Inhibition of primary sperm attachment, identification of egg envelope proteins, and early development of the horseshoe crab, Limulus polyphemus L

Susan Ruttenberg Barnum

Iowa State University

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INHIBITION OF PRIMARY SPERM ATTACHMENT, IDENTIFICATION OF EGG ENVELOPE PROTEINS, AND EARLY DEVELOPMENT OF THE HORSESHOE CRAB, LIMULUS POLYPHEMUS L.

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Inhibition of primary sperm attachment, identification of egg envelope proteins, and early development of the horseshoe crab, Limulus polyphemus L.

by

Susan Ruttenberg Barnum

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department: Zoology
Interdepartmental Major: Molecular, Cellular, and Developmental Biology

Approved:
In Charge of Major Work
Signature was redacted for privacy.
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Members of the Committee:
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Iowa State University
Ames, Iowa
1983
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GENERAL INTRODUCTION

In order for fertilization or fusion of pronuclei to occur, the egg and sperm must undergo numerous structural and metabolic changes. Furthermore, gametes and their components interact during specific time periods. Generally, sperm-egg interactions involve: (1) the interaction of sperm and egg components (e.g. egg jelly or egg vestment) prior to the acrosome reaction; (2) attachment of sperm to the egg envelope or chorion surface after the acrosome reaction; (3) fusion of sperm and egg plasma membranes; and (4) penetration of the sperm nucleus into the egg. These interactions are well-understood with respect to morphology and ultrastructure (Summers and Hylander, 1974; Summers et al., 1975; Austin, 1978; c.f. Longo, 1978; Yanagimachi, 1978); however, many of the biochemical processes involved remain, for the most part, unclear. The majority of studies have utilized the sea urchin to elucidate various aspects of the fertilization process. Effort has generally concentrated on direct sperm-egg or egg component interactions (Epel and Vacquier, 1978; c.f. Shapiro et al., 1980; c.f. Shapiro et al., 1981) as well as physiological changes in the sperm after activation, but prior to egg penetration (c.f. Shapiro et al., 1980; Schackmann and Shapiro, 1981; c.f. Shapiro et al., 1981).

The research for this dissertation utilized the horseshoe crab, Limulus polyphemus L., to examine primary sperm attachment. Limulus possesses several characteristics that are favorable for
studying mechanisms involved in fertilization, and in particular, primary sperm attachment. The species is readily available and easy to maintain in the laboratory and large numbers of gametes can be collected throughout the year from one animal. Most important, however, is that sperm undergo the acrosome reaction only after they attach to the egg envelope. Furthermore, gametes are large and spermatozoa can easily be detected on the egg surface. Another favorable feature is that spermatozoa are nonmotile when spawned and are stimulated only when in close proximity to the egg (Brown, 1976), egg water (Clapper and Brown, 1980 a,b), or a variety of other agents (Clapper and Brown, 1980b).

Section 1 examines the effect of lectins and sugars on primary sperm attachment using a sperm attachment assay. In Section 2, an attempt is made to identify and partially characterize egg envelope proteins and to determine their biological activity using the sperm attachment assay. Determination of saccharide composition of semen and further examination of primary sperm attachment are undertaken in Section 3. Finally, in Sections 4 and 5, early embryonic developmental events are described and a temporal sequence of events is presented.
EXPLANATION OF DISSERTATION FORMAT

The sections of this dissertation are manuscripts that have been or will be submitted to scientific journals. Each section has been modified to conform to the style required by the Iowa State University Thesis Office, and has its own introduction, materials and methods, results, discussion, and references. Following the five sections is a summary-discussion of the entire dissertation.
SECTION 1. EFFECT OF LECTINS AND SUGARS ON PRIMARY SPERM ATTACHMENT IN THE HORSESHOE CRAB, LIMULUS POLYPHEMUS L.
INTRODUCTION

During sperm-egg interactions at least three separate events occur that involve cell surface recognition: (1) interaction of sperm and egg components (i.e., egg jelly or glycoprotein coat) prior to the acrosome reaction; (2) attachment of sperm to the egg envelope surface after the acrosome reaction; and (3) fusion of sperm and egg plasma membranes. In Limulus, the first two events have been described by Brown (1976) and designated as initial attachment (referred to as primary attachment in the present paper) and secondary attachment, respectively. Secondary attachment has been examined in several species of invertebrates and involves the adhesion of the sperm acrosome process and/or acrosome granule contents to the egg vitelline layer or chorion (Shoger and Brown, 1970; Summers and Hylander, 1974, 1975; Summers et al., 1975; Brown, 1976; Schmell et al., 1977; Vacquier and Moy, 1977; Glabe and Vacquier, 1977, 1978; Tsuzuki et al., 1977; Glabe and Lennarz, 1979; Yudin et al., 1979; DeSantis et al., 1980; Kinsey et al., 1980, Clark et al., 1981; Kinsey and Lennarz, 1981). The fusion of gamete membranes has been described in several studies (Bedford and Cooper, 1978; cf. Epel and Vacquier, 1978); however, little is known about the mechanism.

Primary attachment or initial interactions of gametes and the components involved have been examined in several invertebrate species. In sea urchins, when spermatozoa initially contact the
egg jelly coat, the acrosome reaction is induced by a
sulfated-fucose component (SeGall and Lennarz, 1979). This
component may be interacting with an 84 Kd sperm membrane
glycoprotein, since it was found that monovalent antibody to this
glycoprotein blocks the egg jelly induced acrosome reaction in
*Strongylocentrotus purpuratus* spermatozoa (Lopo and Vacquier, 1980
a,b). In ascidians, spermatozoa attach to the chorion prior to the
acrosome reaction (Woollacott, 1977; Dale et al., 1978; Lambert and
Epel, 1979). Rosati et al. (1978) have examined this attachment in
*Ciona intestinalis* and have shown that the surfaces of both the egg
chorion and the sperm share binding sites for concanavalin A and
asparagus pea lectin. In addition, monosaccharide competition
experiments have established that fucose is probably involved in
gamete attachment (Rosati and DeSantis, 1980).

The xiphosurid, *Limulus polyphemus*, was chosen to examine
primary sperm attachment since several favorable features are found
in this species: large numbers of gametes are readily obtained,
sperm attachment is easily observed, egg sections can be utilized
to quantify sperm attachment, and spermatozoa are not motile until
they come in close proximity to eggs (Mowbray and Brown, 1974;
Clapper and Brown, 1980a). Previous studies have demonstrated that
spermatozoa only attach to the outer layer (5 μm) of the egg
envelope (Brown, 1976). The present study examines the effect of
gamete treatment with various lectins, monosaccharides, and
glycosidases on primary gamete attachment in Limulus. Results suggest that a fucose binding protein is present on the sperm surface which interacts with a fucose-containing sperm receptor present on the egg envelope surface.
MATERIALS AND METHODS

Source of Animals

*Limulus polyphemus* L. were obtained from the Florida Marine Biological Specimen Co., Inc., Panama City, Florida. Animals were maintained at 15°C in 150 gal Instant Ocean aquaria containing artificial sea water (ASW) from Jungle Laboratories Corp., Comfort, Texas.

Gamete Collection

Gametes from *Limulus* were artificially spawned by brief electrical stimulation (3-4V, 0.2-1.0mA, ac) of the external region proximal to the genital pores. Semen was collected "dry" and washed in Millipore filtered artificial seawater (MFASW) three times. The washed, concentrated spermatozoa were then diluted with MFASW to obtain a 2.5 (2 x 10^8 spermatozoa/ml) or 5.0% sperm suspension. Spawned eggs were also washed three times in MFASW.

Egg Sections

Eggs were collected, washed, frozen at -20°C with an International Cryostat, Model CTI, and sectioned for quantitative sperm attachment assays. Approximately 20 frozen sections (12-15 µm thick) were mounted on each glass microscope slide. These prepared egg sections adhered well to slides, were able to withstand successive treatments and washings, and could be stored for several weeks at -20°C without losing the ability to bind spermatozoa.
Sperm Attachment Assay

A modification of the assay used by Mowbray and Brown (1974) was employed. Previously prepared egg sections on slides were brought to room temperature (22-23°C), ringed with petrolatum, and subsequently treated with lectins, glycosidases, or MFASW. After treatment, egg sections were washed in a large beaker of MFASW three times, incubated with 0.25 ml of a 2.5 or 5.0% sperm suspension, washed for 15 sec in MFASW, fixed in 10% formalin for 5 sec, rinsed briefly in MFASW, and covered with a glass coverslip before being examined microscopically.

Sperm attachment to egg sections was quantified by selecting two to five sections on each slide and counting attached spermatozoa in the focusing field of a Nikon microscope using a 40x DLL objective lens and a 10x ocular (approximately 1/12 [0.47 mm] the egg circumference). Sperm counts from egg sections treated with MFASW (control) were considered to be 100% attachment (approximately 100 spermatozoa) and all treatments were calculated as a percent of controls. Less than 5% sperm attachment was considered a significant reduction. Micrographs were taken with a Nikon M-35S camera mounted on a WL Zeiss microscope.

To ensure that normal gamete interactions were occurring, sperm motility was examined with a dissecting microscope immediately after mixing spermatozoa and egg sections. If low
motility (< 50%) was observed, 0.10 ml of 340 mM calcium chloride was added to the mixture to enhance or stimulate motility.

**Egg Section Treatment**

Lectins (Con A, APL, WGA, GPL) purchased from Sigma Chemical Co. were used to treat egg sections prior to addition of spermatozoa. Initially, experiments were conducted using lectin concentrations of 500 μg/ml in MFASW. Egg sections were treated with 0.5 ml lectin-MFASW (pH 7.5-8.1) for 1 hr at 4°C and then washed for 30 min in MFASW. Since significant reduction of sperm attachment was observed only when egg sections were treated with APL, a range of concentrations from 26 to 1000 μg/ml was utilized to show the concentration dependence of sperm attachment. As a control, APL-treated egg sections were washed in MFASW containing 60 mg/ml α-L-fucose for 30 min and then in MFASW for 45 min prior to adding spermatozoa. A temperature of 4°C was used when incubating egg sections with lectins since the binding of lectins to cells has been reported to be influenced by temperature and may result in nonspecific binding at room temperature and above (Sharon and Lis, 1975).

Glycosidases were used to remove specific terminal sugar residues. Egg sections were incubated with 0.25 ml of 0.5 U/ml glycosidase for 1 hr following enzyme assay specifications from Sigma (α-L-fucosidase pH 6.5, 24°C; α-D-glucosidase pH 6.8, 37°C; α-D-mannosidase pH 5.0, 23°C; neuraminidase pH 5.0, 37°C). To
ensure that sperm attachment reduction was due only to glycosidase activity and not trypsin contamination, 0.1 mg/ml soybean trypsin inhibitor (SBTI) was included in the incubation mixture. Since a decrease in sperm attachment was observed only with fucosidase treated egg sections, substrate competition controls (2.5 mg/ml p-nitrophenyl α-L-fucopyranoside, p-nitrophenol α-D-galactopyranoside, or p-nitrophenyl α-L-arabinofuranoside) also were performed to demonstrate the substrate specificity of the enzyme.

The optimum pH for α-D-mannosidase activity is 4.5; however, spermatozoa do not attach after egg sections have been exposed to an environment of < pH 4.5 (Mowbray, 1972). Consequently, the pH for this enzyme was increased to 5.0.

Treatment of Spermatozoa

Sugars α-L-fucose, α-D-fucose, D-galactose, N-acetylgalactosamine, N-acetylglucosamine, α-methyl-D-glucoside, D-mannose, α-methyl-D-mannoside, L-mannose, L-glucose, L-galactose, and p-aminophenyl galactopyranoside, were utilized to determine whether they had an effect on sperm attachment. Concentrated spermatozoa (50 μl) were mixed with 1 ml of the specific sugar solution (5% final sperm suspension) for 3 min (22-23°C) and then 0.25 ml of this mixture was added to egg sections. Calcium chloride (57 mM final concentration) was added to enhance sperm motility. Initially, sugar concentrations of 1.0 and 0.5 M were
prepared with either MFASW (the concentration of MFASW was adjusted according to the molarity of the sugar used) or 10 mM Tris-MFASW (pH 8.0); however, data is reported only for MFASW experiments since no difference in sperm attachment was observed between the two types of sugar solutions. Since only L-fucose decreased sperm attachment in preliminary experiments, various concentrations of this sugar (0.05, 0.10, 0.25, 0.50, 1.00 M) were incubated with spermatozoa. To determine whether removal of the sugar increased sperm attachment to control levels, the fucose-treated sperm suspension was centrifuged (1000 x g) and mixed in MFASW prior to being mixed with egg sections.

Since L-fucose reduced sperm attachment to egg sections, the fucose derivative, p-aminophenyl α-L-fucopyranoside (0.25 M), and the fucose-containing polysaccharide, fucoidan (2.5, 5, 15, 30, 60 mg/ml), were incubated with spermatozoa to determine if there also was a decrease in sperm attachment.

Fluorescein-conjugated lectins were used to determine whether the lectins used in sperm attachment experiments actually bound to egg envelopes. Egg sections were treated with FITC-WGA, GPL, APL, or Con A for 1 hr at 4°C and were washed in MFASW. As a control, lectin treated egg sections were washed in MFASW containing 35 mg/ml of the appropriate sugar (WGA, N-acetyl glucosamine; GPL, D-glucose; APL, L-fucose; Con A, methyl-D-mannoside) for 30 min. All egg sections were washed an additional 15 min in MFASW, blotted
dry, and allowed to thoroughly air dry for 1 hr. Immersion oil and a coverslip were added to each preparation and observations were made with a Zeiss RA microscope using a 500 nm barrier filter and BG 12 excitor filter. Micrographs were taken with an Olympus camera.
RESULTS

Egg Section Treatment

Asparagus pea lectin was the only lectin that significantly reduced primary sperm attachment when egg sections were treated with 500 µg/ml Con A, WGA, APL, or GPL (Table 1; Fig. 1a,b). When egg sections were exposed to increasing concentrations of APL ranging from 26-1000 µg/ml, sperm attachment decreased in a concentration dependent fashion (Fig. 2). Practically no spermatozoa attached when 250 µg/ml APL was used (< 5% of controls). In experiments where APL treated egg sections were washed in α-L-fucose, sperm attachment increased to control levels.

A significant reduction of attached spermatozoa was observed when egg sections were incubated with the glycosidase, L-fucosidase, prior to adding spermatozoa (5% of controls; Table 2). As expected, addition of the fucosidase substrate, p-nitrophenyl fucopyranoside (2.5 mg/ml), to the enzyme prevented reduction of sperm attachment (99% of controls). Similar results were seen with p-nitrophenyl arabinofuranoside (97% of controls). However, the addition of the galactosidase substrate, p-nitrophenyl galactopyranoside, to fucosidase failed to prevent any reduction of sperm attachment (5% of controls; Table 2).

The presence of sugars in the egg envelope was demonstrated by treating egg sections with fluorescein-conjugated APL, Con A, WGA or GPL. Fluorescence was observed on the innermost egg envelope
Table 1. Effect of lectin-treated egg sections on primary sperm attachment. Egg sections were treated with 250 μg (0.5 ml of 500 μg/ml) of the selected lectin for 1 hr at 4°C and washed in MFASW

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Number of sections counted</th>
<th>Mean number of attached spermatozoa as a percent of controls ± one S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A</td>
<td>7</td>
<td>95.29 ± 6.05</td>
</tr>
<tr>
<td>GPL</td>
<td>10</td>
<td>92.00 ± 11.65</td>
</tr>
<tr>
<td>APL (250 μg/ml)</td>
<td>10</td>
<td>1.20 ± 1.69</td>
</tr>
<tr>
<td>APL</td>
<td>10</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>WGA</td>
<td>10</td>
<td>91.30 ± 11.00</td>
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Fig. 1. Attachment of spermatozoa to APL and MFASW treated egg sections

(a) Spermatozoa (arrow) readily adhere to the envelope of an egg section treated with MFASW for 1 hr at 4°C. 650x

(b) Spermatozoa do not adhere to envelopes (EE) previously treated with 500 μg/ml (0.75 ml) APL for 1 hr at 4°C. 550x
Fig. 2. Percent sperm attachment as a function of increasing concentrations of APL added to egg sections. Sections were treated with 0.5 ml APL at 4°C for 30 min and washed in MFASW for 15 min. A 2.5% sperm suspension (0.25 ml) was added to treated sections, incubated for 5 min, and rinsed in MFASW prior to being fixed in 10% formalin and examined. Bars denote S.D.
Table 2. Effect of fucosidase-treated egg sections on sperm attachment. Egg sections were incubated with 0.5 U/ml fucosidase pH 6.5 at 24°C for 1 hr and washed in MFASW for 30 min prior to adding spermatozoa. Calcium chloride was added to enhance sperm motility.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of sections counted</th>
<th>Mean number of attached spermatozoa as a percent of controls + one S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>fucosidase + 0.1 mg/ml SBTI</td>
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<td>4.67 ± 5.25</td>
</tr>
<tr>
<td>fucosidase + 2.5 mg/ml p-nitrophenyl galactopyranoside</td>
<td>14</td>
<td>5.43 ± 5.78</td>
</tr>
<tr>
<td>fucosidase + 2.5 mg/ml p-nitrophenyl fucopyranoside</td>
<td>10</td>
<td>99.87 ± 0.63</td>
</tr>
<tr>
<td>fucosidase + 2.5 mg/ml p-nitrophenyl arabinofuranoside</td>
<td>7</td>
<td>97.00 ± 7.08</td>
</tr>
</tbody>
</table>
surface as well as on the outer surface after egg section treatment with FITC-APL (Fig. 3a). Fluorescence disappeared after APL-treated egg sections were washed in L-fucose for 30 min (Fig. 3b). FITC-Con A bound primarily on the outer envelope surface; however, fluorescence was generally faint and patchy areas fluoresced beneath the envelope surface (Fig. 3c). A wash in methyl-D-mannoside for 30 min decreased fluorescence (Fig. 3d). When FITC-WGA was used, the outer 20μm of the egg envelope floresced intensely, while the rest of the envelope was not as bright (Fig. 3e). A loss of fluorescence was observed after the addition of N-acetylglucosamine (Fig. 3f). Fluorescence of FITC-GPL treated egg sections was barely perceptible on the egg cortex and did not appear to be present on the egg envelope (Fig. 3g). No fluorescence was detected after these treated egg sections were washed with D-glucose (Fig. 3h).

