The Mechanism of Sperm Incorporation and an Analysis of Microvilli and Microfilament Organization in Sea Urchin Eggs Examined Through the Use of Cytochalasin B and Ammonium Chloride.

Beverly Dixon Wade

Louisiana State University and Agricultural & Mechanical College

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THE MECHANISM OF SPERM INCORPORATION AND AN ANALYSIS OF MICROVILLI AND MICROFILAMENT ORGANIZATION IN SEA URCHIN EGGS EXAMINED THROUGH THE USE OF CYTOCHALASIN B AND AMMONIUM CHLORIDE

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A Dissertation
Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy
in
The Department of Zoology and Physiology

by
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>x</td>
</tr>
<tr>
<td>CHAPTER 1</td>
<td>1</td>
</tr>
<tr>
<td>OVERVIEW</td>
<td>1</td>
</tr>
<tr>
<td>1. The Fertilization Process in Sea Urchin Eggs</td>
<td>2</td>
</tr>
<tr>
<td>2. Proposed Mechanisms of Sperm-Egg Fusion, Incorporation and Migration.</td>
<td>4</td>
</tr>
<tr>
<td>3. Research Objective</td>
<td>7</td>
</tr>
<tr>
<td>CHAPTER 2</td>
<td>9</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>10</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>12</td>
</tr>
<tr>
<td>a. Collection and Preparation of Gametes</td>
<td>12</td>
</tr>
<tr>
<td>b. Sodium Dodecyl Sulfate Treatment</td>
<td>12</td>
</tr>
<tr>
<td>c. Microscopic Preparations</td>
<td>13</td>
</tr>
<tr>
<td>d. Experimental Design</td>
<td>14</td>
</tr>
<tr>
<td>RESULTS</td>
<td>16</td>
</tr>
<tr>
<td>a. Sperm Incorporation in the Presence of Cytochalasin B.</td>
<td>16</td>
</tr>
<tr>
<td>b. Phase Contrast Microscopy of Sperm Incorporation in the Presence of Cytochalasin B and Ammonium Chloride</td>
<td>19</td>
</tr>
<tr>
<td>c. Time Dependency of Sperm Incorporation.</td>
<td>28</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS (continued)

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DISCUSSION..................................................</td>
<td>36</td>
</tr>
<tr>
<td>CHAPTER 3...................................................</td>
<td>40</td>
</tr>
<tr>
<td>INTRODUCTION................................................</td>
<td>41</td>
</tr>
<tr>
<td>MATERIALS AND METHODS ....................................</td>
<td>44</td>
</tr>
<tr>
<td>a. Cortex Isolation .......</td>
<td>44</td>
</tr>
<tr>
<td>b. Heavy Meromyosin Preparations. .....................</td>
<td>45</td>
</tr>
<tr>
<td>c. Electron Microscopic Preparations. ................</td>
<td>46</td>
</tr>
<tr>
<td>d. Experimental Design. ..................................</td>
<td>46</td>
</tr>
<tr>
<td>RESULTS......................................................</td>
<td>48</td>
</tr>
<tr>
<td>a. The Effect of Cytochalasin B and Ammonium Chloride on Microvilli Organization During Sperm Incorporation.</td>
<td>48</td>
</tr>
<tr>
<td>b. Scanning Electron Microscopic Analysis of Microvilli Elongation in Cytochalasin B and Ammonium Chloride Treated Eggs</td>
<td>55</td>
</tr>
<tr>
<td>c. A Comparative EM Analysis of Microvilli Elongation in Whole Eggs and Isolated Cortices Decorated with Heavy Meromyosin.</td>
<td>64</td>
</tr>
<tr>
<td>DISCUSSION..................................................</td>
<td>76</td>
</tr>
<tr>
<td>CHAPTER 4...................................................</td>
<td>79</td>
</tr>
<tr>
<td>INTRODUCTION................................................</td>
<td>80</td>
</tr>
<tr>
<td>MATERIALS AND METHODS ....................................</td>
<td>82</td>
</tr>
<tr>
<td>a. Deoxyribonucleic Acid Substrate and Deoxyribonuclease 1 Preparations</td>
<td>82</td>
</tr>
<tr>
<td>b. Determination of Deoxyribonuclease 1 Activity.</td>
<td>82</td>
</tr>
<tr>
<td>c. Determination of Deoxyribonuclease 1 Inhibition in Isolated Cortices</td>
<td>83</td>
</tr>
<tr>
<td>TABLE OF CONTENTS (continued)</td>
<td>Page</td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>d. Polyacrylamide Gel Electrophoresis of Protein Preparations</td>
<td>84</td>
</tr>
<tr>
<td>e. Experimental Design</td>
<td>84</td>
</tr>
<tr>
<td>RESULTS</td>
<td>86</td>
</tr>
<tr>
<td>a. The Effects of CB and NH₄Cl on DNA Hydrolysis in Isolated Cortices</td>
<td>86</td>
</tr>
<tr>
<td>b. The Effects of CB and NH₄Cl on Percent Inhibition of DNAase 1 by G-actin</td>
<td>89</td>
</tr>
<tr>
<td>c. SDS-PAGE of Cortical Proteins Isolated from CB and NH₄Cl Treated Eggs</td>
<td>92</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>96</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>99</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>101</td>
</tr>
<tr>
<td>VITA</td>
<td>108</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table                                      Page

CHAPTER 2

2-1. Percent sperm incorporation in L. pictus eggs incubated (10 min) and inseminated in the presence of 2 x 10^{-6} M cytochalasin B ....................................................... 35

CHAPTER 4

4-1. Relative DNA equivalents in extracts of S. purpuratus eggs incubated (10 min) and fertilized in 2 x 10^{-6} M cytochalasin B and 1 mM ammonium chloride. ................................. 88
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CHAPTER 2</strong></td>
<td></td>
</tr>
<tr>
<td>2-1. Transmission electron micrograph of <em>S. purpuratus</em> egg incubated (10 min) and inseminated (30 min) in $2 \times 10^{-6}$ M cytochalasin B.</td>
<td>17</td>
</tr>
<tr>
<td>2-2. Phase contrast observations of <em>S. purpuratus</em> eggs incubated (10 min) and fertilized in the presence of $2 \times 10^{-6}$ M cytochalasin B.</td>
<td>21</td>
</tr>
<tr>
<td>2-3. Phase contrast observations of <em>S. purpuratus</em> eggs incubated (10 min) and fertilized in the presence of $2 \times 10^{-6}$ M cytochalasin B and 1 mM ammonium chloride.</td>
<td>24</td>
</tr>
<tr>
<td>2-4. Phase contrast observations of <em>S. purpuratus</em> eggs incubated (10 min) and fertilized in the presence of 1 mM of ammonium chloride.</td>
<td>26</td>
</tr>
<tr>
<td>2-5. Time dependency of sperm incorporation in <em>S. purpuratus</em> eggs incubated (10 min) and inseminated at 10 min intervals for 60 min in the presence of $2 \times 10^{-6}$ M cytochalasin B and 1 mM ammonium chloride.</td>
<td>29</td>
</tr>
<tr>
<td>2-6. Time dependency of sperm incorporation in <em>S. purpuratus</em> eggs incubated (10 min) and inseminated in the presence of $2 \times 10^{-6}$ M cytochalasin B and 1 mM ammonium chloride for 1, 2.5, 5, 7.5, and 10 min intervals.</td>
<td>32</td>
</tr>
<tr>
<td><strong>CHAPTER 3</strong></td>
<td></td>
</tr>
<tr>
<td>3-1. Scanning electron micrograph of a denuded <em>L. pictus</em> egg at 1 min postinsemination.</td>
<td>49</td>
</tr>
<tr>
<td>3-2. Scanning electron micrograph of the denuded surface of a <em>L. pictus</em> egg incubated (10 min) and inseminated 1 min in $2 \times 10^{-6}$ M cytochalasin B.</td>
<td>51</td>
</tr>
<tr>
<td>3-3. Scanning electron micrograph of a denuded <em>L. pictus</em> egg incubated (10 min) and inseminated 10 min in $2 \times 10^{-6}$ M cytochalasin B and 10 mM ammonium chloride.</td>
<td>53</td>
</tr>
</tbody>
</table>
LIST OF FIGURES (continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-4. Scanning electron micrograph of a denuded <em>L. pictus</em> egg incubated (10 min) and inseminated 30 min in $2 \times 10^{-6}$ M cytochalasin B and 10 mM ammonium chloride.</td>
<td>56</td>
</tr>
<tr>
<td>3-5. Scanning electron micrographs of microvilli (length) of denuded <em>S. purpuratus</em> eggs incubated (10 min) and inseminated for 5, 15, and 30 min in $2 \times 10^{-6}$ M cytochalasin B</td>
<td>59</td>
</tr>
<tr>
<td>3-6. Scanning electron micrographs of microvilli (length) of denuded <em>L. pictus</em> eggs incubated (10 min) and inseminated 10 min in $2 \times 10^{-6}$ M cytochalasin B and 10 mM of ammonium chloride.</td>
<td>62</td>
</tr>
<tr>
<td>3-7. Scanning electron micrographs of microvilli (length) of denuded <em>S. purpuratus</em> eggs incubated (10 min) and inseminated for 5, 15, and 30 min in $2 \times 10^{-6}$ M cytochalasin B and 1 mM ammonium chloride.</td>
<td>65</td>
</tr>
<tr>
<td>3-8. A. Negative stain of rabbit skeletal muscle myosin (HMM) dissolved in 0.5 M KCl (pH 7.0) and extracted in L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK-trypsin) solution (0.5 mg/ml) containing 1 mM HCl for 5 min at 25°C</td>
<td>68</td>
</tr>
<tr>
<td>B. Negative stain of sea urchin egg actin microfilaments decorated with rabbit skeletal heavy meromyosin (HMM).</td>
<td>68</td>
</tr>
<tr>
<td>3-9. Comparative scanning and transmission electron micrographs of <em>L. pictus</em> unfertilized eggs incubated 10 min in $2 \times 10^{-6}$ M CB and 10 mM NH$_4$Cl</td>
<td>70</td>
</tr>
<tr>
<td>3-10. Comparative scanning and transmission electron micrographs of <em>L. pictus</em> zygotes incubated 10 min in $2 \times 10^{-6}$ M CB and 10 mM NH$_4$Cl and fixed at 10 min postinsemination</td>
<td>73</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES (continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAPTER 4</td>
<td></td>
</tr>
<tr>
<td>4-1. Percent inhibition of DNAase 1 by G-actin in extracts of <em>S. purpuratus</em> eggs incubated (10 min) and fertilized in the presence of $2 \times 10^{-6}$ M cytochalasin B and 1 mM ammonium chloride</td>
<td>90</td>
</tr>
<tr>
<td>4-2. Densiometric scans of the protein components of unfertilized and fertilized <em>S. purpuratus</em> egg cortices treated with $2 \times 10^{-6}$ M cytochalasin B and 1 mM ammonium chloride</td>
<td>93</td>
</tr>
</tbody>
</table>
Sperm incorporation in *Lytechinus pictus* and *Strongylocentrotus purpuratus* eggs incubated (10 min) and fertilized in the presence of $2 \times 10^{-6}$ M cytochalasin B (CB) and 1 and 10 mM ammonium chloride ($\text{NH}_4\text{Cl}$) occurs in the presence of limited microvilli and microfilament organization and cortical exocytosis. CB inhibition on sperm incorporation is time related and continues to occur long after sperm-egg fusion. In *S. purpuratus*, at least 30% incorporation is observed in all CB treated eggs. Sperm do not incorporate until 2.5 min after insemination. Maximum incorporation occurs between 7 to 10 min. Percent incorporation approached 60% when eggs were incubated and fertilized in the presence of combined mixtures of CB and $\text{NH}_4\text{Cl}$. In *L. pictus*, sperm incorporation is not seen until 30 min postinsemination and declines from a maximum of 18% to 3% at 50 min postinsemination. In all continuous CB samples in which eggs were reincubated in fresh CB following sodium dodecyl sulfate (SDS) addition and dilution for 60 min, sperm did not migrate beyond the egg cortex. Cortical reactions and chromosome condensation were not seen in eggs reincubated in fresh sea water or test media for at least 60 min. The time patterns of percent incorporation suggest that sperm remain in the cytoplasm and do not undergo pronuclei fusion and condensation. Morphological transmission electron microscopic and scanning electron microscopic comparisons of CB treated
fertilized whole eggs and egg cortices decorated with heavy meromyosin (HMM) reveal short (0.36 to 0.38 μm) microvilli and disorganized actin microfilaments. Eggs incubated and fertilized in the presence of a mixture of CB and NH₄Cl exhibit features transient between CB inhibition and normal responses. At 15 min of insemination, microvilli of CB and NH₄Cl treated eggs elongate to 0.63 μm, approximately one-half the length of untreated control eggs at 10 min postinsemination. Comparatively, isolated cortices exhibit some organization of HMM decorated actin complexes. CB inhibition is also seen in the maintenance of high G-actin pools. Comparative analysis of G-actin and F-actin pools as shown by DNA hydrolysis and SDS-polyacrylamide gel electrophoresis reveals increased percent DNAase I inhibition by G-actin and relatively low DNA hydrolysis in eggs incubated and fertilized in the presence of CB and/or CB and NH₄Cl. Sperm incorporation in the presence of CB and in the absence of an organized microfilament system is suggestive of an alternate mechanism of incorporation.
CHAPTER 1
OVERVIEW
1. The Fertilization Process in Sea Urchin Eggs

