

# IDENTIFICATION OF A SPERM RECEPTOR ON THE SURFACE OF THE EGGS OF THE SEA URCHIN *ARBACIA PUNCTULATA*

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## ABSTRACT

The possibility that the surface of the egg of the sea urchin *Arbacia punctulata* contains a species-specific receptor for sperm has been investigated. The extent of fertilization of eggs of *A. punctulata*, which is proportional to the number of sperm, is unaffected by the presence of either eggs or membranes prepared from eggs of *Strongylocentrotus purpuratus*. In marked contrast, membranes prepared from eggs of *A. punctulata* quantitatively inhibit fertilization of *A. punctulata* eggs by *A. punctulata* sperm. Several lines of evidence indicate that this inhibition is due to the presence of a membrane-associated glycoprotein that binds to the sperm, thus preventing them from interacting with receptor on the surface of the eggs. First, eggs treated with trypsin are incapable of being fertilized, although they can be activated with the  $\text{Ca}^{2+}$  ionophore A23187. Moreover, membranes prepared from eggs pretreated with trypsin do not inhibit fertilization of eggs. Second, receptor isolated in soluble form from surface membranes binds to sperm and thus prevents them from fertilizing eggs; the inhibition by soluble receptor is species-specific. Third, the soluble receptor binds to concanavalin A-Sepharose. Fourth, eggs are incapable of being fertilized if they are pretreated with concanavalin A. The specificity of inhibition, and the affect of trypsin and concanavalin A on intact eggs, suggest that the receptor is a species-specific macromolecule located on the surface of the eggs. The sensitivity of the receptor to trypsin, and its ability to bind to concanavalin A, indicate that it is a glycoprotein.

The process of fertilization is a complex sequence of events which involves the species-specific fusion of the plasma membranes of the sperm and the egg. It seems likely that the initial interactions between the sperm and egg cell are mediated by surface receptors which facilitate sperm-egg recognition and binding. Indeed, Vacquier and Payne (46) have suggested that the saturation kinetics of sperm-egg binding provide evidence for the existence of distinct sperm-binding sites on the egg cell surface. Furthermore, Epel and co-workers (9,

43-45) have presented evidence that these sperm-binding sites are hydrolyzed from the egg cell surface by a protease that is released from the egg upon fertilization.

Although the morphological events involved in sperm-egg fusion have been studied in great detail (12, 14), little is known about the molecular aspects of this process. For many years it was assumed that the jelly coat, or fertilizin, functioned as the sperm receptor during sea urchin fertilization (23). However, several investigators have

shown that the interactions between jelly coat and sperm are not necessarily species-specific (11, 39, 41); thus jelly coat is not the egg surface component responsible for species-specific sperm-egg binding. Aketa and co-workers (1-6, 29, 40) have studied a substance associated with the eggs of several species of sea urchins that is believed to be a receptor for sperm. The results of these studies are difficult to interpret since the assay for sperm binding is at best qualitative. Moreover, many of the experiments were performed with antibodies prepared against receptor substance of unknown purity (cf., Discussion).

This study deals with an attempt to characterize the cell surface component of *Arbacia punctulata* eggs responsible for sperm-egg recognition and binding in fertilization, i.e., the sperm receptor. In approaching this problem the following assumptions have been made about the properties expected of a sperm receptor: (a) the receptor should be a surface component of the egg cell, localized on either the plasma membrane or vitelline layer, but distinctly different than jelly coat; (b) isolated receptor should inhibit fertilization of eggs by binding to sperm cells, thus effectively lowering the titer of sperm cells available for fertilization; and, (c) the processes by which the receptor inhibits fertilization and binds to sperm should be species-specific. Some aspects of this work have been previously reported (33).

## MATERIALS AND METHODS

### Isolation of Gametes

*A. punctulata* were obtained from the following sources: Connecticut Valley Marine Biological Supply Co., Southampton, Mass.; Mr. Norris Hill, Beaufort, N. C.; Gulf Specimen Co., Inc. Panama, Fla.; and Florida Marine Specimen Co., Inc., Panama City, Fla. *Strongylocentrotus purpuratus* were obtained from Pacific Bio-marine Supply Company, Venice, Calif. *A. punctulata* were maintained at 18-19°C, and *S. purpuratus* at 4°C, in aquaria containing commercial artificial seawater (Instant Ocean, Aquarium Systems Co., Eastlake, Ohio). *A. punctulata* eggs and sperm were collected by electrical shock as previously described (18), and *S. purpuratus* gametes were collected by injection of 0.5-1.0 ml of 0.5 M KCl through the peristomal membrane of the body cavity. Eggs were dejellied for all experiments by acid treatment as previously described (32). By this procedure, maximal release of fucose-containing jelly coat is accomplished. Moreover, by histochemical staining with Janus Green B it was found that no jelly coat was evident on the dejellied eggs. All experiments were performed in aquarium water to which penicillin G (35 µg/ml) and

streptomycin sulfate (50 µg/ml) were added after the water was passed through a 0.22-µm cutoff Millipore filter (Millipore Corp., Bedford, Mass.). All experiments were performed with *A. punctulata* unless otherwise noted in the text.