Treatment of Spermatozoa

Treatment of spermatozoa with L-fucose at concentrations of 0.5 M and 1.0 M dramatically reduced sperm attachment to egg sections (Table 3). Fucoidan (5-60 μg/ml) and the fucose derivative, p-aminophenyl fucopyranoside (0.25 M), also significantly reduced attachment. The sugars, D-mannose, L-mannose, methyl-D-mannoside, D-galactose, L-galactose, D-fucose, N-acetylglucosamine, and p-aminophenyl galactopyranoside had no effect (Table 3). The sugars, L-glucose and methyl-D-glucoside
Fig. 3. Egg envelope fluorescence after treatment of egg sections with fluorescein-conjugated lectins

(a) Fluorescein-conjugated APL (100 µg/ml) binds to the outer surface (arrow) and the underlying area of the egg envelope. The inner surface stains more intensely(*). In this photomicrograph, two egg sections are juxtaposed. Arrow, egg envelope surface. 300x

(b) Egg sections treated with FITC-APL and subsequently washed in 35 mg/ml L-fucose for 30 min at 22°C do not show any fluorescence. 325x

(c) FITC-Con A (100 µg/ml) binds primarily to the outer envelope surface (arrow), but some fluorescence is observed in patches in other areas. C, egg cortex. 375x

(d) A wash with methyl-D-mannoside (35 mg/ml) for 30 min at 22°C removes FITC-Con A from the envelope surface. Arrow, egg envelope surface; C, egg cortex. 800x

(e) FITC-WGA (100 µg/ml) binding is observed throughout the egg envelope (EE). However, the outer 20 µm portion fluoresces more intensely. 450x

(f) FITC-WGA treated egg sections washed in N-acetylglucosamine (35 mg/ml) for 30 min at 22°C removes bound lectin from the egg envelope (EE). 575x

(g) After sections are exposed to 100 µg/ml FITC-GPL for 1 hr at 4°C, fluorescence can be detected on the egg surface (arrow) beneath the envelope. 400x

(h) FITC-GPL is removed by a 30 min wash in D-glucose (35 mg/ml) at 22°C. Arrow, egg cortex. 400x
Table 3. The effect of sperm treatment on primary attachment to egg sections. Calcium chloride was added to enhance sperm motility.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Concentration (M)</th>
<th>Number of sections counted</th>
<th>Mean number of attached spermatozoa as a percent of controls ± one S.D.</th>
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</thead>
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<td>L-fucose</td>
<td>1.0</td>
<td>39</td>
<td>5.13 ± 9.60</td>
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<tr>
<td>L-fucose</td>
<td>0.5</td>
<td>10</td>
<td>20.00 ± 16.47</td>
</tr>
<tr>
<td>D-fucose</td>
<td>0.5</td>
<td>10</td>
<td>98.33 ± 3.20</td>
</tr>
<tr>
<td>D-mannoside</td>
<td>0.5</td>
<td>10</td>
<td>97.11 ± 4.28</td>
</tr>
<tr>
<td>D-mannose</td>
<td>0.5</td>
<td>10</td>
<td>98.00 ± 2.88</td>
</tr>
<tr>
<td>L-mannose</td>
<td>0.5</td>
<td>10</td>
<td>98.00 ± 4.73</td>
</tr>
<tr>
<td>D-glucoside(^a)</td>
<td>0.5</td>
<td>10</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>L-glucose(^a)</td>
<td>0.5</td>
<td>20</td>
<td>3.00 ± 4.93</td>
</tr>
<tr>
<td>D-galactose</td>
<td>0.5</td>
<td>10</td>
<td>98.00 ± 3.32</td>
</tr>
<tr>
<td>L-galactose</td>
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<td>20</td>
<td>94.60 ± 9.51</td>
</tr>
<tr>
<td>GluNac(^b)</td>
<td>0.5</td>
<td>10</td>
<td>96.89 ± 5.01</td>
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<tr>
<td>Fucoidan</td>
<td>2.5 mg/ml</td>
<td>10</td>
<td>29.10 ± 10.46</td>
</tr>
<tr>
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<td>4.13 ± 7.04</td>
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<tr>
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</tr>
<tr>
<td>p-aminophenyl fucopyranoside</td>
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<td>20</td>
<td>1.05 ± 2.06</td>
</tr>
<tr>
<td>p-aminophenyl galactopyranoside</td>
<td>0.25</td>
<td>10</td>
<td>90.88 ± 11.14</td>
</tr>
</tbody>
</table>

\(^a\) All spermatozoa become highly motile when mixed with sugar.

\(^b\) N-acetylglucosamine, approximately 20% of spermatozoa become motile when mixed with sugar.
induced intense sperm motility for a very brief period of time so that by the time treated spermatozoa were added to egg sections, they were no longer motile and did not attach to egg sections. When spermatozoa were mixed with N-acetylglucosamine, only approximately 20% of the spermatozoa became motile. This, however, did not affect sperm attachment. Calcium chloride and 5 mM NiCl$_2$ (Clapper and Brown, 1980b) did not stimulate further motility and we assumed that these spermatozoa were no longer capable of attaching.

Sperm attachment was demonstrated to be dependent upon L-fucose concentration (Fig. 4). Approximately 50% sperm attachment occurred when spermatozoa were treated with 0.25 M L-fucose. After these treated spermatozoa were washed in MFASW, attachment increased to practically 100% of controls (Fig. 4).
Fig. 4. Percent sperm attachment as a function of increasing concentrations of L-fucose. Data are expressed as percent of the control mean. Bars denote S.D.
• L-FUCOSE-TREATED SPERM
• L-FUCOSE-TREATED SPERM FOLLOWED BY WASH

PERCENT SPERM ATTACHMENT

L-FUCOSE (M)
A sperm attachment assay was employed to determine whether sugars were involved in primary attachment of *Limulus* spermatozoa to egg envelopes. Egg sections were treated with selected lectins (asparagus pea lectin, wheat germ agglutinin, concanavalin A, garden pea lectin) and the effect on sperm attachment was quantified. In this study, sperm attachment was significantly reduced in egg sections exposed to APL while other lectins had no effect. To further confirm the effect of APL on sperm attachment, glycosidases were incubated with egg sections to specifically remove sugar residues from the envelope surface. A significant reduction in sperm attachment was observed only with fucosidase (0.5 μg/ml, < 5% of controls). Sperm attachment also was reduced greatly after treatment of spermatozoa with p-aminophenyl fucopyranoside (0.25 M, < 5% of controls), L-fucose (0.25 M, 50% of controls), or fucoidan (5 mg/ml, < 5% of controls). Numerous other sugars and sugar derivatives were used, but no reduction in sperm attachment was observed. Furthermore, sperm attachment is perhaps stereo-specific since D-fucose did not reduce the number of attached spermatozoa. Thus, fucosyl sites on the egg envelope most likely play a prominent role in primary sperm attachment.

Sperm receptors comprised of sugars may also be present on egg membranes of the brown algae, *Fucus serratus* (Bolwell et al., 1980)
and the ascidian, *Ciona intestinalis* (Rosati and DeSantis, 1980). Isolated *Fucus* egg membranes have been shown to compete with whole eggs for spermatozoa and as a result, lower the percentage of eggs fertilized (Bolwell *et al.*, 1980). After brief digestion with fucosidase or mannosidase, these membranes no longer competed with intact eggs for spermatozoa. Rosati and DeSantis (1980) showed that the addition of L-fucose to a suspension of *Ciona* eggs and spermatozoa reduced fertilization in competition experiments similar to those carried out in the present study on *Limulus*. However, it was not determined whether the fucose moieties essential for sperm attachment were present on the egg or the sperm.

Lectins have also been utilized to demonstrate the presence of sugars on gamete surfaces. Rosati *et al.* (1978) and Pinto *et al.* (1981) showed that the external surface of both spermatozoa and egg chorion of *Ciona* contains binding sites for fluorescein-conjugated Con A and APL, and although fertilization decreased after lectin treatment of eggs, the results were highly variable. Recently, Honegger (1982) showed that FITC-conjugated and gold-labelled WGA bound to the chorion of the ascidian, *Phallusia mammillata*, and also prevented fertilization. In *Limulus*, FITC-APL, -WGA, and -Con A bound to the envelope surface of the egg; however, only APL decreased sperm attachment. No evidence of FITC-GPL binding was detected except on the egg cortex and GPL
did not inhibit sperm attachment. Kornfeld et al. (1981) demonstrated that fucose residues in the glycoprotein are important for high affinity binding of GPL. However, the components and conformation of the core region of the oligosaccharide chain are essential factors that determine where GPL binds. The conformation of the oligosaccharide chains in the Limulus envelope may be such that GPL cannot bind and, therefore, does not inhibit sperm attachment to GPL-treated egg envelopes.

A previous study of Limulus gamete attachment determined that egg envelope antigen(s) are probably involved since treatment of eggs with either anti-egg envelope serum (AEE) or univalent AEE significantly reduced the number of attached spermatozoa (Mowbray and Brown, 1974). Although L-fucose appears to be essential for primary sperm attachment, high L-fucose concentrations had to be used to reduce attachment. This suggests that sperm membrane 'egg receptors' also recognize other components in addition to the terminal fucose present on egg envelopes. Furthermore, high affinity binding depends primarily on the molecular structure or conformation of the receptor. A fucose derivative, p-aminophenyl fucopyranoside, exhibited greater inhibition of sperm attachment, but at a lower concentration (0.25 M, attachment 1% of controls vs. 0.25 M L-fucose, 50% of controls). This may be due to the longer chain contributed by the phenyl group that mimicked the ring structure of sugars proximal to the terminal fucose. Another
aminophenyl compound, p-aminophenyl galactopyranoside, did not inhibit gamete adhesion which suggests that the primary recognition element is the fucose moiety. A sulfated polymer of L-fucose, fucoidan, was maximally inhibitory at a concentration of 5 mg/ml (5% of controls). The concentration dependence of this inhibitory saccharide can be demonstrated since at 2.5 mg/ml, attachment increased to 30% of controls. Huang et al. (1982) have also demonstrated that fucoidan was a strong inhibitor (100 µg/ml) of spermatozoa-zona pellucida attachment in guinea pig, hamster, and human. The carbohydrate specificity of secondary attachment in sea urchins has been examined by Glabe et al. (1982) and although egg surface polysaccharides were the most powerful inhibitors of bindin-mediated egg agglutination, fucoidan was also a strong inhibitor.

Primary sperm attachment in Limulus may be similar to other systems that have a primary attachment to the egg prior to the acrosome reaction. Evidence suggests that fucose-containing 'sperm receptors' on the egg envelope surface are involved in primary sperm attachment in Limulus. Further characterization of the Limulus egg glycoprotein coat and isolation of the biologically active sperm attachment component(s) are currently being undertaken.
LITERATURE CITED


SECTION 2. ISOLATION, PHYSIOCHEMICAL PROPERTIES, AND THE COMPOSITION OF EGG ENVELOPES FROM LIMULUS POLYPHEMUS EGGS
INTRODUCTION

The Limulus egg envelope is an acellular translucent layer approximately 40 μm thick which envelopes the plasma membrane and egg contents (Dumont and Anderson, 1967; Shoger and Brown, 1970; Brown, 1976). It previously has been demonstrated that the envelope is comprised of two distinct morphological layers, a 5 μm porous outer layer and an underlying 35 μm poreless component (Shoger and Brown, 1970; Brown, 1976). There is also a biochemical dissimilarity between the external and internal egg envelope layers since Brown (1976) demonstrated that spermatozoa only adhere to the outer 5 μm layer. The constituents of the envelope have been briefly examined in several recent studies. Limulus egg envelope cytochemistry by Bennett (1979) demonstrated that both protein and carbohydrates were present. The use of fluorescein-conjugated lectins also has confirmed the presence of various saccharide residues as components of the egg envelope (Barnum and Brown, 1983).

There has been much interest in the structure and composition of egg vestments of other invertebrate species. Several studies have identified proteins and saccharide constituents of the sea urchin vitelline layer (Aketa et al., 1968; Ishihara, 1968; Glabe and Vacquier, 1977, 1978; Tsuzuki, 1977). Soluble vitelline components that inhibit sperm-egg binding and subsequent fertilization have also been obtained during egg activation or by digestion with trypsin.
(Schmell et al., 1977; Glabe and Lennarz, 1979; Kinsey and Lennarz, 1981). Both morphological (Endo, 1961; Wolpert and Mercer, 1961; Vernon et al., 1977) and biochemical modifications of the sea urchin fertilization membrane (Runnstrom et al., 1944; Monroy and Runnstrom, 1948; Foerder and Shapiro, 1977; Vernon et al., 1977; Carroll and Baginski, 1978; Foerder et al., 1978; Hall, 1978; Showman and Foerder, 1979) have been examined. An important biochemical pathway occurring during fertilization that 'hardens' the egg vitelline layer by tyrosine crosslinking was recently elucidated (Foerder and Shapiro, 1977; Foerder et al., 1978; Kay et al., 1982). Egg vestment proteins of other invertebrates also have been studied. Using gel electrophoresis, protein patterns of the Drosophila chorion were determined (King, 1970; Petri et al., 1976). Recently, silkmoth chorion morphology and proteins were studied in an attempt to identify functional roles for some of the structural proteins that make up the chorion (Mazur et al., 1980; Reiger, et al., 1980, 1982).

The zona pellucida of mammalian eggs also has received much attention in recent years. Numerous morphological studies have been undertaken on the mouse (c.f. Baker, 1972; Gwatkin, 1976), hamster (Phillips and Shalgi, 1980; Bousquet et al., 1981), porcine (Dunbar et al., 1978; Menino and Wright, 1979), and human zona pellucida (Hertig and Adams, 1967; Guraya, 1974; Bousquet et al., 1981). Although biochemical investigations have been undertaken (Lowenstein and Cohen, 1964; Mintz, 1962; Soupart and Noyes, 1964; Oikawa et al.,
1973; Nicolson et al., 1975; Gwatkin and Williams, 1976), these have largely been limited to the identification of proteins using gel electrophoresis (Oikawa et al., 1973; c.f. Gwatkin, 1976) and the determination of solubility properties before and after fertilization (Chang and Hunt, 1956; Inoue and Wolf, 1974a, 1974b, 1975).

Recently, proteins of the mouse zona pellucida have been identified and characterized before (Bleil and Wassarman, 1978, 1980a,b,c) and after (Bleil et al., 1981) fertilization.

In the present study (although the precise role of the Limulus egg envelope is unclear) envelope proteins are identified using gel electrophoresis and gel filtration, solubility properties are examined, and saccharide and protein content are determined using quantitative colorimetric tests. The biological activity of protein components is determined using sperm attachment assays.
MATERIALS AND METHODS

Egg Envelope Preparation

Isolation of egg envelopes

Egg envelopes (EE) were removed from freshly spawned, Millipore-filtered artificial seawater (MFASW) washed eggs with watchmaker forceps. The envelopes were torn open and the egg contents were washed out with a jet of double-distilled water (ddH₂O) from a squeeze bottle (4°C). The envelopes were washed extensively to remove any remaining egg contents.

Isolation of egg envelope proteins

Isolated, washed egg envelopes were placed in 3 M urea at 4°C overnight (50 EE in 300 µl 3 M urea). Envelopes were separated from the supernatant (containing EE proteins) by briefly centrifuging in a microfuge. Urea-soluble proteins were either electrophoresed immediately on SDS or acid-urea gels or dialyzed against ddH₂O and stored at 4°C for further use. Precipitated envelope proteins were obtained after urea treated envelopes were dialyzed against MFASW at 4°C for 24 hr. Proteins from the outer 5 µm envelope layer were isolated by rolling washed eggs across a clean glass microscope slide (300 eggs on each slide), adding a small amount of 3 M urea, and scraping the layer loose with a metal spatula. Solubilized proteins then were obtained by collecting the urea solution.
Gel Electrophoresis

SDS gel electrophoresis (PAGE)

Samples were run on 7.5% polyacrylamide slab gels according to the method of Laemmli (1970). Gels were stained with Coomassie Blue for protein detection as described by Laemmli (1970), by the periodic acid-Schiff procedure (Fairbanks et al., 1971) to detect saccharides, or by silver staining (Wray et al., 1981). Molecular weights of proteins were determined from molecular weight standards purchased from BIO-RAD.

