THE EFFECTS OF NICOTINE ON FERTILIZATION IN THE SEA URCHIN, *ARBACIA PUNCTULATA*

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ABSTRACT

The number of sperm incorporated into eggs made polyspermic with varying concentrations of nicotine (0.025–0.25%, v/v) appears to be directly related to the concentrations employed. The cortical response is morphologically equivalent to that observed in control preparations. Shortly after their incorporation all of the spermatozoa undergo structural events normally associated with the development of the male pronucleus in monospermic eggs. During the reorganization of the spermatozoa, sperm asters are formed. The number of male pronuclei that initially migrate to and encounter the female pronucleus is usually one to three. When pronuclei come into proximity to one another the surface of the female pronucleus proximal to the advancing male pronuclei flattens and becomes highly convoluted. Subsequently, the pronuclei contact each other and the outer and inner membranes of the pronuclear envelopes fuse, thereby producing the zygote nucleus. The male pronuclei remaining in the zygote after this initial series of pronuclear fusions continue to differentiate, i.e. they enlarge, form nucleolus-like bodies, and undergo further chromatin dispersion. In approximately 90% of the zygotes, all of the remaining male pronuclei progressively migrate to the zygote nucleus and fuse to form one large nucleus by 80 min postinsemination. Mitosis and cleavage of the polyspermic zygote occurs later than in monospermic eggs.

INTRODUCTION

The pioneering studies of the Hertwigs (1887) and the investigations of Clark (1938) constitute much of what we know concerning agents which induce polyspermy, i.e., the entrance of more than one spermatozoon into an egg. The nature of the mechanism which prevents polyspermy in sea urchins has been studied by numerous investigators (Wilson, 1925; Rothschild and Swann, 1950; Hagström and Allen, 1956, Allen, 1958; Monroy, 1965; Runnström, Hagström, and Perlmann, 1959). Many of the forementioned studies have attempted to correlate the propagation of the block to polyspermy with the morphological events associated with the cortical reaction and the formation of the activation calyx (see Monroy, 1965). These investigators concluded that at the time of insemination the sperm initiates a change in the cortex of the egg which renders it inaccessible to further insemination. Moreover, it is generally held that polyspermy is a result of an alteration in the manner in which this change is propagated over the surface of the egg (Lillie, 1919).

Although polyspermy in the sea urchin is pathological (Harvey, 1956), treatment of eggs with various chemical agents and the entrance of

more than one sperm during insemination adds a further dimension to the elucidation of the many facets of fertilization remaining unsolved. Boveri (1902) was one of the first investigators to take advantage of the additional parameter offered by the polyspermic condition in his elegant experiments on multipolar mitosis in dispermic sea urchin eggs, whereby he conclusively demonstrated that chromosomes are qualitatively distinct and individual in the determinants they carry.

Most studies of polyspermy have been primarily concerned with the initial events of gamete interaction and have paid relatively little attention to its subcellular effects or consequences during later stages of development. The present investigation was undertaken to study the intricate sequence of events involving the egg and the sperm during polyspermy and to compare these with those occurring during monospermy. In this manner it may be possible to obtain more specific information about pronuclear development and fusion by exaggerating the mechanisms associated with these events, and by selecting factors that are common to monospermy and polyspermy. This communication is concerned with an analysis of those events of polyspermy induced by nicotine treatment in the sea urchin, *Arbacia punctulata*.

MATERIALS AND METHODS

Eggs and sperm of *Arbacia punctulata*, acquired from the Marine Biological Laboratory, Woods Hole, Massachusetts, during the months of June and July, were obtained according to procedures previously described (Longo and Anderson, 1968). In an effort to obtain a high percentage of synchronously developing embryos following nicotine treatment, we found it necessary to employ eggs from a single female for each experiment. A similar situation has also been reported by Harvey (1936). After they had been washed in sea water, the eggs were treated with nicotine in concentrations ranging from 0.025 to 0.25% (v/v) for 5 min, fertilized, and permitted to develop at 20°–23°C with constant stirring. Control preparations consisting of eggs treated in the same manner except for the incubation in nicotine were inseminated with the same sperm dilution. Sperm were added to the nicotine-treated and untreated eggs so that the final concentration was approximately 2–5 $\times$ 10^6 spermatozoa/ml (Harvey, 1956). For purposes of clarity, we have referred to insemination as the introduction of the spermatozoa to the egg suspension, and this marks the zero point from which all timing measurements are made.

In some experiments the eggs were washed once in sea water following the nicotine treatment, and then fertilized. To obtain a sequence of development from fertilization to cleavage, eggs were collected and fixed at the following intervals subsequent to insemination: every 15 sec for 2 min; every 2 min for 20 min followed by every 5 min for 70 min. In addition, unfertilized eggs incubated in various concentrations of nicotine (ranging from 0.025 to 0.25%) for periods from 2 to 10 min were collected and fixed. Some eggs were also washed and agitated according to the methods of Lillie (1914) and Harvey (1914) for the removal of the jelly layer, treated with nicotine, and fertilized according to the methods described for jelly-intact eggs. Sperm suspensions, incubated for 5 min in each of the concentrations of nicotine employed, were examined to be sure that they maintained their motility. Observations indicated that all fertilizable eggs were inseminated well within this period.

Eggs and zygotes were prepared for light and electron microscopy according to procedures previously reported (Longo and Anderson, 1968). Cultures, consisting of fertilized eggs, previously treated with various concentrations of nicotine, were also examined with phase microscopy.

RESULTS

Greater than 98% of all the eggs treated in the various concentrations of nicotine were observed to be polyspermic following insemination and to undergo similar morphological events of fertilization and development. In general, a correlation was apparent between the degree of polyspermy and the concentration of nicotine, i.e. di- and trispermy were prevalent at the lower concentrations of nicotine, whereas at higher concentrations fertilization normally involved 10–12 or more sperm. A similar progression was also noted by Clark (1938). The following observations pertain primarily to eggs treated with 0.15% nicotine but apply to all of the concentrations used. Minor morphological differences in zygotes from among the various concentrations of nicotine employed will be noted where pertinent.

Control preparations, consisting of eggs treated in the same manner as the experimentals but not incubated in nicotine, exhibited less than 1% polyspermy and underwent the same structural events as previously described for normal development (Longo and Anderson, 1968; Anderson, 1968). Unfertilized eggs incubated in various concentrations of nicotine for 2–10 min appeared to be morphologically equivalent to untreated eggs at light and electron microscopic levels of observation.

Within 15 sec after insemination, numerous spermatozoa were observed in contact with eggs that had been treated with nicotine (Fig. 1 and *inset*). Adherence of the gametes appeared to be more stable than in the controls or irreversible, for fewer spermatozoa were dislodged from the surface of the egg. The untreated eggs did not accumulate supernumerary spermatozoa. Jelly-free eggs appeared to accumulate more spermatozoa at their surfaces than eggs with the jelly layer intact; no other morphological differences were observed.

When spermatozoa are exposed to nicotine concentrations of 0.15–0.25% for periods in excess of 5 min, their motility eventually diminishes until they are no longer capable of fertilization. The spermatozoa recover their activity if they are put into fresh sea water (see also Hertwig and Hertwig, 1887; Rothschild, 1953; Rothschild and Swann, 1950). There was no indication that the nicotine treatment caused the eggs to become more adhesive to one another or to the glassware.

Since the block to polyspermy has been related to some component(s) of the cortical granules, it is necessary to give a brief account of the cortical reaction (Hagström and Allen, 1956; Rothschild and Swann, 1950; Monroy, 1965). The cortical reaction and the formation of the activation calyx are well in progress 30 sec following insemination, i.e., greater than 50% of the surface of all of the eggs that were activated (about 90%) contained dehiscing cortical granules (Fig. 2, "*CG*"). The release of the cortical granules and the formation of the activation calyx was not initiated at one locus as normally observed in the monospermic condition (see Anderson, 1968) but rather occurred at multiple loci which corresponded to the sites of gamete fusion (Fig. 2, *inset*). At each site of attachment of the sperm to the surface of the egg the vitelline envelope becomes disjoined from the oolemma, producing an incomplete activation calyx and the perivitelline space (Fig. 2 and *inset*). Following the detachment of the vitelline envelope there is a wavelike release of the cortical granules beginning at each site of gamete fusion. The release of the cortical granules in such a manner produces areas of dehiscing granules which eventually meet, thereby forming a continuous perivitelline space and activation calyx (approximately 45 sec following insemination). Following the cortical reaction there is also a release of the rodlike structures from vesicular bodies into the perivitelline space as observed in monospermy (Fig. 4). The rodlike structures form a portion of the hyaline layer (see Anderson, 1968).

