PHYSIOLOGICAL AND MOLECULAR CHANGES OF HAMSTER EGG SURFACES UPON SPERM-EGG INTERACTION

A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN MOLECULAR BIOLOGY IN THE GRADUATE SCHOOL OF THE TEXAS WOMAN'S UNIVERSITY

DEPARTMENT OF BIOLOGY

BY

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DENTON, TEXAS

MAY 1980

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ACKNOWLEDGEMENTS

I wish to thank Dr. Allen E. Waldo, chairman of my graduate committee, for his advice and contribution to this study. And I am grateful to Dr. Kenneth A. Fry, Dr. Michael J. Rudick, Dr. Elaine L. Jacobson and Dr. Alan W. Cockerline for their constructive criticism and advice.

An expression of sincere appreciation to Dr. Prudence Talbot, University of California, Riverside, for her encouragement and suggestion. I extend my thanks to Dr. Gary Martin, University of California, Riverside, for his technical assistance while part of this work was performed there.

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INTRODUCTION

A drastic reduction in number of sperm bound to the zona pellucida (ZP) of hamster egg after fertilization has been observed *in vivo* (Barros and Yanagimachi, 1972). Similarly, *in vitro* fertilized zona-free mouse egg shows a reduction in number of bound sperm on the plasma membrane (Wolf and Hamada, 1979). The cortical reaction has been implicated in altering the binding properties of the egg surfaces which in turn leads to the polyspermy block (Austin and Braden, 1956; Barros and Yanagimachi, 1971; 1972; Gwatkin, 1976; Fukuda and Chang, 1978a). The zona reaction plays a major role in the polyspermy block of hamster egg, whereas the block at the plasma membrane level is relatively weak (Barros and Yanagimachi, 1972). Rabbit egg, however, which does not exhibit a functional zona reaction, relies more on the plasma membrane block (Austin and Braden, 1956; Cooper and Bedford, 1971). Wolf (1978) and Fukuda and Chang (1978a) have recently reported evidence for a strong plasma membrane block in mouse egg which, in addition, exhibits an effective zona reaction.

Lectin labelling of the egg surface has been used to investigate the changes of the lectin-binding sites after

fertilization. The intensity of Concanavalin A (Con A)-peroxidase binding to plasma membrane of mouse egg remains unchanged after fertilization (Solter, 1977). However, fluorescein-Con A labelling indicates a change in the distribution of Con A binding sites from a mosaic pattern before fertilization to a uniformly distributed pattern after fertilization (Johnson et al., 1975).

The present studies use an in vitro reinsemination method to investigate temporal changes in the binding properties and the polyspermy block of hamster zona and plasma membrane surfaces upon sperm-egg interaction. Furthermore, the gold-lectin labelling technique of Horisberger et al. (1975) is used in a scanning electron microscopic analysis of changes in oligosaccharide distribution upon gamete interaction.

REVIEW OF LITERATURE

During mammalian fertilization, sperm undergoes physiological and biochemical changes, a so called "capacitation" (Austin, 1951; Chang, 1951; McRorie, 1974), to acquire fertilizability and to assist in sperm passage through the egg investments. Subsequent interaction of sperm and egg leads to another series of events in egg activation leading finally to the union of sperm and egg nuclei to creat the zygote.

Interaction of gametes includes the primary binding of sperm to egg surface and the eventual fusion of sperm and egg membranes. Gamete binding is a complicated event depending on the maturation stage of sperm as well as that of the egg. Both capacitated and uncapacitated sperm bind to the ZP of homologous eggs, but only capacitated sperm can penetrate the egg (Austin, 1951; Hartmann and Gwatkin, 1971). Sperm binding to egg surface also exhibits a degree of species-specificity (Hanada and Chang, 1972). Capacitated and uncapacitated mouse sperm cannot bind to zonae of hamster eggs (Hartmann and Gwatkin, 1971), suggesting species-specificity of the binding of both capacitated and uncapacitated sperm to the ZP. However,

Bedford (1978) examined binding of uncapacitated sperm to ZP of various mammalian species. Uncapacitated sperm from rabbit, mouse and hamster bind to ZP of eggs from related species as well as to those from species as diverse as human. On the contrary, human sperm fail to bind to ZP of other mammalian species observed except for those from the gibbon. He, thus, concluded that binding, except for human sperm, of uncapacitated sperm to the ZP is not species specific.

Species-specificity plays more prominent role in binding of capacitated sperm to the ZP. Capacitated guinea-pig sperm cannot bind or penetrate hamster ZP (Yanagimachi, 1972) nor can capacitated hamster sperm bind or penetrate mouse ZP (Hartmann et al., 1972). This seems to indicate the existence of species-specific sperm receptors on the zona surface. In contrast, species-specificity of egg plasma membrane varies in different mammalian eggs studied. Zona-free rat and mouse eggs resist penetration by capacitated hamster sperm (Yanagimachi, 1972). Nevertheless, zona-free hamster eggs are penetrable by capacitated sperm from related species such as guinea-pig (Yanagimachi, 1972), rat and mouse (Hanada and Chang, 1972; 1976), and by capacitated sperm from species as distant as human (Yanagimachi et al., 1976).

The kinetics of hamster sperm binding has been studied in a series of investigations by Hartmann and his colleages. In general, the number of sperm bound to the surface is sperm concentration dependent. Uncapacitated hamster sperm bind tightly to zona-intact eggs and mechanically isolated ZP immediately after insemination *in vitro* (Hartmann et al., 1972; Hartmann and Hutchison, 1977a). However, capacitated hamster sperm bind tightly to ZP of zona-intact eggs after a loosely attached period of 30-35 min (Hartmann et al., 1972; Hartmann and Hutchison, 1974a), but bind more readily to mechanically removed ZP (Hartmann and Hutchison, 1976). This seems to implicate the involvement of a factor or factors from the vitellus or perivitelline space in delay binding of capacitated sperm to zona-intact hamster eggs (Hartmann et al., 1972; Hartmann and Hutchison, 1974b). On the other hand, binding of capacitated sperm to hamster egg plasma membrane is immediate (Yanagimachi and Noda, 1970; Usui and Yanagimachi, 1976).

Interaction of capacitated sperm with egg elicits another series of events in egg activation which causes subsequent morphological and physiological changes. An early response to gamete interaction is the cortical reaction (Austin and Braden, 1956) which involves activa-

tion of cortical granules beneath the plasma membrane, fusion of the cortical and plasma membranes and release of their heat labile, trypsin-like cortical content (Gwatkin et al., 1973a; Gwatkin and Williams, 1974). The content released in perivitelline space is, in turn, suggested to alter the conformation of ZP, the so called "zona reaction" (Braden et al., 1954), such that excess sperm cannot bind to the ZP (Austin and Braden, 1956; Barros and Yanagimachi, 1971; Gwatkin et al., 1973a; 1973b). Since zona-free hamster eggs exhibit cortical reactions only upon contact to capacitated hamster sperm and not to homologous uncapacitated sperm (which cannot fuse with or penetrate hamster egg plasma membrane), Gwatkin et al. (1976) suggested that the fusion of capacitated sperm and egg plasma membranes induces the cortical reaction.

An important physiological change upon egg activation is the block to polyspermy; a change which renders the egg impenetrable to excess sperm after entry of the first sperm. A rapid depolarization of egg plasma membranes shortly after fertilization is observed in sea urchin (Jaffe, 1976; Taglietti, 1979), urechis (Gould-Somero et al., 1979) and starfish (Miyazaki and Hirai, 1979). The abrupt shift of membrane potential has been implicated in the mechanism of fast block to polyspermy in these

invertebrates (Jaffe, 1976; Taglietti, 1979; Miyazaki and Hirai, 1979). The development of slow block by activation of cortical reaction occurs later to complete the process of polyspermy block (Jaffe, 1976; Miyazaki and Hirai, 1979).

A rapid block to polyspermy due to depolarization of egg plasma membrane has not been observed in mammalian eggs. Here, the mechanism of polyspermy block has been attributed to the cortical reaction (Austin and Braden, 1956) which occurs within 15 min after sperm contact to the hamster egg plasma membrane (Barros and Yanagimachi, 1972). Therefore, the first block to polyspermy appears to be the zona block initiated by cortical reaction. A second block to polyspermy resides in the egg plasma membrane. Observation from in vivo insemination studies by Barros and Yanagimachi (1972) has shown that the plasma membrane block of hamster is a slow process occurring about 2-3 1/2 hr after sperm penetration. It therefore does not seem to play significant role in hamster polyspermy block. However, mechanisms of polyspermy block vary in mammalian species. Rabbit eggs exhibit weak cortical reaction and the ZP remain penetrable after fertilization (Austin and Braden, 1956). Thus, plasma membrane block plays a more significant role in species

which do not exhibit a cortical reaction. However, existence of plasma membrane block in mouse egg, which also exhibits effective cortical reaction, has been observed in vitro (Pavlok and McLaren, 1972; Wolf and Hamada, 1977; Wolf, 1978; Fukuda and Chang, 1978a).

Changes in binding properties of ZP of fertilized eggs have also been attributed to the cortical reaction. Barros and Yanagimachi (1971) demonstrated that cortical contents released from fertilized zona-free hamster eggs induce zona reaction in unfertilized zona-intact eggs, and in turn greatly reduces the number of sperm bound to the zona surfaces upon further insemination. Similarly, a reduction in sperm receptivity of egg plasma membrane has been demonstrated in fertilized hamster (Usui and Yanagimachi, 1976) and mouse eggs (Wolf and Hamada, 1979).

Heat-soluable ZP preparations from unfertilized hamster (Gwatkin and Williams, 1977) and bovine eggs (Gwatkin and Williams, 1978; Gwatkin et al., 1979) exhibit sperm-receptor activities by interfering with capacitated sperm binding to the ZP of homologous eggs in their presence. In contrast, soluable ZP preparations from eggs which have undergone zona reactions induced by fertilization or electrical stimulation are devoid of such inhibitory effect (Gwatkin and Williams, 1977). Since

mild trypsin or chymotrypsin treatments of hamster ZP prevent capacitated sperm binding, presence of trypsin-sensitive sperm receptors on the ZP is implicated (Hartmann and Gwatkin, 1971). Thus, Gwatkin and Williams (1977) have suggested that a trypsin-like protease released from the cortical contents may destroy the sperm receptors on the ZP. Therefore, soluable ZP preparations from activated eggs do not posses receptor activities. And previously, Oikawa et al. (1973) had suggested that oligosaccharide residues on the ZP may play some role in sperm recognition. Thus, glycoprotein nature of sperm receptors on the ZP is suggested (Gwatkin and Williams, 1977).

Evidence of glycoprotein nature of sperm receptors is derived from studies of osmotically lysed sea urchin egg membranes (Schmell et al., 1977). Presence of membrane preparation inhibits fertilization by competing with receptors on the egg surface. However, membrane preparation from trypsin treated eggs does not affect fertilization. Furthermore, the glycoprotein nature of such preparation is demonstrated by its binding to Con A-sepharose (Schmell et al., 1977). Glabe and Vacquier (1978) made a more direct approach to the problem. They isolated a protease sensitive glycoprotein fraction from ^{125}I-

labelled sea urchin egg surface. The fraction binds specifically to "bindin", a sperm surface protein mediating species-specific recognition and adhesion of sea urchin sperm to egg surface (Vacquier and Moy, 1977). Biochemical analysis for sugar residues reveals that the fraction consists of mannose and galactose but doen not contain sialic acid (Glabe and Vacquier, 1978).