Acid-urea gel electrophoresis

Samples were run on 7.5% acid-urea slab gels according to the method of Panyim and Chalkley (1969). All gels were pre-electrophoresed overnight at 90 V and samples were run at 90 V, 15 mA for 6 hr. Gels were stained with Coomassie Blue or fluorescein-conjugated lectins to identify protein and saccharide constituents, respectively.

Lectin binding to gels

To determine whether there was a difference in the saccharide content of egg envelope glycoproteins, proteins resolved on gels were treated with fluorescein-conjugated lectins. Urea-soluble egg envelope proteins were run on acid-urea gels according to the method of Panyim and Chalkley (1969), but were left unstained. Gels were treated with fluorescein-conjugated lectins according to the method modified from Burridge (1978). Gel lanes were cut out and brought to
neutral pH with buffer (50 mM Tris-HCl, 0.15 M NaCl, 0.1% NaN₃, pH 7.5). The buffer was changed four to five times to equilibrate the gel and the pH was checked with pH paper. After equilibration, gel pieces were totally immersed in a lectin-buffer solution (100 μg/ml FITC-WGA, -Con A, -GPL, or -APL) and placed on a shaker overnight. Calcium and magnesium were added at a concentration of 0.5 mM to aid in lectin binding. Unbound lectin was removed by pouring off the lectin-buffer solution and washing the gel slices in buffer. The buffer was changed five times daily for two days. Gels were then examined with ultraviolet light and photographed. To ensure that the observed fluorescence was due to specific binding of a particular FITC-lectin, control gel lanes were run and equilibrated in the presence of the inhibitory sugar (20 mg/ml) for several hours. The sugar also was present during lectin-buffer incubation and in the subsequent buffer washes.

Scanning Electron Microscopy

Eggs were frozen in liquid nitrogen and fractured with a razor blade. They were then fixed in a 2.5% glutaraldehyde solution (Cacodylate buffer, 4°C) for 12-24 hr. After fixation, eggs were washed, postfixed in OsO₄ for 2 hr, and stored in ddH₂O for 1-5 days. Specimens then were dehydrated in ethanol, cleared in Freon 113, and critical-point dried using liquid carbon dioxide. Preparations were mounted on stubs with silver paint, carbon and gold coated, and examined on a JEOL JSM-35 scanning electron microscope.
Solubility of Egg Envelopes

Envelope proteins in ddH₂O were added to various solutions to determine the solubility in each. The protein-containing solutions were mixed in a ratio of 1:5 (vol/vol) with various reagents and placed at 4°C (except for SDS-containing solutions) for 10 and 30 min. Mixtures were centrifuged for 2 min in a microfuge and examined for precipitation.

Precipitates were collected after urea extracts of egg envelopes were dialyzed against MFASW (4°C) overnight. The solubility of the precipitate was examined in 3 M urea, ddH₂O, MFASW, 1% SDS, or chitinase pH 6.0 (24°C, 3 U/ml).

Sugar and Protein Content of Egg Envelopes

Total neutral sugar content of egg envelopes was determined by the phenol-sulfuric method (Dubois et al., 1956) using mannose and fucose as standards. Methylpentoses were measured by the Dische-Shettles reaction (Dische and Shettles, 1951) using fucose as a standard. Protein concentration of urea-soluble egg envelope proteins (ultra-pure urea) was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard.

Preparation of samples for sugar analysis

Hydrolyzed protein Twenty-five washed envelopes were placed in 16 x 125 mm culture tubes with screw caps containing 2N HCl (2 ml). The tubes were capped after flushing with nitrogen gas to decrease carmelization. The tubes were heated for 8, 12, or 16 hr at 100°C.
and then allowed to cool before neutralizing. Hydrolysates were placed on Dowex 50 (H\(^+\) form) columns and the ddH\(_2\)O eluant was collected. The columns were regenerated between samples. Eluted fractions were then rotary evaporated with a Büchi-Rotavapor-R at 52°C three times to remove any HCl and then resuspended in either one or two ml ddH\(_2\)O.

**Unhydrolyzed protein** Twenty-five washed envelopes were placed in 16 x 126 mm culture tubes with screw caps and the reagents used for the Dische-Shettles and phenol-sulfuric acid sugar tests were added directly.

**Gel Filtration of Urea-Soluble Envelope Proteins and Biological Activity of Fractions**

Sephadex G-100 and G-150 (40-120 mesh, purchased from Pharmacia) were hydrated in 3 M urea at room temperature for 3 days. Columns (250 x 9 mm) were packed at 4°C and blue dextran was utilized to determine void volumes. Urea-soluble proteins from 250 envelopes were separated on columns using 3 M ultra-pure urea as an eluant. One ml fractions were collected and protein was monitored by taking OD\(_{280}\) with a Gilford spectrophotometer. Various fractions then were run on SDS gels to determine the size of proteins eluted. Fractions that showed an increase in absorbance were dialyzed against Ca\(^{++}\)-Mg\(^{++}\) free artificial seawater (Ca\(^{++}\)-Mg\(^{++}\)FSW) for 24 hr at 4°C. Since spermatozoa were able to attach to egg sections in a Ca\(^{++}\)-Mg\(^{++}\)FSW environment and envelope proteins remained soluble in Ca\(^{++}\)-Mg\(^{++}\)FSW,
biological activity of fractions was determined by treating spermatozoa (washed one time in Ca$^{++}$-Mg$^{++}$FSW) with dialyzed fractions, incubating at 4°C for 30 min., and adding washed, treated spermatozoa to freshly sectioned washed eggs as described by Barnum and Brown (1983). Spermatozoa all became vigorously motile after being added to egg sections. The number of attached spermatozoa as a percentage of the control mean was calculated.
RESULTS

Identification of Egg Envelope Proteins

Two major urea-soluble proteins were resolved by both SDS and acid-urea gel (7.5%) electrophoresis (Figs. 1 and 3). A smear was often detected towards the bottom of urea gels (Fig. 1). If gel lanes were overloaded, one or two faint bands were detected above the two major bands (Figs. 1 and 2). Furthermore, material was usually present in the wells indicating that not all of the protein could enter the gel. Similar results were seen using SDS gels, although discrete lower molecular weight bands usually were resolved (Fig. 3). Standards indicated that the two high molecular weight bands had molecular weights of approximately 202,000 (HMWD1) and 193,000 (HMWD2) (high molecular weight doublet). The larger protein stained more intensely on SDS gels; however, the lower molecular weight protein on acid-urea gels stained more intensely (Figs. 1 and 3). The smaller proteins detected toward the bottom third of the gel had molecular weights of approximately 80,000 and 76,000 (D2). Occasionally 38,000 and 36,000 molecular weight proteins (D3) were also seen, but they were usually barely detectable. These proteins were not always resolved into discrete bands and occasionally were smeared (Fig. 4). Protein patterns from envelopes treated with SDS or β-mercaptoethanol were also determined using SDS gel electrophoresis. The high molecular weight doublet (HMWD) was absent or faintly seen after envelopes were soaked in β-mercaptoethanol (Figs. 3 and 5).
Fig. 1. Acid-urea gel electrophoresis (7.5%) of urea-soluble egg envelope proteins. Gel shows that variable amounts of protein are removed from egg envelopes after treatment with 3 M urea. The HMWD and lower molecular weight smear are clearly seen. The lower band (HMWD2) stained more intensely than HMWD1 (lanes 1, 6, 8). Lanes 1, 2, 3, and 8: 100 envelopes were treated with 400 µl 3 M urea and 50 µl were then loaded onto wells; lanes 4, 5, 6, and 7: 50 envelopes were treated with 200 µl 3 M urea and 60 µl were loaded onto wells; lanes 4 and 5: envelopes were first frozen at -70°C for several days prior to adding urea; lane 3, proteins were precipitated and resolubilized.
Fig. 2. Acid-urea gel shows that variable amounts of urea-soluble proteins are obtained from the same number of envelopes. Lanes 1, 2, 3, 4, and 5: 25 envelopes in 100 µl 3 M urea with 80 µl loaded onto wells; lanes 6 and 7: 75 envelopes in 300 µl 3 M urea with 80 and 50 µl loaded onto wells, respectively; lanes 5, 6, and 7 were overloaded to show the presence of a high molecular weight protein(s) above the HMWD.
Fig. 3. Sodium dodecyl sulfate polyacrylamide (7.5%) gels stained with Coomassie blue for protein (lanes 1-6) and for saccharides using PAS staining (lanes 7-13). Lanes 1 and 7 are standards (protein standards stained with PAS do not show up). Urea-soluble egg envelope proteins, lanes 2, 8, and 13; the first H$_2$O wash from freshly collected eggs, lanes 3 and 9; β-mercaptoethanol-soluble proteins, lanes 4 and 10; SDS(2.5%)-soluble envelope proteins, lanes 5 and 11 (SDS and proteins usually precipitate); oviductal fluid, lane 6; washed envelopes soaked in ddH$_2$O for several hr, lane 12.
Fig. 4. SDS gel stained with Coomassie blue (lanes 1 and 2) and PAS (lanes 3, 4, and 5). Low molecular weight proteins are not always resolved on SDS gels (lanes 1 and 2). The HMWD proteins are glycoproteins since they stain positive for saccharides (lanes 3 and 4). Protein standards (lane 5) and low molecular weight proteins (lanes 3 and 4) do not stain with PAS.
Fig. 5. SDS gels stained with Coomassie blue (lanes 1-7) and PAS (lanes 8-12). Lane 1 containing SDS (2.5%)-soluble proteins smeared due to SDS precipitation problems; β-mercaptoethanol-soluble envelope proteins, lanes 2 and 11 (very faint); urea-soluble envelope proteins, lanes 3, 4, and 9; protein standards, lanes 6 and 8; oviductal fluid, lane 7. Envelopes soaked in ddH₂O for several hr show a protein banding pattern identical to lane 2 (not shown). Some glycoproteins do not enter the stacking gel.
The lower molecular weight bands were absent as well. Lanes where SDS-soluble proteins were run always were smeared since SDS usually precipitated out as gels were run. SDS gels in general did not run as well as acid-urea gels since the SDS in Laemmli buffer sometimes precipitated out. Oviductal fluid and the first wash from ovulated eggs were also run on SDS gels. The HMWD was seen faintly as well as a slightly lower molecular weight doublet when the fluid removed from the egg envelope during the first wash was run on gels (Fig. 3). The two lower molecular weight doublets (faint) from urea treated envelopes also were observed. A heavy band near the bottom third of SDS gels was detected in oviductal fluid (Fig. 5) which may correspond to the 80,000 and 76,000 molecular weight proteins seen after urea treatment of envelopes. Not all proteins could enter the stacking gel since material remained in the wells of both SDS and acid-urea gels (Figs. 1, 2, 3, 4, and 5). Also, on SDS gels low molecular weight proteins were not resolved since a band appeared at the bottom of many of the gel lanes (Figs. 3 and 5). Soluble proteins that seemed to correspond to the HMWD and the 38,000 and 37,000 molecular weight proteins were obtained when washed envelopes were soaked in ddH₂O for several hours (Fig. 3). Thus, some envelope proteins may also be water-soluble or are present in residual oviductal fluid that is not washed off the egg. The urea-soluble HMWD proteins also were obtained from only the outer 5 μm porous
envelope layer (Figs. 6, 7, and 8). As was also demonstrated with urea-soluble proteins of whole egg envelopes, HMWD1 was in higher concentration on SDS gels (as indicated by the more intensely stained band), whereas HMWD2 stained more intensely than HMWD1 on acid-urea gels. Lower molecular weight proteins were absent. Thus, the outer envelope layer which specifically binds spermatozoa most likely consists of the HMWD.

Sugar and Protein Content of Egg Envelopes

Periodic acid-Schiff staining of SDS polyacrylamide gels

Periodic acid-Schiff staining of SDS gels demonstrated that the 202,000 and 193,000 molecular weight bands stained positive for saccharides while the other proteins did not (Figs. 3, 4, and 5). PAS stained gels also indicated that not all glycoproteins entered the gel. Furthermore, SDS-soluble glycoproteins precipitated out in the stacking gel and were not detected in the separating gel (Fig. 3).

Fluorescein-conjugated lectin binding to acid-urea gels

The urea-soluble HMWD from whole egg envelopes fluoresced after treatment with all four lectins (FITC-APL, -Con A, -WGA, -GPL). Intensities most likely varied due to differences in the amount of fluorescein dye conjugated to each lectin as well as saccharide concentration differences (Fig. 9). Con A (82.80 mmoles FITC) fluoresced the most intensely while the lane treated with WGA (25.30 mmoles FITC) fluoresced faintly. GPL (8.14 mmoles FITC) appeared to be rather non-specific since the whole gel fluoresced intensely.
Fig. 6. Scanning electron micrograph of the egg envelope shows the 5 μm outer, urea-soluble layer (OL) and the underlying 35 μm poreless layer (UL). 8300x
Fig. 7. SDS gel (7.5%) shows the urea-soluble proteins obtained from the 5 μm outer envelope layer by rolling washed eggs across a glass microscope slide and solubilizing the layer (in 3 M urea) that adhered to the glass. The HMWD seen (no lower molecular weigh proteins are observed) demonstrates that HMWD1 is in higher concentration than HMWD2. The gel background was unevenly stained because it became water marked and torn in the process of being dried on Whatman filter paper.
Fig 8. Acid-urea (a) and SDS (b) gel electrophoresis of urea-solubilized outer envelope proteins on 15% gels. HMWD2 stains more intensely on SDS gels. No lower molecular weight proteins were detected.
Fig. 9. Acid-urea gels showing the presence of saccharides in urea-soluble egg envelope proteins using fluorescein-conjugated Con A (lane 2), APL (lane 3), WGA (lane 4), and GPL (lane 5). Lane 1, Coomassie blue stained proteins. Controls: lane 2', Con A and methyl-mannoside; lane 3', APL and α-L-fucose; lane 4', WGA and N-acetylgalactosamine; lane 5', GPL and α-L-fucose; lane 5'', GPL and α-D-glucose. Two gel lanes were cut in half (lanes 3 and 3', 5' and 5'') so that all treatments and controls could be run on the same gel.
Although the gel lane treated with FITC-APL contained practically as much FITC (30.40 nmoles) as FITC-WGA (25.30 nmoles), the protein fluoresced much more intensely. In addition, APL treated proteins fluoresced almost as much as those treated with Con A, but the APL was conjugated with less than half the amount of fluorescein. Therefore, fucose was either in higher concentration than glucose/ mannose and N-acetylglucosamine or there were more exposed fucose moieties (see results of saccharide tests). A protein larger than the HMWD was detected in both FITC-APL and -Con A treated lanes, while a lower molecular weight smear appeared in all lanes.

**Colorimetric saccharide tests**

The Dische-Shettles sugar test for methylpentoses indicated that there were $193 \pm 63$ ng of fucose in each egg envelope ($n = 25$ EE, repeated 13 times). The phenol-sulfuric acid assay determined that there were approximately $304 \pm 69$ ng of neutral hexoses and $603 \pm 234$ ng of fucose ($n = 25$ EE, repeated four times) in each egg envelope. There was no significant difference in results between the two methods of preparing envelopes (hydrolyzed or unhydrolyzed). One egg envelope weighed 230 µg; thus, the percentage of neutral hexoses was determined to be 0.13 percent of the total dry weight, while fucose (by Dische-Shettles method) was 0.08 percent. The discrepancy in the amount of fucose present in egg envelopes by the two tests was most likely due to the overlap in the absorbance of saccharide molecules at 480 nm and 490 nm (phenol-sulfuric acid test). Thus, measurements
of both fucose and hexose concentrations were probably higher than they actually should be using the phenol-sulfuric acid method.

**Protein content of envelopes**

The amount of egg envelope proteins solubilized in 3 M ultra-pure urea seemed to vary since the amount recovered was 132.39 ± 95.04 μg in 200 μl 3 M urea for 50 envelopes (2.22 ± 2.00 μg from one egg envelope, repeated 27 times). Furthermore, the extent of dissolution in urea also was variable and corresponded to the amount of protein measured. The range of protein values varied from 5.63 to 0.12 μg per envelope. Some envelopes remained unchanged in urea. Gels also confirmed this since band intensities were highly variable (Figs. 1 and 2). Visible differences in egg envelope make-up were not observed; thus, it was not possible to predict which envelopes would enable one to obtain the largest quantity of proteins using urea.