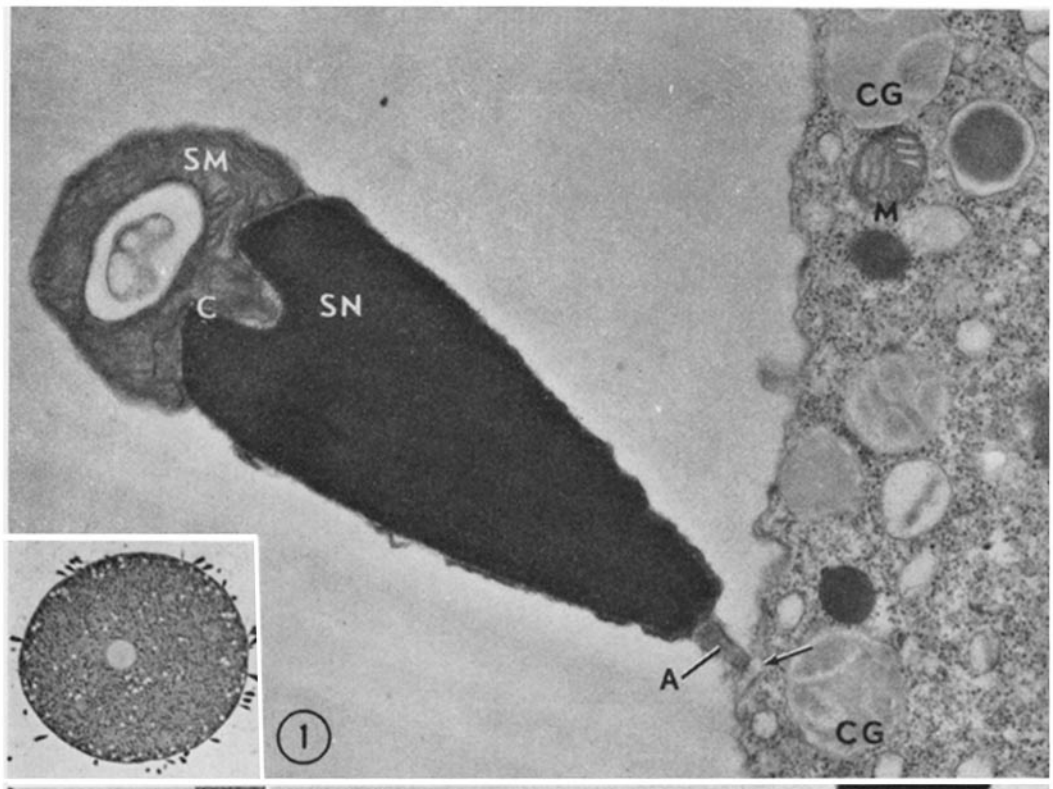
The formation and organization of the activation calyx and the contents of the perivitelline space are the same as found in monospermic eggs; however, there are differences with respect to the time at which certain structures are formed (see Discussion). 1 min after insemination, the activation calyx is fully extended from the surface of eggs treated in nicotine. About 2 min after insemination various areas become thicker and laminated so that the calyx appears to be a more rigid structure (Fig. 3, *AC*). Approximately 6 min after insemination much of the activation calyx appears to be a tough, laminated layer. Approximately 2 min after insemination the materials within the perivitelline space become organized into the hyaline layer (Fig. 3, *HL*).

Incorporation of the Spermatozoa and Formation of the Male Pronucleus and Sperm Aster

At the site of gamete fusion a confluence of the egg and sperm is established and a fertilization

FIGURE 1 Electron micrograph and photomicrograph (*inset*) depiciting the initial contact of the gametes and the aggregation of sperm at the surface of nicotine-treated eggs (*inset*). Note that the cortical reaction has not been initiated. The acrosome (*A*) is associated with some flocculent material at the egg's surface (arrow). *CG*, cortical granule; *M*, mitochondria; *SM*, sperm mitochondrion; *SN*, sperm nucleus; *C*, centriole. Fig. 1, × 25,500; *inset*, × 400, Epon embedded, toluidine blue stained.

FIGURE 2 Electron micrograph and photomicrograph (*inset*) of the cortical reaction and the formation of the activation calyx (*AC*). Two spermatozoa (*S*) are attached to the activation calyx by their acrosomes (*A*). The *inset* shows an egg undergoing the breakdown of cortical granules to either side of attached spermatozoa (arrows). *CG*, cortical granule; *V*, vesicles formed by the fusion of cortical granules ("*CG*") with the oolemma; *, contents of cortical granules which have been discharged into the perivitelline space (*PS*). Fig. 2, × 25,500; *inset*, × 400, Epon embedded, toluidine blue stained.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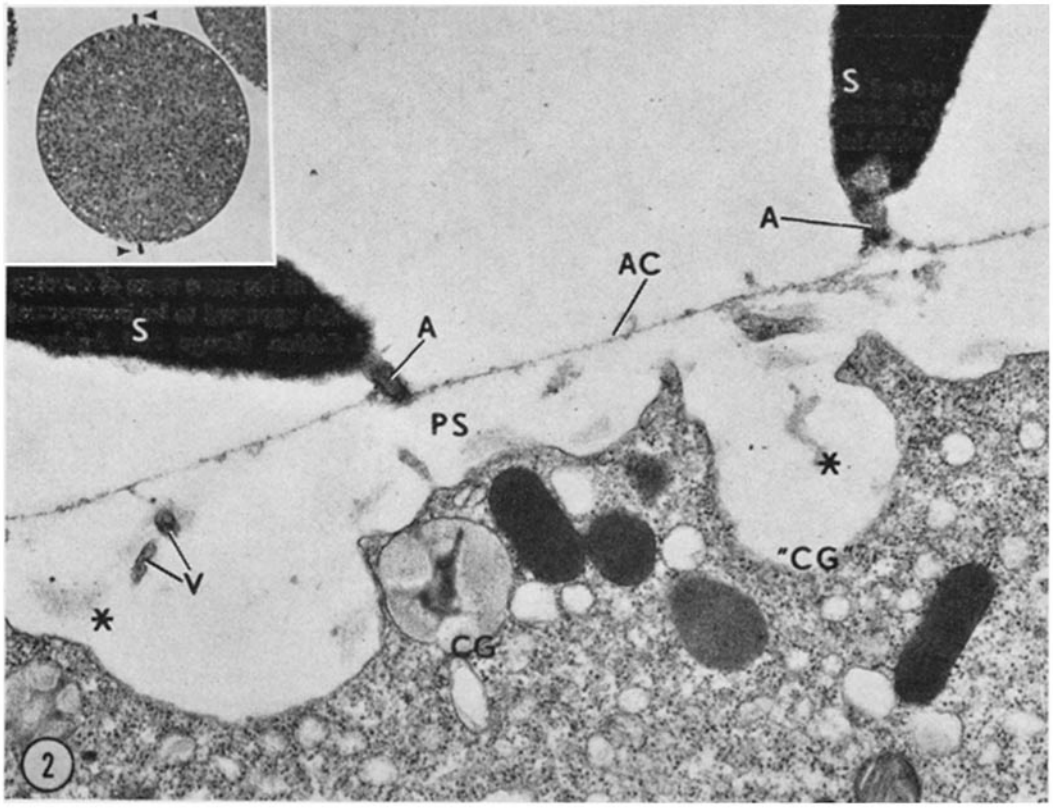
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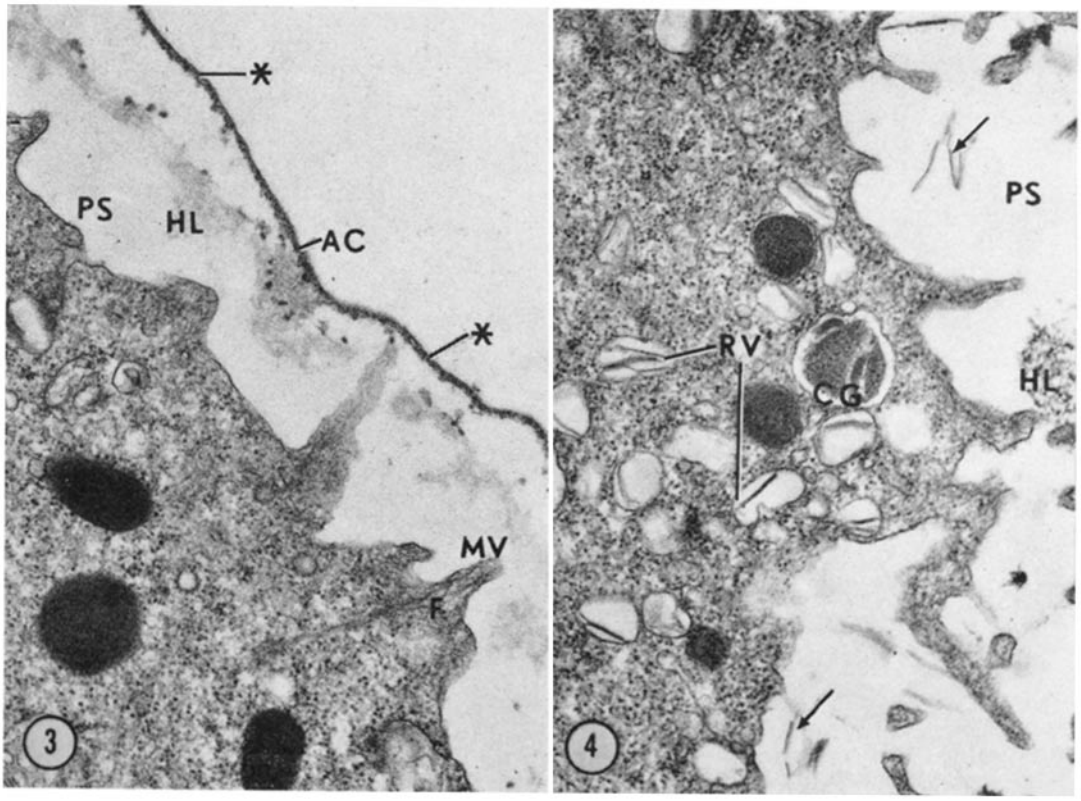