### Fertilization Curves and Inhibition Assays

A fertilization curve was determined for both *A. punctulata* and *S. purpuratus* eggs as follows: varying amounts of sperm (0.2-20 µg protein; 10<sup>4</sup>-10<sup>8</sup> sperm) were added to test tubes containing 0.5 ml of a 1% suspension of eggs (approximately 0.25 mg protein, 5-6 × 10<sup>3</sup> eggs). After 5 min the eggs were washed to remove excess sperm, and examined with phase-contrast microscope. The percentage of fertilized eggs was determined by counting the number of cells with and without fertilization envelopes. In all experiments, greater than 200 cells were counted from each sample. Fertilization curves and inhibition assays (see below) were performed in groups of four to six tubes at a time. The variation between duplicate tubes was ±5%.

To quantitatively determine the effect of various components on sea urchin fertilization, inhibition assays were performed. A particular component was introduced to a 1% suspension of unfertilized eggs (approximately 0.5 mg protein/ml) in a final volume of 0.5 ml. Then a quantity of sperm sufficient to result in 60-70% fertilization of untreated or control eggs was added. After 5 min the eggs were washed, and the percentage of fertilized cells was assessed. The percent inhibition of fertilization was determined by the following formula:  $I = \Delta F/F \times 100$ , where  $I$  is the percent inhibition of fertilization,  $F$  is the control level of fertilization, and  $\Delta F$  is the numerical difference between the control level of fertilization and that in the presence of the component being tested. The following materials were tested for their effect on fertilization: heterologous egg cells; membranes prepared from ghosts of untreated and trypsin-treated eggs; jelly coat; soluble sperm receptor; and various lectins. Pretreatment of sperm with concanavalin A was performed as follows: sperm (4 mg protein/ml) were incubated in the presence or absence of concanavalin A (0.5 mg/ml). After 5 min, fertilization curves were obtained with both concanavalin A-treated and untreated sperm. Since the sperm had to be diluted extensively for the bioassay, the amount of concanavalin A introduced (<0.5 µg) was insufficient to cause inhibition by binding to the eggs.

### Preparation of Membrane-Bound and Soluble Receptor from Eggs and Trypsin-Treated Eggs

Eggs (4 mg protein/ml) were incubated at room temperature in the presence or absence of trypsin (100 µg/ml). After 60 min the cells were washed several times with fresh seawater. Ghosts were prepared from eggs

and trypsin-treated eggs as follows: cells (approximately 64 mg protein) were collected by hand centrifugation. The packed cells were lysed by suspension in 40 ml of distilled water followed by centrifugation for 10 min at 1,000 g. The resulting pellet, which had the appearance of empty sacks or "ghosts," similar to those earlier described (8), was washed with 40 ml of distilled water and centrifuged again for 10 min at 1,000 g for *A. punctulata* cells, and at 36,000 g for *S. purpuratus* cells. *S. purpuratus* ghosts form small vesicles when resuspended, and were therefore collected at higher centrifugal force. The pellets were resuspended in fresh seawater to a final volume of 4 ml and homogenized on ice by 10 passes of a ground glass homogenizer. The resulting ghost-derived membranes were dialyzed overnight against 1 liter of seawater at 4°C. Soluble receptor fraction was prepared from dialyzed membranes by centrifuging the membrane suspension at 100,000 g for 1 h at 0–5°C in a Spinco ultracentrifuge (Spinco Div., Palo Alto, Calif.). The water-clear supernatant material, termed the "soluble receptor fraction," was removed, and the pellet was resuspended in 4 ml of seawater. Both fractions were then assayed for receptor activity (i.e., their inhibitory activity in the fertilization assay). The specific inhibitory activity of the ghost-derived membranes varied between individual preparations. Usually the variations were no more than twofold, but occasionally variations as great as five- to eightfold were observed. These variations appeared to be related to the degree of ripeness of the animals.

#### Miscellaneous Materials and Methods

Concanavalin A, succinyl-concanavalin A, and wheat germ agglutinin were generous gifts of Dr. R. J. Mannino and Dr. M. Burger of the Biozentrum, Basel, Switzerland. Concanavalin A-Sepharose was purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. Trypsin was obtained from Grand Island Biological Co., Grand Island, N. Y. Proteins were determined by the method of Lowry et al. (24).

## RESULTS

### Fertilization Curve

When a constant number of *A. punctulata* or *S. purpuratus* eggs is treated with varying amounts of homologous sperm, and the percent of fertilized cells is subsequently assessed, a fertilization curve is obtained (Fig. 1). Although occasionally curves with a sigmoidal shape caused by low fertilization at low levels of sperm were observed, usually the curve was such that the percentage fertilization was directly proportional to the amount of sperm added.

Experiments with heterologous gametes indi-

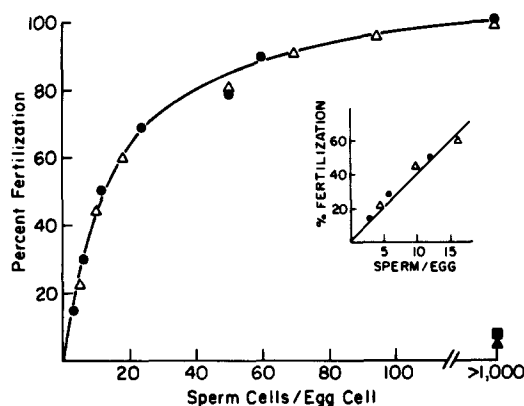


FIGURE 1 Fertilization curves of *A. punctulata* and *S. purpuratus*. Varying amounts of sperm cells ( $0.2\text{--}20\ \mu\text{g}$  sperm protein,  $10^4\text{--}10^6$  sperm) were added to test tubes containing a constant amount of eggs (0.25 mg egg protein, 5,000 eggs) in 0.5 ml of seawater. After 5 min the eggs were pelleted by hand centrifugation and resuspended in 0.1 ml of fresh seawater. In this and all other experiments the percentage of fertilized eggs was quantified as described in Materials and Methods by counting at least 200 cells. As shown, experiments were performed with sperm both homologous and heterologous to the eggs of each species. The inset shows more clearly that percent fertilization is linear, at least to the 50% level, for both species of sea urchins. *A. punctulata* (●); *S. purpuratus* (△); *A. punctulata* sperm X *S. purpuratus* eggs (■); *S. purpuratus* sperm X *A. punctulata* eggs (▲).