Plant lectins which react to specific sugar residues (Sharon and Lis, 1972) have been widely used as a probe for studies of fluidity of cell membrane and the organization of the oligosaccharide residues on the cell surface. Variation in the effects of certain lectins in inducing agglutination and light scattering properties of the ZP of hamster, mouse and rat eggs may reflect different species-specific sperm receptors on the ZP (Oikawa et al., 1975). Further ultrastructural analysis of the ZP and plasma membranes of rodent eggs utilizing ferritin-conjugated lectins reveals differences in distributions of ferritin-conjugated lectins on the ZP and plasma membranes of homologous eggs (Nicolson et al., 1975). Again, the differences in lectin binding to ZP and plasma membranes may also reflect the differences in sperm recognition sites on those two surfaces.

Lectin binding to egg surface before and after fertilization has also been investigated. Ultrastructural observation using Con A-peroxidase technique reveals discontinuous but even distribution of Con A binding sites on the plasma membrane surface of both unfertilized mouse eggs and the embryos (Solter, 1977). However, Johnson et al. (1975) utilizing fluorescein-Con A labelling detected large negatively stained areas on the plasma membranes of unfertilized mouse eggs. But this mosaicism disappears after fertilization so that the fertilized egg is uniformly labelled with fluorescein-Con A.

Fertilization and sperm binding to the egg surface are also affected by lectins. Pretreatments of zona-intact hamster eggs with wheat germ agglutinin (WGA) and ricinus communis agglutinin (RCA) inhibit fertilization whereas pretreatments of dolichos biflorus agglutinin (DBA) and Con A are less effective (Oikawa et al., 1973; 1974). However, only WGA pretreatment prevents binding and fertilization. Oikawa et al. (1974) concluded that N-acetyl-D-glucosamine-like or N-acetyl-neuraminic-acid-like residues may be essential for sperm recognition on hamster eggs. However, extensive cross-linking of sugar residues and lectins possibly masks the sperm receptor sites such that they are no longer accessible. Thus,

lectin induced inhibition of sperm binding or fertilization could also be due to steric hindrance (Gwatkin, 1976; Yanagimachi, 1977).

In addition to the changes revealed by lectin binding, changes on the surface topography of sea urchin egg upon fertilization have been examined. After insemination, the short and rounded papillae on the vitelline surface of unfertilized eggs gradually change in structure and finally become polygonal in shape and more distant from each other (Schroeder, 1979). Drastic changes on the plasma membrane of sea urchin eggs are also observed. The plasma membrane of the unfertilized egg is covered with uniformly distributed short microvilli. After fertilization, the microvilli change in length and eventually the plasma membrane is covered with mostly short microvilli and a few long microvilli (Schroeder, 1979).

Only studies of changes on the plasma membrane surface of mouse egg upon fertilization have been reported in mammalian species. The plasma membrane surface of a zona-free unfertilized mouse egg consists of both microvillar and smooth regions (Eager et al., 1976) creating a polarity of the surface topography. The smooth region overlies the second metaphase spindle and, thus, disappears from the egg surface to the extruded polar body after fertili-

zation. Therefore, the plasma membrane of fertilized mouse egg is highly microvillous (Eager et al., 1976). Structural polarity of the plasma membrane surface of the unfertilized mouse egg has also been reported by Nicosia et al. (1978). But they described the surface to consist of smooth and ruffled configuration. The surface structure correlates with cytoplasmic content. The ruffled surface overlies the region containing cortical granules whereas the smooth surface overlies the region devoid of granules. Furthermore, the microvilli reappear from the smooth and the upper edge of the ruffled surface after fertilization (Nicosia et al., 1978). This suggests a dynamic state of the egg plasma membrane and the relationship of the surface, nuclear and the cytoplasmic events of the egg.

MATERIALS AND METHODS

Preparation of Capacitated Hamster Sperm and Hamster Eggs

Sperm from lacerated cauda epididymedes of golden hamster, Mesocricetus auratus, were collected in normal saline. The concentration of stock sperm suspension was determined by hemocytometer and adjusted to 2×10^8 sperm/ml. Heat-pretreated (60°C, 1 hr) human blood serum was used as capacitation medium (Yanagimachi, 1970; Barros and Garavagno, 1970). A 100 µl drop of serum was placed on a culture dish (Falcon plastic, cat. no. 3001) covered with paraffin oil (Fisher), then 10 µl of stock sperm suspension was inoculated in the serum droplet to the final concentration of 2×10^7 sperm/ml which is an optimum concentration to obtain hamster sperm acrosome reaction in 4 hr (Talbot et al., 1974). The sperm suspension was incubated at 37°C for 3 hr to acquire capacitated sperm.

Mature female golden hamsters were superovulated by injection of 25-30 IU of pregnant mare's serum gonadotropin (Sigma) on day 1 of the estrous cycle. Cumulus masses from the oviducts were taken about 14-16 hr after injection of 25-30 IU of human chorionic gonadotropin (Sigma) on day 3, and were dispersed by 100-150 units/ml

of hyaluronidase (Sigma, Type 1-S) in modified BWW culture medium developed by Bigger, Whitten and Whittingham (1971) containing 94.59 mM NaCl, 4.78 mM KCl, 1.71 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgCl_2.7\ H_2O$, 25.07 mM $NaHCO_3$, 5.56 mM glucose, 0.25 mM pyruvate, 21.58 mM lactate and 1 mg/ml bovine serum albumin (Sigma, fraction V). Following the dispersion, the eggs were washed 3 times in fresh BWW medium and used as zona-intact eggs. Zona-free eggs were prepared by further digestion of washed zona-intact eggs in 50-100 IU/ml of bovine pancreatic trypsin (Sigma, Type III) and washed 3 times in fresh BWW medium.

Preparation for Study of Binding Property Changes of Hamster Egg Surfaces Upon Sperm-Egg Interaction

Changes in Binding Properties of Zona Pellucida

Zona-intact hamster eggs were preinseminated at 37°C with low concentration of $1x10^6$ sperm/ml by inoculation of 50 µl of capacitated sperm (concentration of $2x10^7$ sperm/ml) into 100 µl of BWW medium containing zona-intact eggs. At various times, 5, 20, 40 and 60 min, after preinsemination, the eggs were transferred to new drops of BWW media and washed 3 times through a small diameter (≈150µ) micropipette to free the attached sperm. A single micropipette was used for each experiment. The numbers of bound sperm

were scored by direct count under x200 inverted phase contrast microscope. The eggs were then reinseminated with $1x10^7$ sperm/ml of fresh capacitated sperm by placing washed preinseminated eggs in a drop of 50 µl BWW medium containing 50 µl of fresh capacitated sperm and incubated further at 37°C. At various times, 5, 20, 40 and 60 min, the eggs were sampled, washed through a micropipette, and the numbers of sperm bound were determined. Numbers of sperm bound to zona-intact eggs inseminated with $1x10^6$ and $1x10^7$ sperm/ml without preinsemination were used as controls. The binding of fresh capacitated sperm used for reinsemination at 60 min was also determined and used as control to ensure that binding changes are due to the egg surface rather than sperm surface changes (C_{60}).

Changes in Binding Properties of Egg Plasma Membrane

Zona-free hamster eggs were preinseminated at 37°C with $1x10^5$ sperm/ml by inoculation of 1 µl of capacitated sperm into 200 µl of BWW medium containing zona-free eggs. At 5, 15,30 and 45 min, samples of preinseminated eggs were washed and the numbers of sperm bound were scored as described above. The eggs were then reinseminated with $1x10^6$ sperm/ml of fresh capacitated sperm by placing washed preinseminated eggs in a drop of 200 µl BWW medium

containing 10 µl of fresh capacitated sperm and incubated at 37°C. Numbers of bound sperm at 5, 15, 30 and 45 min were determined. Numbers of sperm bound to zona-free hamster eggs inseminated with $1x10^5$ and $1x10^6$ sperm/ml without preinsemination were used as controls. The binding of fresh capacitated sperm to zona-free eggs used for reinsemination at 45 min was also determined and used as sperm surface change control (C_{45}).

Preparation for Study of the occurrence of Polyspermy Block

Zona Block Zona-intact hamster eggs were preinseminated initially with low concentration of $1x10^6$ sperm/ml as described in the preparation for study of binding property changes to acquire low levels of polyspermy, i.e., low numbers of penetrated sperm per egg. The eggs were reinseminated after 5, 20 40 and 60 min with a concentration of $1x10^7$ sperm/ml which gave high levels of polyspermy and were then incubated further at 37°C for 2-2 1/2 hr. The eggs were prepared under a vaseline supported cover slip and examined under x1000 phase contrast optics to determine numbers of eggs fertilized and levels of polyspermy. Zona-intact eggs inseminated with fresh capacitated sperm used for the reinsemination at

60 min were also prepared as control for the fertilizability of the sperm.

Plasma Membrane Block Zona-free hamster eggs were preinseminated initially with a low concentration of $1x10^5$ capacitated sperm at 5, 15, 30 and 45 min. After 1 1/2 hr of incubation at 37°C, the numbers of fertilized eggs and levels of polyspermy were determined. Controls of zona-free eggs inseminated with $1x10^5$ and $1x10^6$ sperm/ml and of eggs inseminated with $1x10^6$ sperm/ml of fresh capacitated sperm used for reinsemination at 45 min were prepared.

Preparation of Gold-Lectin Labelled Hamster Eggs for the Scanning Electron Microscopy

Colloidal gold (average size of 50 nm) was prepared as described by Frens (1973). Gold labelled with Con A (Sigma), RCA (Sigma) and WGA (Sigma) were then prepared (Horisberger et al., 1975; 1978; Horisberger and Rosset, 1976; 1977).

Zona-intact and zona-free hamster eggs were inseminated with $1x10^6$ and $1x10^5$ sperm/ml, respectively (see methods described previously). Zona-intact eggs before, and 30 and 60 min after, insemination and zona-free eggs before, and 20 and 45 min after, insemination were washed twice in fresh BWW media and fixed for 15 min in modified

Karnovsky's fixative (Karnovsky, 1965). After 3 changes in 0.1 M sodium cacodylate buffer, pH 7.4, the eggs were incubated in excess gold-lectins for 1 hr at room temperature. The controls were incubated in gold-lectin containing 0.2 M specific inhibitors, α-methyl-D-mannoside (Sigma), D(+) galactose (Sigma) and N-acetyl-D-glucosamine (Sigma) for Con A, RCA and WGA, respectively. The eggs were then washed 3 times for 5 min each in 0.1 M sodium cacodylate buffer, pH 7.4, and refixed in modified Karnovsky's fixative for 2 hr. The fixed eggs were washed in 3 changes of 0.1 M sodium cacodylate buffer, pH 7.4, and postfixed for 5 min in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.4. Subsequently, the eggs were washed briefly in 0.1 M sodium cacodylate buffer, pH 7.4, and were dehydrated in a graded series of ethanol (30% through 95%) for 15 min each and 3 changes of 100% ethanol for minimum of 1/2 hr each. The dehydrated specimens were then carbon dioxide critical point dried, coated with gold-palladium, and examined on an AMR 1200 SEM.