Fertilization in sea urchin eggs can be separated into two phases (Epel et al., 1977): A) The early phase which consists of all events that occur during the first 60 sec of development and B) the late phase that begins approximately 5 min of development. The first or "early" phase is initiated by sperm-egg membrane fusion. A prerequisite to fusion is contact by the sperm with the jelly coat surrounding the egg. Fucose sulfate components of the egg jelly induce membrane fusion and protrusion of the acrosomal process of the sperm through a calcium and pH dependent mechanism (Lennarz et al., 1976; Tilney, 1976; Collins and Epel, 1977). The attachment site for the acrosomal process is a receptor protein associated with the vitelline layer that recognizes and binds to a complementary protein on the acrosomal process in a species specific manner (Aketa, 1973; Vacquier and Moy, 1977). Following insemination, up to 2000 sperm bind to the vitelline layer (Vacquier and Payne, 1973).

Sperm-egg plasma membrane fusion leads to a series of ionic changes which result in a variety of developmental events. Immediately following membrane fusion, a rapid influx of Na+ results in depolarization of the egg plasma membrane. The resting potential of the plasma membrane as proposed by Jaffe (1976) changes from between -60 and -10 mV to +10 mV. This depolarization lasts for about 60 sec and prevents additional sperm entry; establishing
an electrically-mediated "fast" block to polyspermy. However, evidence for arguments against this proposal are presented by DeFelice and Dale (1979).

Membrane depolarization is rapidly followed by a transient increase in intracellular Ca++ between 2.5 to 4.5 μM in Lytechinus pictus (Steinhardt et al., 1977). Between 25 to 35 sec following membrane fusion, cortical granules that lie directly below the plasma membrane fuse with the plasma membrane of the egg and exocytose, discharging their contents (proteases, structural proteins, and mucopolysaccharides) into the space between the plasma membrane and the vitelline layer. The vitelline layer lifts and transforms into the fertilization membrane. This transformation is mediated through the products released from the cortical granules; two trypsin-like proteases (Carrol and Epel, 1975) which detach the vitelline layer from the plasma membrane and an ovoperoxidase (Foerder et al., 1977) which catalyzes protein cross-linking. The catalyzing action of ovoperoxidase leads to the formation of tyrosine dimers which result in the harding of the fertilization membrane. Destruction of sperm binding sites and the formation of the fertilization membranes effectively prevents supernumerary sperm from fusing with the egg plasma membrane; thus, the second block to polyspermy is established, the "slow" block (Rothschild, 1953).

Concomitant with these "early" events, the surface topography of the fertilized egg changes dramatically. Short microvilli
(0.5 μM) at the unfertilized egg surface rapidly elongate to as much as 8 to 10 μM (Eddy and Shapiro, 1976; Spiegel and Spiegel, 1977; Schroeder, 1978, 1979). Microvilli elongation appears to be mediated by the development of actin microfilament bundles (Begg and Rebhun, 1979).

During the second or "late" phase of fertilization, approximately 1 min after insemination, egg cytoplasmic pH increases as a result of an efflux of protons. This proton efflux is mediated through a sodium-hydrogen facilitated transport system in which Na+ is exchanged for H+ (Johnson et al., 1976). Alkalization of the egg's cytoplasm is associated with increased protein synthesis (Epel et al., 1975; Epel, 1978), development of new potassium conductance (Steinhardt et al., 1971; Shen and Steinhardt, 1980), amino acid transport (Epel, 1972) and m-RNA polyadenylation stimulation (Wilt and Mazia, 1974).

2. Proposed Mechanisms of Sperm-Egg Fusion and Incorporation

The precise mechanisms of sperm-egg fusion and sperm incorporation into the egg during fertilization are still debatable. Cinemicrophotographic studies on sperm incorporation show that following contact of the sperm at the egg surface, the linear rate of flagellar movement ceases just before entering the egg cortex (Epel et al., 1977). Observations by Nishioka and Cross (1978) suggest that sperm incorporation is not dependent on flagellar motion and could be attributed to either the acrosomal process of
the sperm, the egg cortex or some unknown mechanism. In view of this, two models have been proposed to account for penetration of the spermatozoon into the egg (Epel et al., 1977). First, entry could be a passive event that is a consequence of gamete fusion. Second, actin-like filaments in the sperm or the egg could form a contractile system that is involved in pulling the sperm into the egg. Presently, there is evidence which suggest that the involvement of actin-like filament in this process (Byrd and Perry, 1980; Longo, 1980; Tilney and Jaffe, 1980).

 Passive entry is paralleled with "simple" sperm-egg membrane fusion (Epel, 1975; Epel, 1978). Entry would be a direct passive passage into the cortex similar to the fusion of two oil droplets. Extracellular filaments also may be involved in this fusion process. Thin filaments projecting from the egg surface and surrounding attached sperm have been described (Tegner and Epel, 1976; Schatten and Mazia, 1977). These studies speculate that sperm might be "pulled" in by these surface structures between the fused sperm and egg plasma membrane, and subsequently pass into the egg cortex. These filaments have not been consistently seen and hence cannot be systematically studied.

 Actin-like microfilaments may not only participate in sperm penetration, but also may be involved in egg surface contraction and mechanical changes that occur in the egg cortex. Ultrastructural and biochemical studies have supported the idea that microfilaments (50-70 Å thick) function in many aspects of cell motility (Colwin
and Colwin, 1967; Wessells et al., 1971; Pollard, 1975; Tilney, 1975). In nonmuscle cells they are not rigidly organized and can associate with each other to form bundles of filaments (Brown et al., 1976; Tilney and Jaffe, 1980; Carron and Longo, 1982). Microfilaments in nonmuscle cells differ from skeletal cells in that they only appear situated in localized regions of the cell (Schroeder, 1975). They are frequently transient, appearing at certain times of the cell cycle. Both the structure of microfilaments and their ability to bind heavy meromyosin (HMM) suggest that they are similar in structure and chemistry to muscle actin (Pollard and Weihung, 1974; Mooseker, 1975; Tilney, 1975). These observations have led to the speculation that microfilaments are a major structural component of an actinomyosin-contractile layer that's responsible for many cell surface movements.

Cortical microfilaments underly the plasma membrane of many vertebrate and invertebrate eggs. Bundles of microfilaments identified by HMM binding have been described in isolated sea urchin egg cortices (Burgess, 1977; Begg and Rebhun, 1979). These actin-like filaments also have been found on the cleavage furrow of amphibian eggs (Perry et al., 1971) and in the contractile ring of Arbacia (Schroeder, 1972). Filaments in the acrosomal process of echinoid sperm also are actin-like, as shown by HMM binding in situ and by biochemical characterization (Tilney, 1976). However, the involvement of microfilaments in conveying sperm into the egg has not been established yet.
3. Research Objective

Fertilization of sea urchin eggs results in several biochemical and morphological events: Membrane depolarization and fusion, metabolic activation, topographical alterations, and pronuclear incorporation and migration. Regulation of the morphological events is dependent upon a thin cytoplasmic cortex intimately associated with the plasma membrane. This cortex contains cytoskeletal elements consisting of contractile proteins and microfilaments that play a significant role in the structural rearrangement of the egg surface and related motile processes that occur during fertilization. These contractile elements do not assemble like the cyclic interactions that characterize the actin-myosin system in skeletal muscle. The role of actin microfilament assembly in protrusion of the sperm acrosomal filament has been well documented. However, its function in sperm incorporation and migration in sea urchin eggs is still under extensive study.

One approach to understanding sperm-egg fusion and incorporation has been through studying the effects of microfilament inhibitors on this system. At sufficient levels, cytochalasin B (CB), a mold metabolite blocks sperm incorporation (Byrd et al., 1977; Gould-Somero et al., 1977; Longo, 1977; Byrd and Perry, 1980). The apparent site of drug action seems to be at the level of the plasmalemma and its action related to the disruption of microfilaments in the cell and inhibition of G-actin nucleation (Burgess, 1977; Brown and Spudich, 1979; Flanagan and Lin, 1980).
Therefore, this study examined the distribution of microfilaments in the egg cortex before and after fertilization to determine if sperm incorporation and migration within the egg cortex is actin-microfilament dependent. This was accomplished by an analysis of this system's sensitivity to cytochalasin B coupled with other agents (ammonia) which enhance fertilization. Scanning and transmission electron techniques were used to study the mechanism of incorporation. Microfilament organization and localization was determined through observations of whole eggs as well as actin in isolated egg cortices identified by decoration with heavy meromyosin. Microfilament organization was also compared with an analysis of actin pools in egg extracts through utilization of the DNAase I inhibition assay procedure.
CHAPTER 2

TIME DEPENDENCY OF SPERM INCORPORATION IN CYTOCHALASIN B AND AMMONIUM CHLORIDE TREATED EGGS
INTRODUCTION

The precise mechanism of sperm incorporation in sea urchin eggs is not known. Some insight into this phenomena has been generated by fertilization studies that utilized weak bases and cytochalasin B. Ammonia, nicotine and amine anesthetics have proved to be valuable tools since they enhance sperm-egg fusions or polyspermy (Rothschild, 1953; Longo and Schuel, 1973; Byrd and Collins, 1975; Schuel et al., 1976). Cytochalasin B has been used as a tool for analyzing the motile events associated with the fertilization process. Cytochalasin B's action is associated with its interaction with actin (Spudich and Lin, 1973; Lin and Lin, 1979; Weihung, 1976) and disruption of microfilaments in cellular systems (Wessells et al., 1971).

In the echinoid, *Urechis caupo* (Gould-Somero et al., 1977) and sea urchins (Byrd et al., 1978; Longo, 1978; Byrd and Perry, 1980) sperm incorporation is prevented by the presence of cytochalasin B during insemination. In *L. pictus* and *S. purpuratus* eggs, Byrd and Perry (1978, 1980) found that at low concentrations (1 x 10^{-7} M) did not. The latter concentration was sufficient to inhibit cleavage, but not egg activation as judged by a cortical reaction and an increase in protein synthesis and respiration. Other investigators have reported similar findings (Gould-Somero et al., 1977; Longo, 1978). These effects are reversible upon washing or dilution of CB incubated gametes (Byrd
and Perry, 1980; Gundersen et al., 1980). Migration of the sperm pronucleus within the egg cortex also is inhibited in the presence of high concentrations of CB. The effect of CB on sperm activity prior to fertilization is minimal (Sanger and Sanger, 1975).

In contrast to reports on the effects of CB on the fertilization process, I have found that high concentrations of CB do not completely inhibit sperm incorporation and that ammonia can enhance incorporation. In this chapter, the mechanism of sperm incorporation will be examined through the use of both cytochalasin B and ammonium chloride.
MATERIALS AND METHODS

a. Collection and Preparation of Gametes:

Lytechinus pictus and Strongylocentrotus purpuratus were used for these studies. L. pictus was collected from St. Andrews State Park, Panama City Beach, Florida and S. purpuratus was collected from the San Diego Bay, San Diego Bay, California. Both species were maintained at 16°C in aquarium tanks containing marine biological sea water (MBL-SW) (Cavanaugh, 1956) and fed ad libitum a diet of seaweed.

