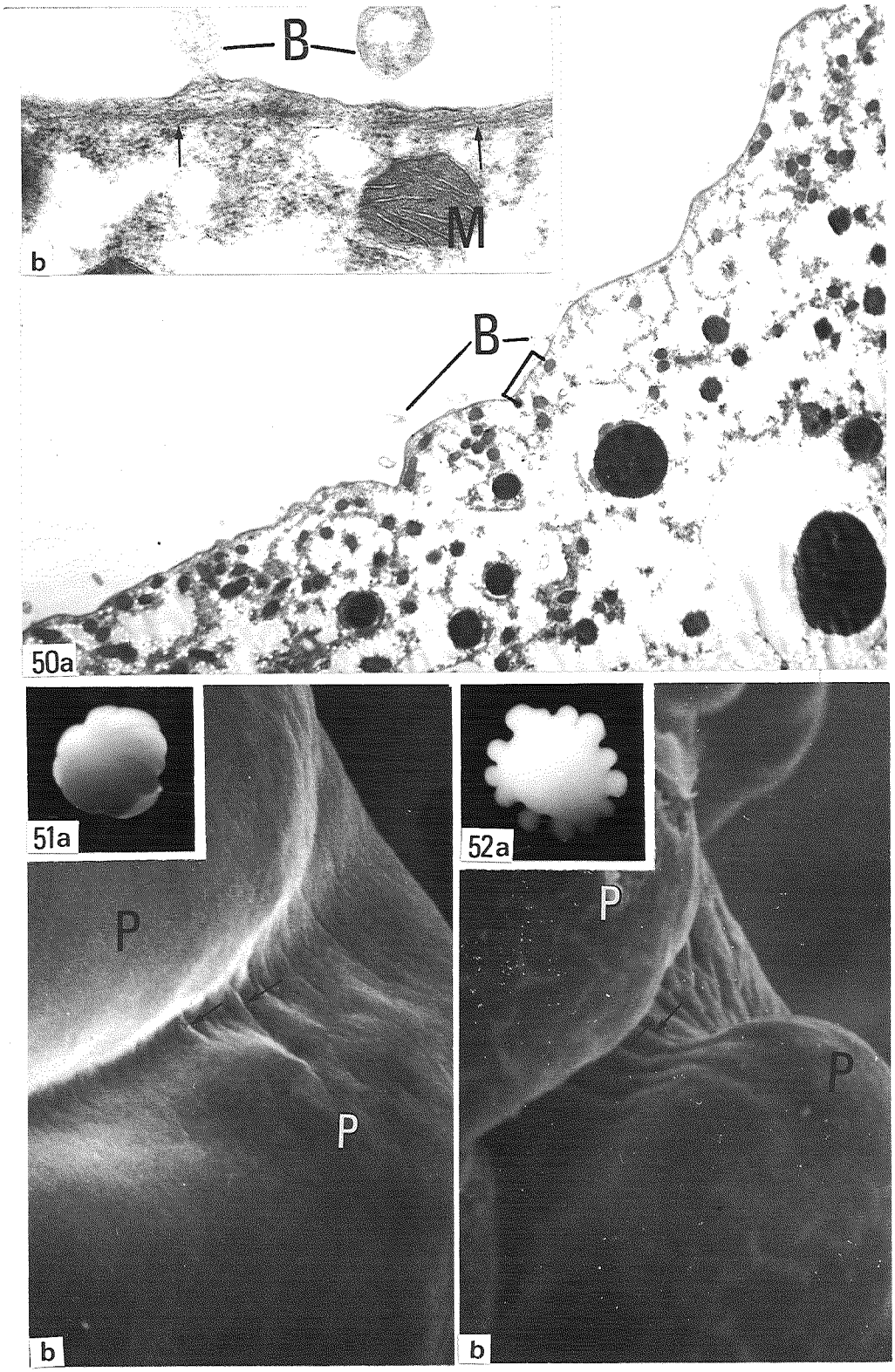
Explanation of Plate 15

Figs. 50-51. Cytochalasin-treated egg undergoing regressed deformation movement. The egg was treated with 50 μg/ml cytochalasin B at early I-3 stage for 40 minutes and was thoroughly washed in the normal culture medium.

Fig. 50. (a) Peripheral region at the bottom of a shallow groove. Note the distinct cortical layer and the blebs (B). x5000. (b) Detail of the area indicated in Figure 50a. Microfilaments (arrows) are arranged parallel to the egg surface. x40000.

Fig. 51. (a) Live egg viewed at angle of about 45° from the animal pole. x35. (b) Scanning electron micrograph showing the surface of the equatorial region. Several folds (arrows) are seen on the surface of a groove. P, protuberance. x1000.

Fig. 52. The egg in climax phase of the second deformation movement. (a) Live egg viewed from the animal pole. x35. (b) Scanning electron micrograph of the surface of the equatorial region. Note the constriction at the proximal part of the protuberance (P) and intense folds (arrows) on the groove surface. Compare with Figure 51b. x1000.

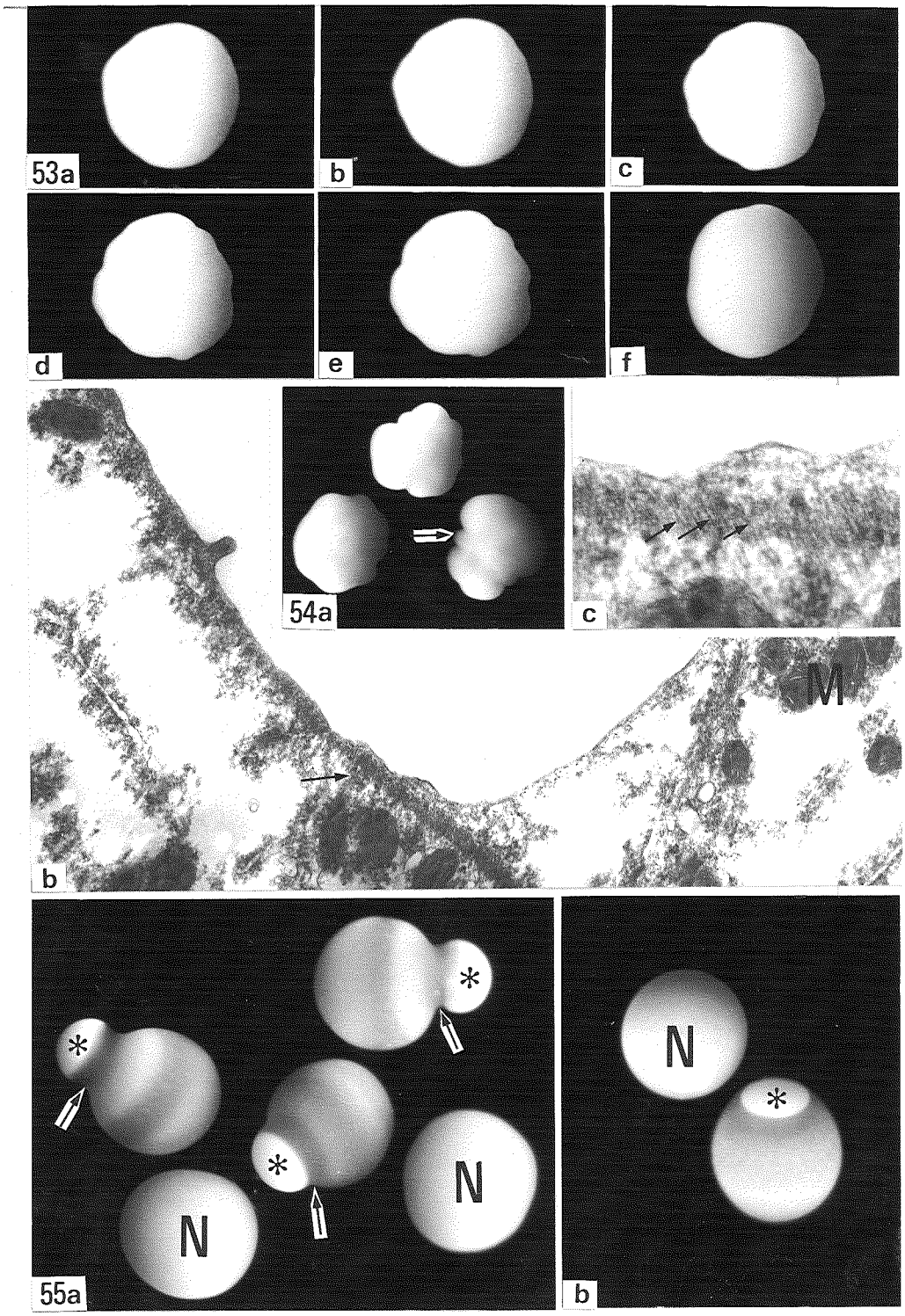

B
M
b
B
50a
51a
P
P
b
52a
P
P
b

Explanation of Plate 16

Fig. 53. Serial photographs showing behavior of an egg treated with 5 μg/ml ionophore in Ca^{++}-Mg^{++}-free medium at the beginning of stage I-5. Animal pole view. Note grooves on the equatorial surface. x40.