FIGURES 3 and 4 Small portions of the surface of activated eggs, 2 and 8 min after insemination, respectively, showing the hyaline layer (*HL*) and the perivitelline space (*PS*). Arrows indicate rodlike elements which have been discharged into the perivitelline space from the rod containing vesicles (*RV*). *, indicate regions of the activation calyx (*AC*) with a laminated architecture. *F*, filaments located within microvilli (*MV*); *CG*, cortical granule. Figs. 3 and 4, × 25.500.

cone is produced. Although the fertilization cone appears to be larger in polyspermic eggs, its morphology and ontogeny is similar to that previously described for monospermic eggs (Longo and Anderson, 1968). Gamete attachment and fusion takes place within the first 45 sec following insemination, for sperm incorporation was not observed at any time following this period.

Usually the spermatozoon rotates soon after entering the egg; however, occasionally spermatozoa either fail to rotate or remain for extended periods (2–4 min) in the fertilization cone. In the latter cases, the spermatozoa appear to be involved in various phases of morphogenesis normally associated with the reorganization of the sperm nucleus, i.e., the vesiculation of the sperm nuclear envelope and dispersion of the sperm chromatin. Sperm which failed to rotate or commenced pronuclear development within the fertilization cone were at the same stage of development as those which appeared to be incorporated in the normal fashion (Longo and Anderson, 1968).

Development of the male pronucleus in polyspermic eggs follows the same course as observed in monospermic eggs (Longo and Anderson, 1968). All of the male pronuclei that developed within polyspermic eggs appeared to be associated with a sperm aster, a sperm mitochondrion, and flagellum throughout the later stages of development (Fig. 5 and *inset*).

Migration and Fusion of the Pronuclei

Nicotine treatment does not alter the morphology of the female pronucleus (Figs. 1 and 5, *insets*),

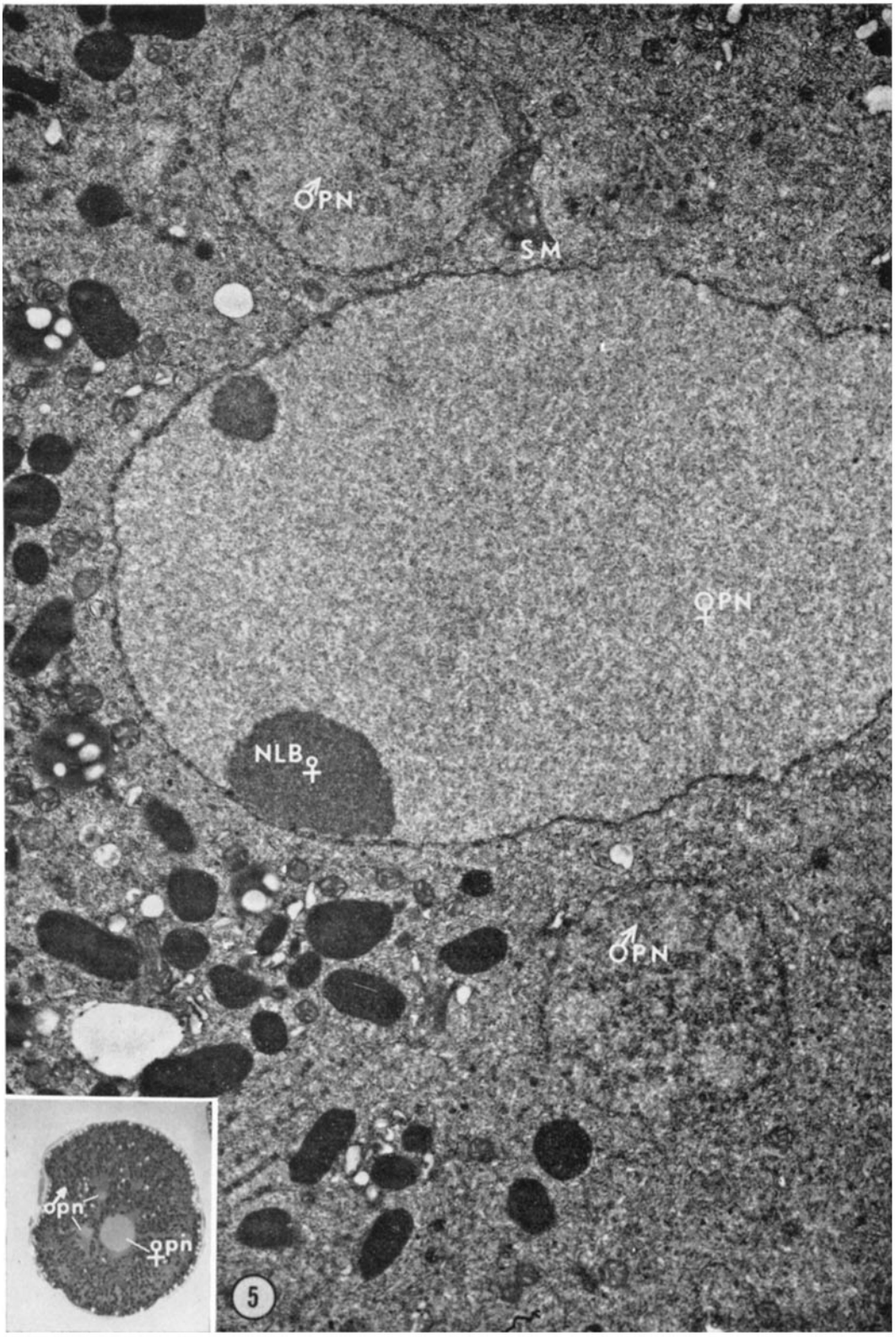


FIGURE 5 Two male pronuclei (♂ *PN*) associated with a female pronucleus (♀ *PN*). The *inset* is a photomicrograph of two male pronuclei (♂ *pn*) migrating to the female pronucleus (♀ *pn*). *NLB*♀, nucleolus-like body located within the female pronucleus; *SM*, sperm mitochondrion. Fig. 5, × 13,000; *inset*, × 400. Epon embedded, toluidine blue stained.

for it remains unchanged from the time of insemination until its encounter with male pronuclei (see Longo and Anderson, 1968).

About 10 min after insemination some of the male pronuclei have migrated to the vicinity of the female pronucleus which, in most cases, has moved to the center of the zygote. The association of the male and female pronuclei involves the intermixing of the constituents of the sperm aster(s) with cytoplasmic matrix surrounding the female pronucleus. As in monospermic eggs the regions of the female pronucleus facing the advancing male pronuclei become flattened and highly irregular (Fig. 5). Subsequently, the outer and the inner lamina of the male and female pronuclear envelopes contact and fuse, thereby forming a zygote nucleus (Fig. 6, *ZN*) (Longo and Anderson, 1968).

