

# SPERM ASTER IN RABBIT ZYGOTES: ITS STRUCTURE AND FUNCTION

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## ABSTRACT

Microscope observations of rabbit zygotes demonstrate that a sperm aster forms in association with the male pronucleus approximately 1 h postinsemination and consists of two regions. One, the centrosphere, contains a dense aggregation of cisternae of smooth endoplasmic reticulum and microtubules. The second consists of fascicles of microtubules which emanate from the centrosphere. Fertilized rabbit eggs were cultured in medium containing colcemid in order to determine its effects on various events of fertilization, such as movements of the male and female pronuclei and DNA synthesis. No evidence was obtained to indicate that a sperm aster is formed in colcemid-treated zygotes. In addition, migration and close apposition of the pronuclei do not take place. Breakdown of the pronuclear envelopes and condensation of the maternally and paternally derived chromosomes occur even though the pronuclei fail to migrate centrad. Autoradiographic analysis of the synthesis of DNA by both pronuclei demonstrates that their migration into close apposition to one another is not required for the incorporation of tritiated thymidine.

Pronuclear migration involves the movements of the male and female pronuclei from their site of formation to a region within the zygote where they become associated and form the embryonic genome. There is relatively little information concerning the nature of the vectors, structures, and cellular mechanisms which bring about the migration of the pronuclei. Early studies by a number of investigators employing the gametes of invertebrates, indicated that the sperm aster, which is formed in association with the incorporated spermatozoon, may be involved in the movements of the male pronucleus to the male female pronucleus (4, 9). More recent investigations have ascertained the ultrastructural features of the sperm aster in several species of invertebrate zygotes (cf. 10). These studies have shown that the sperm aster

consists of essentially two compartments: (a) a central region, the centrosphere, and (b) a cortical area, which gives the body its stellate appearance. Both regions are richly endowed with microtubules and cisternae of smooth endoplasmic reticulum. There is some evidence to indicate that the microtubules associated with the sperm aster are directly involved in the migration of the pronuclei (20, 21).

The role of the sperm aster in mammalian zygotes is unknown. In fact, the presence of a sperm aster, as a general feature of all mammalian zygotes, is an open question (see Discussion in reference 18). A structure, reminiscent of the sperm aster found in invertebrates, has been observed in the rabbit, ewe, and sow with the light microscope (18). Szollosi and Hunter (17) have observed mi-

croton tubules in association with the developing male pronucleus in zygotes of the domestic pig and suggest that they may be a portion of the sperm aster.

The present communication describes, at the light and electron microscope levels of observation, the structure of the sperm aster in rabbit zygotes. Investigations are also described which demonstrate a function of the sperm aster and the relationship of pronuclear migration to subsequent events of fertilization.

## MATERIALS AND METHODS

Fertilized eggs of Dutch Belted does, superovulated according to the method of Kennelly and Foote (9), were obtained at hourly intervals from 13 to 21 h postcoitus and fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 at 4°C. The zygotes were washed in cacodylate buffer overnight, postfixed in 1% OsO<sub>4</sub> in phosphate buffer, dehydrated in increasing concentrations of ethanol, and embedded in Epon.

Zygotes were also flushed from the oviducts of superovulated does at 13 and 14 h postcoitus with Ham's F-10 medium. Half of the zygotes were incubated in Ham's F-10 medium (controls) while the remaining half were cultured in Ham's F-10 medium containing colcemid (experimentals). Zygotes were cultured at 37°C for 24 h in varying concentrations of colcemid ( $5 \times 10^{-7}$ – $5 \times 10^{-4}$  M) to determine the most effective dosage to be employed. Samples were also treated with colcemid for 8 h, washed eight times, and cultured in fresh medium in order to determine whether or not the effects of colcemid treatment could be reversed. At 1-h intervals after the initiation of culture, samples were removed from the experimental and control groups and fixed and processed as described above.

Some cultures of controls and experimentals were also incubated in the medium of Brackett and Williams (2) containing 5  $\mu$ C/ml of [<sup>3</sup>H]thymidine (New England Nuclear Corp., Boston, Mass.). Samples were taken at 2-h intervals from 15 to 23 h postcoitus and processed for autoradiography.