Gametes were induced to spawn by a 0.5 ml injection of 0.5 M KCl. Eggs were collected in marine biological sea water (MBL-SW), jelly coats removed by passage through a silk bolting cloth or Nitex mesh (180-253 μm mesh), followed by acidification of the MBL-SW to a pH of 5.0 - 5.5 with 0.1 N HCl for approximately 2 min. The pH was then readjusted to 8.0 by titration with 1 M Tris-HCl buffer (pH 8.0) and washed several times following gravitational settling in fresh MBL-SW. Only eggs showing a 95-100% fertilization response were used. Sperm were collected "dry" (i.e., undiluted) and refrigerated until used.

b. Sodium Dodecyl Sulphate Treatment:

Supernumerary sperm of fertilized egg suspensions were killed by the addition of an equal volume of 0.0075% sodium dodecyl sulfate (SDS) in MBL-SW for 5 sec (Byrd and Collins, 1975). This
suspension was then diluted with a four-fold volume of artificial sea water (AWS) and allowed to gravity settle followed by three additional washes. Spermicidal activity and egg viability were monitored by the addition of SDS to unfertilized egg suspensions, followed by fertilization for 15 sec and the absence of egg vitelline layers observed by light microscopy. Egg and sperm were then diluted four-fold with MBL-SW, washed three times and fresh sperm added with vitelline membrane elevations observed.

c. Microscopic Preparations:

Light microscopy. Samples were fixed in Carnoy's fixative (3 parts 95% ethanol:1 part glacial acetic acid) with fresh fixative added twice for at least 3 hr. Leuco Fuschin staining was followed by decantation of the fix and addition of 2 ml of cold 1 N HCl. Egg samples were then centrifuged (clinical) for 8 sec, the supernatant decanted and the eggs mixed with 2 ml of 1 N HCl and maintained at 60°C for 8 min. These steps were followed by the addition of 2 ml of Leuco Fuschin stain for 2 hr with frequent resuspension. Prior to phase contrast microscopic observations, samples were suspended in an acid rinse (1% potassium metabisulfite and 1 N HCl) for 20 min in a fume hood and resuspended in 45% acetic acid for 5 min to allow pronuclei to swell.

Transmission electron microscopy. One volume of eggs was fixed for transmission electron microscopy (TEM) in 5 volumes of a 3% gluteraldehyde solution (0.1 M sodium phosphate and 0.45 M
sucrose, pH 7.3) containing 0.1% tannic acid for at least 90 min at 4°C with resuspension in fresh fixative 30 min after initial fixation time. Samples were then washed in two 10 min changes in 0.1 M phosphate buffer - 0.45 M sucrose rinse (pH 7.3), post-fixed in 1% osmium tetroxide (containing the phosphate-sucrose buffer) solution on ice for 60 min, and washed three times in distilled water. The eggs were resuspended in 0.5% uranyl acetate for 12 hr and washed again three times. Samples were then dehydrated in increasing concentrations of ethyl alcohol (ETOH), transferred through three separate 10 min changes of 100% ETOH and resuspended in three 15 min changes of 100% acetone. This was followed by suspension in 30% Epon:Araldite - 70% acetone for 1 hr, 70% Epon:Araldite - 30% acetone for 3 hr and 100% Epon:Araldite overnight. Samples were embedded in Beem capsules with fresh 100% resin, placed under vacuum for 12 hr and polymerized at 70°C for at least 18 hr. They were thin-sectioned on a Porter Blum MF-2 ultramicrotome, poststained for 5 min in uranyl acetate and 7 min in lead citrate and viewed with a Jeol 100CX electron microscope.

d. Experimental Design:

A 1.5% (V:V) egg suspension was added 1) to an equal volume of cytochalasin B (final concentration of $2 \times 10^{-6}$ M), 2) to an equal volume of ammonium chloride (1 mM final concentration), or 3) a mixture of cytochalasin B (CB) and ammonium chloride ($\text{NH}_4\text{Cl}$), to give a final concentration of $2 \times 10^{-6}$ M CB and 1 mM $\text{NH}_4\text{Cl}$ for 10 min pulse treatment time. The eggs were fertilized by adding
2 ml of a 10% sperm suspension to each test sample. Supernumerary sperm were killed by the addition of 1 ml of 0.00075% SDS for 1 min. Eggs were washed by a four-fold dilution in MBL-SW. Following dilution of SDS with sea water, eggs were allowed to incubate for 12.5 min at 16°C before fixation in 95% ethanol - glacial acetic acid (3:1). Samples receiving continuous CB and NH₄Cl treatment were reincubated in fresh test media following SDS application for up to 60 min. The time between SDS dilution and reincubation in fresh test media did not exceed 4 min. At least 50 eggs per test sample were scored by phase microscopy for the presence of sperm pronuclei. To determine precise sperm-egg fusion times, samples were fertilized and processed as above with the addition of SDS at 1, 2.5, 5, 7.5, and 10 min intervals.
RESULTS

a. Sperm Incorporation in the Presence of Cytochalasin B:

Sperm incorporation is normally prevented in sea urchin eggs preincubated and fertilized in the presence of $2 \times 10^{-6}$ M cytochalasin B (Byrd and Perry, 1978; Longo, 1978). However, if *S. purpuratus* eggs are continually monitored for the presence of sperm pronuclei by either light or transmission electron microscopy, by 30 min postinsemination, some sperm pronuclei are seen within the innermost area of the egg cytoplasm (Fig. 2-1). TEM observations of these eggs show the plasma surface to have short (or contracted) microvilli with underlying cortical granules. The migration pattern of sperm pronuclei appears disoriented and sperm pronucleus migration distance from the egg surface was less than that which normally occurs during in vitro fertilization. In contrast, in untreated controls, sperm and egg pronuclei are usually fused by 30 min after insemination.

Sperm incorporation in the presence of CB was unexpected and infrequently observed. How CB affects sperm incorporation is not known. At this point, one could speculate that CB was either affecting sperm-egg fusion or the contractile mechanism of the cell. Sperm remain bound at the surface, but subsequent entry into and migration within the cortex usually could be inhibited by the presence of CB. Possibly, the incorporation of sperm might be dependent on the "time" at which sperm successfully contact the
Figure 2-1. Transmission electron micrograph of *S. purpuratus* egg incubated (10 min) and inseminated (30 min) in $2 \times 10^{-6}$ M cytochalasin B. Contracted microvilli at egg surface (arrow); cortical granules in cortex (CG); sperm pronucleus in cytoplasm (SP). 8,448X.
egg surface. In addition, if CB does not affect sperm-egg fusion, then conditions that enhance fusion could enhance fusion rates in the presence of CB.

b. Phase Contrast Microscopy of Sperm Incorporation in the Presence of Cytochalasin B and Ammonium Chloride:

To determine the timing of sperm-egg fusion in _S. purpuratus_, samples were exposed to the spermicidal agent, sodium dodecyl sulfate (SDS) at 5 min intervals for 60 min. Spermicidal agents have been used in other studies to determine the timing of sperm-egg fusion (Hagstrom, 1954; Byrd and Collins, 1975). Sperm fusion is inhibited within 1 sec of its addition. The egg is unaffected by this treatment as is post fusion development. Sperm nuclei which fuse before SDS addition, enter the egg's cytoplasm and undergo condensation as in normal controls. Following SDS addition, eggs were washed, reincubated in fresh sea water and fixed 12.5 min later. Eggs receiving continuous treatment of CB and/or NH₄Cl were reincubated in fresh test media after SDS treatment for up to 60 min before fixation.

In this study, cytochalasin B did not completely block sperm incorporation. Several CB treated eggs not only contained incorporated sperm, but optical dense cortical granules organized beneath the plasma membrane (Fig. 2-2). In normal _in vitro_ studies, sperm incorporation is immediately followed by cortical granule exocytosis, which results in elevation of the fertilization membrane. Cortical granules and the absence of a fertilization
membrane also were observed in all unfertilized CB controls (Fig. 2-2, A). However, condensed sperm pronuclei were observed in the immediate cortex of eggs incubated and fertilized in CB for 10 min (Fig. 2-2, B). In CB treated eggs at 30 min postinsemination, sperm could be seen deeper in the cytoplasm (Fig. 2-2, C). Longer incubation time, without the addition of fresh test media may have resulted in reduced effects of CB on microfilaments, thus allowing sperm to move out of the egg cortex. Eggs receiving continuous CB exposure (CB is readded within 4 min of SDS dilution) also revealed sperm pronuclei at 50 min post-insemination, but just beneath the plasma membrane in the egg cortex (Fig. 2-2, D). Shorter migration distances in these samples may reflect the continued inhibition of CB by reincubation in fresh test media. Microfilament formation would be inhibited and sperm would not be able to move out of the egg cortex. The time between SDS dilution and reincubation in CB (maximum of 4 min) would not be adequate time to allow for normal migration recovery when compared to the 15 min recovery time that was observed by Byrd and Perry (1980) in S. purpuratus eggs fertilized in the presence of $5 \times 10^{-7} \text{M}$ CB. The effect in this study should be even greater since eggs were pretreated (10 min) in CB and at a higher concentration ($2 \times 10^{-6} \text{M}$).

Eggs fertilized in the presence of combined mixtures of CB and NH$_4$Cl produced results similar to both CB treated and NH$_4$Cl treated eggs. In CB and NH$_4$Cl treated unfertilized controls,
Figure 2-2. Phase contrast observations of S. purpuratus eggs incubated (10 min) and fertilized in the presence of $2 \times 10^{-6}$ M cytochalasin B.

Supernumerary sperm were killed by the addition of SDS (0.0075%) in sea water at the indicated postinsemination times. Eggs were washed and reincubated in fresh sea water for 12.5 min and then fixed in Carnoy's. Eggs receiving continuous CB treatments were reincubated in fresh CB following SDS addition and dilution for up to 60 min. A, unfertilized, CB treated control; B, CB treated egg at 10 min postinsemination with one sperm pronucleus (arrow); C, sperm pronucleus (arrow) in CB treated egg at 30 min postinsemination; D, sperm pronucleus (arrow) in egg with repeated replacement of CB at 50 min postinsemination. Bar = 5 μm.
eggs appeared unactivated; no cortical exocytosis (Fig. 2-3, A). At 10 min postinsemination, these eggs were polyspermic (Fig. 2-3, B). Sperm migration distances in the cytoplasm appeared to be equal to migration distances in CB treated eggs at 30 min postinsemination. This suggests that the egg is less sensitive in the presence of NH$_4$Cl and sperm migration recovery occurs in less time. Recovery time was even less at 30 min postinsemination (Fig. 2-3, C). Sperm pronuclei approached the middle axis of the egg. Eggs receiving continuous treatment at 50 min postinsemination (Fig. 2-3, D) contained pronuclei, but near the egg cortex. How NH$_4$Cl might be affecting sperm migration can not be determined at this time. However in addition to its influence on polyspermy, another effect may be on the microfilament system in the fertilized egg.

Unfertilized ammonium chloride treated (10 min) eggs were compared to fertilized ammonium chloride eggs (Fig. 2-4, A). At 30 min postinsemination, untreated control eggs had entered into the late prophase of the mitotic cycle (Fig. 2-4, B). When compared to ammonium treated eggs at 30 min postinsemination, polyspermic and early prophase conditions were seen (Fig. 2-4, C). Continuous ammonium treated eggs at 30 min postinsemination also revealed polyspermic conditions, a double mitotic spindle and decondensed sperm pronuclei (Fig. 2-4, D). The fertilization membranes of all ammonium treated eggs were slightly elevated. In this study, ammonium's effect on influencing sperm-egg fusion appears to parallel polyspermy.
Figure 2-3. Phase contrast observations of *S. purpuratus* eggs incubated (10 min) and fertilized in the presence of $2 \times 10^{-6}$ M cytochalasin B and 1 mM ammonium chloride.