Fig. 54. (a) Eggs treated with 10 ug/ml ionophore in Ca^{++}-Mg^{++}-free medium at the beginning of stage I-5. Thirty-five minutes after beginning of treatment. Animal pole view. Note distinct protuberances (arrow). x20. (b) Transmission electron micrograph showing bottom of groove in an egg treated like those in (a). An arrow indicates cortical microfilaments. M, mitochondria. x21000. (c) Detail of cortical layer of groove-bottom shown in (b). Note highly organized microfilaments (arrows) oriented obliquely to the egg surface. x52000.

Fig. 55. Eggs centrifuged at 30 minutes after beginning of I-3 stage. (a) Eggs treated with ionophore in Ca^{++}-Mg^{++}-free medium. Thirty minutes after beginning of treatment. Note grooves (arrows) on the protoplasmic layer. (b) Control eggs immersed in Ca^{++}-Mg^{++}-free medium containing 0.1% dimethyl sulfoxide. No grooves are observed and eggs are spherical. N, eggs placed in the normal culture medium gum arabic system but not centrifuged. Asterisks, centripetal lipid layer. x40.


53a
b
c
d
e
f
54a
c
M
b
*
*
*
N
N
55a
N
*
b

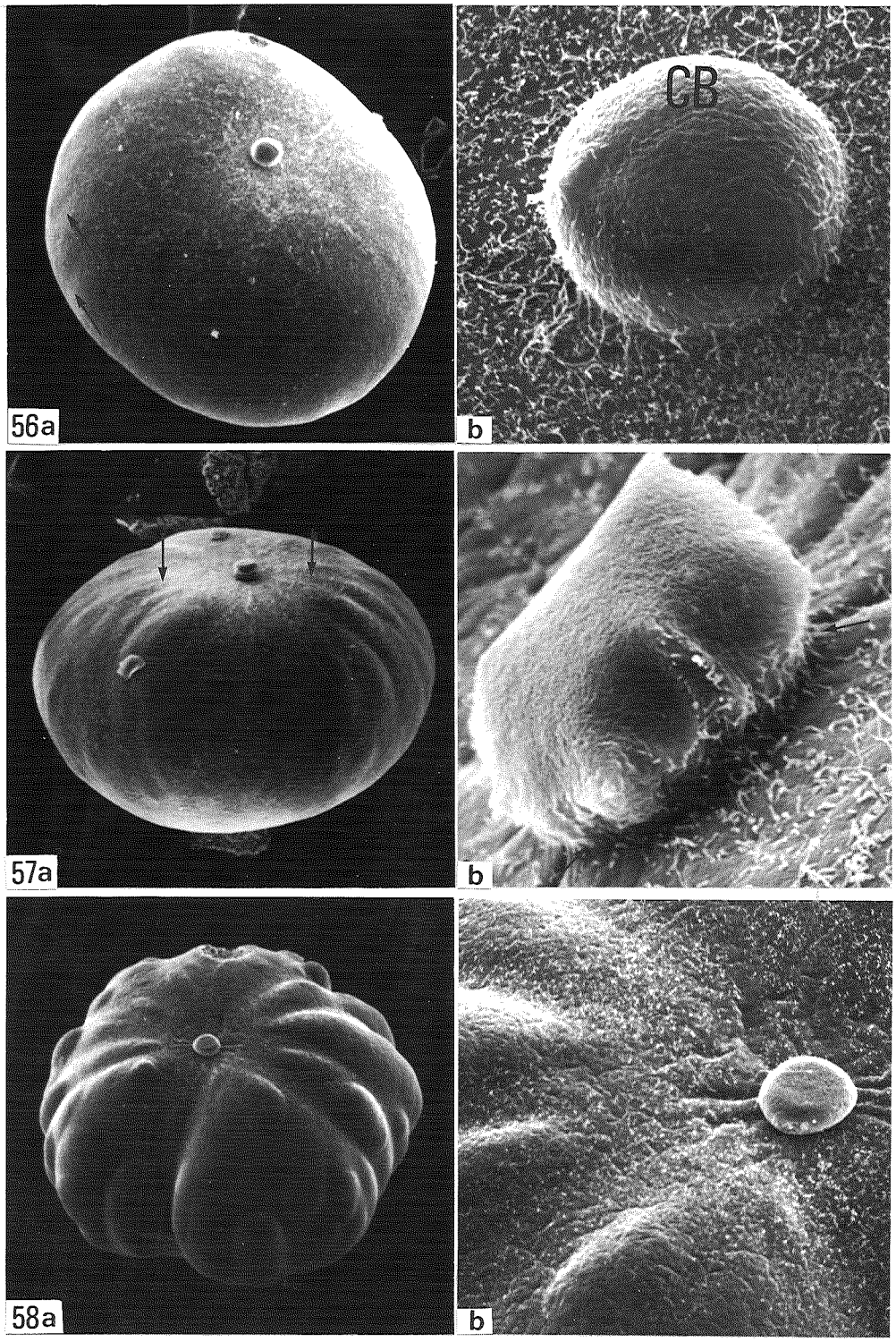
Explanation of Plate 17

Figs. 56-58. Scanning electron micrographs of eggs undergoing the first deformation movement.

Fig. 56. The beginning of the movement. Note shallow grooves on the equatorial surface (arrows in Fig. 56a). Cytoplasmic bulge (CB) of the first polar body (Fig. 56b) is of maximum size. Numerous microvillous blebs on the animal pole region characterize this phase of the movement. a, x120; b, x1100.

Fig. 57. Ten minutes after the beginning of the movement. Distinct grooves are found on the animal hemisphere as well (arrows in Fig. 57a). Note the proximal constriction of the cytoplasmic bulge (arrows in Fig. 57b). a, x120; b, x1850.

Fig. 58. Twenty minutes after the beginning of the movement. Deepening of the grooves is conspicuous (Fig. 58a). Note the radially arranged folds around the animal pole (Fig. 58b). a, x120; b, x400.