## RESULTS

### *Structure of the Sperm Aster*

The sperm aster forms in association with the male pronucleus approximately 1 h postinsemination. Structurally, it is similar to the sperm asters that have been described for several invertebrates (Fig. 1) (cf. 10). Two regions of the sperm aster of the rabbit zygote can be discerned microscopically: (a) a medullary region which is referred to as the centrosphere and (b) a cortical region consisting of radiating columns of organelles. The centrosphere consists of a dense aggregation of microtubules

and elements of smooth endoplasmic reticulum (Fig. 2). This array of cisternae and tubules surrounds the male pronucleus. The microtubules of the centrosphere are frequently seen in close association with the male pronucleus. It has not been possible to observe any direct connection between the pronuclei and the tubules. From the centrosphere emanate loosely organized fascicles of microtubules and smooth endoplasmic reticulum (Fig. 1). Because the fascicles that emanate from the centrosphere are not composed of large numbers of microtubules which are always arranged in parallel, the stellate nature of the sperm aster of the rabbit zygote is not always apparent and may be appreciated best at low magnification (Fig. 1, inset).

Unlike the sperm aster observed in the fertilized eggs of invertebrates studied to date, the centrosphere of the rabbit sperm aster does not appear to contain centrioles. Portions of the sperm tail are frequently found within this region.

The sperm aster increases in size until its volume fills much of the zygote (Fig. 1, inset). It will remain this size throughout the centrad migration of the male and female pronuclei. Migration of the pronuclei is concluded with their close association in the center of the zygote (Fig. 3, inset), approximately 4 h postinsemination (cf. 10, 12).

With the association of the pronuclei the sperm aster begins to disintegrate. This occurs principally through the disappearance of many of the microtubules that make up this body. The sperm aster eventually metamorphoses into an area composed of considerable amounts of cisternae of endoplasmic reticulum and Golgi complexes that surrounds the associated pronuclei (Fig. 3). Relatively few microtubules are observed within this region at this stage of fertilization.

### *Colcemid Treatment of Rabbit Zygotes— Effects on the Sperm Aster*

The possible role of the sperm aster in rabbit zygotes was investigated by incubating in vivo fertilized eggs in culture medium containing colcemid. To determine the most suitable dosage to be employed during the course of these studies, zygotes were isolated approximately 1–2 h postinsemination and cultured in Ham's F-10 medium containing various concentrations of colcemid. Colcemid concentrations of  $5 \times 10^{-7}$ – $5 \times 10^{-4}$  M were effective in preventing the first cleavage division of fertilized rabbit eggs. Concentrations of