Supernumerary sperm were killed by the addition of SDS (0.0075%) in sea water at the indicated postinsemination times. Eggs were washed and reincubated in fresh sea water for 12.5 min and then fixed in Carnoy's. Eggs receiving continuous CB and NH$_4$Cl treatments were reincubated in fresh CB and NH$_4$Cl following SDS addition and dilution for up to 60 min. A, unfertilized CB and NH$_4$Cl treated control egg; B, two sperm pronuclei (arrows) in CB and NH$_4$Cl treated egg at 10 min postinsemination; C, sperm pronucleus in CB and NH$_4$Cl treated egg at 30 min postinsemination (arrow); D, sperm pronucleus (arrow) at 50 min postinsemination in continuous CB and NH$_4$Cl treated egg. Bar = 5 μm.
Figure 2-4. Phase contrast observations of *S. purpuratus* eggs incubated (10 min) and fertilized in the presence of 1 mM of ammonium chloride.

Supernumerary sperm were killed by the addition of SDS (0.00075%) in sea water at the indicated postinsemination times. Eggs were washed and reincubated in fresh sea water for 12.5 min and then fixed in Carnoy's. Eggs receiving continuous NH$_4$Cl treatments were reincubated in fresh NH$_4$Cl following SDS addition and dilution for up to 60 min. A, unfertilized control egg; B, chromosome condensation (arrow) in untreated zygote at 30 min postinsemination; C, polyspermy in NH$_4$Cl treated egg at 30 min; D, sperm pronucleus and double mitotic spindle (arrow) in egg receiving continuous NH$_4$Cl treatment at 30 min postinsemination. Bar = 5 µm.
c. Time Dependency of Sperm Incorporation:

To establish the time of incorporation, we also utilized SDS to block further sperm-egg interactions. Under normal fertilization conditions eggs were inseminated, then SDS was added at indicated times to block further sperm incorporation. Following SDS treatment, eggs were allowed to incubate in sea water for 12.5 min, fixed, and scored for the presence of sperm pronuclei. In control eggs, the maximum number of pronuclei per egg (approximately 1 sperm/egg) was reached within the first few minutes of insemination (Fig. 2-5, A). Since these eggs exhibited a normal cortical reaction, it was apparent that successful sperm fused prior to the cortical reaction. Since pronuclear fusion occurs within 25 to 30 min, there were no sperm pronuclei observed in eggs after 20 min of insemination.

In CB treated eggs, the number of sperm pronuclei/egg reached a maximum by 10 min and then remained constant (Fig. 2-5, A). This suggests that sperm successfully interact for at least 10 min. However, the total number of eggs fertilized remains low, approximately 30% (with no polyspermy); indicating that sperm incorporation is reduced in these treated eggs. Assuming each CB egg has one successfully fused sperm, there are two possible modes of action: 1) Sperm continue to penetrate the egg cortex at different times of development (so that some are decondensing and fusing, while others remain in the egg cytoplasm) or 2) Sperm that fuse with the egg by 10 min enter the cytoplasm where they remain and do not fuse with the female pronucleus. It is possible to
Figure 2-5. Time dependency of sperm incorporation in _S._ purpuratus eggs incubated (10 min) and inseminated in the presence of $2 \times 10^{-6}$ M cytochalasin B and 1 mM ammonium chloride.

Abscissa: time (min) of SDS addition after insemination;

Ordinate: percent of eggs scored showing sperm pronuclei.

Supernumerary sperm were killed by the addition of SDS (0.0075%) in sea water at the indicated time intervals after insemination and allowed to incubate 12.5 min before fixation in Carnoy's. Eggs receiving continuous treatment were reincubated in fresh test media following SDS addition and dilution for up to 60 min. A: Pulse CB treated eggs, O. B: Continuous CB treated eggs. C: Pulse CB and NH$_4$Cl treated eggs, O. D: Continuous CB and NH$_4$Cl treated eggs. Δ, Untreated control eggs; ▲, NH$_4$Cl treated control eggs.
distinguish between these two possibilities by utilizing the previous observations of Byrd and Perry (1980). They found that the re-addition of $2 \times 10^{-6}$ M CB within 1 min after the sperm has fused with the egg inhibits migration and fusion with the female pronucleus. Therefore, if further fusion is inhibited by SDS and sperm pronuclei that have already made successful fusion continue to enter with time, there would be more sperm pronuclei; the longer the time of incubation. Since there was no increase in the maximum number of sperm pronuclei after 10 min, it would appear that the second possibility is the correct one. Continuous incubation in CB (reincubation of eggs in CB after SDS treatment up to 60 min) showed a similar sperm incorporation pattern, with percent sperm incorporation into eggs declining by approximately 5% (Fig. 2-5, B). Maintenance of eggs in continuous treatments of CB enhances the effect of its action on this system. This was also demonstrated in the previous experiment involving phase contrast microscopy.

Eggs were also treated with ammonium chloride. Ammonium is a known polyspermic agent which increases the number of successful sperm-egg fusions prior to the cortical reaction. As seen in (Fig. 2-5, C), percent sperm incorporation approximated those of the untreated controls. However, a marked increase in percent sperm incorporation could be seen when ammonium is combined with cytochalasin B. Percent sperm incorporation approached 60% in CB and $\text{NH}_4\text{Cl}$ pulse samples (Fig. 2-5, C) and declined to 30% (Fig. 2-5, D) in continuous treated samples. While addition of $\text{NH}_4\text{Cl}$ to CB increased the number of sperm pronuclei/egg, it did not alter
Figure 2-6. Time dependency of sperm incorporation in *S. purpuratus* eggs incubated (10 min) and inseminated in the presence of $2 \times 10^{-6}$ M cytochalasin B and 1 mM ammonium chloride for 1, 2.5, 5, 7.5 and 10 min intervals. Abscissa: time (min) of SDS addition after insemination; Ordinate: percent of eggs scored showing sperm pronuclei.

A: Pulse treated eggs in which supernumerary sperm were killed by the addition of SDS (0.0075%) in sea water at the indicated time intervals after insemination and allowed to incubate 12.5 min before fixation in Carnoy's. B: Eggs receiving continuous treatment reincubated in fresh test media following SDS addition and dilution for up to 60 min. Δ, untreated control eggs; ∆, NH$_4$Cl treated control eggs; O, CB and NH$_4$Cl treated eggs; ●, CB treated eggs.
Sperm Pronuclei / Egg

Time of Addition (min)
the shape of the curve or the timing of sperm fusion when compared to CB samples.

To precisely determine the timing of sperm-egg fusion, the same experiments were repeated using shorter time intervals, less than 10 min (Fig. 2-6). Again, it is apparent that the cortical reaction by one minute blocks further sperm penetration in control eggs. As was seen earlier, maximum incorporation of sperm in CB treated eggs occurs approximately 10 min after insemination. However, in untreated and NH₄Cl treated controls, incorporation occurred within seconds of insemination and continued linearly with respect to the indicated incubation times. In cytochalasin B and ammonium chloride treated eggs, maximum incorporation was not observed until approximately 10 min after insemination.

Earlier data obtained of *L. pictus* eggs fertilized on the presence of cytochalasin B (Wade, 1979) were compared with observations on *S. purpuratus* (Table 2-1). In *L. pictus*, sperm incorporation was not observed until 30 min postinsemination. This could be a result of species differences or some experimental artifact. Percent incorporation at 30 min postinsemination remained relatively constant for 10 min, but declined as the egg approached normal cleavage time. The pattern of eggs containing sperm observed in this species differ from that seen in *S. purpuratus*. The decline in percent incorporation might represent egg activation; pronuclei fusion or condensation. However, neither fusion nor condensation were observed in this study.
Table 2-1. Phase contrast observations of _L. pictus_ eggs incubated (10 min) and inseminated in the presence of $2 \times 10^{-6}$M cytochalasin B. Fertilization was achieved by using a $10^4$ sperm-egg ratio.

<table>
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<tr>
<th>Postinsemination Time (Min)</th>
<th>Total Eggs Observed</th>
<th>% Eggs With Visible Sperm Pronucleus</th>
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<td>90</td>
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DISCUSSION

In contrast to previously published reports, high concentrations of cytochalasin B do not completely inhibit sperm incorporation (Gould-Somero et al., 1977; Longo, 1978; Byrd and Perry, 1978, 1980). At least 30% incorporation was observed in all CB treated eggs. Percent sperm incorporation increased when eggs were incubated and fertilized in the presence of combined CB and NH₄Cl mixtures.

The effect of CB on the time of sperm incorporation appeared to depend on the time of successful sperm-egg membrane fusion. Sperm did not incorporate in CB treated eggs until 2.5 min after insemination. Maximum sperm incorporation occurred between 7 to 10 min. The addition of NH₄Cl to CB did not make a significant difference. However, CB did appear to have some affect on sperm-egg fusion as judged by the percent incorporation seen at 10 min of insemination. The overall low percent sperm incorporation rates, the delay of incorporation time and the absence of polyspermy in CB treated eggs suggest the entry into the egg may be dependent on some activity that occurs after sperm-egg fusion.

In view of the extensive citations on CB inhibition of actin microfilament development, it is conceivable that incorporation may largely be dependent on this contractile process. Sperm may successfully bind, but incorporation into the egg and migration out of the cortex may be actin dependent. If the contractile
system represents the major vehicle for sperm entry, then impairment of this system would reduce sperm incorporation accordingly. Since sperm were observed within the cortex and cytoplasm, then entry may be a result of reduced CB sensitivity in this system or entry by some other mechanism such as "passive" incorporation. However, once inside, passage through the cortex could still be filament dependent. This dependence is likely since, in CB and CB and NH$_4$Cl treated eggs, sperm migration was associated with longer than normal insemination time with continued treatments of these agents. In all continuous CB treated samples, incorporated sperm failed to migrate beyond the cortex. This observation is also supported by Byrd and Perry's (1980) data on sperm migration rates in the presence of high and low CB concentrations. The CB effect appears to occur during the initial time of migration when the sperm is in the cortex. In _S. purpuratus_, sensitivity to the drug ($1 \times 10^{-7}$ to $5 \times 10^{-7}$ M CB) occurs within 5 to 10 min postinsemination in $2 \times 10^{-7}$ M CB. These data suggest species sensitivity differences, which might account for later sperm incorporation as shown in Table 2-1 for _L. pictus_.

Morphologically, the concentrations of CB and NH$_4$Cl used in this study often produced structural alterations that were not seen in untreated control eggs; fragmented surfaces and grainy cytoplasm. In addition, an effect was seen on cortical exocytosis. Cytochalasin B and CB and ammonium chloride eggs showing incorporated sperm did not produce the cortical reactions (i.e.,
cortical granule exocytosis and fertilization membrane formation) that were observed in inseminated untreated control eggs of this species. These observations on cortical exocytosis were similar to those reported by Longo (1978) and Byrd and Perry (1980) in which partial cortical reactions, with persistent attachment of the vitelline layer to the plasma membrane were described in CB inseminated eggs. Normally, cortical reactions occur within 20 sec of insemination. The partial reactions that were observed in these studies were delayed up to 60 sec.

Morphological determination of egg activation by light microscopy is difficult if not impossible if inseminated eggs do not show cortical reactions and pronuclei fusions. In this study, sperm incorporation into CB and NH₄Cl treated eggs was not accompanied by cortical reactions or chromosome condensation. Therefore, it can not be concluded at this time that eggs showing incorporation were activated. However, the pattern of the time dependency of sperm incorporation curve (Fig. 2-5) does suggest that due to the continued inhibition effects of CB, sperm which entered eggs remained in the cytoplasm and that pronuclear fusion and condensation would not occur.