Initially, the zygote nucleus has a number of internuclear bridges which reflect the number of male pronuclei that have fused with the female pronucleus over a given period (Fig. 8, inset). The diameter of the internuclear bridges are initially small; however, they gradually increase in diameter, thereby forming a spheroidal or ellipsoidal zygote nucleus. Subsequently, the paternal chromatin is recognized as dense fibrillar masses at the various sites of fusion (Figs. 6, 8, and 11; *PC*). Later, this material diffuses throughout the whole of the zygote nucleus (Fig. 11, inset).

The number of male pronuclei which initially fuse with the female pronucleus is usually one to three. While the initial series of pronuclear fusions is occurring the remaining male pronuclei are located within the peripheral cytoplasm or are in various stages of migration towards the zygote nucleus (Fig. 7 and inset).

Differentiation of the Male Pronuclei

Subsequent to the initial series of pronuclear fusions the remaining male pronuclei continue to develop. This development yields male pronuclei which, in time, resemble a female pronucleus (Fig. 9). Moreover, there appears to be a direct relation between the period a male pronucleus remains in the zygote cytoplasm and its resemblance to a female pronucleus (see Discussion). Male pronuclei, having structural characteristics similar to female pronuclei, have been observed in eggs treated with 0.15% nicotine as late as 90 min following insemination.

During the differentiation of the male pronucleus there is a continued dispersion of the chromatin which appears as a reduction in its electron opacity and a further dissipation of its fibrillar matrix (Figs. 9 and 10). Concomitantly, the male pronucleus increases in volume and may eventually become larger than a female pronucleus, i.e., approximately 12 μ in diameter (Fig. 9, *inset* B).

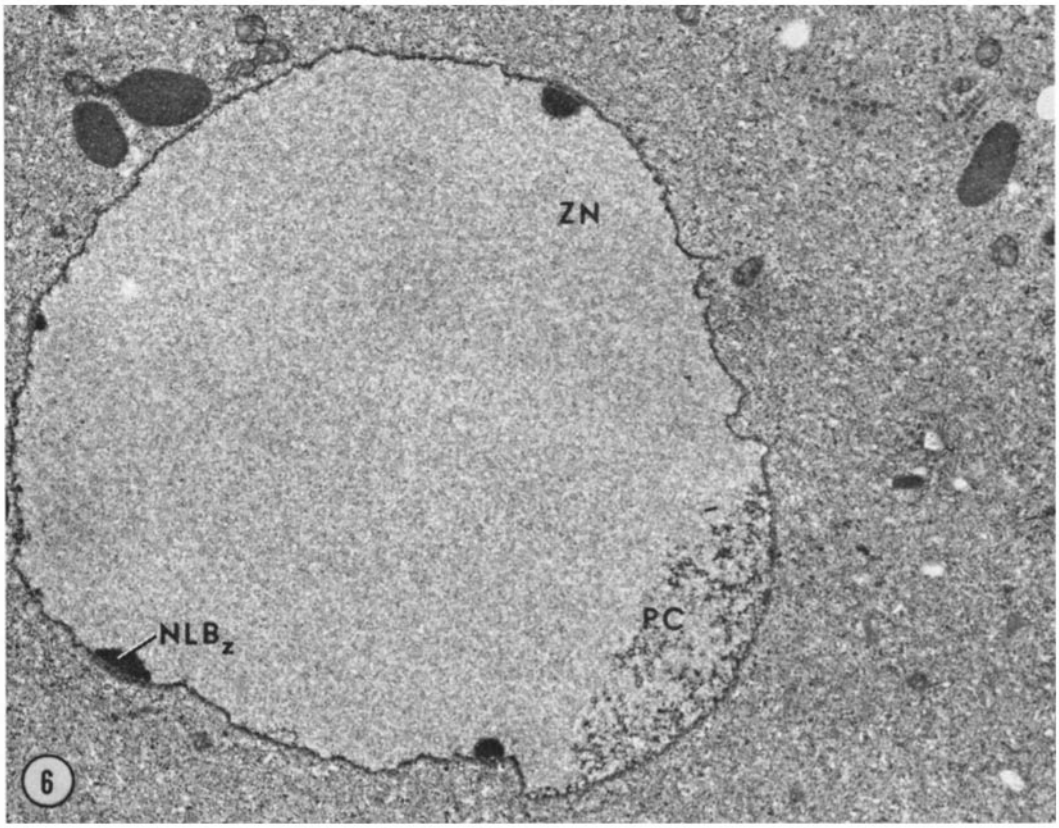
Enlargement of the male pronucleus involves an increase in the pronuclear envelope. The increase of the male pronuclear envelope appears to occur throught the aggregation and fusion of vesicles (see Longo and Anderson, 1968).

Prior to the initial series of pronuclear fusions, male pronuclei were not observed to contain nucleolus-like bodies; however, subsequent to this phase of fertilization these intranuclear structures appear (approximately 14 min postinsemination) (Figs. 7, 9, and 10). At first the nucleolus-like bodies are rather small and sparse; later they become larger and more numerous Structurally, the nucleolus-like bodies possess a fine texture material and appear to be similar to those found in the female pronucleus or the zygote nucleus. Nucleolus-like bodies have not been observed in the male pronucleus of monospermic eggs (Longo and Anderson, 1968). However, they have also been observed in eggs made polyspermic by chemical agents such as urethane and chloral hydrate (F. J. Longo and E. Anderson, unpublished observations).

A continual migration of male pronuclei toward the zygote nucleus occurs from approximately 15 to 75 min postinsemination in cultures treated

FIGURE 6 Zygote nucleus (*ZN*) containing nucleolus-like bodies (NLB_Z) and a region occupied by paternal chromatin (*PC*). Fig. 6, × 17,500.

FIGURE 7 Electron micrograph and photomicrograph (*inset*) of a male pronucleus (♂ *pn*) and a sperm aster*s* (*a*) migrating to a zygote nucleus (*zn*) 16 min following insemination. *NLB* ♂, nucleolus-like body of the male pronucleus; *C*, centriole; *M*, mitochondrion. Fig. 7, × 25,500; *inset*, × 400, Epon embedded, toluidine blue stained.



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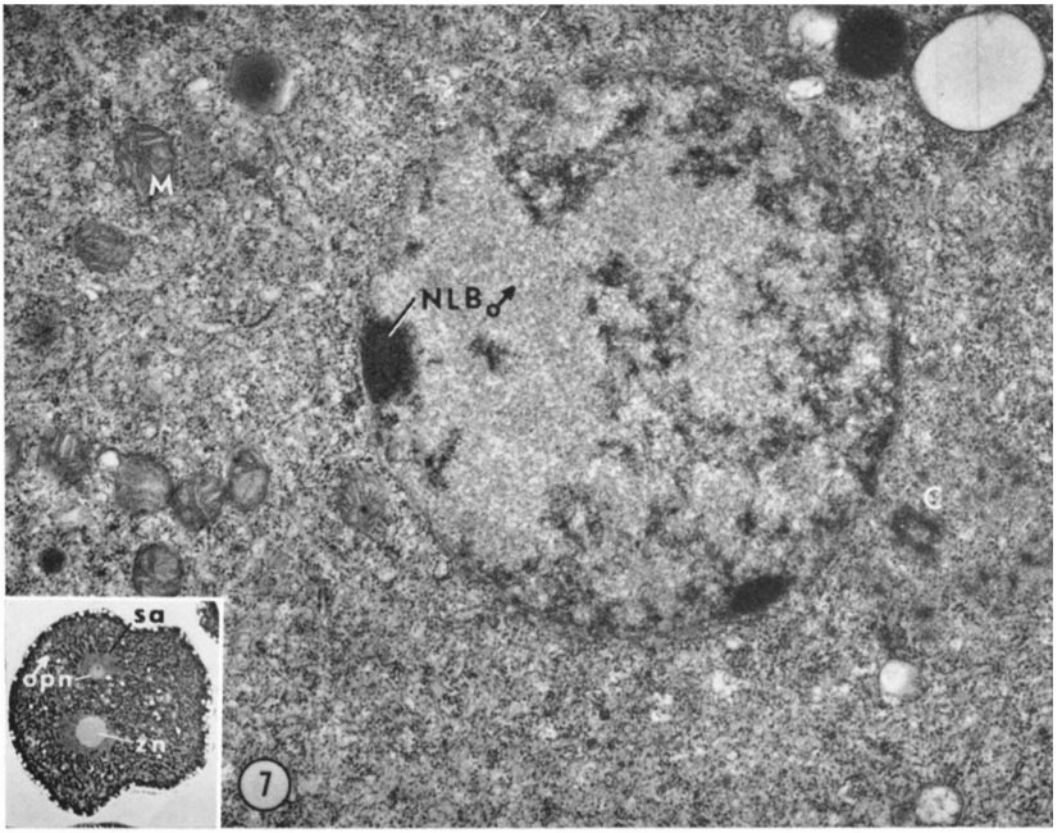

