THE FINE STRUCTURE OF EARLY EMBRYONIC DEVELOPMENT OF A TELEOST, BRACHYDANIO RERIC

by

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INTRODUCTION

The period of embryonic development, from fertilization to self-sustenance, is the most rapid and dynamic period during the individual existence of an organism. In a relatively brief period of the life cycle, a single undifferentiated cell with maternally supplied nutrients proceeds by cell multiplication and cell differentiation to form tissues and organs necessary for independent, adult existence. The interest of the scientific community and they layman has been centered largely upon the events leading to anomalies of existence. However, anomalies which occur in early development prior to the period of tissue and organ formation can also preclude development into the period of tissue and organ formation. Hence, early deviations in embryonic development would not lead to an adult organism and thus would not affect the interest of the community.

The period of early development, from the fertilized one-cell stage to the multicellular embryo, is the base upon which all remaining development rests. Satisfactory establishment of such a base is necessary for the attainment of the adult organism.

An organism suitable for study is the developing embryo of Brachydanio rerio (Hamilton-Buchanan), the Zebrafish. A cypridant, indigenous to the fresh water of India, this teleost is popular as a domestic, tropical fish. Scientific

merits include its rapid development from fertilization to hatching in 72 hours at 26°C (Hisaoka and Battle, 1958), production of large numbers of closely fertilized viable eggs (50-500 eggs, Hisaoka and Firlit, 1962a), and a small egg size (0.5 mu) suitable for observation with bright-field (Lewis and Roosen-Runge, 1942), phase-contrast (Hisaoka et al., 1957), Nomanski interference contrast optics (Hamano, 1964) or for subsequent electron microscopy (Thomas, 1964a,b). The suborder teleostei, of which B. rerio is a member, exhibits more evident characteristics of development, e.g. gastrulation (Devillers, 1961), than seen in other commonly used embryonic systems (e.g. chick, amphibia). Other embryological justifications for the use of teleostei and comparisons of teleostei to the embryologically well studied amphibia are given in the review of Devillers (1961). For these reasons, the early development of the vertebrate embryo, B. rerio, was considered a very appropriate organism of study.

The ovaries of B. rerio are located laterally and ventrally to the posterior portion of the gastro-intestinal tract in the female. Mature oocytes are first evident in the female at 3-4 months when first breeding is possible (Hisaoka and Firlit, 1962a). After a spawn, a period of only 5 days must elapse before mature eggs are again available. The histological details of the ovary and a classification of the oocyte

developmental stages are given by Hisaoka and Firlit (1962b). The localization of nucleic acids (Hisaoka and Firlit, 1962b) and the formation of yolk in the ovary as followed by histochemical methods (Malone and Hisaoka, 1963) will be subsequently discussed where appropriate for consideration of events in the embryo. The oocytes of B. rerio have been shown to be dependent upon organs of the adult for production of materials included in the yolk of the oocyte (Korfsmeier, 1966). Preliminary observations of the ultrastructure of B. rerio oocytes (Thomas, unpublished observations) show an elaborate interdigitation of oocyte cell membrane with surrounding nurse cells forming a zona radiate around the oocyte. Between the microvillar elaborations of the oocyte, an extracellular deposit of material is laid down which becomes the chorion of the embryo after fertilization. Similar membrane elaborations and extracellular deposits between oocytes and nurse cells have been reported in amphibia (Hope et al., 1963), teleosts (Jollie and Jollie, 1964), mammals (Adams and Hertig, 1964), and invertebrates (Rebhun, 1962).

The B. rerio egg has been used as an advantageous test system by Hisaoka (1958a,b), Hisaoka and Battle (1952), and Hisaoka and Hopper (1957) to study the effects of carcinogens and by Jones and Huffman (1957) to study the effects of various plant derivatives on embryonic development. The normal developmental stages have been enumerated and timed (Hisaoka

and Battle, 1958) by means of timelapse cinematography with phase contrast optics (Hisaoka et al., 1957) and correlated to hematoxylin and eosin stained paraffin sections (Hisaoka and Firlit, 1960). Marrable's study (1965) of B. rerio provides data of mitotic index, cell number, and cell volume during early development.

Upon fertilization the teleost egg, unlike the amphibian egg, undergoes a separation of cytoplasm and yolk (Devillers, 1961) that does not end in B. rerio until several cell cleavages have occurred (see films by Lewis and Roosen-Runge, 1942; Hisaoka et al., 1957). Roosen-Runge's early work (1938) on bipolar differentiation, where he notes an egg contraction or pulsation during the yolk-cytoplasm separation, is the first use of B. rerio as a test organism. In further investigations, Roosen-Runge (1939) studied karyokinesis and reported the occurrence of chromosomal vesicles in telophase, a short duration of the telophase period and cell cycle in general, the lack of nucleoli and "the absence of a typical spireme formation in the prophase" (p. 91). These reports cover the gametes and the development of the Zebrafish embryo to the end of bipolar differentiation.

This study will examine the structural events occurring in B. rerio from the completion of bipolar differentiation into the period of gastrulation and epibolic movements of the blastula cells. At the initial point in this study, two

things are prominent in the embryo, the embryonic yolk and the mitotically active cells lying upon the embryonic yolk. The transfer, distribution, and fate of yolk and the structure of the mitotic cell will be examined and discussed in relation to the progression of a B. rerio embryo through early development.

MATERIALS AND METHODS

Mature Zebrafish, Brachydanio rerio (Hamilton-Buchanan), were obtained from commercial hatcheries. In the laboratory, the fish were kept in balanced aquaria at 25°C with a day-period of fourteen hours (tungsten illumination). Both live brine shrimp (Artemia salina), hatched in the laboratory from dry eggs, and dry fish food were fed to the fish daily.

For breeding, a gravid female and several mature males were confined in a plastic breeding trap in an aquarium without sand or vegetation (kept at 26°C). Breeding usually occurred, if at all, within the first hour of "day" illumination (Hisaoka and Firlit, 1962a). Fifty to three-hundred closely developing eggs were found on the bottom of the spawning tank after breeding. Spawning will not occur if the period from the previous spawn has been less than five days or longer than forty days for a given female (Hisaoka and Firlit, 1962a). In the first case, prior to the fifth day eggs are assumed to be maturing; after ten days, two periods of maturation, the eggs become overripe, and the females become eggbound and are difficult to breed.

Eggs were pipetted from the spawning tank into a medium-sized dish for observation and use. The use of very closely developing populations of eggs was accomplished by further selection from a given batch of eggs of sub-spawns chosen by taking eggs which progressed from one very early developmental

stage to the next, that is, one-cell to two-cell stage within a sixty-second period. The developmental stages of B. rerio enumerated and timed by Hisaoka et al., (1957) and Hisaoka and Battle (1958) for 26°C incubation by timelapse cinematography have been used.

Embryos from early high blastula (stage 8, Hisaoka and Battle, 1958) to closure of the blastopore (stage 17) were fixed for studies of normal morphology (Table 1) or exposed to experimental milieu (Table 2) and subsequently fixed. Experimental treatments included 49.5% deuterium oxide in aquarial water, 10^{-3} molar colchicine (Calbiochem, MW=399.4) or either 0.95 gamma per ml. or 0.2 gamma per ml. of vincristine (VCR) in aquarial water (Eli Lilly Research Laboratories, Indianapolis, Indiana). Table 2 is a summary of eggs exposed, stage of exposure, and stage of controls at termination of exposure. Parallel sub-spawn samples of eggs with chorions rented to provide freer access of solutions were exposed to the abnormal milieu to determine the availability of the milieu to the embryo. For the materials noted, the chorion did not hinder availability to the embryo. In all cases, some of the controls were permitted to hatch to affirm the viability of the spawn.

Fixation of the eggs was carried out in one of three ways: 1) 1% osmium tetroxide with either 3×10^{-3} or 10^{-2} molar calcium chloride buffered with veronal acetate (sodium

Table 1. Developmental stages and the times of their occurrence from fertilization and the fixatives used to preserve material in this study

Hisaoka and Battle (1958) stage number and description		Time in minutes from fertilization at 26°C	Fixative used
#1	recently fertilized egg	0	
#2	1-cell blastodisc	25	
#3	2-celled ovum	35	
#4	4-celled ovum	43	
#5	8-celled ovum	71	
#6	16-celled ovum	90	
#7	32-celled ovum	105	
#8	late cleavage	110	
#9	early high blastula	131	OsCa[a]
#10	late high blastula	153	OsCa
#11	flat blastula	212	HAA-cac[b]
#12	very late blastula	247	OsCa, HAA-cac, HAA-PO4[c]
#13	early gastrula	296	HAA-cac, HAA-PO4
#14	one-third epiboly	348	OsCa, HAA-cac
#15	one-half epiboly	405	OsCa
#16	three-quarter epiboly	462	OsCa
#17	closure	600	OsCa

[a] OsCa is 1% osmium tetroxide with 3 X 10^{-3}M calcium chloride in veronal acetate buffer at pH 7.5

[b] HAA-cac is 1% hydroxyadipaldehyde buffered with sodium cacodylate at pH 7.6 fixation followed by 1% osmium tetroxide buffered with veronal acetate at pH 7.6

[c] HAA-PO4 is 1% hydroxyadipaldehyde buffered with Millonig phosphate at pH 7.5 fixation followed by 1% osmium tetroxide buffered with veronal acetate at pH 7.5

Table 2. Apparent developmental advancement of B. rerio embryos when observed with low power light microscope optics

Stage #4	6	8	10	12	14	16	18
Duration of Exposure							
3 Hours	---------- Expected Development at 26°C						
	---------- Control						
	X Colchicine (Periblast Enlarged)						
	---------- D_2O						
6 Hours	---------- Expected						
	---------- Control						
	X Colchicine						
	---------- D_2O Embryos Disintegrating						
12 Hours	---------- Expected						
	---------- Control						
	X Colchicine						
4 Hours	---------- Expected						
	---------- Control						
	---------- Colchicine						
	---------- D_2O						
9 Hours	---------- Expected						
	---------- Control						
	------- Colchicine						
	------- D_2O						
	---------- VCR (0.95 gamma/ml)						
	---------- VCR (0.2 gamma/ml)						

----- = Period of developmental advancement (i.e., from left to right, initial point of exposure to termination.

X = No apparent advancement.

barbitol-sodium acetate) at pH 7.5 for thirty minutes, 2) 1% hydroxyadipaldehyde buffered with Millonig phosphate at pH 7.5 for thirty minutes, rinsed in Millonig phosphate buffer at pH 7.5 for five minutes, and post-fixed in 1% osmium tetroxide buffered with Millonig phosphate at pH 7.5 for thirty minutes (Sabatini et al., 1963), or 3) 1% hydroxyadipaldehyde buffered with sodium cacodylate at pH 7.6 for thirty minutes, rinsed in sodium cacodylate buffer at pH 7.6 for thirty minutes, and post-fixed in 1% osmium tetroxide buffered with veronal acetate at pH 7.6 for thirty minutes (Sabatini et al., 1963). Experimentally treated material was fixed for thirty minutes only in 1% osmium tetroxide with 10^{-2} molar calcium chloride buffered with veronal acetate at pH 7.5.

Dehydration was achieved by passing the fixed eggs through an ascending series of graded ethanols. After dehydration in absolute ethanol, tissue was passed into propylene oxide and embedded in Epon resin according to the following procedure modified from Luft (1961): instead of preparing two Epon mixtures (Epon-nadic methyl anhydride (NMA) and Epon-dodecyl succinic anhydride (DDSA)) as Luft suggests, a suitable mixture was found by using Luft's two mixture procedure, then the total components necessary for the final mixture were calculated. A representative embedding mixture, Epon 44 ml., DDSA 37 ml., and NMA 19 ml. to which 2 ml.

DMP-30 catalyst (Rohm and Haas, Philadelphia) is added, is thoroughly mixed and used for embedding immediately. This mixture is the equivalent to Luft's six parts (Epon-DDSA) and four parts (Epon-NMA). The added convenience of this modification is the omission of two mixing steps and the reliability of using room-temperature anhydrous stock components. The Epon mixture was polymerized at 60°C for eighteen hours.

Thick sections were hand-cut with razor blades or with glass knives on a Reichert ultramicrotome OMU-2 for preliminary observations and correlation of stage morphology to in vivo observations just prior to fixation. Hisaoka and Firlit's study (1960) of sectioned paraffin-embedded B. rerio eggs was used as a basis of comparison. Sections were observed and photographed under Zeiss Zernicke phase contrast optics or Zeiss Nomarski interference contrast optics. Selected embryos were sectioned at 40-65 mu on an LKB Ultrotome with a Dupont diamond knife or on a Reichert ultramicrotome OMU-2 with glass knives and mounted on 75 x 300-mesh unfilmed copper grids. All sections for electron microscopy were stained with lead citrate (Reynolds, 1963) prior to observation on an RCA EMU-3F electron microscope operated at either 50 or 100 kv. Standard photographic procedures were used for the processing of negatives and prints.

YOLK

That soluble and insoluble nutrients in the form of yolk platelets, granules, or particles are provided to all eggs is a generally agreed concept. Of the two types of eggs recognized, the determinative egg, by definition, is absolutely dependent upon all materials provided by the maternal gamete (Raven, 1958) as shown by cytoplasm and/or cell removal studies. Organelle distribution and localization of enzymes have been studied to explain the necessity of all maternal contributions for development of regulative eggs (Humphreys, 1962, 1964; Raven, 1958; Berg and Long, 1964; Berg and Humphreys, 1960).

Regulative eggs, however, can have, by definition, one or several cells or a quantity of yolk removed without hindering normal development (Devillers, 1961). Embryos from such eggs have been studied by using nuclear transfers (Briggs and King, 1959) and X-irradiation which was designed to stop any nuclear activity (Neyfakh, 1959) and thus determine the point of initial regulation of the cytoplasm by the nucleus. Both Neyfakh (1959) and Briggs and King (1959) agree that nuclear activity is minimal, if not non-existent, until the initiation of gastrulation in a regulative egg. During early development when the nucleus is inactive, mitosis is occurring, and maternally provided yolk is assumed to be used by the embryo (Devillers, 1961).

Observations

At early high blastula (see Table 1 for stage number and embryo age), a group of cells, the blastomeres, is situated on the yolk mass (Figure 1a). Yolk, as round opaque particles, is visible at low magnifications with phase contrast optics and is located at the periphery of all cells (Figure 1a and b). A survey electron micrograph (Figure 3) shows a large yolk particle (4u maximum dimension) in the cytoplasm as well as yolk particles equal in size or smaller than the 0.5u mitochondria.

Below the blastomeres in the periblast, cytoplasm that is contiguous with the yolk mass (Figure 1b and 4d) contains large closely packed particles of yolk. Between the yolk particles and in the cytoplasm of the periblast, and the blastomeres, ribosome-like particles can be seen in high numbers. Each yolk particle is surrounded by a membrane if an appropriate cross-section is observed (Figure 4b,m). The interior of the yolk particle appears uniform under phase contrast optics (Figures 1a and 1b) and at the electron microscopic level of observation except that membranous vesicles can occasionally be seen internally (Figure 4b,v).

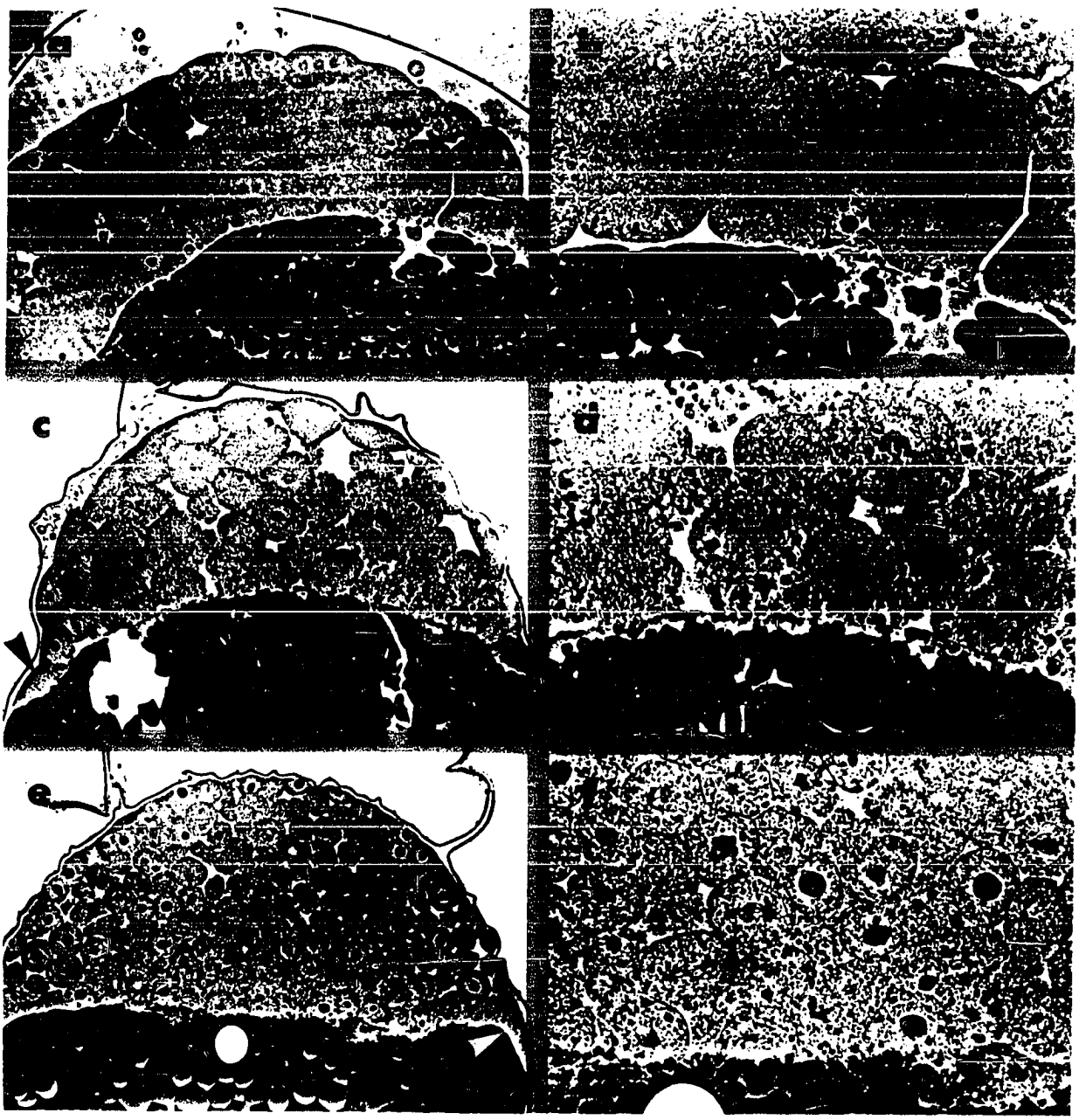
In cells of the late high blastula, yolk particles are located peripherally to the nucleus or mitotic apparatus (Figure 1c). The number of yolk particles in cells near the periblast is higher than in cells toward the forming epidermic

Figures 1a and 1b. Early high blastula embryos have uniformly distributed yolk after bipolar differentiation. Note the absence of small yolk particles in the periblast syncitium.

Figures 1c and 1d. Late high blastula embryos have smaller volume cells. Yolk particles in cells near the periblast are more numerous than in cells away from the periblast. The marginal periblast is now evident. (black arrow, Figure 1c).

Figures 1e and 1f. Flat blastula embryos have smaller volume cells and the marginal periblast is more evident (white arrow, Figure 1e). Note several mitotic spindles in Figure 1f.

Figures 1a, c, and e are X 150; Figures 1b, d, and f are X 370.


c
d
e

Figures 2a and 2b. Very late blastula cells near the periblast contain the majority of yolk particles. Mitotic figures in various phases are frequent.

Figures 2c and 2d. At early gastrula stage, the blastoderm is lower. Small yolk particles in the periblast and in cells near the periblast are higher in number than in cells away from the periblast.

Figures 2e and 2f. One-third epiboly stage embryos still show small yolk particles in the periblast and in cells near the periblast.

Figures 2g and 2h. At one-half epiboly stage, the marginal periblast has several prominent nuclei. Small yolk particles in the periblast are absent. Only scattered cells above the periblast contain yolk particles.

Figures 2a, c, e and g are X 150;
Figures 2b, d, f and h are X 370.

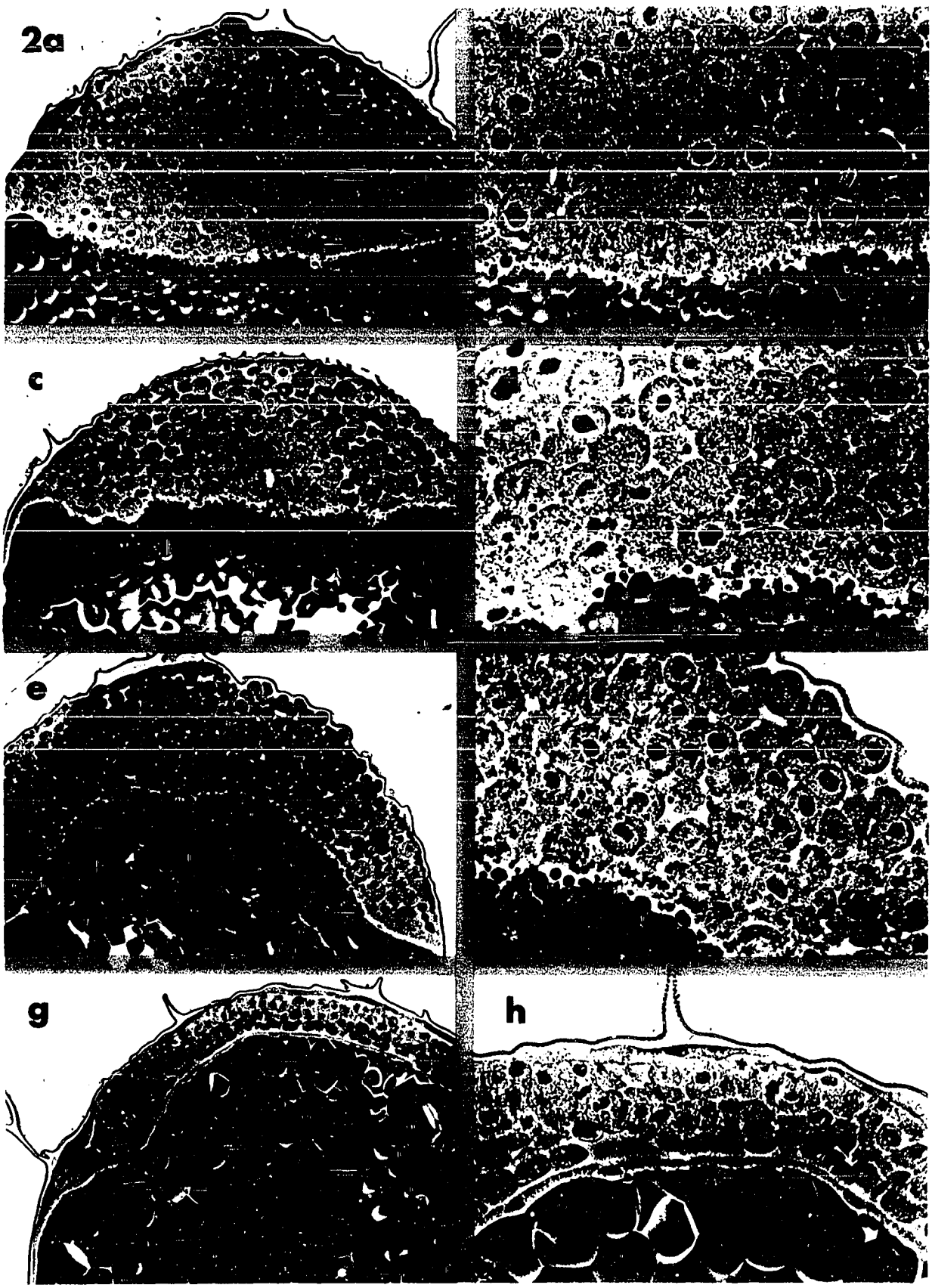
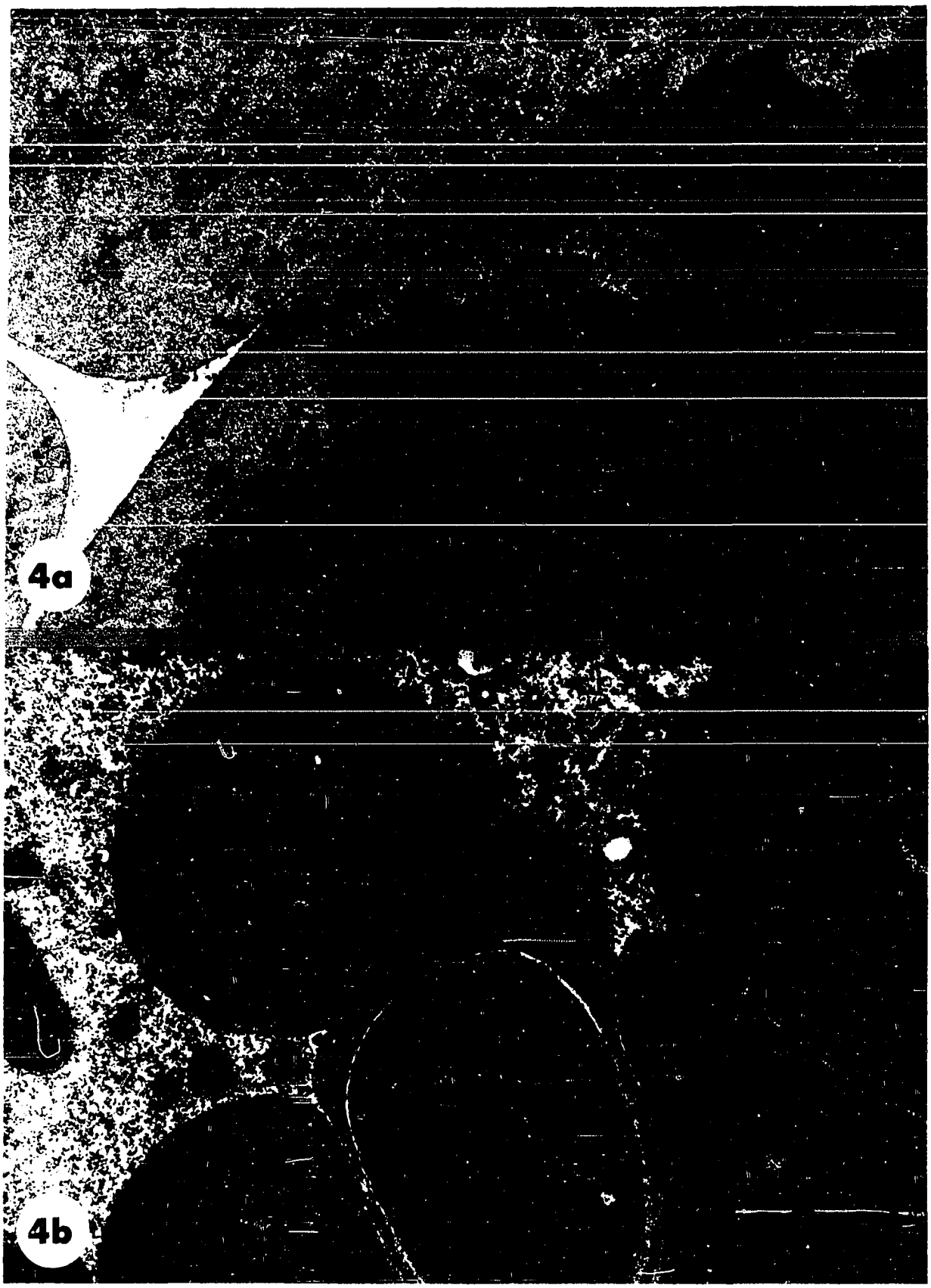

2a
c
e
g
h

Figure 3. A survey micrograph of an early high blastula embryo shows a 4.7-micron yolk particle but other yolk particles are smaller in diameter than the mitochondria. Anaphase chromosomes can be found in the lower left corner of the micrograph. Osmium tetroxide with calcium fixation; lead citrate stained. X 7700


3

Figure 4a. Periblast yolk of the early high blastula embryo is mainly large membrane-bound yolk particles. Cytoplasm can be seen between some yolk particles in the periblast. Osmium tetroxide with calcium fixation; lead citrate stained. X 6300.