Cytochalasin B inhibition on sperm incorporation may be time related and occur long after initial sperm-egg membrane fusion. Normally, sperm incorporation may be affected through CB action on the microfilament system. Incorporation may be microvilli related, but not totally dependent. To be conclusive
on these points, it was necessary to examine the effects of CB and
NH₄Cl on microvilli and cytoskeletal structure and function.
CHAPTER 3

THE EFFECTS OF CYTOCHALASIN B AND AMMONIUM CHLORIDE ON MICROVILLI ELONGATION AND CORTICAL ORGANIZATION DURING FERTILIZATION: AN ELECTRON MICROSCOPIC ANALYSIS OF WHOLE EGGS AND HEAVY MEROMYOSIN DECORATED MICROFILAMENTS IN ISOLATED EGG CORTICES
INTRODUCTION

The unfertilized sea urchin egg surface contains numerous short finger-like projections called microvilli which represent approximately 22,000 \( \mu m^2 \) of surface area (Schroeder, 1979). Microvilli elongate following fertilization and midway through the first cell division. In unfertilized Lytechinus pictus eggs, microvilli are less than 0.5 \( \mu m \) long and by 2 min postinsemination, extend more than 1.5 times their original length (Kidd and Mazia, 1979). Similar microvilli extension can also be seen in Arbacia punctulata, Strongylocentrotus droebachiensis and Strongylocentrotus purpuratus (Schroeder, 1978; Longo, 1980, Tilney and Jaffe, 1980). Microvilli elongation at fertilization represent the mechanism by which the egg surface increases when the oolemma takes up excess membrane introduced by cortical granules.

Microvilli extension and the formation of a fertilization cone at the egg surface during sperm incorporation has been well-documented in A. punctulata, L. pictus, and L. variegatus (Longo and Anderson, 1968; Longo, 1980, Schatten and Schatten, 1980). The fertilization cone has been identified as an extension of actin filaments within microvilli at the plasma membrane. This extension begins at the point of contact between the acrosomal process of the sperm and egg surface. The cone develops within 1 min of insemination and following maximal extension within 2 min becomes reabsorbed as the spermatozoon makes its complete
entry into the egg cortex. Descriptions of a developing fertilization cone have not been consistent in S. purpuratus.

Evidence that sea urchin eggs contain contractile proteins has led to an analysis of actin microfilament function and microvilli elongation to determine their relevance to the fertilization process. Actin associated with the cortex polymerizes concomitant with microvilli elongation (Begg and Rebhun, 1979; Spudich and Spudich, 1979; Tilney and Jaffe, 1980; Carron and Longo, 1982). Thin microfilaments begin to appear 1 min after fertilization. They are evenly dispersed in microvilli and in the cortex as a network attached to the plasma membrane. Within 2 min they become rearranged into bundles which appear to support microvilli elongation.

As reviewed in Chapter 2, cytochalasin B effects microfilament structure. A secondary effect of treating cells with cytochalasin B is disorganization of microvillar structure. This effect varies from cell type to cell type. Whereas; microvilli of intestinal epithelial cells and pseudocleaving mouse cells are unaffected (Mak et al., 1974; Wasserman et al., 1977), microvilli in the sea urchin system lengthen in direct proportion to microfilament organization (Longo, 1978; Flanagan and Lin, 1980; Longo, 1980).

In this chapter, cytochalasin B effects on the distribution and arrangement of microvilli and microfilaments during normal fertilization and development is reported. The structural and functional relationship between cytoskeletal contractile elements
during fertilization require adequate identification techniques. When intact sea urchin eggs are fixed by standard electron microscopic methods that preserve microfilaments in other cells, 50-70 Å filaments are not always clearly visible. Tannic acid-glutaraldehyde fixation following heavy meromyosin (HMM) incubation of whole eggs or isolated egg cortices was used to preserve actin structure and permit accurate identification. Isolated egg cortices facilitated the penetration of HMM as well as identification of cortical elements void of excess cytoplasmic substances. Ammonia which is known to enhance sperm-egg fusion also was used as a probe for understanding the events associated with fertilization.
MATERIALS AND METHODS

Collection and preparation of *L. pictus* and *S. purpuratus* gametes followed the procedures described in Chapter 2, Materials and Methods.

a. Cortex Isolation:

Cortices were isolated from denuded (vitelline layer removed) unfertilized and fertilized eggs. To remove the egg's vitelline layer (VL), an equal volume of 20 mM dithiothreitol (DTT, pH 9.1) was added to each unfertilized egg suspension, to give a final concentration of 10 mM DTT. Suspensions were allowed to incubate at 20°C for 5 min and mixed frequently with Pasteur pipettes. Eggs were then diluted four-fold with MBL-SW, pH 8.0, gravity settled and washed several times to remove DTT. Vitelline layer removal was monitored by light microscopic observations of fertilized eggs.

Cortical isolation followed the previously described method of Beg and Rebhun (1979). The isolation medium (IM) contained 0.35 M glycine, 0.1 N N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES), pH 6.5, 5.0 mM EGTA, 1 mM dithiothreitol (DTT), 5.0 mM MgCl₂, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 mg/ml soybean trypsin inhibitor (SBTI) to reduce proteolysis. To remove divalent cations, eggs were washed once in Ca++/Mg++-free sea water (pH 7.8) followed by resuspension in IM at 4°C. All eggs were settled by hand centrifugation. They
were resuspended in fresh IM as a 10% suspension and lysed in a Dounce homogenizer by two passes of the pestle or until 98% lysis was observed through light microscopic examinations. Lysed eggs were sedimented at 200 g for 5 min in 15 ml conical centrifuge tubes. The upper cortical layer was pipetted and washed in fresh IM at 4°C, with repeated centrifugation (200 g for 5 min) three times.

b. Heavy Meromyosin Preparation:

Myosin preparation was based on modifications of the techniques described by Kielley and Harrington (1959) and Szent-Gyorgyi (1974). Skeletal muscle was extracted from a Black and White Dutch male rabbit. Extraction procedures were performed at 4°C. Heavy meromyosin (HMM) was separated from light chain meromyosin by concentration of the protein mix in 0.1 M phosphate buffer, pH 7.0, containing 0.5 M KCl. Ten volumes of the solution were digested with one volume of L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK-trypsin) (Worthington Biochemical Corp., Freehold, N. J.) solution (0.5 mg/ml) containing 1 mM HCl for 5 min at 25°C with constant stirring. Trypsin inhibitor (1 mg/ml) in water at pH 7.4) was added to stop the reaction. The digest was dialyzed overnight against 7.0 mM potassium phosphate buffer, pH 7.0 and the resulting insoluble material was removed by centrifugation at 100,000 g for 1 hr. The supernatant containing the HMM yield (3.74 mg/ml) was checked by negative staining and SDS gel electrophoresis and stored as 1-2 mg/ml at 0°C in 5% glycerol containing 7 mM potassium phosphate buffer.
c. Electron Microscopic Preparations:

Scanning electron microscopy. Gametes were fixed for scanning electron microscopy (SEM) at 22°C in a 2.5% glutaraldehyde (GTA) solution containing 0.1 M phosphate buffer + 0.45 M sucrose, pH 7.3. After two changes in the GTA fixative within 90 min, each sample was washed for four 10 min intervals in the phosphate-sucrose buffer, washed identically in tap water, transferred to 2,2-dimethoxypropane (DMP) acidified with HCl and allowed to warm up to room temperature. Following DMP dehydration, samples were transferred to reagent grade acetone, placed in modified Beem capsules capped with nucleopore filter and dried at the critical point of carbon-dioxide. All samples were coated with 200 Å of gold-palladium and viewed on a Hitachi (S-500) scanning electron microscope.

Transmission electron microscopy. Materials and Methods were those described in Chapter 2.

d. Experimental Design:

Twenty milliliters of a 1% L. pictus or S. purpuratus egg suspension (dejelled) were incubated in either 2 x 10^{-6} M of cytochalasin B (CB) dissolved in 1% dimethyl sulfoxide (DMSO), 10 mM ammonium chloride (NH₄Cl), or 2 x 10^{-6} M of CB and 10 mM of NH₄Cl for 10 min, fertilized with 1 ml of a 0.5% sperm solution and fixed for TEM and SEM at 1, 5, 10, 15, and 30 min intervals. Controls included unfertilized eggs, unfertilized eggs incubated in CB, NH₄Cl, DMSO, and CB and NH₄Cl for the same time intervals.
as the test groups. Eggs were maintained in suspension with periodic mixing with Pasteur pipettes (Epel, 1972).

Five ml of a 5% egg suspension were sampled at each indicated time interval and egg cortices were prepared as described. Isolated cortices were decorated with HMM by the addition of an equal volume of the protein (3.74 mg/ml) for 30 min at room temperature, washed three times and processed for TEM.
RESULTS

a. The Effects of Cytochalasin B and Ammonium Chloride on Microvilli Organization During Sperm Incorporation:

Microvilli extension in the formation of a fertilization cone can be clearly seen in denuded L. pictus eggs. Removal of the vitelline layer by treatment with dithiothreitol (DTT) leaves the egg surface unobscured and permits direct observation of surface structures. At one minute postinsemination, microvilli surrounding the point of sperm entry can be seen as rigid structures whose lengths approximately double other surface microvilli (Fig. 3-1). The fertilization cone becomes reabsorbed by the plasma surface after the spermatozoon enters the egg cytoplasm. Insemination of eggs pretreated (10 min) in cytochalasin B prevents fertilization cone formation response. CB treated eggs fixed at 1 min of insemination did not exhibit surface activity that would be typical of sperm incorporation (Fig. 2-3). Sperm can be seen at the egg surface, but microvilli elongation and fertilization cone development is not observed.

Eggs preincubated (10 min) in cytochalasin B and ammonium chloride and then inseminated produced different results. Several eggs fixed at 10 min postinsemination revealed sperm attached at the egg surface by an elongated acrosomal process (Fig. 3-3). Actin polymerization in formation of the sperm acrosomal process is
Figure 3-1. Scanning electron micrograph of a denuded *L. pictus* egg at 1 min postinsemination. Receding fertilization cone (arrow) at the site of sperm entry. 15,500X.
Figure 3-2. Scanning electron micrograph of the denuded inactivated surface of an _L. pictus_ egg pretreated (10 min) cytochalasin B (2 x 10^{-6} M) egg fixed at 1 min postinsemination. Several spermatozoon can be seen at egg surface containing short microvilli. 6,000X.
Figure 3-3. Scanning electron micrograph of a denuded *L. pictus* egg incubated (10 min) and inseminated (10 min) in 2 x 10^{-6} M cytochalasin B and 10 mM ammonium chloride. Surface contortions (arrows); binding of sperm acrosomal process (AP). Bar = 5 μm.
insensitive to CB (Sanger and Sanger, 1975; Schatten and Schatten, 1980). The entire egg surface appears distorted, as is typical of CB and NH₄Cl treated eggs. Microvilli do not elongate and remain as short projections over the entire surface. However, at 30 min postinsemination, sperm incorporation aided by a well developed fertilization cone is occasionally observed (Fig. 3-4). Elongated microvilli can be seen covering the entire egg surface. Microvilli around the penetrating sperm are longer and appear to project from the fertilization cone to cover the sperm head. These observations correlate with results on the time of sperm incorporation in Chapter 2 (Table 2-1) on L. pictus. Sperm pronuclei were not visible in this species until 30 min after insemination in CB treated eggs. In S. purpuratus, sperm incorporation can be seen within 2 min of insemination, but, fertilization cones are not observed. Again, this may reflect differences between species and/or an NH₄Cl effect in influencing microvilli elongation. In view of the low probability of direct observations of sperm and egg fusion, the distribution and localization of microfilaments and microvilli elongation during these events were analyzed further.

b. Scanning Electron Microscopic Analysis of Microvilli Elongation in Cytochalasin B and Ammonium Chloride Treated Eggs:

Average microvilli lengths were computed from photomicrographs of whole mounts of 9,000 X. Five cm² surface areas from four different egg batches were selected at random. Previous studies
Figure 3-4. Scanning electron micrograph of a denuded *L. pictus* egg incubated (10 min) and inseminated (30 min) in $2 \times 10^{-6}$ M cytochalasin B and 10 mM ammonium chloride. Well developed fertilization cone surrounding a penetrating sperm. 22,000X.
on microvilli dimensions have employed similar manual measurements as well as the electronic record of a stage micrometer printed on videotape (Schroeder, 1979; Schatten and Schatten, 1980). The determination of microvilli dimensions have been relative with respect to "shrinkage" factors due to specimen preparations, surface clarity and angle of measurement.