cate that fertilization of *A. punctulata* and *S. purpuratus* eggs is a species-specific process. Fertilization of *A. punctulata* eggs by *S. purpuratus* sperm or *S. purpuratus* eggs by *A. punctulata* sperm is not observed at titers of sperm sufficient to obtain 100% fertilization with homologous gametes. Even at titers of sperm 10–100 times higher, less than 10% cross-fertilization is observed (Fig. 1).

Although the results in Fig. 1 show that *A. punctulata* and *S. purpuratus* gametes cannot cross-fertilize, it was of interest to determine whether the eggs of *S. purpuratus* interact with the sperm of *A. punctulata* so as to impair their ability to fertilize homologous eggs. When *A. punctulata* eggs are fertilized in the presence of varying amounts of *S. purpuratus* eggs, ranging from an equal number to a 20-fold excess, no effect on the extent of fertilization of the *A. punctulata* eggs is observed. Thus, not only is there no cross-fertilization between these two species of sea urchins, but there also appears to be no irreversible interaction between the heterologous sperm and egg cells.

### Trypsin Treatment of Intact *A. punctulata* Eggs

It is well known that brief treatment of *A. punctulata* eggs with trypsin prevents elevation of the fertilization envelope (42) but has no apparent effect on the formation of the hyaline layer when the trypsinized eggs are subsequently fertilized. As shown in Table I, prolonged incubation of *A. punctulata* eggs with high levels of trypsin renders greater than 80% of the egg cells unfertilizable, as judged by the absence of both fertilization envelopes and hyaline layers. Furthermore, less than 20% of the trypsinized cells cleave 90 min after fertilization, whereas greater than 70% of untreated cells reach the two-cell stage within a similar period of time. Thus, the trypsin-treated cells are indeed incapable of being fertilized by sperm. Other workers have shown that the  $\text{Ca}^{2+}$  ionophore A23187 induces the early morphological changes involved in normal fertilization, i.e., the cortical reaction, and elevation of the fertilization envelope and hyaline layer (38). With this ionophore, it was found that cells remain intact and viable after trypsin treatment, since greater than 95% of them are still capable of ionophore-induced activation, as judged by the appearance of a hyaline layer.

Although the results presented in Table I show that trypsin-treated eggs cannot be fertilized, it was of interest to determine whether sperm cells interact with or bind to the trypsin-treated cells. When untreated eggs were fertilized in the presence of an equal number of trypsin-treated eggs, no effect on the extent of fertilization of the untreated eggs was observed. Thus, the trypsin-treated eggs not only cannot be fertilized, but apparently are also incapable of binding sperm. In confirmation of this conclusion, it was observed that no sperm cells can be seen associated with the surface of trypsin-treated eggs, whereas upon insemination of untreated eggs numerous sperm cells are seen adhering to the egg surface.

### Inhibition of Fertilization by Ghost-Derived Membranes

The results described above suggest that a trypsin-sensitive protein on the surface of eggs can serve as a receptor for sperm. To prepare a simpler system containing the receptor, eggs were lysed and membranes derived from ghosts were prepared as described in Materials and Methods.  $^{125}\text{I}$ -Labeling experiments indicate that the ghost-

derived membrane fraction is enriched fivefold in cell surface membranes (reference 33, and unpublished results of E. Schmell and W. J. Lennarz). It was assumed that the sperm receptor associated with these membranes would inhibit fertilization in the inhibition assay described in Materials and Methods by competing with eggs for the limited number of sperm cells added to the assay mixture. As shown in Fig. 2, membranes prepared from *A. punctulata* egg ghosts do indeed inhibit the fertilization of *A. punctulata* eggs. Furthermore, membranes prepared from trypsin-treated eggs have no

TABLE I  
Effect of Trypsin Pretreatment on *Arbacia* Eggs

Pretreatment	Fertilization by sperm	Activation by Ionophore
	%	%
None	>95	>95
Trypsin	<20	>95

Separate aliquots of untreated eggs and eggs treated with trypsin as described in Materials and Methods were tested for fertilizability by sperm or activation by  $\text{Ca}^{2+}$  ionophore A23187 (38). Fertilization or activation of untreated cells was assessed by determining the number of cells that raised a fertilization envelope as described in Materials and Methods. With trypsin-treated eggs, fertilization or activation was determined by the presence of a hyaline layer.