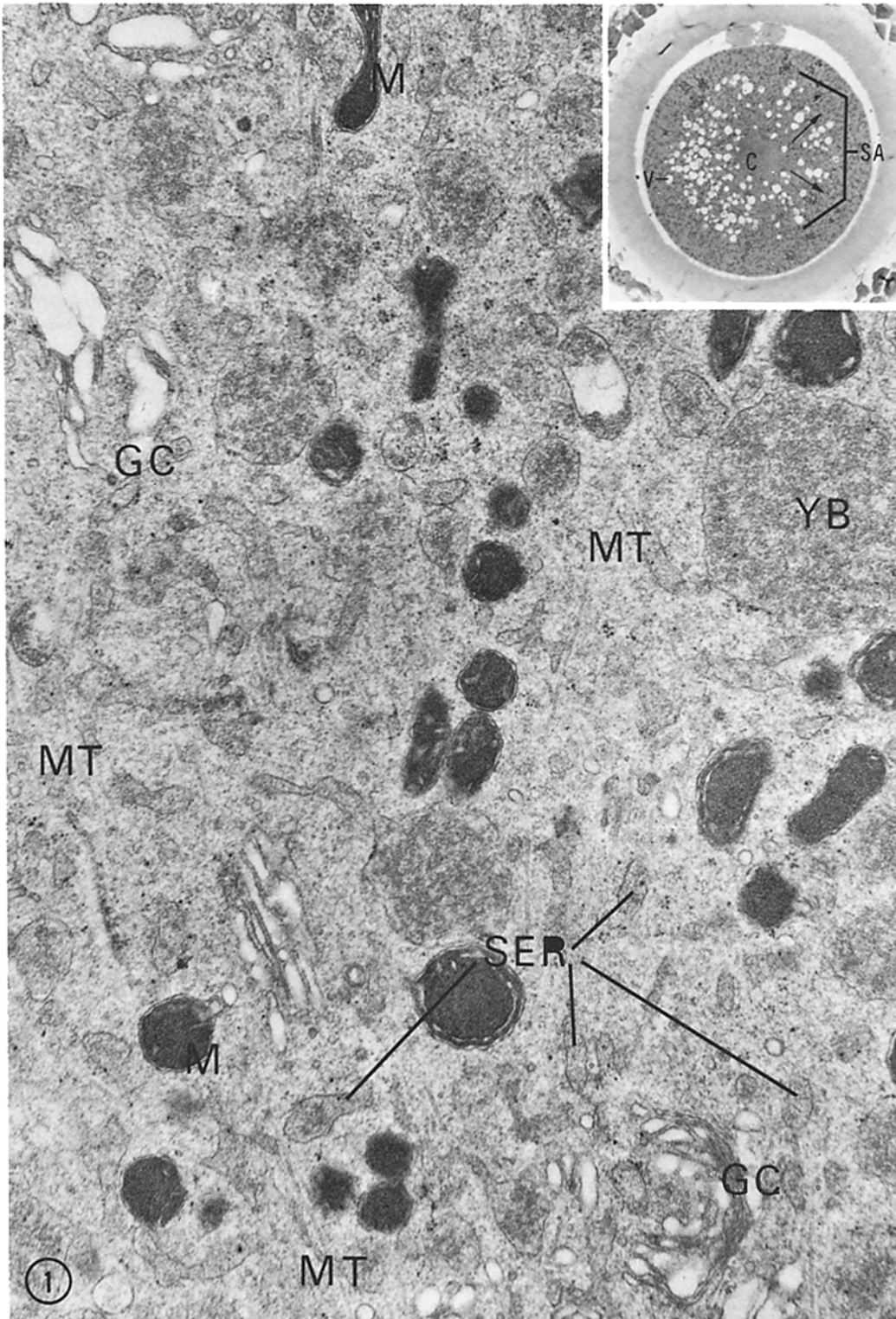


FIGURE 1 Sperm aster (SA) of rabbit zygotes. The centrosphere (C), illustrated in the *inset*, appears as a relatively clear area which is surrounded by radiating fascicles composed of vesicles (V) and particulate organelles (e.g., mitochondria). In the electron micrograph, a portion of the peripheral aspect of the sperm aster is shown, containing smooth endoplasmic reticulum (SER), microtubules (MT), mitochondria (M), Golgi complex (GC), and yolk bodies (YB). These organelles become aligned into parallel arrays as depicted by the arrows in the inset.  $\times 42,000$ ; *inset*,  $\times 400$ .