**Solubility of Egg Envelope Proteins**

The solubilities of egg envelope proteins in various reagents are shown in Table 1. Proteins were insoluble in salts (including buffers), non-ionic detergents, and MFASW. However, proteins remained soluble in SDS, ddH₂O, Ca⁺⁺-Mg⁺⁺FSW, and reagents (such as urea and guanidine-HCl) which disrupt hydrogen bonds. Gels demonstrated that urea-soluble proteins were present in the precipitate and absent or in concentrations too low to be detected in the supernatant (Fig. 10). Protein determinations further showed that 0.16 ± 0.06 μg protein was present in the supernatant while 2.22
Table 1. The effects of various treatments on the solubility of water-soluble egg envelope proteins

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Solubility^a</th>
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<tbody>
<tr>
<td>10, 20 mM Borate pH 8.0</td>
<td>--</td>
</tr>
<tr>
<td>Ca^{++} - Mg^{++} FSW</td>
<td>++^b</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>++^c</td>
</tr>
<tr>
<td>1 mM EDTA pH 8.0</td>
<td>--</td>
</tr>
<tr>
<td>1 mM EDTA-1% SDS pH 8.0</td>
<td>++</td>
</tr>
<tr>
<td>Freezing, -20°C</td>
<td>--</td>
</tr>
<tr>
<td>3 M Guanidine-HCl</td>
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</tr>
<tr>
<td>MFASW</td>
<td>--</td>
</tr>
<tr>
<td>1 mM, 10 mM NaCl</td>
<td>--</td>
</tr>
<tr>
<td>1 mM NaCl-1% Triton X-100</td>
<td>--</td>
</tr>
<tr>
<td>10 mM, 1 mM NaHCO₃ pH 8.0</td>
<td>--</td>
</tr>
<tr>
<td>1% Sarcosyl</td>
<td>++</td>
</tr>
<tr>
<td>1% SDS</td>
<td>++</td>
</tr>
<tr>
<td>10% TCA</td>
<td>--</td>
</tr>
<tr>
<td>1 mM, 10 mM Tris pH 8.0</td>
<td>--</td>
</tr>
<tr>
<td>3 M Urea</td>
<td>++</td>
</tr>
</tbody>
</table>

^a++ indicates that solubility was maintained.

^bAfter prolonged dialysis at 4°C, some protein precipitation may occur.

^cSmall amounts of precipitation may be observed (particularly at 4°C).
Fig. 10. Acid-urea gel electrophoresis of urea-soluble envelope proteins. Lanes 1 and 3, urea treatment of envelopes; lane 2, the supernatant after urea-soluble proteins were precipitated by dialyzing against MFASW at 4°C; lane 4, proteins obtained from the resolubilized (in 3 M urea) precipitate.
+ 0.05 µg protein was measured in MFASW-precipitated protein (n = 50, repeated seven times). Therefore, proteins were removed from envelopes and maintained in solution by reagents that disrupted hydrogen bonds (e.g. urea). Proteins also remained soluble in ddH₂O and divalent cation-free seawater. Both high concentrations of sodium (in Ca⁺⁺-Mg⁺⁺FSW) and the absence of sodium (in ddH₂O) maintained solubility, while precipitates formed in low sodium (1 mM, 10 mM NaCl). Precipitated protein could not be resolubilized in ddH₂O, MFASW, 1% SDS, or chitinase, although precipitates almost totally dissolved in 3 M urea.

Although the outer 5 µm envelope layer generally was readily soluble in 3 M urea, the underlying layer was never completely soluble. The underlying layer also was unchanged by 1-5% SDS, β-mercaptoethanol, and 6 M urea (the 5 µm layer exhibited dissolution only in urea). Although this layer did not dissolve in 2N HCl (the outer layer did), 0.2N NaOH dissolved the entire envelope. Upon neutralization, a white, flocculent precipitate appeared.

Gel Filtration of Urea-Soluble Proteins and Their Biological Activity

Gel Filtration

SDS gels demonstrated the banding pattern from fractions eluted from Sephadex G-100 and G-150 columns (Figs. 11 and 12). The HMWD as well as protein that did not enter the stacking gel (in most cases) were observed in all fractions (both G-100 and G-150 columns) that
Fig. 11. SDS gel electrophoresis of eluted envelope proteins from a Sephadex G-100 column. Lanes 1-4 and 13-16 are silver stained while lanes 5-8 and 9-12 are stained with Coomassie blue. Solubilized proteins prior to gel filtration, lanes 1 (10 μl), 5 (35 μl), and 9 (75 μl); fractions from the eluant peak at OD$_{280}$ (fraction 8), lanes 2 (15 μl), 6 (45 μl), 12 (125 μl), and 14 (25 μl); fraction 11, lanes 3 (15 μl), 7 (80 μl), 11 (100 μl), and 15 (30 μl); fraction 13, lanes 4 (15 μl), 8 (80 μl), 10 (80 μl), and 16 (30 μl); fraction 30 where no increase in OD$_{280}$ was measured, lane 13 (150 μl). Proteins stained with Coomassie blue are smeared, although silver stained proteins in lane 14 indicate the presence of the HMWD and a larger protein.
Fig. 12. SDS gels of eluted envelope proteins from a Sephadex G-150 column stained with Coomassie blue. Lanes 1 and 9, protein standards; lane 2, fraction 22 where no increase in OD$_{280}$ was measured (160 μl); lane 3, fraction 19 where no increase in OD$_{280}$ was measured (150 μl); lane 4, fraction 13 where no increase in absorbance was measured (150 μl); lane 5, fraction 11 where there was no increase in OD$_{280}$ (150 μl); lanes 6 and 13, eluant peak at OD$_{280}$ (fraction 6, 110 and 130 μl, respectively); lanes 7 and 14, fraction 5 (110 and 150 μl, respectively); lanes 8 and 15, fraction 4 (120 and 160 μl, respectively); lane 10, fraction 10 (145 μl); lane 11, fraction 8 (140 μl); lane 12, fraction 7 (140 μl). Proteins appear to be complexed since both high and low molecular weight proteins are detected in eluted fractions. The HMWD proteins are not resolved into two bands.
had an increased absorbance from the blank. These fractions had protein patterns practically identical to those obtained from urea-soluble proteins of whole egg envelopes, although bands were not well-resolved. Envelope proteins appeared to be complexed or degraded since the HMWD could not be resolved into two bands and separation of low and high molecular weight proteins could not be achieved even on the G-150 column (Fig. 12). Absorbance appeared to correspond to the amount of protein loaded on gels since higher absorbances corresponded to more intensely stained bands (Figs. 13 and 14).

**Biological activity of fractions**

During dialysis of fractions against Ca\(^{++}\)-Mg\(^{++}\)FSW, a residue of protein generally formed along the walls of the dialysis bag. Thus, proteins were lost prior to determining biological activity of various fractions by the sperm attachment assay (Fig. 13). The most protein precipitation (determined by the amount of precipitate seen) formed in fractions that had the highest OD\(_{280}\); however, these fractions still were able to decrease sperm attachment to egg sections when added to spermatozoa (Table 2). Sephadex G-150 fractionated proteins also decreased sperm attachment. However, during dialysis most of the proteins in the peak fraction may have precipitated out since a large precipitate was seen and sperm attachment was not significantly reduced from controls. Other fractions near the peak had little or no precipitation during dialysis and sperm attachment ranged from 25-75 percent of controls.
Fig. 13. Typical elution profile of urea-soluble envelope proteins before (—) and after (—) dialysis against Ca\(^{++}\)-Mg\(^{++}\) FSW at 4°C. The effect of several fractions on sperm attachment demonstrates the activity of eluting protein (*). Gel filtration was performed on a column of Sephadex G-100 equilibrated in 3 M urea. \(V_o\), void volume.
PERCENT SPERM ATTACHMENT (\(*\))

ELUTION VOLUME (ML)
Fig. 14. Typical elution profile of urea-soluble envelope proteins on a Sephadex G-150 column prior to dialysis against Ca$^{++}$Mg$^{++}$FSW. $V_0$, void volume
Table 2. Biological activity of urea-soluble egg envelope proteins after Sephadex G-100 fractionation

<table>
<thead>
<tr>
<th>Fractions(^a)</th>
<th>Total Number (N) of Sections Counted</th>
<th>Sperm Attachment as a Percentage of the Control Mean(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before gel filtration(^c)</td>
<td>30</td>
<td>81.6 ± 16.5</td>
</tr>
<tr>
<td>Peak(^d,e) (Fraction 8)</td>
<td>30</td>
<td>30.0 ± 24.6</td>
</tr>
<tr>
<td>Fraction 11</td>
<td>30</td>
<td>11.3 ± 9.7</td>
</tr>
<tr>
<td>Fraction 13</td>
<td>30</td>
<td>55.9 ± 17.2</td>
</tr>
</tbody>
</table>

\(^a\) Compare with Figures 11 and 13.

\(^b\) Fractions that had no absorbance at 280 nm were used as controls (N = 28, 52.2 ± 7.3 spermatozoa attached to egg sections). Assays also were run with spermatozoa treated only with Ca\(^{++}\)-Mg\(^{++}\) FSW. Sperm attachment was approximately 50% of MFASW counts which agreed with results of control fractions. Thus, approximately 50 spermatozoa attached to controls rather than 100.

\(^c\) Protein residue was observed during dialysis against Ca\(^{++}\)-Mg\(^{++}\) FSW.

\(^d\) Most sections had no spermatozoa attached; however, areas with the highest attachment were chosen for counts.

\(^e\) The largest precipitation of protein during dialysis against Ca\(^{++}\)-Mg\(^{++}\) FSW occurred in fractions with the highest OD\(_{280}\).
Fractions outside the absorbance curve did not inhibit sperm attachment to egg sections. Since it was not known how much protein precipitated out in each fraction, the number of spermatozoa that attached to egg sections was not always correlated with the amount of protein that remained soluble in each fraction. Thus, the fractions that initially had the highest $\text{OD}_{280}$ reading did not always inhibit sperm attachment as much as fractions that had a lower absorbance prior to and after dialysis against Ca$^{++}$-Mg$^{++}$FSW. It appeared that the activity was in fractions eluting later than where most of the HMWD proteins eluted (see Table 2, Figs. 11 and 13). The most important conclusion to be made from these experiments is that envelope protein-containing fractions were actually able to decrease the number of protein treated spermatozoa that attached to egg sections.
DISCUSSION

In this study, urea-soluble egg envelope proteins were identified using gel electrophoresis. Two major proteins had molecular weights of approximately 202,000 and 193,000. This doublet most likely was from the 5 \( \mu \)m outer layer of the envelope since these two high molecular weight bands were seen when solubilized proteins from the outer layer were run on gels. Periodic-acid Schiff staining of SDS gels indicated that they were glycoproteins. Since solubility studies showed that envelope proteins were soluble in Ca\(^{++}\)-Mg\(^{++}\)FSW, the sperm attachment assay using a Ca\(^{++}\)-Mg\(^{++}\)FSW environment was used to determine the effect of envelope protein treatment of spermatozoa on egg section attachment. Sperm attachment to egg sections was approximately 32 percent (average of all assays using protein-containing fractions) of controls when urea-soluble proteins, separated on Sephadex G-100 columns, were used. Attachment was 82 percent of controls when urea-soluble proteins were utilized prior to running them on a G-100 column. The activity probably is present in fractions that elute later than the early-eluting proteins (HMWD).

The HMWD could be separated from other envelope components by urea disruption of noncovalent bonds. The two high molecular weight bands were resolved on acid-urea gels (if not overloaded), but often ran as a smear or thick band on SDS gels. Furthermore, a fraction of the HMWD glycoproteins may differ slightly from the rest of the HMWD population since more protein was found in HMWD1, than in HMWD2 on
SDS gels, but on acid-urea gels, more protein was in HMWD2. Protein mobility differences indicate that there actually may be three populations of proteins in the HMWD. One may be glycosylated differently or is a subunit of one of the HMWD proteins. Lower molecular weight proteins were seen on gels after envelopes were treated with SDS and β-mercaptoethanol only after proteins had first been solubilized by urea. It was not known why only the HMWD from urea-soluble proteins of the 5 μm envelope layer appeared on SDS gels. The layer may only consist of these two proteins or perhaps the concentration of protein obtained was so low that lower molecular weight proteins were not detected. The protein pattern from the outer 5 μm layer corresponded to the high molecular weight doublet and may be sperm attachment 'receptor' proteins since spermatozoa no longer attached to the egg after removing the outer layer or to 3 M urea treated eggs. Furthermore, both sugar tests and FITC-lectin binding studies demonstrated that envelope components consist of glycoproteins (since saccharides were present). The high molecular weight bands were the only proteins which were PAS positive (on SDS gels). Lectin binding showed that saccharides also were present in the low molecular weight smear on acid-urea gels; however, these proteins may not be resolved on SDS gels. This agrees with a previous study by Barnum and Brown (1983) which showed that the egg envelope component(s) involved in sperm attachment is a saccharide (fucose)-containing component.
The 5 μm envelope layer appears to be very soluble in urea, acid, and base. However, the solubility of envelope proteins in 3 M urea was quite variable as determined by the amount of soluble protein obtained as well as by observing the dissolution of envelopes. The reason for this is unknown since eggs from one female can have both urea-soluble and -insoluble envelopes. This made it very difficult to predict the amount of protein one could obtain from urea treatment of a certain number of egg envelopes. The underlying layer was shown to be highly insoluble except in base (NaOH). This suggests that very few hydrophobic interactions of components exist or are exposed since 6 M urea could not even dissolve or disrupt this layer. Since sodium hydroxide breaks covalent bonds, solubility in base may indicate that the underlying layer consists of covalently linked proteins that may even be crosslinked. Insolubility in acid suggests that there may be a high concentration of hydrophobic residues (uncharged polar groups) such as tyrosine (pK_R^ć = 10.07). Tyrosyl crosslinks are responsible for hardening the Drosophila chorion (Mindrinos et al., 1980) and the sea urchin fertilization membrane (Foerder and Shapiro, 1977; Hall, 1978). The polypeptide components of these vestments are insoluble in various denaturing solutions (2% SDS, 8 M urea, 6 M guanidine hydrochloride) which do not disrupt covalent bonds.

In order for an assay to be developed to determine biological activity (ability of proteins to inhibit or decrease sperm attachment to egg sections), it was necessary to find a medium where envelope
proteins remained soluble and spermatozoa would still be viable and attach to egg envelopes. Although some protein did precipitate out during dialysis (residue formation along the sides of the bag), enough soluble protein remained to inhibit sperm attachment during assays using envelope protein treated spermatozoa. Since higher activities of various fractions did not always correspond to absorbance levels, there may have been an active component present in the later eluting fractions in addition to precipitation differences and variation in the assay. Unfortunately, it was not possible to determine the amount of protein added to spermatozoa since there was not enough material collected in each fraction to run gels, run protein determinations before and after dialysis, and to perform sperm attachment assays. Elution profiles of proteins from Sephadex G-100 and G-150 columns differ since more protein was eluted in the void volume on the G-150 column. Furthermore, proteins eluted in fewer fractions. Some proteins may undergo a conformational change (perhaps a change in shape as the molecules moved through the dextran columns) or stick to the gel. Perhaps the use of polyacrylamide gel would eliminate the variation in profiles as well as resolve proteins better.

Intact 'sperm receptors' from sea urchin eggs have been isolated by digesting eggs with trypsin (Schmell et al., 1977; Glabe and Lennarz, 1979; Kinsey and Lennarz, 1981). Although data were not included in this section, trypsin digestion of the egg envelope surface was undertaken in an attempt to obtain glycopeptides that...
contained a soluble, intact 'sperm receptor'. However, results were highly variable (0-100 percent reduction of sperm attachment to egg sections) and if egg envelope surface digestion proceeded for over 20-30 min, the digest initiated sperm motility.

The major envelope proteins isolated in the present study may be similar to the fibronectins. These proteins have been implicated in adhesion of cells and cell-cell recognition (c.f. Hynes and Yamada, 1982; c.f. Ruoslahti et al., 1982). Cold insoluble globulin (CIG) belongs to this class of adhesive surface proteins which are high molecular weight glycoproteins (two subunits of approximately 200,000-250,000). CIG which is immunologically similar to fibronectin (Ruoslahti and Vaheri, 1975), has been shown to be insoluble in the cold and can be recovered in the cryoprecipitate (Morrison et al., 1948). If Limulus egg envelope proteins isolated in this study are similar to CIG, precipitation problems may be due in part to their being insoluble in the cold (also when frozen). Dialysis was always performed at 4°C to decrease the possibility of proteolysis. Further studies would be needed to determine whether these proteins are indeed related to CIG.

Further studies need to be undertaken to determine the role of the egg envelope in fertilization. The function of the envelope protein complex should also be further examined to more fully characterize the HMWD glycoproteins (differences in amino acid content/sequence and also glycosylation). An additional control
could be included during the biological activity studies. Since fucosidase treatment of the egg envelope surface decreased sperm attachment (Barnum and Brown, 1983), fractions also could be digested with this glycosidase and the activity compared with untreated fractions. Furthermore, biological activity (decrease in sperm attachment) must be correlated with protein concentration as well as saccharide concentration and composition if further experiments are to be undertaken.
LITERATURE CITED


Bradford, M. M. 1976. A rapid and sensitive method for the


SECTION 3. SPERM ATTACHMENT TO AN AGAROSE BEAD EGG MODEL AND THE USE OF LECTINS TO DETERMINE SACCHARIDE COMPOSITION OF SEMINAL FLUID AND THE SPERMATOZOAN SURFACE
INTRODUCTION

During fertilization, a complex series of events take place which enable spermatozoa to become motile, recognize and bind to the egg surface, and penetrate so as to eventually unite with the female pronucleus. Recently, research has been undertaken to determine what activates invertebrate spermatozoa after release from the male and the effect of activation on spermatozoa physiology (Hathaway, 1963; Ohtake, 1976; Collins and Epel, 1977; Schackmann et al., 1978; Kinsey et al., 1979; Hansbrough and Garbers, 1981; Schackmann and Shapiro, 1981).