56a
CB
b
57a
b
58a
b

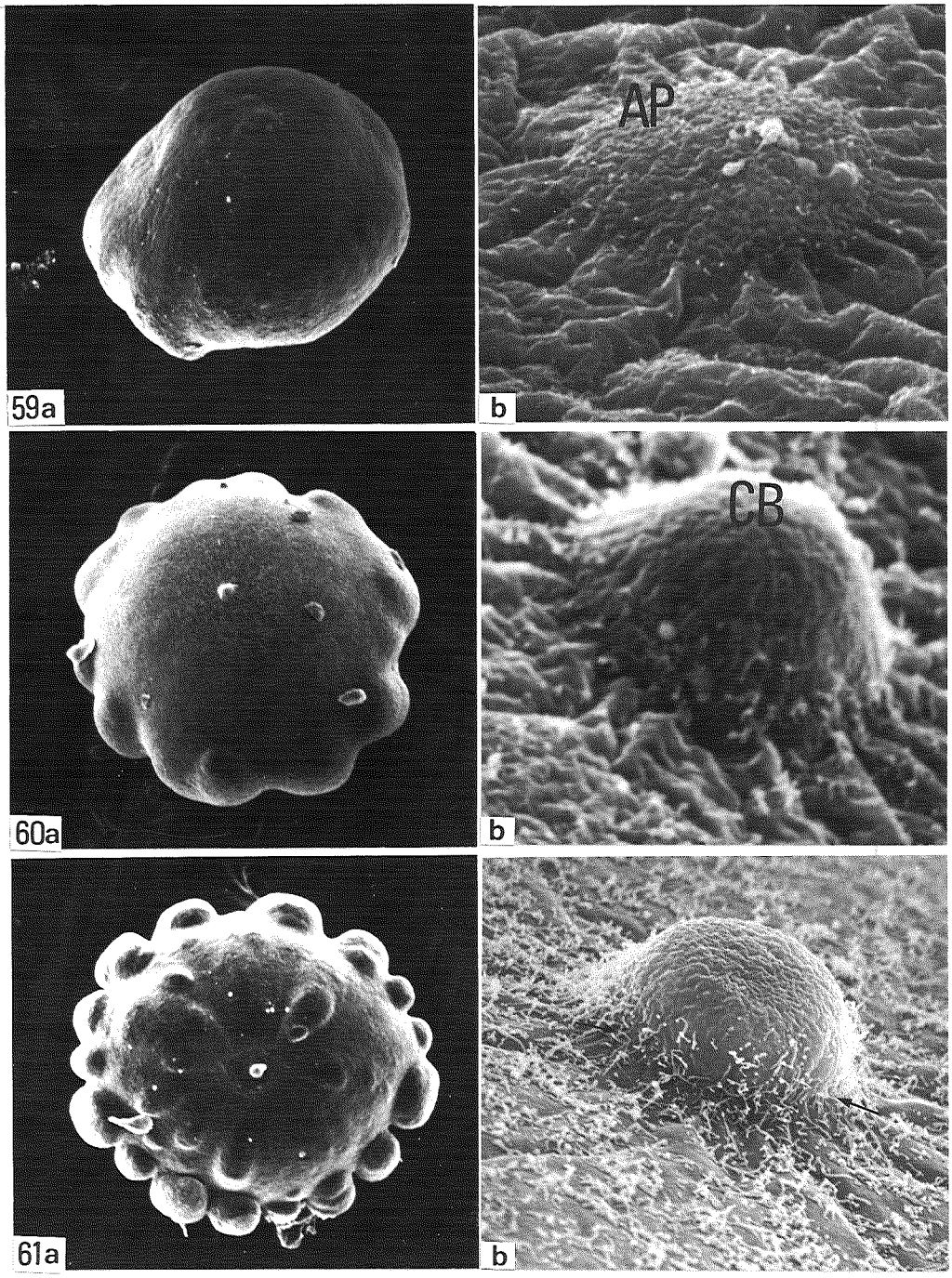
Explanation of Plate 18

Figs. 59-61. Scanning electron micrographs of eggs undergoing the second deformation movement.

Fig. 59. Shallow grooves are seen on the equatorial surface (shallow groove phase; Fig. 59a). No cytoplasmic bulge is seen on the animal pole (AP). The surface of the animal pole, which will be extruded later for the formation of the second polar body, can be distinguished from the rest of the egg surface by its characteristic texture (Fig. 59b). a, x120; b, x2800.

Fig. 60. Twenty minutes after the beginning of the movement. Conspicuous grooves are found on the equatorial region (Fig. 60a). The proximal constriction of the cytoplasmic bulge (CB) is not yet seen (Fig. 60b). a, x120; b, x2100.

Fig. 61. Forty minutes after the beginning of the movement. Note clear grooves on the animal hemisphere (Fig. 61a) and the proximal constriction of the cytoplasmic bulge (arrows in Fig. 61b). a, x120; b, x1800.


59a
AP
b
60a
CB
b
61a
b

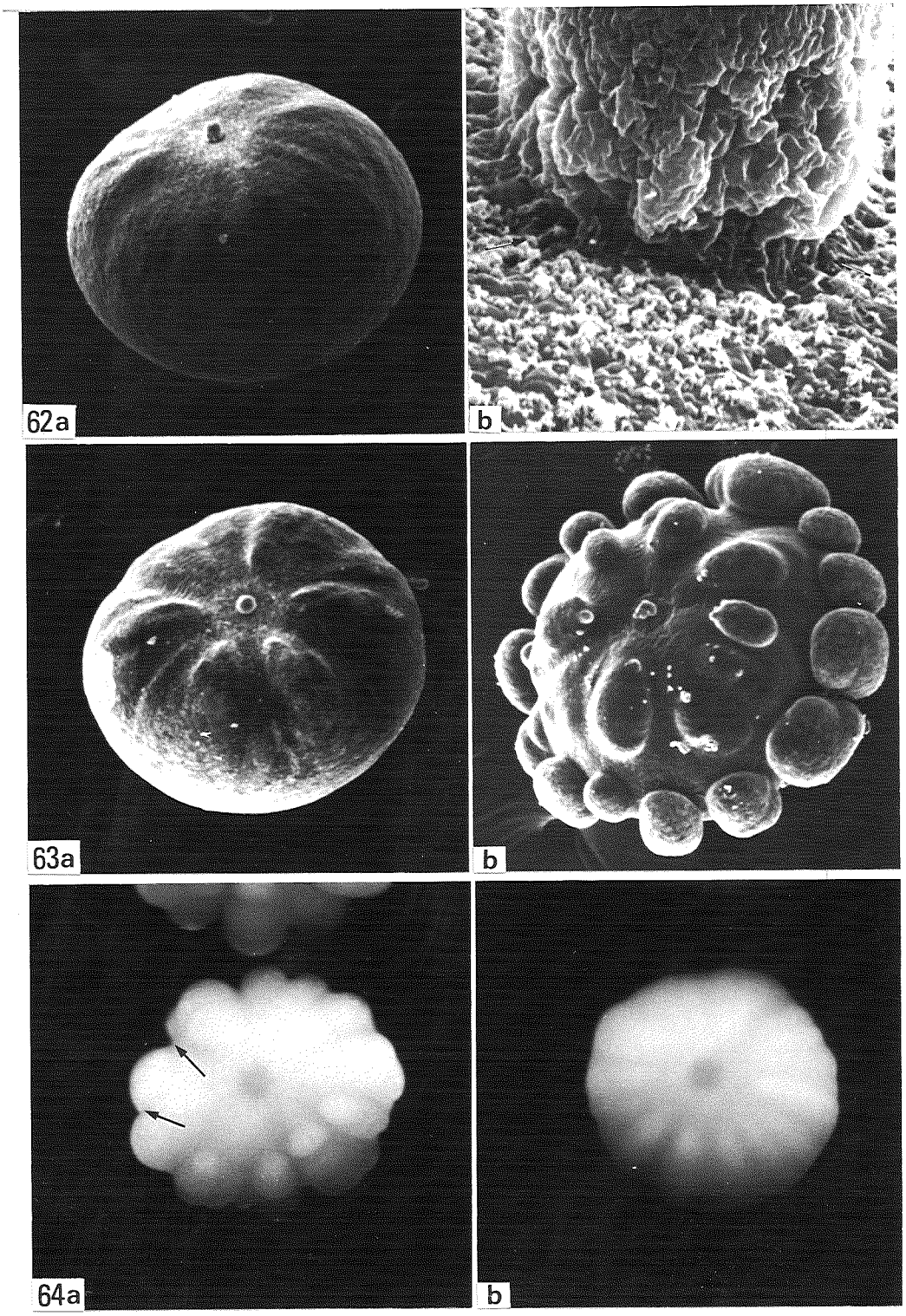
Explanation of Plate 19

Figs. 62-64. Eggs treated with dinitrophenol for 20 minutes in the early phase of the second deformation movement. Figs. 62-63, scanning electron micrographs; Fig. 64, photomicrograph.

Fig. 62. Twenty-five minutes post-treatment. Note the presence of shallow grooves on the animal hemisphere (Fig. 62a). No grooves are seen on the equatorial surface. The proximal region of the cytoplasmic bulge is apparently constricted (arrows in Fig. 62b). a, x130; b, x2800.

Fig. 63. Thirty-five minutes post-treatment (Fig. 63a). At this time, the synchronously developing control egg is in the climax phase of the movement (Fig. 63b). x130.