Figure 4b. At a higher magnification, the membranes (m) around the yolk particles are readily apparent. Some yolk particles are within others but separated by their own membranes. Vesicles (v) can be seen in some yolk particles. Osmium tetroxide with calcium fixation; lead citrate stained. X 16,600.


4a
4b

stratum, the surface of the embryo. The periblast contains a large variety of yolk-particle sizes (Figure 1d) as compared to those of the periblast in early high blastula (Figure 1b).

Later, the marginal periblast (arrow, Figure 1e) is well developed at flat blastula, whereas it was only suggested at late high blastula (Figure 1e, arrow). Yolk particles, that are more numerous in cells close to the periblast (Figures 1e and 1f), may be seen in the montage of electron micrographs of a flat blastula (Figure 5). The periblast contains yolk particles of varying sizes in late high blastula (Figures 1f, 5, and 6a).

Yolk particles are found to be more numerous in cells close to the periblast and to be of various sizes in the periblast syncitium from late high blastula to the one-third epiboly stage[1] (Figures 2c and 2f). However at one-half epiboly (Figures 2g and h), only randomly scattered cells have definite yolk particles. The periblast yolk of the one-half epiboly embryo (Figure 2h) appears similar to that of the early high blastula embryo (Figure 1b). Various-sized yolk particles present in the periblast of embryos epiboly from late high blastula to one-third epiboly stage are absent at the one-half epiboly stage.

Throughout this entire developmental period, yolk

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[1] The one-third epiboly stage of an embryo is when the blastomeres have reached one-third the distance to the vegetal pole by cell migration (epiboly).

Figure 5. Yolk distribution in the late high blastula is evident in this montage. The periblast syncitium contains numerous yolk particles. Fewer yolk particles are present in cells further away from the periblast. Hydroxyadipaldehyde buffered with sodium cacodylate fixation; lead citrate stained. X 1350.

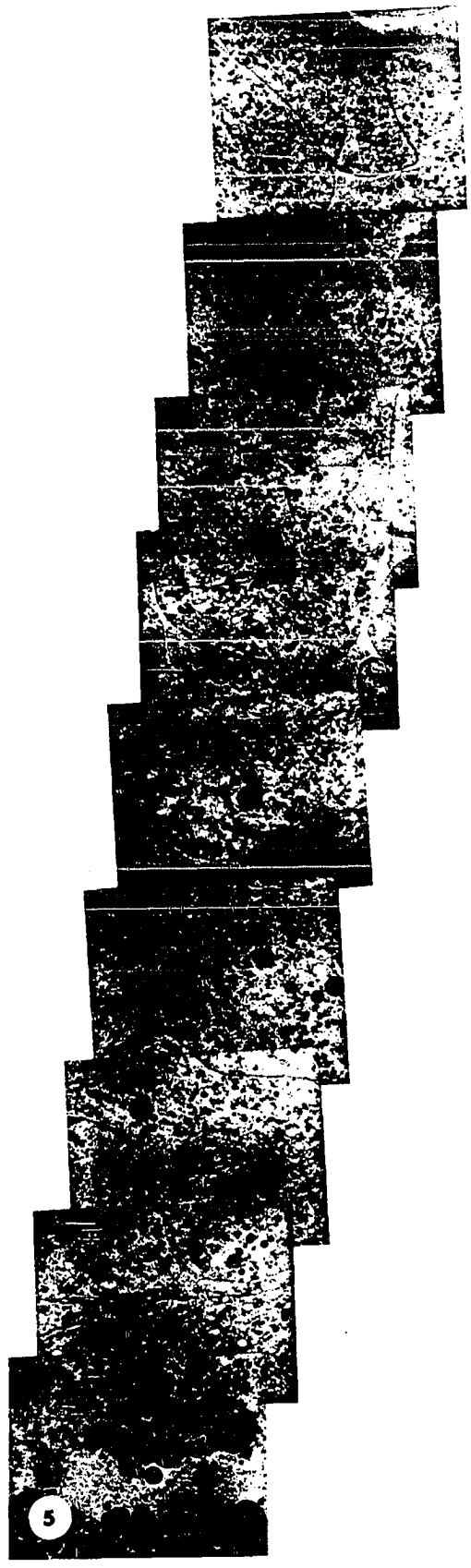
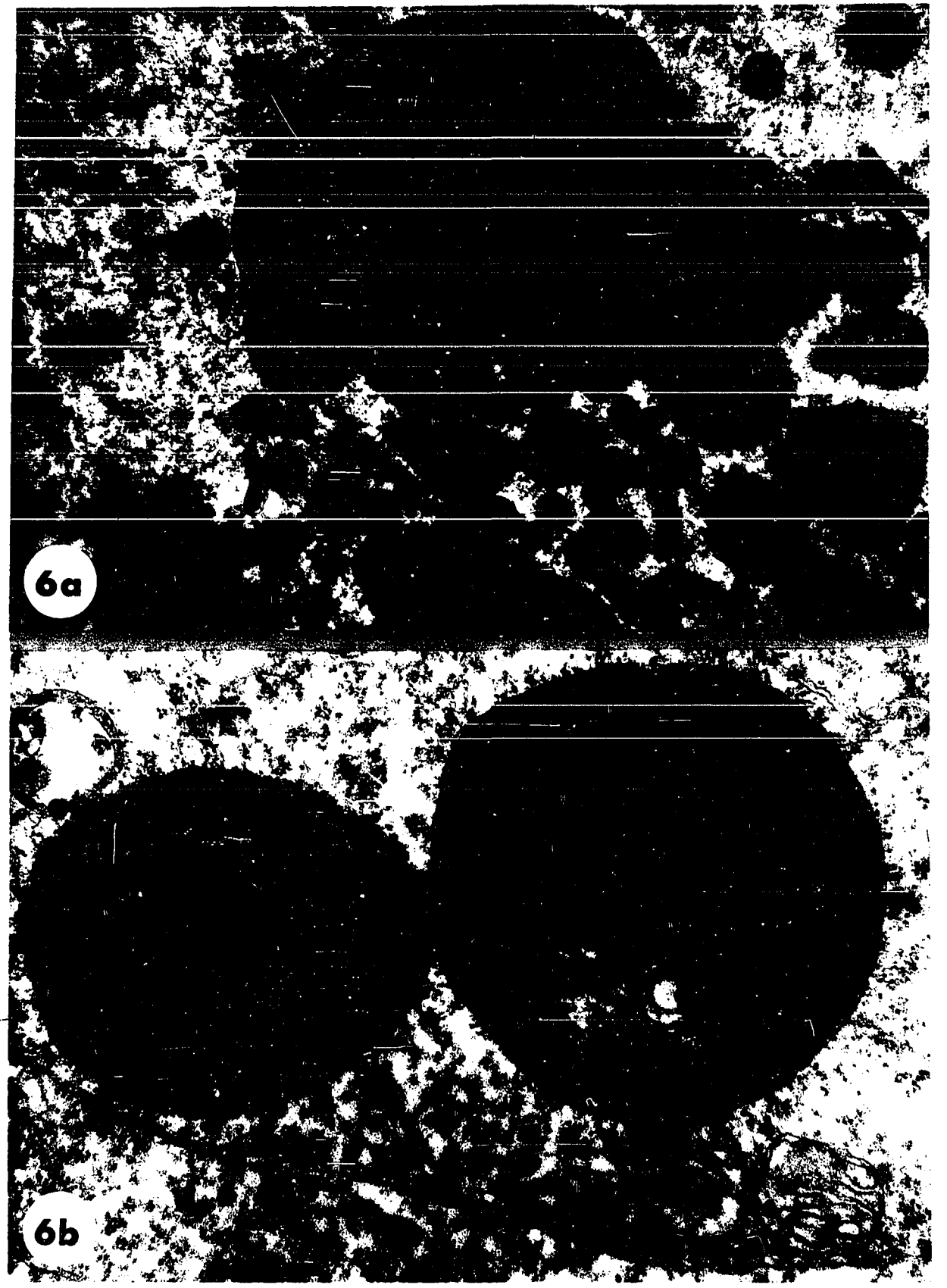

5

Figure 6a. Membrane-bound yolk particles within yolk particles are found in the periblast of the late high blastula embryo. Hydroxyadipaldehyde buffered with sodium cacodylate fixation; lead citrate stained. X 14,600.

Figure 6b. Yolk particles have a uniform granular appearance at high magnification except where membranous vesicles are within the yolk. Osmium tetroxide with calcium fixation; lead citrate stained. X 42,100.

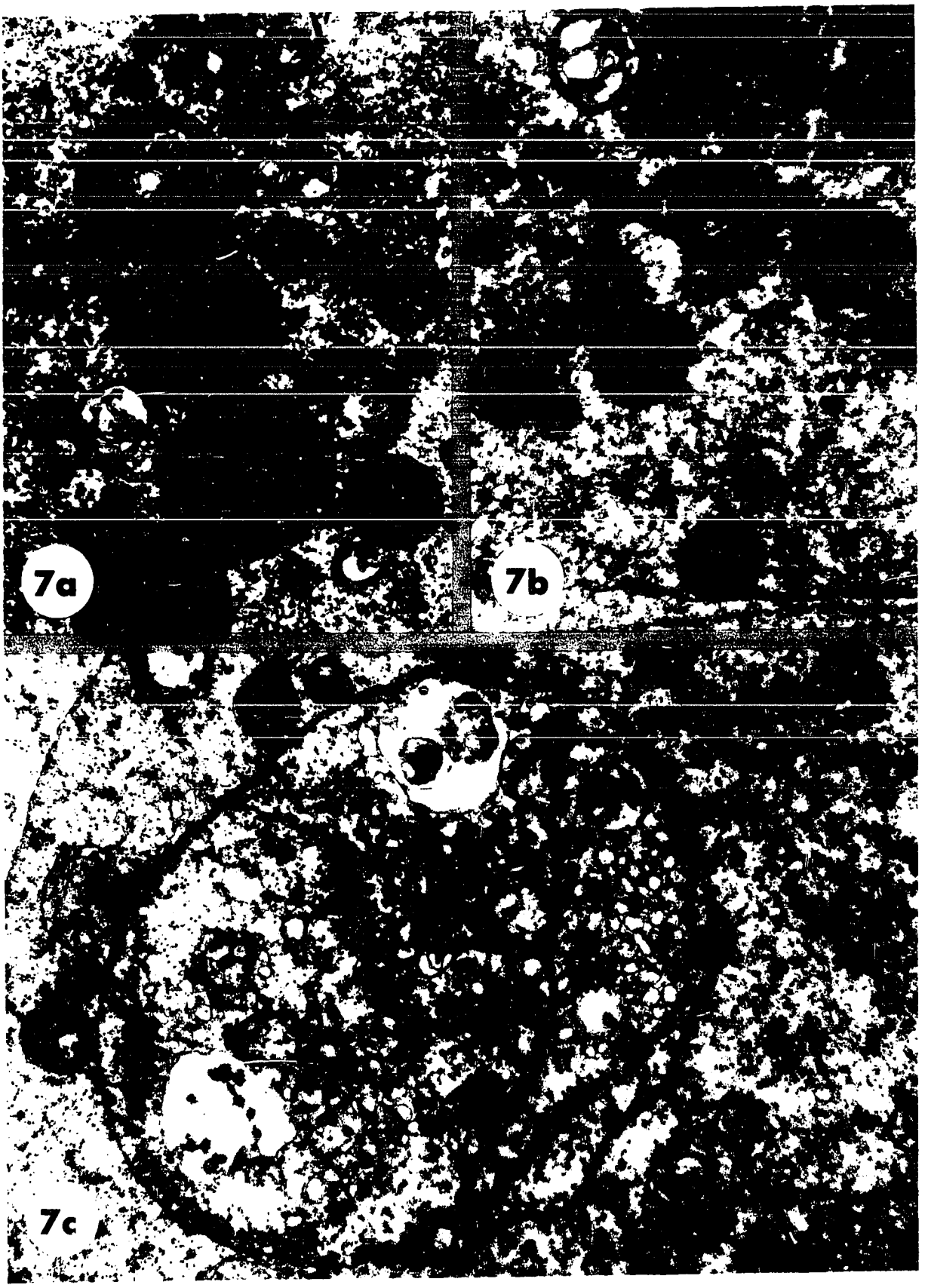

6a
6b

particles that were first seen in all cells of the early high blastula are missing from the cells furthest from the periblast until one-half epiboly, when only randomly scattered cells contain visible yolk particles. In cells of all stages from late high blastula to one-half epiboly, some yolk particles (0.5-1.5u in diameter) are seen with various ratios of yolk and centrally located membranous elements and ribosome-like particles (Figures 6b-7c). Varying amounts of yolk surround the membranous elements and ribosome-like particles, or alternately larger quantities of membranous elements and ribosomes can be seen within the membrane-bound yolk particles of cells in the one-third epiboly embryo (Figures 6b, 7a and 7b). A cytoplasmic structure (4.7u average diameter) found in a single cell (Figure 7c) is suggestive of the ultimate complexity and fate of a yolk particle. The interior of this complex structure contains numerous membranous vesicles and ribosome-like particles. Separated from the cytoplasm by a membrane, a thin layer of material similar in density and structure to a yolk particle (arrows) is deliniated from the interior by a second membrane. Another micrograph of this complex structure (Figure 17b) shows its position in a telophase cell and the suggestion of a mitochondrion-like structure in the interior.

Figure 7a. Yolk particles are seen with varying amounts of membranous material and ribosome-like particles. A sequence of these yolk particles may be used to describe the order of yolk utilization. Osmium tetroxide with calcium fixation; lead citrate stained. X 13,700.

Figure 7b. The yolk particle in the center of the micrograph contains ribosome-like particles similar to ribosomes of the cytoplasm. Osmium tetroxide with calcium fixation; lead citrate stained. X 15,000.

Figure 7c. The ultimate fate of a yolk particle may be the structure which dominates this micrograph. Membrane-bound yolk, similar to yolk in a smaller particle (arrows), is separated from the interior of the particle by a second membrane. The interior of the particle appears as the cytoplasm outside the yolk particle. Osmium tetroxide with calcium fixation; lead citrate stained. X 21,500.


7a
7b
7c

Discussion

Regulative eggs by definition may have one or several cells removed without altering normal development. A meridional transection of the two-cell stage of teleost embryos yields within experimental limits separate twin embryos. The work of Oppenheimer (1934, 1936) and Tung (1955, see also Devillers, 1961) suggests that an embryo such as Fundulus, a marine teleost, from early cleavage (two-cell stage) is more dependent upon the amount of periplast yolk included in an explant than is an embryo that is more advanced prior to explantation. In other words, "if it [the embryo] is explanted before a so-called critical stage, the blastoderm turns into a hyperblastula [a non-differentiated mass of cells]; if it is explanted after that, it undergoes differentiation. The critical stage corresponds to 8 blastomeres in Carassius, 32 in Fundulus, and the young blastula in "Salmo" (Devillers, 1961, p. 391). Devillers, in summary, states that "these results mean that a substance indispensable for differentiation exists in the yolk sphere:..." (p. 392). He continues, "On the other hand, how this hypothetical material may reach the blastoderm needs to be explained. The base of the blastoderm is in direct communication with the periblast in the early stages; later on, the syncitium 'buds' off blastomeres that add themselves to the embryonic disc" (p. 397). He then asks the question "but then how can one explain that in later stages the

syncitium can still impose an orientation on the germ? Are diffusing organizing substances involved?" (p. 397)

The relatively young age of the B. rerio egg at fertilization as compared to other species such as Carassius and Fundulus has been noted by Devillers (1961). The developmental events observed in these two species may occur later in the developmental age of the B. rerio embryo, but what is stated for Carassius and Fundulus may be extended to B. rerio.

On the basis of these (Oppenheimer, 1934, 1936; Tung, 1955; Devillers, 1961) and other studies, the distribution of yolk and the fate of yolk particles will be discussed in B. rerio in an effort to find information pertinent to Devillers' questions.

Bipolar differentiation, that continues into late cleavage stages of B. rerio, results in all cells having a complement of yolk particles. We may assume that the yolk in any given cell will be utilized almost as readily as in a neighboring cell. In this study, mitotically active cells with peripherally located yolk were found on the central periblast of the yolk mass. Marrable's data (1965) showing that B. rerio has a high mitotic index (83%) and a uniform distribution of mitosis at blastula stages would suggest, excluding the occurrence of yolk transfer from the periblast, that uniform distribution and usage of intracellular yolk should be expected. However, yolk is diminished in cells

away from the periblast, and yolk particles are consistently seen in high concentration in cells near the periblast.

Two possibilities may be considered for the occurrence of yolk in cells near the periblast. Yolk particles were never found extracellularly nor was cell-to-cell transfer of cytoplasm and yolk observed. Therefore, the direct transfer of yolk from cell to cell is excluded as a possible mechanism. The alternate explanation is that mitosis from the periblast contributes blastomeres and causes the small yolk particles of the periblast to be a natural marker of the periblast cytoplasm. Mitosis from the periblast has not been observed but has been assumed as a mechanism of cell contribution from the periblast (Devillers, 1961). This hypothesis of mitosis from the periblast though assumed as valid for this study must be investigated further.

As suggested by Oppenheimer (1934, 1936), Tung (1955), and Devillers (1961), some substance in the yolk of the embryo carrys information (or material) necessary for the differentiation processes. Identification of the components of yolk should provide important information on the nature of the 'yolk-substance' necessary for differentiation.

Yolk particles as seen in this study in one-third epiboly cells have been observed initially to have only amorphous yolk material surrounded by a unit membrane, then to have membranous elements and ribosome-like particles in the center of

membrane-bound yolk material, and to appear finally as a highly complex particle appearing as cytoplasm surrounded by little yolk and a unit membrane. These yolk particles containing various quantities and types of inclusions could be arranged as suggested above in a sequential pattern of utilization. Bellairs (1958) has seen a similar, but more complex, sequential ordering of yolk particles in the developing chick embryo. Strikingly, the final member of the sequence (not observed or mentioned by Bellairs) appears as an isolated portion of cytoplasm surrounded by a thin layer of yolk and an external unit membrane. That a supply of maternal membranous elements and ribosomes be the determining factor from the periblast necessary for permitting differentiation at later developmental stages is a highly suggestive hypothesis.

The possibility of ribosomes in yolk must be considered by reviewing the formation of yolk during oogenesis in B. rerio. Hisaoka and Firlit (1962b) suggest the inclusion of RNA in a masked form in yolk of the mature oocyte. Ribonucleic acid content, by methyl green-pyronin-Y staining, is high (intense staining) as cell size and yolk material increase until oocyte stage 5. They state (Hisaoka and Firlit, 1962b, p. 206): "Throughout oogenesis the intravesicular yolk does not contain RNA. However, RNA is present in the extravesicular yolk of all stages except stage 5"

(mature oocytes). After fertilization, RNA remains constant in all cells during cleavage and blastula stages except for regional decreases during interphase and prophase (Hisaoka and Firlit, 1961).

In Rana pipiens, Brown and Caston (1962) report the binding and masking of exogenous isotope-labeled ribosomes to yolk in studies of in vitro protein synthesis during early development. Until Shumway stage 21-23 in Rana pipiens, Karasaki (1963) notes the decrease in yolk in the embryo, but he does not report ribosome-like particles within Rana yolk platelets. Rounds and Flickinger (1958) conclude, however, from their study of neural induction in R. pipiens that yolk breaks down in chordamesoderm with a subsequent release of RNA. Bellairs (1958) reports micro-particles in yolk similar to cytoplasmic micro-particles now known as ribosomes.

Oogenesis in most species is essentially synthesis and sequestering of material for the period from fertilization to nuclear activity. Formation of the insect fat body, for the metamorphosis of larva to pupa, is a similar period. Therefore, the work of Locke and Collins (1965) on the structure and formation of protein granules in an insect fat body deserves critical study. In summary, they found an intracytoplasmic isolation of mitochondria and rough, endoplasmic reticulum into cytolysome-like structures. Eventually, the mitochondria and rough endoplasmic reticulum of the insect fat

body are encompassed by a unit membrane and accumulate protein. The membranes and ribosomes within the cytolysome loose their identity among the accumulated protein and appear similar to the yolk material within the yolk particles seen in the periblast of B. rerio. "Protein + RNA granules" of the insect fat body often contain crystalline protein with a repeat spacing of 90 to 160 Å similar to crystals with an average spacing of 70 Å found in Rana yolk by Karasaki (1963). Golgi vesicles, in the fat body, also accumulate proteinaceous material and appear as "yolk". Locke and Collins find isolation of membranous material and ribosomes into membrane-bound, protein-containing granules; in contrast, B. rerio yolk particles, which appear as the protein granules of the insect fat body, give rise to membranous material and ribosome-like particles during breakdown. In the sequestering fat body, granules fuse to form larger "fat" particles within cells; in contrast, in the periblast of B. rerio during yolk utilization, large yolk particles appear to "pinch-off" into smaller yolk particles.

Yolk, in Rana pipiens embryos, has been reported to contain RNA. In B. rerio oocytes, RNA appears to be masked in extravesicular yolk. In an insect fat body, ribosomes and membranous material are packaged in "protein granules" similar to yolk particles. Microparticles found in the yolk of the chick are similar to ribosomes in the cytoplasm of the

developing chick embryo. Therefore, the conclusion is strongly suggested that membranous material and ribosome-like particles in the yolk of B. rerio originate maternally and are necessary for differentiation. This hypothesis might be further tested by explanting an embryo which should hyper-blastulate and adding either 1) maternal yolk, or 2) embryonic or maternal ribosomes (without messenger-RNA), or 3) embryonic or maternal membranous material, or 4) both ribosomes and membranous materials to the explanted embryo. Thus yolk, besides providing soluble nutrients to the embryonic cells, could be tested for its contribution of the substance of yolk necessary for future differentiation.

MITOSIS

The next prominent event after yolk utilization during early development is the production of additional cells by mitosis. The oocyte is a center of synthesis and storage of material, but after fertilization, an ordering of material by bipolar differentiation occurs. Some protein synthesis does occur in developing sea urchin eggs (Gross and Cousineau, 1963), but control of protein synthesis is extranuclear (Tyler, 1966). The non-involvement of the nucleus in the physiological activities of the early developing egg is indicated also by both the work of Briggs and King (1959) and Neyfakh (1959). A high rate of cell division immediately after fertilization demands that the material to be used during early development be produced or programmed in the oocyte. What, then, are the differences between an undifferentiated cell and a nucleus-activated, partially differentiated cell during mitosis?

The study of mitosis in general has been a focal point of man's curiosity because of the involvement of mitosis in all aspects of life (Mazia, 1961). In the last decade, the study of the mitotic apparatus by the use of electron microscopy has proceeded from confirmation of the filamentous nature of the mitotic apparatus (Roth et al., 1960; Roth and Daniels, 1962; Harris, 1961, 1962) to general descriptions of amenable systems such as cultured cells

(Robbins and Gonatas, 1964; Barnicot and Huxley, 1965) and to some experimental electron microscope investigations (vincristine: George et al., 1965; cold, colchicine, urea: Roth, 1964). Some investigators have approached the study of mitosis with histochemical and isolation-biochemical techniques. From the assumption (Mazia, 1961) that adenosine triphosphate (ATP) is the energy source for mitosis, attempts have been made to localize ATPase activity in the mitotic apparatus (Miki, 1963, 1964); or from the suggestion of Rapkine (1931) that sulphydryl groups may be significantly involved in the mitotic process, sulfydryl reactions have been examined by histochemical methods (Kawamura and Dan, 1958; Kawamura, 1960). Following the pioneering work of Mazia et al. (1961), many investigators have physically-isolated the mitotic apparatus of some marine invertebrate blastomeres, especially from sea urchins. These techniques have resulted in fruitful but conflicting data. To the present, investigators state that ATPase activity is both present or absent in the mitotic apparatus (Miki, 1964; Stephens, 1965, respectively) and state varying values for the size of mitotic apparatus, spindle protein subunits (Zimmerman, 1960; Kane, 1965; and Sakai, 1966). This work, however, has centered around a single type of organism, the sea urchin, although investigators have used different species of urchins.