^{125}I-Con A Labelling of Hamster Egg Plasma Membrane Surface

Briefly fixed zona-free hamster eggs before, and 45 min after, insemination (see methods described previously) were washed in 3 changes of 0.1 M sodium cacodylate buffer,

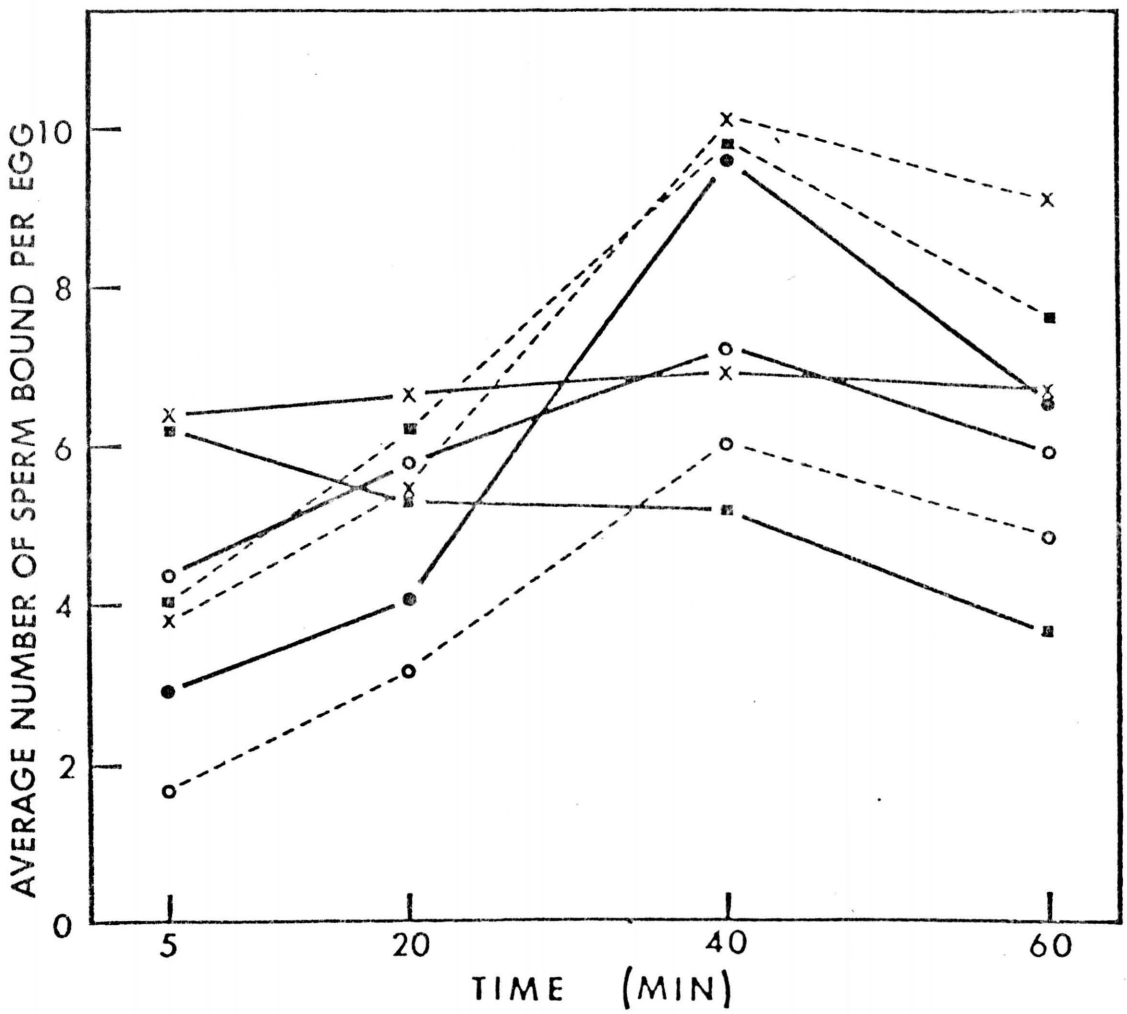
pH 7.4, and 1 change of sodium phosphate buffer, pH 7.5. The eggs (20) were then incubated for 1 hr in 50 µl of 50 µg/ml ^{125}I-Con A specific acitivity of 0.5 µCi/µg (New England Nuclear). Controls were incubated in ^{125}I-Con A with addition of 0.2 M α-methyl-D-mannoside. After incubation, the eggs were washed 6 times in sodium phosphate buffer, pH 7.5, and dissolved overnight in 20 µl of 90% formic acid. Radioactivity was determined by scintillation counting of the samples in 5 ml of Ready-To-Use II aqueous scintillation fluid (Eastman) in a Beckman LS 9000 liquid scintillation counter.

RESULTS

Changes of the Binding Properties of Hamster Egg Surfaces Upon Sperm-Egg Interaction

The number of capacitated sperm bound to the ZP of zona-intact hamster eggs at various times after insemination with $1x10^6$ and $1x10^7$ sperm/ml, which represent the low and high concentration controls respectively, follow the same pattern (Fig. 1). The low concentration control consistently gives lower number of bound sperm/egg at each time interval. The average number of bound sperm/egg from high concentration control increases linearly to the maximum of 10.1±1.82 SEM at 40 min after insemination. The number of sperm bound to control C_{60} (see materials and methods) eggs at each time interval closely resembles ($p>0.05$) that of the high concentration control. Similarly, number of bound sperm/egg at each time interval of zona-intact eggs preinseminated for 5 min does not differ significantly ($p>0.05$) from that of the high concentration control. Alteration of the binding properties of the ZP is observed in eggs preinseminated for 20 min. The number of bound sperm/egg reaches a lower ($p<0.05$) maximum than that of the high concentration control at 40 min (Fig. 1).

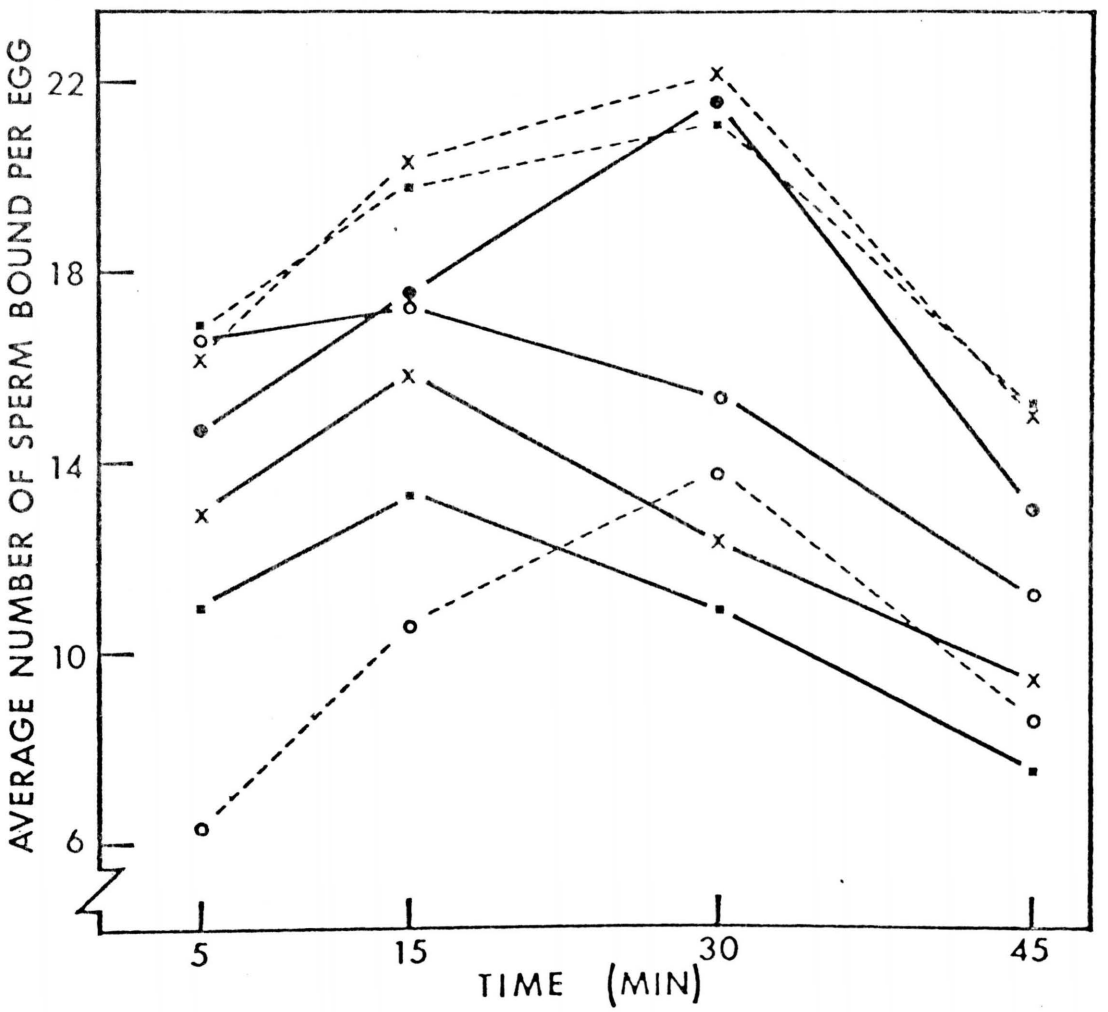
Figure 1. Average number of capacitated sperm bound per egg to the ZP of preinseminated zona-intact hamster eggs at various times after reinsemination. •——•, o——o, x——x and ▪——▪ represent number of sperm bound per egg to eggs preinseminated with $1x10^6$ sperm/ml for 5, 20, 40 and 60 min, respectively, followed by reinsemination with $1x10^7$ sperm/ml. •---•, x---x and ▪---▪ are controls representing number of sperm bound per egg to zona-intact hamster eggs inseminated with $1x10^6$, $1x10^7$ sperm/ml and with $1x10^7$ sperm/ml of fresh capacitated sperm used for reinsemination at 60 min after preinsemination, respectively.


AVERAGE NUMBER OF SPERM BOUND PER EGG
0
2
4
6
8
10
5
20
40
60
TIME (MIN)

Drastic changes in the binding properties of zona-intact eggs preinseminated for 40 and 60 min are observed. There is no increase in number of bound sperm/egg ($p>0.05$) by reinsemination of eggs preinseminated for 40 min, i.e., there is no addition of new bound sperm by reinsemination (Fig. 1). Furthermore, eggs preinseminated for 60 min show a steady decline in the number of bound sperm (Fig. 1).

Capacitated sperm bind more readily to egg plasma membrane than they do the the ZP. Again, the number of capacitated sperm bound to plasma membranes of zona-free hamster eggs at various times after insemination with $1x10^5$ and $1x10^6$ sperm/ml (low and high concentration controls respectively) follow the same pattern, except for the consistent lower number of bound sperm/egg from those inseminated with the lower sperm concentration (Fig. 2). The number of bound sperm/egg increases linearly with time and reaches a maximum of 22.1±1.96 SEM by 30 min after insemination of zona-free hamster eggs with $1x10^6$ sperm/ml. The control C_{45} (see materials and methods) gives the same ($p>0.05$) number of bound sperm/egg as that from the high concentration control at each time interval (Fig. 2). The number of sperm bound to the plasma membrane of eggs pre-

Figure 2. Average number of capacitated sperm bound per egg to plasma membranes of preinseminated zona-free hamster eggs at various times after reinsemination. ●——●, O——O, x——x and ▮——▮ represent number of sperm bound per egg to zona-free hamster eggs preinseminated with $1x10^5$ sperm/ml for 5, 15, 30 and 45 min, respectively, followed by reinsemination with $1x10^6$ sperm/ml. O---O, x---x and ▮---▮ are controls representing number of sperm bound per egg to zona-free hamster eggs inseminated with $1x10^5$, $1x10^6$ sperm/ml and with $1x10^6$ sperm/ml of fresh capacitated sperm used for reinsemination at 45 min after preinsemination, respectively.