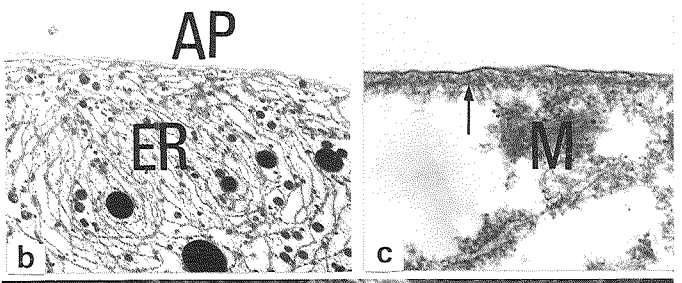
Fig. 64. Live eggs at 30 (b) and 45 (a) minutes after treatment. Viewed from the animal pole. Note the disappearance of grooves on the animal hemisphere seen in Figure 64b and the reappearance of equatorial grooves (arrows in Fig. 64a). x70.

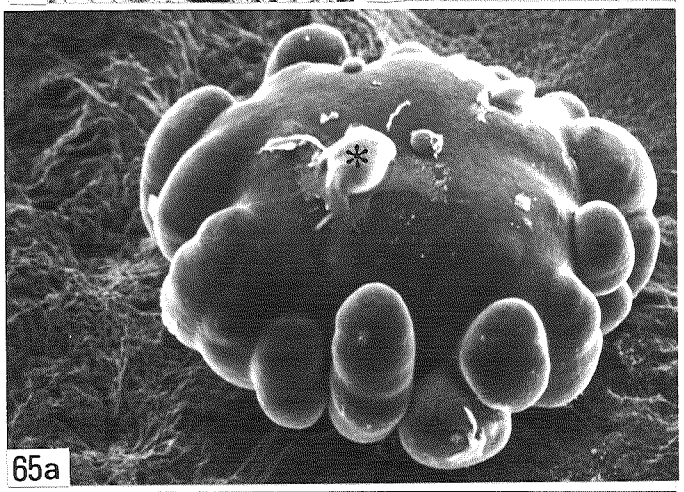

62a
b
63a
b
64a
b

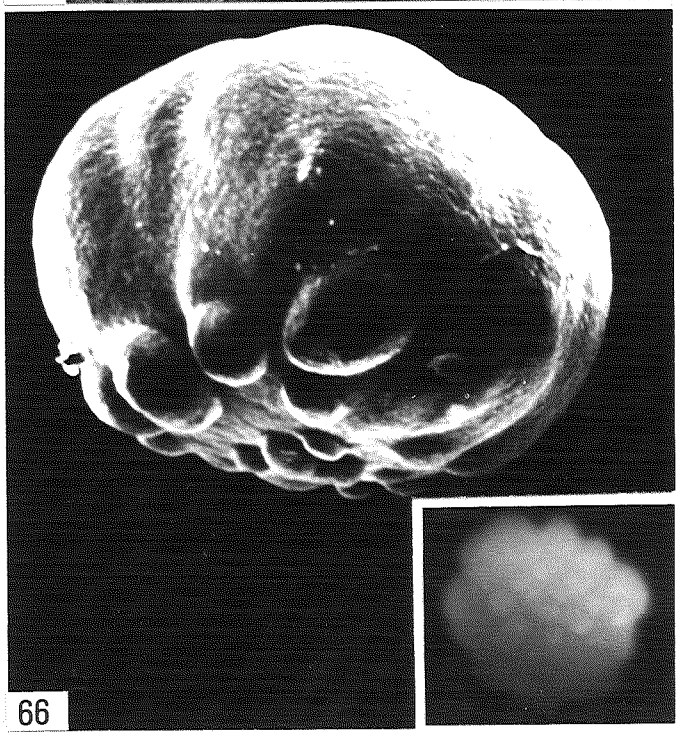
Explanation of Plate 20

Fig. 65. Scanning (a) and transmission (b and c) electron micrographs of eggs treated with colchicine as indicated in Text-figure 10D. Eggs were fixed at 10 (Figs. 65b and c) and 75 (Fig. 65a) minutes after the beginning of the second deformation movement. Figures 65b and c show meridional sections passing through the egg axis. After colchicine treatment no microtubules are observed in the animal pole and animal hemisphere. The meiotic apparatus has been destroyed; endoplasmic reticula (ER) and some yolk granules are found in the region where the meiotic apparatus has been present (Fig. 65b). The microfilamentous cortical layer (arrow) of animal hemisphere appears to be normal (Fig. 65c). The surface of the animal hemisphere is smooth and no grooves are detectable, while the equatorial grooving is conspicuous and normal (Fig. 65a). AP, animal pole; M, mitochondria. a, x150; b, x1300; c, x20000.

Fig. 66. Scanning electron micrograph of an egg successively treated with colchicine and dinitrophenol as indicated in Text-figure 10E. Fixed at 45 minutes after treatment. Note the unusually large blebs formed on the vegetal hemisphere and the shallow equatorial grooves. Inset is a live egg viewed from the vegetal pole. x170; inset, x50.