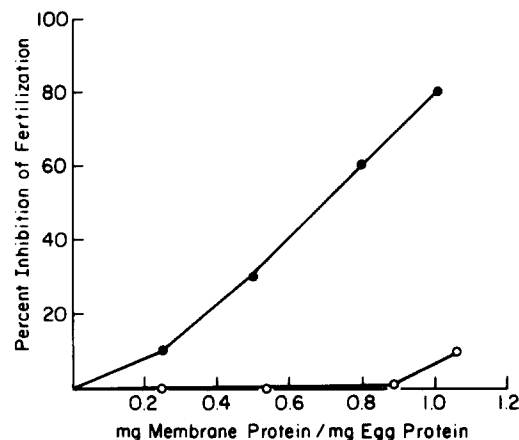


FIGURE 2 Inhibition of fertilization of *A. punctulata* eggs by ghost-derived membranes. Eggs were incubated with or without trypsin for 60 min, and membranes were prepared as described in Materials and Methods. Aliquots of membranes from trypsin-treated eggs (○) and from untreated eggs (●) were tested for their ability to inhibit fertilization as described in Materials and Methods.

effect on fertilization (Fig. 2), as expected if trypsin treatment cleaves the sperm receptor from the egg cell surface. The inhibition of fertilization is species-specific; membranes prepared from *A. punctulata* eggs are capable of inhibiting fertilization of *A. punctulata* eggs, but membranes from *S. purpuratus* eggs exhibit only very low inhibitory activity at high concentrations (Fig. 3 A). Conversely, membranes prepared from *S. purpuratus* inhibit fertilization of *S. purpuratus* eggs, but *A. punctulata* membranes do not (Fig. 3 B). As expected (Fig. 3 A), when this type of experiment is performed with sperm from a strain of *A. punctulata* from North Carolina that does cross-fertilize eggs of *A. punctulata* from Florida, inhibition by membranes of *A. punctulata* from Florida is observed.

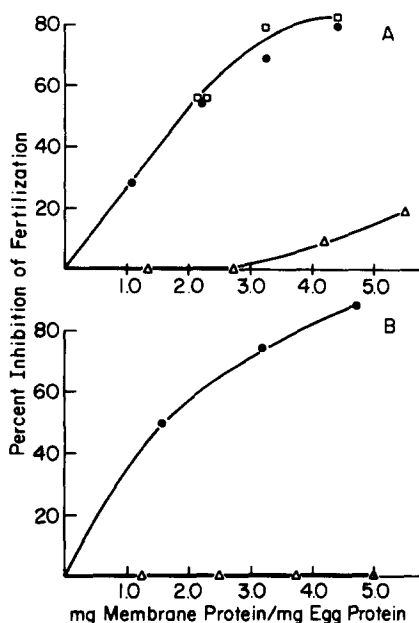


FIGURE 3 Species specificity of the inhibition of fertilization by membranes. Separate 1% suspensions of *A. punctulata* and *S. purpuratus* eggs were prepared as described in Materials and Methods. (A) Inhibition of fertilization of *A. punctulata* eggs by membranes prepared from either *A. punctulata* eggs (●) or from *S. purpuratus* eggs (△). In a separate experiment (□), with sperm from a strain of *A. punctulata* from North Carolina that does cross-fertilize with *A. punctulata* from Florida, similar results were obtained. (B) Inhibition of fertilization of *S. purpuratus* eggs by membranes prepared from either *S. purpuratus* eggs (●) or *A. punctulata* eggs (△). In both A and B membranes were prepared and assayed for their inhibitory activity as described in Materials and Methods.

TABLE II  
Development of Eggs Fertilized in the Presence and Absence of Ghost-Derived Membranes

Addition to eggs	Fertilization		Cleavage
	5 min	90 min	cleaved cells/total cells
None	58	60	45*
Membranes	20	21	14*

Eggs were fertilized with sufficient sperm to obtain 60% fertilization in the absence of membranes. The inhibition of fertilization by membranes, calculated as described in Materials and Methods, was 65% at both time-points.

\* Approximately 70% of the fertilized cells in both samples cleaved after 90 min; thus, the percent cleavage is lower than the percent fertilization. If inhibition of fertilization is calculated on the basis of percent cleavage after 90 min, the extent of inhibition is 68%.

The membrane fraction prepared from 0.4 ml of packed eggs (16 mg of total cellular protein) was found to contain 4.0 mg of protein. The hexose content of this fraction was 2.36  $\mu\text{mol}$  hexose/mg protein. The membrane fraction prepared from trypsin-treated cells was decreased by 20% in protein content and by 73% in hexose content; it contained 0.78  $\mu\text{mol}$  hexose/mg protein.

To establish that the inhibitory effect of membranes during insemination is indeed inhibition of fertilization, and not merely inhibition or retardation of fertilization envelope elevation, eggs were fertilized in the presence or absence of membranes. After 5 min, one-half of each sample was assessed for the percentage fertilization and the other half was incubated at room temperature. After 90 min, samples were examined in the phase-contrast microscope. As shown in Table II, in both samples the percentage of cells fertilized, i.e., the percentage of cells with fertilization envelopes, was the same whether fertilization was assessed after 5 or 90 min. Furthermore, if inhibition of fertilization is determined by assessing the percentage of cleaved cells after 90 min, approximately the same level of inhibition by membranes is obtained. In the sample treated with membranes, none of the cells lacking fertilization envelopes were observed to be in the two-cell stage after 90 min. Thus, membranes clearly inhibit fertilization, since both envelope elevation and cleavage do not occur.

Since, as noted above, the membranes em-

ployed represent one-fifth of the total cellular protein, it might be expected that 50% inhibition would be observed when the ratio of egg protein to membrane protein is 5:1. However, as shown in Figs. 2 and 3, four to eight times as much membrane is required. This apparent decrease in the inhibitory activity of the isolated membrane fraction could result from loss of receptors during preparation, vesiculation of the membranes, or a decrease in the binding affinity of the membrane receptors.