Microvilli organization in DTT treated S. purpuratus eggs occasionally appeared fused and irregular in length and diameter (Fig. 3-5). Although variations occurred between egg batches, irregularity in length and diameter was always constant. Inseminated control egg microvilli elongated from 0.48 μm at 1 insemination time to 0.81 μm at 5 min postinsemination and 1.7 μm at 15 min postinsemination (Fig. 3-5, A-C). Due to further extension and usual overlap, measurements at 30 min postinsemination were not possible. However, microvilli appeared to extend twice their length at 15 min postinsemination (Fig. 3-5, D). Microvilli of eggs incubated 10 min and inseminated in cytochalasin B (2 x 10^{-6} M) at identical time intervals did not differ appreciably from inactivated control eggs, indicating inhibition of elongation (Fig. 3-5, E-H).

Previous work (Chapter 2) using ammonium in addition to cytochalasin B resulted in enhanced sperm incorporation. Could ammonium also be influencing microvilli structure? To explore this possibility, the effects of ammonium chloride (10 mM) on microvilli elongation were examined next. Microvilli of non-
Figure 3-5, A-H. Scanning electron micrographs of microvilli (length) of denuded S. purpuratus eggs incubated (10 min) and inseminated for 5, 15, and 30 min in 2 x 10^{-6} M cytochalasin B. A. Untreated and noninseminated control egg (0.48 μm): B. Inseminated control egg at 5 min (0.81 μm): C. Inseminated control egg at 15 min (1.7 μm): D. Inseminated control egg at 30 min: E. CB treated noninseminated control egg (0.36 μm): F. CB treated egg at 5 min postinsemination: G. CB treated egg at 15 min postinsemination: H. CB treated egg at 30 min postinsemination. Bar = 5 μm.
inseminated control eggs showed typical "inactivated" surfaces (Fig. 3-6, A-D). Microvilli length of unfertilized control eggs measured approximately 0.38 μm (Fig. 3-6, A). Microvilli of cytochalasin B treated unfertilized eggs measured 0.31 μm (Fig. 3-6, B), cytochalasin B and ammonium chloride treated 0.38 μm (Fig. 3-6, C), and ammonium chloride treated, 0.38 μm (Fig. 3-6, D). Microvilli of unfertilized DMSO treated eggs were similar to untreated controls.

At 10 min postinsemination, pronounced changes in microvilli length were observed between untreated and treated eggs (Fig. 3-6, E-H). In the untreated fertilized egg, microvilli length increased to approximately 1.14 μm (Fig. 3-6, E). This increase corresponds to the increase in length that occurred within 6 min postinsemination as seen by Longo (1979). A similar increase (1.13 μm) was also seen in ammonium chloride treated eggs (Fig. 3-6, H). However, eggs incubated in cytochalasin B and cytochalasin B and ammonium chloride (10 min) and fertilized 10 min did not differ appreciably from unfertilized treated control eggs. Microvilli length in both samples ranged between 0.35 and 0.38 μm (Fig. 3-6, F and G).

When untreated fertilized eggs are compared with cytochalasin B and cytochalasin B and ammonium chloride treated fertilized eggs, a varied effect on the time course of microvilli elongation can be seen (Fig. 3-7, A-L). At 5, 15, and 30 min postinsemination intervals, microvilli of untreated control eggs elongated from
Figure 3-6, A-H. Scanning electron micrographs of microvilli (length) of denuded *L. pictus* eggs incubated (10 min) and inseminated (10 min) in $2 \times 10^{-6}$ M cytochalasin B and 10 mM of ammonium chloride. A, untreated and non-inseminated control (0.38 µm); B, non-inseminated CB control (0.31 µm); C, CB and NH$_4$Cl noninseminated control (0.38 µm); D, noninseminated NH$_4$Cl control (0.38 µm); E, untreated inseminated control (1.14 µm); F, CB inseminated (0.35 µm); G, CB and NH$_4$Cl inseminated (0.38 µm); H, NH$_4$Cl inseminated (1.13 µm). Bar = 5 µm.
approximately 0.5 μm to well over 1.13 μm by 30 min (Fig. 3-7, A-D). Microvilli of eggs incubated and inseminated in cytochalasin B did not increase beyond their original length of 0.38 μm at 15 min (Fig. 3-7, E-G). However, at 30 min postinsemination, diameter rather than length appeared to be affected (Fig. 3-7, H). Cytochalasin B combined with ammonium chloride produced changes transient between untreated controls and cytochalasin B treated eggs (Fig. 3-7, I-L). Microvilli prior to fertilization measured approximately 0.38 μm (Fig. 3-7, I) and increased to 0.63 μm at 5 min and 15 min postinsemination (Fig. 3-7, J and K). At 30 min postinsemination, microvilli elongated to lengths intermediate between untreated and cytochalasin B treated controls (Fig. 3-7, L).

c. A Comparative EM Analysis of Microvilli Elongation in Whole Eggs and Isolated Cortices Decorated with Heavy Meromyosin:

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were utilized to compare changes at the surface and within the cortex of *L. pictus* eggs during fertilization. The entire cortex of the sea urchin egg can be isolated as a single unit from denuded eggs by using a high glycine medium (Begg et al., 1978; Begg and Rebhun, 1979).

In this study, heavy meromyosin (HMM) extracted from rabbit skeletal muscle was used as the actin probe. Extraction in 0.5 M KCl produced typical bipolar thick myosin aggregates formed in a head-to-head association when viewed by negative staining (Fig. 3-8, A). The width of the central zone of the filaments was
Figure 3-7, A-L. Scanning electron micrographs of microvilli (length) of denuded *S. purpuratus* eggs incubated (10 min) and inseminated for 0, 5, 15, and 30 min in 2 x 10^{-6} M cytochalasin B and 1 mM ammonium chloride. A. Untreated noninseminated control egg (0.5 µm): B. Inseminated control egg at 5 min: C. Inseminated control egg at 15 min: D. Inseminated control egg at 30 min (1.13 µm): E. CB treated noninseminated control egg (0.36 µm): F. CB treated egg inseminated for 5 min: G. CB treated egg inseminated for 15 min: H. CB treated egg inseminated for 30 min: I. CB and NH₄Cl treated and non-inseminated control egg (0.38 µm): J. CB and NH₄Cl treated egg inseminated for 5 min (0.63 µm): K. CB and NH₄Cl treated egg inseminated for 15 min (0.63 µm): L. CB and NH₄Cl treated egg inseminated for 30 min. Bar = 5 µm.
measured. Length could not be determined because aggregate end points could not be distinguished. The addition of egg actin in 0.5 M KCl (pH 6.8) to a solution of rabbit skeletal muscle HMM produced typical arrowhead structures (Fig. 3-8, B). Each filament showed definite polarity with respect to the alignment of repeated barbed and pointed ends.

Transmission electron microscopic observations of noninseminated control eggs closely paralleled scanning electron observations. Short microvilli were seen at the egg surface when viewed by TEM or SEM (Fig. 3-9, E-H). Intact cortical granules also were seen beneath the plasma membrane in the cortex of the egg (Fig. 3-9, A-D). However, several cytochalasin B control eggs showed a slightly elevated vitelline membrane (Fig. 3-9, B). This may represent spontaneous activation that has previously been reported in some species incubated in CB (Perry, unpublished) or a fixation artifact.

Observations on noninseminated isolated egg cortices decorated with HMM produced results compatible with both SEM and TEM observations. The plasma membrane of all control eggs contained membrane folds rather than distinct microvilli. These eggs exhibited various degrees of microfilament organization (Fig. 3-9, I-L). Untreated control egg cortices revealed a loose network of 50 to 70 Å actin filaments linearly arranged (Fig. 3-9, I). Several were seen randomly attached to the plasma membrane. Cytochalasin B treated eggs exhibited no structurally identifiable microfilaments (Fig. 3-9, J). These cortices appeared partially
Figure 3-8, A. Negative stain of rabbit skeletal muscle myosin (HMM) dissolved in 0.5 M KCl (pH 7.0) and extracted in L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK-trypsin) solution (0.5 mg/ml) containing 1 mM HCl for 5 mM at 25°C. Typical bipolar thick filament (arrow). 32,500X.

B. Negative stain of sea urchin egg actin microfilaments decorated with rabbit skeletal heavy meromyosin (HMM). Note typical arrowhead structures (arrow). 46,500X.
Figure 3-9, A-L. Comparative scanning and transmission electron micrographs of *L. pictus* unfertilized eggs incubated 10 min in $2 \times 10^{-6}$ M CB and 10 mM NH$_4$Cl. TEM observations of egg cortex: (A) unfertilized control, 9,000X; (B) CB treated, 13,000X; (C) CB and NH$_4$Cl treated, 15,500X; (D) NH$_4$Cl treated, 15,000X. SEM observations (4,500X) of microvilli: (E) unfertilized control; (F) CB treated; (G) CB and NH$_4$Cl treated; (H) NH$_4$Cl treated. Microfilaments in isolated cortices decorated with HMM: (I) unfertilized control, 22,500X; (J) CB treated, 28,600X; (K) CB and NH$_4$Cl treated, 22,900X; (L) NH$_4$Cl treated, 30,000X.
degraded with semblances of disrupted microfilaments. CB and 
NH₄Cl treated cortices produced an unorderly array of microfilament 
structures loosely arranged throughout the entire cortex (Fig.
3-9, K). Actin filaments developed, but did not form bundles. 
Ammonium chloride control cortices produced linear aggregates 
similar to those of the untreated controls (Fig. 3-9, L).

Transmission EM observations of control and "test" eggs 
fixed at 10 min postinsemination showed different stages of egg 
activation (Fig. 3-10, A-D). In all inseminated untreated eggs, 
activation was recognized as lifting of the vitelline membrane, 
exocytosis of cortical granules, and extension of microvilli at 
the plasma surface (Fig. 3-10, A). In most eggs, several micro-
villi appeared in different planes within the perivitelline space. 
The surface of cytochalasin B treated inseminated eggs appeared 
distinctly different (Fig. 3-10, B). In all eggs observed, 
 microvilli were shorter and cortical granules remained intact 
within the cortex. Several cytochalasin and ammonium chloride 
treated eggs appeared activated (Fig. 3-10, C). Vitelline 
membranes had lifted and cortical granules were absent. Eggs 
incubated and inseminated in ammonium chloride resembled the 
activated state of untreated controls. Comparative surface views 
can be seen in SEM micrographs (Fig. 3-10, E-H).

Cortices isolated and decorated with HMM 10 min post-
insemination differed according to different incubation media 
(Fig. 3-10, I-L). With the exception of cytochalasin B treated
Figure 3-10, A-L. Comparative scanning and transmission electron micrographs of *L. pictus* zygotes incubated 10 min in $2 \times 10^{-6}$ M CB and 10 mM NH$_4$Cl and fixed at 10 min postinsemination. TEM observations of egg cortex: (A) untreated control, 11,200X; (B) CB treated, 4,000X; (C) CB and NH$_4$Cl treated, 8,700X; (D) NH$_4$Cl treated, 13,000X. SEM observations (4,500X) of microvilli: (E) untreated control; (F) CB treated; (G) CB and NH$_4$Cl treated; (H) NH$_4$Cl treated. Microfilaments in isolated cortices decorated with HMM: (I) untreated control, 23,600X; (J) CB treated, 41,700X; (K) CB and NH$_4$Cl, 27,000X; (L) NH$_4$Cl treated, 25,000X.
samples, elongated microvilli were seen projecting from the egg surface. Microfilament bundles could be seen in each microvillus core. Organized microfilament bundles appeared within the cortex and also attached to the plasma membrane. None appeared attached to the tip of microvilli. Within the cortex of cytochalasin B treated eggs, microfilament development was minimal (Fig. 3-10, J). In some areas, these filaments were short and disrupted. Some organization was seen in those that appear to extend from the plasma membrane.
DISCUSSION

The effects of cytochalasin B on the fertilization process were consistently observed. It is apparent that CB exerts its effects by influencing the contractile system of the egg. The structural organization of microvilli and microfilaments during fertilization in sea urchin eggs is actin mediated and dependent. The results presented demonstrate that actin is associated with the cortex in a nonfilamentous form which at fertilization can be induced to polymerize. Cytochalasin B inhibits this response, but the presence of ammonium chloride at longer incubation times reduces this inhibition.