While the histochemical localizations and isolation-biochemical analysis has provided some information, still much must be learned of mitosis *in situ* in both vertebrate and invertebrate cells. Systems studied to date have either been invertebrate (protozoan or embryonic invertebrate) or dedifferentiated tissue culture cells with remnants of the differentiated state. Zebrafish blastomeres which are mitotically active (83% mitotic index) and have a short cell cycle (15-18 minutes, Roosen-Runge, 1939; Marrable, 1965) are an ideal source of mitotic cells. Therefore, the morphological characteristics of the undifferentiated early embryonic, mitotic cell, at a time when little or no nuclear control is involved, will be compared to the physiologically active, nucleus-controlled, mitotic cell. This study is a considerably more complete examination of mitosis during early development in *B. rerio* than those presented earlier (Thomas, 1964a,b).

Observations

Mitosis has been followed in cells from embryo at early high blastula to the one-third epiboly stage. The cells in this period are more favorable for study because of their smaller size as compared to the large volume of cells found through early high-blastula.

Interphase

The early embryonic, interphase cell is undifferentiated (Figure 8). The nucleus (n) is spherical except that it contains a cytoplasmic cleft. The nucleoplasm has a uniform, fibrillar appearance following osmium-tetroxide fixation; nucleoli are absent. Adjacent to (Figure 8) or completely surrounding the centriole (Figure 9a) is a 'halo' of fine material which together with the centriole forms the centrosome. Golgi material (g) is located directly opposite the centriole and the nucleus. Spherically shaped mitochondria are scattered throughout the cytoplasm and concentrated characteristically next to the nuclear envelope. Ribosome-like particles are ubiquitous, and yolk particles vary in quantity and size from cell to cell.

Prior to attenuation of the nuclear envelope during prophase, spindle microtubules (sp) are seen adjacent to the centriole (Figure 9b) but do not form a detectable aster during mitosis. The nucleus surrounding the cleft appears as finger-like projections toward the centriole (Figures 9a and c) which is most evident when both centrosome regions are in the same plane of section (Figure 9c). From the centrosome (cs), a spindle microtubule (arrow) can be seen leading toward the nuclear cleft (Figure 10a).

Figure 8. An interphase cell contains a spherically shaped nucleus (n) with numerous mitochondria along the nuclear envelope. A centriole is situated between the Golgi apparatus (g) and the nucleus. Osmium tetroxide with calcium fixation; lead citrate stained. X 11,300.

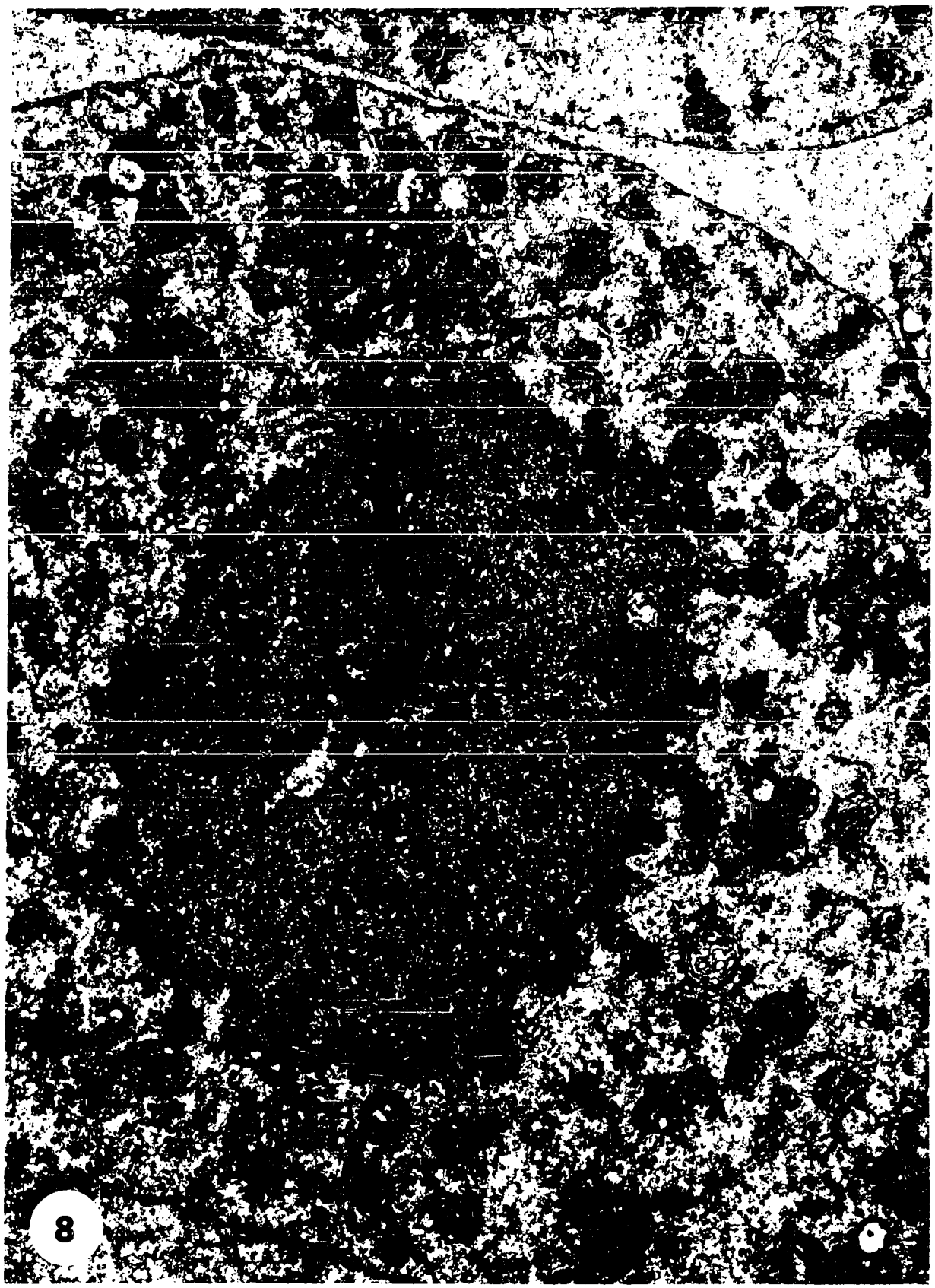
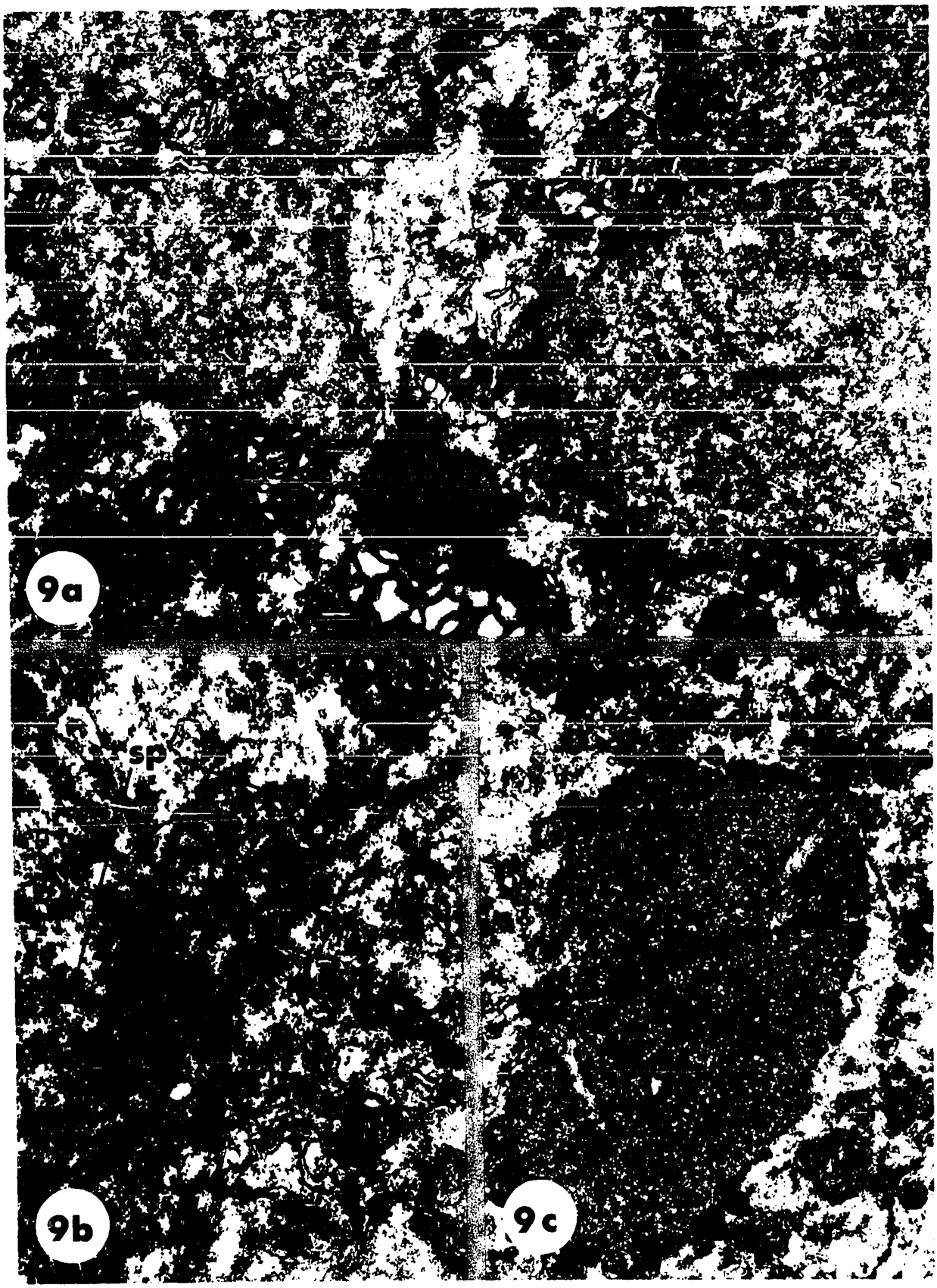

8

Figure 9a. A cross-sectioned centriole showing cartwheel-arranged triplets is in a matrix forming the centrosome. The centrosome characteristically is at the edge of the nuclear cleft. Osmium tetroxide with calcium fixation; lead citrate stained. X 32,000

Figure 9b. Prior to nuclear envelope interruption, spindle microtubules (sp) radiate from the centrosome. Osmium tetroxide with calcium fixation; lead citrate stained. X 26,800

Figure 9c. During early prophase, the nucleus is ellipsoidally shaped with few mitochondria along the nuclear envelope. Two nuclear clefts and two centrosomes are evident. The nucleoplasm is uniform; the chromosomes have not yet condensed. Osmium tetroxide with calcium fixation; lead citrate stained. X 7500


9a
SP
9b
9c

Prophase and Prometaphase

Condensation of chromosome material (c), attenuation of the nuclear envelope (ne), and the presence of spindle microtubules (among the chromosomes and nuclear envelope fragments) characterizes the transition into prophase (Figure 10b). Mitochondria are not as concentrated along the nuclear envelope as was seen in interphase cells. Nuclear envelope fragments with nuclear pores (np) showing in cross and tangential section, mitochondria, ribosome-like particles, chromosomes, and spindle microtubules can be seen intermingled at a higher magnification (Figure 11a).

As chromosomes approach alignment on a metaphase plate (Figure 11b), large nuclear envelope fragments persist at the periphery and within the forming mitotic spindle. The typical tangential view of spindle microtubules is difficult to discern because much fine material is close to them. Yolk particles and mitochondria are excluded from the spindle, and Golgi vesicles can be seen at one of the poles. A cross-section through a similar pro-metaphase figure illustrates the exclusion of mitochondria and yolk from the forming mitotic apparatus (Figure 12a). Cross-sections of spindle microtubules (arrow) are found in groups adjacent to and removed from the chromosomes but are closely associated with the nuclear envelope fragments (Figure 12b,c). The spindle microtubules are 19-21 mu in diameter whether

Figure 10a. A spindle microtubule (white arrow) can be seen between the centrosome (cs) and the nucleus. Numerous nuclear pores can be seen along the edge of the nucleus. Osmium tetroxide with calcium fixation; lead citrate stained. X 38,700

Figure 10b. The nuclear envelope (ne) appears intact when chromosomes (c) are condensed at prophase. Osmium tetroxide with calcium fixation; lead citrate stained. X 9300

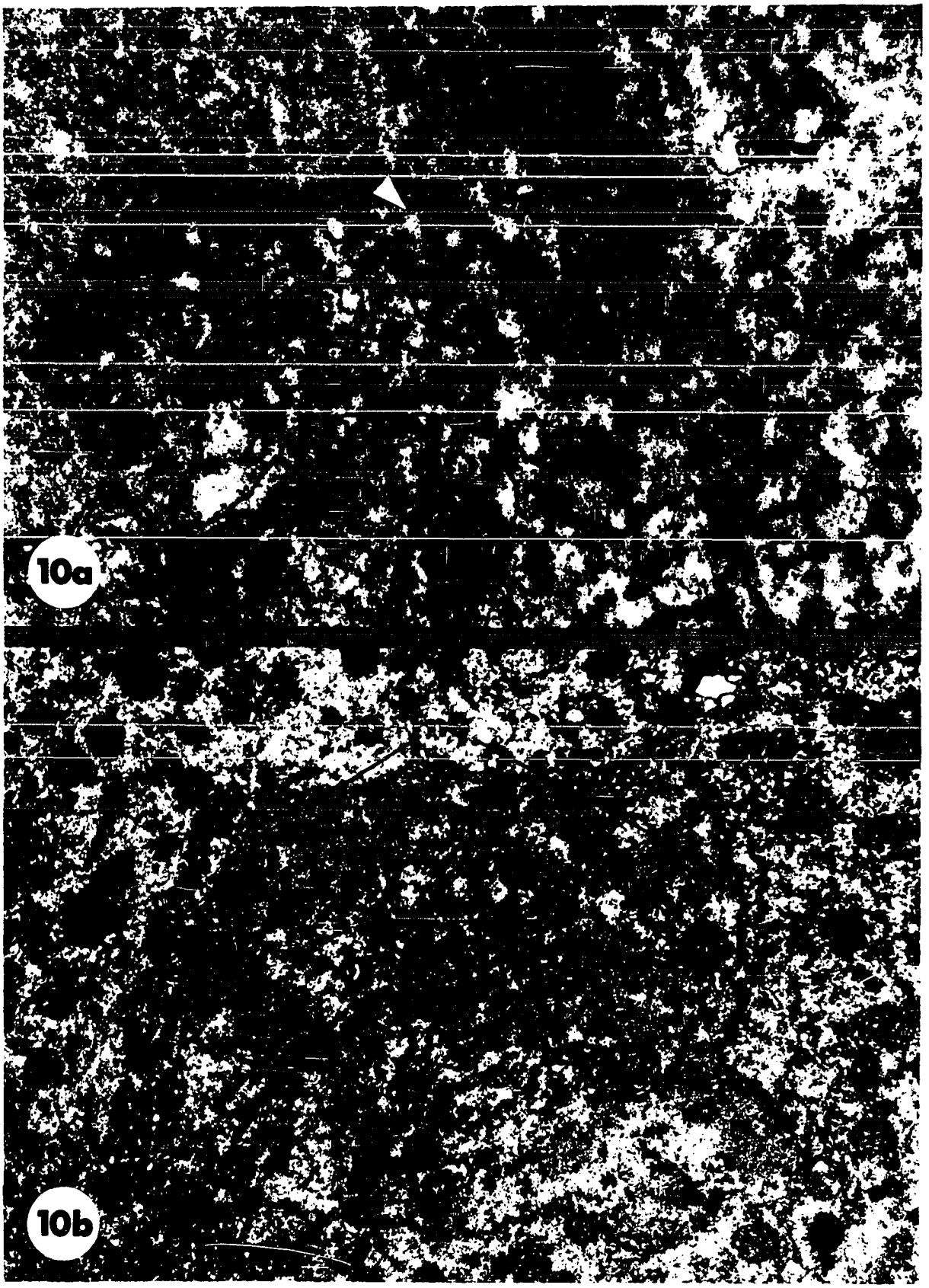

10a
10b

Figure 11a. At a higher magnification, spindle microtubules can be seen in the prophase nucleus. The nuclear envelope can be seen in cross-section and tangential section where nuclear pores (np) are prominent. Osmium tetroxide with calcium fixation; lead citrate stained. X 21,800

Figure 11b. When chromosomes appear almost aligned at prometaphase, nuclear envelope fragments remain around the formed mitotic apparatus. Golgi apparatuses can be seen at both ends of the mitotic figure. Hydroxyadipaldehyde buffered with phosphate fixation; lead citrate stained. X 7600

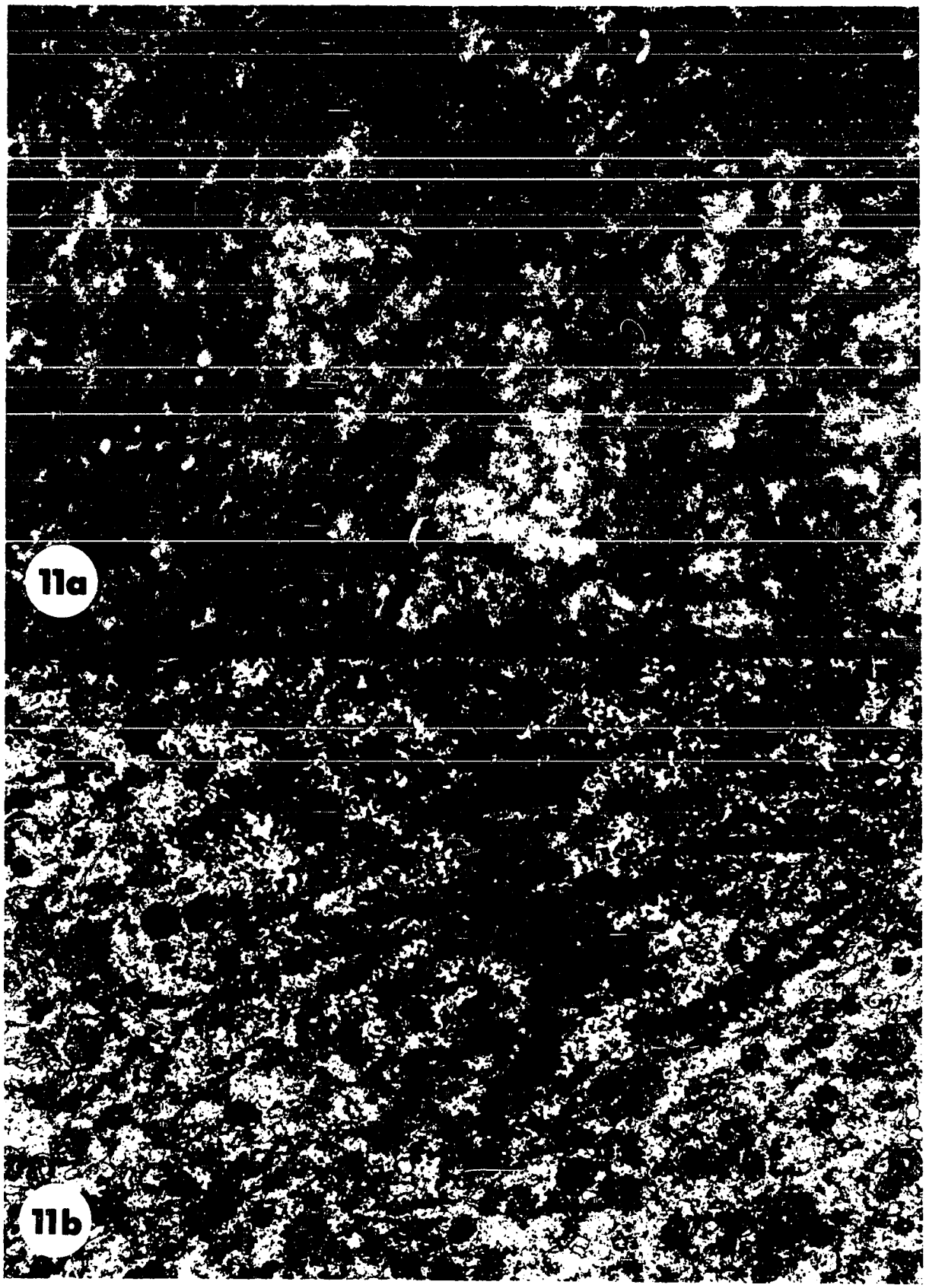
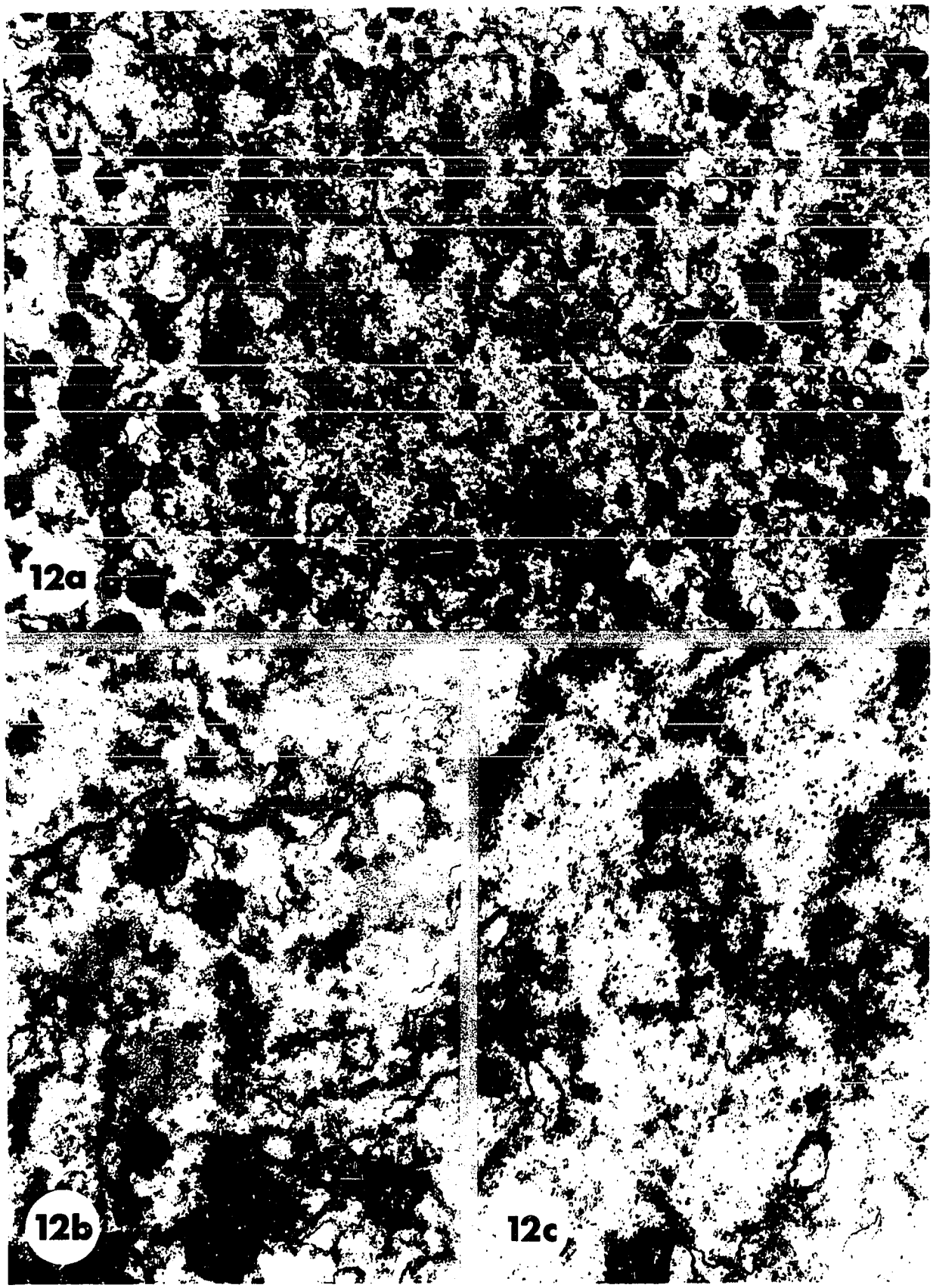

11a
11b

Figure 12a. A cross-sectioned prometaphase mitotic figure shows the exclusion of mitochondria and yolk from the formed spindle. Hydroxyadipaldehyde buffered with phosphate fixation; lead citrate stained. X 7400

Figure 12b. Spindle microtubules in groups (arrows) are near either chromosomes or nuclear envelope fragments. Hydroxyadipaldehyde buffered with phosphate fixation; lead citrate stained. X 18,400

Figure 12c. At higher magnifications, the circular cross-section of spindle microtubules (arrows) preserved by hydroxyadipaldehyde is evident. Hydroxyadipaldehyde buffered with phosphate fixation; lead citrate stained. X 39,000


12a
12b
12c

spindle microtubules are preserved by osmium-tetroxide with calcium or by hydroxyadipaldehyde.