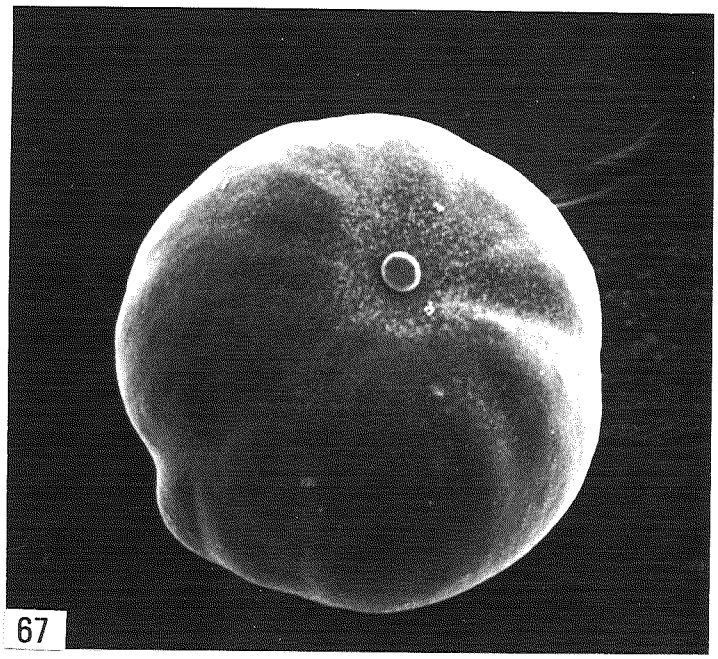

AP
ER
b
M
c


*
65a


66

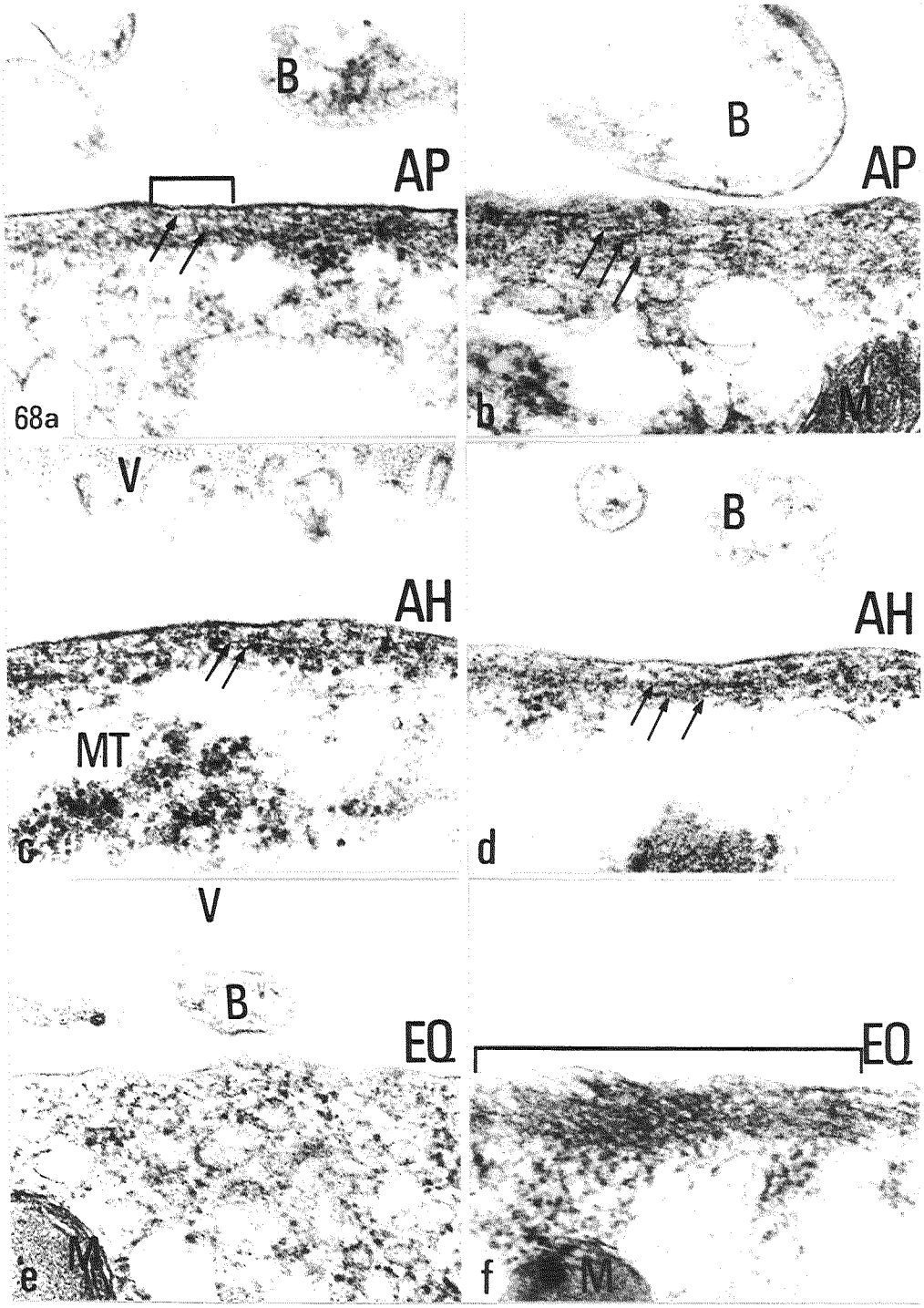
Explanation of Plate 21

Fig. 67. Scanning electron micrograph of an egg treated as indicated in Text-figure 10C. The second deformation movement began at 100 minutes post-treatment. Fixed at 50 minutes after the beginning of the "retarded" movement. Equatoril grooves are shallow but the animal hemisphere grooves and the proximal constriction of the cytoplasmic bulge appear to be normal. x170.


67

Explanation of Plate 22

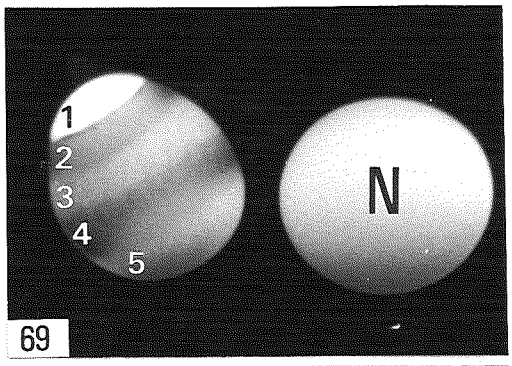
Fig. 68. Transmission electron micrographs showing the cortical layer of the animal pole (AP), animal hemisphere (AH) and equatorial region (EQ) in the dinitrophenol-treated (Figs. 68a, c and e) and control (Figs. 68b, d and f) eggs. Eggs were fixed immediately after treatment (see Text-fig. 10A). Some microfilaments (arrows) running parallel to the egg surface, though smaller in number than control eggs, are seen in the animal pole and animal hemisphere of the treated eggs (Figs. 68a and c). Figure 68e shows the region corresponding to the bottom of previous equatorial groove; note complete disappearance of microfilaments in the equatorial region of the dinitrophenol-treated egg. Large number of microfilaments that are arranged obliquely to the egg surface are seen at the bottom region of the groove in the control egg (Fig. 68f). (For counting the number of microfilaments, brackets indicated in Figures 68a and f show the size of divided cortical area.) B, bleb; M, mitochondria; MT, microtubule; V, vitelline membrane. x60000.


B
AP
68a
B
AP
b
M
V
B
AH
MT
c
AH
d
V
B
EQ
e
M
EQ
f
M

Explanation of Plate 23

Figs. 69-71. Eggs immediately after centrifugation at early I-3 stage. Stratifications are marked with numerals (Fig. 69). Separation of components in the egg interior is clear (Figs. 70-71). 1, lipid layer; 2, protoplasmic layer; 3, large yolk granule layer; 4, opaque plasma layer; 5, small yolk granule layer. ER, endoplasmic reticula; M, mitochondria; N, non-centrifuged control egg. Fig. 71, toluidin blue preparation. Figs. 69 and 71, photomicrographs; Fig. 70, transmission electron micrograph. Fig. 69, x50; Fig. 70, x3000; Fig. 71, x150.

Fig. 72. Section of an egg centrifuged at early I-3 stage and left to itself for 60 minutes. Compare with Figure 71. It is evident that there is not remarkable redistribution of egg components. ER, endoplasmic reticula; M, mitochondria. Numerals (1-5) correspond to those in Figure 71. Toluidin blue preparation. x150.



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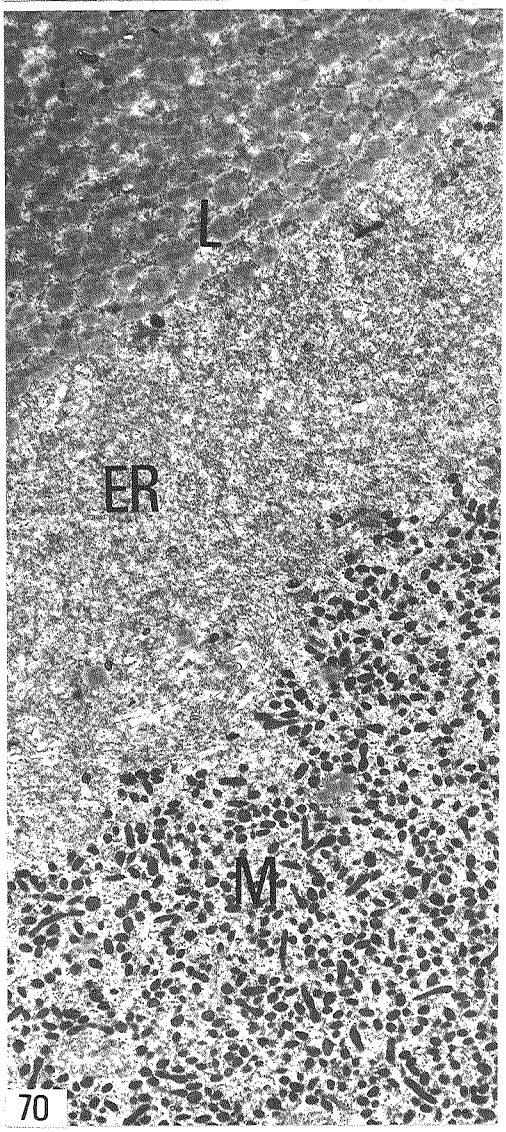
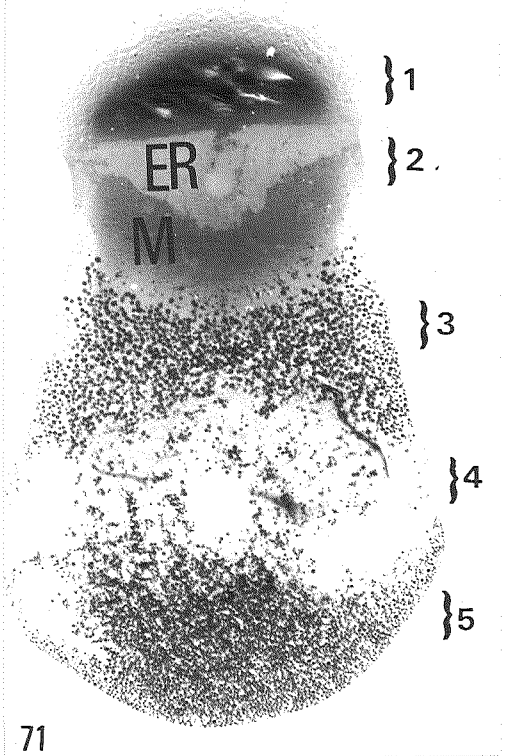