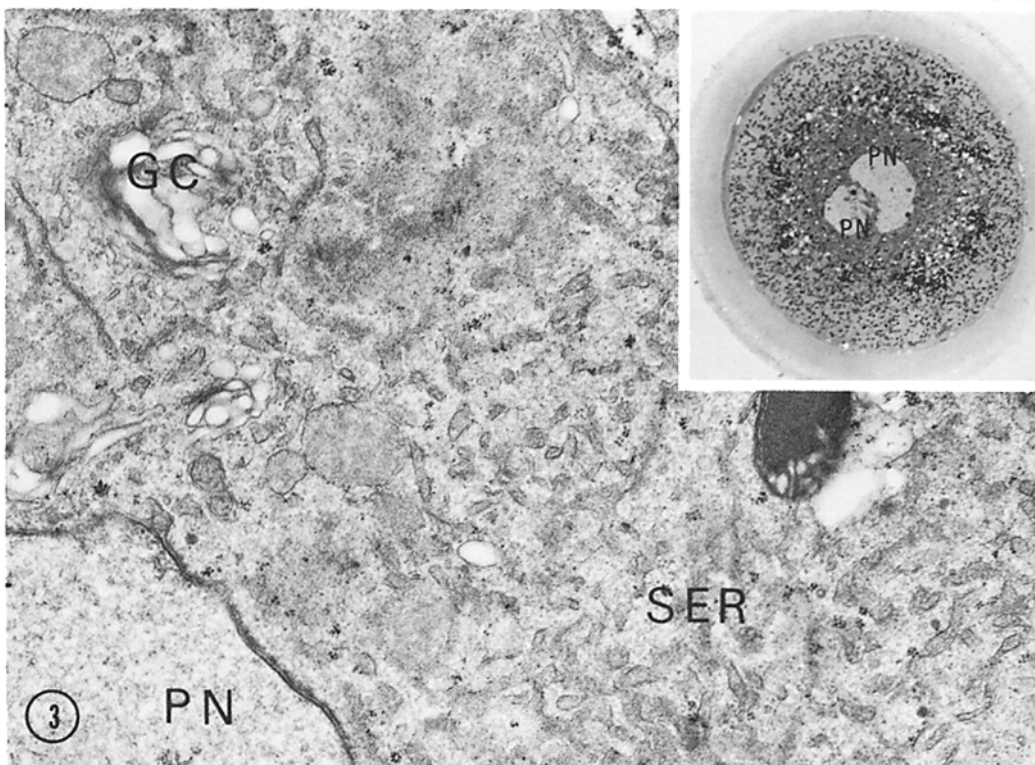
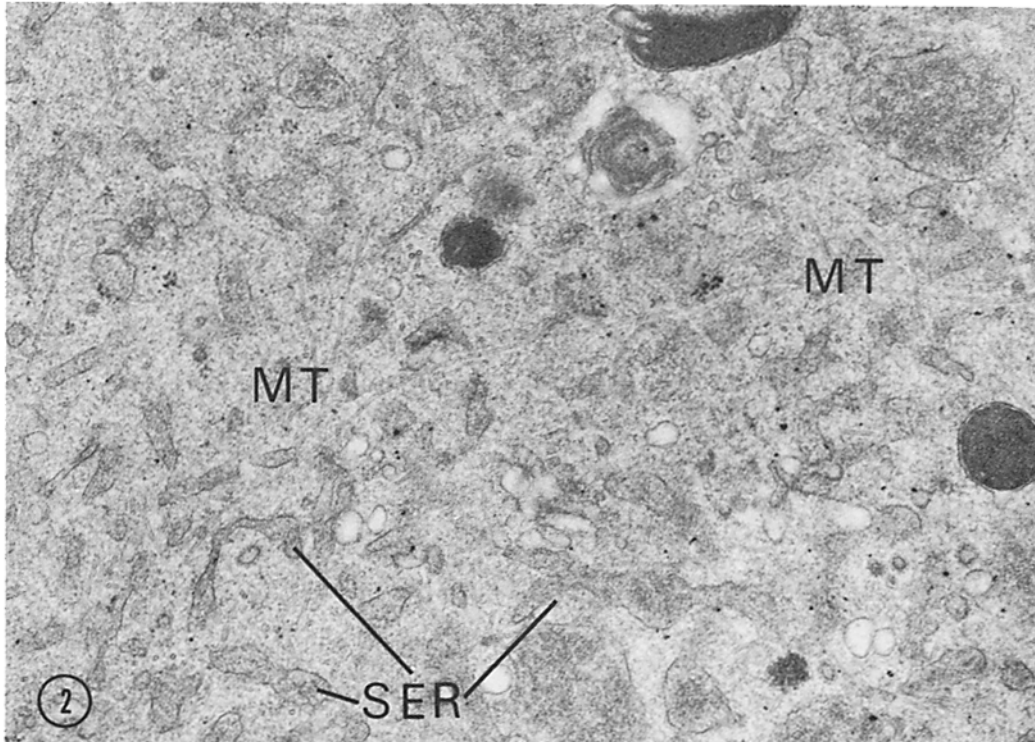


FIGURE 2 Centrosphere of a rabbit sperm aster illustrating the abundant microtubules (*MT*) and smooth endoplasmic reticulum (*SER*) that fill this region.  $\times 40,000$ .