Few studies have been published concerning activation of spermatozoa from the horseshoe crab, Limulus polyphemus. Previous investigations have been limited to the formation of the acrosomal process during the acrosome reaction (Tilney, 1975; Tilney et al., 1979, DeRosier et al., 1982). Recently, however, the mechanism of motility initiation and acrosome reaction induction has been investigated (Clapper and Epel, 1982b). Spermatozoa from the horseshoe crab, Limulus polyphemus, differ from other invertebrates commonly studied such as the sea urchin since nonmotile spermatozoa become motile only after contact with a sperm motility initiating factor (SMI) that is released from the egg (Shoger and Bishop, 1967; Clapper and Brown, 1980a,b; Clapper and Epel, 1982a,b). An increase in intracellular pH and an influx of specific ions have been ruled out as direct mediators of SMI-initiated motility (Clapper and Epel,
1982b). Thus, the mechanism of spermatozoa activation and factors involved in the induction of the acrosome reaction in *Limulus* are still unknown.

In this study, sperm interactions with an egg model that consisted of fucopyranoside-conjugated agarose beads were observed. These experiments were conducted to confirm earlier studies that suggested a fucose-mediated sperm attachment to egg envelopes. Saccharide content of seminal fluid was determined and the effect of lectins on sperm motility and egg section attachment also was studied to gain insight into the properties of the spermatozoan surface and seminal fluid. The effect of seminal fluid on sperm motility was also examined and its possible role in sperm activation is discussed.
MATERIALS AND METHODS

Sperm Attachment to Agarose Beads

Attachment assay

Agarose beads were washed extensively in cold Millipore-filtered artificial seawater (MFASW) for 30 min by filtering and stirring occasionally. The washed beads then were resuspended in MFASW. These beads were placed on clean microscope slides and several drops of a 2.5% sperm suspension were added. Sperm behavior was immediately noted prior to and after motility initiation by CaCl$_2$ (34 mM). A coverslip with clay supports was added after 10 min. Observations were made before and after motility initiation and photomicrographs were made using a Nikon microscope.

Agarose beads used for the attachment assay

Selectin 8, immobilized p-aminophenyl α-L-fucopyranoside, was purchased from Pierce Chemical Company (fucopyranoside-conjugated agarose beads). Selectin 8 was used in attachment assays before and after digestion with 0.5 U/ml α-L-fucosidase pH 6.0, 24°C (from Boehringer Mannheim Biochemicals) for 1 hr. Soybean trypsin inhibitor (2.5 mg/ml) and p-aminophenylbenzamidine (2.5 mg/ml) were added to the incubation mixture to inhibit proteases. After fucosidase digestion, the treated beads were washed extensively in cold MFASW before adding spermatozoa. Selectin 8 also was treated with asparagus pea lectin (APL) purchased from Sigma (125 μg/ml). The
lectin and agarose bead suspension was mixed for 30 min at 4°C. The beads were washed by filtering for 30 min in MFASW.

Fucosylex beads, immobilized fucose-binding protein from Lotus tetragonolobus (APL), were purchased from Miles Laboratory (APL-conjugated agarose beads).

Dialysis of semen

Spectra-por dialysis tubing (molecular weight cutoff of 12,000-14,000) was used to dialyze semen against MFASW at 4°C with several changes of seawater. Fluorescein-conjugated APL was added to spermatozoa as described previously to determine whether fucose existed freely and could be dialyzed away or was complexed with other components.

Fluorescence microscopy

Photomicrographs were taken using a Zeiss fluorescence light microscope mounted with an Olympus camera. A 500 nm barrier filter and BG 12 excitor filter were used.

Effect of Lectins on Spermatozoa

Concanavalin A (Con A), wheat germ agglutinin (WGA), and asparagus pea lectin (APL) were purchased from Sigma Chemical Company and diluted to 100 μg/ml in MFASW. Freshly collected semen was either diluted to a 10% sperm suspension and centrifuged once to concentrate spermatozoa or raw, undiluted semen was used in experiments directly.
Lectin treatment

Immediately after lectin was added to spermatozoa (10% suspension or undiluted semen), a small sample was examined to determine whether agglutination and/or motility of spermatozoa occurred. After lectin incubation (10 min, 4°C), unbound lectin was removed by centrifuging spermatozoa and resuspending in MFASW. Spermatozoa were diluted to make a 2.5% suspension and several drops were placed on a clean microscope slide. Calcium chloride was added to induce motility. Sperm behavior after lectin treatment was noted before and after motility initiation. A coverslip was added after 10 min to prevent evaporation of MFASW so that observations could be made until sperm motility terminated. Photomicrographs were taken; however, only documentation is provided for fluorescein-conjugated lectins since the results were the same for both types of treatments. Lectin treated spermatozoa (5% suspension) were also added to freshly sectioned Limulus eggs to determine if attachment occurred. The attachment assay utilized was a modification of Mowbray and Brown (1974) as described by Barnum and Brown (1983).

Detection of Saccharides in Semen using Fluorescein-conjugated Lectins

Fluorescein-conjugated (FITC) Con A, WGA, and APL were purchased from Sigma Chemical Company and were diluted to 100 μg/ml in MFASW. Freshly collected semen was either diluted to a 10% sperm suspension prior to low speed centrifugation to remove seminal fluid (then spermatozoa were collected by sedimentation) or undiluted semen was used.
Fluorescein-conjugated lectin treatment

After spermatozoa were incubated with FITC-lectin for 30 min at 4°C, they were sedimented and resuspended in MFASW two times to remove unbound lectin. Several drops of a 15% sperm suspension were placed on a clean microscope slide and allowed to air dry for 20 min. Either 1 M sucrose or immersion oil was added prior to adding a coverslip. Spermatozoa were examined with both ultraviolet and transmitted light.

Colorimetric saccharide tests

The presence of saccharides in seminal fluid was substantiated using the phenol-sulfuric acid assay for neutral sugars (Dubois et al., 1956) and the Dische-Shettles test for methylpentose (Dische and Shettles, 1951).
RESULTS
Sperm Attachment to Agarose Beads

Spermatozoa adhere to fucopyranoside-conjugated agarose beads by the sperm apex (Fig. 1a). Many, however, did not appear to attach very tightly since some detach from the bead surface and swim away until confronting another bead. During observation of sperm behavior near agarose beads, the microscope slides were gently shaken so as to dislodge any loosely bound sperm. Thus, Figures 1a and b show spermatozoa that were bound to beads tightly. The acrosome reaction did not occur since the acrosome vesicle remained intact on the bead surface (Fig. 1b). Spermatozoa that did adhere loosely were detained briefly and during that time they spun vigorously as they remained attached to the bead surface. Tightly bound spermatozoa behaved similarly, although they were not able to detach. Spermatozoa did not adhere to fucopyranoside-conjugated agarose beads treated with α-L-fucosidase to remove fucopyranoside residues (Fig. 1c).

Sperm behavior also was observed when spermatozoa were mixed with Lotus tetragonolobus (APL)-conjugated agarose beads. Surprisingly, although many did not adhere, spermatozoa attached by their tails (Fig. 1d). These attached spermatozoa were highly motile and spun rapidly by their tails. Few sperm were able to detach which suggests that the affinity was stronger than sperm apex-fucopyranoside interactions mentioned above. Spermatozoa tail attachment was
Fig. 1. Sperm attachment to agarose beads

a) Spermatozoa attach to fucopyranoside-conjugated agarose beads by their apical tips. 300x

b) Primary sperm attachment to an agarose bead. Acrosomes remain intact. 1000x

c) Spermatozoa do not attach to fucosidase digested fucopyranoside-conjugated agarose beads. 625x

d) Spermatozoa adhere to asparagus pea lectin-conjugated agarose beads by their tails. 560x
difficult to photograph since the sperm head was generally elevated from the agarose bead and neither the bead nor the sperm could be in focus simultaneously. Spermatozoa added to APL treated fucopyranoside-conjugated agarose beads, also adhered by their tails (Figs. 2a,b,c), but in somewhat higher numbers than that found in APL-conjugated beads. Similar behavior was exhibited in this interaction as described above for APL-conjugated beads. These observations suggest that spermatozoa acrosome tips and tails recognize fucose residues and fucose-binding protein, respectively.

Effect of Lectins on Spermatozoa and Detection of Saccharides in Semen using Fluorescein-Conjugated Lectins

Asparagus pea lectin agglutinated spermatozoa immediately after it was added to either raw or diluted semen (Figs. 2d, 3a). Many seminal particles and spermatozoa were present in each agglutinated mass. Although spermatozoa were still able to become highly motile, they were not able to adhere to the envelopes of egg sections since they were anchored by their tails and could not break away. If motility were initiated prior to adding APL, there was less agglutination and large masses of spermatozoa were not detected. Fucose appeared to be a major saccharide constituent of semen since fluorescein-conjugated (FITC) APL treated semen fluoresced intensely (Fig. 3b). This was substantiated by the Dische-Shettles and phenol-sulfuric acid saccharide tests. The constituents of seminal fluid rather than spermatozoa appeared to fluoresce most intensely.
Fig. 2. Sperm attachment to agarose beads

a) Spermatozoa adhere to asparagus pea lectin treated fucopyranoside-conjugated beads by their tails. Arrows show where spermatozoa acrosomes are clearly seen. 400x

b) Spermatozoa tail attachment to asparagus pea lectin treated fucopyranoside-conjugated beads. Arrows show where spermatozoa acrosomes are clearly seen. 1100x

c) Spermatozoa tails attached to asparagus pea lectin treated fucopyranoside-conjugated beads. Arrow, spermatozoa tails. 1300x

d) Tails agglutinate when spermatozoa are treated with asparagus pea lectin. 1100x
Fig. 3. Spermatozoa treated with fluorescein-conjugated asparagus pea lectin

a) Unwashed spermatozoa agglutinate in the presence of asparagus pea lectin. Both seminal fluid-particles and spermatozoa are seen in the agglutinated mass. 440x

b) Fluorescence microscopy shows that the entire mass fluoresces intensely. It appears from this micrograph that seminal particles/fluid fluoresce most intensely while isolated spermatozoa do not fluoresce. 440x

c) Light micrograph of unwashed, agglutinated spermatozoa. Seminal fluid and particles are present among the clumped sperm mass. 440x

d) Micrograph of agglutinated sperm (treated with APL). These unwashed spermatozoa fluoresce most intensely where seminal particles are present (arrow). Isolated spermatozoa do not appear to fluoresce. 440x

e) Washed spermatozoa agglutinate after APL-treatment. Most agglutination occurs where seminal particles exist (arrow). 560x

f) Fluorescence microscopy shows that there is faint fluorescence where APL-agglutinated spermatozoa and seminal particles are seen with light microscopy. A more intensely fluorescing area exists where there is a large seminal particle (arrows; compare with fig. 3e). 560x

g) Light micrograph showing agglutinated seminal particles which include a few isolated sperm after treatment with FITC-APL. 520x

h) Fluorescence microscopy of seminal particles treated with FITC-APL. Several particles fluoresce faintly (arrow); spermatozoa do not appear to fluoresce. 520x
Figures 3c and d demonstrate APL induced agglutination of unwashed, diluted spermatozoa; however, there was no fluorescence of spermatozoa that were not clumped. A comparison of the light and fluorescence micrographs shows that only components of the seminal fluid seemed to fluoresce (Figs. 3c,d; 3e,f). Spermatozoa agglutinated less after they were washed in MFASW, although as previously seen, wherever seminal particles were present, agglutination occurred (Fig. 3e). Fluorescence also occurred only in the region where seminal components were observed (Fig. 3f) and no fluorescence was detected where individual spermatozoa were present. When spermatozoa and seminal fluid were completely separated by centrifugation, APL did not agglutinate spermatozoa; however, seminal particles clumped together (Fig. 3g). Furthermore, fluorescence was detected only where seminal particles agglutinated (Fig. 3h). Dialysis of semen against MFASW for 48 hr did not prevent agglutination when APL was added; therefore, fucose is probably complexed with another component.

Con A and WGA did not agglutinate spermatozoa as shown in Figures 4a and c, respectively. However, these lectin treated spermatozoa became highly motile upon the addition of CaCl₂ and could attach to egg sections. Little fluorescence could be detected when Con A or WGA treated spermatozoa were examined (Figs. 4b,d). Spermatozoa did not appear to fluoresce, although some seminal components may contain glucose, mannose, and N-acetylglucosamine in addition to fucose.
Fig. 4. Spermatozoa treated with fluorescein-conjugated lectins

a) Unwashed spermatozoa do not agglutinate when treated with concanavalin A. 800x

b) Faint fluorescence of semen (most likely seminal fluid) after treatment with fluorescein-conjugated Con A. 800x

c) Unwashed spermatozoa treated with fluorescein-conjugated wheat germ agglutinin do not agglutinate. 720x

d) Faint fluorescence of semen after treatment with fluorescein-conjugated wheat germ agglutinin (arrow). The fluorescence is most likely due to seminal fluid or particles since no fluorescence of spermatozoa is detected. 720x
DISCUSSION

The behavior of *Limulus* spermatozoa was examined in the presence of fucopyranoside-conjugated agarose beads and after treatment with lectins (APL, Con A, WGA). It was found that spermatozoa readily attached to these saccharide-conjugated beads; however, after fucosidase digestion of the beads, no spermatozoa bound. Spermatozoa tails adhered to APL-conjugated beads or APL treated with fucopyranoside-conjugated beads. APL also agglutinated nonmotile unwashed spermatozoa; however, motile or washed spermatozoa did not agglutinate as readily. Con A and WGA did not agglutinate spermatozoa (washed or unwashed) either before or after initiation of motility. Furthermore, lectin (Con A, WGA) treated spermatozoa readily attached to egg sections. fluorescein-conjugated lectins and the colorimentric saccharide tests showed that fucose is a major saccharide in semen.

In the present study, we have attempted to observe spermatozoa in the presence of an egg model. Aketa et al. (1979) showed that spermatozoa were able to adhere to agarose beads conjugated with sperm binding factor isolated from two species of sea urchin. However, we were unsuccessful in conjugating isolated *Limulus* surface proteins to agarose beads due to the insolubility of the proteins in the presence of even small amounts of salt. Attempts to bind spermatozoa to egg surface proteins separated on acid-urea gels equilibrated in MFASW also resulted in failure since the majority of
spermatozoa examined were immotile even in the presence of CaCl₂ or SMI. Fortunately, saccharide-conjugated agarose bead experiments met with some success.

Agarose bead experiments demonstrated that spermatozoa adhered to fucopyranoside-conjugated beads by their apical tips even though a strong attachment was not always observed since many could detach or be knocked off by shaking the microscope slide. This still suggests, however, that fucose or fucose derivatives must be present in order for spermatozoa to attach. Other moieties also may play a role as well as molecular conformations of the receptor constituents in order for high affinity binding to occur. These data are consistent with an earlier study by Barnum and Brown (1983) which showed that spermatozoa did not attach to APL treated egg sections since fucose moieties were blocked by the lectin. However, unlike the sperm tail attachment to APL-conjugated agarose beads or APL treated fucopyranoside beads, egg sections treated with APL did not exhibit sperm tail binding. This may be due to the orientation of 'sperm receptors' on the egg so that each divalent APL molecule binds to two fucose sites. Thus, no APL binding sites would be available to spermatozoa tails. This may not happen with the agarose beads. More sperm tails attached to APL treated fucopyranoside-conjugated beads than to agarose beads conjugated with APL. The difference in the number of sperm bound may reflect a difference in the conformation and/or chain length of the residues since when APL was added to
fucopyranoside-conjugated garose beads, the added lectin could ultimately result in a longer chain of moieties protruding from the agarose beads.

Numerous studies have been published which have used lectins to probe the spermatozoan surface (c.f. Koehler, 1981). Modifications in lectin binding have been studied with regards to agglutinability, site distribution, and in particular, changes in the sperm surface during mammalian sperm capacitation. In the present study, lectins were used to determine what sugars were present in seminal fluid and on the spermatozoan surface by using fluorescence, agglutination, and attachment to egg sections as indicators. Fluorescein-conjugated lectins determined that the major saccharide component of seminal fluid was fucose. Fucose was responsible for agglutinating spermatozoa in the presence of APL, but could be removed by low-speed centrifugation. Fluorescence of spermatozoa was not apparent after treatment with FITC-APL, -Con A, or -WGA; however, resolution of the present studies may not be refined enough to be able to detect saccharides present on the spermatozoan surface. Fucose-containing seminal particles may bind to the spermatozoan surface which may be the reason antibodies to sperm antigens (obtained by washing with Tris) agglutinated spermatozoa tails and seminal particles (Cooper and Brown, 1972). The present study demonstrates that fucose is a prominent saccharide in seminal fluid and fucose-containing particles reversibly bind to spermatozoa tails. Although tails agglutinated
in the presence of seminal fluid and APL, the agglutination response became less apparent as the seminal fluid was diluted. In lower concentrations of seminal fluid (2-3% sperm suspension) and after treatment with APL, sperm still agglutinated, but now tail-to-tail clumping was apparent. When seminal fluid was washed out, spermatozoa agglutinated less; however, seminal fluid and particles were not always completely removed. If APL was added after motility had been initiated in a 2-3% sperm suspension, agglutination was much less pronounced and only a few spermatozoa clumped tail-to-tail. Evidence seems to suggest that fucose-binding moieties reside on the sperm apical tip and fucose is present in seminal particles which may bind to tails. The tip may have a lower affinity for the tail than to the egg envelope due to a difference in the conformation of residues or components that make up the receptors. However, it has been noted that spermatozoa in high concentration (raw semen to a > 10% sperm suspension) do agglutinate head-to-tail after initiation of motility.