70



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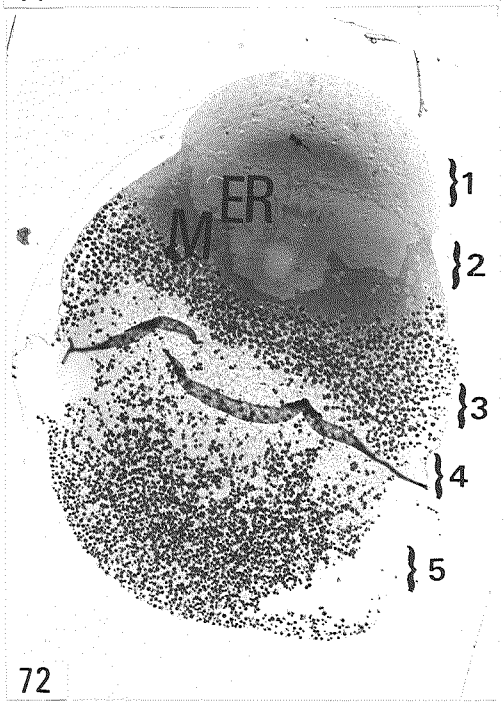
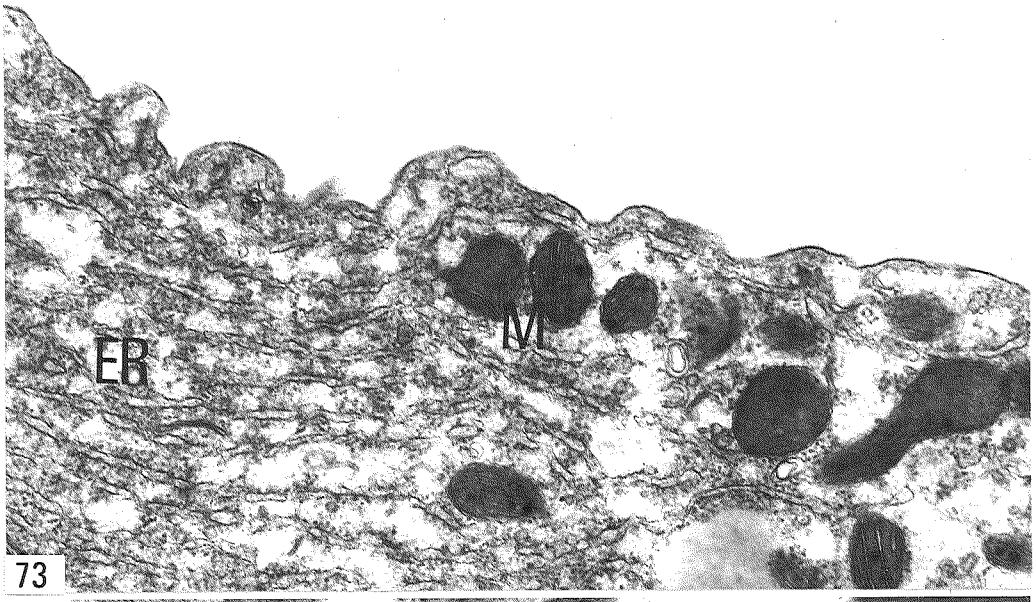


72

Explanation of Plate 24

Figs. 73-74. Transmission electron micrographs showing the peripheral portions of an egg immediately after centrifugation at early I-3 stage. Figure 73 shows the boundary between the endoplasmic reticula (ER) and mitochondria (M) zones of the protoplasmic layer. These organelles are also seen in the large yolk granule (Y) layer (Fig. 74). Note the thin cortical layer. Mt, microtubule. x30000.



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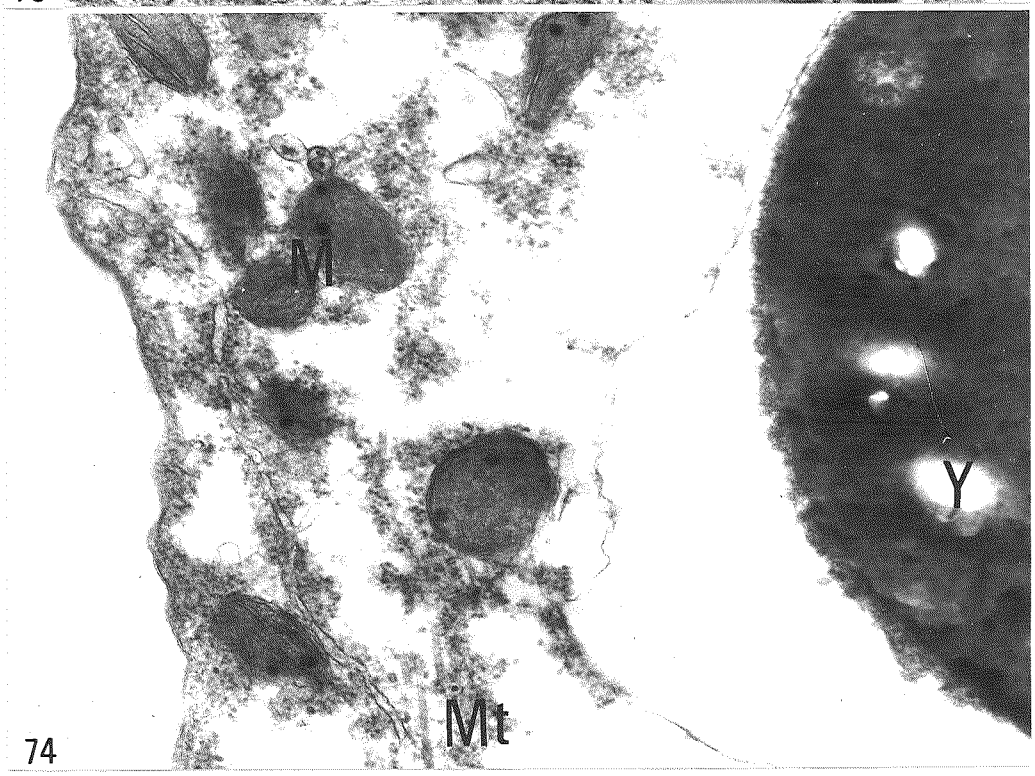


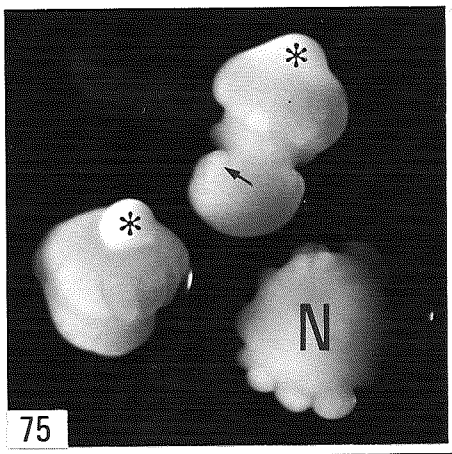
74

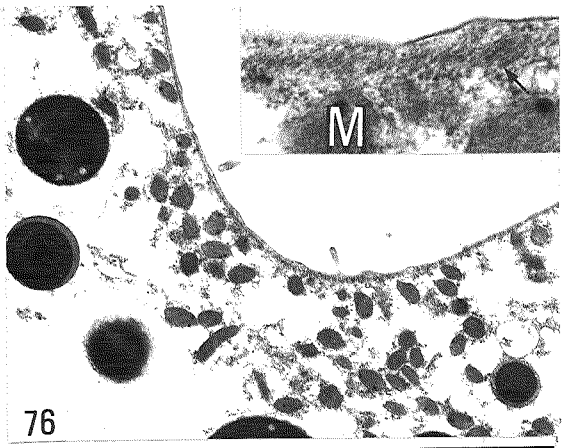
Explanation of Plate 25

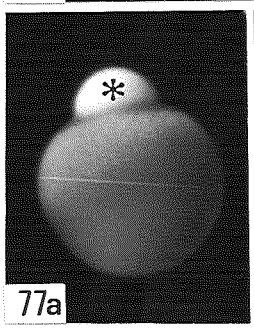
Fig. 75. Photomicrograph showing the grooving activity in the centrifuged eggs. Eggs were centrifuged at early I-3 stage and left to themselves; photographs were taken at the time when the control non-centrifuged egg (N) underwent the second deformation movement (I-4 stage). Centripetal lipid layer is indicated by asterisks. Note grooves on the large and small yolk granule layers (arrow). x40.