AVERAGE NUMBER OF SPERM BOUND PER EGG
22
18
14
10
6
5
15
30
45
TIME (MIN)

inseminated for 5 min at each time interval is also similar ($p>0.05$) to that of the high concentration control and, thus, reaches the same maximum ($p>0.05$) at 30 min after reinsemination. However, zona-free hamster eggs preinseminated for 15 min clearly show alteration of the binding properties of the plasma membrane. The number of bound sperm/egg reaches the maximum of 17.3±2.05 SEM by 15 min after reinsemination, which is lower ($p<0.05$) and earlier than that of the high concentration control. Eggs preinseminated for 30 and 45 min have a similar pattern. There are fewer additional sperm bound following reinsemination and the number of bound sperm/egg reaches a lower ($p<0.05$) and earlier (15 min) maximum than that of the high concentration control (Fig. 2).

Occurrence of Polyspermy Block

The percentages of fertilized hamster eggs in the present studies range from 95 to 100. Increase in levels of polyspermy by reinsemination with higher sperm concentration is used to evaluate the occurrence of polyspermy block. Dispersion of penetrated sperm nuclei in the egg cytoplasm is used as criterion for fertilization (Figs. 3 and 4).

Minimum and maximum polyspermy control levels of

Figure 3A. Phase contrast micrograph of zona-intact, fertilized hamster egg. Dispersed, penetrated sperm nucleus (arrow) and the zona pellucida (ZP) are shown. x650.

Figure 3B. High magnification phase contrast micrograph of dispersed sperm nucleus (arrow) in the zona-intact hamster egg. x1,500.

Figure 3C. High magnification phase contrast micrograph of zona-intact, fertilized hamster egg. Dispersed, penetrated sperm nuclei (arrow) and zona pellucida (ZP) are shown. x2,000.

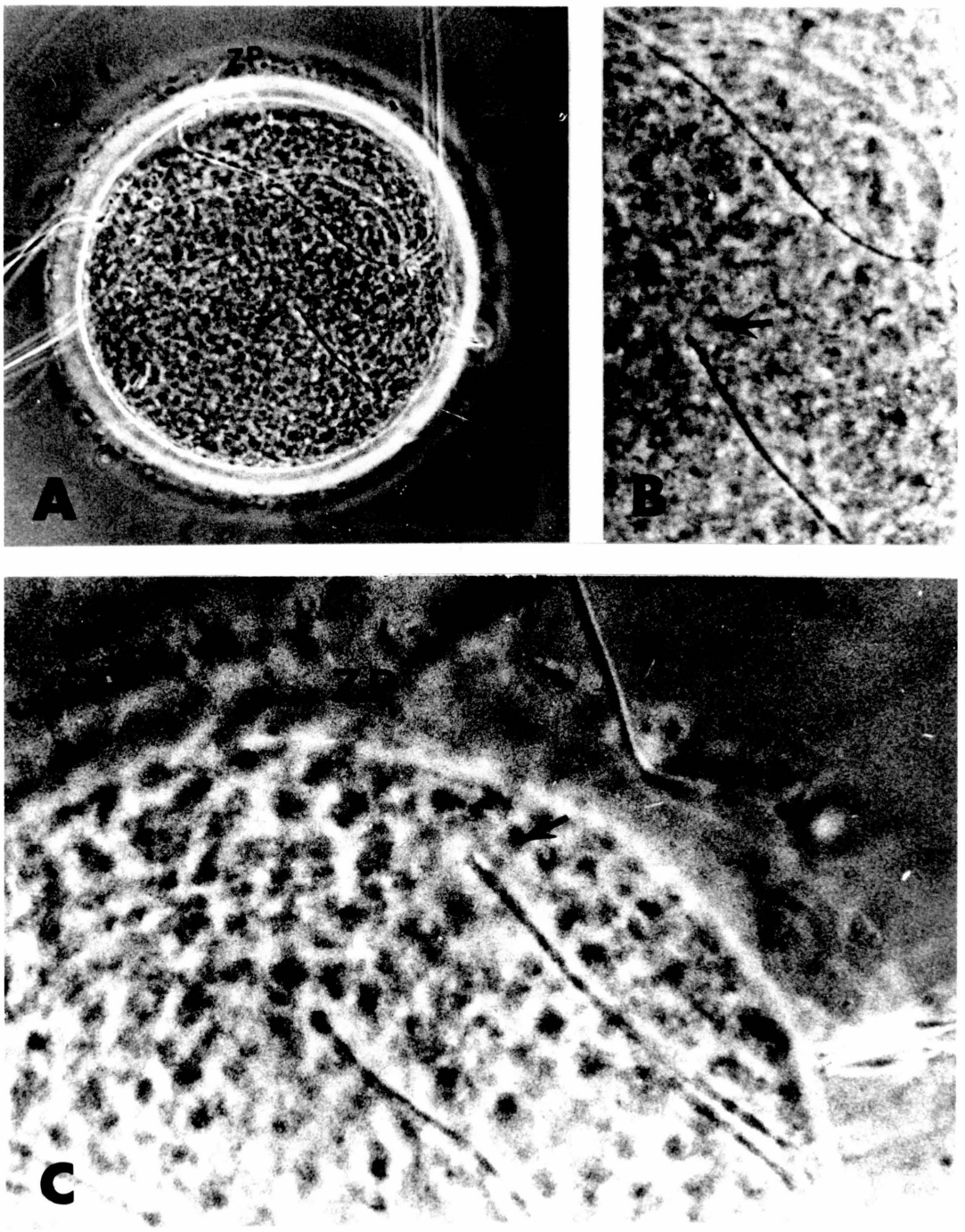
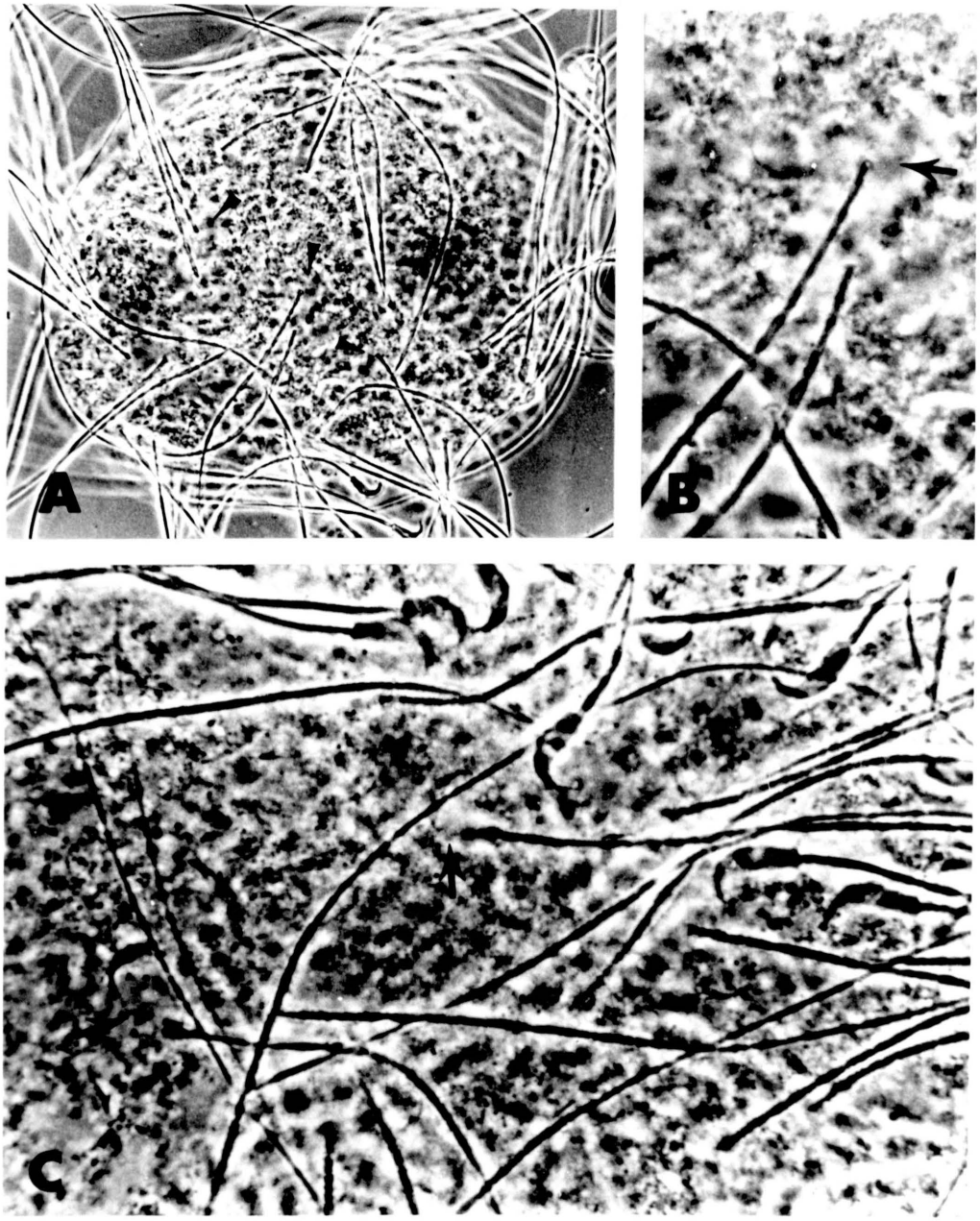

ZP
A
B
C

Figure 4A. Phase contrast micrograph of zona-free, fertilized hamster egg. A large number of penetrated sperm with dispersed nuclei in the egg cytoplasm are seen (arrows). x750.

Figure 4B. High magnification phase contrast micrograph of dispersed sperm nuclei (arrow) in the zona-free hamster egg. x1,500.

Figure 4C. High magnification phase contrast micrograph of zona-free, fertilized hamster egg. Dispersed, penetrated sperm nuclei (arrows) at different stages are seen. x2,000.