#### *Inhibition of Fertilization by Soluble Receptor Fraction*

To study the properties of the receptor in more detail, a soluble form of the receptor was prepared from the dialyzed membrane fraction as described in Materials and Methods. As shown in Fig. 4, the soluble receptor fraction obtained from untreated egg cells inhibits fertilization in the inhibition assay described. However, soluble receptor fraction prepared from membranes of eggs pretreated with trypsin has no effect on fertilization (Fig. 4). The receptor activity present in the soluble receptor fraction varies from 40 to 60% of the total activity present in the dialyzed membrane preparation; the remainder is recovered still bound to the membrane pellet after centrifugation.

The receptor has the properties of a glycoprotein because it binds to concanavalin A-Sepharose

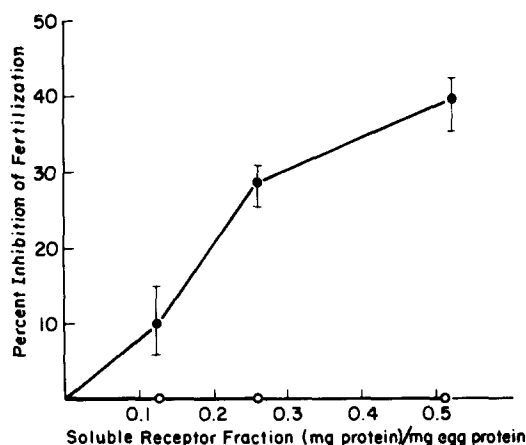


FIGURE 4 Inhibition of fertilization by soluble receptor fraction. Soluble receptor fraction was prepared from the membranes of untreated eggs (●) and eggs pretreated with trypsin (○), and inhibition of fertilization by both fractions was determined as described in Materials and Methods.

(see below), and can be eluted in an active form by  $\alpha$ -methyl mannose. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the soluble receptor fraction revealed the presence of multiple macromolecular components. Seven major bands, ranging in apparent mol wt from 80,000 to greater than 150,000, and staining for both protein and carbohydrate, were observed. The ratio of hexose to protein in the soluble receptor fraction was found to be 1.17  $\mu$ mol hexose/mg protein. The yield of soluble receptor fraction from 0.4 ml of packed eggs (16 mg of total cellular protein) was 0.75 mg of protein.

#### *Binding of Soluble Receptor to Sperm*

The receptor assay is based on the assumption that the receptor inhibits fertilization by binding to sperm and, in effect, lowering the concentration of sperm capable of binding to receptor on the surface of the eggs. Several experiments were performed to test this assumption. In one experiment, sperm and eggs were pretreated with soluble receptor prepared as described in Materials and Methods. As shown in Table III, only the sperm were affected by pretreatment with the receptor; pretreatment of eggs did not lower their ability to be fertilized. Another experiment was performed to determine whether the inhibitory effect of receptor on sperm was because of its binding to sperm, or whether it was the result of the action of an enzyme in the receptor preparation that causes an alteration of the cell surface of the sperm. Soluble receptor was incubated with high concentrations of sperm (i.e., 400- to 500-fold excess over that present in the inhibition assay). As shown in Table IV, after removal of the sperm cells by centrifugation, the soluble fraction no longer inhibited fertilization. These results suggest that the receptor inhibits fertilization by binding to the sperm, since it can be "titrated" out of solution by excess sperm.

#### *Inhibition of Fertilization by Soluble Receptor Fraction is Species-Specific*

To determine whether the soluble receptor inhibits fertilization in a species-specific manner, *A. punctulata* and *S. purpuratus* eggs were fertilized under standard inhibition assay conditions in the presence of 500  $\mu$ g of soluble receptor fraction prepared from *A. punctulata*, ghost-derived membranes. Under these conditions, the soluble receptor caused 33% inhibition of fertilization of *A.*

TABLE III  
Pretreatment of Sperm and Eggs with Soluble Receptor

Pretreatment	Treatment during fertilization	Inhibition of fertilization %
Experiment A: Pretreatment of sperm		
100 $\mu$ g receptor fraction	None	30
None	None	0
None	100 $\mu$ g receptor fraction	25
None	100 $\mu$ g receptor fraction from trypsin-treated eggs	0
Experiment B: Pretreatment of eggs		
100 $\mu$ g receptor fraction	None	0
None	None	0
None	100 $\mu$ g receptor fraction	25
None	100 $\mu$ g receptor fraction from trypsin-treated eggs	0

Either sperm (0.5  $\mu$ g protein) or eggs (0.5 mg protein) in 0.5 ml of seawater were pretreated with a solution containing 100  $\mu$ g of soluble receptor fraction prepared as described in Materials and Methods. After 5 min the cells were collected by centrifugation and resuspended in fresh seawater. In experiment A, after pretreatment as shown, the sperm were collected by centrifugation at 1,000 g for 5 min and added to untreated eggs (0.5 mg protein) in 0.5 ml of seawater. In experiment B, eggs were pretreated as shown, centrifuged, washed with seawater, and then fertilized with untreated sperm (0.5  $\mu$ g sperm protein) in 0.5 ml of seawater. After 5 min the inhibition of fertilization was determined as described in Materials and Methods. As a control for the activity of the receptor in both exp. A and B, 100- $\mu$ g of receptor was added to a mixture of untreated sperm and eggs as in the standard receptor assay described in Materials and Methods.

*punctulata* eggs, but had no inhibitory effect on the fertilization of *S. purpuratus* eggs. Thus, the soluble form of the receptor retains its species specificity in the inhibition assay.