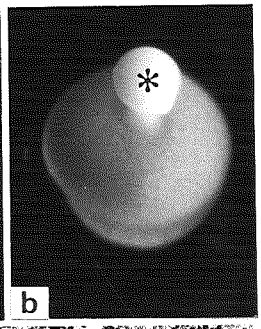
Fig. 76. Transmission electron micrograph of the groove-bottom in a centrifuged egg showing the grooving activity as in Figure 75. The surface is lined with electron-dense cortical layer, showing highly organized microfilaments (arrows). x6000; inset, x30000.

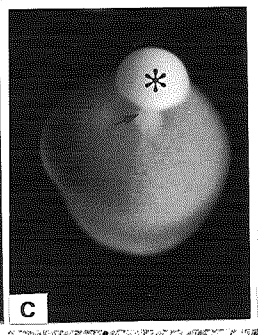
Figs. 77-78. Centrifuged eggs showing the grooving activity. Eggs were centrifuged at alte I-3 stage. Figure 77 shows an egg photographed at 6 (a), 30 (b), 40 (c) and 60 (d) minutes after centrifugation. Note a constriction (arrow in Fig. 77c) on the protoplasmic layer and grooves (arrow in Fig. 77d). Figure 78 is a transmission electron micrograph showing a constricted portion of an egg fixed at the time corresponding to Figure 77b. The constriction is indicated by an open arrow. Note redistribution of mitochondria (M) and endoplasmic reticula (ER). Asterisk, lipid layer. Fig. 77, x50; Fig. 78, x1200.


*
*
N
75


M
76


*
77a


*
b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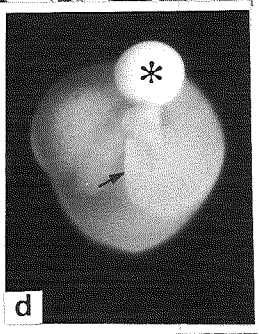
c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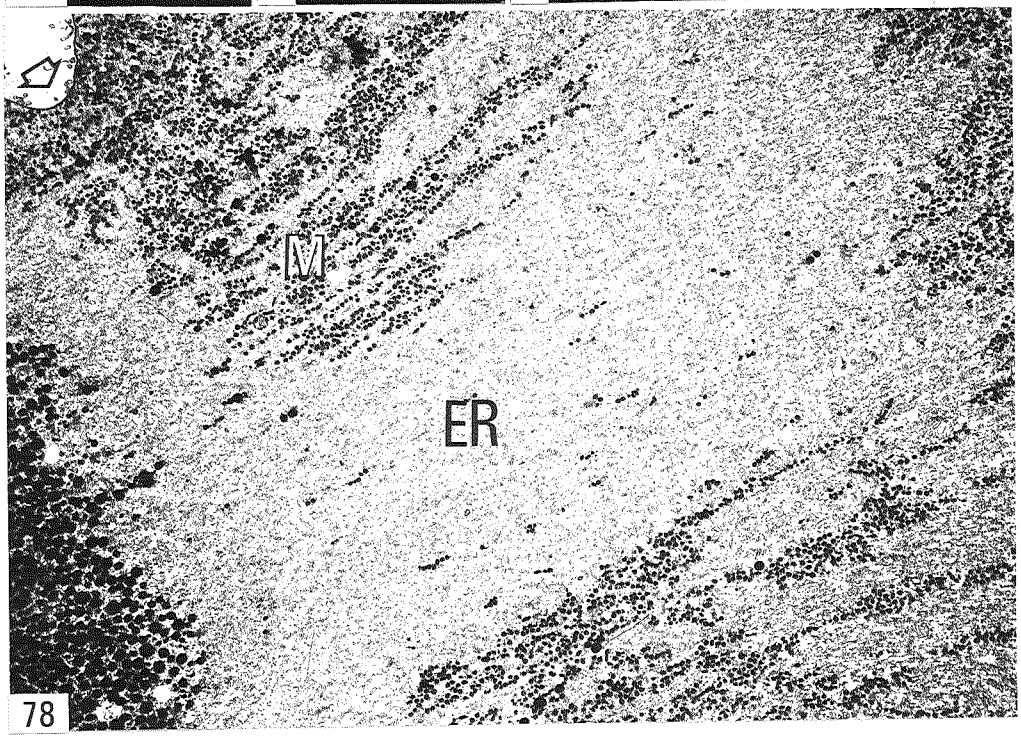
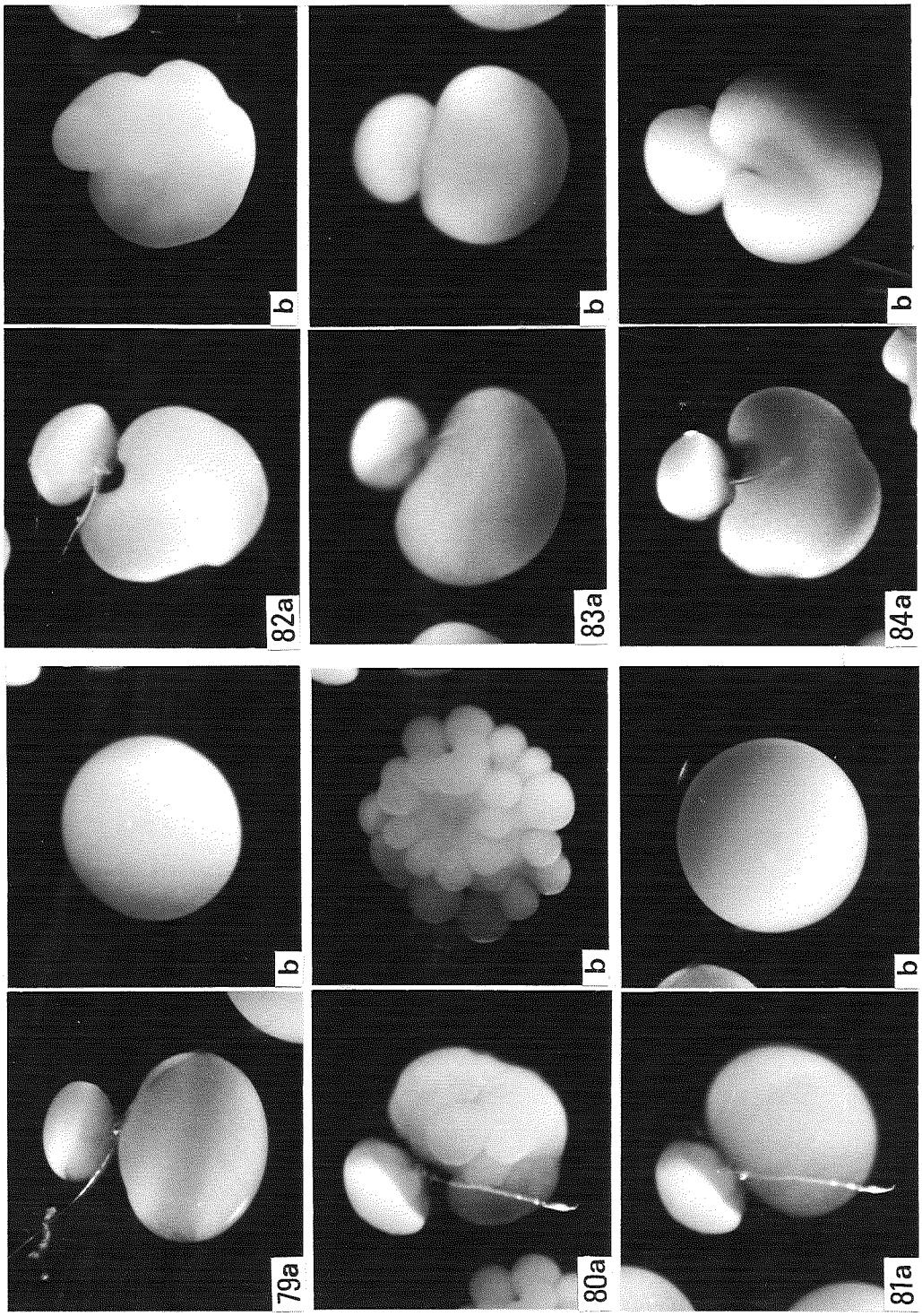
d


M
ER
78

Explanation of Plate 26

Figs. 79-84. Photomicrographs showing the shape change in non-nucleated egg fragments (a) and control eggs (b). Centrifuged eggs were constricted by silk thread and separated into small nucleated and large non-nucleated egg fragments. Fig. 79b, I-3 stage (immediately after production of egg fragments); Fig. 80b, I-4 stage (the second deformation movement); Fig. 81b, I-5 stage; Fig. 82b, the first cleavage stage; Fig. 83b, two-cell stage; Fig. 84b, the second cleavage stage. Note the groove-forming activity in egg fragments shown in Figures 80a, 82a and 84a. x55.