Metaphase

At metaphase (Figure 13), chromosomes are aligned on an equatorial plate with spindle microtubules extending to and between chromosomes. At the poles, Golgi vesicles (g) are present, but astral filaments are absent; the nuclear envelope fragments are gone (compare with Figure 11b). Microtubules are arranged in groups often ending at a kinetochore-like structure (k) at the chromosomes or go between the chromosomes (arrow, Figure 14a).

Anaphase

An early anaphase figure in tangential section shows the interzone (IZ) between daughter chromosome sets (Figure 14b). The daughter chromosomes are seen at varying separations, but the poles are at a relatively constant distance of 15u (Figures 15, 16a). At the initiation of cytokinesis, the pole-to-pole separation (the chromosomes are at the poles) varys up to a maximum of 24u (Figure 16b). Table 3 is a summary of pole-to-pole distances of the phases of mitosis from measurements of phase contrast and electron micrographs. Groups of microtubules extend to the chromosomes, and the poles and microtubules are found in the interzone (Figure 15); no asters are present. Membranous vesicles and elements,

Figure 13. At metaphase, the chromosomes are neatly aligned on a plate between Golgi apparatuses (g). Membranes identifiable as nuclear envelope fragments are absent. Compare Figure 13 to Figure 11b. Hydroxyadipaldehyde buffered with phosphate fixation; lead citrate stained. X 10,800

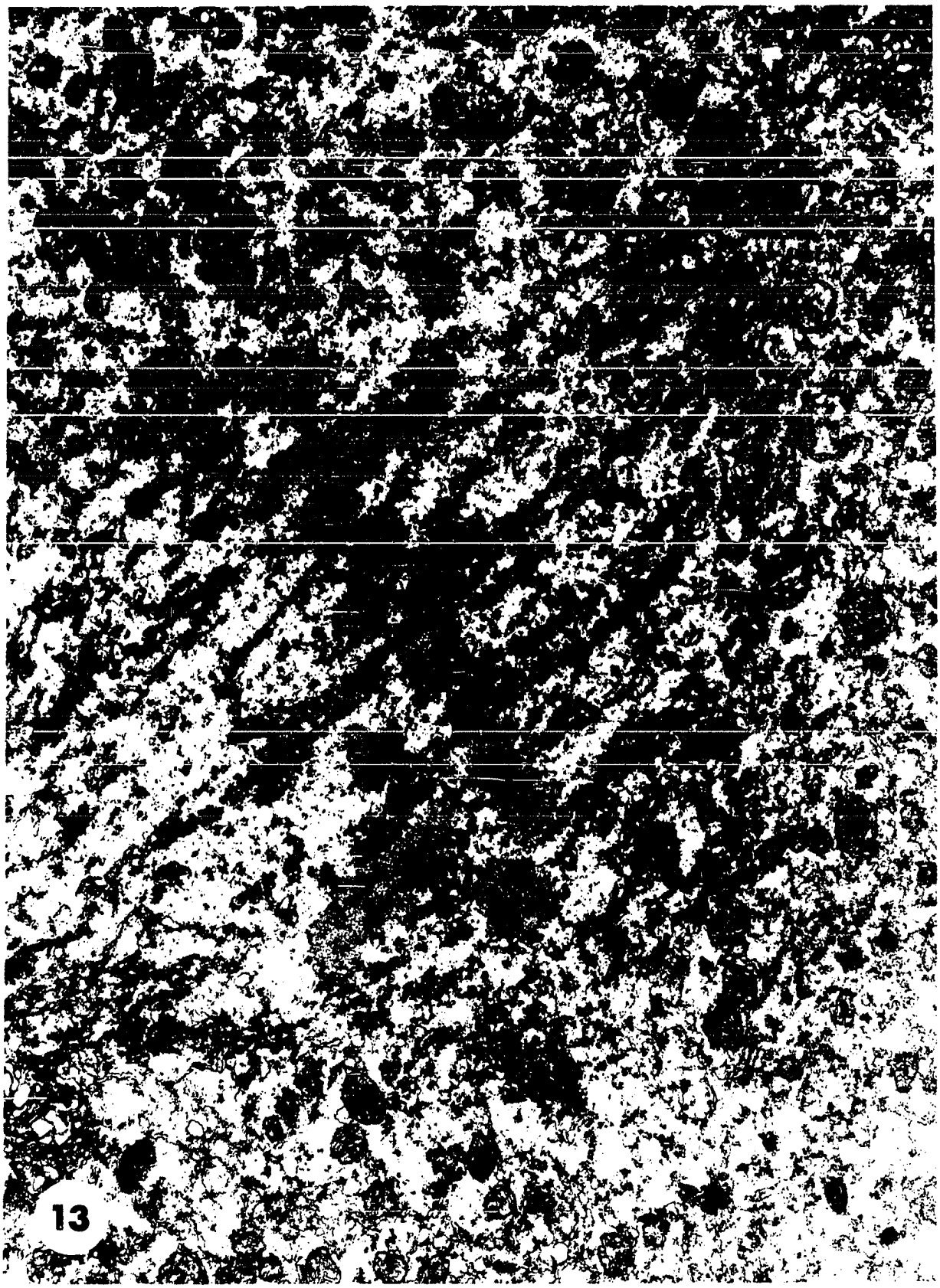

13

Figure 14a. Spindle microtubules in groups end at the chromosomes forming a kinetochore (k). A spindle microtubule can also be seen between chromosomes. Osmium tetroxide with calcium fixation; lead citrate stained. X 15,900

Figure 14b. An interzone (IZ) formed by the separation of daughter chromosomes signals the initiation of anaphase. Spindle microtubules and ribosomes are within the interzone but membranous elements and yolk are absent. Hydroxyadipaldehyde buffered with sodium cacodylate fixation; lead citrate stained. X 9900

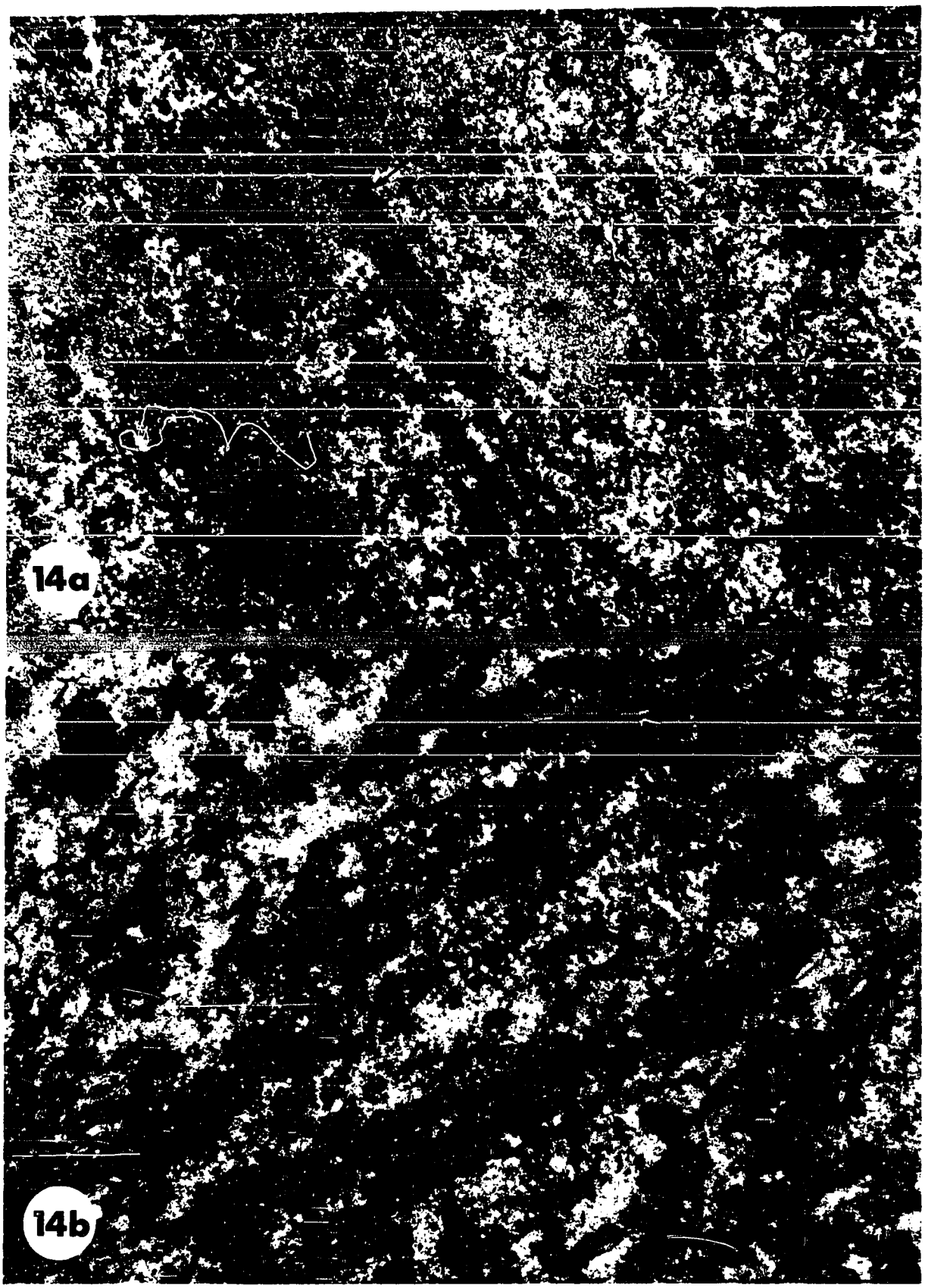

14a
14b

Figure 15. At late anaphase, the chromosomes are at the poles. The interzone is free of membranous elements but contains continuous spindle microtubules and some small yolk particles. Asters are absent and the cytokinetic furrow has not yet appeared. Osmium tetroxide with calcium fixation; lead citrate stained. X 7400

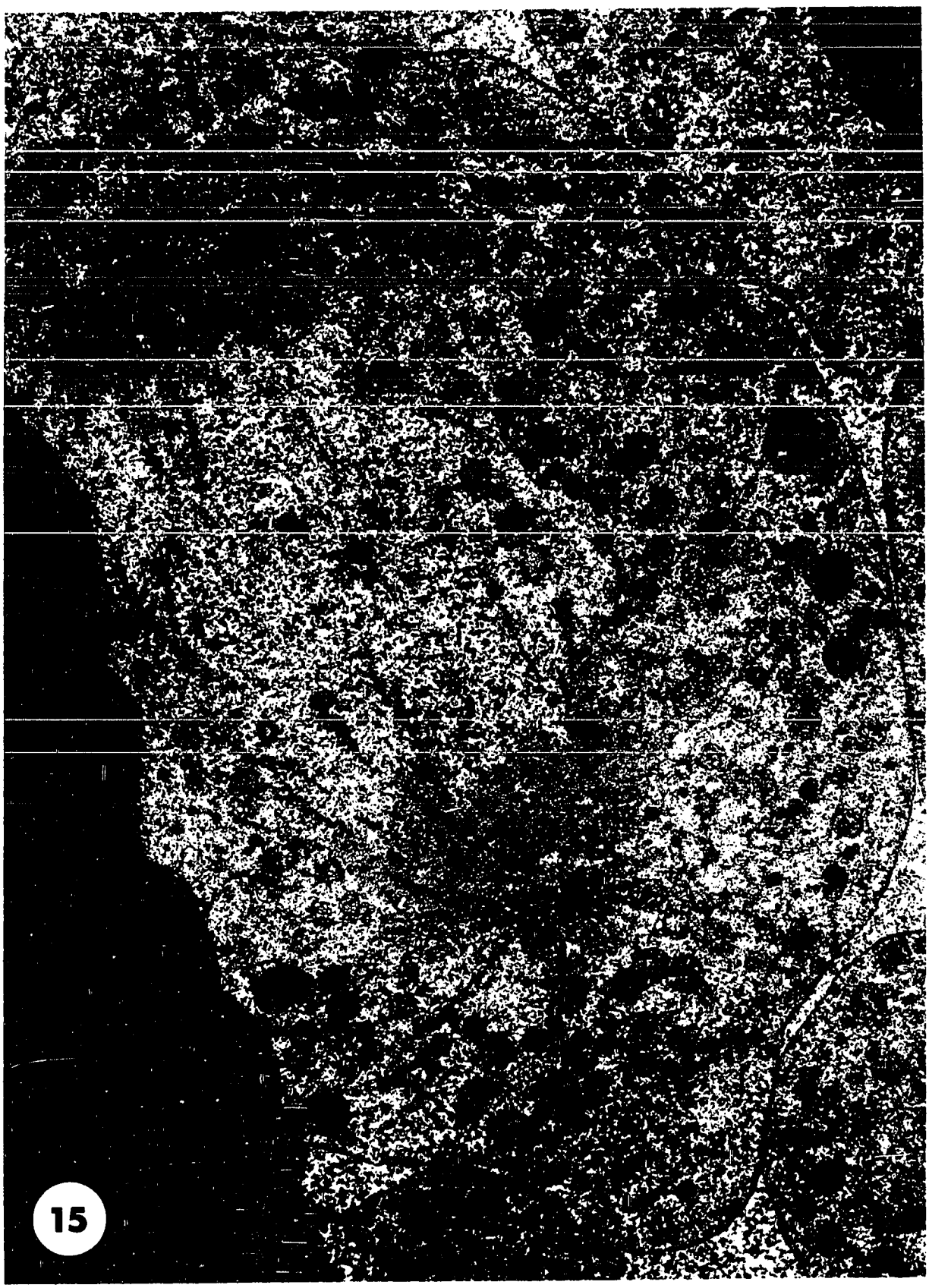

15

Table 3. Pole-to-Pole Distances of Mitotic Figures at Various Developmental Stages

Stage #	Metaphase	Anaphase Without Furrow	Anaphase With Furrow and Telophase
10	15.5 ± .7 u	15.6 ± .4 u	22.9 ± .8 u
12	14.7 ± .5 u	15.5 ± .4 u	20.2 ± .4 u
13	14.8 ± .4 u	15.7 ± .4 u	20u ±
14[a]	11 u (one measurement)	12.5 ± 1.6 u	20.5 ± 2.5 u
Total	15.0 ± .6 u	15.5 ± 1.6 u	21.5 ± 1.6 u

[a] Cell diameter at one-third epiboly stage is much less than cells in other stages. Mitotic index is also lower hindering observation of mitotic figures.

absent from the interzone (Figures 15, and 16a), are found in the remainder of the cytoplasm and against the leading edge of the daughter chromosome sets.

Telophase

Telophase is classically described as the reconstruction of the interphase state. The chromosomes, which had membranous material on their leading edges during anaphase, are now completely surrounded with membranous elements (Figures 16b and 17a). Individual chromosomes or groups of chromosomes are surrounded with membranous material with nuclear pores (nuclear envelope) forming either micronuclei which become interconnected or a single nuclear structure.

In cells of large volume, through early or late high blastula (Figure 18a), the chromosomes are physically separated from each other so that each has an envelope around it. These chromosomal vesicles fuse, enlarge, and form the interphase nucleus often with the result that remnants of nuclear envelope are found within the nucleus (Figure 18a). At the later stage of very late blastula, cell volume is reduced and telophase chromosomes are physically together (Figures 15-17b, and 19a). Although each chromosome does have membranous elements on its poleward surface, the nucleus does not form chromosomal vesicles, that is individual chromosomes surrounded by nuclear envelope. Rather a simple daughter nuclear structure is seen with the light microscope

Figure 16a. Before the appearance of the cytokinetic furrow, the chromosomes are about 15 microns apart. The interzone is free of large particles and appears 'clear'. Hydroxyadipaldehyde buffered with phosphate fixation; lead citrate stained. X 3800

Figure 16b. The chromosomes have membranous elements on their surfaces when the cytokinetic furrow appears. Hydroxyadipaldehyde buffered with sodium cacodylate fixation; lead citrate stained. X 4300

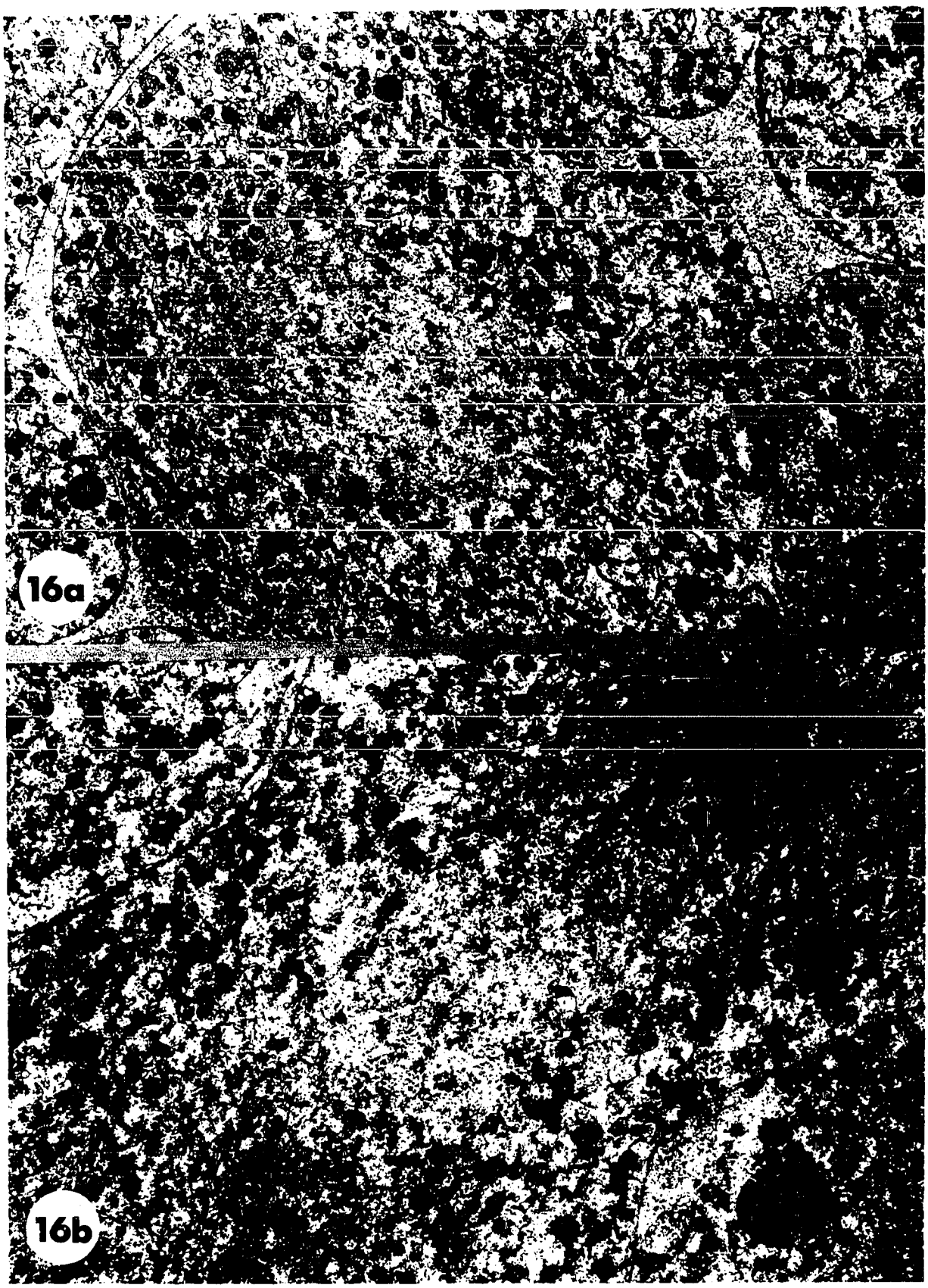

16a
16b

Figure 17a. The interzone disappears as the cytokinetic furrow deepens and the connection between daughter cells becomes smaller. Hydroxyadipaldehyde buffered with phosphate fixation; lead citrate stained. X 3850

Figure 17b. The width of the connection between daughter cells decreases as cytokinesis continues and longitudinal elements suggestive of spindle microtubules appear between the furrow edges. Also in the micrograph, a yolk particle suggested as the last step in yolk fate is seen near the nucleus. Osmium tetroxide with calcium fixation; lead citrate stained. X 11,400

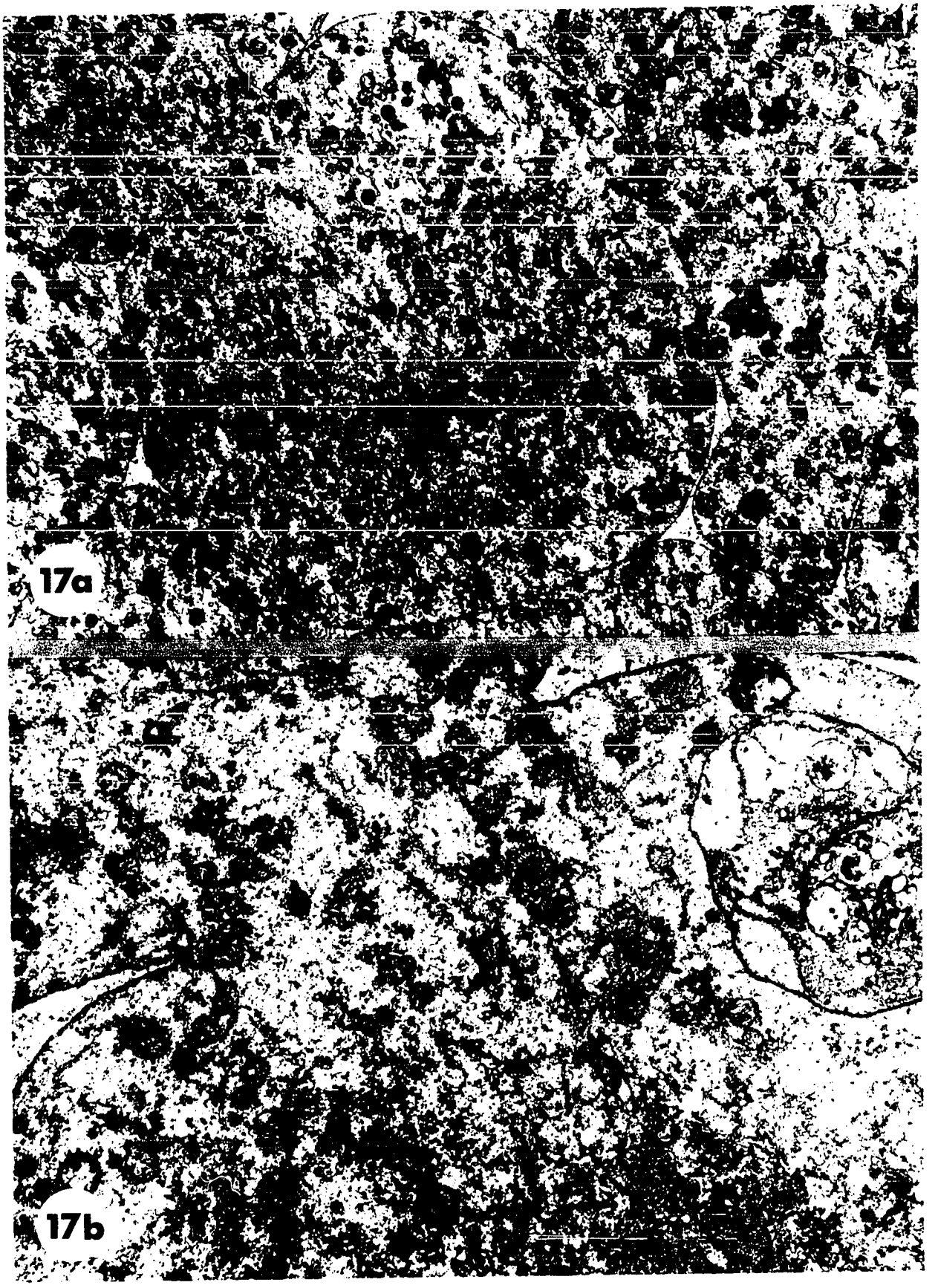
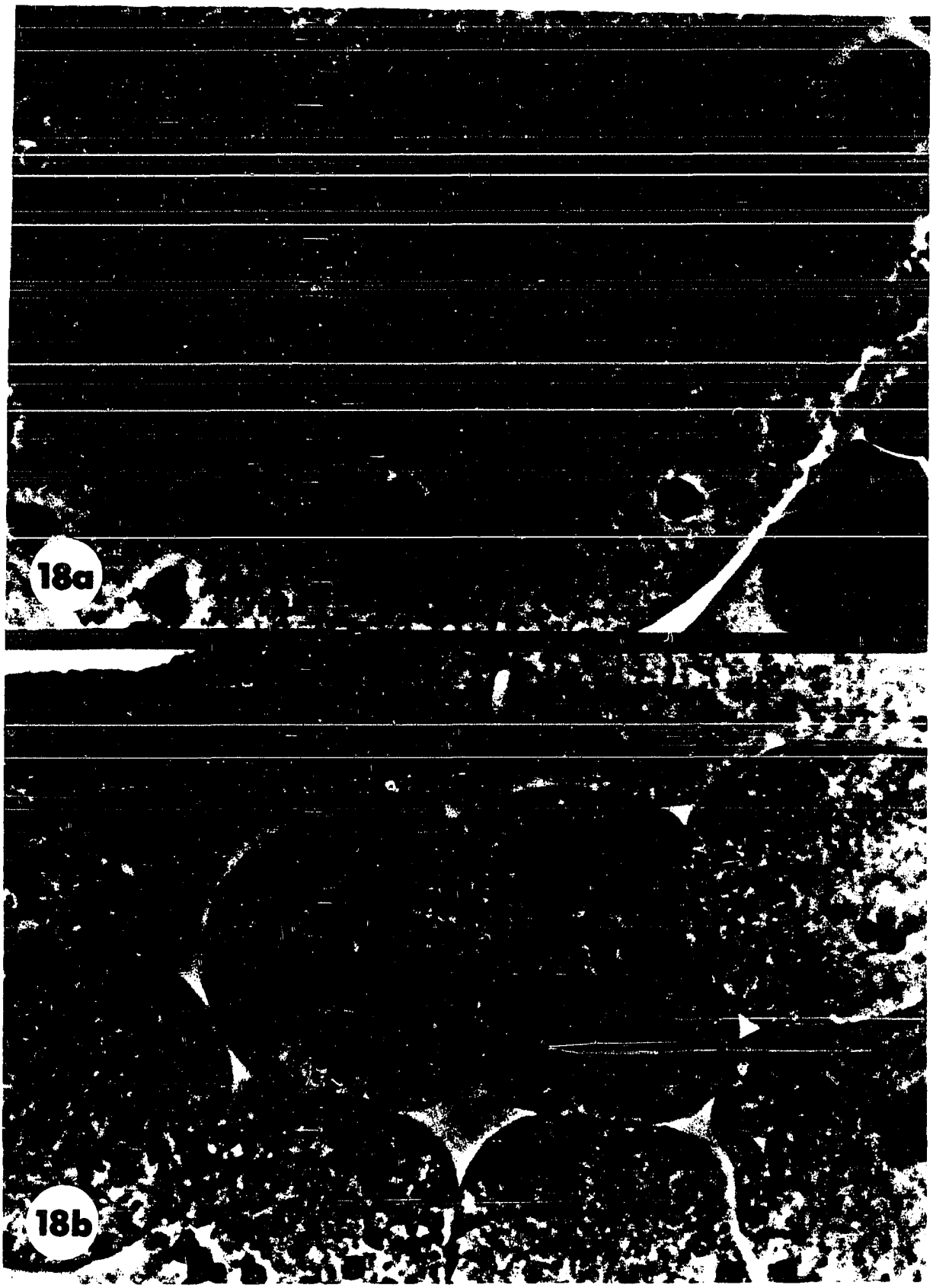

17a
17b

Figure 18a. At early high blastula, fused chromosomal vesicles forming a 'partitioned' nucleus are often seen.