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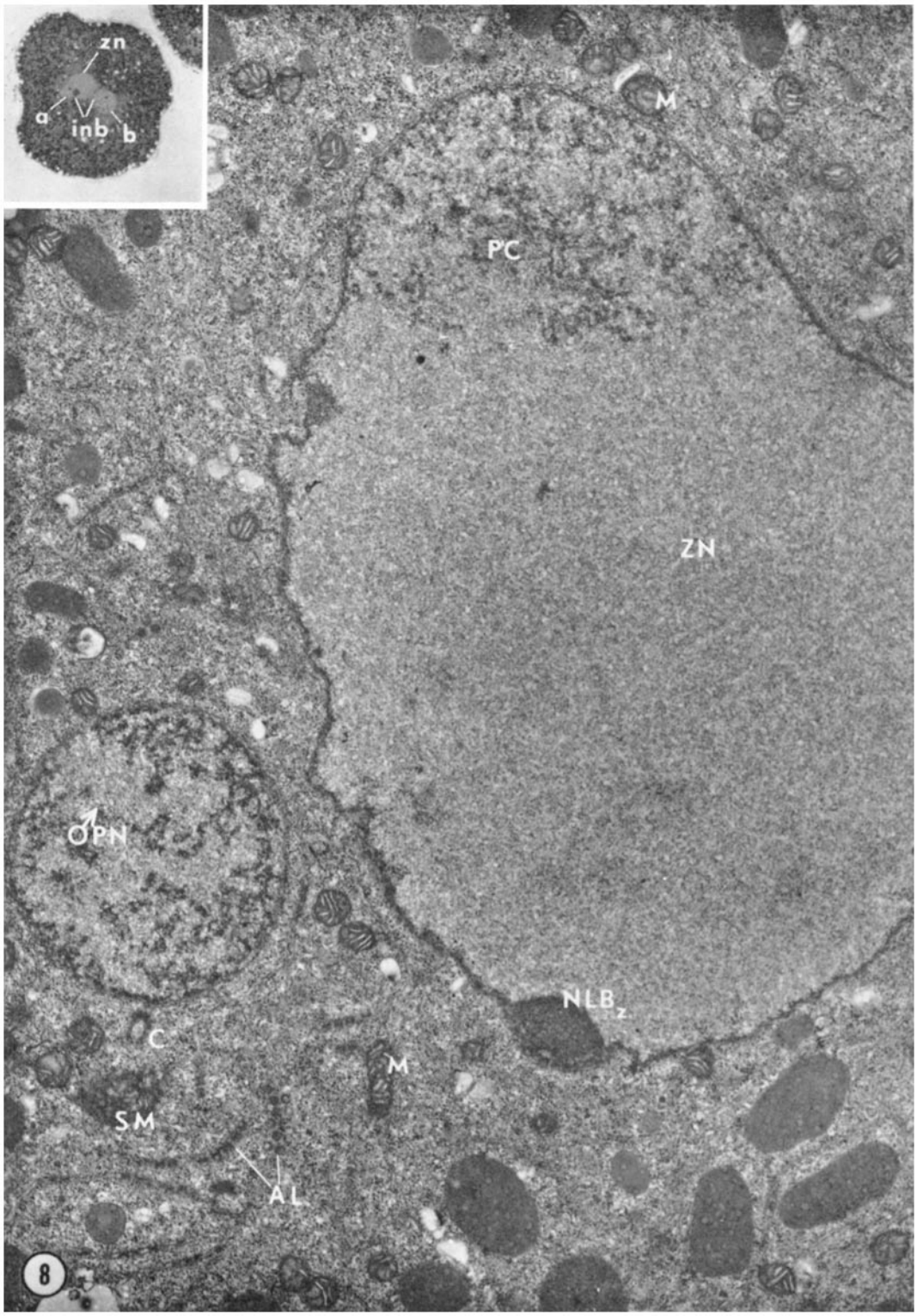


FIGURE 8 Male pronucleus (♂ *PN*) prior to its fusion with the zygote nucleus (*ZN*). *M*, mitochondria; *AL*, annulate lamellae; *C*, centriole; *SM*, sperm mitochondrion; *NLB*$_z$, nucleolus-like body of the zygote nucleus; *PC*, paternal chromatin. The *inset* is a photomicrograph depicting two male pronuclei (*A* and *B*) which are associated with the zygote nucleus (*zn*) via intranuclear bridges (*inb*). Fig. 8, × 17,500; *inset*, × 400. Epon embedded, toluidine blue stained.

with 0.15% nicotine. During this period many male pronuclei are closely associated with one another (Fig. 9, *inset* A). Although male pronuclei were not observed fusing with one another, they have been observed in various stages of fusion with regions of the zygote nucleus that were formerly portions of male pronuclei (Fig. 10).

Eggs treated with 0.15% nicotine undergo synchronous development for about the first 60 min following insemination. Subsequent to this period, many zygotes enter mitosis and cytokinesis while others continue to undergo morphogenic events reminiscent of the later stages of fertilization, i.e., pronuclear migration and fusion. From 60 to 75 min postinsemination male pronuclei are not observed in mitotic embryos whereas nonmitotic zygotes invariably contain at least one male pronucleus. From 75 to 90 min postinsemination the number of mitotic and cleaving embryos increases (up to 90–95%), and concomitantly there is a decrease in the number of zygotes possessing male pronuclei. By 85 to 90 min postinsemination almost all of the zygotes are in the latter stages of mitosis or cleaving, while in a few embryos (5–10%) the zygote nucleus and several remaining male pronuclei are in prophase (see also Hertwig and Hertwig, 1887; Wilson, 1902 and 1925). As far as we were able to determine, in approximately 90% of the eggs treated with nicotine all male pronuclei that develop in the cytoplasm eventually fuse with the female pronucleus or zygote nucleus by 80 min postinsemination. There is no indication that male pronuclei degenerate, i.e., all sperm that enter the egg differentiate into male pronuclei.

Differentiation of the Sperm Aster

During the period in which the male pronuclei differentiate and migrate to the zygote nucleus, the sperm asters enlarge presumably by the acquisition of microtubules, endoplasmic reticulum, and annulate lamellae (Fig. 7, *inset*). This enlargement appears to be proportional to the concentration of nicotine used or more specifically the number of incorporated spermatozoa (see below). Frequently, two or three pronuclei are located at the periphery of the zygote associated with one large sperm aster (Fig. 9, *inset* A). The presence of such an association suggests that several sperm asters have presumably coalesced since all male pronuclei possessed sperm asters prior to the initial series of pronuclear fusions.