Although the role of fucose in semen is unknown, both fucose and seminal fluid appear to depress (delay) motility (unpublished observation). Thus, seminal fluid may prevent spermatozoa from being activated prematurely so that motility can only be initiated after dilution of seminal fluid in seawater. This may indeed be the case since immediately after release from the male and dilution into seawater, spermatozoa have been shown to undergo a brief surge (< 60
sec, short duration) of motility after which they are nonmotile until spermatozoa are exposed to the sperm initiating factor or CaCl₂ (long duration) (Clapper and Epel, 1982b). Motility appeared to be enhanced by dilution of seminal fluid with MFASW (unpublished observations). High concentrations of seminal fluid (from raw semen to a 15% sperm suspension) delayed motility; however, vigorous motility was gradually seen as semen was diluted with MFASW. Fucose (0.5 to 1.0 M) seemed to have a similar effect since motility was delayed for 1-2 min (2-15% sperm suspension). Calcium chloride or sperm motility factor must be added to the suspension to stimulate motility to control levels.

In most animals, spermatozoa become motile either before or at the time of ejaculation (c.f. Bishop, 1962). Limulus spermatozoa, however, remain nonmotile in seawater until encountering a sperm motility initiating substance released from eggs (Shoger and Bishop, 1967; Clapper and Brown, 1980a,b; Clapper and Epel, 1982a,b). The only other organism previously studied that utilizes a motility initiating factor associated with eggs is the herring (Yanagimachi, 1957); however, the substance could not be isolated from the egg.

In several species of starfish, motility of spermatozoa upon dilution into seawater was described as immotile to slightly motile (Glaser, 1914; Metz, 1945; Fujii et al., 1955) and although it is unknown when or how motility is initiated, egg substances have been shown to increase motility (Glaser, 1914; Chambers, 1923; Metz,
1945). In sea urchins, spermatozoa become motile upon dilution of semen into seawater and a peptide associated with the egg maintains motility (Kopf et al., 1979; Hansbrough and Garbers, 1981; Suzuki et al., 1981).

In Limulus, Clapper and Epel (1982a) showed that sperm motility was unaffected by protease digestion. However, we have found that fucosidase digestion prevented motility and subsequent egg section attachment (unpublished observation). Since neither increase in pH nor influx of specific ions initiate motility (Clapper and Epel, 1982b), a peptide (protease-resistant)-receptor interaction or perhaps interaction with various saccharides or glycopeptides may be involved in sperm motility initiation (long duration). We have previously shown that glucose or methyl-D-glucopyranoside induced an immediate and vigorous burst of sperm motility (Barnum and Brown, 1983). It is not yet known whether this is an unnatural occurrence or if it is biologically significant. The use of calcium chloride to induce sperm motility may bypass the normal motility initiation mechanisms. Thus, glucose or SMI motility initiation experiments using APL, Con A, or untreated spermatozoa (both washed and unwashed) and spermatozoa (both washed and unwashed) in the presence of fucose or seminal fluid should be undertaken.
LITERATURE CITED


SECTION 4. POSTFERTILIZATION EVENTS IN THE HORSESHOE CRAB,

*LIMULUS POLYPHEMUS* L.
INTRODUCTION

In the horseshoe crab, *Limulus polyphemus*, the cleavage process is complex and the details are not entirely clear despite a recent study by Scholl (1976, 1977). In the present study, cleavage is divided into two aspects: 1) intralecithal cleavage (Anderson, 1973) and 2) 'total' cleavage. The first part consists of a sequence of nine discrete surface events which includes intralecithal nuclear division and nuclear migration (Stages 1 and 2). Sekiguchi et al. (1982) and Brown and Clapper (1980, 1981) have very briefly described some postfertilization events; however, a detailed sequence of events has not adequately been described. The second phase of cleavage resembles holoblastic cleavage when observed with a dissecting microscope. Serial sections, however, have demonstrated that cleavage furrows are incomplete. Although the emphasis of this aspect of cleavage is on the nine surface phenomena, the entire cleavage process has been examined.

Although numerous studies concerning the embryology of the American horseshoe crab, *Limulus polyphemus* (Scholl, 1976, 1977; c.f. Anderson, 1973, 1980), and closely related species (Sekiguchi, 1973) have been published, few studies have been reported on development occurring during early cleavage. Early investigators (Kingsley, 1892; Brooks and Banks, 1885; Osborne, 1885; Iwanoff, 1933) examined early development in several species of horseshoe
crabs, and with the exception of Kingsley (1892), apparently did not observe the postfertilization events examined in this study. They did, however, describe furrow formation. Kingsley (1892) reported the appearance of columnar-like fields (granulation in the present study) which appeared and disappeared within a few hours after fertilization, but did not pursue any further studies of this event possibly due to the difficulty of preparing embryos for sectioning. He also briefly noted the presence of nuclei during early cleavage and their movement to the surface. In later studies, Sekiguchi (1960, 1966, 1973) examined the early developmental stages in *Tachypleus tridentatus*, but did not describe surface and cortical modification prior to the appearance of nuclei near the surface.

In order to illustrate the numerous surface changes that occur during development, this study focuses primarily on the nine events (Stage 1) prior to the appearance of nuclei near the surface (Stage 2). These are documented with light micrographs of whole and sectioned *Limulus* embryos cultured at 22-23°C. Also included are brief descriptions of Stages 3 and 4 involving the appearance of cleavage and blastula formation, respectively. Time-lapse cinematography was used to determine temporal relationships and to supplement surface descriptions.
MATERIALS AND METHODS

Source of Animals

Specimens of *Limulus polyphemus* L. were obtained from the Florida Marine Biological Specimen Co., Inc., Panama City, Florida. Animals were maintained at 15°C in 150 gal Instant Ocean Aquaria containing artificial seawater (ASW) from Jungle Laboratories Corporation, Comfort, TX.

Gamete Collection, Fertilization, and Early Development

Gametes were collected by brief electrical stimulation (3-4V, 0.2-1.0 mA, ac) in a region proximal to the genital pores. Semen was diluted with ASW to a 10% sperm suspension (10^9 spermatozoa/ml) and was usually used within a few minutes. Approximately 10-25 freshly spawned eggs were collected and placed with wooden applicators in a plastic Petri dish containing ASW. The dish was swirled to disperse ovidual fluid, one or two drops of a sperm suspension were added, and the dish was swirled again to ensure mixture of gametes. The ASW was subsequently aspirated and fresh ASW was added. To enhance surface features, some embryos were stained (Sekiguchi, 1960) for about 2 hr shortly after fertilization using vital stains: nile blue 0 or neutral red (2.5 mg/ml). Embryos were observed with a Wild M-5 dissecting microscope and photographed using a Nikon camera. The temperature during development and for observations was maintained at 22-23°C.
Histology

Aliquots of embryos at specific times were removed and fixed in ice cold 2.5% glutaraldehyde buffered with 0.2 M sodium cacodylate. To facilitate penetration of the fixative, within 10-40 min after immersion in fixative, egg envelopes were teased away with watchmaker forceps. Embryos frequently became brittle when left in fixative more than 40 min and consequently were often damaged when the envelopes were removed. Egg envelopes of unfertilized and recently fertilized eggs (0-10 min) were often only partially removed since they adhered tightly to the egg surface and complete removal of the envelope generally damaged the egg surface. After removal (or partial removal) of egg envelopes, specimens were placed in fresh fixative and stored overnight at room temperature prior to clearing with ethyl alcohol and embedding in Polysciences JB-4 water-soluble plastic embedding medium. Embryos were then serial sectioned using a Sorvall M-1, stained with 1% toluidine blue in 1% borax, and subsequently examined and photographed with a Nikon camera mounted on a Zeiss WL compound microscope.

Time-lapse Cinematography

The timing of events and stages were augmented by using time-lapse cinematography. Developing embryos were photographed with an Arri-S 16 mm camera equipped with an Arri single-frame motor which exposed one frame at a time upon receiving an
electrical pulse from an intervalometer. A disk intervalometer was used for filming at rates of one frame every 5 sec for the first 3 hr and one frame every 15 sec thereafter. In addition, a clock intervalometer was used for filming at the rate of one frame per min. The Petri dish containing embryos was placed on a sheet of black plastic and the ASW was layered with oil to prevent evaporation. The dish was illuminated at an angle of 20 degrees with an incandescent ribbon filament microscope lamp.
RESULTS

The following descriptions and times are based on a sequence of events and stages observed during early development in *Limulus*. Although variation exists in the time of appearance of events and stages, those used in this study represent the times a particular event or stage was most often observed. Micrographs of living embryos were utilized from egg batches obtained from two mating pairs of adults since an adequate sequence of micrographs from one egg batch was not obtained. The micrographs representing histological sections of embryos are also from egg batches obtained from two mating pairs.

Stage 1, Postfertilization Events and Intraeclithal Cleavage

This stage represents nine discrete surface modifications which are referred to as 'events'. This stage terminates when nuclei are observed near the surface with a dissecting microscope. Although the first four events have been previously described using ultrastructural methods (Bannon and Brown, 1980; Brown and Clapper, 1980), their inclusion here is necessary if an accurate developmental sequence is to be presented.

Event 1, unfertilized egg

After spawning, the egg surface (beneath the egg envelope) appears smooth (Figs. 1, 2). Indentations in the egg (including the egg envelope) may be present as a result of storage in the ovary and oviduct; however, these slowly disappear after spawning.
The distinct periplasm (cortex) is approximately 5-10 μm thick and is similar in appearance throughout the egg periphery (Fig. 2).

**Event 2, appearance of pits**

Approximately 10-15 min after fertilization, pits become obvious across the entire surface of the embryo (Fig. 3) and slowly increase in size. In sections (Fig. 4), pits are seen as depressions that extend into the periplasm. The thickness of the periplasm around the embryo is highly variable (5-25 μm). The yolky layer has been displaced by pit formation.

**Event 3, coalescence of pits**

As the pits enlarge, they coalesce with neighboring pits and form large crater-like areas (Fig. 5). The thickness of the periplasm has become more uniform (5-15 μm), although the embryonic surface is still very irregular (Fig. 6).

**Event 4, smooth surface after the cortical reaction**

By approximately 45-60 min after fertilization, the surface becomes smooth (Figs. 7, 8) and will remain so until the first granulation is initiated. The periplasm has become even thinner (5-10 μm) and is now primarily a thin, homogeneous peripheral layer around the embryo (Fig. 8). Few irregularities in the periplasm can be observed.

**Event 5, first granulation**

The first granulation appears about 2.5-3.0 hr after fertilization (Figs. 9, 10) and lasts for approximately 1.5 hr
STAGE 1. Post fertilization Events and Intralecithal Cleavage.
The approximate range of time after fertilization in
which the event and/or stage appears is placed in the
upper left hand corner of each whole embryo light
micrograph. The stage and/or event is designated on
micrographs of sections.

Event 1. Unfertilized egg

Fig. 1. The egg surface appears smooth and the egg
envelope is a translucent layer surround the egg. Bar = 1.0 mm

Fig. 2. The cortex (arrow) is seen as a thin
peripheral layer directly beneath the egg
envelope (ee) where no yolk platelets are
present. Bar = 0.1 mm

Event 2. Appearance of pits

Fig. 3. Pits appear uniformly and simultaneously over
the entire embryo surface

Fig. 4. Pits are quite obvious and show definite
displacement of yolk platelets. The
periplasm is more variable in thickness than
in the unfertilized egg

Event 3. Coalescence of pits

Fig. 5. The large crater-like areas continue to grow
and eventually coalesce

Fig. 6. In this and the following sectioned embryos,
the egg envelope has been completely
removed. The embryonic surface is quite
irregular and still shows considerable
displacement of yolk platelets

Event 4. Completion of the cortical reaction

Fig. 7. The embryonic surface may still appear
uneven in areas, but pits and craters are
absent

Fig. 8. The surface is smooth and no craters or pits
are visible
before the surface becomes smooth again. Granules appear simultaneously over the embryo surface and form a columnar-like layer (Fig. 10). Although these granules are somewhat variable in size and shape, most measure approximately 60-75 μm in length and 25-35 μm in diameter. The contents of these granules (yolk platelets and other components) are continuous with the rest of the embryo as seen in JB-4 sections. Furthermore, the periplasm is greatly reduced and is continuous with the granules. Event 5 terminates when the granules simultaneously disappear.

**Event 6, smooth surface after the first granulation**

The surface of the embryo again becomes smooth 3.5-4.0 hr after fertilization (Figs. 11, 12). The periplasm is once again obvious (5-10 μm thick) and is now associated with numerous cytoplasmic areas (5-10 μm wide) which are free of yolk platelets and extend approximately 30-35 μm into the cytoplasm (Fig. 12). This event lasts approximately 1.5 hr and ends when the second granulation is initiated.

**Event 7, second granulation**

At 5.0-5.5 hr after fertilization, granules reappear simultaneously across the entire embryo surface (Figs. 13, 14). These granules morphologically resemble those in event 5 (70-85 μm in length and 25-40 μm in diameter). The clear cytoplasmic areas that are seen in event 6 are no longer detected and the periplasm is obscure (Fig. 14). Three hours after granules first appear,
Event 5. First granulation

Fig. 9. Granules are observed over the entire embryo surface

Fig. 10. Granules are columnar-like and are continuous with the cytoplasm. Each granule contains numerous yolk platelets as well as other unidentified cytoplasmic components

Event 6. Smooth surface after the first granulation

Fig. 11. The granules have completely disappeared and the embryo once again is smooth

Fig. 12. A smooth surface and irregularly-shaped cytoplasmic areas (arrow) characterize this event. The periplasm reappears as granules disappear

Event 7. Second granulation

Fig. 13. This event is morphologically similar to the first granulation; however, granules remain for a longer period of time than those visible in event 5

Fig. 14. Granules present in this event are larger than those in event 5

Event 8. Smooth surface after the second granulation

Fig. 15. The smooth surface reappears and although similar to the two previous smooth events (E4 and E6), occasional isolated 'droplets' are detected

Fig. 16. The clear cytoplasmic areas observed in event 6 are absent
they gradually decrease in size until the embryonic surface is smooth.

**Event 8, smooth surface after the second granulation**

By 8.5-9.0 hr after fertilization, except for a few scattered granules which resemble oil droplets, the surface becomes smooth for the third time (Figs. 15, 16). This smooth event persists for approximately 5 hr. The periplasm is approximately 5.8 μm thick and again forms a thin peripheral layer around the embryo (see event 4; Fig. 16). The morphology of embryos observed during this event resemble those described during event 4.

Event 8 was the earliest time that intralecithal nuclei were observed. This identification was possible since these ovoid nuclei (8 x 10 μm) were located within yolk platelet-free cytoplasmic masses (25-30 μm in diameter). In one serial sectioned embryo, 15 such nucleo-cytoplasmic masses were counted. These were located approximately 300 μm (one sixth of diameter) from the surface and were distributed fairly equally in the embryo.

**Event 9, third granulation and contraction wave**

Fourteen to 15 hr after fertilization, the third granulation begins (Figs. 17, 18). It appears locally at one end of the embryo and slowly spreads across the remaining surface (takes 1 hr). Sections made through the spreading edge of the granulation (Fig. 18) show that the granules are heterogeneous, although when fully formed, they resemble granules seen in events 5 and 7 (columnar)
except that they are smaller (25-30 μm in height). When the granules cover about one-third of the embryo, a contraction wave forms near the origin of the granulation and also moves in the same direction as the granulation. This contraction wave had been previously overlooked in our studies until time-lapse cinematography was employed. The contraction wave takes approximately 1 hr to move over the entire embryonic surface and generally is quite obvious.

Although the appearance of granules in this third granulation is readily detected, when they disappear has not been fully established. Apparently, these granules may persist throughout event 9 and into Stage 2 and possibly Stage 3. During this time, the periplasm becomes thin and indistinct. In sectioned embryos, 13 nucleo-cytoplasmic masses have been observed approximately 300 μm from the surface and are similar to those in event 8.

Stage 2, Appearance of Intralecithal Nuclei Near the Surface

At approximately 18-19 hr after fertilization, 12-16 intralecithal nuclei appear near the surface (Figs. 19, 20). These nuclei may appear alone or in pairs. In sections (Fig. 20), the nucleo-cytoplasmic masses are associated with the surface and cytoplasmic extensions extend 52-62 μm into the embryo. In one sectioned embryo, 13 nucleo-cytoplasmic masses were observed near the surface while one remained near the center of the embryo. The
fate of these surface nuclei is not presently known although they appear to undergo continuous divisions near the embryonic surface. These surface nuclei are not to be confused with structures which appear on the surface and resemble oil droplets with dark inclusions (Fig. 20).

Stage 3, 'Total' Cleavage

Cleavage commences when a deep irregular groove or furrow appears on the embryo surface which initially resembles holoblastic (total) cleavage. However, sections of an approximately 16 "blastomere" embryo (Fig. 21) indicate that the cleavage pattern is superficial (Fig. 22). Nevertheless, each blastomere is associated with an inner nucelo-cytoplasmic mass (cleavage energid). Sections of 4 and 8 blastomere embryos (not shown in this study) reveal 4 and 8 nuclei, respectively, associated with the blastomeres. These are not to be confused with intralacithal surface nuclei described in Stage 2. Surface nuclei are still present in Stage 3 and frequently can be observed in furrows between blastomeres, particularly when vital staining is used.