#### Concanavalin A Inhibition of Fertilization

Several investigators have reported that sea urchin fertilization is inhibited or impaired by the addition of concanavalin A to sperm and egg cells (7, 19, 22). To determine whether concanavalin A affects eggs, sperm, or both, the gametes of *A. punctulata* were pretreated separately with concanavalin A, and then tested with the other (untreated) gamete. As shown in Fig. 5, when eggs were pretreated with increasing amounts of concanavalin A, subsequent fertilization of the eggs was inhibited.

The data presented in Fig. 5 also demonstrate that monovalent succinyl-concanavalin A does not inhibit *A. punctulata* fertilization. A similar observation was reported by Howe and Metz (19) using

monovalent concanavalin A prepared by papain digestion. Furthermore, wheat germ agglutinin also has no effect on fertilization, although the levels of agglutinin tested quantitatively agglutinated type O human erythrocytes. When sperm cells were treated with an equivalent amount of con-

TABLE IV  
Binding of Receptor to Sperm

Addition to preincubation mixture	Inhibition of fertilization by supernate %
75 $\mu$ g receptor fraction	12
75 $\mu$ g receptor fraction and sperm	0
75 $\mu$ g receptor fraction from trypsin-treated eggs	0
125 $\mu$ g receptor fraction	27
125 $\mu$ g receptor fraction and sperm	0
125 $\mu$ g receptor fraction from trypsin-treated eggs	0

The soluble receptor fraction (75 or 125  $\mu$ g) was incubated in 0.5 ml of seawater in the presence or absence of 50  $\mu$ g of sperm. After 5 min the sperm were centrifuged at 36,000 g for 30 min, and the supernatant material was assayed for receptor activity as described in Materials and Methods.

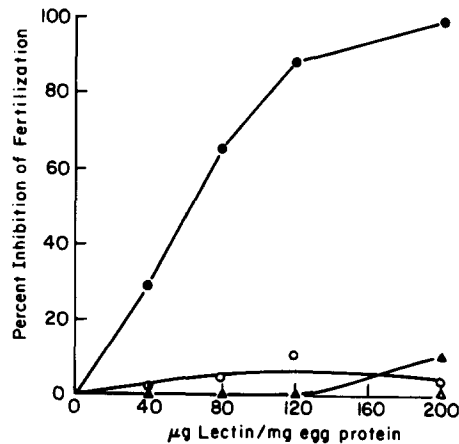


FIGURE 5 Effect of pretreatment of eggs with concanavalin A, succinyl-concanavalin A, and wheat germ agglutinin. Egg suspensions (0.5 mg protein/ml) were incubated with varying amounts of lectin (0-50  $\mu$ g protein) in a final volume of 0.5 ml. After 5 min the eggs were washed with 5 ml of fresh seawater, fertilized, and inhibition of fertilization was determined as described in Materials and Methods. Concanavalin A (●), succinyl-concanavalin A (○), wheat germ agglutinin (▲), and ovalbumin (△).

canavalin A (based on the ratio of concanavalin A to the surface area of the cell), there was no effect on their ability to fertilize eggs. Thus, concanavalin A inhibits fertilization in *A. punctulata* by binding to the egg surface. This inhibition is reversed by  $\alpha$ -methyl mannoside. When eggs were treated with sufficient concanavalin A to inhibit fertilization by 50% and then washed with seawater containing  $\alpha$ -methyl mannoside (0.5 mg/ml), 100% of the washed cells could be fertilized. When the concanavalin A-inhibited cells were washed in seawater alone, the inhibition was not reversed.

#### *Binding of Soluble Receptor to Concanavalin A-Sepharose*

In view of the finding that concanavalin A binds to the surface of the egg and inhibits fertilization, it was of interest to determine whether the soluble receptor also binds concanavalin A. The receptor fraction was incubated with concanavalin A-Sepharose beads for 15 min. The beads were then removed by centrifugation, and the receptor fraction was assayed for inhibition of fertilization. As shown in Table V, after exposure to concanavalin A-Sepharose the receptor fraction no longer inhibited fertilization. This result indicates that the soluble receptor binds to concanavalin A. It is well known that jelly coat, or fertilizin, agglutinates sperm and inhibits fertilization (23, 42). However, the receptor can be distinguished from jelly coat since, unlike the receptor, the inhibitory activity of jelly coat does not bind to concanavalin A (Table V).<sup>1</sup> Moreover, no aggregates of sperm were observed when inhibition assays were carried out using the soluble or membrane-bound form of the receptor.

#### DISCUSSION

The species-specific recognition process that must occur before fusion of the plasma membranes of sperm and egg cells during fertilization represents a highly specialized case of cell-cell recognition and adhesion. The biochemical mechanism(s) of cell-cell interactions has received considerable at-

<sup>1</sup> Furthermore, unpublished experiments performed in this laboratory by Mr. F. Kuhajda have provided direct chemical evidence that jelly coat does not bind to concanavalin A-Sepharose. In these experiments, lack of binding was established by monitoring the major carbohydrate constituent of jelly coat, fucose.