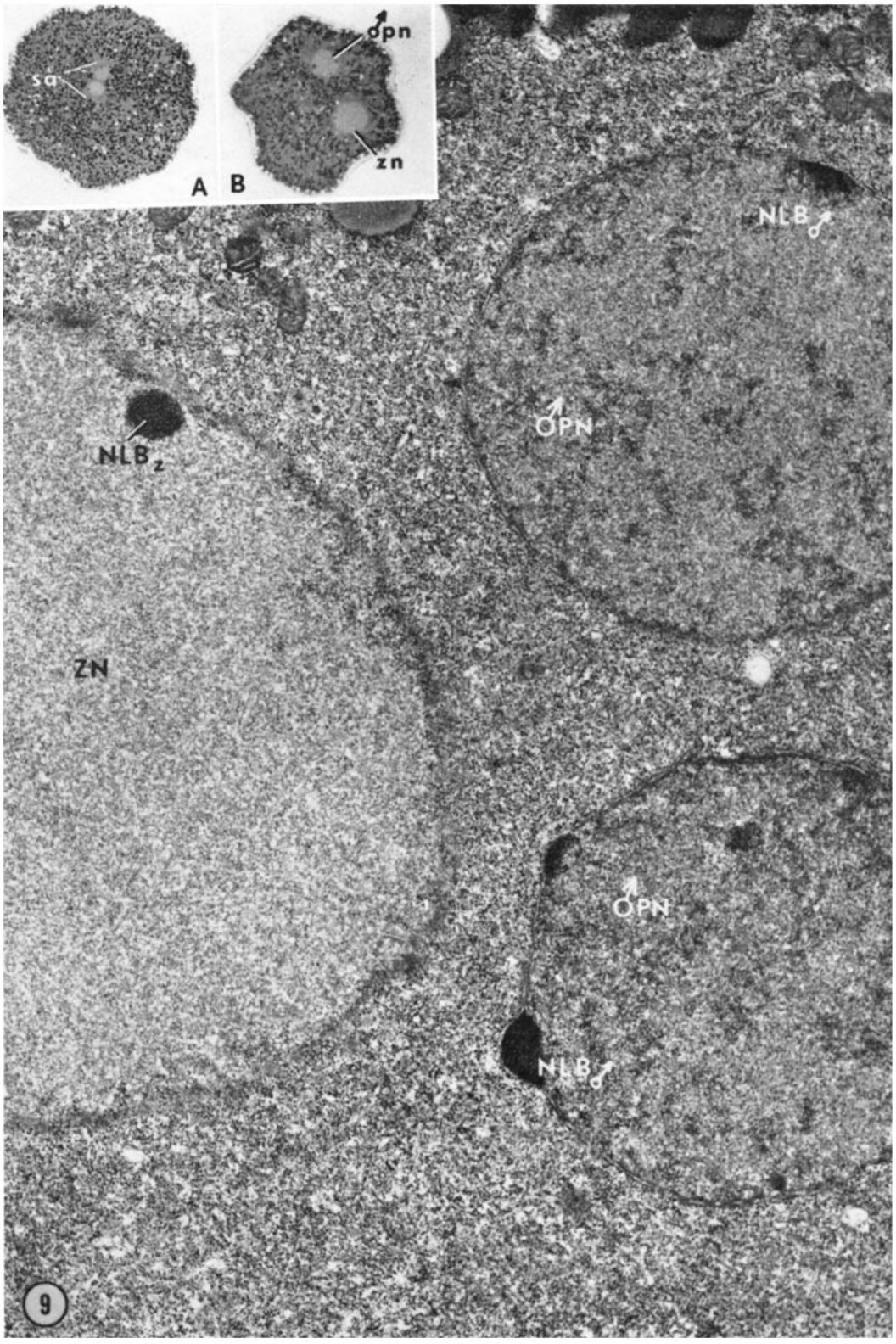
Fusion of Male Pronuclei with the Zygote Nucleus

Subsequent to the initial series of pronuclear fusions, in approximately 90% of the embryos, all of the remaining male pronuclei progressively migrate to the center of the zygote and fuse with the zygote nucleus. In cultures treated with high concentrations of nicotine (0.15–0.25%), as many as six male pronuclei have been observed in the vicinity of the zygote nucleus. The fusion of male pronuclei with the zygote nucleus during the early stages of development is structurally similar to that previously described for the male and female pronuclei (Fig. 8). However, during later stages of development, and presumably after many male pronuclei have fused, the zygote nucleus no longer exhibits the flattening and the extension of nucleoplasmic projections in the direction of the advancing male pronucleus (Fig. 9). Nevertheless, the zygote nucleus (and also the female pronucleus) appears to be receptive for fusion with male pronuclei along any portion of its envelope in either a sequential or simultaneous fashion.

Fusion of the male pronuclei and the zygote nucleus brings about the incorporation of the male pronuclear envelope into the zygote nuclear envelope, including those specialized portions that were derived from the sperm nuclear envelope, e.g., the apical end and centriolar fossa region (Fig. 11, arrow).

With continued development the zygote nucleus increases in size until it is a large spheroid structure, presumably due to the fusion of many male pronuclei (Fig. 11 and *inset*). The size of the zygote nucleus following the fusion of all of the male pronuclei appears to vary directly with the concentration of nicotine employed. For example, in eggs treated with 0.15% nicotine the zygote nucleus attains a diameter of about 30 μ. The zygote nucleus of monospermic fertilization is approximately 13 μ in diameter.

Concomitantly, the size and the number of nucleolus-like bodies increases as does the cytoplasmic area surrounding the zygote nucleus. Within this cytoplasmic area are microtubules, annulate lamellae, and endoplasmic reticulum, much of which is apparently derived from the sperm asters (Fig. 11). A number of centrioles (as many as six have been observed) and sperm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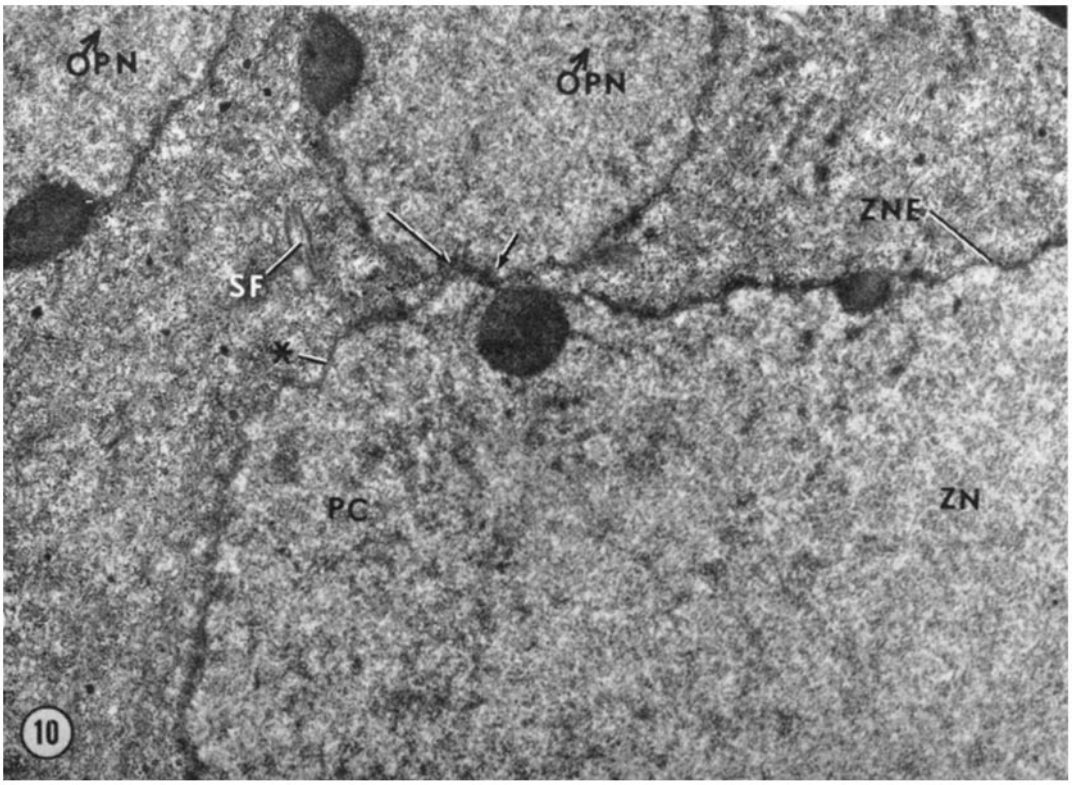


FIGURE 10 Electron micrograph of a male pronucleus (♂ *PN*) fusing with the zygote nuclear envelope (*ZNE*) at a region (arrows) that once was a portion of a male pronuclear envelope (*). *PC*, paternal chromatin within the zygote nucleus (*ZN*); *SF*, portion of a sperm flagellum. Fig. 10, × 17,500.

mitochondria and flagella are also located within this region (Figs. 10 and 11).

DISCUSSION

The data presented in this paper have demonstrated that nicotine induces the eggs of the sea urchin *Arbacia punctulata* to become polyspermic, and that this effect appears to be primarily a result of the agent's action on the female gamete. It has been generally held that polyspermy-inducing agents act by retarding the rate at which the cortical change is propagated over the surface of the egg from the site of sperm entry (Lillie, 1919). Although this may be the simplest and most easily visualized mechanism to explain the observed effects, results from the present study and from others (Clark, 1938; Rothschild, 1953; Hagström and Allen, 1956) are difficult to reconcile with this idea and suggest that the action of nicotine may have an entirely different basis.