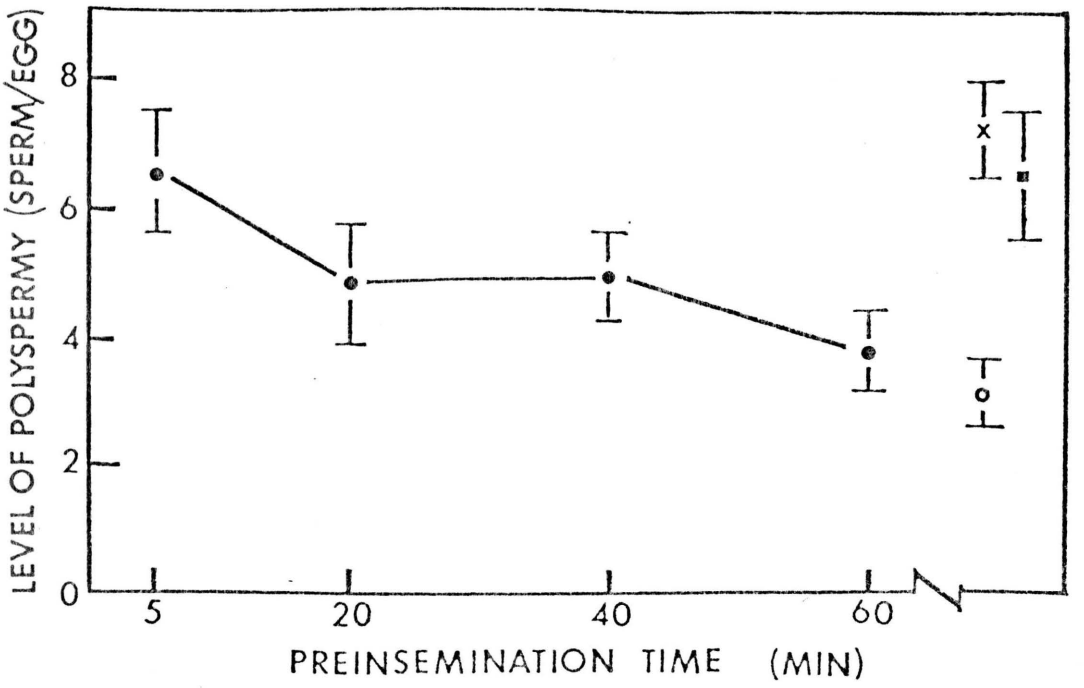

A
B
C

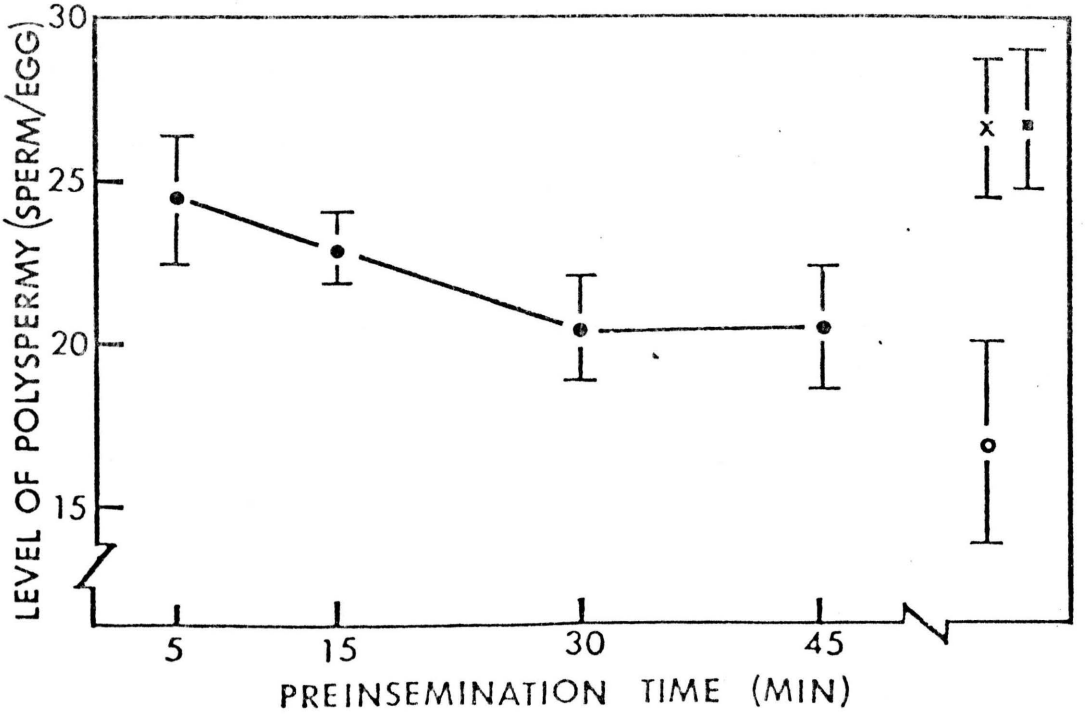
zona-intact hamster eggs inseminated with $1x10^6$ and $1x10^7$ sperm/ml are 3.0±0.57 SEM and 7.2±0.92 SEM sperm/egg, respectively (Fig. 5A). Level of polyspermy of zona-intact eggs preinseminated for 5 min does not differ significantly ($p>0.05$) from that of maximum control (Fig. 5A). On the other hand, levels of polyspermy of eggs preinseminated for 20 and 40 min drop significantly ($p<0.05$) to 4.8±0.78 SEM and 4.9±0.63 SEM sperm/egg, respectively, and the polyspermy levels of eggs preinseminated for 60 min reaches that of the minimum control ($p>0.05$). There is no significant difference ($p>0.05$) between levels of polyspermy of maximum control and that of the capacitated sperm control used for reinsemination at 60 min (Fig. 5A).

Zona-free hamster eggs inseminated with $1x10^5$ and $1x10^6$ sperm/ml give minimum and maximum polyspermy control levels of 16.9±3.13 SEM and 26.6±2.27 SEM sperm/egg, respectively. Zona-free eggs preinseminated for 5 min give the same ($p>0.05$) level of polyspermy as that of maximum control (Fig. 5B). However, the polyspermy level of eggs preinseminated for 15 min is significantly lower ($p<0.05$) than that of the maximum control. The polyspermy levels of eggs preinseminated for 30 and 45 min gradually

Figure 5A. Levels of polyspermy of zona-intact hamster eggs preinseminated for various times followed by reinsemination with higher sperm concentration. Each point represents the average level of polyspermy±standard error of means of 6 different experiments. ○, x and ▪ represent levels of polyspermy of zona-intact eggs inseminated with $1x10^6$ and $1x10^7$ sperm/ml and with $1x10^7$ sperm/ml of fresh capacitated sperm used for reinsemination at 60 min after preinsemination, respectively.

Figure 5B. Levels of polyspermy of zona-free hamster eggs preinseminated for various times followed by reinsemination with higher sperm concentration. Each point represents the average level of polyspermy±standard error of means of 6 different experiments. ○, x and ▪ represent levels of polyspermy of zona-free eggs inseminated with $1x10^5$ and $1x10^6$ sperm/ml and with $1x10^6$ sperm/ml of fresh capacitated sperm used for reinsemination at 45 min after preinsemination, respectively.


LEVEL OF POLYSPERMY (SPERM/EGG)
0
2
4
6
8
5
20
40
60
PREINSEMINATION TIME (MIN)


LEVEL OF POLYSPERMY (SPERM/EGG)
15
20
25
30
5
15
30
45
PREINSEMINATION TIME (MIN)

decline to a level close ($p>0.05$) to that of minimum control (Fig. 5B). There is no significant difference ($p>0.05$) between levels of polyspermy of maximum control and that of the capacitated sperm control used for reinsemination at 45 min (Fig. 5B).

Gold-Lectin Distributions on Unfertilized and Fertilized Hamster Egg Surfaces

Gold-lectin labelling of zona-intact and zona-free hamster eggs reveal different distributions of lectin receptors on the zona and plasma membrane surfaces Gold-RCA distributes uniformly over the entire zona surface of the unfertilized egg (Fig. 6A). The labels are seen as tiny spots of gold residue (arrows, Fig. 6A). There is no significant observable change in intensity of gold-RCA labelling on the zonae of eggs 30 (Figs. 6B and 6D) and 60 min (Fig. 6C) after insemination. Zona-intact eggs labelled with gold-RCA in the presence of 0.2 M D-galactose are devoid of gold labelling (Figs 6E and 6F). Gold-WGA binds intensely to the zona surface of unfertilized hamster egg and appears as a thick coating (Fig. 7A). Intense gold-WGA binding is also observed on the ZP of eggs 30 (Figs. 7B and 7D) and 60 min (Fig. 7C) after insemination. Gold binding is not found in eggs labelled

Figure 6A. Gold-RCA treated zona-intact, unfertilized hamster egg. There are spots of gold-RCA (arrows) over the entire zona surface. x5,000.

Figure 6B. Gold-RCA treated zona-intact hamster egg 30 min after insemination. The gold labels (arrows) are found over the entire zona surface and on the sperm head (arrow). x5,000.

Figure 6C. Gold-RCA treated zona-intact hamster egg 60 min after insemination. There is no significant change in gold-RCA distribution and intensity of binding on the zona. The gold labels (arrows) are found uniformly distributed over the entire zona. x5,000.

Figure 6D. Lower magnification scanning electron micrograph of gold-RCA treated zona-intact hamster egg 30 min after insemination. Gold-RCA is evenly distributed over the entire egg surface. Non-specific large gold aggregates (arrow) are found occasionally. x2,000.

Figure 6E. Zona-intact, unfertilized hamster egg treated with gold-RCA in the presence of D-galactose. There is absence of gold-RCA binding. Few non-specific gold aggregates (arrow) are found. The zona surface contains fibrous networks and porous structures. x5,000.

Figure 6F. Zona-intact hamster egg 30 min after insemination treated with gold-RCA in the presence of D-galactose. There is absence of gold binding. Changes in the zona structure are evident; large "depressions" (small arrows) and distortions of the ZP (large arrow) can be seen. x4,500.

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Figure 7A. Gold-WGA treated zona-intact, unfertilized hamster egg. Individual gold spots are indistinguishable due to the intense labelling over the entire zona surface; the gold-WGA labelling appears as a thick coat on the network structures (arrow). x5,000.

Figure 7B. Gold-WGA treated zona-intact hamster egg 30 min after insemination. The ZP remains densely labelled with gold-WGA. x5,000.

Figure 7C. Gold-WGA treated zona-intact hamster egg 60 min after insemination. No significant change in the intensity of gold binding is seen; the thick coat of gold label remains. x5,000.

Figure 7D. Lower magnification scanning electron micrograph of gold-WGA treated zona-intact hamster egg at 30 min after insemination. The entire egg is covered with a thick coat of gold-WGA x2,000.

Figure 7E. Zona-intact hamster egg 30 min after insemination treated with gold-WGA in the presence of N-acetyl-D-glucosamine. Note lack of gold labelling. Changes of the zona structure include large "depressions" (small arrows) and distortions of the zona surface at the region of sperm penetration (large arrow). The acrosome of detached sperm is found on the zona surface (a). x1,800.

Figure 7F. Zona-intact hamster egg 60 min after insemination treated with gold-WGA in the presence of N-acetyl-D-glucosamine. The ZP does not show gold labelling; a penetrating sperm in a "channel-like" structure can be seen. x5,000.

A
D
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a

Figure 8A. Gold-Con A treated zona-intact, unfertilized hamster egg. The ZP exhibits scattered gold-Con A distribution (arrows). x1,800.

Figure 8B. Gold-Con A treated zona-intact hamster egg 30 min after insemination. A scattered distribution of gold-Con A is seen (arrows). x2,000.

Figure 8C. Gold-Con A treated zona-intact hamster egg 60 min after insemination. The ZP shows a slight increase in the intensity of gold-Con A labelling. x1,800.

Figure 8D. Higher magnification scanning electron micrograph of gold-Con A treated zona-intact, unfertilized hamster egg. A scattered gold-Con A distribution of the ZP is observed. x5,000.

Figure 8E. Zona-intact, unfertilized hamster egg treated with gold-Con A in the presence of α-methyl-D-mannoside. Note the absence of gold labelling. Only a few non-specific gold residues (arrow) are found. The zona surface has well defined fibrous networks and porous structures. x8,000.