TABLE V  
*Binding of Jelly Coat and Receptor to Concanavalin A-Sepharose*

Inhibitor	Pretreatment	Inhibition of fertilization by supernate %
100 $\mu$ g receptor fraction	None	30
200 $\mu$ g receptor fraction	None	55
100 $\mu$ g receptor fraction	Concanavalin A-Sepharose	0
200 $\mu$ g receptor fraction	Concanavalin A-Sepharose	0
None	Concanavalin A-Sepharose	0
8.0 $\mu$ g jelly coat*	None	33
16.0 $\mu$ g jelly coat	None	50
8.0 $\mu$ g jelly coat	Concanavalin A-Sepharose	33
16.0 $\mu$ g jelly coat	Concanavalin A-Sepharose	55
None	Concanavalin A-Sepharose	0

Jelly coat was prepared by dejellying eggs with acid as previously described (32). The first two acid washes, which contain greater than 90% of the releasable jelly coat, were pooled. The pH of the jelly coat fraction was brought to about 8.0 with 0.2 N NaOH and then dialyzed overnight against 250 vol of fresh seawater. The final concentration of jelly coat, expressed in terms of its protein content, was 640  $\mu$ g/ml. Soluble receptor fraction (100 or 200  $\mu$ g protein) and jelly coat (8 or 16  $\mu$ g protein) were incubated with 0.2 ml of concanavalin A-Sepharose in 0.5 ml of seawater. After 5 min the concanavalin A-Sepharose beads were removed by centrifugation, and the supernatant material was assayed for inhibition of fertilization as described in Materials and Methods.

\* On a dry weight basis, rather than a protein basis, the specific inhibitory activity of jelly is actually much lower since less than 17% of the dry weight of jelly coat is protein.

tention in recent years, and the cell surface components involved in this process(es) have been identified in several biological systems (21, 25, 30, 35, 47). All of these studies strongly suggest that cell surface glycoproteins and/or carbohydrate binding proteins are involved in cell-cell recognition.

Little is known about the biochemical events involved in sperm-egg recognition and fusion during fertilization. More than sixty years ago, Lillie (23) first described the jelly coat of echinoid eggs, and proposed the fertilizin-antifertilizin theory of sperm-egg interaction. This theory has dominated the thinking of most 20th-century embryologists in dealing with sperm-egg binding in sea urchins. The theory is based on two major observations: first, jelly coat (i.e., fertilizin) reversibly agglutinates homologous sperm cells; second, jelly coat induces the acrosomal reaction in sperm of many species of echinoids.

The fact that jelly coat binds sperm and induces the acrosomal reaction led many early investigators to believe that it is the sperm receptor. However, subsequent investigations have cast doubt on this conclusion. Tyler (41) has reported on jelly



coat-sperm interactions and found little or no correlation between cross-fertilizability and cross-agglutinability. He concluded that "the specificity of the fertilizin-antifertilizin reaction does not in itself account for the specificity of fertilization." Dan (11) later provided evidence that the induction of the acrosomal reaction by jelly coat is not necessarily species-specific. More recently, Summers and Hylander (39) have demonstrated conclusively that induction of the acrosomal reaction and sperm-egg binding are two independent processes; only the latter process manifests the species specificity found in the process of echinoderm fertilization. Thus, although the jelly coat may indeed play a role in the initial stages of sperm-egg interaction, it is not the egg surface component responsible for species-specific sperm-egg binding.

Aketa and co-workers (1-6, 29, 40), working with several species of sea urchins from the Japanese coast, have published a series of papers pertaining to a sperm-binding protein isolated from eggs and presumed to be a component of the vitelline membrane. Initial studies, involving *Hemicentrotus pulcherrimus* gametes, revealed "sperm adherence to bubbles made in a solution of the substance" (1). This observation was interpreted as evidence that the sperm-binding protein binds to sperm, and was later used as an assay for this process (2, 40). Antibodies prepared against this material have been shown to bind to the egg cell surface and prevent fertilization (4, 5). However, this inhibition is not species-specific (4). It is unclear whether the sperm-binding protein inhibits fertilization by binding to sperm, since all experiments were performed by mixing sperm, eggs, and the sperm-binding protein together in solution (6). Experiments designed to demonstrate that sperm-binding protein inhibits fertilization in a species-specific manner are inconclusive (6). Although sperm-binding protein prepared from heterologous species does not inhibit *H. pulcherrimus* fertilization, it has not been shown that these other sperm-binding proteins inhibit fertilization of eggs of their own species. The sperm-binding protein appears to be rather complex in composition, since it contains protein, carbohydrate, and phospholipid (3). No rigorous criteria for the purity, solubility, or surface localization of this sperm-binding protein have been reported. The sperm-binding protein has not been distinguished from jelly coat, and the evidence that it binds to sperm is, at best, qualitative. Furthermore, most of the data pertaining to sperm-bind-

ing protein has been derived from experiments performed with antibodies prepared against material of unknown purity containing multiple antigenic components (29). For these reasons we feel that the studies by Aketa and co-workers do not provide conclusive evidence for a sperm receptor protein.

To understand more clearly the molecular basis of sperm-egg recognition during fertilization, we have investigated the possibility that a sperm receptor exists on the surface of eggs of *A. punctulata*. As a first step, a quantitative competition assay for inhibition of fertilization was developed. Using this competition assay, we have obtained evidence for the presence of a sperm receptor on the surface membrane of the eggs of *A. punctulata*.