FIGURE 3 Closely apposed male and female pronuclei (*PN*) of a rabbit zygote (*inset*). Note the concentric layers of organelles that surround the apposed pronuclei in the photomicrograph. The cytoplasmic region surrounding the pronuclei (*PN*) is filled with elements of smooth endoplasmic reticulum (*SER*) and Golgi complexes (*GC*) (Fig. 3). Few microtubules are observed in the perinuclear cytoplasm at this stage of fertilization.  $\times 32,000$ ; *inset*,  $\times 400$ .

colcemid less than  $10^{-7}$  M had little or no observable effect on in vitro development of rabbit zygotes. After 8 h incubation in media containing colcemid, zygotes were washed eight times and recultured in fresh media lacking colcemid. Recovery, as evident by the cleavage of treated zygotes, was greater than 90% in all of the cases and at all of the concentrations employed.

Fertilized eggs were recovered 1–2 h postinsemination and cultured in  $5 \times 10^{-6}$  M colcemid. The initiation of culturing in medium containing colcemid corresponded to the time just before or immediately after the completion of the second meiotic division of the maternally derived chromatin. The results of colcemid treatment differed depending on whether the zygotes were cultured at the time of the development of the female pronucleus or immediately afterwards (6). When the zygotes were cultured during the development of

the female pronucleus, colcemid disrupted the development of the female pronucleus. Usually two "female pronuclei" were formed (Fig. 4) (cf. 14). In other systems, namely *Spisula*, colcemid inhibits polar body formation and a multinucleate zygote is formed (F. J. Longo, personal observations).

In most instances, zygotes were incubated in colcemid after the formation of the female pronucleus, and the development and fate of both the male and female pronuclei were followed. No evidence was obtained to indicate that a sperm aster forms in colcemid-treated zygotes; neither microtubules nor clustering of cisternae of smooth endoplasmic reticulum were observed in the vicinity of the male pronucleus.

Pronuclear migration did not occur in colcemid-treated zygotes (Fig. 5). The male and female pronuclei failed to move centrad and become