Figure 8F. Zona-intact hamster egg 60 min after insemination treated with gold-Con A in the presence of α-methyl-D-mannoside. The ZP is completely devoid of gold labelling. Marked change on the zona structure is observed. Large "channel-like" structures (arrows) are evident. The fibrous networks become less well defined and portions of the zona surface become convoluted (*). x5,000.

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with gold-WGA in the presence of 0.2 M N-acetyl-D-glucosamine (Figs. 7E and 7F). Gold-Con A usually forms aggregates (seen as larger gold spots) in a scattered distribution over the zona surface of unfertilized egg (Figs. 8A and 8D). There is no significant change in the gold-Con A distribution and intensity on the zona surface 30 and 60 min after insemination (Figs. 8B and 8C). No labelling, or only some non-specific labelling, is observed in control zona-intact eggs labelled with gold-Con A in the presence of 0.2 M α-methyl-D-mannoside (Figs. 8E and 8F).

Gold-lectin residues are usually bound to the microvilli of zona-free hamster eggs, and these microvilli are relatively indistinguishable from each other in comparison with those from the controls labelled in the presence of specific inhibitors. This effect is possibly due to cross linking of the gold lectins such that microvilli are bound together.

Gold-RCA labels the entire plasma membrane surface of unfertilized zona-free hamster eggs (Fig. 9A). Dense distributions of gold-RCA over the plasma membrane 20 min after insemination is also observed (Figs. 9B and 9D). The intensity of gold-RCA binding declines slightly by 45 min after insemination, at which time a drastic change in the surface topography is also observed (Fig. 9C). Gold-

WGA binds intensely to the entire plasma membrane surface of both unfertilized eggs (Fig. 10A) and of eggs 20 min after insemination (Figs. 10B and 10D). Although there is drastic change in surface topography of plasma membrane by 45 min after insemination, gold-WGA labelling along the upper edges of the ruffled surface is detected (arrow, Fig. 10C). Gold-Con A does not distribute uniformly on the plasma membrane surface and patches of label are seen on unfertilized eggs (Fig. 11A). The labelled area is small 20 min after insemination (Figs. 11B and 11D), and by 45 min after insemination the gold label is normally not detected (Fig. 11C), although occasionally a small amount of labelling is observed (not shown). All controls in the presence of appropriate specific inhibitors are devoid of gold binding (Figs. 9E, 9F, 10E, 10F, 11E and 11F).

Changes of Surface Topography of Hamster Eggs Upon Fertilization

In the course of these studies, changes in the surface topography of zona and plasma membrane have been observed following fertilization. The various control samples can be used to illustrate these changes.

The zona surface of unfertilized hamster egg has well defined fibrous networks and porous structures (Figs. 6E

Figure 9A. Gold-RCA treated zona-free, unfertilized hamster egg. Uniform distribution of gold labelling over the entire plasma membrane surface is seen. x5,000.

Figure 9B. Gold-RCA treated zona-free hamster egg 20 min after insemination. The plasma membrane shows dense labelling of gold-RCA. Also note the spots of gold residues along the sperm tail. x5,000.

Figure 9C. Gold-RCA treated zona-free hamster egg 45 min after insemination. The plasma membrane surface which previously had long-slender microvilli now has a ruffled appearance. Spots of gold-RCA (arrows) are found in a scattered distribution over the plasma membrane surface and on the sperm head. x5,000.

Figure 9D. Lower magnification scanning electron micrograph of gold-RCA treated zona-free hamster egg at 20 min after insemination. Dense gold-RCA labels the entire egg. x1,800.

Figure 9E. Zona-free hamster egg 20 min after insemination treated with gold-RCA in the presence of D-galactose. The plasma membrane surface is devoid of gold labelling. The microvilli on the surface still retain a long-slender shape. Note the smooth region (*) lacking microvilli. Fusion of microvilli over the post-acrosomal region of penetrating sperm head is occasionally seen (arrow). x5,000.

Figure 9F. Zona-free hamster egg 45 min after insemination treated with gold-RCA in the presence of D-galactose. The plasma membrane surface shows complete absence of gold binding (cf. Fig. 9C) and the surface topography has become ruffled. x5,000.

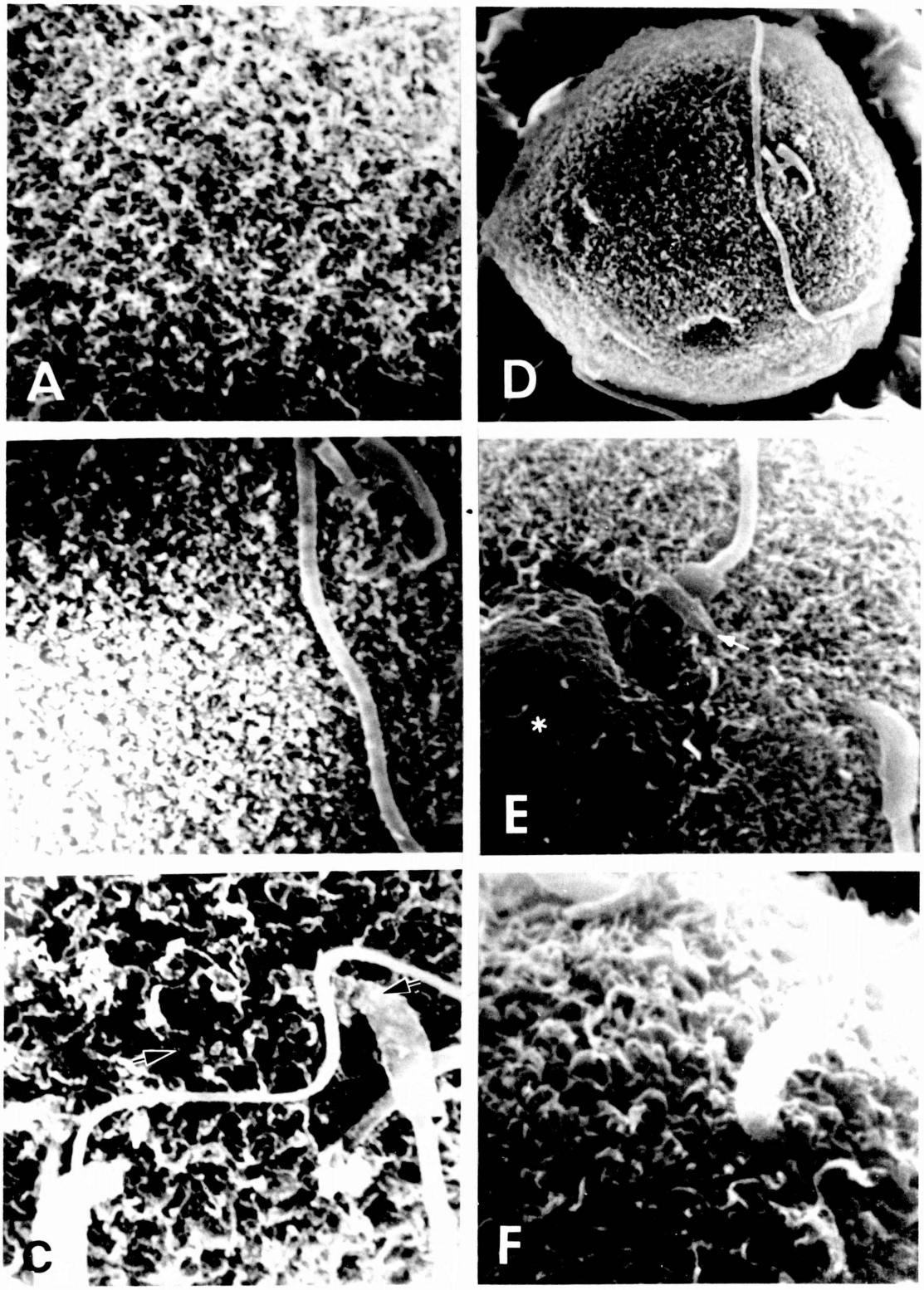
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Figure 10A. Gold-WGA treated zona-free, unfertilized hamster egg. Dense binding of gold-WGA over the microvilli of the entire egg surface is observed (cf. Fig. 10E). x5,000.

Figure 10B. Gold-WGA treated zona-free hamster egg 20 min after insemination. The plasma membrane surface retains intense gold label. x5,000.

Figure 10C. Gold-WGA treated zona-free hamster egg 45 min after insemination. There are drastic changes in the plasma membrane morphology but gold-WGA labelling (arrow) along the upper edges of the ruffled surface remains. x5,000.

Figure 10D. Lower magnification scanning electron micrograph of gold-WGA treated zona-free hamster egg 20 min after insemination. Intense gold binding over the entire egg is seen. x2,000.

Figure 10E. Zona-free, unfertilized hamster egg treated with gold-WGA in the presence of N-acetyl-D-glucosamine. There is absence of gold binding. Long-slender microvilli and a smooth region (*) devoid of microvilli are observed. x5,000.

Figure 10F. Zona-free hamster egg 45 min after insemination treated with gold-WGA in the presence of N-acetyl-D-glucosamine. Gold binding is absent. The microvilli on the surface become shortened at this stage. x5,000.

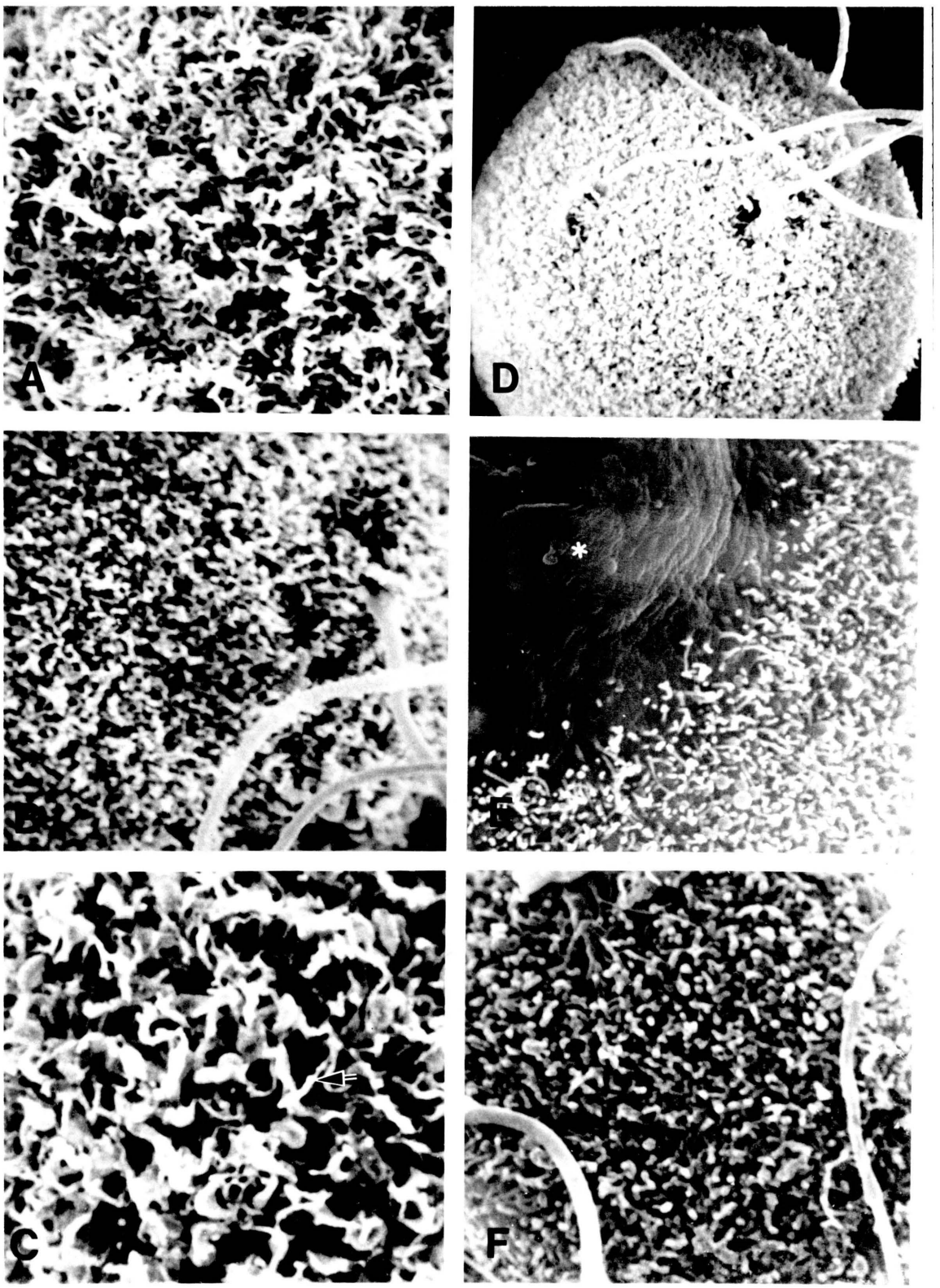
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Figure 11A. Gold-Con A treated zona-free, unfertilized hamster egg. Patches of gold labelling (arrows) are found on the plasma membrane surface. x2,000.