Figure 18b. At very late blastula, cell volume is reduced and chromosomes form a single nuclear structure.

Figures 18a and 18b are X 1960.


18a
18b

(Figure 18b) or closely associated chromosomes surrounded by nuclear envelope are seen with the electron microscope (Figure 19a).

The end of karyokinesis and cytokinesis is summarized in Figure 19b. The edge of an interphase nucleus surrounded by mitochondria can be seen at the left. A midbody (mb), the remnant of the cleavage furrow, has a closely gathered group of microtubules and is the last connection between daughter cells.

Cytokinesis

Although the occurrence of a cytokinetic furrow is common at the one-third epiboly stage in B. rerio (Figures 17a,b and 19b), both cytokinesis by the formation of a furrow and by fusion of a row of vesicles (phragmoplast) has been observed in the same blastula and after the use of more than one fixative. A row of vesicles transversing the entire cleavage plane is the indication of the phragmoplast (Figure 20). Often at the center of the row an area lacking vesicles between daughter cells contains material suggestive of spindle microtubules and the formation of a midbody (mb, Figure 21). The indication of the mitotic phase is given by the nuclear morphology which is similar to a telophase nucleus. There is no indication of fine material on the cytoplasmic side of the cell membrane at the end of the vesicle row (Figures 20 and 21). However, a cytokinetic

Figure 19a. A very late blastula telophase nucleus appears as closely associated, interconnected chromosomal vesicles. Hydroxyadipaldehyde buffered with phosphate fixation; lead citrate stained. X 18,000

Figure 19b. A midbody (mb) with gathered microtubules is the last connection between daughter cells. The nucleus, upper left, has mitochondria along the nuclear envelope characteristic of interphase. Osmium tetroxide with calcium fixation; lead citrate stained. X 11,400

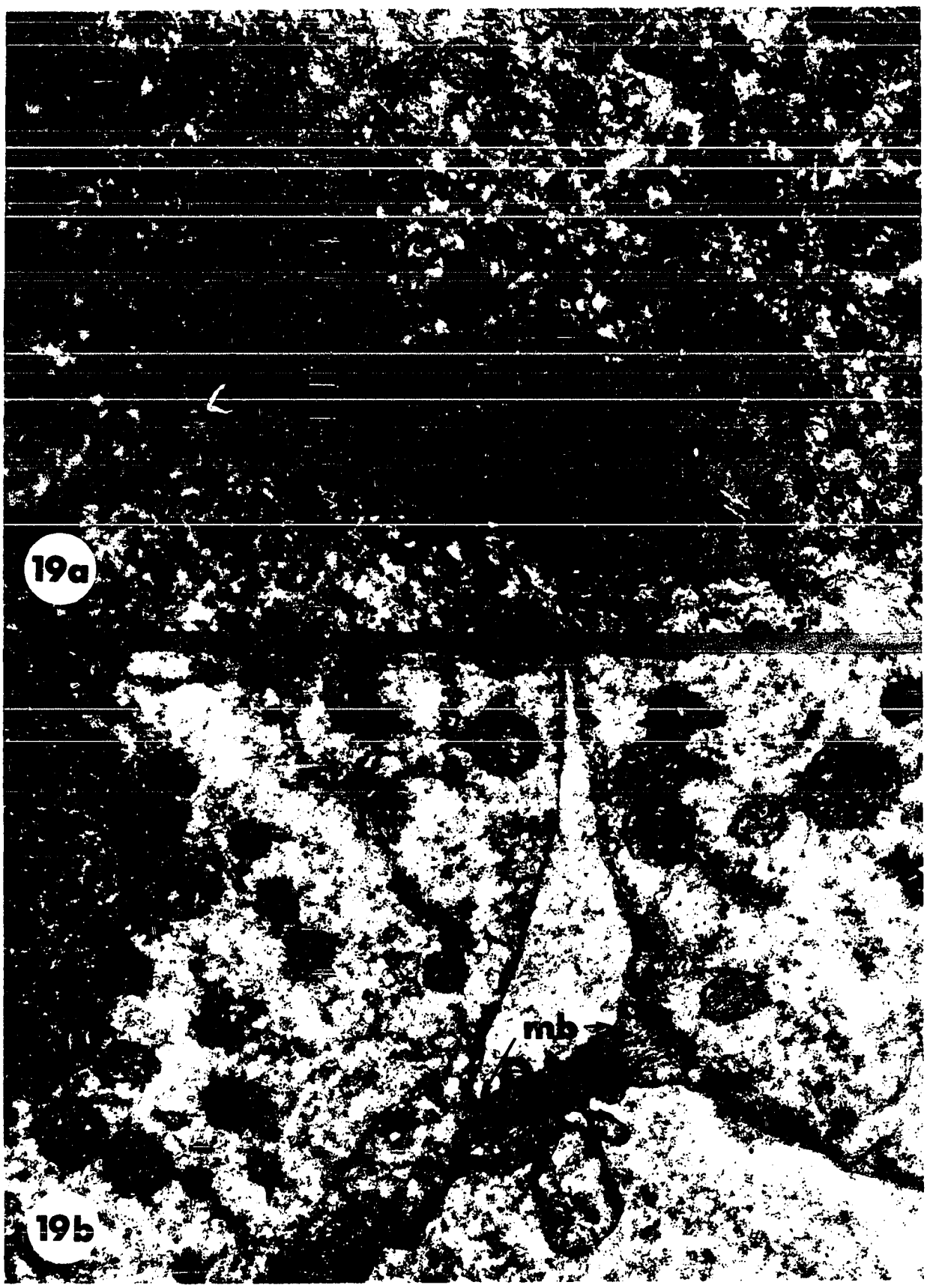

19a
mb
19b

Figure 20. A row of ordered vesicles separates the daughter cells in a late high blastula embryo. Hydroxyadipaldehyde buffered with sodium cacodylate fixation; lead citrate stained. X 13,200

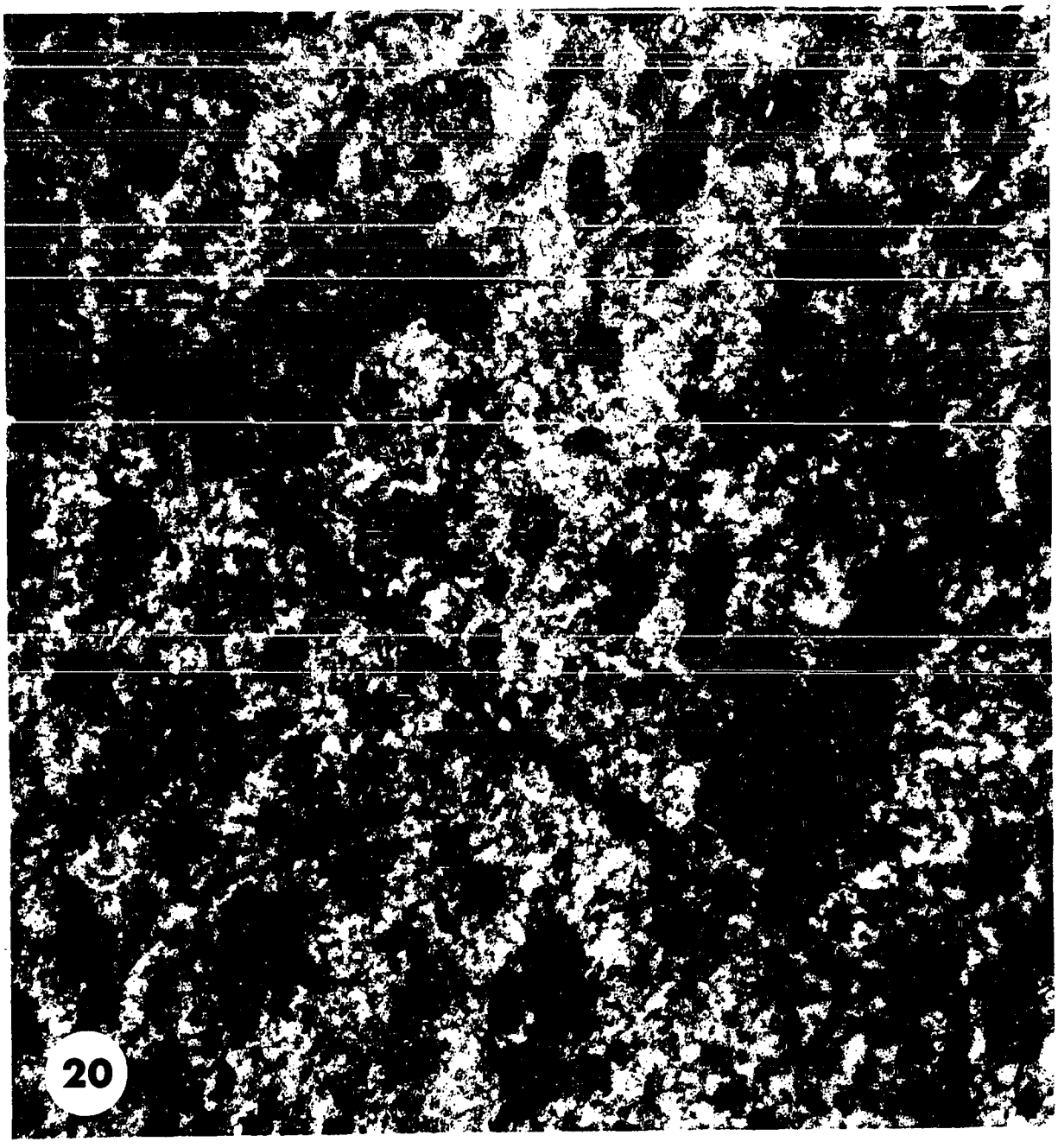

20

Figure 21. A midbody-like structure (mb) is at the center of a row of vesicles separating daughter cells in a late high blastula embryo. A characteristic telophase nucleus in one daughter cell can also be seen. Hydroxyadipaldehyde buffered with sodium cacodylate fixation; lead citrate stained. X 8400


21

furrow almost bisecting two daughter cells is also seen in the same blastula (Figure 22). Even at low magnification (Figure 22), a deposit appears along the edge of the furrow and is not present along the cell membrane that is not in the furrow area (arrows indicate contrasting areas, Figure 22). This fine material is a layer about 60-mu thick and appears as dense fibrillar material (fm) under a cross-sectioned plasma membrane (Figure 23a). The midbody has gathered spindle microtubules in a 'neck' between the daughter cells (Figures 23b-d). The 'neck' of the midbody has accessory membrane projections (ap) with membranous vesicles within the bulbous projections extending into the extracellular space. The center of the midbody 'neck' contains closely packed microtubules which have a very dense band (db).

Still later at very late blastula, both the formation of a vesicle-type cleavage (Figure 24) and the formation of a definitive furrow with fine material along the leading edge can be found (Figure 17a). However, at the later stage of one-third epiboly, only cytokinetic furrows with fine material on the leading edge are observed (Figure 17b). The midbody formed at one-third epiboly (Figure 19b) resembles closely the midbody observed in cells in late high blastula (Figures 23 b-d).

Figure 22. Cytokinetic furrows are also seen cleaving cells in the same late high blastula embryo. The furrow membrane has an inner layer of material which is absent from the remainder of the cell membrane (arrows). Hydroxyadipaldehyde buffered with sodium cacodylate fixation; lead citrate stained. X 8400


22

Figure 23a. A sixty-millimicron layer of fine material (fm) is found along the furrowing cell membrane. Figure 23a is a higher magnification of the furrow in Figure 24. Hydroxyadipaldehyde buffered with sodium cacodylate fixation; lead citrate stained. X 49,000

Figures 23b-d. Serial sections through a midbody of late high blastula cells show a dark band (db) in the center of the midbody 'neck'. From the midbody, bulbous membranous extensions into the intercellular space contain some membranous vesicles. These bulbous extensions may be called accessory projections (ap). Hydroxyadipaldehyde buffered with sodium cacodylate fixation; lead citrate stained. X 17,000

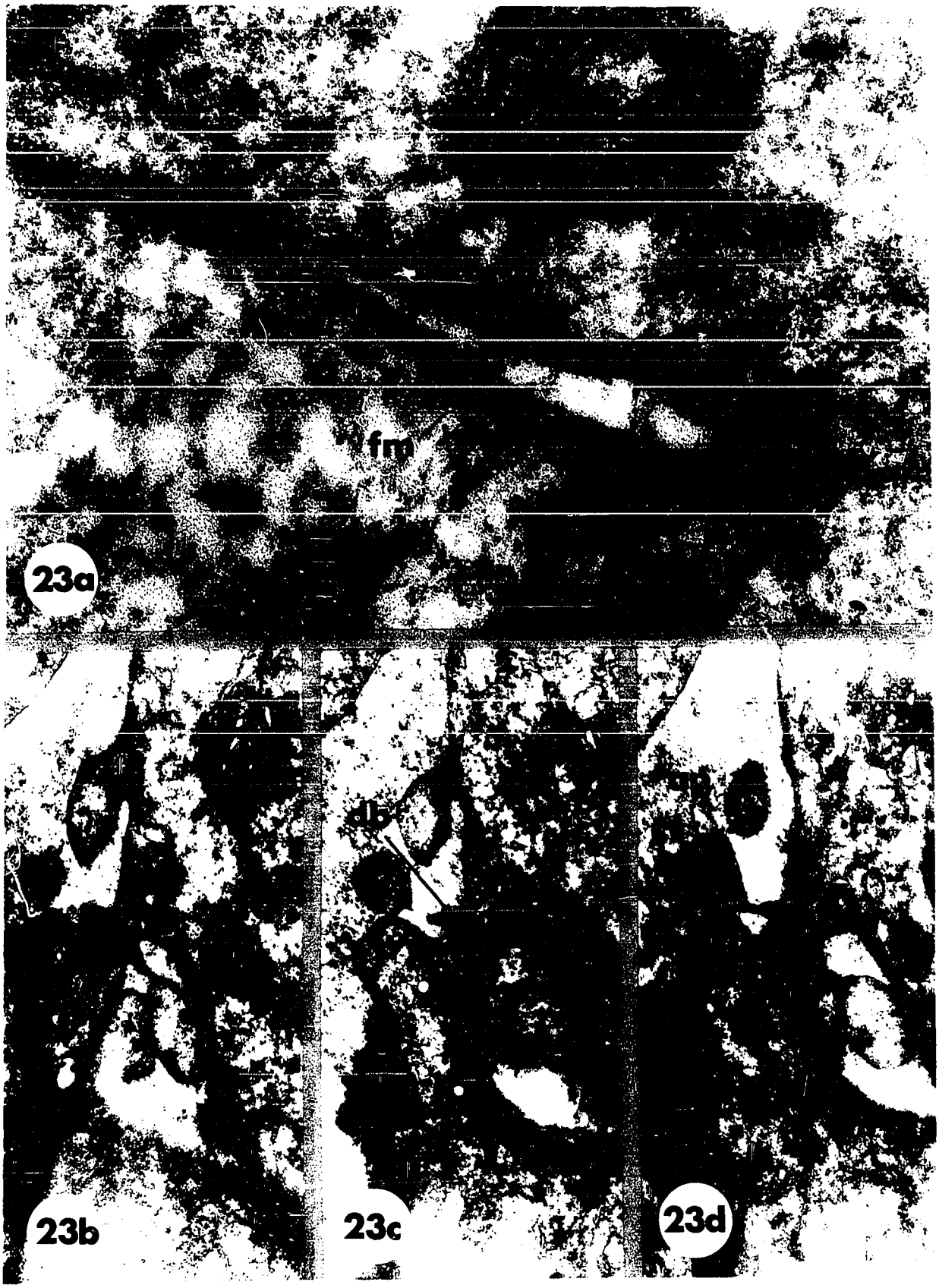
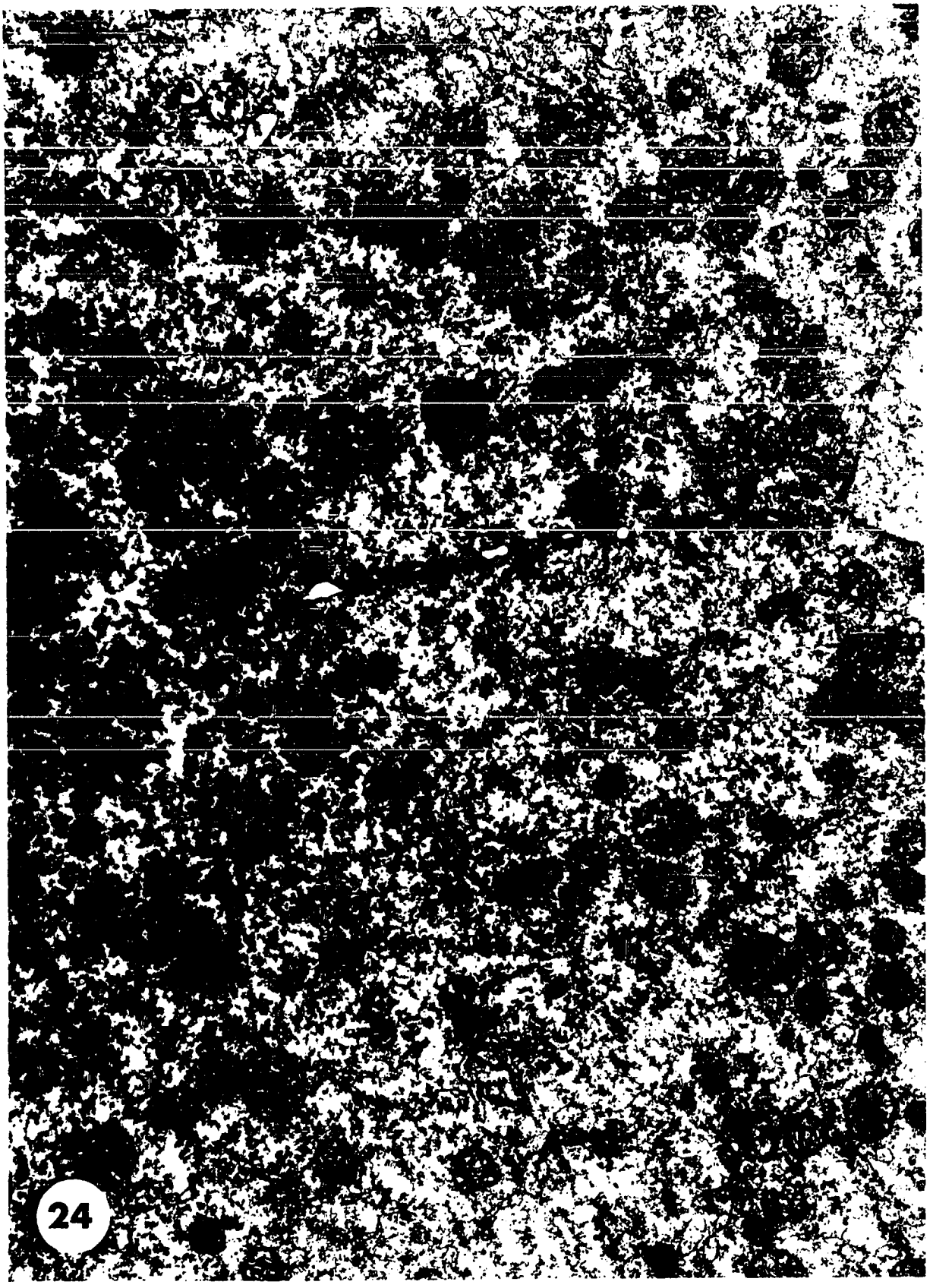

fm
23a
23b
23c
23d

Figure 24. Vesicle-cleavage, an ordered row of vesicles, is seen in a very late blastula embryo although furrow-cleavage does still occur (see Figure 17a). Osmium tetroxide with calcium fixation; lead citrate stained. X 12,200


24

Discussion

Electron microscopic studies of the mitotic portion of the cell cycle have previously been directed largely toward the establishment of the fibrillar nature of the mitotic spindle or the involvement of specific organelles in partially differentiated cells. Prophase events such as the formation of the mitotic apparatus and nuclear envelope dissolution, anaphase spindle elongation and chromosome movements, telophase nucleus reconstruction, and cytokinesis have been difficult to study, and little has been reported. A developing embryo such as B. rerio with its high mitotic index and rapid cell cycle provides a cell population amenable for study of these events. Therefore, the preservation of spindle microtubules, the location of spindle microtubule formation and nuclear envelope dissolution, anaphase 'movements', chromosomal vesicles, and cytokinesis as seen in the developing embryo of B. rerio will be discussed.

Preservation of Spindle Microtubules

The filamentous nature of the mitotic apparatus was first characterized as a circular profile of about 15 mu by Roth et al. (1960) in the mitotic apparatus of amebae and by Harris (1961) in the sea urchin embryo. Both investigators noted the necessity of including divalent cations such as calcium to osmium tetroxide fixatives at alkaline pH's

(pH 7.4-7.6) as also used in this study. The introduction of dialdehyde fixation (Sabatini et al., 1963) permitted the preservation of microtubules of the mitotic spindle at alkaline pH's without divalent cations (Dales, 1963; Roth et al., 1963; and many others). The dialdehyde of choice for preservation of microtubules is considered to be glutaraldehyde, 1,5-pentadial. However, as investigations of microtubules preserved by glutaraldehyde were reported, microtubule diameter varied from 15 to 26 mu from laboratory to laboratory (Dales, 1963; Robbins and Gonatas, 1964; Barnicot and Huxley, 1965).

Hydroxyaldehyde (2-hydroxy-1,6-hexenedial), suggested by Sabatini et al., (1963) as the preferred fixative for subsequent localization of adenosine triphosphatase (ATPase) and other enzymes, was used in an unsuccessful attempt to localize ATPase in the mitotic apparatus of B. rerio. Mitotic spindle microtubules were found well preserved with diameters of 19-21 mu. Fixation of Zebrafish blastomeres with osmium tetroxide with divalent cations resulted in the preservation of spindle microtubules with the same diameters.

Location of Spindle Microtubule Formation and Nuclear Envelope Dissolution

The extremely short cell cycle and mitotic period in B. rerio make study of rapidly occurring events easy since the mitotic index is high. About one-sixth of the mitotic

period is generally considered to be prophase (Mazia, 1961). In B. rerio, Roosen-Runge (1939) reports a prophase period of 18.5% of the mitotic period with interphase time excluded or 16.5% with interphase time included. Events are occurring rapidly if the 15-18 minute time period for the cycle is considered.

From light microscope observations, Roosen-Runge (1939) simply states that chromosomes become visible immediately before breakdown of the 'nuclear membrane', and chromosomes appear at the metaphase plate while still within the nuclear 'membrane'. As observed with the electron microscope in this study, the interphase nucleus is seen as a large cup near the small centrosome. The center of the cup is a cleft remaining from telophase reconstruction of the nucleus. During prophase and prometaphase, spindle microtubules are seen 1) near the centrosome at the lip of the cup or cleft prior to nuclear envelope interruption, 2) in the cleft pointing toward the center of the cup, and 3) within an interrupted (but relatively complete) nuclear envelope. Toward the end of prometaphase, large nuclear envelope fragments still surround the formed mitotic apparatus. The shape of the nuclear envelope progresses during pro-prometaphase from 1) a sphere with a single cleft, to 2) an ellipsoid with two clefts, and then to 3) an interrupted ellipsoidal shape with spindle microtubules within the volume defined by the nuclear envelope

fragments. During the period when the shape of nuclear envelope is being altered, mitochondria are found 1) in high numbers along the spherical interphase nucleus, 2) around the ellipsoidal nucleus (with its condensed chromosomes), in lower number, to 3) none within the formed mitotic apparatus as the nuclear envelope completes dissolution.