Induction of Polyspermy

Studies have indicated that the induction of polyspermy by nicotine may be brought about by one

FIGURE 9 Two male pronuclei (♂ *PN*) adjacent to the zygote nucleus (*ZN*) approximately 30 min postinsemination. NLB_Z, and $NLB_{♂}$, nucleolus-like bodies of the zygote nucleus and male pronuclei, respectively. *Inset* A is a photomicrograph of two male pronuclei located within one large sperm aster (*sa*). *Inset* B is a photomicrograph depicting the large size attained by many male pronuclei (♂ *pn*) during the latter stages of fertilization (approximately 40 min postinsemination). *zn*, zygote nucleus. Fig. 9, × 17,500; *insets* A and B, × 400, Epon embedded, toluidine blue stained.

or more of the following: (*a*) a decrease in the rate of the cortical reaction (Rothschild and Swann, 1950) or its failure (Hagström and Allen, 1956); (*b*) partial fertilization of the eggs (Hagström and Allen, 1956); (*c*) abnormal hyaline layer development (Hagström and Allen, 1956); (*d*) an elimination or retardation of the fast partial block to polyspermy (Rothschild, 1953; 1954); and (*e*) an alteration of the gamete's surface in such a manner as to increase the probability of a successful sperm-egg collision, e.g., the development of a more stable adherence of the sperm to the egg (Rothschild and Swann, 1950; Hagström and Allen, 1956).

Observations made in our study are most easily reconciled, although not entirely, with the suggestion that nicotine promotes a more stable adhesion of the gametes either in a qualitative or quantitative manner and therefore allows more sperm to fuse with the egg than normal. Several facts tend to support such a contention: (*a*) nicotine appears to cause an irreversible adhesion of the sperm to the egg's surface, i.e. when the spermatozoa contact the egg many do not appear to be dislodged, and (*b*) experiments by Werle and Schievelbein (1965) have shown that rabbit blood platelets aggregate in the presence of nicotine and that this aggregation may be due to a change in the electrical properties of the platelet plasma membrane. We recognize the fact that platelets are *portions* of megakaryocytes; however, a similar alteration of the electrical or other properties of the oolemma may also occur upon treatment of the egg with nicotine which would facilitate gamete adhesion. Morphological alterations of the oolemma and of material comprising the vitelline layer in nicotine-treated eggs have not been observed with the electron microscope.

Although the mechanisms of gamete plasma membrane fusion are unknown, it is likely that this process requires a rather stable adhesion of the two gametes. Once the gametes firmly adhere to one another, they would then be free to fuse. Various factors, such as sperm-sperm interference, electrostatic forces, Brownian movements, etc. (see Hultin and Hagström, 1956; Curtis, 1967; Colwin and Colwin, 1967 *a* and *b*; Allen and Hagström, 1955; Hultin, 1956; Hagström and Allen, 1956), may impede or completely destroy the attainment of the proper state of adhesion for gamete fusion. Therefore, not all of the sperm that contact the egg fuse or even adhere, and in order for sperm incorporation to take place the various parameters associated with adhesion and fusion must be fulfilled. With so many and specific constraints on the adhesion and fusion processes, the likelihood of polyspermy is reduced.

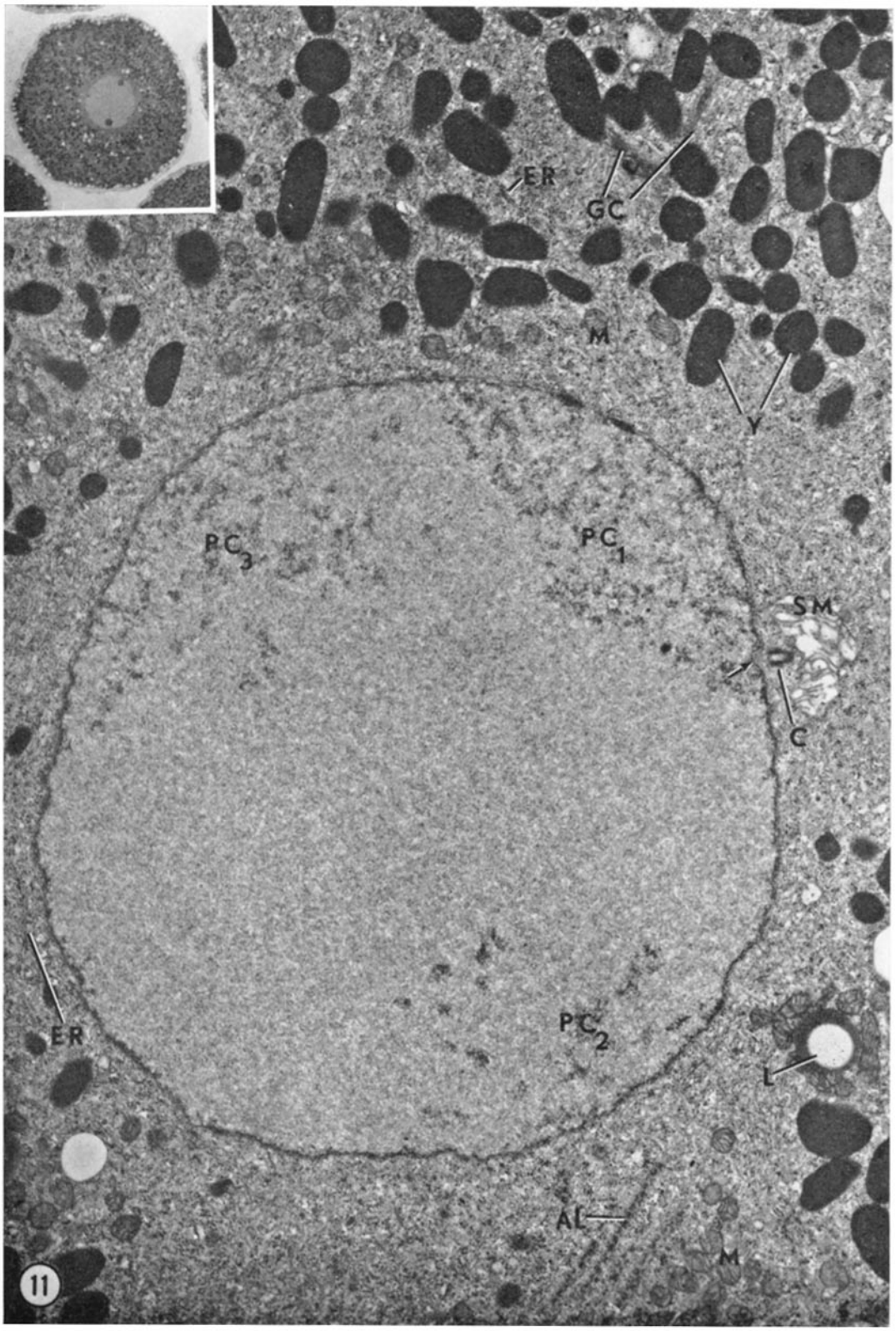
Under normal conditions, i.e. monospermy, at 45 sec following insemination approximately 50% of the egg's surface has undergone the cortical reaction. By 60 sec the cortical reaction is completed. The formation of the activation calyx and the presence of dehiscing cortical granules no doubt disturb the association of the sperm and egg, thereby reducing the probability of many gamete fusions. Micrographs demonstrating spermatozoa apparently adhering to the activation calyx as it is separating from the surface of the egg support this suggestion.

The Cortical Reaction and Formation of the Activation Calyx

Temporally there does not appear to be a decrease in the rate at which the cortical response is propagated and the way the activation calyx and hyaline layer are formed in nicotine-treated eggs. However, these processes are completed before equivalent events in untreated eggs. This decrease in the duration of the cortical response may be a direct consequence of the nicotine itself and/or of the fusion of more than one sperm to the egg's surface, thereby initiating the cortical reaction from many loci.

Anderson (1968) has observed that the development of the laminar structure of the activation calyx occurs at the time of pronuclear fusion (approximately 10–12 min following insemina-

FIGURE 11 Electron micrograph and photomicrograph (*inset*) of zygote nuclei following multiple pronuclear fusions. PC_1, PC_2, and PC_3 indicate regions of the zygote nucleus where three male pronuclei have fused. The arrow indicates a region of the zygote nuclear envelope that was formerly a portion of the sperm nuclear envelope that was associated with the centriolar fossa. *SM*, sperm mitochondrion; *C*, centriole; *AL*, annulate lamellae; *L*, lipid; *Y*, yolk; *M*, mitochondria, *ER*, endoplasmic reticulum; *GC*, Golgi complex. Fig. 11, × 13,000; *inset*, × 400, Epon embedded, toludine blue stained.