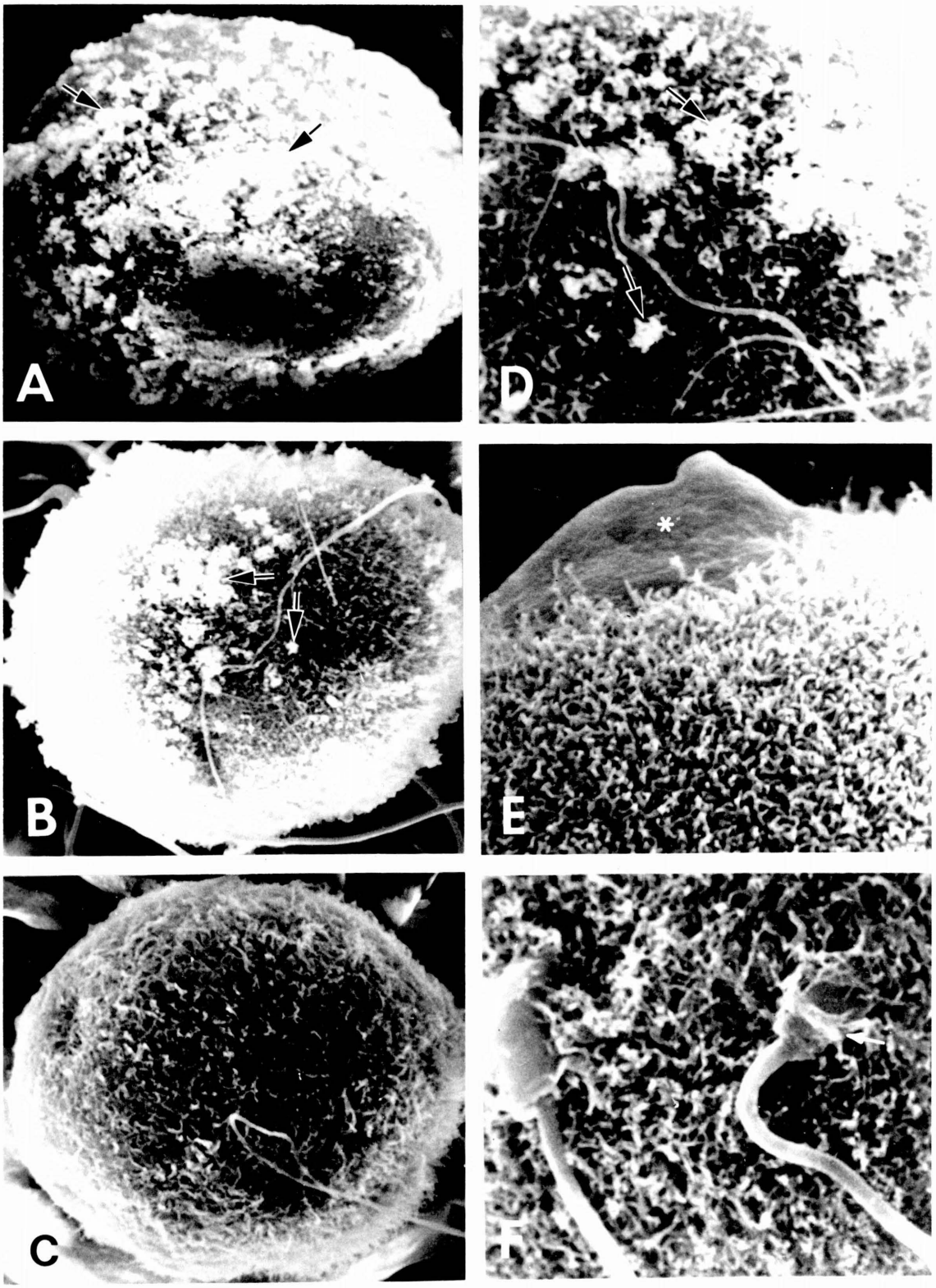
Figure 11B. Gold-Con A treated zona-free hamster egg 20 min after insemination. The plasma membrane surface exhibits a reduction in gold-Con A binding. Note the small patches of gold-Con A (arrows) on the surface. x2,000.

Figure 11C. Gold-Con A treated zona-free hamster egg 45 min after insemination. There is no binding of gold-Con A at this stage. x2,000.

Figure 11D. Higher magnification scanning electron micrograph of gold-Con A treated zona-free hamster egg 20 min after insemination. There are patches of gold-Con A (arrows). Some areas of the plasma membrane surface are devoid of gold labelling. x5,000.

Figure 11E. Zona-free, unfertilized hamster egg treated with gold-Con A in the presence of α-methyl-D-mannoside. There is no gold binding. The plasma membrane surface has a dense distribution of long-slender microvilli. Note the smooth region (*) without microvill. x5,000.

Figure 11F. Zona-free hamster egg 20 min after insemination treated with gold-Con A in the presence of α-methyl-D-mannoside.. The plasma membrane surface is absent of gold binding. The microvilli remain densely distributed. Fusion of the microvilli (arrow) over the post-acrosomal region of penetrating sperm head is seen. x5,000.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and 8E). Eggs 30 min after insemination still retain the fibrous networks with additional large "depressions" (arrows, Figs. 6F and 7E). Occasionally, distortion of the zona structure at the region of sperm penetration is observed (large arrows, Figs. 6F and 7E). The number of "depressions" increases by 60 min after insemination. By this time, some regions of the surface have less well defined fibrous networks or a convoluted surface (Figs. 7F and 8F). Penetrating sperm in a "channel-like" structure is seen occasionally (Fig. 7F).

The plasma membrane surface changes more drastically upon fertilization. With the exception of a smooth region (Figs. 10E and 11E), which is possibly overlying the second meiotic spindle, unfertilized, zona-free hamster egg has a uniform distribution of closely arranged long-slender microvilli over the entire plasma membrane surface (Figs. 10E and 11E). There is no significant change in microvilli structure by 20 min after insemination (Figs. 9E and 11F). Occasionally, fusion of microvilli around the post-acrosomal region of the head of incorporating sperm is seen (Figs. 9E and 11F). There is a marked alteration of the plasma membrane surface by 45 min after insemination of zona-free hamster eggs. The microvilli become shortened (Fig. 10F) or the plasma membrane is

altered to a ruffled surface (Fig. 9F also see Figs. 9C and 10C).

Changes of ^{125}I-Con A Labelling of Hamster Egg Plasma Membrane after Fertilization

The amount of ^{125}I-Con A bound to the plasma membrane of zona-free hamster egg is 120.9 cpm/egg in one instance and 3625.1 cpm/egg the other instance. By 45 min after fertilization, the amount of ^{125}I-Con A is reduced to 70.5 cpm/egg. The ^{125}I-Con A binding is specific since the controls of unfertilized and fertilized zona-free hamster eggs labelled in the presence of 0.2 M α-methyl-D-mannoside show 2.9 and 2.6 cpm/egg, respectively.

DISCUSSION

Sperm concentration is a major factor influencing sperm binding (Hartmann and Hutchison, 1977b; Wolf and Inoue, 1976), fertilization rate (Fraser and Maudlin, 1978; Fukuda and Chang, 1978a). The present studies employ sperm concentrations which provide fertilization rate of 95-100%. Thus, only the variation in levels of polyspermy is taken into account for quantitation.

The binding properties of hamster ZP differs from that of the plasma membrane for capacitated sperm. Though a lower sperm concentration is used to inseminate zona-free eggs, comparatively higher number of sperm bind immediately to the plasma membrane of zona-free eggs. Similarly, immediate binding of a high number of capacitated mouse sperm to the plasma membrane of mouse egg has also been noted (Wolf and Hamada, 1979). The difference in the binding properties of egg zona and plasma membrane may reflect differences in the sperm receptors on both surfaces.

In the present studies, numbers of sperm bound to the ZP and the plasma membrane of hamster egg increase as a function of time and then decline at 40 and 30 min after

insemination of zona-intact and zona-free eggs, respectively. This indicates temporal changes and loss in sperm receptivity on the egg surfaces at the early stages of fertilization. Most previous studies observed a loss of sperm receptivity of the ZP after fertilization at later stage of fertilization as in hamster eggs obtained 5 hr after insemination in vivo (Barros and Yanagimachi, 1972) and in mouse eggs recovered 2 hr after mating (Inoue and Wolf, 1975). Similarly, a decrease in sperm receptivity of the hamster egg plasma membrane was observed at the pronuclei stage (Usui and Yanagimachi, 1976). However, kinetic analysis of mouse sperm binding to the homologous egg plasma membrane indicates that detachment of sperm on the egg surface occurs 12.4 min after first sperm penetration (Wolf and Hamada, 1979).

Temporal changes of the binding properties of the ZP and plasma membrane at the early period of fertilization are demonstrated in the present reinsemination studies. Alteration of the binding properties of the ZP is first detected in zona-intact eggs preinseminated for 20 min; the binding pattern as a function of time begins to deviate from that of the control, i.e., the number of sperm bound does not reach the same maximum as that of the control. Alteration of the binding properties of the ZP

is more drastic in the eggs preinseminated for 40 min, as there is no addition of new sperm bound to the surface by reinsemination. Furthermore, only detachment of previously bound sperm is observed in eggs preinseminated for 60 min. Alteration of the binding properties of the plasma membrane after fertilization is first detected in zona-free eggs preinseminated for 15 min. Again, eggs preinseminated for longer periods of time show more drastic loss in sperm receptivity. Such changes in the binding properties of egg surfaces may represent the early aspect of the polyspermy block (Austin and Braden, 1956; Wolf and Hamada, 1979).