Sperm entry observed in *L. pictus* was associated with well developed fertilization cones. Undirectionally polarized actin filaments decorated with myosin subfragment 1 in both fertilization cones and microvilli have been previously described (Burgess and Schroeder, 1977; Begg et al., 1978; Begg and Rebhun, 1979; Tilney and Jaffe, 1980; Carron and Longo, 1982). In this study, fertilization cones were not observed in any species treated with CB or in *S. purpuratus* control eggs. Schatten and Schatten (1980) report that eggs treated with CB fail to form a cone at insemination and quickly resorb the cone when CB is added after insemination.

Microvilli extension, increasing with time (0.5 μm before fertilization to +1.7 μm at 30 min of insemination) in fertilized CB plus NH₄Cl controls (0.40 μm) were intermediate between the
length of the unfertilized and untreated control eggs (0.50 μm) and those of the unfertilized CB treated controls (0.36 μm). At 15 min after insemination, CB and NH₄Cl eggs showed unequal, but elongated (0.63 μm) microvilli. Comparatively, isolated cortices decorated with HMM also exhibited several organized HMM complexes in the cortex and within developing microvilli. In view of the difference in the effect of CB alone and the effect of CB in the presence of NH₄Cl, a consideration of how NH₄Cl may be influencing these differences is given.

Acid-efflux data reported by Tilney and Jaffe (1980) appeared to be consistent with other investigations (Tilney et al., 1978; Begg and Rebhun, 1979), which indicate that a change in internal pH may be the regulating factor; the greatest change occurring at the same time (0.5 to 2.5 min) as actin filaments first appear in the cortical cytoplasm. However, other ions such as Ca++ also have been suggested as regulators of actin assembly (Tilney, 1976; Spudich and Spudich, 1979; Carron and Longo, 1982).

If Ca++ and pH are considered to be the in vivo components that control microvilli and microfilament organization, then the CB and NH₄Cl responses can be interpreted accordingly. Actin organization in the sea urchin egg may be a two-step process. At fertilization, increase in intracellular Ca++ may be the prerequisite for initiating actin organization in the cortex and microvilli, and subsequent pH increases may be required for reorganization of actin into bundles of microvilli core filaments (Begg et al., 1982). Therefore, in the absence of egg activation,
neither CB nor NH$_4$Cl should induce microvilli elongation or actin polymerization, as is shown in this study. However, enhanced levels of sperm-egg fusions in the presence of a CB and NH$_4$Cl mixture may induce actin organization through the release of enough Ca$^{++}$ to initiate actin organization.

At this point, only morphological descriptions of the effect of CB and NH$_4$Cl on this system have been presented. To correlate the structural effect of these agents with the functional aspect of the protein controlling these events, I analyzed relative actin concentrations under identical experimental conditions.
CHAPTER 4

THE DETERMINATION OF G-ACTIN AND F-ACTIN POOLS
BY DNAase I INHIBITION IN CYTOCHALASIN B AND
AMMONIUM CHLORIDE TREATED EGG EXTRACTS
INTRODUCTION

Actin molecules with highly conserved amino acid sequences appear to be ubiquitous in most cells. Isolated, actin is a monomeric protein of approximately 42,000 Daltons containing one mole of bound nucleotide/mole (ATP) and one mole of divalent cation/mole (Ca++ or Mg++). The globular form (G-actin) polymerizes by a condensation type mechanism. Under conditions that promote polymerization (> ionic strength), G-actin nucleation centers form slowly and elongate into double-stranded helical filaments of F-actin. In the process, ATP is hydrolyzed to ADP with each subunit containing tightly bound ADP (Oosawa and Asakura, 1975; Flanagan and Lin, 1980; Korn, 1981). Following polymerization, stability of actin microfilaments is regulated through crosslinking by other proteins.

Microfilament organization in nonmuscle cells is based on ionic controlled states of actin polymerization and depolymerization. Actin has been extracted and purified from several sea urchin species (Hatano et al., 1969; Kane, 1975; Begg and Rebhun, 1978; Vacquier and Moy, 1979). In *L. pictus* unfertilized eggs, actin represents approximately 12 to 27% of the total cortical protein and in zygotes isolated 40 min after insemination, 50 to 65% (Vacquier and Moy, 1980). Actin polymerization to form microfilaments during fertilization in sea urchins as shown by the data presented in Chapter 3, is inhibited by cytochalasin B.
Therefore, in this study, the correlation between the action of cytochalasin B and actin polymerization has been limited to electron microscopic observations. The deoxyribonuclease 1 (DNAase 1) inhibition assay which determine polymerized and unpolymerized actin pools in cell extracts provided a rapid method for correlating CB action and actin polymerization.

In muscle cells, G-actin binds rapidly to DNAase 1 forming a stable 1:1 complex. The polymerization of actin is blocked and the activity toward deoxyribonucleic acid (DNA) is inhibited (Lindberg, 1967; Lazarides and Lindberg, 1974; Hitchcock et al., 1976). DNAase 1 also complexes with filamentous action (F-actin) but at a much slower rate, shifting the equilibrium between F- and G-actin states. This difference in binding rates serves as a basis for the selectivity of this assay. The mechanism of actin depolymerization by DNAase is unknown. DNAase inhibition activity has been described in extracts of human platelets, lymphocytes and HeLa cells (Blinstad et al., 1978). In this Chapter, polymerized and unpolymerized actin states during the normal fertilization process as well as in treated eggs were examined.
MATERIALS AND METHODS

Collection and preparation of *S. purpuratus* gametes followed the procedures described in Chapter 2, Materials and Methods.

a. Deoxyribonucleic Acid Substrate and Deoxyribonuclease I Preparations:

Deoxyribonucleic acid (DNA) from calf thymus (type I: Sigma) and noncrystalline deoxyribonuclease I (DNAase I) from beef pancreas (DN 100: Sigma) were prepared according to the method of Blikstad et al., (1978). DNA fibers were cut into fine pieces and dissolved in 0.1 M Tris-HCl (pH 7.5), 4 mM MgSO₄, and 1.8 mM CaCl₂ (40 μg/ml). The mixture was slowly stirred at room temperature for 48 hr, millipore filtered and stored at 4°C until used. DNAase I was dissolved at 0.1 mg/ml in 50 mM Tris-HCl (pH 7.5), 0.01 phenylmethyl-fulfonyl fluoride (PMSF) and 0.25 mM CaCl₂. The enzyme solution was prepared fresh, maintained at 4°C and used within 8 hr of preparation.

b. Determination of Deoxyribonuclease I Activity:

In the DNAase I assay, 1.25 ml (50 μg) of the DNA substrate were mixed with increasing concentrations of the DNAase I enzyme solution and resulting hydrolysis of DNA measured by observing its hyperchromicity at 260 nm on a Beckman 35 UV-Visible spectrophotometer. The slope of the linear part of the absorbance increased relative to the amount of enzyme solution added. Complete
digestion of 50 μg DNA was obtained with 0.80 μl of DNAase solution containing 8.0 μg of enzyme.

c. Determination of Deoxyribonuclease 1 Inhibition in Isolated Cortical Proteins:

Preparation of egg cortices for actin isolation followed the procedure described in Materials and Methods (Chapter 3). Isolated cortices were diluted 4-fold with 0.6 M KCl containing 0.01 M PIPES (pH 6.8) for 30 min. The mixture was centrifuged for 20 min at 40 g. Solubilized egg actin in the supernatant versus total cortical protein composition was determined by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Actin represented approximately 46% of the proteins contained in the supernatant.

For determination of inhibitor (actin) activity, a standard amount of DNAase 1 (40 μl containing 1 mg of enzyme) was mixed with 0 - 200 μl of cortical protein and 1.25 ml (50 μg) of the DNA substrate. The interval between mixing and substrate addition was kept below 5 sec. This minimizes the contribution of F-actin to DNAase 1 inhibition (see Discussion). The standard amount of DNAase 1 used represented the activity that produced 50% digestion of DNA. The decrease in DNAase 1 activity in the control sample was directly proportional to the amount of cortical protein added (independent of dilution).

Total cortical protein (mg/ml) was measured by the Bio-Rad Protein Standard 1. Assay (Bradford, 1976), using bovine serum
albumin standard. Total G-actin concentrations in each sample were determined by UV absorption at 260 nm after a 1:1 dilution of cortical suspensions with a depolymerization buffer containing 1.5 M guanidine HCl, 1 M sodium acetate, 1 mM CaCl₂, 1 mM ATP, 20 mM Tris-HCl, pH 7.5, and incubation for at least 5 min at 4°C with frequent agitation.

d. Polyacrylamide Gel Electrophoresis of Protein Preparations:

Soluble extracts were incubated at 70°C for 15 min in a small amount of sample buffer containing 5% SDS, 50% sucrose, 0.5 M Tris-HCl (pH 8.0), 5 mM EDTA, 200 mM DTT and 25 µg/ml pyronin Y. Ten percent polyacrylamide running gels containing 0.1% SDS were used (Laemmli, 1970). Approximately 25 µg of sample protein were loaded in each well; adjacent wells contained 25 µg of a mixture of polypeptide molecular weight standards ranging from 200K to 45K. Actin showed a molecular weight of 42K by comparison to standard. Gels were stained for protein with Coomassie blue (Weber et al., 1972), destained in 0.1% glacial acetic and relative protein concentrations determined by densitometric scanning.

e. Experimental Design:

Three different S. purpuratus egg batches were prepared for the DNAase 1 inhibition assay and SDS-PAGE by the procedures described in Chapter 3 (Materials and Methods). Cytochalasin B and ammonium chloride treated fertilized egg protein samples were assayed at 5, 10, 15, and 30 min intervals. Controls
consisted of unfertilized eggs, unfertilized eggs incubated in CB, a mixture of CB and NH$_4$Cl, and NH$_4$Cl for 10 min and untreated and ammonium treated fertilized eggs at the above sample time intervals and respective concentrations. Each control and test sample (5 to 200 µl cortical protein) was analyzed for DNAase inhibition by using the determined standard amount (4.0 µg) of the DNA enzyme that resulted in 50% inhibition. Maximum levels of DNAase activity was determined for each sample and absorbance calculated based on 2.0 µg/ml of cortical proteins. A two-point incubation time assay was used for all samples measured; immediately after mixing the reactants and periodically for 1 min. Total G-actin concentrations for each sample's corresponding volume was determined by a 1:1 dilution of the test sample with the depolymerization buffer.
RESULTS

a. The Effects of CB and NH$_4$Cl on DNA Hydrolysis in Isolated Cortices:

Morphological changes in F-actin microfilament development can be related to G-actin inhibition of DNAase hydrolysis of DNA. Upon hydrolysis, DNA's optical absorbance at 260 nm increases. Bovine pancreatic deoxyribonuclease cleaves DNA linkages between purine and pyrimidine bases, releasing free 3'-OH groups and 5'-phosphate groups. Inhibition of hydrolysis results from DNAase's ability to bind rapidly with G-actin. This affinity for G-actin in a cellular system is specific and does not appear to involve competitive control mechanisms.

In this study, DNA hydrolysis by DNAase 1 proceeded relative to G-actin contents in 2.0 µg/ml of proteins in extracts of isolated egg cortices. DNA equivalents paralleled G- and F-actin concentrations before and after insemination (Table 4-1).

At insemination, DNA equivalents of all control samples ranged between 10.40 ± 2.0 to 12.00 ± 3.1 µg. DNA hydrolysis in untreated fertilized controls decreased within 5 min after insemination. DNA levels increased from 12.0 ± 3.1 µg at insemination to 14.0 ± 4.0 µg 5 min after insemination. However, at 10 min postinsemination, levels fell from 12.80 ± 1.8 µg to 11.86 ± 6.0 µg at 15 min. The large difference in standard error recorded at 5 min (±4.0) and at 15 min (±6.0) is probably reflective of the low sampling used for this study.
DNA levels observed in CB treated eggs were consistently lower than untreated controls at all tested time intervals. However, levels decreased by 1.2 units 5 min after insemination, returned to control levels at 10 min and remained relatively constant for 30 min. The fluctuations observed at 5 min are probably insignificant. The return to constant levels at 10 min reflects the expected consistency not observed in untreated control eggs.