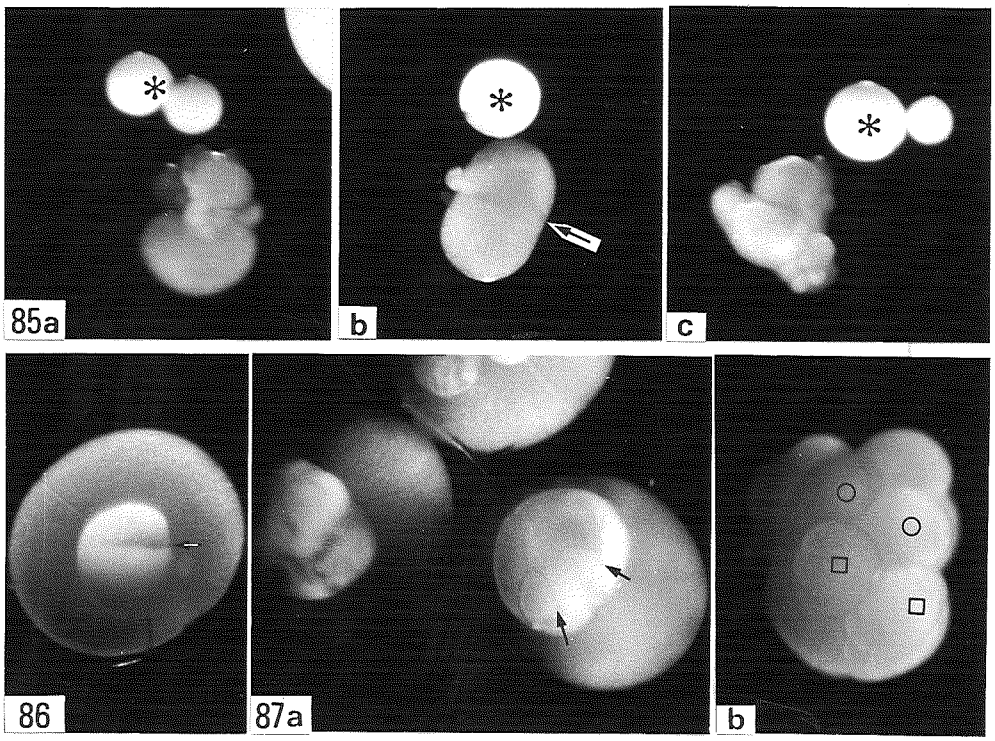

79a
b
80a
b
81a
b
82a
b
83a
b
84a
b

Explanation of Plate 27

Fig. 85. Photomicrographs showing the surface behavior of live nucleated egg fragment. Fragment was produced at I-3 stage (see Text-fig. 3) and was removed from the enveloping vitelline membrane at I-5 stage of the control eggs. The egg fragment was photographed at the stages of the first cleavage (a), two-cell (b) and the second cleavage (c) in the control eggs. During the grooving activity, the portion of the lipid layer (asterisks) was separated from the rest of the fragment. An arrow in Figure 85b indicates that the fragment is consisted of two cells. Note the deformation of the fragment at the time of division (Figs. 85a and c). x80.

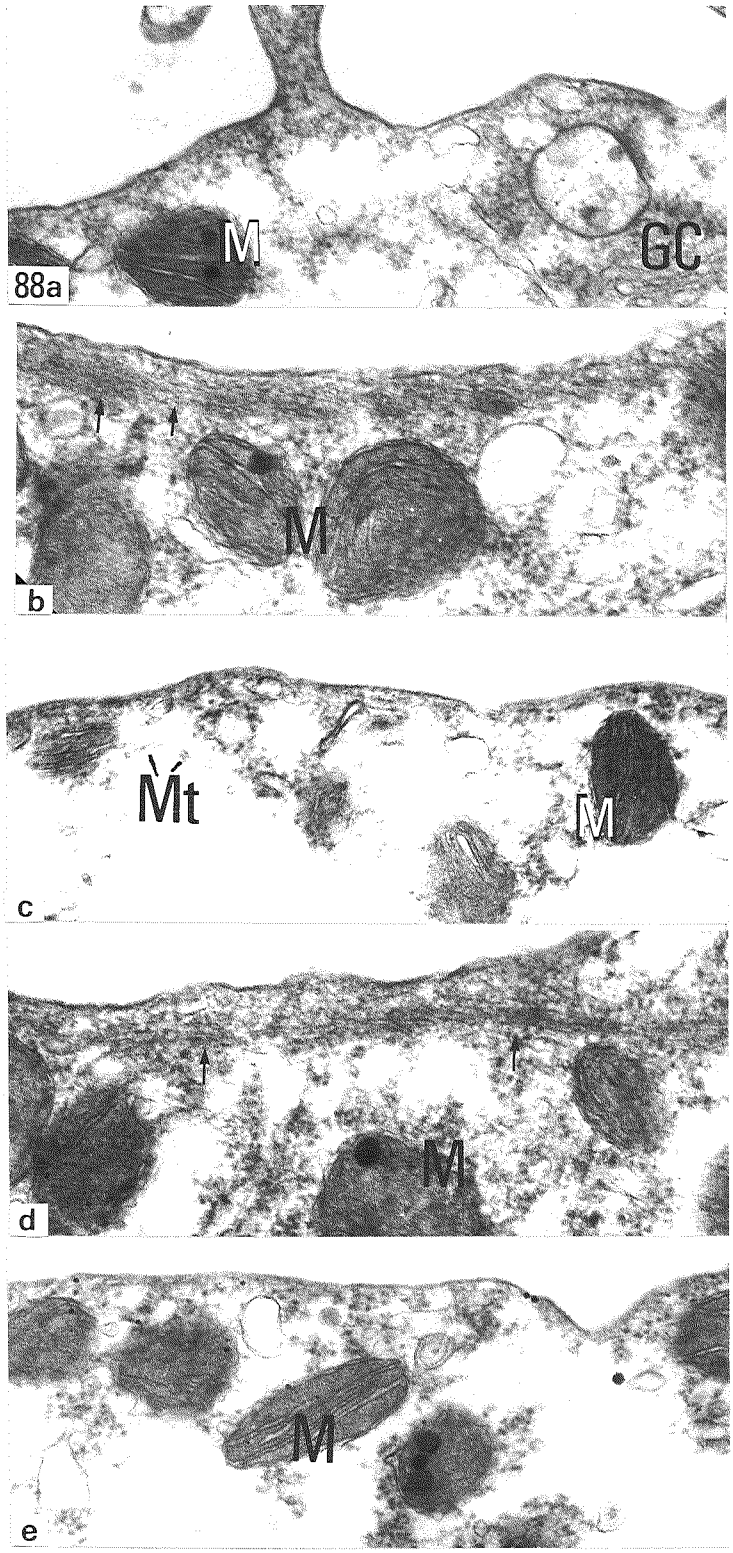
Fig. 86. Photomicrograph showing division of live nucleated egg fragment at the time when control egg is at the first cleavage. An arrow indicates cleavage furrow. x70.

Fig. 87. Egg fragments (a) and control egg (b) two days after production of the fragments. Nucleated fragment is consisted of several cells (arrows). The control egg has developed into late blastula possessing ectoteloblasts (open squeres) and mesoteloblasts (open circles). x70.


85a
b
c
86
87a
b

Explanation of Plate 28

Fig. 88. Transmission electron micrographs showing the cortical layer of the non-nucleated egg fragments produced at I-3 stage. Egg fragments were fixed at 10 (a; see Fig. 79a), 100 (b; see Fig. 80a), 160 (c; see Fig. 81a), 400 (d; see Fig. 82a) and 460 (e; see Fig. 83a) minutes after production of egg fragments. Note the exclusive presence of microfilaments in the cortical layer of the egg fragments showing grooving activity (Figs. 88b and d). GC, Golgi complex; Mt, microtubule. x40000.


M
GC
88a
M
b
Mt
M
c
M
d
M
e