The present studies also provides evidence for the occurrence of polyspermy block on both the ZP and the plasma membrane surfaces at the early period of fertilization. The level of polyspermy of zona-intact eggs preinseminated for 20 min is significantly lower than that of the control. This seems to indicate the occurrence of a zona block as early as 20 min after sperm contact to the ZP. The block is completed by 60 min at which time the level of polyspermy of the preinseminated eggs reaches that of the minimum control, i.e., there is no further sperm penetration after reinsemination. The plasma membrane block of hamster eggs occurs 15 min after insemina-

tion of zona-free eggs at which time the level of polyspermy of preinseminated eggs is significantly lower than that of the control. Subsequently, eggs preinseminated for 45 min exhibit a polyspermy level close to that of the minimum control.

Hamster plasma membrane block is delayed until 2 1/2-3 hr after insemination in vivo (Barros and Yanagimachi, 1972). In contrast, occurrence of the plasma membrane block at an earlier stage is indicated in the present studies. The disparity may be due to the difference in the fertilization conditions. The tubal environment affects the binding of mouse sperm to the ZP of the egg (Wolf and Inoue, 1976), however, the observation of hamster plasma membrane block by Barros and Yanagimachi (1972) only included the period later than 2 hr after insemination in vivo. The plasma membrane block may be a transient mechanism; masking of the plasma membrane by cortical contnet is diminished at later stage of fertilization but then is overcome by permanent alteration of the plasma membrane at a very late stage of which observed by Barros and Yanagimachi (1972). An early plasma membrane block of zona-free mouse eggs fertilized in vitro is also evident; the block develops about 40 min after sperm contact to the plasma membrane (Wolf, 1978), which time

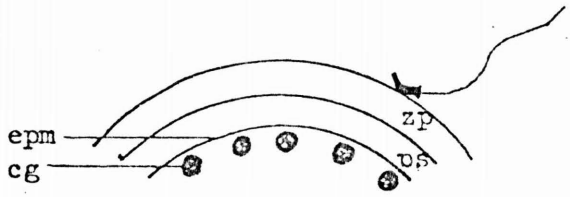
correlates with the cortical reaction of the mouse eggs.

The present studies also reveal a correlation of the temporal changes in binding properties of the ZP and the plasma membrane of hamster eggs after fertilization and the occurrence of polyspermy block. Thus, both physiological changes possibly are related to the same mechanism. The cortical reaction has been implicated in the zona reaction (Austin and Braden, 1956; Barros and Yanagimachi, 1971; 1972; Gwatkin et al., 1973a), and alteration of the zona binding subsequently leads to the block of polyspermy (Austin and Braden, 1956). The time correlation of these two events in the present studies supports this idea. Furthermore, physiological changes observed here are clearly related to the cortical reaction. In fact, the hamster cortical reaction takes place 5-15 min after sperm contact with the plasma membrane (Barros and Yanagimachi, 1972; Gwatkin et al., 1976; Fukuda and Chang, 1978b) or 17-20 min after sperm contact to the ZP (Barros and Yanagimachi, 1972). A slight variation in the time of the cortical reaction is expected, since occurrence of the cortical reaction is sperm concentration dependent: the higher the sperm concentration, the earlier the cortical reaction (Fukuda and Chang, 1978b).

Beside the effect on the ZP, the cortical reaction may play some role in plasma membrane block. Good correlation in time of the occurence of plasma membrane block in the present studies and the occurence of cortical reaction seems to indicate the involvement of the cortical reaction in the hamster plasma membrane block. Crude cortical content from fertilized mouse eggs reduces penetration of zona-free and zona-intact mouse eggs (Wolf and Hamada, 1977) suggesting that cortical content does, indeed, modify both the ZP and plasma membrane of mouse eggs. The sequence of the physiological events observed in the present studies is illustrated (Fig. 12).

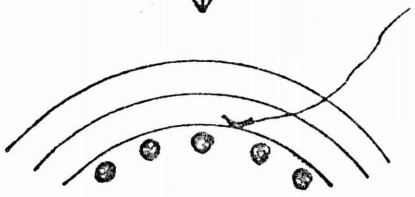
Observations of gold-lectin labelling on the hamster egg surfaces reveal the oligosaccharide distributions on the outer layers of the egg surfaces. Since the size of gold particles (≈50 nm) observable by scanning electron microscopy does not allow good penetration of the marker due to the narrow spacing of the oligosaccharide brushes, Only terminal sugars or those close to the terminal portion of the oligosaccharide chains are effectively labelled. Reduction of the gold marker size to those observable by transmission electron microscopy yields more effective labelling (Horisberger et al., 1978).

Figure 12. Diagram of the sequence of physiological events after sperm contact to the zona pellucida. zp, zona pellucida; ps, perivitelline space; epm, egg plasma membrane; cg, cortical granule.



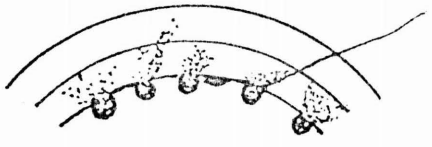
sperm contact to the ZP

after 5'



ZP penetration

after 15'



cortical reaction

after 20'

alteration of the binding properties of the ZP and plasma membrane

occurrence of the zona and plasma membrane blocks

Dense and uniform distributions of the terminal or close to terminal D-galactose and N-acetyl-D-glucosamine residues on both zona and plasma membrane surfaces are revealed by the dense labelling of gold-RCA and gold-WGA, respectively, on the entire zona and plasma membrane surfaces. The scattered labelling of gold-Con A on hamster zona surface in the present studies does not rule out the existence of the mannose residues at the unlabelled area, but rather, it indicates that those residues are probably located at the proximal portions of oligosaccharide chains. Therefore, only the scattered distribution of oligosaccharide chains with terminal mannose residues are revealed. In fact, the sparse labelling of ferritin-Con A throughout the hamster zonae has been reported (Nicolson et al., 1975). In contrast, the terminal mannose residues are densely distributed on the hamster plasma membrane surface.

It has been established that hamster zonae change their binding properties after fertilization in vivo (Barros and Yanagimachi, 1971) and in vitro (present studies) and that there is a change in their surface topography (see further discussion). Despite this, no dramatic change in gold-lectin labelling of the zona can be seen after fertilization, although there is a very

slight increase in labelling in some cases. Apparently, the physiological alterations on the ZP do not involve extensive alteration of the terminal oligosaccharide residues. The oligosaccharides on the hamster ZP have been reported to be quite stable, as there is no change in agglutination (induced by various lectins) of zona-intact hamster eggs before and after fertilization. Furthermore, there is no detectable change of fluorescein-labelled lectins on the hamster zonae from before fertilization up to the blastocyst stage (Yanagimachi and Nicolson, 1976).

Changes of gold-lectin binding to the plasma membrane surface are drastic. Slight decline in D-galactose and N-acetyl-D-glucosamine residues accompanying the surface changes occurs as late as 45 min after insemination. In fact, the superimposed surface topography changes make it difficult to evaluate whether the binding changes are a result of actual alterations of the sugar residues or of the gross structural alterations.

A better correlation of physiological and molecular changes on the hamster egg surface after fertilization is revealed by gold-Con A labelling of the plasma membrane surfaces of zona-free hamster eggs. Here the gold-Con A binding of eggs 20 min after insemination declines significantly, and the binding is almost absent by 45 min after

insemination. Reduction of Con A binding is confirmed in ^{125}I-Con A binding of hamster egg plasma membranes. There is approximately a 40% reduction of the amount of ^{125}I-Con A binding to the plasma membranes of hamster eggs 45 min after insemination in one instance and a 98% reduction in another instance. The 98% reduction agrees well with the scanning electron microscopic observations of gold-Con A labelling. The binding of the lectin is specific since low radioactivities are detected in the controls.

Variations of Con A binding changes on egg plasma membranes after fertilization have been reported. Increase in Con A binding to egg plasma membranes after fertilization is observed in rabbit (Gordon et al., 1975) and sea urchin eggs (Veron and Shapiro, 1977), while Con A binding to plasma membrane surface is unchanged in fertilized mouse eggs (Solter, 1977) and decreases in hamster blastocysts (Yanagimachi and Nicolson, 1976). Correlation of the reduction of Con A binding to the plasma membrane with the physiological changes tends to indicate the involvement of the cortical reaction on the early molecular changes of the hamster egg plasma membrane surface after insemination. The trypsin-like, cortical content (Gwatkin et al., 1973a) released may cleave off the glycoproteins containing terminal mannose residues on the

plasma membrane.

Structural change in zona surface after fertilization has not previously been reported. The "depressions" observed on the zona surface of eggs after insemination may be due to the zona digestion by bound sperm which were removed during processing for scanning electron microscopy. The "depressions" later become deep "channel-like" structures, sperm penetrating through these "channel-like" structures are occasionally observed in eggs 60 min after insemination. Yanagimachi (1977) utilizing transmission electron microscopy, has observed slit-like structures in the zona which he believes to be caused by penetrating sperm. These probably correspond to the "channel-like" structures observed in the current studies. In addition, fibrous networks of the zona are slightly swollen or distorted at some region on the egg surface after 60 min of insemination. These later changes seem to indicate a physical distortion of the zona structure from the inside which may be a consequence of the cortical reaction.

Drastic alteration of the plasma membrane topography of mouse egg after fertilization has been reported. The plasma membrane of unfertilized egg, which is partially covered with microvilli, becomes entirely covered with microvilli after fertilization (Eager et al., 1976). The

appearence of microvilli on both smooth and ruffled surface after fertilization of mouse egg has been reported by Nicosia et al. (1978). In the present studies, there is no obvious change in plasma membrane surface topography in zona-free eggs 20 min after insemination, but the plasma membrane surface changes drastically by 45 min after insemination. The shortening of the microvilli or the complete alteration of the microvillous plasma membrane of unfertilized hamster egg to a ruffled surface is always observed. Such late change does not seem to indicate a direct correlation with the cortical reaction, which occurs earlier, but it may be the consequence of the the completion of the cortical reaction or may be related to other early developmental events.

SUMMARY

(1) Temporal changes of the binding properties of both ZP and plasma membranes of hamster eggs are observed after sperm-egg interaction.

(2) Decline in sperm receptivities of hamster ZP and egg plasma membranes are first detected at 20 and 15 min after insemination of zona-intact and zona-free hamster eggs, respectively.

(3) Polyspermy block at both ZP and plasma membrane levels of hamster eggs are observed at an early period after insemination *in vitro*.

(4) Hamster zona and plasma membrane blocks exert their effects at 20 and 15 min and are completed at 60 and 45 min after insemination of zona-intact and zona-free eggs, respectively.

(5) Time correlation of the binding property changes and the occurrence of polyspermy block on both ZP and plasma membranes with the cortical reaction suggests the involvement of the cortical reaction in both observed physiological changes.

(6) There is no detectable alteration of D-galactose, N-acetyl-D-glucosamine and mannose residues on the ZP at

early period after insemination *in vitro* using the gold-lectin labelling technique.

(7) Slight reduction of terminal D-galactose and N-acetyl-D-glucosamine on the egg plasma membrane at 45 min after insemination of zona-free hamster eggs is detected by the gold-lectin labelling technique.

(8) Significant reduction of terminal mannose residues, as revealed by gold-Con A labelling, is observed as early as 20 min after insemination of zona-free hamster eggs. Mannose residues are almost totally absent by 45 min after insemination, as revealed by both gold-Con A and ^{125}I-Con A labelling.

(9) "Channel-like" structures in the fibrous networks of the ZP and distortion of some regions of the zona are observed after insemination.

(10) Plasma membrane surface topography alters drastically from a microvillous to a ruffled surface by 45 min after insemination of zona-free hamster eggs.

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