ER
GC
M
Y
PC3
PC1
SM
C
ER
PC2
L
AL
M
11

tion), and as the embryo continues to develop the stratum increases in thickness. In eggs treated with nicotine, differentiation of the activation calyx occurs at about 2–4 min after insemination and continues as the polyspermic embryos developed.

The Male Pronucleus

As previously stated, all incorporated sperm appear to differentiate into male pronuclei and were never observed in cleaving zygotes or in blastomeres. This situation is unlike that found in the pig where the majority of sperm nuclei in highly polyspermic eggs experience little if any swelling (Hunter, 1967). Hunter (1967) suggests that differentiation of the sperm nucleus is initiated by an interaction between elements of the spermatozoon and the egg which are present in limited amounts. Thus, failure of the large proportion of sperm nuclei to undergo development may be due to a deficiency in some specific component(s) in the cytoplasm, which is exhausted during the formation of a small number of male pronuclei. The existence of a similar ooplasmic component(s), limited in quantity, which interacts with the sperm to bring about its differentiation is not so readily evident as in the case of polyspermic *Arbacia* eggs, since all incorporated spermatozoa differentiate into male pronuclei. However, spermatozoa which have inseminated *oocytes* (germinal vesicle stage) of *Arbacia* do not develop into pronuclei and form sperm asters (Franklin, 1965). This lack of development has been attributed to the absence of a specific cytoplasmic component(s) necessary for the morphogenesis of the male pronucleus (Lillie, 1914; Longo and Anderson, 1968). Therefore, a cytoplasmic component(s) exists in the mature egg which interacts with the incorporated sperm and is responsible, at least in part, for the development of the male pronucleus and the sperm aster. Experiments with polyspermic eggs, however, demonstrate that this cytoplasmic component(s) is not present in limited amounts, at least according to the methods we have employed.

Development of male pronuclei in eggs made polyspermic with nicotine is fairly well synchronized, indicating that sperm incorporation occurs during a given time period. In connection with this, Anderson (1969) has found that there is a simultaneous incorporation of thymidine-^{3}H into DNA in all of the male pronuclei observed in zygotes made polyspermic with nicotine.

Increase in the size of the male pronuclei and dispersion of the sperm chromatin persists throughout all stages of development. Similar changes have also been noted by Gurdon (1967, 1968) in nuclei transplanted into *Xenopus* eggs. In nuclear transplantation experiments it is uncertain to what extent the increase in volume is necessary for subsequent changes in nucleic acid synthesis (Gurdon, 1967). Gurdon (1968) states that: "... nuclear swelling does not itself induce any one kind of nuclear change, but should be regarded as a process of derepression, the result of which is to make chromosomes more reactive to the particular cytoplasmic environment in which they happen to lie" (see also Gurdon and Woodland, 1968).

Enlargement, further chromatin dispersion, and the acquisition of nucleolus-like bodies yield male pronuclei which possess many characteristics observed in the female pronucleus. An example of a naturally occurring situation where the male pronucleus attains the same or similar structural characteristics as the female pronucleus is found in the *Ascaris* type of fertilization (Wilson, 1925; Longo and Anderson, 1969 *a* and *b*). Wilson (1925) suggested that fertilization may take essentially two forms, depending upon the morphology of the male and female pronuclei when they become associated. The two forms are: (*a*) The sea urchin type of fertilization and (*b*) the *Ascaris* type of fertilization.

One of the characteristics of the *Ascaris* type of fertilization is the large size attained by the male pronucleus, in many cases becoming as large as the female pronucleus (see Longo and Anderson, 1969 *a* and *b*). The sea urchin type of fertilization is characterized by the dramatic inequality of the pronuclei at the time of fusion (see Longo and Anderson, 1968). This difference in the size of the male pronuclei is closely correlated with the meiotic state of the egg and the time it is normally fertilized (Wilson, 1925). Artificial prolongation of the interval between the entrance of the sperm and the fusion of the pronuclei in the sea urchin egg may cause the phenomena of the sea urchin type of fertilization to take on, more or less completely, the character of the *Ascaris* type of fertilization (Wilson, 1902). The observations presented here confirm this suggestion.

Development of the Sperm Aster and Pronuclear Migration

The increase in size of the sperm asters with continued development appears to be due to the

accumulation of large quantities of microtubules, endoplasmic reticulum, and annulate lamellae. Although the egg is richly endowed with a random dispersal of annulate lamellae and endoplasmic reticulum, very few microtubules are found other than those associated with the sperm aster. The origin and method of assembly of these lamellar-tubular structures is unknown.

Concomitant with the enlargement of the sperm aster, the male pronuclei appear to be confined to the peripheral aspect of the zygote. The peripheral displacement of the male pronuclei may be due to the impedence of their migration which is a result of a physical hindrance by the enlarged sperm asters. Such an association would account for the asynchrony that occurs at the later stages of development and the delay in mitosis observed in nicotine-treated embryos. Studies of polyspermy in frog eggs have indicated that pronuclei may be restricted during their movements due to the physical obstruction and growth of adjacent sperm asters (see Morgan, 1927; Rothschild, 1956), and lend support to this suggestion.

Our findings indicate that in most cases the female pronucleus moves from a peripheral position to a more central one following insemination. Concomitantly, the male pronuclei appear to migrate to the center of the zygote following their development. It is possible that the pronuclei are primarily "attracted" to the center of the zygote and secondarily to each other. The movement of the female pronucleus from a peripheral to a central location in the egg following activation with sperm or chemical agents (M. Sachs and E. Anderson, unpublished observations) tends to support this suggestion (see also Wilson, 1925; Tyler, 1955). How pronuclear movement is affected is unknown; however, agents which inhibit microtubule development (e.g. colcemid) delay pronuclear migration and fusion (F. J. Longo and E. Anderson, unpublished observations; Zimmerman and Zimmerman, 1967).

Multiple Pronuclear Fusion

Multiple nuclear fusions normally occur in plants where there is (*a*) a fusion of a male nucleus with the female nucleus and (*b*) the fusion of a second male nucleus with the fused polar nuclei, yielding the triploid nucleus of the endosperm (Jensen, 1964). The fusion of more than two haploid nuclei in the case of the sea urchin *Arbacia punctulata* is pathological.

Although we could not determine unequivocally whether or not male pronuclei fuse with one another, they appeared to fuse sequentially or simultaneously at any locus along the female pronucleus or zygote nucleus and along regions of the zygote nucleus that were once portions of the male pronuclear envelope. These observations testify to the lack of structural or spatial specificity of the fusion process. Austin (1961) has observed that in the polyandrous rat egg the male pronuclei may approach and contact each other as frequently as a male and a female pronucleus, therefore indicating the lack of specificity in the forces that draw the pronuclei together.

In contrast to the situation that exists in many urodeles, reptiles, birds, and insects where the eggs are normally polyspermic (Rothschild, 1954), in most instances all pronuclei fuse together to form one zygote nucleus in nicotine-treated *Arbacia* eggs. Fankhauser (1948), investigating polyspermy in salamanders, showed that the male pronucleus closest to the female pronucleus following meiosis becomes associated with it to produce the diploid state of the embryo. Subsequently, the supernumerary male pronuclei degenerate at various times according to their distance from the associated male and female pronuclei.

Although our findings suggest that the proximity of the male pronucleus and its associated structures induces morphological alterations of the female pronucleus, there appears to be a gradual decrease in the response of the zygote nucleus as greater numbers of male pronuclei fuse with it (see also Longo and Anderson, 1969 *a* and *b*).

Mitosis and Cleavage

The delay of mitosis and cleavage may be due to an "inhibitory effect" of the male pronuclei. The concomitant increase in the number of sperm incorporated and the delay of cleavage supports such a suggestion. These results are in direct contrast to those reported by Rothschild (1953) who states that polyspermic sea urchin eggs (*Paracentrotus lividus*) undergo their first cleavage markedly earlier than monospermic eggs. The reason for this discrepancy is not clear; however, it should be pointed out that the experimental design and organisms employed in both cases differed. One cannot exclude the possibility that nicotine is causing the delay in cleavage directly; however, this appears to be unlikely since other polyspermy-inducing agents (e.g., urethane and chloral hydrate) also delay cleavage in a similar

manner (F. J. Longo and E. Anderson, unpublished observations).

Despite the treatment and the conditions imposed by polyspermy, the sperm and egg organelles continue to undergo specific morphological processes normally observed during monospermy. The results of this study indicate the high degree of independence of the cellular operations and organelles which allow for their individual modification and modulation with only a temporary disturbance of the equilibrium. Hence, the events of fertilization are affected by nicotine primarily quantitatively and not qualitatively.

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