Stage 4, Early Blastulation

Near the end of cleavage, 48-50 hr after fertilization, when approximately 128 blastomeres are present (Fig. 23), the surface of each blastomere is so granular that internal features are obscured.
Event 9. Third granulation

Fig. 17. This granulation differs from the previous two since it originates at one end of the embryo and slowly spreads across the surface. A contraction wave (not shown) commences shortly thereafter and also moves over the surface

Fig. 18. An area of the surface showing the leading edge of the granulation

STAGE 2. Appearance of Intralecithal Nuclei Near the Surface

Fig. 19. Nuclei (arrows) are visible near the surface

Fig. 20. A nucleus (arrow) is observed near the surface and is surrounded by cytoplasm

STAGE 3. 'Total' Cleavage

Fig. 21. Approximately 16 blastomeres are present. Although this stage is initiated 23-24 hr after fertilization, this particular embryo is about 30 hr old

Fig. 22. A cross-section through one blastomere reveals that the cleavage furrow is not complete. A nucleus (arrow) and its cytoplasm (energid) is beneath the embryonic surface

STAGE 4. Early Blastulation

Fig. 23. Nuclei are seen within blastomeres at this stage

Fig. 24. Nuclei (arrows) are surrounded by cytoplasm and are enclosed within individual blastomeres
However, as these granules slowly recede, a prominent nucleo-cytoplasmic mass becomes apparent within each blastomere. This represents the beginning of Stage 4 and the formation of the early blastula. These blastomeres vary in size and shape and areas of very small blastomeres can frequently be seen. In sections (Fig. 24), nuclei beneath the surface can be observed and although difficult to detect, blastomere boundaries exist.
DISCUSSION

The sequence of postfertilization changes during early embryonic development of *Limulus* has been examined. These changes are quite distinct and are demonstrated in the present study using whole and sectioned embryos. Efforts have been made to relate these surface changes to cleavage, a process in *Limulus* and other xiphosurids which has not been thoroughly understood. The following classification of cleavage is used: 1) intralecithal cleavage which includes all events occurring in Stages 1 and 2, and 2) 'total' cleavage which includes Stage 3 and terminates with Stage 4 during blastula formation. As a result of the present study, insight into this process is developing and additional studies should follow that further clarify xiphosurid cleavage.

The following surface changes occurring during intralecithal cleavage have been emphasized in this study: the cortical reaction, appearance and disappearance of granules, a contraction wave, and nucleo-cytoplasmic movement toward the surface. The cortical reaction in horseshoe crabs was overlooked by earlier investigators and only recently has been described (Bannon and Brown, 1980, Brown and Clapper, 1980). The cortical reaction not only involves the periplasm but also affects the upper yolky layer since a displacement of approximately 70 μm of this layer occurs in the area of pit formation. This is a dynamic process since cortical vesicles which are present in the periplasm fuse with the plasma
membrane and as a result, undergo numerous morphological modifications.

The three granulations represent a sequence of changes that involves the periplasm and the upper portion of the yolky cytoplasm. The first two granulations (E-5 and E-7) are morphologically similar and granules appear on the surface suddenly in both events. Although the significance of these granulation cycles has not been determined, some form of membrane reorganization or synthesis of precursors of the extra-embryonic shell may be occurring since an increase in amino acid uptake occurs during this period (Bannon et al., 1981). Evidence from studies of other arthropod species suggests that the granulations may be related to intralecithal cleavage and nuclear migration. In species which undergo a superficial cleavage such as Drosophila, surface events appear to be related to nuclear division and migration. Fullilove et al. (1978) have demonstrated that shallow invaginations between nuclei appear and disappear several times prior to the formation of actual cleavage furrows and during this time, microvilli form above each nucleus. In spiders, polygonal fields have been shown to develop on the surface of the embryo during intralecithal cleavage (Holm, 1941, 1954; Seitz, 1966). In general, it has been demonstrated that when synchronous nuclear divisions occur in insect eggs, the egg and its contents undergo rearrangements (c.f. Counce, 1973).
The third granulation (S-1, E-9) is particularly interesting since granule formation appears at one end of the embryo and a contraction wave follows soon after. Although no significance can presently be attributed to this event, unpublished studies in our laboratory indicate that the future embryonic disc develops at the end opposite the contraction wave origin. Perhaps the axis of the embryo is established during this time or segregation of embryo components occurs during contraction. In a recent study on the ascidian, *Ciona intestinalis*, Sawada and Osanai (1981) suggest that ooplasmic segregation in embryos (a mosaic egg) is related to a cortical contraction that occurs within a few minutes after fertilization. Contraction waves also have been described in other species. In barnacles, "contraction rings" occur shortly after fertilization and precede cleavage. Their disruption by chemical inhibitors has been demonstrated to cause abnormal development (Lewis, 1977). In *Xenopus*, the egg undergoes a "surface contraction wave" just prior to the first cleavage (Hara et al., 1980). This event also has been observed in artificially activated eggs, merogones, and in fertilized eggs that have been prevented from undergoing cleavage. A biological clock mechanism may be involved since the waves are timed with the normal cleavage cycle. Cyclic cytoplasmic contractions have also been recorded in sea urchins (Yoneda et al., 1978), the ascidian, *Ciona intestinalis* (Bell, 1962), and in insect embryos (Miyamoto and van der Meer,
1982). The detection of cyclic contractions in artificially activated eggs, merogones, and fertilized eggs of numerous species has elicited interest in the relationship of contraction waves to cleavage and the movement of cytoplasmic components. Further investigation into this event in Limulus could lead to a better understanding of the contraction wave itself, the determination of embryo formation, and the movement of cytoplasmic components during the contraction wave.

The appearance of intralecithal nuclei near the surface in Limulus marks the end of Stage 1 and the beginning of Stage 2. The ultimate fate of these nuclei is presently unknown, although they remain near the surface and can be observed between blastomeres during Stage 3. Using vital stains, Sekiguchi (1960) also described the appearance of nuclei in embryos of Tachypleus. These nuclei have been observed to divide several times both by our laboratory in Limulus and by Sekiguchi (1960) in Tachypleus. In sections of one embryo during S-2, 13 nucleo-cytoplasmic masses were observed near the surface while one was more centrally located. Although the pattern of nuclear movement during intralecithal cleavage in the Limulus embryo is not well-understood, the zygotic nucleus in the fertilized egg probably divides four times during intralecithal cleavage and then all nuclei but one migrate to the surface. Investigations on related species such as spiders show that karyokinesis occurs for as many
as 3-4 divisions before nuclear migration. This may be a common phenomenon in chelicerates since it has been reported in scorpions and ticks as well (c.f. Anderson, 1973).

A similar process is observed in *Drosophila* (Fullilove et al., 1978), a mandibulate, where numerous intralecithal cleavage nuclei form a syncytial blastoderm. They then migrate to the surface from within the yolk. Nuclei are seen within cytoplasmic islands (cleavage energids) similar to those reported in the present study. A number of nuclei still reside in the yolk where they become yolk nuclei or vitellophages. In an orthognath spider, Holm (1954) also observed nuclei within the yolk mass after cleavage nuclei with surrounding cytoplasm were on the surface. In *Limulus*, perhaps the central nucleus within the yolk is a yolk nucleus similar to those seen in *Drosophila* and in other chelicerates and/or may give rise to the nuclei later detected within each blastomere. At the present time, however, there is no evidence to support these speculations. The tracking of nuclei using traditional staining methods (Feulgen staining) has been unsuccessful due to the nonspecificity of the stain. However, a fluorescent method of staining the DNA (e.g. Hoescht dye) may yield better results.

The Chelicerata are the least understood embryologically of the three major arthropod groups (Uniramia, Crustacea, Chelicerata). The xiphosurids of the large subphylum Chelicerata
share close early developmental relationships with most arachnids (c.f. Anderson, 1973). Round, yolky eggs, characteristic of most chelicerates, undergo a modified cleavage when compared to smaller eggs with a reduced amount of yolk (certain mites, pseudoscorpions, and the viviparous scorpions) where total cleavage occurs (c.f. Anderson, 1973). Two of the best studies that describe cleavage in large, yolky eggs (0.5-1.9 mm) of chelicerates are on the primitive liphistiid, *Heptathela kimurai* (Yoshikura, 1954, 1955), and on the angelenid spider, *Agelena labyrinthica* (Holm, 1952), which is typical of advanced spiders. In both, the nuclear divisions form eight cleavage energids (nuclei enclosed within a cytoplasmic mass) distributed equally in the fertilized egg. This is followed by the formation of several yolk pyramids. Further nuclear divisions occur, and at the same time, nuclei migrate toward the egg surface. More divisions of yolk pyramids follow, although the actual relationship between nuclear division and yolk pyramid divisions is unclear. When the energids merge with the periplasm at the embryo surface (surface of the yolk pyramids), the blastoderm is formed. Further cell divisions at the surface then form a uniform blastoderm around the egg mass. Scattered blastoderm cells later reinvade the yolk mass and differentiate as vitellophages and later give rise to epithelium of the midgut. In various species of *Limulus*, Kishinouye (1891), Kingsley (1892), and Iwanoff (1933) showed that the first few nuclear divisions are intralecithal;
however, a furrowing of the surface then occurs which is reminiscent of total cleavage. The egg becomes divided into large yolky cells with central nuclei and divisions then take place primarily in the periphery which gives rise to a blastoderm. Thus, although cleavage is not superficial, total cleavage (in the classical sense) also does not take place since furrows are not complete. Anderson (1973) speculates that central cells, which remain undivided, give rise to the midgut epithelium. However, early studies remain unclear and details of observations presented in the present study were not detected by previous investigators. Obviously, further studies of the ultimate fate of the central nucleus, surface nuclei, and nuclei observed during Stage 3 ('blastomere' nuclei) need to be completed before an accurate description (and comparison with arachnids) of early developmental events in *Limulus polyphemus* can be made.
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SECTION 5. MODIFICATION OF THE FERTILIZED EGG SURFACE FOLLOWING THE CORTICAL REACTION IN *LIMULUS POLYPHEMUS* L.
INTRODUCTION

The surface of the fertilized egg of *Limulus polyphemus* undergoes a series of discrete modifications during cleavage and prior to the formation of a blastula (Brown and Barnum, 1983). Development of *Limulus* has been separated into 21 stages (Brown and Clapper, 1981) which is in agreement with the stages described for the Japanese horseshoe crab by Sekiguchi (1973) and Sekiguchi et al. (1982). Stage 1 consists of the cortical reaction and three intermittent granulation events, that terminates with the appearance of intralecithal nuclei near the embryonic surface (Stage 2) approximately 20 hrs after fertilization. Thus, Stages 1 and 2 include intralecithal cleavage which is followed by cleavage of the embryo (Stage 3) and blastula formation (Stage 4). In a previous study, Stage 1 has been divided into 9 events that emphasizes major surface and cortical changes using light microscopy (Brown and Barnum, 1983). The first four events (cortical reaction) have been adequately described ultrastructurally by Bannon and Brown (1980) and Brown and Clapper (1980). However, a thorough examination of the granulation process has not been undertaken.

Granulation events have been previously reported in xiphosurids (*Limulus*, Kingsley, 1892; *Tachypleus tridentatus* and *Limulus*, Sekiguchi et al., 1982). Although the significance of
these granulation cycles is not clear, similar modifications have been observed in other chelicerates. In spiders, a few hours after the eggs are laid (during intralecithal cleavage) the periplasm or cortex becomes subdivided into numerous polygonal fields (Balbiani, 1873; Locy, 1886; Kishinouye, 1891; Kautzsch, 1910; Holm, 1941, 1954; Ehn, 1963, Seitz, 1966). These fields disappear when intralecithal nuclei reach the surface (periplasm). Similar surface modifications have been observed in other arachnid eggs during intralecithal cleavage (ticks, Aeschlimann, 1958, 1961; opilionids, Juberthie, 1961; 1964).

These morphological modifications appear to be unique to chelicerates and may be related to intralecithal cleavage and subsequent migration of nuclei to the surface (c.f. Anderson, 1973). Since the granulation process has not previously been closely examined, the modification of granule and microvillar structure through 11 hr is examined with scanning electron microscopy in the xiphosurid, Limulus.
MATERIALS AND METHODS

Specimens of Limulus polyphemus L. were obtained from the Florida Marine Biological Specimen Co., Inc., Panama City, Florida, and maintained at 15°C in 150 gal Instant Ocean Aquaria containing artificial seawater (ASW) from Jungle Laboratories, Inc., Comfort, Texas.

Gamete Collection and Insemination

Gametes were collected by brief electrical stimulation (3-4 v, 0.2-1.0 mA, ac) below the genital pores. Semen was diluted with ASW to obtain a 1-5% sperm suspension ($10^8$ spermatozoa/ml). Approximately 50 eggs were collected with wooden applicators and placed in a 10 cm plastic Petri dish containing 35 ml of filtered ASW. The dish was swirled to disperse oviductal fluid, two drops of the sperm suspension were added, and the dish was swirled again to assure insemination of all eggs. The ASW was removed after 10 min and fresh ASW was added. At 21-22°C, approximately 90% of the fertilized eggs developed to swimming larvae.

Histology

Aliquots of embryos at 3 and 5 hr after fertilization were removed and fixed in cold 2.5% glutaraldehyde buffered with 0.2 M cacodylate. Within 10-40 min after immersion in fixative, egg envelopes were teased away as much as possible with watchmaker
forceps. Specimens then were placed in fresh fixative and stored overnight prior to clearing with ethyl alcohol and embedding in Polysciences water-soluble JB-4 plastic embedding medium. Embryos were serial-sectioned using a Sorvall M-1, stained with 1% toluidine blue in 1% borax, and subsequently examined and photographed with a Nikon camera mounted on a Zeiss WL compound microscope. From photographs (8 x 10) of JB-4 sections of embryos at 3 and 6 hr after fertilization, the length and width of granules were measured.

Scanning Electron Microscopy (SEM)

Fertilized eggs were examined with a Wild dissecting microscope. When the appropriate event was reached, aliquots of embryos (4-5) were removed and placed directly into 2.5% glutaraldehyde (0°C) buffered with 0.2 M sodium cacodylate. Egg envelopes were then teased away with watchmaker forceps. Specimens were placed in fresh fixative for a total of 45 min, post-fixed in 1% osmium tetroxide for 2 hr, and stored in ddH₂O for several days. Embryos then were dehydrated in ethanol, cleared in Freon 113, critical point dried using liquid carbon dioxide, and mounted on stubs with silver paint prior to being coated with gold-palladium and examined on a JEOL JSM-35 scanning electron microscope.
RESULTS

Stage 1 consists of 9 events (E) beginning with the cortical reaction (E-1 to E-4) and followed by three intermittent granulation events and two smooth events. The present study describes the following events after the cortical reaction has occurred: E-5 at 3 hr (first granulation); E-6 at 4 hr (smooth surface after the first granulation); E-7 at 5, 7, and 8 hr (second granulation); and E-8 at 9 and 11 hr (smooth surface after the second granulation). The final events of Stage 1 consist of a contraction wave and a third granulation; however, these are not included in this study.

E-5, First Granulation

First granulation at 3 hr

When the embryo is examined 3 hr after fertilization with the dissecting microscope, the surface appears granular. This modification occurs simultaneously over the entire embryonic surface (Fig. 1a). Numerous granules can be seen when the embryo is examined during this time period with SEM (Fig. 1e). Although granule size is rather heterogeneous, the mean length and width was determined from JB-4 sections made from 3 hr embryos (micrograph not shown; 68 ± 8 x 27 ± 8 μm). Granule microvilli are present (Fig. 1f), but the smooth granule surface can be clearly seen and impressions of underlying yolk droplets can be detected. In furrows separating each of the granules, larger microvilli exist
(Fig. 1f). The width of the microvilli appears uniform throughout the embryo.

E-6, Smooth Surface

The granules have disappeared and the surface appears smooth when examined with the dissecting microscope 4.5 hr after fertilization (Fig. 1b). SEM shows that numerous microvilli protrude from the embryo and that yolk droplets are present beneath the surface (Fig. 1g). The embryo in Fig. 1g is entering the second granulation event (the embryo is almost 5 hr old) and granules may be forming since the surface appears to be irregular and the outline of granules can be seen.