The events outlined above agree with the early observations of Roosen-Runge (1939) and some of the observations agree with other investigations of mitosis in centriole-containing cells. The occurrence of microtubules at or near the centrioles (centrosomes) prior to interruption of the nuclear envelope has also been documented by Harris and Mazia (1962) in sea urchin embryos, by Robbins and Gonatas (1964) in HeLa cells, and by Barnicot and Huxley (1965) in tissue culture cells of the newt heart and human fibroblasts. In general, these studies agree that spindle microtubules are first seen before the nuclear envelope is interrupted and always in close proximity to the centrioles (centrosomes). Harris and Mazia (1962), Robbins and Gonatas (1964), and this study agree that microtubules are within the nuclear cleft. The direction of spindle microtubules toward the nucleus (and the condensed chromosomes) suggests that the consideration of the centrioles as a fiber organizing center (Schrader, 1953; Mazia, 1961) is most probable. Jokelainen (1965) in his study of kinetochore differentiation in rat embryonic

cells reports that spindle microtubules attach to the kinetochores rather than microtubules originating from the kinetochores. Therefore, it may be concluded that the formation of the mitotic apparatus originates at the centrosome and proceeds toward the condensed chromosomes.

The occurence of mitochondria around the interphase nucleus may be only a secondary result of telophase nucleus expansion. From a compact telophase condition, the chromosomes during their expansion may push the nuclear envelope outward against the nearby mitochondria. A change in the nucleus shape, due for example to chromosome condensation during prophase, would result in mitochondria being separated from the nuclear envelope. This is in fact what has been observed in this study. Such a hypothesis would, however, negate the hypothesis of Barer et al. (1958) that the mitochondria are involved as producers of enzymes for the dissolution of the nuclear envelope. It is most probable that the mitochondria are not involved in production of enzymes for nuclear envelope dissolution because the nuclear envelope is intact after the mitochondria are removed.

The nuclear envelope retains most of its integrity during the formation of the mitotic apparatus. As seen by Roosen-Runge (1939) and in this study, the nuclear envelope (or rather large fragments of the nuclear envelope) remain around the condensed chromosomes. From the first observation

of spindle microtubules within the nuclear cleft to prometaphase when the chromosomes are almost aligned upon the metaphase plate, the spindle microtubules appear between nuclear envelope fragments from the centrosome to the condensed chromosomes. Jokelainen's (1965) observations and suggestion of a mechanism of spindle to kinetochore attachment may be aided by the observations of this study. The restriction of mitochondria and yolk or particles of similar size from a mitotic apparatus, as is generally seen (Mazia, 1961), would be most reasonable by the persistence of the nuclear envelope fragments until spindle-kinetochore attachment is completed.

In summary, the site of spindle microtubule formation appears to at the centrioles (centrosomes) before the nuclear envelope is interrupted. Nuclear envelope fragments persist until late prometaphase, when the mitotic spindle is formed, and restrict mitochondria, yolk, and particles of a similar size from the formed spindle.

Anaphase 'Movements'

During the developmental period of late high blastula to one-third epiboly, metaphase pole-to-pole distance is a relatively constant 15 u. Polar separation remains constant until the daughter chromosome sets have reached their respective poles. Only after the chromosomes have reached the poles and the first indications of a cytokinetic furrow are evident is polar separation found at 21 u. During both

periods of anaphase chromosome movement, interzonal microtubules are present.

Dettlaff (1963) reports that in the first mitotic period in either sturgeon or trout eggs there is a simultaneous chromosomal movement to the poles and separation of the poles. In reviewing the voluminous literature of anaphase movements, Mazia (1961, p. 280) concludes that there are two anaphase "movements: 1) an elongation of the central spindle, describable as a pushing apart of the poles, and 2) a movement of the chromosomes toward the poles. The relative contributions of these in the separation of sister chromosomes varies from one type of cell to another as do their time relations."

The movements of chromosomes during anaphase as observed in B. rerio are similar to other cells. In addition, this study shows that both movements occur during anaphase in this species. The initial movement is the daughter chromosomal movement of about 7 u from the metaphase plate and then the poles move apart from 15 u separation to about 21 u.

Chromosomal Vesicles

Chromosomal vesicles, karyomeres, or micronuclei, i.e., individual chromosomes or groups of chromosomes surrounded by nuclear envelope, have been noted as a significant characteristic of early cleavage cells or early embryonic cells in several species (trout and sturgeon, Dettlaff, 1963; sea urchins, Harris, 1961; molluscs, Raven, 1958; and Zebrafish,

Roosen-Runge, 1939). They have also been noted in plant cells with divergent mitosis (Mazia, 1961). Chemical treatments, such as a short exposure of tissue culture cells to colchicine (Starr, 1963), also result in the formation of micronuclei by permitting the dispersal of chromosomes. Starr (1963) has used the formation of micronuclei as a test system for the localization of viruses to specific chromosomes, but unsuccessfully.

Only in cells with large volume, where chromosomes are well separated, or in cells where chromosomes are separated artificially by disruption of the mitotic apparatus do chromosomal vesicles or micronuclei form. As cell volume is reduced by repeated mitosis and chromosomes are restricted within a smaller volume during anaphase movement, chromosomes tend to act as a single chromosome mass rather than as individual entities.

It can be concluded that the formation of chromosomal vesicles, karyomeres, or micronuclei in cells of large volume is the result of physical separation of chromosomes during anaphase movement to the poles. Likewise, the formation of a single telophase nucleus in a cell of small volume would be the result of chromosomes acting as a single entity during the anaphase movement to a pole, except when chromosomes are dispersed by artificial means. Therefore, nuclear reconstruction whether as chromosomal vesicles or a single intact

nucleus appears to be dependent not upon nuclear control but rather upon physical restrictions of cell volume.

Cytokinesis

Rappaport (1966) with an excellent experimental design arrives at the conclusion that the studies of Mitchison (1953) and others on the cleavage of animal cells that occurs not by a furrow may only be characteristic of a few species. Cytokinesis in botanical material has been well documented as the fusion of a row of vesicles (e.g., Whaley et al., 1966). Animal cells have been characterized as having mainly furrow cytokinesis (Robbins and Gonatas, 1964; Buck and Krishan, 1965); however, vesicle cleavage has also been reported (Buck and Tisdale, 1962; Humphreys, 1964). Rappaport's conclusions (1966) state that either cleavage-type is mutually exclusive.

Zotin (1964) from observations of the first cleavages of large-celled teleost eggs reports that vesicle-cleavage is visible with the light microscope. Mitchison's study (1953) suggests the occurrence of vesicle-cleavage in some sea urchin eggs. During this study, early developmental stages such as late high blastula or very late blastula were characterized by the occurrence of both vesicle and furrow cleavage. Only at later developmental stages such as one-third epiboly were cleavage furrows alone found.

Vesicle cleavage has been characterized as an ordered row of vesicles that transverse the cell and ultimately fuse,

although fusion may not necessarily occur (Humphreys, 1964). In general, vesicle cleavage occurs in cells with large volumes (Selman and Waddington, 1955; Zotin, 1964). Furrow cleavage is characterized initially by a slight constriction of the cell membrane with a distinct intracellular layer of fine material along the furrow area. The furrow edges are seen closer together until finally the interzonal microtubules are gathered to form a midbody. A simple connection remains between cells until it is assumed broken. The furrow-type cleavage has been described most frequently in animal cells which are usually smaller than the first cleavage cells of teleost or sea urchin eggs. Furrows have even been found in the minute cells of the blue-green algae (Pankratz and Bowen, 1963).

It may be concluded from the observation of both vesicle- and furrow-cleavage in the same blastula of B. rerio that the two cleavage types are not mutually exclusive as suggested by Rappaport (1966). The diminution of cell volume (Marrable, 1965) and the concomitant disappearance of vesicle cleavage in B. rerio suggests strongly that the method of cleavage used in animal cells is largely dependent upon cell volume. The mitotic apparatus has often been considered necessary for the initiation and continuation of cytokinesis (Mazia, 1961).

The occurrence of the cleavage furrow with asters absent

or the mitotic apparatus removed (Hiramoto, 1956, 1965) substantiates Wolpert's conclusion (1960) that the astral growth theory of Gray (1924) is inadequate to explain the entire phenomenon of cytokinesis. The spindle elongation theory, which also requires the presence of asters (K. Dan, 1943b; J. C. Dan, 1948) to explain initiation of the cleavage furrow, is also erroneous. More suggestive is the hypothesis of Marsland and Landau (1954) of a 'cortical' contraction. Buck and Krishan (1965) have shown that a cell anchored by desmosomes, as in amphibian epithelial cells that they studied, will still form a cleavage furrow but without breaking the desmosomes. Therefore, the cleavage furrow area must be a site of membrane growth or stretching of the plasma membrane. Kuno-Kojima (1957) demonstrates, in the cleavage furrow area in sea urchin eggs (Hemicentrotus pulcherrimus), 'regional differentiation' that is 'stiffer' than the remainder of the cell membrane. Krishan and Buck (1965) report a dense material on the cytoplasmic side of the cell membrane of incompletely cleaved, insect spermatocytes. Mammalian cells, rat ameloblasts, and human mammary carcinoma also show dense staining of the cleavage furrow with tannic acid-phospho-molybdic acid-amido black technique (Kallenbach, 1964) but not in the rat thymus or testis. Kimoto (1964) also reports staining of furrow areas by 'connective tissue' stains such

as Mallory's method. Richmond[1] reports an increase in sulfhydryl-group staining that is in the area of the furrow in B. rerio and is similar to the localizations of Kallenbach and Kimoto. With the electron microscope, several investigators have shown dense material along the furrow during cleavage (Psammechinus miliaris, Mercer and Wolpert, 1958; Weinstein and Hebert, 1964; HeLa cells, Robbins and Gonatas, 1964; chick, Allenspach and Roth, paper in preparation).

Though electron micrographs are static portrayals of cell function, the material along the cell membrane of cleavage furrows, as even found in B. rerio, is similar in appearance to the cytoplasmic filaments found by Cloney (1966). He suggests that the cytoplasmic filaments are the active contraction material which permits an ascidian embryo to contract and absorb its entire tail into the main body by contraction of epidermal cells. That such similar fibrillar material is found in a contraction system as the ascidian epidermal cell is possibly coincidental, but it suggests that either membrane growth or an active contraction of the cell membrane at the furrow site are significant for furrow cleavage. The dense fibrillar material along the cleavage furrow may either be contractile fibrils, similar to fibrillar

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[1] Richmond, Paul, Minneapolis, Minnesota. Observations of B. rerio cytokinesis. Private Communication. 1966.

material seen by Cloney (1966), or material to be added to the cell membrane at the cleavage furrow, respectively. Until the localization of membrane growth can be detected by isotopic labeling techniques, as may be expected by the present work of Mitchison and Cummins (1966), the question of the mechanism of cleavage furrowing may be narrowed to two possibilities: either active contraction of the furrow area or insertion of intact membranous materials. Nevertheless, this study of B. rerio cytokinesis has shown that both types of cytokinesis may occur in a single animal cell population and may be found at the same time in a blastula.

EXPERIMENTAL TREATMENTS

In an effort to disrupt the early developmental processes, eggs were exposed to colchicine (the classic spindle disruptor, Eigsti and Dustin, 1955) or vincristine sulfate (a 'carcinostatic' agent, George et al., 1965) or deuterium oxide (Gross and Spindel, 1960a,b). Colchicine, though arresting mitosis in the blastula of Fundulus eggs, still permits cell migration (epiboly) and closure of the blastopore (Kessel, 1960). Vincristine sulfate, successful in the treatment (arrest) of mitotically active carcinomas, was used as a 'mitotic' inhibitor that is similar to colchicine (George et al., 1965). Deuterium oxide at low concentrations such as 45-50% slows mitosis and development and thus permits the detailed observation of developmental processes.

Observations

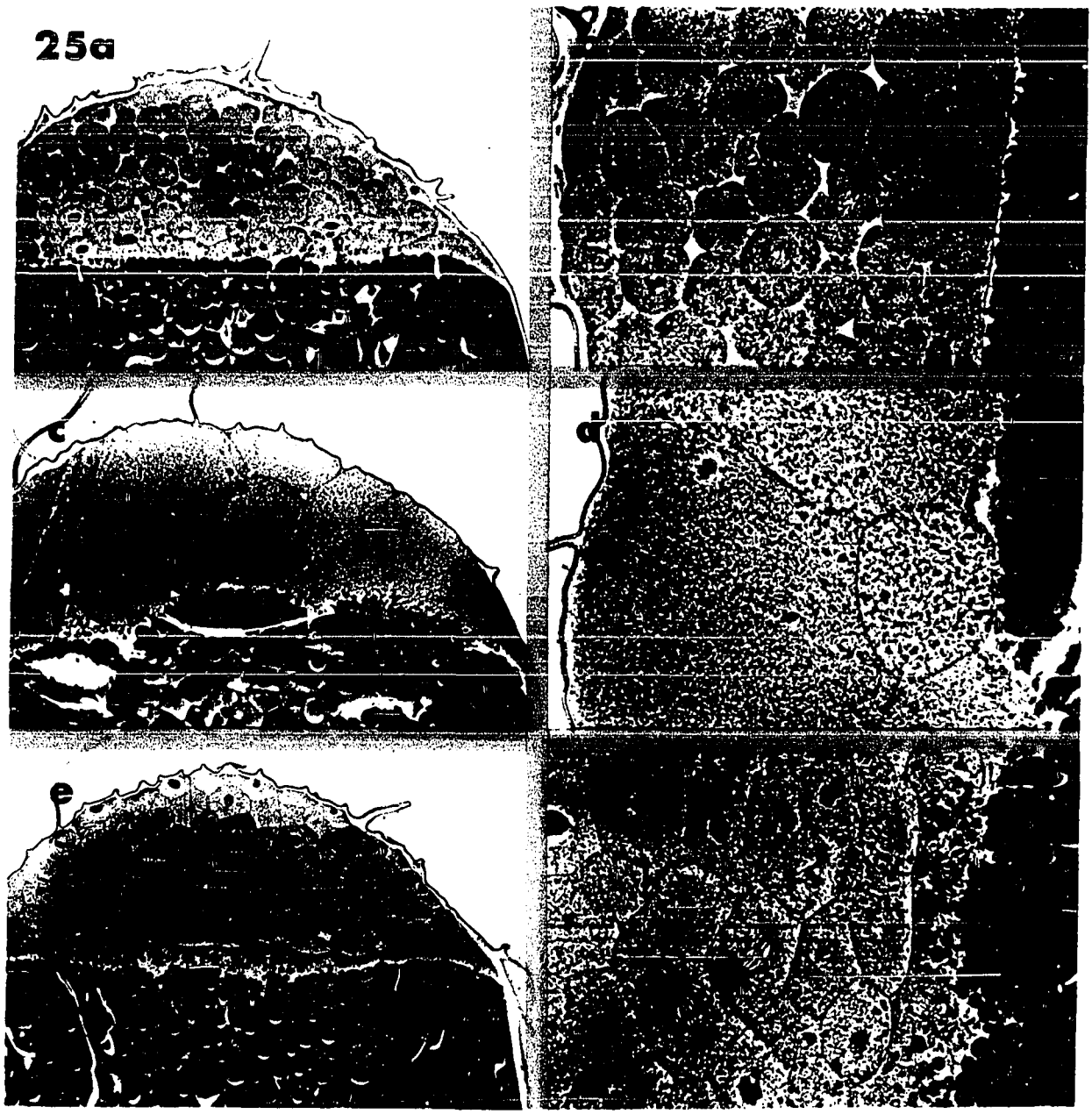
The experimental design and parameters are summarized in Table 2. Eggs at the 8-cell stage were exposed to either 10^{-3} molar colchicine or 49.5% deuterium oxide. In a period of three hours, the control embryos progressed to the flat very late blastula (Figures 25a,b). The gross morphology of the control embryos is indistinguishable from other material at the same stage used in this study (Figures 1e, f, and 2a, b).

Figures 25a and 25b. A flat to very late blastula embryo is developed from the eight-cell stage after three hours. Note the yolk particles in cells near the periblast. This embryo is a control for embryos shown in Figures 25c-f.

Figures 25c and 25d. An eight-cell embryo exposed to colchicine for three hours does not advance developmentally. Yolk particles are infrequent and smaller in size.

Figures 25e and 25f. Embryos exposed to deuterium oxide for three hours advance to late high to flat blastula stages when cell size is compared to normally developed eggs. Intercellular spaces are absent and the periblast is enlarged. Mitotic figures have prominent aster-like structures.

Figures 25a, c, and e are X 150; Figures 25b, d, and f are X 370.


25a

Colchicine exposure results in immediate mitotic arrest (Figures 25c and d). Some yolk particles are seen within the cells but nuclei or chromosomes cannot be seen. The yolk particles are fewer and smaller than those seen in an early high blastula embryo. The periblast is greatly enlarged and small yolk particles are near the yolk mass. Low magnification electron micrographs show large expanses of cytoplasm but no chromosomes or nuclei are readily seen in large expanses of cytoplasm (Figure 26). Some flattened membranous elements are present, but the cytoplasm is as expected for an 8-cell embryo. The cell membrane has a smooth profile and numerous intercellular foldings.

After three-hours exposure to deuterium oxide, the gross morphology of the blastula is similar to that at flat or very late blastula, but the cells have a diameter like that of late high or flat blastula embryos (Compare Figures 25e and f to the normal cell size in Figures 1c-f). Intracellular spaces are absent (Figures 25f and 27). Many mitotic figures with prominent poles are present. The quantity of yolk particles in cells near the periblast appears the same as yolk particles in the control. The periblast is enlarged with more small yolk particles in the periblast than is seen in the control embryos (Compare Figures 25f to control Figure 25b).

Six hours after initial exposure to experimental milieu, the control embryos (Figures 28a, b) are at one-third epiboly

Figure 26. The cytoplasm of the colchicine-exposed eight-cell embryo contains scattered mitochondria, some yolk particles and flattened membranous elements characteristic of large undifferentiated cells. Osmium tetroxide with calcium fixation; lead citrate stained. X 3600

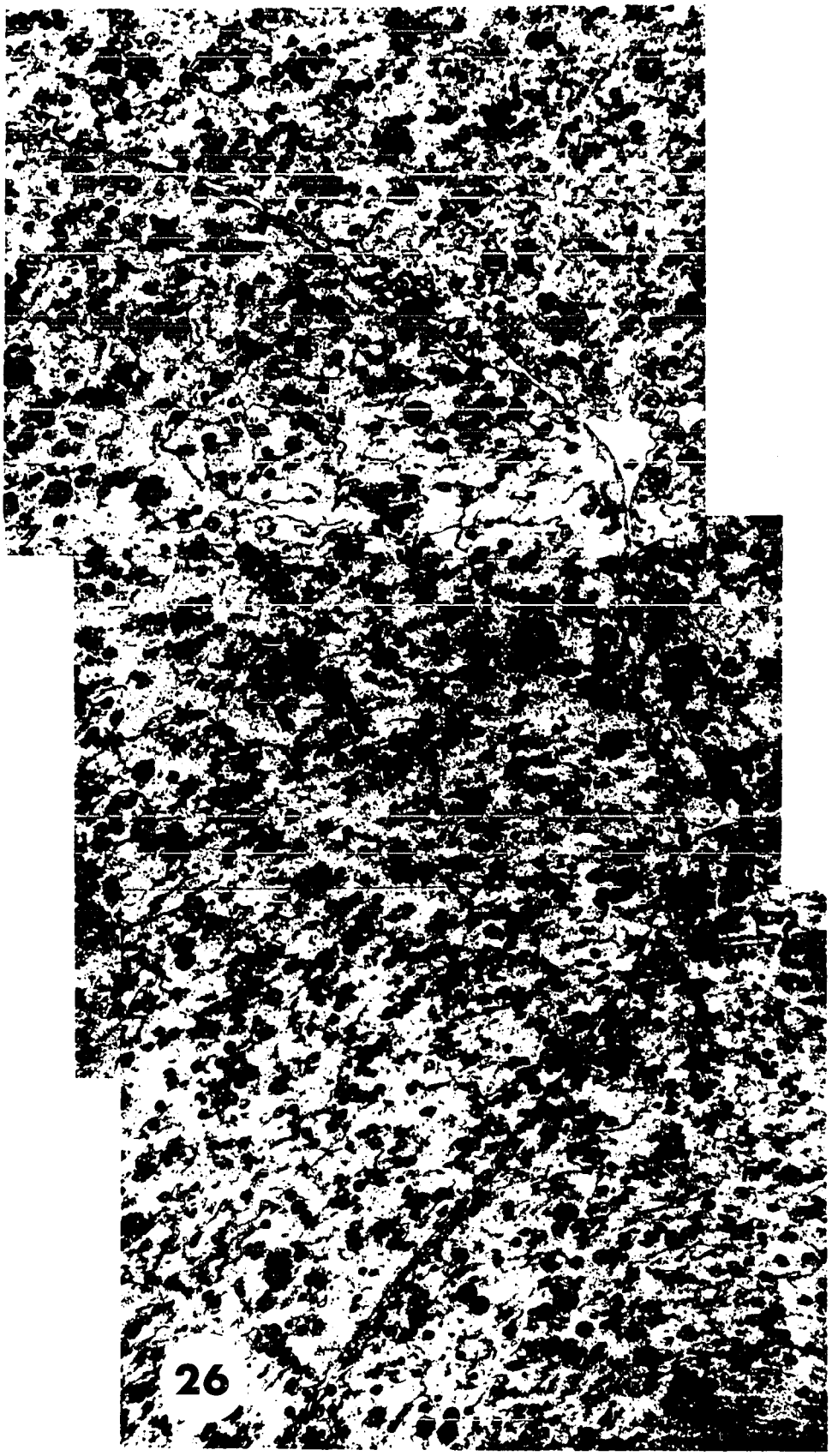
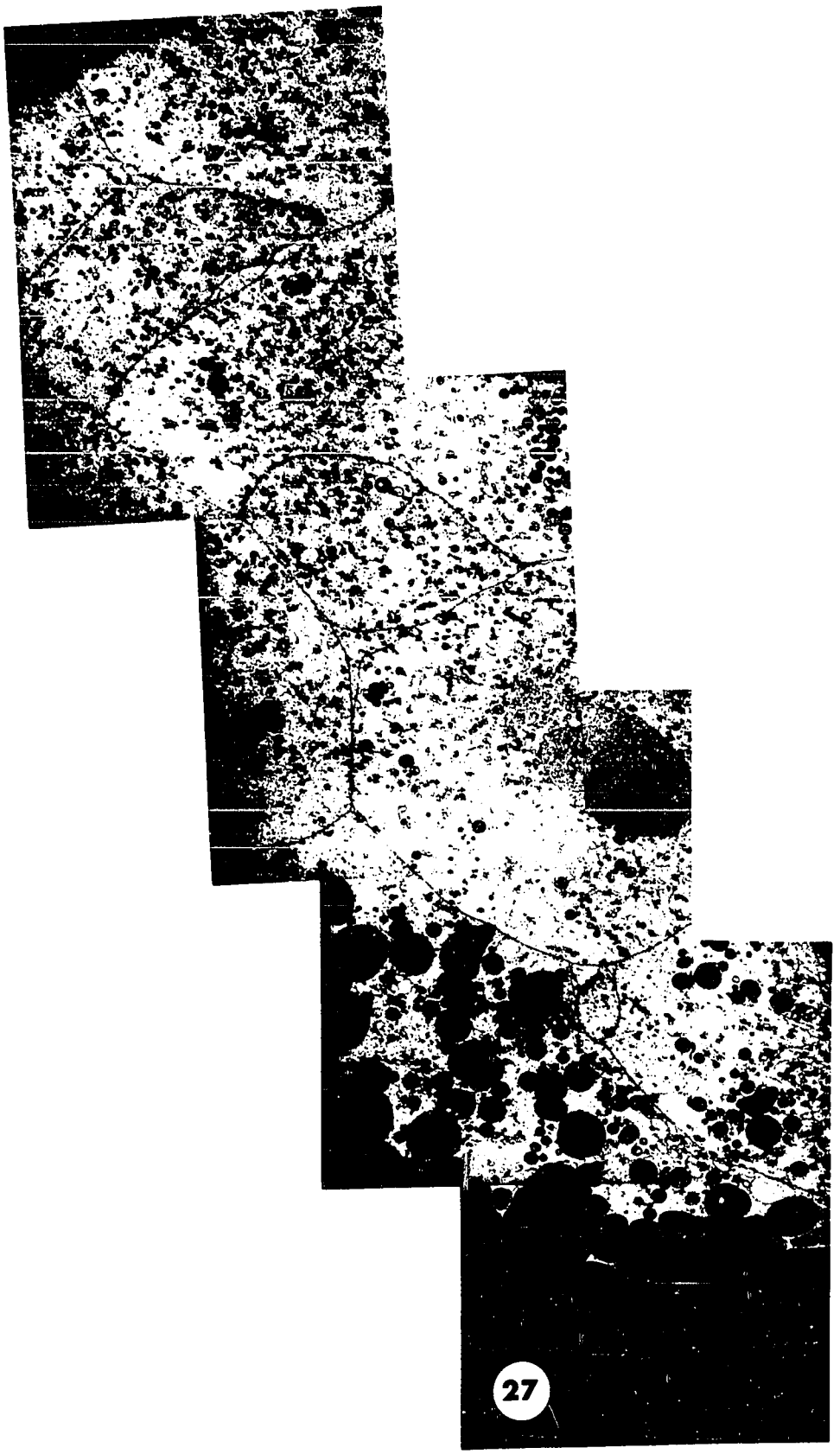

26

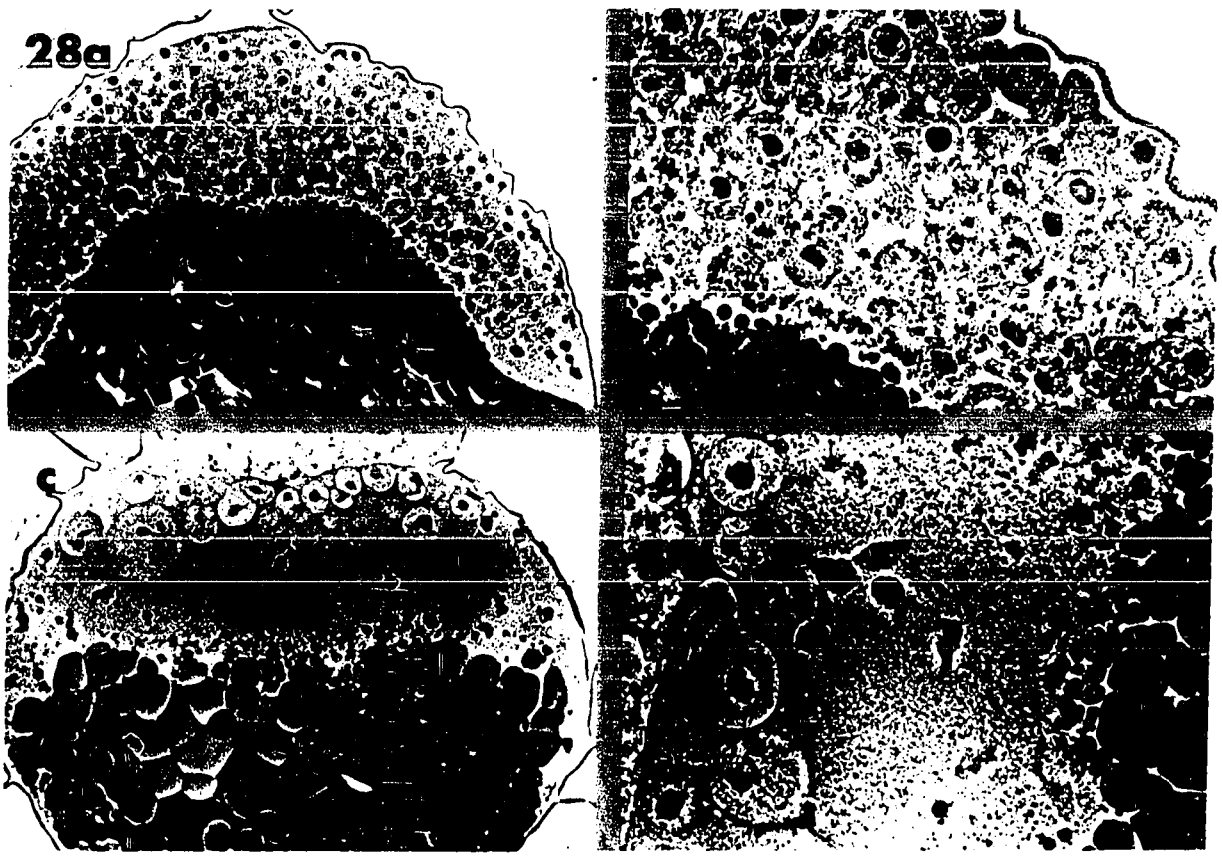
Figure 27. Intercellular spaces are absent in embryos exposed to 49.5% deuterium oxide for a three hour period. The periblast contains some small yolk particles. Osmium tetroxide with calcium fixation; lead citrate stained. X 2050


27

Figures 28a and 28b. The control embryo for six-hour exposure to deuterium oxide proceeds to the one-third epiboly stage.