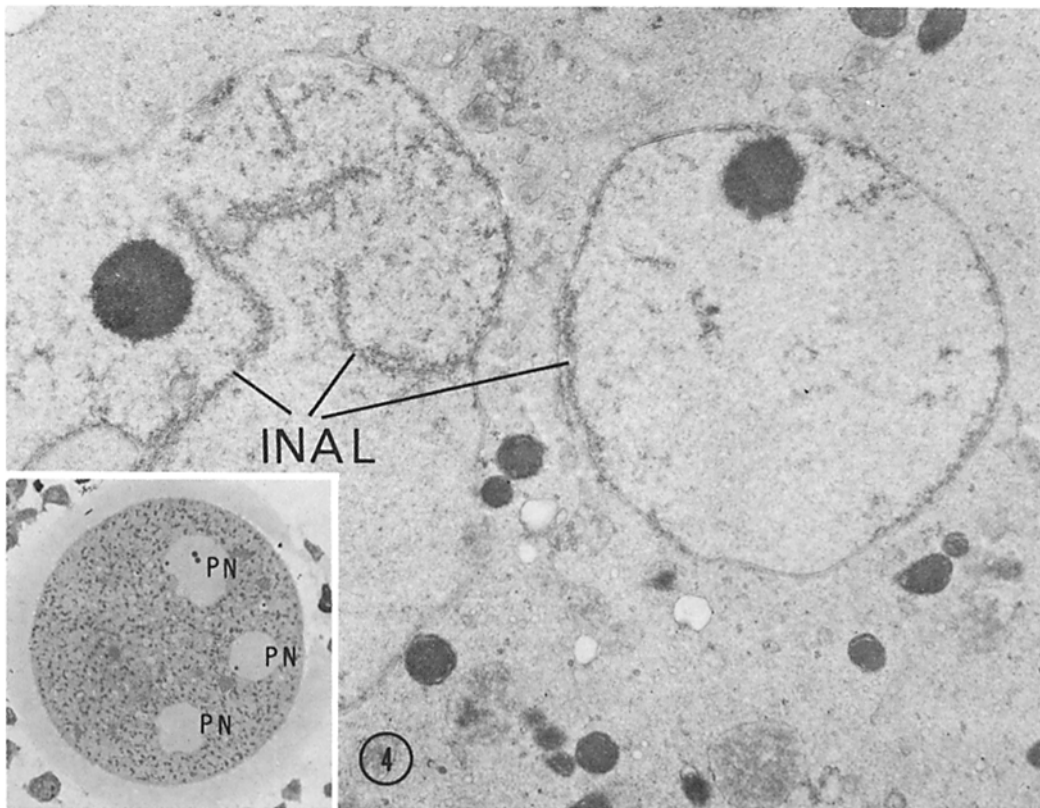


FIGURE 4 Rabbit zygotes incubated for 4 (Fig. 4) and 8 h (*inset*) in medium containing colcemid. Colcemid treatment in these cases was initiated just before telophase of meiosis and has interfered with the formation of the female pronucleus. Two female pronuclei have formed containing abundant intranuclear annulate lamellae (*INAL*). In the presence of colcemid, the female pronuclei and male pronucleus (*PN*) fail to migrate centrad (*inset*).  $\times 20,000$ ; *inset*,  $\times 400$ .