Initial studies with intact cells revealed that *A. punctulata* and *S. purpuratus* gametes do not cross-fertilize, and that *A. punctulata* sperm do not interact with heterologous eggs. These findings supported the working assumption that an inhibition assay could be used to detect a receptor that would inhibit fertilization in a species-specific manner by competing with eggs for the limiting amount of sperm. As expected, if there were a receptor for sperm on the cell surface, membranes prepared from ghosts of *A. punctulata* inhibit fertilization. This inhibition is not merely the result of the presence of membrane fragments in the fertilization assay mixture, since membranes prepared in a similar fashion from *S. purpuratus* eggs have no inhibitory effect. Furthermore, membranes prepared from trypsin-treated *A. punctulata* eggs do not inhibit fertilization, as they presumably lack sperm receptors. The loss of fertilizability of trypsin-treated eggs, and the absence of an inhibitor associated with membranes prepared from these eggs, are consistent with the conclusion that a trypsin-sensitive sperm receptor is localized on the egg cell surface.

Upon extraction of ghost-derived membranes with sea water, a soluble form of the receptor is obtained. Preincubation of either sperm or eggs with the soluble form of the receptor showed that the receptor inhibits fertilization by binding to sperm.

Several workers have reported that concanavalin A inhibits sea urchin fertilization (7, 19, 22). We have confirmed and quantified this observation, and have shown that in *A. punctulata* concanavalin A inhibits by binding to eggs. Assuming that one of the surface components of the egg that

binds concanavalin A might be the sperm receptor, the ability of the soluble receptor to bind to concanavalin A was investigated. The results showed that, indeed, the receptor does bind to concanavalin A. In contrast, jelly coat, which might be expected to bind to sperm and inhibit fertilization, does not bind to concanavalin A. Thus, based on this finding, and the fact that all studies were performed using dejellied eggs, it is highly unlikely that the receptor under study is jelly coat.

It was reported earlier that monovalent concanavalin A prepared by papain treatment binds to eggs but has no inhibitory effect on fertilization (19). Using monovalent succinyl concanavalin A, we have confirmed this observation. These findings indicate that concanavalin A does not inhibit fertilization by binding to the region of the receptor that is involved in sperm binding. However, since the soluble receptor does bind to concanavalin A, it is clear that the sperm receptor contains  $\alpha$ -glucosyl or  $\alpha$ -mannosyl units. The differing effects of monovalent and native concanavalin A may be explained in two ways. Although both forms of the lectin may bind to the glycosyl moiety of the sperm receptor, perhaps only the more bulky native lectin sterically hinders the binding of sperm to the receptor. Alternatively, binding of sperm to the egg surface may require local rearrangements of receptors or a "capping" phenomenon, i.e., sperm-egg binding may involve the confluence of multiple surface sperm receptors in order to anchor the sperm cell to the egg surface. Native lectin may immobilize the sperm receptors and, in this way, inhibit fertilization. Monovalent concanavalin A, which cannot cross-link receptors, would not be expected to prevent receptor migration and thus has no inhibitory effect on fertilization. This explanation is consistent with the fact that migration of surface receptors has been postulated in several other systems (13, 17, 28, 31).

It is interesting to compare our results with those of Glaser and co-workers (16, 26, 27) involving cell-cell interactions in various neuronal tissues of the chick embryo. In this system the surface component(s) involved in the tissue-specific process of cell adhesion was detected by monitoring inhibition of adhesion of intact single cell suspensions. In this manner, it was determined that surface membranes prepared from cells of various tissues bind to intact cells of the original tissue, but not cells of another tissue (26). This

binding results in specific inhibition of adhesion of homologous intact cells (16). The plasma membrane component(s) involved in the binding and inhibition has been solubilized and shown to have properties consistent with those of the membrane-bound molecule (27). Although the process of fertilization differs significantly from the relatively simple process of cell adhesion, it is not unreasonable to assume that at least the initial events of cell-cell recognition may involve similar molecular mechanism in many biological systems.

On the basis of our experiments with intact eggs, membranes derived from ghosts of eggs, and a soluble fraction derived from the membranes, we conclude that there is a sperm receptor associated with the surface of the egg. It inhibits fertilization in a species-specific manner and binds to sperm. However, a number of very important questions about the receptor remain to be answered. For example, is it associated with the plasma membrane, with the vitelline layer that is believed to coat the external surface of the plasma membrane, or with both components of the cell surface? Indeed, it has been suggested that both the plasma membrane and the vitelline layer contain sperm receptors, but that only the vitelline layer receptors are species-specific (45). Our results do not preclude this possibility.

Another important question is related to the isolation of receptor in soluble form. In the procedure used, membranes are prepared by lysing the cells in distilled water. The soluble form of the receptor is then obtained by extracting the ghost-derived membrane with seawater. This treatment is extraordinarily mild when compared to the more usual conditions for solubilization of membrane proteins, i.e., treatment with detergents or chaotropic agents. One possible explanation is that exposure to distilled water so drastically alters the structure of the membrane that subsequent extraction with seawater results in release of the receptor. Release of a portion of the insulin receptor in a soluble form from membranes of fat and liver cells by extraction with neutral buffer has been reported (10). A second possible explanation is that the soluble receptor is, in fact, a biologically active fragment of the complete receptor that is cleaved from the ghost by an endogenous protease during the extraction process. Precedent for this possibility is found in the case of the membrane-bound cytochrome  $b_5$  of liver microsomes (20, 36, 37). In this context, it should be noted that Epel and co-workers (9, 43-45) have

reported on a proteo-esterase activity in the eggs of *S. purpuratus* that is believed to cleave the sperm receptor from the egg cell surface after fertilization. A similar enzyme is believed to exist in *A. punctulata* eggs (34). Thus, the possibility that the receptor is enzymatically cleaved from the membrane during the extraction procedure must be investigated more closely. Hopefully, isolation and purification of the sperm receptor should enable us to answer many of these questions.

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