Increased DNA equivalents in CB and NH₄Cl samples compared to the increased in DNA levels observed in the untreated control samples. Concentrations measured 10.40 ± 2.0 μg before fertilization and peaked to 15.20 ± 3.7 μg 15 min after fertilization. At 30 min after insemination, DNA levels fell to 11.20 ± 8.2 μg. Comparative DNA equivalents in CB samples and samples treated with a combined mixture of CB and NH₄Cl did not change significantly until 10 min after insemination. CB levels remained constant, but CB and NH₄Cl levels increased and then declined at 30 min postinsemination. DNA concentrations in ammonium chloride samples reached a maximum at 10 min postinsemination, slightly exceeding twice the concentration of the unfertilized control.

With the exception of ammonium treated samples, all DNA levels paralleled expected G-actin concentrations in the presence and absence of cytochalasin B during fertilization in this system. In vivo synthesis of DNA did not appear to affect equivalents measured in these samples. In normal controls, DNA is not appreciably increased until pronuclei fusion (Matsumoto and Piko, 1971; Longo and Plunkett, 1973).
Table 4-1. Relative DNA equivalents in extracts of *S. purpuratus* eggs incubated (10 min) and fertilized in $2 \times 10^{-6}$ M cytochalasin B and 1 mM ammonium chloride. DNA quantities were determined by incubation of 2.0 μg/ml of egg cortical proteins in the presence of 4.0 μg of DNAase 1 and 50 μg of DNA.

<table>
<thead>
<tr>
<th>Insemination Time (min)</th>
<th>DNA EQUIVALENTS (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated Control</td>
</tr>
<tr>
<td>0</td>
<td>12.00 ± 3.1</td>
</tr>
<tr>
<td>5</td>
<td>14.00 ± 4.0</td>
</tr>
<tr>
<td>10</td>
<td>12.80 ± 1.8</td>
</tr>
<tr>
<td>15</td>
<td>11.86 ± 6.0</td>
</tr>
<tr>
<td>30</td>
<td>18.40 ± 2.5</td>
</tr>
</tbody>
</table>
b. The Effects of CB and NH₄Cl on Percent Inhibition of DNAase 1 by G-actin:

Percent DNAase inhibition was equated to percent of total actin measured as G-actin in each protein sample as determined by utilization of a guanidine-HCl depolymerization buffer. The optical density indicative of DNA/DNAase activity under both depolymerized and nondepolymerized conditions was compared (Fig. 4-1). In untreated fertilized controls, a marked decrease from 99 to 30% inhibition was observed at 5 min postinsemination, a decrease at 15 min to 7%, and maximum increase to 16% at 30 min. Inhibition at 5 min represented the lowest percent inhibition observed in all samples tested. Percent inhibition in NH₄Cl samples also declined from approximately 97% to 10 and 12%, respectively by 5 and 10 min postinsemination. At 30 min, an increase to 27% was observed.

Within 30 min of insemination, cytochalasin B treated samples exhibited maximal percent inhibition (97%). However, absorbance fluctuations that were observed during DNA hydrolysis can also be seen here between 5 and 15 postinsemination. Inhibition in the presence of CB did not fall below 85% at any time interval tested. Cytochalasin B and ammonium chloride treated samples revealed maximum inhibition at 10 min postinsemination (92%) and decreased to 61% by 30 min.
Figure 4-1. Percent inhibition of DNAase 1 by G-actin in
*S. purpuratus* egg extracts. Abscissa: insemination
time (min) of cytochalasin B (2 x 10⁻⁶ M) and
ammonium chloride (1 mM) treated eggs. Ordinate:
percent inhibition (total actin measured as G-actin)
= sample G-actin ÷ total sample G-actin. Optical
density at 260 nm. △, Untreated controls; ●, CB
treated; ○, CB and NH₄Cl treated; ▲, NH₄Cl treated.
Error bars indicate SEM for 3 different egg batches.
c. SDS-PAGE of Cortical Proteins Isolated from CB and NH₄Cl Treated Eggs:

Electrophoresis of sea urchin egg extracts reveal that actin is a major component of cortical proteins. Incubation of isolated cortices in 0.6 M KCl and centrifugation at 40 g in this study failed to separate actin from other cortical proteins. However, the results reported here indicate a differential pattern of relative actin concentrations in the supernatant of unfertilized and fertilized eggs. Comparative data have been provided from earlier studies on electrophoretic scans of the affect of cytochalasin B and D on actin concentrations in cell lysates. The amount of actin associated with preincubation of lysates of thrombin-stimulated platelets in cytochalasin D resulted in dramatic decreases in their actin concentrations in centrifuged pellets (Casella et al., 1981). Vacquier and Moy (1980) also demonstrated that when sea urchin cortices are resuspended in an isolation medium containing cytochalasin B, most of the actin protein becomes soluble in the supernatant at 40 g.

Electrophoretic analysis of relative cortical actin contents in this study confirms its differential concentrations in the cortex of fertilized and unfertilized eggs (Fig. 4-2). Relative actin amounts were determined by measuring the length of each scan peak. In the unfertilized egg, actin represented approximately 46% of the proteins isolated. However, when compared to the fertilized state, actin occurred in relatively small amounts. At 15 min of fertilization in untreated controls, actin amounts
Figure 4-2. Densiometric scans of the protein components of unfertilized and fertilized *S. purpuratus* egg cortices treated with $2 \times 10^{-6}$ M cytochalasin B and 1 mM ammonium chloride. Arrow marks the 42K action band of supernatant. A, untreated control; B, untreated control at 15 min postinsemination; C, untreated control at 30 min postinsemination; D, CB treated (10 min) control; E, CB treated at 15 min postinsemination; F, CB treated at 30 min postinsemination; G, CB and NH$_4$Cl treated (10 min) control; H, CB and NH$_4$Cl treated at 15 min postinsemination; I, CB and NH$_4$Cl treated at 30 min postinsemination; J, NH$_4$Cl treated (10 min) control; K, NH$_4$Cl treated at 15 min postinsemination; L, NH$_4$Cl treated at 30 min postinsemination. +, positive pole; -, negative pole.
increased by approximately 3.6X that of the unfertilized controls and approximately 4.4X at 30 min postinsemination. A marked difference can be seen in cortices of CB treated fertilized and unfertilized eggs. Actin in these cortices appeared to be relatively constant. No significant change could be seen in either fertilized or unfertilized eggs. Actin amounts in cytochalasin B and ammonium chloride treated fertilized eggs did not reveal the increase pattern that was observed in either the untreated fertilized controls nor the CB fertilized eggs. At 15 min, a 4-fold increase in actin was observed. By 30 min, the amount decreased by approximately 50%. A more stable pattern was observed in CB and NH₄Cl samples. At 15 min postinsemination, actin amounts increased by 50% and did not change beyond twice its original amount.
DISCUSSION

DNAase 1 inhibition and electrophoretic analysis of actin contents in isolated egg cortices confirmed, in part, results obtained through electron microscopic observations. This study showed that actin comprised a relatively large amount of the detectable proteins in the unfertilized egg cortex. In the unfertilized cortex, actin is in an unpolymerized form (G-actin). Actin stability following fertilization involves polymerization of G-actin to F-actin. Such a change appears to be required for the elongation of surface microvilli that was observed in Chapter 3.

Presently, it is not clear what regulates the polymerization of actin in the cell, but Ca++ and pH are suggested (Tilney, 1976; Spudich and Spudich, 1979; Carron and Longo, 1982). However, pH alone through the use of ammonium chloride in this study failed to induce high levels of F-actin in the unfertilized egg. This study also confirms microscopic observations of the affect of cytochalasin B on contractile proteins in this system. The mechanism of CB action on inhibition of microfilament development appears to be directly correlated to its induction and maintenance of high G-actin pools (Brown and Spudich, 1979; MacLean-Fletcher et al., 1980, Korn, 1981).

Comparative G- and F-actin pools in unfertilized and fertilized eggs shown by relative DNA equivalents and percent DNA inhibition are significantly different. Nonpolymerized actin in the
unfertilized egg in all controls was demonstrated by relatively low DNA concentrations. G-actin monomers are unnucleated and free to bind rapidly to introduced DNAase I, resulting in less DNA hydrolysis. Electrophoretic scans showed comparatively low actin levels in the unfertilized egg.

The DNAase I analysis also supports the stimulation of F-actin polymerization at fertilization, which resulted in low G-actin levels. DNA levels in untreated eggs increased with increased F-actin concentrations, reaching maximum concentrations at 30 min postinsemination. DNAase inhibition by G-actin decreased parallel reduced G-actin pools. Therefore, as absorbance increased, percent inhibition (total actin measured as G-actin) decreased. Electrophoretic gel scans also show that cortical egg actin increased at fertilization and continued to increase for 30 min. Similar increases were also reported by Begg and Rebhun (1979) and Vacquire and Moy (1980). This suggests that in addition to cortical G-actin, cytoplasmic actin may also participate in the formation of filaments following fertilization.

Cytochalasin B treated eggs produced contrasting results. Percent inhibition increased rather than decreased, indicating high G-actin levels. These results were consistent with data describing cytochalasin D reversal of thrombin induced actin polymerization in human platelet extracts (Casella et al., 1981). DNA hydrolysis would be inhibited by the enzyme binding to G-actin monomers. While G-actin levels fluctuated between 5 and 15
min postinsemination, at 30 min, G-actin levels approximated those of the unfertilized controls. Further support to this pattern was seen in CB treated gel scans. At 15 min postinsemination, actin levels increased approximately 23% and remained elevated at 28% at 30 min. If actin levels increase as a result of an increase in cytoplasmic actin following fertilization, then CB's inhibition on the fertilization response would be expected to inhibit this increase.

DNA hydrolysis in CB and NH₄Cl treated eggs were more typical of the CB response; high percent DNAase inhibition and relatively low hydrolysis. The increase in absorbance that was observed at fertilization could suggest either an abnormal increase in cytoplasmic actin or increased F-actin levels as a result of the pH effect of ammonium chloride. Electrophoretic scans demonstrate this increase to be approximately 50% within 30 min of fertilization. The return to low absorbance at 30 min which approximated the controls, could result from a reduced affect of ammonium chloride, which would increase the inhibition affect of cytochalasin B on actin polymerization.
SUMMARY

The mechanism of sperm incorporation and the organization of microvilli and microfilaments in sea urchin eggs were examined through the use of cytochalasin B and ammonium chloride.

The following conclusions can be made:

1. In contrast to previously published reports, high concentrations (2 x 10^{-6} M) of cytochalasin B do not completely inhibit sperm incorporation.

2. Cytochalasin B's block to the normal time of sperm incorporation, rotation, and migration increases with longer and continuous incubations.

3. Contractile processes in the egg cortex appear to be the major mechanism for sperm incorporation. Microvilli extension and microfilament assembly is dose dependent and seems to be associated with the time of successful sperm-egg fusion and sperm migration in the cortex.

4. Successful sperm-egg fusion in the presence of cytochalasin is dependent upon its interaction with the microfilament system in the cell. Microfilament formation and hence microvilli organization are inhibited in the presence of this drug.

5. Sperm incorporation in the presence of cytochalasin B (30%) and in the absence of an organized microfilament system is suggestive of an alternate mechanism for incorporation.
6. Ammonium chloride in the presence of cytochalasin B increases sperm incorporation, sperm migration, and polyspermy; in addition, microvilli and microfilament organization in inseminated eggs is initiated. The ammonium chloride effect on these responses can not be explained at this time.

7. Cytochalasin B and combined cytochalasin B and ammonium chloride inhibited cortical exocytosis, which normally occurs immediately after insemination.

8. One effect of cytochalasin B inhibition on the fertilization process is the maintenance of high G-actin pools. G-actin is a major component of the cortical proteins in the unfertilized egg. At fertilization, G-actin polymerizes to form F-actin bundles.
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VITA

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EXAMINATION AND THESIS REPORT

Candidate: Beverly Dixon Wade

Major Field: Zoology

Title of Thesis: The mechanism of sperm incorporation and an analysis of microvilli and microfilament organization in sea urchin eggs examined through the use of cytochalasin B and ammonium chloride.

Approved:

[Signature]
Major Professor and Chairman

[Signature]
Dean of the Graduate School

EXAMINING COMMITTEE:

[Signature]

[Signature]

[Signature]

[Signature]

Date of Examination: June 25, 1982