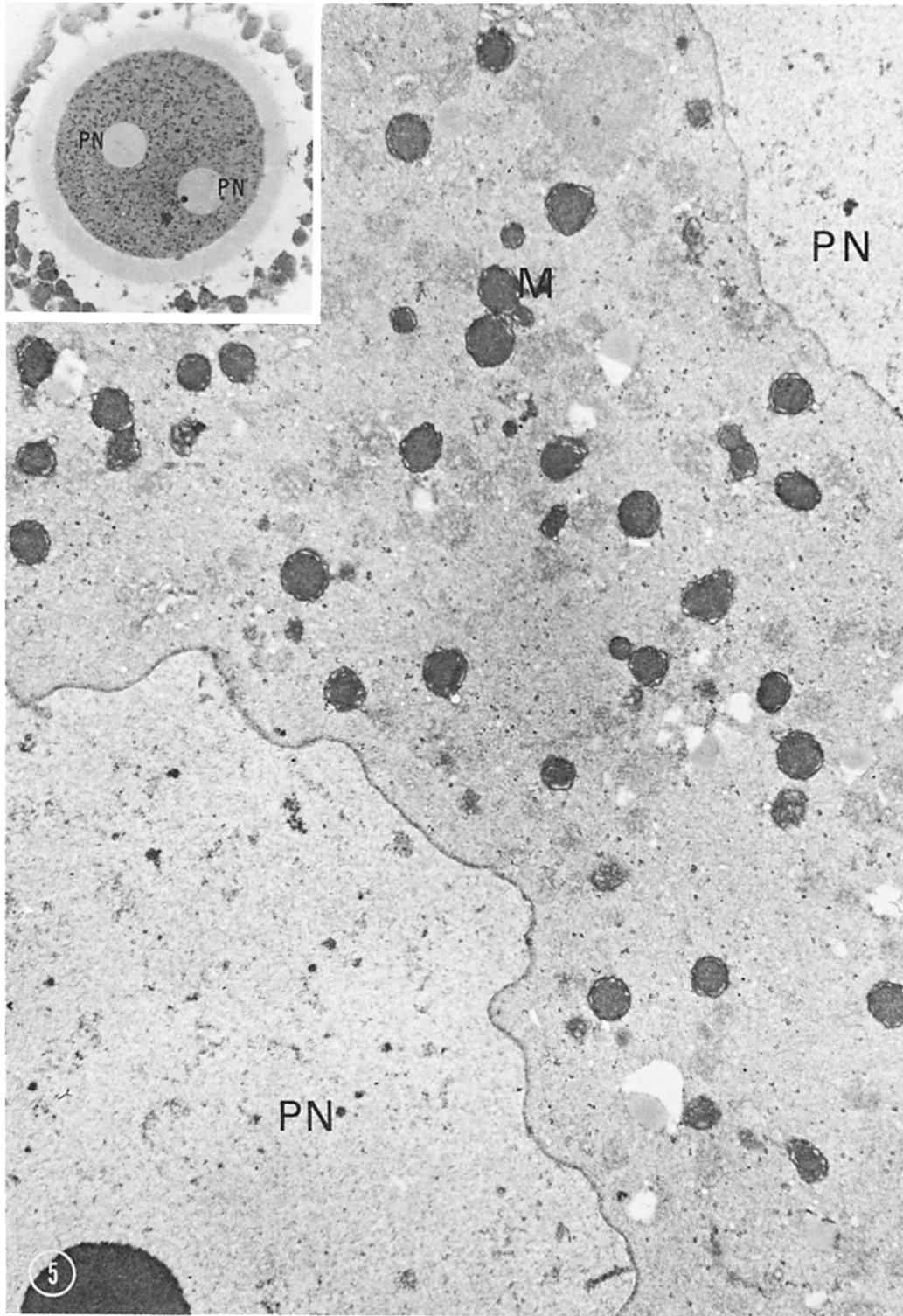


FIGURE 5 Rabbit zygote incubated for 8 h in medium containing colcemid. The male and female pronuclei (*PN*) have not migrated into close apposition to one another. Note that the cytoplasmic organelles surrounding the pronuclei are randomly dispersed and do not form the concentric layers observed in untreated specimens. (Compare with Fig. 3, *inset*). *M*, mitochondria.  $\times 15,000$ ; *inset*,  $\times 400$ .

closely associated in a manner typical of controls. The pronuclei remained separated during the entire period of treatment. When embryos were washed with fresh medium 8 h after culturing, the pronuclei migrated centrad and development, consisting of the latter events of fertilization and cleavage, ensued.

#### *Colcemid Treatment of Zygotes— Effects on Postmigration Events*

If fertilized eggs are incubated in colcemid for greater than 8 h, the male and female pronuclei undergo processes similar to those described for untreated specimens (10, 12). As far as we were able to determine, the nucleoli in both pronuclei disappear at about the same time. In addition, there is also the concomitant breakdown of the pronuclear envelopes and the condensation of the chromosomes within each pronucleus. These events take place along the cortex of the zygote and independently within each pronucleus, i.e., while the pronuclei are separated from one another. In untreated specimens a spindle develops and the condensed chromosomes from both pronuclei become aligned to form a metaphase plate (12). However, due to the presence of colcemid in experimentally treated specimens, microtubular elements fail to form and the condensed chromosomes are scattered within the cytoplasm. Radio-

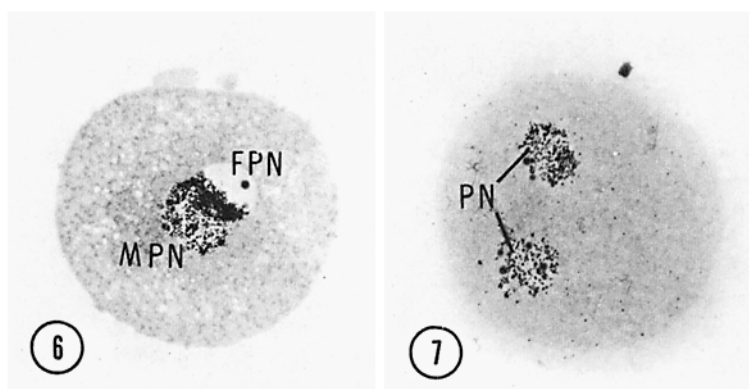
autographic analysis of tritiated thymidine incorporation indicates that pronuclei from control and experimentally treated specimens initiate DNA synthesis at about the same time (Figs. 6 and 7).

#### DISCUSSION

The results presented here demonstrate that a sperm aster, structurally similar to those observed in fertilized eggs of invertebrates, forms in rabbit zygotes. The sperm aster of fertilized rabbit eggs consists of two regions: (a) the centrosphere, containing a dense aggregation of smooth endoplasmic reticulum and microtubules, and (b) a cortex, consisting of fascicles of microtubules which emanate from the centrosphere.

The results of earlier published observations suggest that the sperm aster is involved in the migration of the male and female pronucleus (19). Rothschild (15) postulated that elements of the sperm aster push against the inner aspect of the plasma membrane and thereby move the male pronucleus to the female. We have searched for such an association of the sperm aster with the cortex of the zygote, e.g., microtubules of the sperm aster in close proximity to the plasma membrane, in the surf clam, sea urchin and the rabbit, but have not observed one (10; F. J. Longo, unpublished observations).