Figures 28c and 28d. A tangential section of an embryo exposed to deuterium oxide for six hours shows broken cell membranes (arrows, Figure 28d) and many nuclei in the periblast.

Figures 28a and c are X 150; Figures 28b and d are X 370.


28a

stage (Compare to Figure 2f); the colchicine exposed embryos did not advance morphologically, and deuterium oxide exposed embryos (Figures 28c, d) showed cytolysis (breakdown of the cell membrane). The tangential section of deuterium oxide exposed cells (Figures 28c and 28d) shows numerous periblast nuclei and broken cell membranes (Figure 28d, arrows). Deuterium oxide exposure was terminated because of the disintegration of the embryos.

As the controls continued to closure, twelve hours after initial exposure of the experimental embryos, the colchicine exposed embryos remained unchanged. There was no indication of cell movement or epiboly.

Other eggs were initially exposed at a later developmental stage, early high blastula, to either colchicine or deuterium oxide for a period of four hours. During the period of exposure, the control eggs progressed to the very late blastula stage (Figures 29a, b).

Colchicine-exposed blastulae (Figures 29c, d) have cells of varying diameters, and mitotic figures are absent. The periblast has many small yolk particles. The cell size variance is evident in the montage of electron micrographs (Figure 30) which can be compared to a similarly staged embryo (Figure 5). Yolk particles are absent in the larger volume cells. Both intact nuclei (Figure 31a) and free chromosomes (Figure 31b) are found throughout the blastula.

Figures 29a and 29b. The control embryo for embryos exposed to colchicine or deuterium oxide at early high blastula is at the very late blastula stage after four hours.

Figures 29c and 29d. A colchicine-exposed embryo has a blastoderm of varying cell sizes. Yolk particles are absent in the large cells near the free surface of the embryo.

Figures 29e and 29f. Deuterium oxide-exposed embryos have progressed to the late high to flat blastula stage when cell diameters are compared to normally developed embryos. Yolk in cells away from the periblast is diminished.

Figures 29a, c, and e are X 150; Figures 29b, d, and f are X 370.

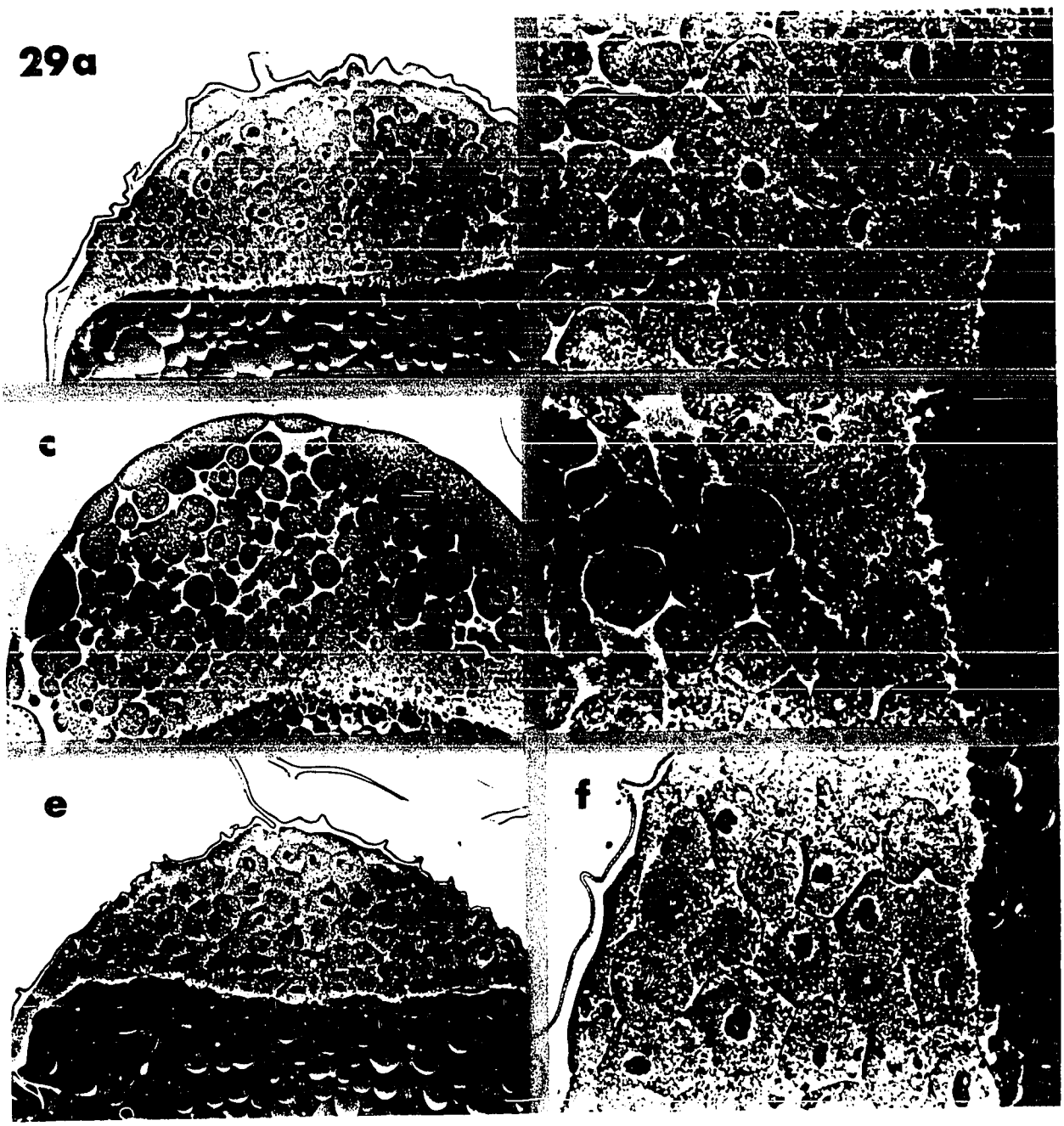

29a
c
e
f

Figure 30. A survey of an embryo at early high blastula exposed to colchicine shows the various cell sizes. Small cells near the periblast contain yolk and occassionally a nucleus. Other cells, toward the free surface of the embryo, have chromosomes scattered throughout the cytoplasm. Osmium tetroxide with calcium fixation; lead citrate stained. X 840

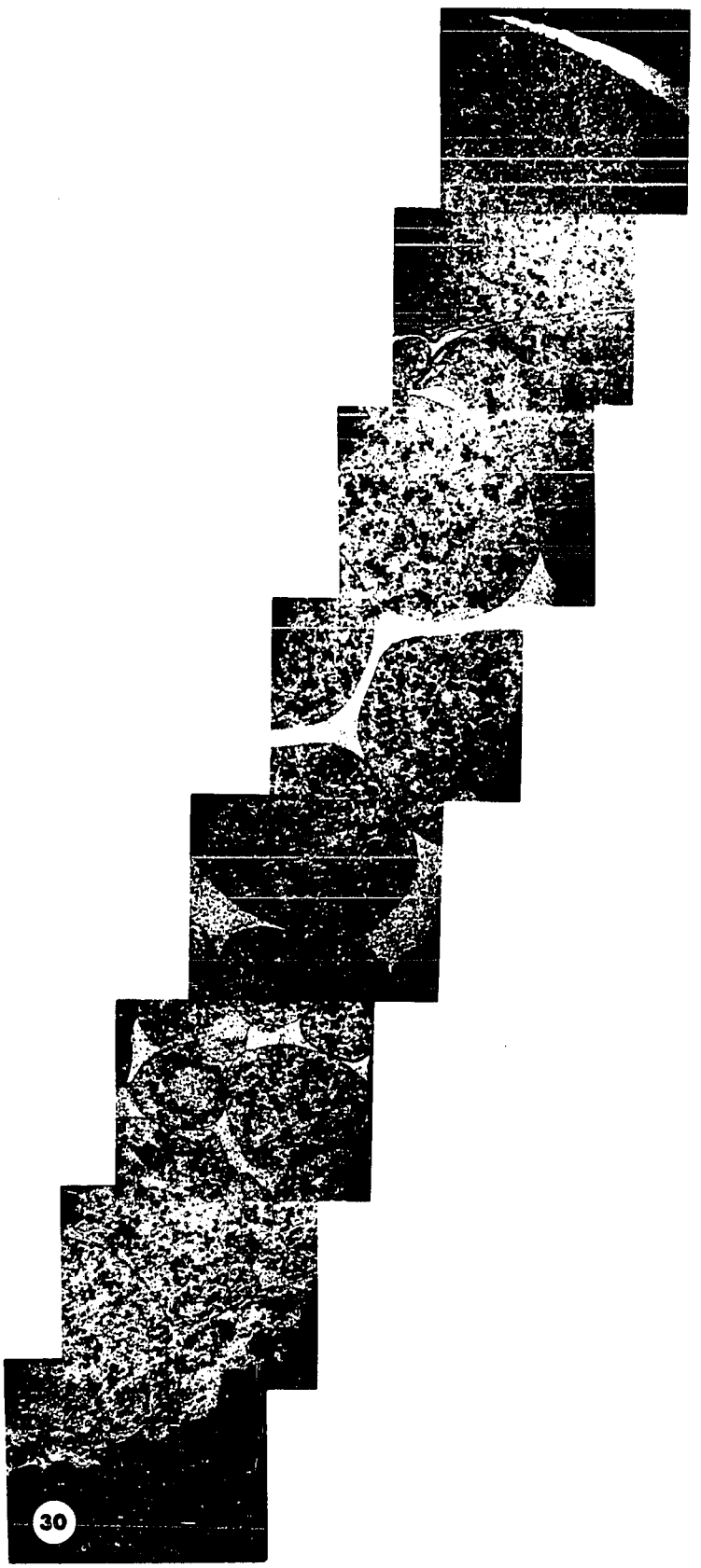
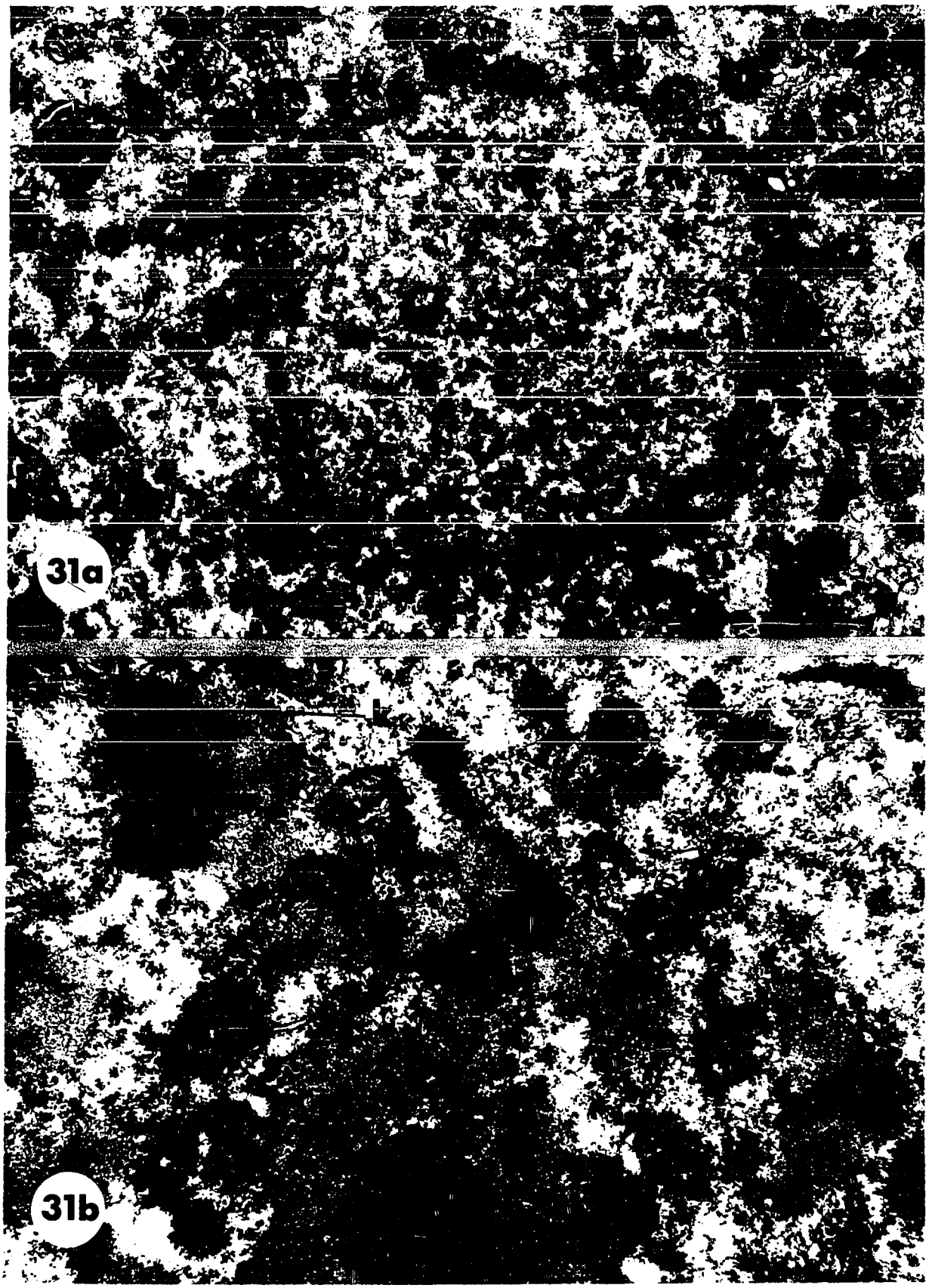

30

Figure 31a. An interphase nucleus in a four-hour colchicine-exposed early high blastula embryo has mitochondria along the nuclear envelope and normal appearing cytoplasm. The nucleus appears extracted when compared to other similarly fixed interphase embryonic nuclei. Osmium tetroxide with calcium fixation; lead citrate stained. X 10,300

Figure 31b. Free chromosomes of colchicine -exposed cells have prominent kinetochore-like structures (k) on or within them. Osmium tetroxide with calcium fixation; lead citrate stained. X 21,000


31a
31b

Prominent in the free chromosomes are large 'kinetochore' structures which appear as either a single or double fibrillar unit at the edge of a chromosome (k, Figure 31b). The general cytoplasm appears unchanged from the controls.

Deuterium oxide-exposed eggs progressed to the late high, flat blastula stage as judged by cell diameters (Figures 29e, f). Intracellular spaces are absent, and yolk particles are less frequent in all cells (Figure 29f) as also in the control embryos (Figure 29b and 2b) as compared to an earlier control embryo (Figure 1d). However, the amount of yolk is less in the deuterium oxide-exposed cells when compared to an embryo with a similar cell diameter (Compare Figure 29f to Figures 1d, f).

Late high blastula embryos were exposed to either colchicine, deuterium oxide, or vincristine sulfate for a period of nine hours. During the period of exposure, the control embryos progressed to the one-half epiboly stage (Figure 32a, b). The marginal periblast (Figure 32a) is at the equator of the embryo and an epidermic stratum covers loose cells of the embryo. Yolk particles are in scattered cells and the periblast does not have any small yolk particles; only small periblast nuclei (Figure 32b, arrow) and large yolk particles of the main yolk mass comprise the periblast. At higher magnifications, the control cells appear as previously described interphase cells (Figure 33a).

Colchicine-exposed embryos treated as above (Figures 32c, d) are arrested at late high to flat blastula stage (Figure 1d, f). Mitotic figures are absent although a homogeneous area unlike normal cytoplasm is centrally located in some cells (Figure 32d, arrow). Cells are almost completely free of yolk particles. Twin nuclei surrounded by high numbers of mitochondria (Figure 33b) or free chromosomes scattered throughout the cytoplasm among many flattened membranous elements (Figure 34a) are seen at higher magnifications. Kinetochores are very prominent on the free chromosomes (k, Figure 34b).

After nine hours exposure to deuterium oxide, an embryo exposed at late high blastula has progressed only to the one-third epiboly stage (Compare Figures 32e, f to Figures 2e, f). Cells contain significantly less yolk than a similarly advanced embryo. The periblast has small yolk particles. With the electron microscope, cell membrane breaks and cytoplasmic components in the intracellular space are commonly seen (Figures 35a, b). Interphase nuclei are surrounded by many mitochondria (Figures 35a), whereas the periblast contains a high number of small yolk particles (Figure 35b). Normal appearing mitotic figures are often found (Figure 36a).

The vincristine-sulfate-exposed embryos appear as the control embryos both in developmental stage and cell size (Compare Figures 32g, h to Figures 32a, b). Interphase cells

Figures 32a and 32b. Control embryos after twelve hours from late high blastula were all at one-half epiboly stage. Small yolk particles are absent from the periblast cytoplasm. Nuclei are easily seen in the periblast. (arrow, Figure 32b).

Figures 32c and 32d. Colchicine-exposed late high blastula embryos are arrested with cell diameters of cells of the late high to flat blastula. Nuclei are absent, but diffuse areas unlike the general cytoplasm are present (arrows, Figure 32d). Note the almost complete absence of yolk particles in cells near the periblast.

Figures 32e and 32f. Deuterium oxide-exposed embryos have progressed to only the one-third epiboly stage. Little yolk is present in the cells of the embryo.

Figures 32g and 32h. Vincristine-sulfate-exposed late high blastulae are at the one-third epiboly stage. No cytological alterations are evident.

Figures 32a, c, e, and g are X 150; Figures 32b, d, f, and h are X 370.

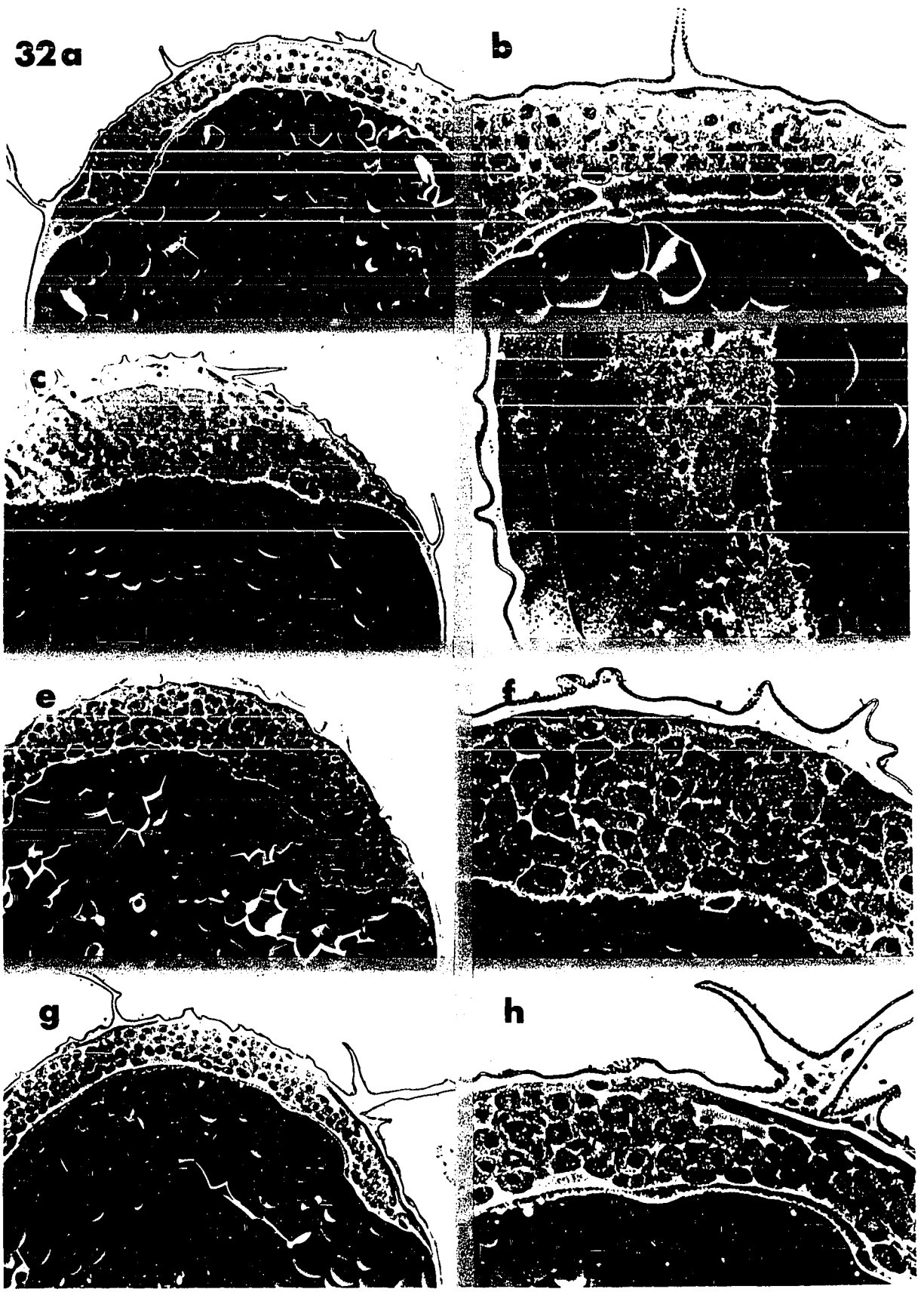

32a
b
c
e
f
g
h

Figure 33a. The one-half epiboly stage interphase cell appears as other interphase cells. The nucleus contains a single cleft. Numerous small yolk particles are present, both intact and partially utilized. Osmium tetroxide with calcium fixation; lead citrate stained. X 10,000

Figure 33b. Twin nuclei or chromosome masses surrounded by numerous mitochondria are often seen in colchicine-treated late high blastula embryos. Osmium tetroxide with calcium fixation; lead citrate stained. X 5800