E-7, Second Granulation

Whole embryos resemble those examined at 3 hr after fertilization since the surface is again covered with numerous granules (Fig. 2a). These granules, although similar morphologically to those of the first granulation event (E-5), differ in average length and width as determined by JB-4 sections of embryos 6 hr after fertilization (78 ± 7 x 35 ± 8 μm; Fig. 2b). When an embryo is fractured and observed with SEM, the columnar nature of the granules is demonstrated (Fig. 2c). Long microvilli protrude from deep within furrows (furrow microvilli). Some microvilli appear to exit from an opening on the granule surface (Fig. 2d). Yolk droplet impressions are still clearly visible on the granules.
Figure 1. Egg surface morphology during early development of *Limulus polyphemus*

a A light micrograph of an embryo 4 hours after fertilization (E-5). The entire surface is granular. 21x

b By 4 hours after fertilization (E-6), the granules have disappeared and a smooth surface is observed with the dissecting microscope. 21x

c Five hours after fertilization (E-7), granules have reappeared on the surface of the embryo. 21x

d A light micrograph of an embryo 9 hours after fertilization (E-8) shows that the granules are no longer well-defined. 21x

e SEM of an embryo 3 hours after fertilization. The egg envelope has been removed so that the underlying granular surface can be seen (arrow). 53x

f Granules covered with short microvilli are seen 3 hours after fertilization. Furrow microvilli are present between granules and impressions of yolk droplets are observed on the granule surface. 1375x

g SEM of an embryo 4.5 hr after fertilization shows that the granules have disappeared and the surface is smooth. 5400x
Fig. 2. The egg envelope has been removed in all preparations

a  SEM of a whole embryo 5 hr after fertilization demonstrates the granular nature of the surface. 54x

b  JB-4 section of an embryo 6 hr after fertilization shows prominent columnar-shaped granules. 41x

c  SEM of a 5-hour embryo (E-7) that has been broken away so that the underlying yolk region is exposed. Columnar-shaped granules (arrow) are present at this stage. 400x

d  SEM of a furrow (F) and adjacent granules 5 hours after fertilization. A microvillus similar to that seen in furrows (FM) protrudes from a granule through an opening or circular structure. Impressions of yolk droplets are seen beneath the surface (YD). 6842x
Granules persist from 5-7 hr after fertilization with few modifications when whole embryos are observed with the dissecting microscope (Fig. 1c) or SEM (Fig. 3a). Granules of embryos at 7 hr, however, have become even more irregularly shaped and rather flattened than those seen previously. Inspection at higher magnification shows little difference between granule morphology at 3 hr and those seen at 7 hr (Fig. 3b). Few microvilli protrude from the granule surface as seen at 5 hr; however, long furrow microvilli are absent. The furrow microvilli that are seen are flattened and even club-shaped (Fig. 3b). Impressions of yolk droplets still are evident on the granule surface.

Second granulation at 8 hr

The appearance of granules has changed considerably and are no longer dome-shaped as seen at both 5 and 7 hr (Fig. 3c). Short, thick microvilli sparsely populate granule surfaces and are no longer filamentous as previously seen (Fig. 3d). Furthermore, numerous blebs protrude from the granule surface. Furrow microvilli are present and although they are longer than those emanating from granule surface membranes, they are also flattened and club-shaped.

E-8, Smooth Surface

Smooth surface at 9 hr

By 9 hr after fertilization, the surface again appears smooth when observed with a dissecting microscope (Fig. 1d). However,
Figure 3. The egg envelope has been removed in all preparations

a SEM of an embryo at 7 hours (E-7). Granules appear to be more flattened and their shape less distinct than those seen at 3 and 5 hours after fertilization. 50x

b Irregularly-shaped granules are present on the surface of the embryo 7 hours after fertilization. Fewer microvilli are present on these granules (GM) than on granules in event 5. Furrow microvilli (FM) are observed, but their morphology differs from those seen at 5 hours. Yolk droplets (YD) are beneath the surface. 1571x

c Eight hours after fertilization (E-7), granules that were previously present have become flattened and more irregularly-shaped as shown by SEM. Furrow (FM) and granule (GM) microvilli are observed. 450x

d The granule surface facing a furrow contains thickened microvilli (GM) and blebs (B). Microvilli adhere from one granule to microvilli protruding from the nearest granule (E-7). Yolk droplets are beneath the surface (YD). 4385x
examination with SEM reveals a flattened, wrinkled embryo surface (Fig. 4a,b). Higher magnification discloses a ridge-like surface that is formed by short, thick blebs and club-like microvilli (Fig. 4c). Some filamentous microvilli are seen in furrows, but club-shaped microvilli are more numerous. Material presumably from cortical granule exudate adheres to furrow regions, blebs, and clubs.

Smooth surface at 11 hr

A ridge-like network is still present 11 hr after fertilization; however, numerous changes have occurred. Filamentous microvilli are more numerous and appear to originate from the ridges (Fig. 4d). The blebs that make up the ridges seem to have coalesced into smooth, irregular mounds and are covered by more microvilli than at 9 hr. Exudate is still clinging to ridge microvilli, but is absent from furrows. However, this difference may be due to the fixation procedure. Numerous microvilli are also present in furrows. These are more filamentous than those seen at 9 hr, although club-shaped microvilli are still observed.
Figure 4. The egg envelope has been removed in all preparations

a SEM of a fertilized egg 9 hours after insemination (E-8). The surface appears less granular than earlier stages examined with electron microscopy. 60x

b At higher magnification, furrows have disappeared and the surface appears wrinkled. 480x

c A furrow between extensively modified granules shown by SEM at 9 hours after fertilization. Blebs (B) and club-shaped microvilli are present on granules that now resemble ridges. Material (S) of unknown origin adheres to furrows and ridges. 3208x

d Eleven hr after fertilization, numerous microvilli are seen protruding from the surface and from ridges comprised of blebs. 1556x
DISCUSSION

In the present study of _Limulus_ development, surface modifications of the embryo after the cortical reaction have been described using SEM. Three hours after fertilization, the embryonic surface becomes granular and microvilli sparsely populate the granule surface (E-5). By 4 hr, the surface becomes smooth (E-6) and resembles embryos 2 hr after fertilization (Brown and Clapper, 1980). Numerous filamentous microvilli cover the embryo surface as is also reported in 2 hr embryos by Bannon and Brown (1980) and Brown and Clapper (1980). One to two hours later, granules reappear, but now are longer than those previously seen at 3 hr (E-7). Few granule microvilli are observed, but those present are filamentous. Although the surface appears smooth with the dissecting microscope 9 hr after fertilization (E-8), the morphology of egg plasma membrane extensions is actually greatly modified and the extensions now resemble blebs and clubs which are densely packed in irregular ridges. Ridges are still present 11 hr after fertilization, but numerous filamentous microvilli have appeared. Blebs that previously formed the ridges have coalesced forming smooth, irregular mounds.

Although a number of recent studies have examined surface modifications during and immediately after the cortical reaction (Eddy and Shapiro, 1976; c.f. Epel and Johnson, 1976; Nicosia et al., 1977; Hart et al., 1977; Iwamatsu and Keino, 1978; Schroeder,
few studies have been concerned with surface reorganization throughout early development. Furthermore, few informative studies exist concerning surface modifications during early development in chelicerates (c.f. Anderson, 1973; 1980) and most include only schematics. Early studies have briefly mentioned surface modifications during early development (formation of polygonal fields during intralecithal cleavage) in various chelicerates such as arachnids (c.f. Anderson, 1973); however, these have not been closely examined and ultrastructural studies have not been undertaken. Granulation events similar to those observed in the present study have been reported in earlier studies on xiphosurids (Limulus, Kingsley 1892; Tachypleus and Limulus, Sekiguchi et al., 1982; Limulus, Brown and Barnum, 1983); however, these also have not been examined in detail. A thorough description (SEM and light microscopy) of development of the mandibulate, Drosophila, recently has been published by Fullilove et al. (1978). They report that raised microvillar covered mounds form above each nucleus during intralecithal cleavage and nuclei migration. These disappear and reappear several times prior to actual cleavage furrow formation. This may be similar to polygonal fields and granulations. The significance of these modifications and the relationship to
intralecithal cleavage, nuclei migration, and to other aspects of cleavage is unknown.

Microvillar modification in fertilized eggs has been examined by numerous investigators. An increase in the diameter of microvilli takes place during the cortical reaction in *Limulus* (Bannon and Brown, 1980; Brown and Clapper, 1980). Monroy and Baccetti (1975) described microvillar morphology and modification during *Xenopus laevis* development. They discovered that extensive rearrangement of the surface occurred through the 8-cell stage. A number of studies have been concerned with the origins of new plasma membrane during cleavage (Bluemink and Delaat, 1973; Arnold, 1974; Singal and Sanders, 1974a,b; Sanders, 1975). Studies indicate that initial microvillar elongation of the egg plasma membrane may be a mechanism by which new membrane from cortical vesicles is incorporated into the plasma membrane (Vacquier, 1975; Eddy and Shapiro, 1976; Schroeder, 1978, 1979). In *Limulus*, membrane which is contributed during the cortical reaction (Bannon and Brown, 1980) may be conserved after vesicle-plasma membrane fusion and forms numerous microvilli or granules. As granules appear, microvilli become smaller or disappear, whereas microvilli elongate as granules disappear. The plasma membrane and underlying cortex appear to be a dynamic complex that is in a continuous state of flux following cortical vesicle and plasma membrane fusion.
Surface modifications may not only represent additional membrane reserves, but may also be a result of egg activation. It has been demonstrated that physiological and biochemical processes are activated following fertilization in sea urchin eggs (Jaffe, 1976; Steinhardt et al., 1977; Jaffe et al., 1978; Shen and Steinhardt, 1978; c.f. Epel, 1978, 1979). Microvillar elongation and actin polymerization have been observed during fertilization and egg activation in the absence of cortical breakdown (Mazia et al., 1975; Spiegel & Spiegel, 1977; Begg and Rehbun, 1979; Kidd and Mazia, 1980). Changes in the cell cycle have also been shown to correlate with alterations in surface topography. In the mouse egg after in vivo fertilization, changes in the cell cycle have been associated with modifications in the surface (Jackowski and Dumont, 1979). Blebs, mounds, and microvilli present on the unfertilized egg surface are modified upon insemination and as the cell cycle proceeds. The surface and cortex of the mouse embryo appear to be highly labile and dynamic, not unlike what is observed in Limulus. Thus, topographical changes observed upon fertilization through 11 hr are probably the result of numerous processes activated at fertilization. Modifications in the fertilized egg surface and cortex may be due to egg activation, changes in the cell cycle, formation of intralecithal cleavage nuclei and their migration to the surface, or the anticipation of cleavage (approximately 25 hr after fertilization) by increasing surface area through new
membrane incorporation. The dynamic nature of the early embryonic plasma membrane may be a reflection of the many biochemical changes occurring after fertilization. Granules and microvilli observed during cleavage all affect the surface area and may represent membrane reserves and/or reorganization of the surface and underlying cortex. A vast amount of membrane is needed during cleavage when the total egg surface area increases tremendously. Although Schroeder (1979) has shown that cortical vesicle membrane added to the surface during the cortical reaction in sea urchins is mostly reabsorbed, increased membrane incorporation during the cortical reaction may be maintained for future use during cleavage in Limulus. New membrane presumably could arise by the addition of precursors from a pool present in the egg, growth of new membrane, or by fusion of vesicles from the egg cortex.

The surface area should be measured at various times after fertilization to determine if there is an increase after the cortical reaction and whether it is conserved or reabsorbed. Furthermore, migration of intralecithal nuclei needs to be closely correlated with surface modifications.
LITERATURE CITED


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SUMMARY-DISCUSSION

In this dissertation research, primary sperm attachment in *Limulus* during fertilization was examined, egg envelope glycoproteins were identified, and early developmental events were carefully described.

The apical regions of motile *Limulus* spermatozoa have been shown to adhere to the outer layer of the egg envelope (Shoger and Brown, 1970; Brown, 1976). Shortly after primary sperm attachment, the acrosome reaction occurs, resulting in a stronger adhesion or secondary attachment (Shoger and Brown, 1970; Brown, 1976). In Section 1, a sperm attachment assay that quantified the number of spermatozoa attached to egg sections was utilized to identify components involved in primary attachment. Egg sections treated with asparagus pea lectin (250 μg/ml) bound significantly fewer spermatozoa as compared to those exposed to wheat germ agglutinin, concanavalin A, and garden pea lectin. Furthermore, sperm attachment was also greatly reduced when egg sections were first incubated with the glycosidase, α-L-fucosidase (< 5% of controls). Treatment of spermatozoa with α-L-fucose, fucoidan, or p-aminophenyl fucoside also reduced sperm attachment when compared to Millipore-filtered artificial seawater controls. This evidence suggests that the methylpentose, α-L-fucose, plays an important role in primary sperm attachment in *Limulus*. 
Urea-soluble egg envelope proteins were identified in Section 2 using 7.5% acid-urea (Panyim and Chalkley, 1969) and SDS (Laemmli, 1970) gel electrophoresis. Periodic acid-Schiff staining of SDS gels (Fairbanks et al., 1971) and colorimetric saccharide tests (Dische and Shettles, 1951; Dubois et al., 1956) showed that the egg envelope constituents were glycoproteins. The two major urea-soluble glycoproteins detected had molecular weights of approximately 202,000 and 192,000 as determined from standards run during SDS gel electrophoresis. Gel filtration of urea-soluble glycoproteins on Sephadex G-100 and G-150 columns followed by SDS gel electrophoresis of various fractions demonstrated that the two high molecular weight glycoproteins could not be separated and at least two lower molecular weight proteins may be complexed with the larger ones since these were seen on SDS gels from fractions where the two large glycoproteins eluted. These lower molecular weight proteins were not seen on acid-urea gels. Fractions from the high molecular peak were collected from a G-100 column, against Ca^{++}-Mg^{++}FSW, and were tested for their ability to decrease primary sperm attachment to egg sections. Attachment ranged from 11.3 ± 9.7 to 55.9 ± 17.2 percent of the control mean. This suggests that a soluble component(s) isolated from egg envelopes is capable of blocking sperm attachment to egg envelopes.

In Section 3, experiments that examined sperm attachment to p-aminophenyl fucoside-conjugated agarose beads confirmed previous studies from Section 1 which showed that exposed fucose moieties or
fucose derivatives on the egg envelope were important for sperm attachment. Surprisingly, however, the surface of spermatozoa tails contains fucose as well. This is based on studies using APL-conjugated and APL treated p-aminophenyl fucoside-conjugated agarose beads which demonstrated that spermatozoa adhered by their tails. Lectins (APL, WGA, Con A) added to spermatozoa also confirmed this observation since APL agglutinated MFASW-diluted spermatozoa tail-to-tail. By using fluorescein-conjugated (FITC) lectins, seminal fluid was found to consist of fucose-containing particles which appeared to be responsible for agglutination in the presence of APL. Based on motility studies in the presence and absence of either seminal fluid or fucose, spermatozoa appear to become motile more quickly and reach maximum velocity sooner than unwashed spermatozoa (in seminal fluid) or spermatozoa in the presence of fucose. The function of fucose in seminal fluid or fucose moieties on spermatozoa tails remains unclear, although it can be speculated that seminal fluid may act to depress spermatozoa so that premature activation is prevented. Fucose-containing particles may bind to the tail surface and inhibit sperm motility until it is diluted in seawater.

Finally, early development of Limulus was examined using both light and SEM in Sections 4 and 5, respectively. The nine embryonic surface events that occur during Stage 1 were carefully described and a chronological sequence of developmental events was obtained.
In Section 4, the first four of twenty-one developmental stages were described as outlined for the Japanese horseshoe crab, *Tachypleus tridentatus* by Sekiguchi (1973) and for *Limulus* by Brown and Clapper (1980). The cleavage process consists of two aspects: 1) *intralecithal* cleavage and 2) 'total' cleavage. The study presented in this dissertation emphasized the nine postfertilization events that occurred during *intralecithal* cleavage in Stages 1 and 2. Stage 1 included fertilization, the egg cortical reaction, three granulation events, and *intralecithal* cleavage. After the cortical reaction, a granulation event occurred at each of the following approximate times after fertilization: 2.5, 5, and 14 hr. The first two granulation events were distinguished by the simultaneous appearance of granules over the entire surface of the fertilized egg. The third granulation event differed from the first two since granules appeared locally on the embryo and gradually progressed across the surface. This event was further characterized by a contraction wave that also moved over the surface. Stage 2 was distinguished by the appearance of *intralecithal* nuclei near the surface. Stages 3 and 4 involved 'total cleavage' and initial formation of the blastula, respectively.

Section 5 described the changes in surface morphology during the first 2 granulations and alternating smooth events. Events through 11 hr after fertilization were examined using SEM and light microscopy. The first granulation occurred 3 hr after fertilization
at 22°C and lasted approximately 1 hr. Granules completely covered the surface and were approximately 68 x 27 μm. Short, thinly dispersed microvilli were found on granules while longer ones extended from furrows between granules. After the granules receded, a microvillar surface was observed that resembled the smooth surface seen 2 hr (S-2) after fertilization (Bannon and Brown, 1980; Brown and Clapper, 1980). A second granulation appeared approximately 5 hr after fertilization; however, the granules were larger than those previously observed and were practically devoid of microvilli. This second granulation lasted for 4 hr and during this period, granule and microvillar modifications were observed. The surface again became smooth as the granules receded and the morphology of the microvilli was described. Thus, the embryonic surface and underlying cortex undergo numerous changes and rearrangements through at least 11 hr after fertilization. Possible roles in development were discussed.

The biochemical events involved in Limulus fertilization are complex and have not been studied until recently. The results reported in this dissertation indicate that fucose or fucose derivatives are implicated in primary sperm attachment to eggs and may also play a role in sperm motility initiation. However, the function of the egg envelope in fertilization, the mode of action of the sperm motility initiation factor, and the factors involved in the induction of the acrosome reaction after sperm attachment are
important aspects of fertilization that need to be elucidated. Furthermore, motility initiation by glucose and glucose derivatives should be studied further since this does not entirely agree with the study published by Clapper and Epel (1982). Further characterization of the fucose-containing 'sperm receptors' on the egg and the fucose present on spermatozoa tails and in seminal fluid should be undertaken to determine the importance of this abundant saccharide during fertilization.
LITERATURE CITED


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