That colcemid simultaneously prevents the appearance of the sperm aster and the migration of



FIGURES 6 and 7 Autoradiographs of colcemid treated (Fig. 7) and untreated (Fig. 6) rabbit zygotes. The closely apposed male and female pronuclei (*MPN* and *FPN*) of the untreated zygote have undergone DNA synthesis as evident by the presence of silver grains over the nuclei. One of the pronuclei (*FPN*) typically demonstrates a polarity in the distribution of silver grains. This is in agreement with the results of Oprescu and Thibault (13). The presence of silver grains over the proximal portion of one of the pronuclei in this manner corresponds to localization of Feulgen-positive material as described by Dautier and Thibault (5) for the female pronucleus. The male and female pronuclei (*PN*) of the colcemid-treated zygote have incorporated tritiated thymidine but have not migrated centrad.  $\times 400$ .

both the male and the female pronuclei indicates that the sperm aster is involved in the centrad movements of the pronuclei via microtubules (20, 21). (a) The implied role of microtubules in the movements of nuclei and other organelles in a wide variety of biological systems (3) and (b) the presence of a sperm aster concurrently with the movement of the pronuclei and its disappearance at the conclusion of migration support the purported role of the sperm aster during fertilization.

How microtubules bring about the movements of the pronuclei is not known. This problem is similar to that which has been considered in other cellular systems for the movement of various cellular inclusions (3). Two general mechanisms have been considered in these cases: (a) Microtubules actually supply the motive force or (b) they merely function as tracks for movement, the tubules serving in this case only as a supportive framework for some force-generating elements. It has been postulated that in some cases dynein-like bridges move structures over the surface of the microtubules (3). Direct connection between nuclei and microtubules has not been found in zygotes or in somatic cells where nuclear migration has been studied (8). Dense aggregations of microtubules have been observed in the region separating the pronuclei in the brine shrimp, *Artemia* (1). Although there is little evidence to substantiate such a claim, it is possible that these tubules are instrumental in bringing the pronuclei together.

Even though there are a number of similarities between the transport of various cytoplasmic organelles as reported in somatic cells and the movement of pronuclei in zygotes, there are major differences that must be considered in the formulation of any scheme to account for the centrad migration of both the male and female pronuclei. For example, unlike the situation in many somatic cells where cytoplasmic components are moved along parallel arrays of microtubules, in the zygote both the sperm aster and the male and the female pronuclei are transported centrad. Furthermore, neither of the general mechanisms cited above are adequate to explain how microtubules are responsible for the simultaneous movement of the sperm aster and the male and female pronuclei.

It is possible that the fascicles of microtubules in the sperm aster establish forces which move organelles within the cytoplasm separating the two pronuclei and not the nuclei *per se*. Translocation of the intervening cytoplasmic constituents may

ultimately draw the sperm aster and the pronuclei together. In the case of rabbit zygotes, such forces may also be responsible for the establishment of the concentric layers of organelles that come to surround the associated pronuclei (12).

The suggestion that pronuclear migration may be brought about by a streaming of cytoplasmic components, directed by the sperm aster, is not new. Early investigators such as Conklin, Boveri, and Chambers made similar proposals based on microscope observations of living zygotes (cf. 4). These early observations differ from those made more recently of fused BHK cells where nuclei migrate in an orderly and directed manner to the center of the syncytium. Holmes and Choppin (8) conclude that nuclei are not carried by cytoplasmic streaming in BHK syncytia.

Not all movements of the male and female pronuclei are similar or depend exclusively upon the function of a sperm aster. For example, in artificially activated rabbit and sea urchin eggs the female pronucleus migrates centrad (11, 16). The elements necessary for this movement have not been ascertained. These observations do not prove, however, that movement of the female pronucleus in fertilized eggs is not associated with those forces responsible for the migration of the male pronucleus.

Although colcemid inhibits pronuclear movements, it appears to have little, if any, effect on subsequent events of fertilization. That is, the morphogenesis of the pronuclei, characteristic of the concluding events of fertilization, continues to take place. Other than the fact that aspects involving the breakdown of the pronuclear envelope, the condensation of chromosomes and the loss of nucleoli do not take place in closely associated pronuclei, they are morphologically similar to events taking place in control specimens. In colcemid-treated zygotes, the chromosomes that condense within each pronucleus are "lost" to the cytoplasm since a spindle fails to form. Such a result is to be expected on the basis of the known effect of colcemid on microtubules. These results indicate that if cooperative nuclear interaction between the male and the female pronuclei is necessary for the terminal events of fertilization in rabbit zygotes, it does not appear to depend upon their close physical proximity (cf. also 7).

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