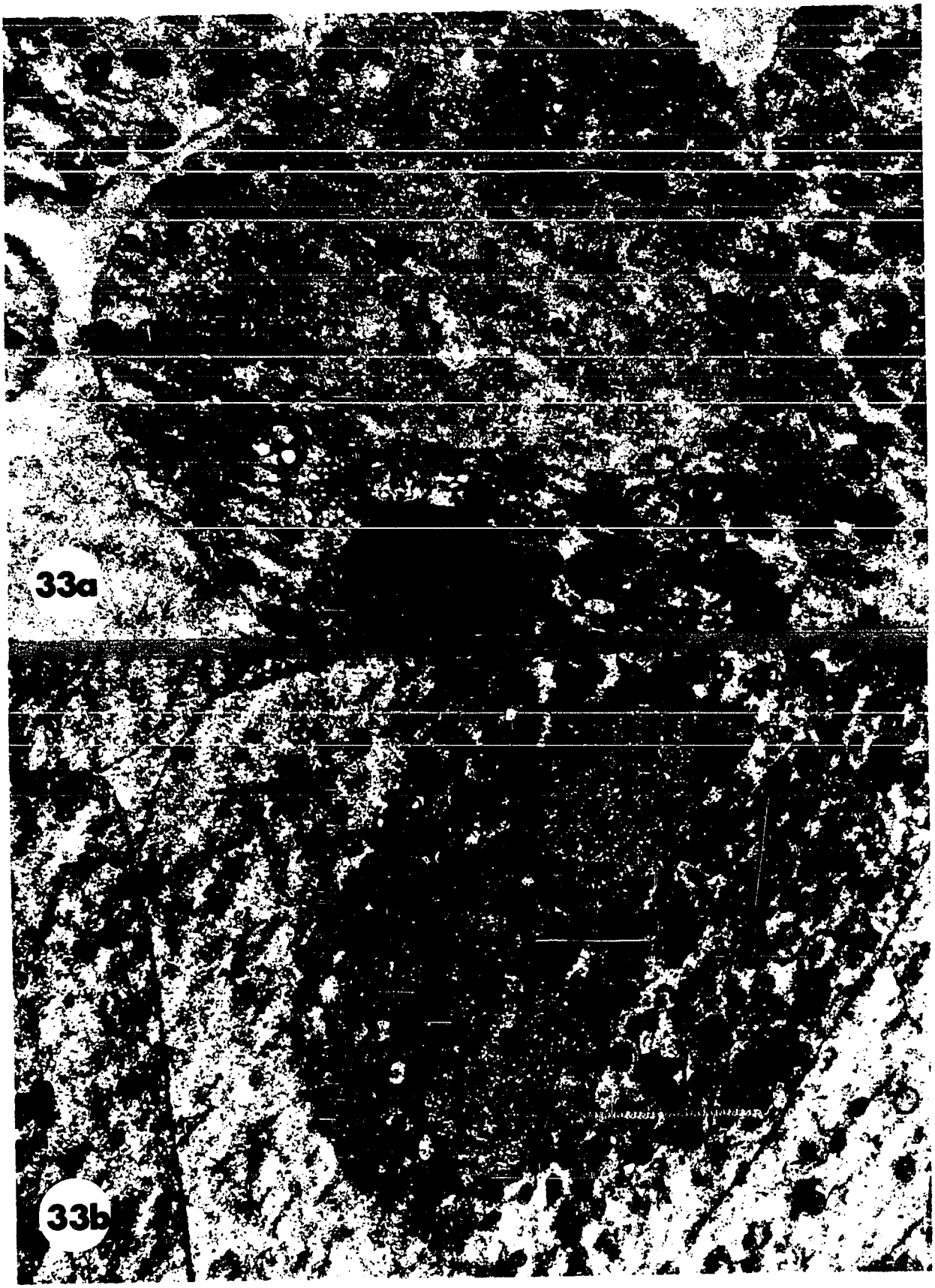

33a
33b

Figure 34a. Free chromosomes scattered throughout the cytoplasm among flattened membranous elements and mitochondria can be seen in colchicine-arrested cells. Osmium tetroxide with calcium fixation; lead citrate stained. X 8000

Figure 34b. At higher magnifications, chromosomes of colchicine-arrested cells have elaborate kinetochore-like structures (k) on or within the chromosomes. Osmium tetroxide with calcium fixation; lead citrate stained. X 24,000

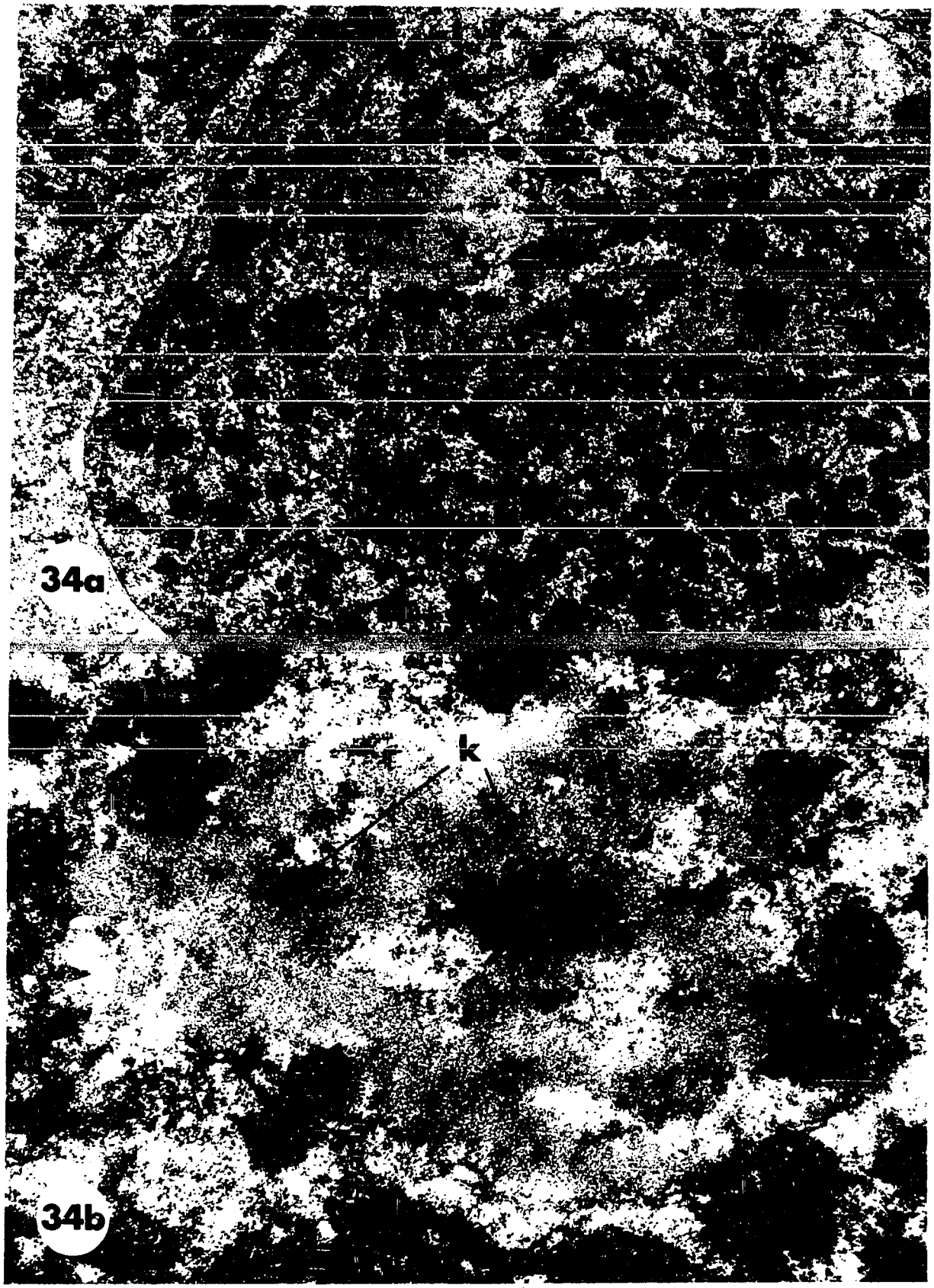

34a
k
34b

Figure 35a. After nine hours exposure to deuterium oxide, a late high blastula embryo which has progressed to the one-third epiboly stage, shows normal nuclei with mitochondria around it. However, the cell membranes are broken. Osmium tetroxide with calcium fixation; lead citrate stained. X 4000

Figure 35b. Deuterium oxide-exposed embryos contain numerous yolk particles in the periblast. Osmium tetroxide with calcium fixation; lead citrate stained. X 3740

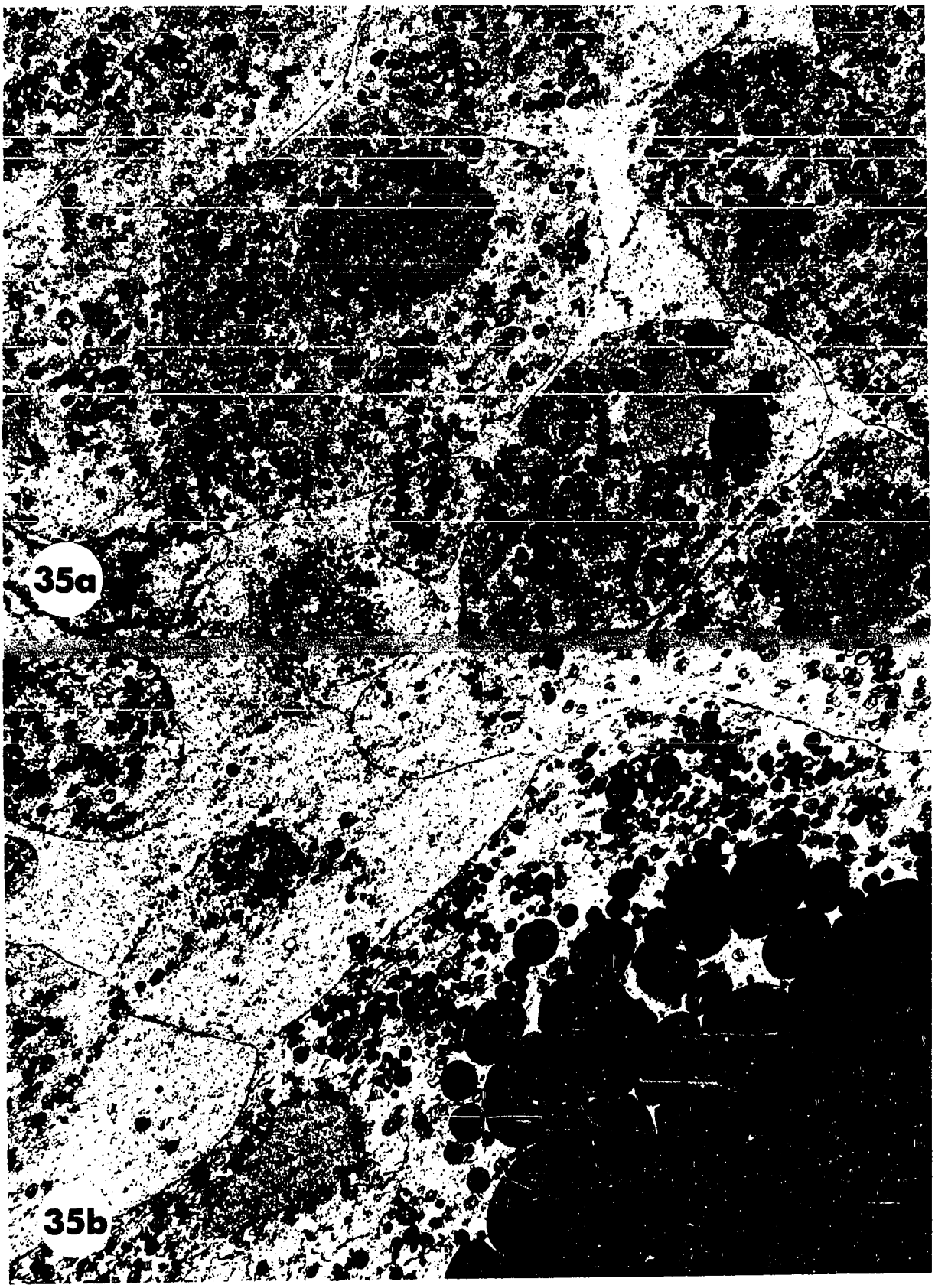
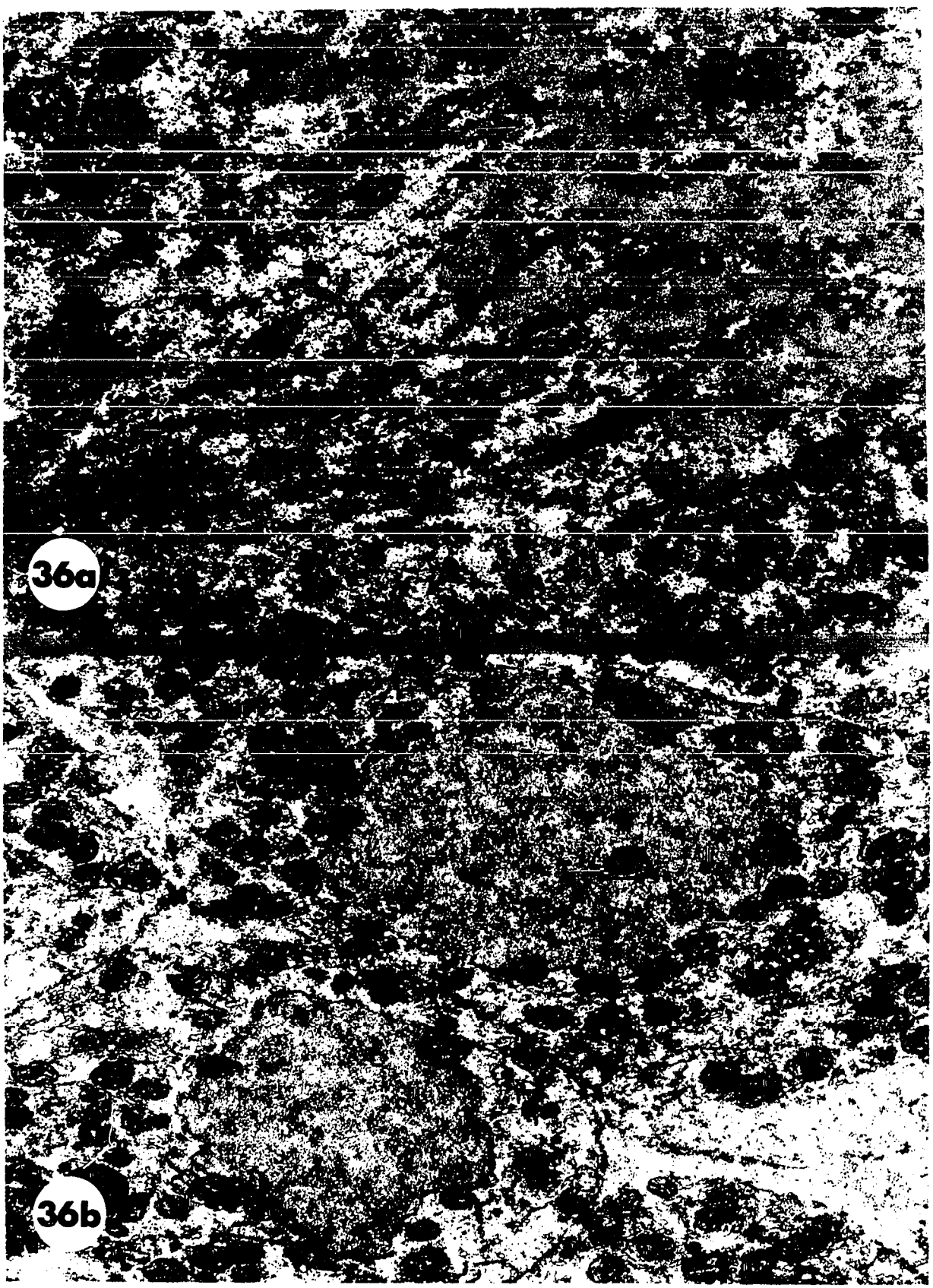

35a
35b

Figure 36a. Mitotic figures in deuterium oxide-exposed embryos appear almost normal. More membranous material appears within the mitotic spindle and at the poles. Osmium tetroxide with calcium fixation; lead citrate stained. X 14,300.

Figure 36b. Vincristine sulfate-exposed interphase cells appear as the control cells. Osmium tetroxide with calcium fixation; lead citrate stained. X 10,000.


36a
36b

are indistinguishable from control cells at higher magnification (Figure 36b).

Discussion

The exposure of B. rerio embryos to deuterium oxide, vincristine sulfate, and colchicine permits the observation of structures or events which are not readily apparent in normal rapidly developing embryos. The effect of deuterium oxide and vincristine exposure and the effects of colchicine on the appearance of kinetochores and on yolk transfer and mitosis will be discussed.

Deuterium Oxide

Deuterium oxide, although differing from water by the substitution of deuterium (atomic weight = 2) for hydrogen (atomic weight = 1), exhibits a phenomenally large 'isotope effect' (Gross and Spindel, 1960a, b). Mitotic cells immersed in a high concentration of deuterium oxide (70-96%), that is deuterium oxide substituted for hydrogen oxide, are immediately arrested regardless of progress through the mitotic cycle (Gross and Spindel, 1960a, b; Marsland and Zimmerman, 1963, 1965).

In this study, exposure of B. rerio embryos to concentrations of deuterium oxide (49.5%) which would retard but not arrest mitosis allowed gross normal development of all

embryos. The effect of deuterium oxide upon the mitotic apparatus in B. rerio is similar to the observations of Gross and Spindel (1960a,b). Developmental advancement when cell volume is considered (Marrable, 1965) was always retarded; deuterium oxide exposure resulted in blastomeres that were swollen and had reduced or missing intracellular spaces, and mitotic apparatuses that had prominent astral regions. The effect is, therefore, similar to that described by Gross and Spindel (1960a,b).

Exposure of 8-cell embryos resulted in otherwise normal development, even yolk utilization, until cytolysis occurred. Early high blastula embryos exposed to deuterium oxide lagged developmentally and showed fewer yolk particles in the blastoderm as compared to the control. Late high blastula embryos proceeded to the one-third epiboly stage as the control embryos proceeded to the one-half epiboly stage. Significantly, the cells of deuterium oxide-exposed embryos did not contain as many yolk particles in the cells as in similarly advanced embryos.

The developmental lag effected by exposure to deuterium oxide, although possibly caused by slowing of the mitotic cycle, would probably not cause cessation of yolk utilization. If yolk utilization did continue in the blastomeres without a contribution of yolk-containing cells to the blastoderm by the periblast, the finding of cells with less yolk in the

embryo would be expected. Indeed, the lack of yolk in deuterium oxide-exposed eggs did occur. Therefore, the conclusion may be advanced that mitosis from the periblast does occur and results in yolk appearing in cells near the periblast. However, this hypothesis as tested by exposure of the embryos to deuterium oxide is inconclusive because of cytolysis in all embryos exposed for four hours or longer.

Vincristine Sulfate

In hindering development or mitosis in B. rerio, vincristine sulfate was ineffective. Sufficient information is not available to explain this negative result when compared to the observations of George et al. (1965) who noted vincristine mitotic spindle disruption in HeLa cells.

Colchicine and Kinetochores

Exposure of B. rerio embryos to colchicine, a known mitotic inhibitor (Eigsti and Dustin, 1955; Taylor, 1965), causes an abrupt cessation of development regardless of exposure stage. Dispersed chromosomes are found in all embryos exposed to colchicine. Kinetochores or structures at spindle-chromosome connections during normal mitosis were only rarely seen in this study. The dispersed chromosomes have a bipartite, filamentous structure on or within them. Similar, more elaborate structures, have been characterized

as kinetochores in HeLa cells (Robbins and Gonatas, 1964), in Chinese hamster, colchicine-treated, tissue-culture cells (Brinkley, 1965) and in embryonic rat cells (Jokelainen, 1965). The assumption may be made that the structures on the chromosomes of colchicine-arrested B. rerio cells are kinetochores, but the absence of similarly structured kinetochores during normal mitosis in B. rerio opens many questions. These questions, though suggested by information from this study, cannot be answered with the information available: 1) is the obviousness of kinetochore structure dependent upon the time on exposure of chromosomes to the cytoplasm, 2) is the elaborate kinetochore either on chromosomes of colchicine-treated cells or in some tissue culture line anomalous when compared to kinetochores on chromosomes of untreated mitotic cells or in in situ mitotic cells, respectively, or 3) are none of the above questions valid and another alternative possible?

Colchicine and Periblast Mitosis

Developmental arrest can be seen to occur rapidly by the non-advancement of an embryo to the next developmental stage. Some colchicine-exposed blastulae with a thick blastoderm are found with large, surface cells but smaller deep cells. A surface cell is immediately exposed to colchicine, but a cell deep within the blastoderm may be affected by colchicine

later due to penetration difficulties. This phenomenon of rapid exposure is most apparent at the 8-cell stage when all cells are immediately exposed or at late high blastula when the blastoderm is thin; early high blastulae have a significantly thicker blastoderm which would present a problem of rapid penetration. In embryos which have cells that are considered rapidly exposed to colchicine, no interphase nuclei are evident. However, in the early high blastula embryo where exposure is considered variable because of penetration, interphase nuclei are found.

Rapid cessation of mitosis by colchicine could be expected to affect any mitosis that might occur from the periblast but not affect yolk utilization. Therefore, the question of yolk transfer from the periblast syncitium to the blastoderm can be subjected to experimentation. If yolk is transferred to the cells of the blastoderm by any means other than mitosis, yolk could be expected in some blastomeres in spite of their mitotic arrest. However, in embryos rapidly affected by colchicine, the 8-cell and late high blastula embryos, yolk is relatively absent. If yolk is transferred by periblast mitosis, colchicine-arrested cells near the periblast should contain a reduced amount of yolk as compared to untreated cells near the periblast. However, if a yolk complement was given to cells during bipolar differentiation and subsequently utilized and new cells with yolk were formed

from the periblast, we would expect to find cells without yolk in untreated cells furthest from the periblast and cells close to the periblast with a complement of yolk. This situation is observed in normally developing embryos but is not seen in embryos where mitosis has been stopped. Therefore, the conclusion is drawn that mitosis from the periblast does occur and substantiates the hypothesis of yolk distribution outlined in a previous chapter.

Summary of Experimental Conclusions

The experimental treatments of the B. rerio embryo at various times in its developmental period and for various exposure times showed that 1) 49.5% deuterium oxide causes a slowing of development, a swelling of the blastula cells, and eventually cytolysis; 2) colchicine causes immediate cessation of mitosis and uniform developmental arrest (if penetration is rapid), dispersal of chromosomes, and permits appearance of elaborate kinetochore-like structures on the chromosomes; and 3) vincristine sulfate, a colchicine-like agent, does not appear to affect the early development. Colchicine-caused cessation of mitosis in the B. rerio embryo does show that mitosis from the periblast providing blastomeres with periblast yolk is highly probable.

SUMMARY

1. Yolk is found evenly distributed in all cells of the blastoderm after bipolar differentiation at the early high blastula stage. Small yolk particles are common in the periblast until the one-third epiboly stage. Cells further away from the periblast during this period contain yolk particles which are decreased in number and smaller in size. At one-third epiboly to one-half epiboly stage, small yolk particles in the periblast are absent, and only scattered cells of the embryo contain yolk. It is inferred that mitosis from the periblast provides new blastomeres with a yolk complement and is the mechanism of yolk transfer to the blastoderm.

2. Exposure of B. rerio embryos to 49.5% deuterium oxide solution causes a slowing of normal development, a swelling of blastula cells, and ultimately cytolysis. Although development and mitosis are slowed down, yolk utilization continues. Cells away from the periblast contain smaller and fewer yolk particles than cells of a control embryo with similar cell diameters. Mitosis from the periblast is slowed down by deuterium oxide and could not then provide yolk-containing cells to the blastoderm as rapidly. From this observation, the inference of mitosis from the periblast providing a yolk complement is reaffirmed.

3. Colchicine causes immediate cessation of mitosis and

uniform developmental arrest if penetration is rapid. Arrested cells are found with much less yolk than control cells of equal size. Cessation of mitosis and the absence of yolk transfer is consistent with the hypothesis that cells with a yolk complement originate from the periblast by mitosis.

4. Yolk particles found in B. rerio cells are seen initially to have only amorphous yolk material surrounded by a unit membrane, then to have membranous elements and ribosomes in the center of membrane-bound yolk material, and to appear finally as a highly complex particle that appears to have cytoplasm surrounded by a little yolk and a unit membrane. The above order is suggested as a sequential pattern of yolk utilization.

5. Yolk, besides providing soluble nutrients to the embryonic cells, contributes maternal membranous material and ribosomes to the blastomeres.

6. Vincristine sulfate, a colchicine-like agent, does not affect early development of B. rerio under the conditions used in this study.

7. Hydroxyadipaldehyde as used in this study preserves mitotic spindle microtubules with diameters of 19-21 mu as well as does osmium tetroxide with calcium chloride fixation.

8. The presence of mitotic spindle microtubules at the centrosome and in the nuclear cleft prior to nuclear envelope interruption suggests that the formation of the mitotic

apparatus occurs at the centrosome and proceeds toward the condensed chromosomes.

9. During prophase and prometaphase, the shape of the nuclear envelope progresses from a sphere with a single cleft, to an ellipsoid with two clefts, and then to an interrupted ellipsoidal shape. The persistence of nuclear envelope fragments until the establishment of the mitotic spindle hinders large particles, such as mitochondria or yolk, from being included in the mitotic spindle.

10. That mitochondria, although lying close to the interphase nucleus probably due to telophase nucleus enlargement, are not lying near the prophase nucleus in significant numbers when the nuclear envelope is interrupted suggests that mitochondria are not 'enzymatically' involved in nuclear envelope dissolution. The nuclear shape change during this period suggests that absence of mitochondria is due to nuclear envelope shape change.

11. During early anaphase, the chromosomes move about 7 microns to the poles which remain 15 microns apart; later when a cytokinetic furrow is forming, the poles and chromosomes move to a 21-micron separation.

12. Kinetochore-like structures are seen on chromosomes of colchicine-treated cells and are significantly more elaborate than kinetochores seen during normal mitosis.

13. The mode of nuclear reconstruction, whether as chromoso-

mal vesicles or a single intact telophase nucleus, appears to be dependent not upon nuclear control but rather upon physical restrictions of cell volume during anaphase.

14. In the B. rerio embryo during early development, either a row of vesicles or a furrow can be found to separate daughter cells. Both cleavage types occur within a single blastula under any fixation condition utilized.

15. The cleavage furrow of B. rerio is characterized by a 60-mu layer of fine material under the plasma membrane only in the furrow area and is similar to the cleavage furrows seen by other investigators.

16. The last remnant of the cleavage furrow when an interphase nucleus is also present is a midbody with gathered spindle microtubules, with a dark band in the center of the midbody, and with membranous projections.

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